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EFFECTS OF MECHANICAL STIMULATION ON SIGNAL TRANSDUCTION PATHWAYS,
INTEGRINS AND CYTOSKELETAL PROTEINS IN OSTEOLAST-LIKE CELLS In Vitro

BY

ROBERTO S. CARVALHO

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba
in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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to Elsa

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ABSTRACT

Mechanical stimulation has been shown to play an important role in the regulation and maintenance of bone cell phenotype. The equilibrium between strain-induced mechanisms is critical for determining the behavior of bone cells. Hence, an understanding of strain at the cellular level, including strain transduction events into cellular function is of crucial importance in bone related physiological processes. The aim of this work was to study the effects of strain on: 1) signal transduction pathways, 2) behavior of cytoskeletal proteins and 3) regulation of integrin subunit proteins in bone cells *in vitro*. To study these strain-induced mechanisms of bone cell regulation, we have used the Flexercell System apparatus. This system is capable of controlling strain parameters delivered to the cells through deformation of the cell-substrate relationship.

During the course of this study, rat and human bone cells were intermittently strained for different periods of time, and unstrained cells were used as controls. Preliminary observations confirmed that strain at the cellular level by demonstrating the ability of the Flexercell apparatus to effectively change cell shape and "footprint" area. Signaling transduction pathways in stimulated cells indicated that the phosphatidylinositol pathway responds much quickly, contrary to what was originally regarded as a cAMP-regulated response. We suggested that this pathway may be more useful in transducing mechanical signals intracellularly. The role of the cytoskeleton in our studies has indicated that strain plays an active role in the rearrangement of focal contact protein relationships. Increases in strain-induced vinculin and talin synthesis and their immunolocalization distribution changes appeared to indicate that a primary response to strain may lay in an augmentation of focal contact plaques, thus increasing cell-substrate attachment. Rearrangement of actin microfilaments and vimentin intermediate filaments also suggested an active role of the cytoskeleton in strain-mediated bone response. Finally, activation of strain pathways could be partly explained by the selective stimulation of integrin subunit receptor proteins. As a result of some of these studies, a model for strain-induced response in bone cells has been proposed.



Chapter ONE

General Introduction



GENERAL INTRODUCTION

Mechanical stimulation applied to the skeleton partly determines the metabolic response of bone cells. Discrete and large adaptations of osteoprogenitor cells and/or osteoblasts to physiological usage are responsible for overall homeostasis of the skeleton. Studying the effects of mechanical strain in living tissue is a means of investigating the well known concept "function dictates form", also known as Wölff 's Law (Wölff, 1892). First described by Wölff, this concept of mechanical function correlated to bone adaptation postulates that form and function of bone will depend on changes of its internal architecture according to self-ordered mathematical rules (Rubin and Hausman, 1988). Since then such changes have been accepted as physiological modifications; meaning that mechanical strain may be responsible for remodeling changes in bone structure due to function.

Modifications in bone architecture or bone remodeling are thought to be continually influenced by the level and distribution of functional strains generated within the bone (Rubin, 1984; Rubin and Lanyon, 1985; Frost, 1988; Rubin and Hausman, 1988; Frost, 1990; Lanyon, 1991). This applies to specific morphologic variations that are present in different areas of the body, and the degree in bone formation is dependent on the load bearing characteristics of each area. Thus, the morphology of different skeletal components seems to be continuously under load tension.

The general overall strain applied to the skeleton has some morphologic directives or derivations, such as bending moments (Rubin, 1984) degree of bone curvature (Rubin *et al.*, 1990) and behavior modifications such as gait (Rubin and Lanyon, 1982; Rubin *et al.*, 1990). In addition, morphologic aspects of bone such as bone curvature seem to accentuate the skeletal strain. It appears, however, that minimization of strain is not ideal for bone adaptation (Rubin *et al.*, 1990), but a resultant form of strain or a cell responsive strain frequency. Consequently, if the cells are indeed more sensitive to a specific strain frequency, the remodeling process would primarily depend on changes of stimulation from the same area.

Changes in bone architecture are attributed to three mechanisms: growth, modeling and remodeling (Frost, 1988). It appears that these mechanisms respond to mechanical usage, however, little is known about how they do it. As bone is loaded, the minimum expected threshold for adaptive changes determines which mechanism will take place. Such observations describe the changes in volume and area of bone. Three main parameters are present in bone stimulation: magnitude, frequency and periodicity of the applied force or load. However, force application within bone tissue is characterized by the phenomenon of strain, defined as deformation per unit of original area. Earlier studies suggested that the quantity of new bone formation is directly proportional to strain applied to the tissue (Langrot *et al.*, 1969; Hert *et al.*, 1971; O'Connor *et al.*, 1982). Although several studies have reported that an increase in functional activity will lead to an increase in bone mass (Chamay and Tschantz, 1972; Goodship *et al.*, 1979; Lanyon *et al.*, 1982; Lanyon, 1987; Rubin and Hausman, 1988), bone architectural changes do not generally seem to be associated with areas of highest strains, or peak strains (Rubin *et al.*, 1990; Brown *et al.*, 1990). However, it has been observed that minimum underloading strains caused remodeling changes, while overloading caused modeling adaptation (Frost, 1986; Frost, 1988). Cellular processes for bone adaptation also appear to be "saturated" at relatively low levels of strain (Rubin and Lanyon, 1985; Lanyon, 1991). Consequently, there may be a strain derivative in the cells to determine a range of stimulation to which the cells may respond and establish a continuation of the message. Rubin and Hausman (1988) have suggested that this range or "frequency" would be transmitted to the canalicular system to which bone cell membranes are susceptible, in the form of low pulse fluid fluxes. In fact it appears that a maximum response can be triggered by a relatively short "osteogenic" stimulation rather than a repetitive cumulative stimulus (Rubin and Hausman, 1988; McLeod *et al.*, 1990; Gordon, 1991).

The nature of applied strain in biological systems has been primarily divided into continuous and intermittent. Application of static loads does not elicit significant response

(Klein-Nullend *et al.*, 1986; Rubin *et al.*, 1990), and in some cases these loads are ignored as a stimulus (Rubin and Hausman, 1988; Rubin *et al.*, 1990; Brown *et al.*, 1991). The optimal cell response appears to be favored by an intermittent rather than continuous mechanics (Lanyon *et al.*, 1986; Yen *et al.*, 1989; Rubin *et al.*, 1990; Lanyon, 1991). In contrast, normal architectural changes in bone tissue appear to be continuous in character, and in spite of activation by biomechanical stimulation (i.e. baseline growth), mechanical usage induces an interrupted response as the tissue is stimulated. Incidentally there seems to be a dual behavior in which the tissue may or may not respond to mechanical usage, and when in fact a response occurs it may proceed at variable rates (Frost, 1988). Even though, this could be partly explained by the fact that values of strain under threshold levels do not seem to elicit a response (Frost, 1986), bone cell recognition of the stimulus and response to "normal" functional usage is critical to basic processes of bone tissue adaptation. However, molecular mechanisms of cell response to mechanical strain are poorly understood. Studies on parameters of bone adaptation that are mostly influenced by mechanical strain and those of strain-induced signal transduction mechanisms are invaluable to understanding the relationship between function and cellular response.

There are a variety of sensory functions such as touch, hearing, baroreception, proprioception and gravity sensation which function as input to modify the range of unconscious smooth and coordinated motion (Wang *et al.*, 1993). Some of the mechanisms underlying these phenomena may possibly involve direct matrix-cell and cell-cell stimulation by mechanical strain via specialized mechanical transduction mechanisms. To assume that mechanical strain messages are transmitted to cells, some mechanism must convert these mechanical messages into signals to which cells can perceive and respond. It is likely though, that several different "transducers" may be present and some may potentiate or inhibit the others. To date the best characterized receptor-activated signal transducers are the adenylate cyclase and phosphatidylinositol pathways.

Adenylate cyclase pathway through G-protein regulation (guanine nucleotide binding protein) is involved in several second messenger responses. This pathway is responsible for production of cyclic adenosine monophosphate (cAMP) which acts in a variety of biological processes. According to Mills *et al.* (1990) the repetitive deformation of the cell membrane by mechanical stimulation may affect adenylate cyclase and/or G-proteins directly. This system may also be a modulator of cell growth (Letsou *et al.*, 1990) and in osteoblasts may be potentiated by prostaglandins (E₁ and E₂) and parathyroid hormone (PTH) (Binderman *et al.*, 1988; Sandy *et al.*, 1989a; Sandy *et al.*, 1991). In a similar fashion, the guanylate cyclase pathway produces cyclic guanosine monophosphate (cGMP), but its function remains unclear (Bourne *et al.*, 1989). The phosphatidylinositol (PI) pathway is also a G-protein dependent pathway. Enzymes such as phospholipase C (PLC) and phospholipase A₂ (PLA₂) are important for PI degradation. Phospholipase C acts primarily on phosphatidylinositol-4,5 bisphosphate (PIP₂) to generate inositol-1,4,5 trisphosphate (IP₃) and diacylglycerol (DG). IP₃ can mobilize intracellular Ca²⁺ while DG can activate protein kinase C (PKC) (Jones and Schlüßers, 1987; Jones *et al.*, 1991; Sumpio, 1991). In addition, the IP₃ pathway may provide Ca²⁺ for the stimulation of PLA₂. The action of the latter causes hydrolysis of arachidonic acid which is a precursor of a variety of eicosanoid mediators which may regulate cellular response (Sumpio, 1991).

Second messenger molecules such as the cyclic nucleotides, cAMP and cGMP have long been considered as primary signaling molecules (White *et al.*, 1969; Rodan *et al.*, 1975). These "messengers" have been suggested to convert membrane stimulation into cellular activity (Hardman and Sutherland, 1969; Rodan *et al.*, 1975). Since the regulation of their activity reflects enhanced cellular metabolism (White *et al.*, 1969; Hardman and Sutherland, 1969; Rubin and Hausman, 1988; Burger *et al.*, 1992), their concentration might participate in the mechanical strain-induced cell response. However, studies suggest that cyclic nucleotides are not the primary mediator to respond and activate cellular response (Jones and Schlüßers, 1987; Sandy *et al.*, 1989b; Jones *et al.*, 1991). Despite their

intracellular activity, their ubiquitous distribution may not be indicative that a particular response is taking place, rather than an overall increase in cellular activity. Jones and Schlüßers (1987) suggested that the PI pathway, through PLC and PKC, may be activated by mechanical strain prior to any changes in cyclic nucleotides (Jones *et al.*, 1991; Sandy *et al.*, 1993). Furthermore, PLC-dependent IP₃ increase has been reported in endothelial cells exposed to shear stresses (Nollert *et al.*, 1990) and the cycle PI-PLC-PKC has also been shown to stimulate prostaglandin synthesis (Jones *et al.*, 1991). IP₃ as well as its counterpart DG, acts not only as second messenger but also as an intermediate for many different cellular cycles (Sumpio, 1991; Sandy *et al.*, 1993). In any case, the mechanism by which mechanical stimulation activates cellular metabolism appears to be initiated by a deformation of the plasma membrane which takes place prior to a secondary activator or "messenger" of cellular response.

Potential to respond to cell membrane deformation appears to be a characteristic of cells subjected to mechanical constraints, independently of the type of stimulation. Assuming that the receptor-activated cell response has a chemical component in the form of second messengers, one may hypothesize that the process of strain transduction may reside in both a chemical and a physical mechanism. Since any form of second messenger response appears to take place downstream of this initiation of cell activation, the question arises as to what component is the real transducer of strain.

There is some evidence for the presence of specialized channels called mechano-transducers (Guharay and Sachs, 1984). In fact a role for these components in controlling cell division has been reported in oocytes (Yang and Sachs, 1987). When compared to other similar transduction mechanisms, the idea of these channels refers to a specific sensory system which potentiates extracellular stimulation as usable energy (Sachs, 1988). The latter can be seen clearly in cells such as those of the vestibular system of the inner ear (Bialek, 1987). These cells, capable of detecting minimal physical energy, are specialized epithelial cells or hair cells containing tufts of mechanically sensitive cilia (Ding *et al.*,

1991). If mechano-transducers in fact exist, they might behave as stretch activated channels. There are reports that ion membrane channels behave as strain-sensitive channels (Guharey and Sachs, 1984; Lansman *et al.*, 1987; Jones *et al.*, 1991; Davidson, 1993). Sachs (1988) classified ion channels into three distinct categories based on the dominant source of energy for gating: voltage-sensitive, ligand-sensitive and tension-sensitive channels. Some of the evidence that the so-called mechano-transducers are in fact responsive to strain, comes from patch-clamp experiments, which demonstrated that in long periods of activity the only possible source of energy to open the channels is the stretch derived from membrane strain (Guharey and Sachs, 1984; Sachs, 1988; Sokabe *et al.*, 1991; Davies, 1991; Sigurdson *et al.*, 1993; Yang and Sachs, 1993).

Since Rodan *et al.* (1975) have demonstrated that mechanical strain stimulated not only second messenger systems, but also the movement of relatively impermeable cations such as Ca^{+2} and K^{+} through bone cell membrane, there have been a number of studies showing the presence of so-called mechanotransducer channels. Channels sensitive to K^{+} have been demonstrated in endothelial cells (Davies, 1991), astrocytes (Bowman *et al.*, 1992), myocytes (Bustamante *et al.*, 1991; Ruknudin *et al.*, 1993) and osteoblasts (Jones *et al.*, 1991; Ypey *et al.*, 1992; Davidson, 1993). It seems that these channels control the transmembrane flux of K^{+} ions as a function of shear strain (Davies, 1991) and may also represent an adaptation of strain associated with mechanical loading (Davidson, 1993). In addition, changes in extracellular Ca^{2+} concentration have been shown to modulate behavior of stretch-activated channels (Cooper *et al.*, 1986). The latter seems due to a partial blockage of Ca^{2+} in Na^{+} and K^{+} binding (Sachs, 1988; Ruknudin *et al.*, 1993). Although, there is some evidence that Ca^{2+} concentration increases intracellularly before changes in other signaling pathways (Jones *et al.*, 1991, Sigurdson *et al.*, 1993; Arora and McCulloch, 1994), Ca^{2+} alone may not constitute a distinct signaling mechanism (Guharey and Sachs, 1985; Wirtz and Dobbs, 1990; Charles *et al.*, 1991; Jones *et al.*, 1991). For instance, mechanical strain has been shown to increase ion permeability through membrane

deformation (Sigurdson and Morris, 1987; Sigurdson *et al.*, 1992; Sachs, 1988; Davidson, 1993; Ruknudin *et al.*, 1993; Sigurdson *et al.*, 1993; Bell and Holmes, 1994), even though intracellular communication does not require an increase in Ca^{2+} concentration (Charles *et al.*, 1991). Mechanically-induced Ca^{2+} flux may also be regulated by IP_3 and tyrosine kinase signaling mechanisms (Charles *et al.*, 1991; Arora and McCulloch, 1993), as most of the intracellular Ca^{2+} is released by IP_3 from intracellular stores after mechanical strain (Charles *et al.*, 1991). IP_3 is also important in cell-cell signaling through gap junctions (Sanderson *et al.*, 1990; Charles *et al.*, 1991; Schirrmacher *et al.*, 1992). Ca^{2+} , however, might be considered as a second messenger in helping maintain the channels in an open state, similar to cGMP inactivation of Na^+ channels in light activated rod retinal cells (Bourne *et al.*, 1989). Be that as it may, the concentration of Ca^{2+} , Na^+ and K^+ seems to undoubtedly influence channel behavior. Changes in intercellular Na^+ concentration may lead to the key stimulation for stretch channels in cases of skeletal and cardiac muscle hypertrophy (Vandenburgh and Kaufman, 1979; Vandenburgh and Kaufman, 1981; Sachs, 1988; Bustamante *et al.*, 1991).

The idea of channels as mechano-transducers is appealing. However, as Cooper *et al.* (1986) pointed out, channel conductance seems to be independent of membrane tension, which would exclude the possibility of a direct mechanical stimulation of the channel, forcing it to open. On the other hand, some degree of pressure may be present by changes in ion flow (Sachs, 1988). Considering the mechanical strain-induced membrane tension in cells from tissues like bone and muscle, the activity of the so-called mechanotransducers may indeed participate on the processes of strain transduction. However, in spite of any linkage between membrane tension and strain-sensitive channels, the assumption that stretch-channels are entirely responsible for strain transduction is at best, premature. Sokabe *et al.* (1991) indicated that channel activity can be measured as a function of the proportionality between pressure and tension (Sokabe *et al.*, 1991). While this relationship has been described as in the case of an elastic model (Sachs, 1989 for a review) there is no

data available on the direct measurement of such tension. Thus, there are few conclusive results to show which biological processes may be regulated by any stretch-activated ion changes (Watson, 1991). Studies have suggested that the patch-clamping technique may enhance cell permeability, producing stretch-activated channels measurable as artifacts (Morris and Horn, 1991). However, an absence of inducible Ca^{2+} flux in mechanically stimulated cells without such channels suggests that ion permeability constitutes a specific response to strain (Sigurdson *et al.*, 1992). Notwithstanding the merits of stretch-channels, additional work is required before the role of such structures can be precisely determined.

Strain-induced cell signaling is not, however, solely present at the membrane level, but may affect the entire cell. Stretch-channels have not been investigated here; their study requires special techniques not available to us. Their potential role, however, cannot be ruled out in the milieu of cellular response. An indication for this is based on the evidence for coupling of stretch-activated channels to the cytoskeleton (Sokabe *et al.*, 1991; Bibby and McCulloch, 1994). Changes in channel-induced intracellular osmolarity have also shown that swelling of certain cell types results in the reversible disassembly of cytoskeleton proteins (Waston, 1991; Bibby and McCulloch, 1994). Therefore, interaction of both extracellular and intracellular mechanisms may play an important role in maintenance of cell activity and conformation. In particular, we emphasize the relationship of cell-cell and cell-extracellular matrix (ECM) in mechanically-induced cellular response.

ECM participates actively in processes of strain transduction and consequently may influence the extent and nature of the cellular response (Rodan *et al.*, 1985; Lanyon, 1987; Rubin and Hausman, 1988; Ingber, 1991; Ingber, 1993). It is, however, the culmination of a cascade of connecting proteins and biochemical pathways that will determine the strain signals. Mechanical strain-mediated cell response constitutes a unique form of cellular activity, what may potentially be regulated by many factors. It has been observed that attachment of cells in a variety of substrata initiates mechanically-induced signal

transduction (Dunn and Brown, 1986; Ben-Ze'ev, 1991; Hush and Overall, 1991; Watson, 1991; Oakley and Brunette, 1993). In such a situation, although there is no obvious agonist-receptor binding at the membrane level of bone cells, such as in hormone-induced response (Pead *et al.*, 1988), cell-ECM attachment receptors may provide a link between ECM and cell deformation and cellular function (Meghji, 1992). The best characterized mechanisms of strain transduction through the cell membrane into cell function appears to involve the integrins and associated proteins (Tidball, 1991; Ingber, 1991, Watson, 1991; Ben-Ze'ev, 1991; Hynes, 1992; Meghji, 1992; Ingber, 1993; Vouri and Ruoslahti, 1994). Integrins are ubiquitous receptors that span the cell membrane as heterodimers (Hynes, 1992 for a review). These dimers are formed by $\alpha\beta$ subunits and according to their combination confer cell specificity to a variety of ECM proteins such as fibronectin (Clover *et al.*, 1992) and vitronectin (Fath *et al.*, 1989) to name but a few. Presently, a total of more than 20 combinations have been identified, from the dimerization of one of 14 different α units with one of 8 distinct β subunits (Virtanen *et al.*, 1990; Hynes, 1992; Vouri and Ruoslahti, 1994). This large variety of specific receptors has transformed the potential function of integrins from simply "integrating molecules of ECM and cytoskeleton", to a complex structure capable of regulating multiple cellular functions. The interaction of regulatory intracellular and extracellular proteins with integrin receptors has characterized not only a physical link in tissue formation, but also a pathway for signal transduction mechanisms activating the expression of genes involved in processes of growth, development and maintenance of cellular phenotype.

Integrins connect the cytoskeleton through specialized areas of the membrane termed focal contacts (Burrige *et al.*, 1988; Fath *et al.*, 1989; Burrige *et al.*, 1990; Dowrick *et al.*, 1991). These areas form strong "adhesive" contacts connecting the ECM with the terminal end of cytoskeletal actin fibers (Mueller *et al.*, 1989; Burrige *et al.*, 1990; Pavalko *et al.*, 1989 for a review). Abundantly present in focal contacts, integrins associate most prominently with proteins such as vinculin (Burrige and Mangeat, 1984;

Geiger *et al.*, 1985) and talin (Burrige and Mangeat, 1984; Fath *et al.*, 1989; Burrige *et al.*, 1990; Dowrick *et al.*, 1991). Other important proteins are α -actinin (Tidball, 1991), tensin (Davies *et al.*, 1991; Hynes, 1992), paxillin (Turner *et al.*, 1990; Stossel, 1993) and filamin (Langanger *et al.*, 1984; Pavalko *et al.*, 1989). Interaction of integrins with the underlying cytoskeletal proteins may potentially determine the transmembrane transduction of signals from the extracellular environment to cellular functional regulatory mechanisms.

The properties of integrin-associated signal transduction have been described in detail (Ingber, 1991; Watson, 1991; Damsky and Werb, 1992; Ingber, 1993; Sastry and Horwitz, 1993; Ingber, 1993). The close association of integrin receptors with the cytoskeleton has been shown in many biological processes (Benya *et al.*, 1988; Geiger, 1989; Mueller *et al.*, 1989; Ingber, 1990; Ben-Ze'ev, 1991 for a review). For instance, areas of focal contacts possess several signal transduction molecules, such as tyrosine kinases, PKC, Ca^{2+} -dependent proteases and various proto-oncogenes and oncogene products (Beckerle, 1990 for a review). Moreover, β integrin subunit and the focal contact proteins talin, vinculin and paxillin can be modified through phosphorylation mechanisms (Fath *et al.*, 1989; Beckerle, 1990; Ben-Ze'ev, 1991; Derventzi *et al.*, 1992). Furthermore, the closeness of the integrin-cytoskeleton interaction is further supported by studies showing that propagation of intracellular signals is impaired if a discontinuity occurs between these components. The latter was observed in studies of cell migration and chemotaxis (Mueller *et al.*, 1989; Letsou *et al.*, 1990; Ben-Ze'ev, 1991; Meghji, 1992). Mechanical strain is followed by cytoskeletal rearrangement and cell-shape changes (Hong and Brunette, 1987; Buckley *et al.*, 1988; Sumpio and Banes, 1988; Ben-Ze'ev, 1991; Iba and Sumpio, 1991; Pender and McCulloch, 1991), and such changes would involve integrins. Thus, coordination of cellular functions by mechanical stimulation will likely involve the development, assembly and maintenance of specific cytoskeletal protein networks and the respective integrin-ECM interactions. Furthermore, taking into account the variety of mechanical stimulation transmitted through cell membranes, underlying

mechanisms responsible for the corresponding cellular functions must have a degree of specialization and specificity to deal with individual responses. This may also be applicable to a receptor mediated nuclear response as a regulatory mechanism, in addition to any combinations of ion channel/second messenger/structural deformation which might be taking place concomitantly.

In summary, there is strong evidence to suggest that mechanical deformation of cell structure initiates cell specific signal transduction (Meikle *et al.*, 1989 for a review). Moreover, binding of attachment receptors to ECM proteins can elicit changes in cellular shape as a consequence of cellular biochemical mechanisms (Watson, 1991). In fact the parallel between mechanisms of strain transduction and the mechanism of ligand binding to specific cell membrane receptors which have been well characterized is clear. However, the complexity of the latter pathways suggests that mechanical strain transduction may be equally complex. More importantly, the diverse range of information which has arisen from studies of ligand-receptor interactions suggests that a similar wealth of information may be derived from examination of the mechano-receptor route. Thus, both the vital importance and complexity of strain-induced cell response clearly requires further investigation.

The overall hypothesis in this series of studies is that transduction of mechanical strain by bone cells occurs through specific signaling transduction pathways involving structural receptor and cytoskeleton proteins. The main objectives are: 1°) to study the regulation of mechanical stimulation of AC and PI pathways in bone cells, 2°) to study the effects of strain on changes in distribution and synthesis of cell cytoskeleton proteins, 3°) to study the effects of strain on changes in distribution and synthesis of integrins and 4°) to study the regulation of strain-induced molecular response of distinct integrin subunits.

REFERENCES

- Arora PD and McCulloch CAG 1994 Intracellular calcium responses of periodontal fibroblasts to mechanical stretch. *J Dent Res* **73**:1326.
- Beckerle MC 1990 The adhesion plaque protein, talin, is phosphorylated *in vivo* in chicken embryo fibroblasts exposed to a tumor -promoting forbol ester. *Cell Regul* **1**:227-36.
- Bell J and Holmes MH 1994 A note on modeling mechano-chemical transduction with an application to a skin receptor. *J Math Biol* **32**:275-285.
- Ben-Ze'ev A 1991 Animal cell shape changes and gene expression. *BioEssays* **13**:207-211.
- Benya PD, Brown PD and Padilla SR 1988 Microfilament modification by dihydrocytochalasin B causes retinoic acid-modulated chondrocytes to reexpress the differentiated collagen phenotype without a change in shape. *J Cell Biol* **103**:1021-1031.
- Bialek W 1987 Physical limits to sensation and perception. *Annu Rev Biophys Chem* **16**:455-1987.
- Bibby KJ and McCulloch CA Regulation of cell volume and Ca^{2+} in attached human fibroblasts responding to anisosmotic buffers. *Am J Physiol* **266**:1639C-1649C. 1994
- Binderman I, Zor U, Kaye A, Shimshoni Z, Harell A and Sömjem D 1988 The transduction of mechanical force into biochemical events in bone cells may involve activation of phospholipase A_2 . *Calcif Tiss Intl* **42**:261-266.
- Bourne H, Cohen P, Hardie G, Koshland D, Levitzki A, Mishel R, Mudge A, Schramm M, Sellinger Z and Vanaman T 1989 Cell Signaling. In Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD, eds. *Molecular Biology of the Cell*, 2nd ed., New York, NY:Garland Publishing Inc.

- Bowman CL, Ding JP, Sachs F and Sokabe M 1992 Mechanotransducing ion channels in astrocytes. *Brain Res* **584**:272-286.
- Brown T, Petersen D, Gray M, Brand R and Rubin CT 1990 Toward an identification of mechanical parameters initiating periosteal remodeling: a combined experimental and analytical approach. *J Biomechanics* **23**:893-905.
- Buckley MJ, Banes AJ, Levin LG, Sumpio BE, Sato M, Jordan R, Gilbert A, Link GH and Tran Son Tray R 1988 Osteoblast increase their rate of division and align response to cyclic, mechanical tension *in vitro*. *Bone Min* **4**:225-236.
- Burger EH, Klein-Nulend J and Veldhuijzen JP 1992 Mechanical stress and osteogenesis *in vitro*. *J Bone Min Res* **7**:397S-401S.
- Burridge K and Mangeat P 1984 An interaction between vinculin and talin. *Nature* **308**:744-755.
- Burridge K, Fath K, Kelly T, Nuckolls G and Turner C 1988 Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Ann Rev Cell Biol* **4**:487-525.
- Burridge K, Nuckolls G, Otey C, Pavalko F, Simon K and Turner C 1990 Actin-membrane interactions in focal adhesions. *Cell Differ Dev* **32**:337-42.
- Bustamante JO, Ruknudin A and Sachs F 1991 Stretch-activated channels in heart cells: relevance to cardiac hypertrophy. *J Cardiovasc Pharmacol* **17**:110S-113S.
- Chamay A and Tschantz P 1972 Mechanical influence in bone remodeling: experimental research in Wolff's law. *J Biomech* **5**:173-180.
- Charles AC, Merrill JE, Dirksen ER and Sanderson MJ 1991 Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* **6**:983-992.
- Clover J, Dodds RA and Gowen M 1992 Integrin subunit expression by human osteoblasts and osteoclasts *in situ* and *in culture*. *J Cell Sci* **103**:267-271.

- Cooper KE, Tang JM, Rae JL and Eisenberg RS 1986 A cation channel in frog lens epithelia responsive to pressure and calcium. *J Membr Biol* **93**:259-265.
- Damsky CH and Werb Z 1992 Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* **4**:772-781.
- Davidson RM 1993 Membrane stretch activates high-conductance K⁺ channel in G292 osteoblastic-like cells. *J Membr Biol* **131**:81-92.
- Davies PF 1991 Mechanical sensing mechanisms: shear stress and endothelial cells. *J Vasc Surg* **13**:729-731.
- Derventzi A, Rattan RIS and Clark BFC 1992 Phorbol ester-induced reorganization of the cytoskeleton in human fibroblasts during ageing *in vitro*. *Biochem Biophys Res Comm* **182**:1423-1428.
- Ding JP, Salvi RJ and Sachs F 1991 Stretch-activated ion channels in guinea pig outer hair cells. *Hear Res* **56**:19-28.
- Dowrick PG, Prescott AR and Warn RM 1991 Catter factor affects changes in the cytoskeleton organization of epithelial cells. *Cytokine* **3**:299-310.
- Dunn GA and Brown AF 1986 Alignment of fibroblasts on grooved surfaces described by a single geometric transformation. *J Cell Sci* **83**:313-340.
- Fath KR, Edgell CJS and Burridge K 1989 The distribution of distinct integrins in focal contacts is determined by the substratum composition. *J Cell Sci* **92**:65-75.
- Frost HM 1986 In: Frost HM, ed. *Intermediary organization of the skeleton*. Vols. I and II. CRC Press, Boca Raton, Florida.
- Frost HM 1988 Vital biomechanics: Proposed general concepts or skeletal adaptations to mechanical usage. *Calcif Tissue Int* **42**:145-156.
- Frost HM 1990 Skeletal structural adaptations to mechanical usage (SATMU): 4. Mechanical influences on intact fibrous tissues. *Anat Rec* **226**:433-439.

- Geiger B, Avnur Z, Volberg T and Volk T 1985 Molecular domains of adherens junctions. In: Edelman GM and Thiery JP, eds. *The Cell in Contact*. Wiley, New York, pp. 461-89.
- Geiger B 1989 Cytoskeletal associated cell contacts. *Curr Opin Cell Biol* **1**:103-109.
- Goodship AE, Lanyon LE and McFie H 1979 Functional adaptations of bone to increased stress. *J Bone Jt Surg* **61**:539A-546A.
- Gordon GA 1991 Stress reactions in connective tissues: a molecular hypothesis. *Med Hypoth* **36**:289-294.
- Guharey F and Sachs F 1984 Stretch activated K⁺ channels in muscle cell membranes as mechanotransducers? *J Physiol (Lond)* **352**:685-701.
- Guharey F and Sachs F 1985 Mechano-transducer ion channels in chick skeletal muscle: The effect of extracellular pH. *J Physiol* **363**:119-124.
- Hardman JG and Sutherland EW 1969 Guanidylate cyclase, an enzyme catalyzing the formation of guanosine 3', 5'-monophosphate from guanosine triphosphate. *J Biol Chem* **237**:6363-6370.
- Hert J, Liskova M and Landa JL 1971 Reaction of bone to mechanical stimuli: Part I. Continuous and intermittent loading of tibia in rabbit. *Folia Morphol* **19**:290-317.
- Hong HL and Brunette DM 1987 Effect of cell shape on proteinase secretion by epithelial cells. *J Cell Sci* **87**:259-267.
- Hush JM and Overall RL 1991 Electrical and mechanical fields orient cortical microtubules in higher plant tissues. *Cell Biol Int Rep* **15**:551-560.
- Hynes RO 1992 Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**:11-25.
- Iba T and Sumpio BE 1991 Morphological response of human endothelial cells subjected to cyclic strain *in vitro*. *Microvasc Res* **42**:245-254.

- Ingber D, Prusty D, Frangioni JV, Cragoe EJ, Lechene C and Schwartz M 1990 Control of intracellular pH and growth by fibronectin in capillary endothelial cells. *J Cell Biol* **110**:1803-1811.
- Ingber DE 1991 Integrins as mechanochemical transducers. *Curr Opin Cell Biol* **3**:841-848.
- Ingber DE 1993 Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* **104**:613-627.
- Jones DB and Schlüßbers JG 1987 Evidence that phospholipase C mediates the mechanical stress response in bone. *Calcif Tiss Int* **41**:4-10.
- Jones DB, Nolte H, Scholubers JG, Truner E and Veltel D 1991 Biochemical signal transduction of mechanical strain in osteoblast-like cells. *Biomaterials* **12**:101-110.
- Klein-Nullend J, Veldhuijzen JP and Burger EH 1986 Increased calcification of growth plate cartilage as a result of compressive force *in vitro*. *Arthrit Rheum* **29**:1002-1009.
- Langanger G, Demey J, Moeremans M, Daneels G, Debrabander M and Small JV 1984 Ultrastructural localization of alpha-actinin and filamin in cultured cells with the immunogold staining (IGS) method. *J Cell Biol* **99**:1324-1334.
- Langrot B, Hert J and Liskova M 1969 Influence on the long term continuous bending on the bone. An experimental study on the tibia of the rabbit. *Folia Morphol* **17**:389-399.
- Lansman JB, Hallam J and Rink TJ 1987 Single stretch activated ion channels in vascular endothelial cells as mechanotransducers? *Nature* **325**:811-813.
- Lanyon LE 1987 Functional strain in bone tissue as an objective, and controlling stimulus for adaptive bone remodeling. *J Biomech* **20**:1083-1093.
- Lanyon LE, Goodship AE and Pye C 1982 Mechanic adaptive bone remodeling. A quantitative study on functional adaptation in the radius following ulna osteotomy in sheep. *J Biomech* **15**:141-154.

- Lanyon LE, Rubin CT and Baust GE 1986 Modulation of bone loss during calcium insufficiency by controlled dynamic loading. *Calcif Tiss Int* **38**:209-216.
- Lanyon LE 1991 Biomechanical properties of bone and response of bone to mechanical stimuli: Functional strain as a controlling influence on bone modeling and remodeling behavior. In: Hall BK (ed) *Bone Matrix and Bone Specific Products*. Volume 3, Boca Raton, FL, CRC Press. pp. 79-108.
- Letsou GV, Rosales O, Maitz S, Vogt A and Sumpio BE 1990 Stimulation of adenylate cyclase activity in cultured endothelial cells subjected to cyclic stretch. *J Cardiovasc Surg* **31**:634-639.
- McLeod KJ and Rubin CT 1990 Frequency specific modulation of bone adaptation by induced electric fields. *J Theor Biol* **145**:385-396.
- Meghji S 1991 Bone remodeling. *Br Dent J* **172**:235-242.
- Meikle MC, Heath JK, Atkinson SJ, Hembry RM and Reynolds JJ 1989 Molecular biology of stressed connective tissues at sutures and hard tissues *in vitro*. In: Burstone C and Norton L, eds. *The Biology of Tooth Movement*, Boca Raton, FL, CRC Press. pp. 71-86.
- Mills I, Letsou G, Rabban J, Sumpio, BE and Gewertz H 1990 Mechanosensitive adenylate cyclase activity in coronary vascular smooth muscle cells. *Biochem Biophys Res Comm* **171**:143-147.
- Morris CE and Horn R 1991 Failure to elicit neuronal macroscopic mechanosensitive currents anticipated by single-channel studies. *Science* **251**:1246-1249.
- Mueller C, Kelly T, Dai M, Dai H and Chen WT 1989 Dynamic cytoskeleton-integrin associations induced by cell binding to immobilized fibronectin. *J Cell Biol* **109**:3455-64.
- Nollert MU, Eskin SE and McIntire LV 1990 Shear stresses increase inositol triphosphate levels in human endothelial cells. *Biochem Res Comm* **170**:281-287.

- O'Connor JA, Lanyon LE and McFie H 1982 The influence of strain rate on adaptive bone remodeling. *J Biochem* **15**:767-781.
- Oakley C and Brunette DM 1993 The sequence of alignment of microtubules, focal contacts and actin filaments in fibroblasts spreading on smooth and grooved titanium substrata. *J Cell Sci* **106**:343-354.
- Pavalko FM, Otey CA and Burrige K 1989 Identification of a filamin isoform enriched at the ends of stress fibers in chicken embryo fibroblasts. *J Cell Sci* **94**:109-18.
- Pead MJ, Skerry TM and Lanyon LE 1988 Direct transformation from quiescent to bone formation in the adult periosteum following a single brief period of bone loading. *J Bone Min Res* **3**:647-656.
- Pender N and McCulloch AG 1991 Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching. *J Cell Sci* **100**:187-193.
- Rodan GA, Bourett LA, Harvey A and Mensi T 1975 Cyclic AMP and cyclic GMP: mediators of the mechanical effects on bone remodeling. *Science* **189**:467-469.
- Rubin CT and Lanyon LE 1982 Limb mechanics as a function of speed and gait. *J Exp Biol* **107**:321-327.
- Rubin CT 1984 Skeletal strain and the functional significance of bone architecture. *Calcif Tissue Intl* **36**:11S-18S.
- Rubin CT and Lanyon LE 1985 Regulation of bone mass by mechanical strain magnitude. *Calcif Tissue Intl* **37**:411-417.
- Rubin CT and Hausman MR 1988 The cellular basis of Wölff's law. transduction of physical stimuli to skeletal adaptation. *Rheum Dis Clin North America* **14**:503-517.
- Rubin CT, McLeod KJ and Lanyon LE 1989 Prevention of osteoporosis by pulse electromagnetic fields. *J Bone Jt Surg* **71**:411-417.
- Rubin CT, McCleod KJ and Bain SD 1990 Functional strains and cortical bone adaptation: epigenetic assurance of skeletal integrity. *J Biomech* **23**:43-54.

- Ruknudin A, Sachs F and Bustamante JO 1993 Stretch-activated ion channels in tissue-cultured chick heart. *Am J Physiol* **264**:960H-972H.
- Sachs F 1988 Mechanical transduction in biological systems. *Crit Rev Biomed Eng* **16**:141-169.
- Sanderson MJ, Charles AC and Dirksen ER 1990 Mechanical stimulation and intercellular communication increases intracellular Ca^{2+} in epithelial cells. *Cell Regulation* **1**:585-596.
- Sandy JR, Meghji S, Farndale RW and Meikle MC 1989a Dual elevation of cyclic AMP and inositol phosphates in response to mechanical deformation of murine osteoblasts. *Biochim Biophys Acta* **1010**:265-269.
- Sandy JR, Meghji S, Scutt AM, Harvey W, Harris M and Meikle MC 1989b Murine osteoblasts release bone-resorbing factors of high and low molecular weights. *Bone Min* **5**:155-166.
- Sandy JR, Meikle MC, Martin BR and Farndale RW 1991 Leukotriene B4 increases intracellular calcium concentration and phosphoinositide metabolism in mouse osteoblasts via cyclic adenosine 3'-5'-monophosphate-independent pathways. *Endocrinology* **129**:582-590.
- Sandy JR, Farndale RW and Meikle MC 1993 Recent advances in understanding mechanically induced bone remodeling and their relevance to orthodontic theory and practice. *Am J Ortho Dentofac Orthop* **103**:212-222.
- Sastry SK and Horwitz AF 1993 Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr Opin Cell Biol* **5**:819-831.
- Schirmacher K, Schmitz I, Winterhager E, Traub O, Brummer F, Jones D and Bingmann D 1992 Characterization of gap junctions between osteoblast-like cells in culture. *Calcif Tiss Int* **51**:285-290.

- Sigurdson WJ and Morris CE 1987 Stretch activation of a K⁺ channel in molluscan heart cells. *J Exp Biol* **10**:582-588.
- Sigurdson WJ, Ruknudin A and Sachs F 1992 Calcium imaging of mechanically induced fluxes in tissue-cultured chick heart: role of stretch-activated ion channels. *Am J Physiol* **262**:1110H-1115H.
- Sigurdson WJ, Sachs F and Diamond SL 1993 Mechanical perturbation of cultured human endothelial cells causes rapid increases of intracellular calcium. *Am J Physiol* **264**:1745H-1752H.
- Sokabe M, Sachs F and Jing Z 1991 Quantitative video microscopy of patch clamped membranes stress, strain, capacitance, and stretch channel activation. *Biophys J* **59**:722-728.
- Sumpio BE and Banes AJ 1988 Prostacycline synthetic activity in cultured aortic endothelial cells undergoing cyclic mechanical deformation. *Surgery* **104**:383-389.
- Sumpio BE 1991 Hemodynamic forces in the biology of the endothelium: signal transduction pathways in endothelial cells subjected to physical forces *in vitro*. *J Vasc Surg* **13**:744-746.
- Tidball JG 1991 Force transmission across muscle cell membranes. *J Biomechan* **24**:43-52.
- Vandenburgh H and Kaufman S 1979 *In vitro* stretch-activated hypertrophy of skeletal muscle. *Science* **203**:265-268.
- Vandenburgh H and Kaufman S 1981 Stretch-induced growth of skeletal myotubules correlates with activation of the sodium pump. *J Cell Physiol* **109**:205-210.
- Virtanen I, Korhonen M, Karineimi A, Gould VE, Laitinen L and Ylännä J 1990 Integrins in human cells and tumors. *Cell Diff Dev* **32**:215-228.
- Vouri K and Ruoslahti E 1994 Association of insulin receptor substrate-1 with integrins. *Science* **266**:1576-1578.

- Wang NW, Butler JP and Ingber DI 1993 Mechanotransduction across the cell surface and through the cytoskeleton. *Science* **260**:1124-1127.
- Watson PA 1991 Function follows form: generation on intracellular signals by cell deformation. *FASEB J* **5**:2013-2019.
- White AA, Aurbach GD and Carlson SF 1969 Identification of guanidylate cyclase in mammalian tissues. *Fed Proc* **28**: 473-476.
- Wirtz HR and Dobbs LG 1990 Calcium mobilization and exocytosis after one mechanical stretch. *Science* **250**:1266-69.
- Wölfel JL 1892 The law of bone remodeling. In: Maquet P and Furlong R, eds. (Translation, 1986) *Das Gesetz der Transformation der Knochen*, Springer-Verlag, Berlin.
- Yang XC and Sachs F 1987 Stretch-activated channels in several tissues. *Biophys J* **51**:252A-256A.
- Yang XC and Sachs F 1993 Mechanically sensitive, nonselective cation channels. *EXS* **66**:79-92.
- Yen EHK, Yue CS and Suga DM 1989 Effect of force level on synthesis of type III and type I collagen in mouse interparietal suture. *J Dent Res* **68**:1746-1751.
- Ypey DL, Weidema AF, Hold KM, Van der Laarse A, Ravesloot JH, Van der Plas A and Nijweide PJ 1992 Voltage, calcium and stretch activated ionic channels in intracellular calcium in bone cells. *J Bone Min Res* **7**:377S-387S.



Chapter TWO

*Characteristics of the Degree of Stretch
Applied to Muscle, Bone and Lung Cells
Cultured on Silastic Membranes*

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INTRODUCTION

Traditionally culture techniques have employed cell monolayers attached to a negatively charged substrate to study metabolic responses to hormones or factors. These techniques have provided valuable information on cellular metabolic requirements and interactions that could be expected to occur in the intact tissue. Nevertheless, extrapolation from the *in vitro* situation to the corresponding *in vivo* environment is problematic in many tissues since, other than passive stimuli acting on the tissue, many organs and their constituent tissues also experience mechanical forces. Without doubt, these forces affect their function. Compression and tension forces on bone (Bellows *et al.*, 1986; Frost, 1990; Jones and Schlüßbers, 1987; Lanyon, 1991), contraction forces on muscles (Vilman *et al.*, 1985; Weinraub *et al.*, 1989) and expansion forces on lung cells (Riley *et al.*, 1990) are likely to play important roles in cell function (Wirtz and Dobbs, 1990). Numerous recent studies which use mechanical inducers to study force effects on tissues indicate a wide interest in the role of force application in cell function. Introduction of a computer-controlled vacuum apparatus (Flexcell Corporation, McKeesport, PA) has greatly improved the ability of investigators to simulate *in vivo* mechanical strain situations. This system is used in conjunction with multiwell culture plates with a flexible silastic membrane to which the cells attach and subsequently will replicate and grow. However a major criticism of this apparatus is that cells which are initially attached to the silastic membrane may not, during strain of the membrane, in fact be strained at all. Rather any measurable effect may only be due to localized damage to the cell membrane if the cells separate from the well substrate. Using three different cell types under investigation in our laboratory, this study was therefore undertaken firstly to determine if straining of the silastic membrane to which the cells are attached alters cell membrane permeability as measured by trypan blue staining and the active uptake of a fluorescent dye. Secondly the extent of cell strain was demonstrated and measured under the same conditions as applied above.

MATERIALS, METHODS AND RESULTS

Adult male Sprague-Dawley rats were used as the source of bone cells after Bellows *et al.* (1986) while New Zealand White timed-pregnant rabbits were used to isolate fetal lung type II cells (Scott *et al.*, 1983; Scott, 1992; Scott *et al.*, 1993) and fetal muscle cells (Anderson *et al.*, 1993). Figure 1A-1C shows the typical uptake and distribution of diacetyl fluorescein/ethidium bromide by fetal rabbit cells. Living cells are able to hydrolyze diacetyl fluorescein and therefore fluoresce green (Rotman and Papermaster, 1966) (Figure 1A). In contrast, ethidium bromide binds to DNA of dead or highly permeable cells which fluoresce red (Figures 1B and 1C). Strain did not alter the apparent incidence of dead cells in monolayers of any of the three cell types used herein (compare Figures 1A and 1B), whereas brief exposure to glutaraldehyde produced uniform red fluorescence (Figure 1C). All cultures, including those which were not strained contained occasional dead cells as determined by their red fluorescence (Figure 1B). Fluorescent dye uptake was identical in the muscle and bone cells and are therefore not shown. Trypan blue uptake was not altered by straining the cells for 24 hours. Strain did not alter the general histologic appearance of bone, or fetal muscle or lung cells *in vitro* (Figure 1D-1F). Comparison of photographs of strained and unstrained cell monolayers showed that size changes were very difficult to detect on a subjective basis. However microscopic examination as vacuum was applied to the silastic membrane clearly showed that the monolayer was being strained as cells were appreciably enlarged during strain. Morphometric analyses (Table 1) confirmed that deformation of the silastic membrane was reflected in strain of the cells. Measurements of individual cells showed that circumference was significantly increased ($p < 0.01$) by 4-6 % while area was approximately 7 % greater ($p < 0.01$) while the cells were under strain. Diameters of individual cells of each type measured in the direction of the radius of strain from the periphery towards the center of the membrane were significantly increased ($p < 0.01$) by approximately 3 % compared to the

corresponding control cells (Table 1). In addition diameters measured at right angles to the radius also showed a significant increase ($p < 0.01$) in size.

DISCUSSION

Many cell types experience mechanical perturbations which may affect their functional characteristics. While it is not entirely clear how compressing or straining a cell induces changes, preliminary studies have implicated several transduction pathways in transmitting deformation stimuli to the cell interior (Riley *et al.*, 1990). The recent development of cell isolation procedures together with the introduction of the computer-controlled Flexercell apparatus (Flexcell Corporation, McKeesport, PA.) has enabled straining of monolayers of single cell types as well as single cells and thereby allowed investigation of cellular responses to mechanical forces. This has particular significance in study of the three cell types chosen for this study. Bone cells experience deformation at low rates which may affect the rate of mineralization, tissue mass or architecture (Lanyon, 1991). Differentiating muscle cells undoubtedly undergo strain during fetal movements after 8 weeks gestational age (Arey, 1965). Similarly fetal lung cells *in utero* experience expansion-compression forces which may occur at very high rates (up to 49 cycles per minute, Lanyon and Rubin, 1984; Lanyon, 1991). Previous methods of modelling the effects of these forces using materials which apply a constant but low frequency force have been difficult to control. In addition, particularly in the case of muscle and lung cells, these methods have been unable to model either the sporadic nature or the high frequency of the *in vivo* strain conditions. Using the apparatus in the present study we have presented preliminary evidence that strain induces changes in metabolic functions in these three cell types (Yen *et al.*, 1992; Anderson *et al.*, 1993; Scott *et al.*, 1993). However from these studies, it remained a possibility that the cells were not undergoing mechanical deformation but rather the observations were the result of damage to the cell membrane. The present results show that as strain is applied to the silastic membrane to which the cells are attached, a corresponding degree of strain is experienced by the cells. Furthermore straining does not change the trypan blue staining or the fluorescent dye uptake of the cells which, should these be altered, would indicate cell damage.

Recent studies by Wirtz and Dobbs (1990) using a single strain of adult alveolar type II cells *in vitro* showed that cell surface area was increased approximately 25 % upon straining however the pressure used to bring about this degree of strain was not indicated. The present study shows that the distention applied to cells using 20 kPa (kilopascals), which is quoted as applying less than 1 % distention to cells (Flexcell Corporation), actually induces a measurable increase of approximately 3 % at a point midway between the center and periphery of the culture dish which is in close agreement with calculations done on the membranes of these dishes assuming a mean chord length increase of 1 % and average vertical displacement of 0 cm. The small differences in strain-induced size of the three cell types may reflect functional differences or the types of intercellular attachments established within the cell monolayers. Indeed shape differences are frequently used as initial identifying criteria and are readily apparent between the trapezoidal shape of the bone cells, the irregular muscle myotubes and the typical cobble-stone appearance of the epithelial cells. In addition that stage of development of the fetal cells used herein may also be reflected in the ability to respond to strain in one direction. For example, a cell type that typically attaches and is elongated *in vivo* in a proximo-distal orientation such as skeletal muscle, may differentiate to withstand or adapt to lengthwise strain between tendons while strain at right angles may induce cell damage. By comparison an epithelial cell such as the alveolar type II cells would likely attach around the perimeter to seal an epithelial layer and may be more adapted to distribute strain forces in a radial distribution. Bone cells *in vivo* would be expected to distribute mechanical forces over their entire area to provide compressive strength. Furthermore as the present study was carried out on three cell types under strain for 24 hours, it is not known whether the same degree of deformation would be demonstrated in cells strained for longer time periods. The response to chronic strain may promote further distinctions in cell orientation to strain and cell differentiation (Anderson *et al.*, 1993) in addition to the effects of greater or lesser differences in the degree of cell strain.

We conclude that using the apparatus described in the present study, bone, lung and muscle cells attach to the collagen-coated silastic membrane sufficiently to allow membrane-applied strain to be transmitted to the cells. As shown within the parameters used here, strain does not damage the cells. This system will be used in subsequent studies using these cell types to examine strain effects on cellular and biochemical development and metabolic function.

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REFERENCES

- Anderson JE, Lentz DL and Johnson RB 1993 Recovery from disuse osteopenia coincident to restoration of muscle strength in mdx mice. *Bone* **14**:625-634..
- Arey LB 1965 A reference table of correlated human development. In: Arey LB, ed. *Developmental Anatomy*, W. B. Saunders Co., Philadelphia, pp. 1-695.
- Bellows CG, Aubin JE, Hersche HNM and Autosz ME 1986 Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria cell populations. *Calcif Tiss Int* **38**:143-154.
- Frost HM 1990 Skeletal structural adaptations to mechanical usage (SATMU): 4. Mechanical influences on intact fibrous tissues. *Anat Rec* **226**:433-439.
- Jones DB and Schlüßers JG 1987 Evidence that phospholipase C mediates the mechanical stress response in bone. *Calcif Tiss Int* **41**:4-10.
- Lanyon LE and Rubin CT 1984 Static versus dynamic loads as an influence on bone remodeling. *J Biomech* **17**:897-906.
- Lanyon LE 1991 Biomechanical properties of bone and response of bone to mechanical stimuli: Functional strain as a controlling influence on bone modeling and remodeling behavior. In: Hall BK, ed. *Bone Matrix and Bone Specific Products*. Volume 3, Boca Raton CRC Press, pp. 79-108.
- Ott L 1977 *An Introduction to Statistical Methods and Data Analysis*. North Scituate, Duxbury Press.
- Patrick J, Fetherstone W, Vick H and Voegelin R 1978 Human fetal breathing movements and gross fetal body movements at weeks 34 to 35 gestation. *Am J Obstet Gynecol* **130**:693-699.
- Patrick JK, Campbell L, Carmichael R, Natale B and Richardson A 1980 A definition of human fetal apnea and the distribution of fetal apneic intervals during the last 10 weeks of pregnancy. *Am J Obstet Gynecol* **136**:471-477.

- Riley DJ, Rannels DE and Low RB 1990 Effect of physical forces on lung structure, function and metabolism. *Am Rev Respir Dis* **142**:910-14.
- Rotman B and Papermaster BW 1966 Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc Natl Acad Sci USA* **55**:134-141.
- Rubin T and Hansman MR 1988 The cellular basis of Wölff's Law. Transduction of physical stimuli to skeletal adaptation. *Rheum Dis North America* **14**:503-517.
- Schaffler MB, Radin EL and Burr DB 1989 Mechanical and morphological effects of strain rate on fatigue of compact bone. *Bone* **10**:207-214.
- Scott JE, Possmayer F and Harding PGR 1983 Alveolar pre-type II cells from the fetal rabbit lung. Isolation and characterization. *Biochim Biophys Acta* **753**:195-204.
- Scott JE 1992 Phosphatidylcholine synthesis, secretion and reutilization during differentiation of the surfactant-producing type II alveolar cell from fetal rabbit lungs. *Exp Lung Res* **18**: 563-580.
- Scott JE, Yang SY, Stanik E and Anderson JE 1993 Influence of stretch on [³H]thymidine incorporation, surfactant-related phospholipid synthesis and cAMP levels in fetal type II alveolar cells. *Am J Respir Cell Mol Biol* **8**:258-265.
- Vilman H, Juhl M and Kirkeby S 1985 Bone-muscle interactions in the muscular dystrophic mouse. *Eur J Orthod* **7**:18-192.
- Weinraub M, Rodan GA and Thompson DD 1989 Osteopenia in the immobilized rat hind limb is associated with increased bone resorption and decreased bone formation. *Bone* **10**:187-194.
- Wirtz HR and Dobbs LG 1990 Calcium mobilization and exocytosis after one mechanical stretch. *Science* **250**:1266-69.
- Yen EHK, Carvalho RS and Suga DM 1992 Intermittent levels and alveolar bone formation *in vitro*. *J Dent Res* **71**:613.

Figure 1. Bone cells were isolated from mandibles and maxillae as described by Bellows *et al.* (1990) and plated into 5.00 cm² Flexcell collagen-coated multiwell plates (Flexcell Corporation, McKeesport, PA.). Cells were grown to confluence (7 days). Fetal skeletal muscle cells were isolated and grown as described (Anderson *et al.*, 1993), plated into identical multiwell plates and incubated for 5 days. Fetal lung type II alveolar cells were isolated as described previously (Scott *et al.*, 1983; Scott, 1992; Scott *et al.*, 1993), and grown to confluence (2-3 days) in 10 % carbon-stripped fetal bovine serum. Viability of the cells after straining was established by exclusion of trypan blue dye and fluorescent dye uptake (Rotman and Papermaster, 1966). After reaching confluence, cells were strained at 20 kPa at 3 cycles per minute (cpm, 10 seconds strain, 10 seconds relaxed) for muscle and bone cells or 50 cpm (0.6 s/0.6 s) for lung cells. The rate for the lung cells was chosen since it approximates the breathing rate of the human fetus (Patrick *et al.*, 1978; Patrick *et al.*, 1980). Identical unstrained monolayer cultures served as controls. After 24 hours, membranes (with attached cells) were incubated with diacetyl fluorescein/ethidium bromide (DF/EB; 25 µg/ml; 2.5 µg/ml) as described by Rotman and Papermaster (1966) and viewed under a Zeiss fluorescence microscope. Living cells fluoresce green (495 nm excitation wavelength, BG12 filter; 525 nm emission wavelength) while nuclei of dead cells appear red (495-520 nm excitation wavelength, BP546/7 filter; 580 nm emission wavelength). (A) Typical pattern of DF/EB uptake in cells strained for 24 hours. This field shows confluent fetal rabbit type II alveolar cells exposed to straining at 50 cpm. Cells were photographed under blue light (excitation 495 nm, Zeiss BG12 filter; emission 525 nm). Virtually all cells display green fluorescence indicating they are viable, X 200. (B) Typical red fluorescence (495-520 nm excitation, Zeiss BP546/7 filter; 580 nm emission) in cultured fetal rabbit cells. Sporadic dead

cells are discernible in the field (arrow). Other cells which took up the diacetyl fluorescein dye, representative of living cells are not visible under this wavelength of light. Unstrained monolayers also showed sporadic dead cells, X 50. (C) Fetal type II alveolar cells exposed to 1 % glutaraldehyde in phosphate buffer for 2 minutes prior to incubation with DF/EB and photographed under green light displayed red fluorescence (ethidium bromide binding) indicating that the cells are dead. No fluorescence was visible under blue light, X 50. (D) Typical appearance of fetal rabbit muscle cells isolated on the 24th gestational day, grown for 5 days on silastic membranes, strained at 3 cpm for 24 hours and viewed under phase contrast. Note the multinucleated character of the cell (arrow) as well as the cell in mitosis (double arrow), X 240. (E) Fetal alveolar type II cells (arrow) isolated on the 24th gestational day, grown for 2-3 days *in vitro* on silastic membranes, strained at 50 cpm for 24 hours and viewed under phase contrast. Few fibroblasts are present in the culture (double arrow), X 240. (F) Mixed mandibular and maxillary alveolar bone cells from adult rats after growing for 7 days *in vitro* on silastic membranes and straining for 24 hours at 3 cpm. Bone cells displaying typical trapezoidal shape are indicated (arrow), X 240.

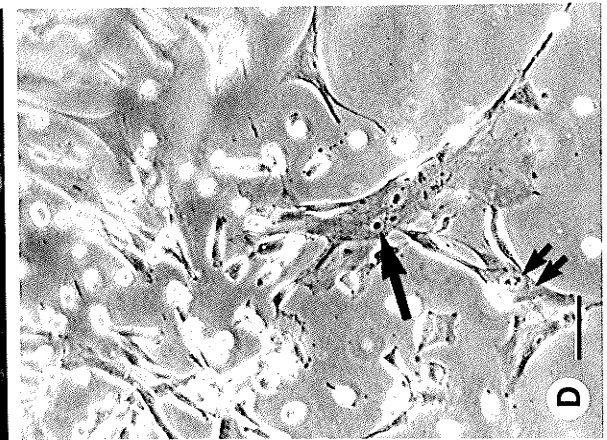
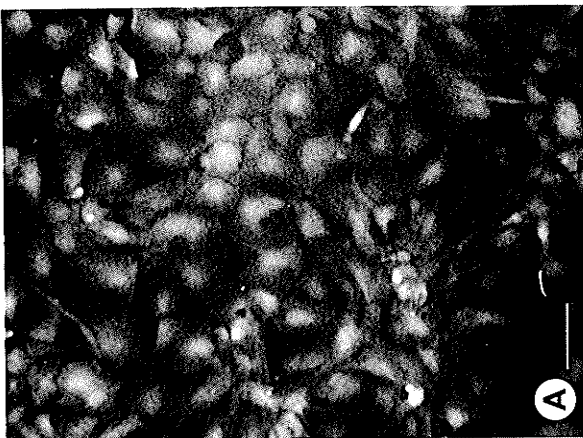
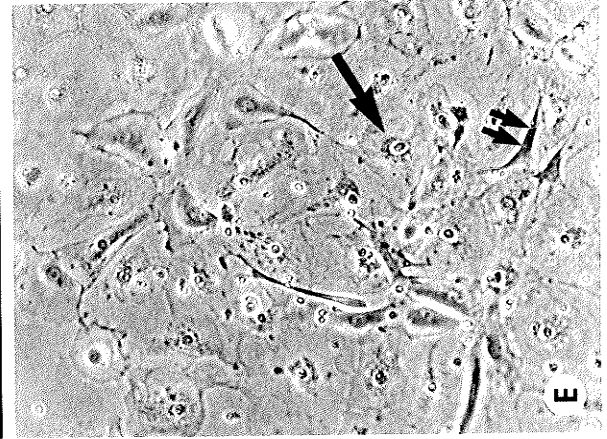
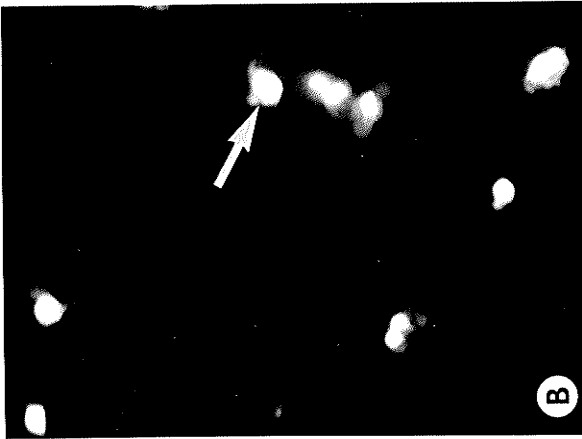
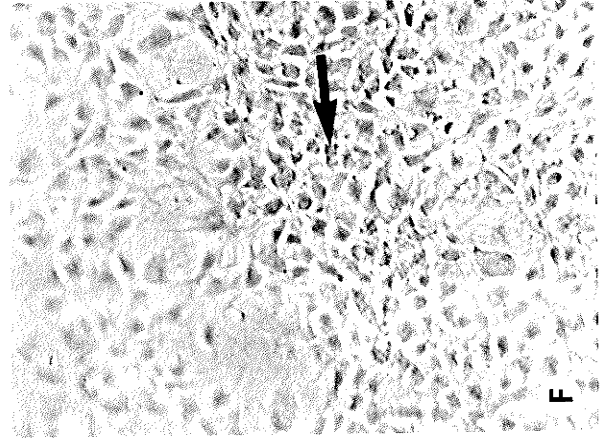
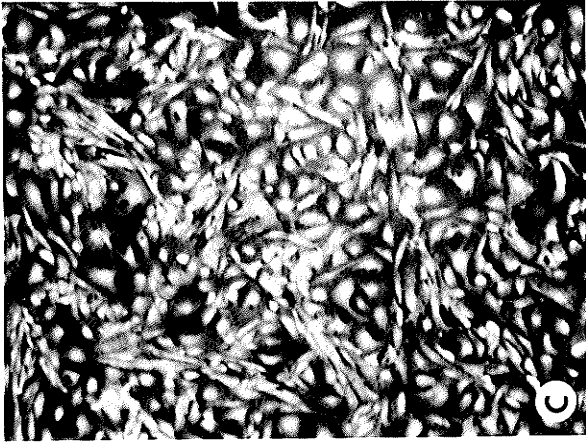


Table 1. Effect of strain on circumference, area and radial and circumferential cell diameters (Mean percentage increase under strain conditions \pm SD).^a

Measurement	Muscle	Lung	Bone
A. <i>Circumference</i>	4.44 \pm 1.32*	6.25 \pm 0.22	6.95 \pm 1.20
<i>Area</i>	7.89 \pm 4.20	8.12 \pm 3.21	6.52 \pm 0.42
Diameters			
	<i>Radial</i>	<i>Circumferential</i>	
B. <i>Lung cells</i>	3.45 \pm 0.10*	3.62 \pm 0.11	
<i>Muscle cells</i>	3.40 \pm 0.06	3.98 \pm 0.06	
<i>Bone cells</i>	3.64 \pm 0.10	5.90 \pm 0.50	

^aIn all measurements, cells were used at confluence. Flex plates were mounted in a specially designed adaptor constructed of Rubbertex (LewisCraft, Toronto, ON) which held a single 6-well culture plate. Vacuum (20 kPa, 3 PSI), which provides over 10 % deformation at the periphery was applied to plates exactly as used in the Flex mat during culture. Regions of the membrane at the midpoint between the center and lip of the plastic dish were viewed at 100X and photographed under unstrained and strained conditions. Prints (10X enlargements) were prepared and analyzed with the SigmaScan morphometrics program (Jandel Scientific, San Rafael, CA). Cell circumferences, diameters (along radius and perpendicular to radius) and areas were measured. A minimum of 9 measurements

were done. A. Circumference and area refer to the outlined distance and computed area of the same cell under strained and unstrained conditions. B. Radial refers to a cell diameter in the direction of a radius from the periphery to the center point of the membrane. Circumferential refers to the cell diameter at a right angle to the radial diameter.

*all statistical evaluations, calculated using the raw data, showed that measurements of cells under strain were significantly greater ($p < 0.01$) compared to the unstrained control cells as determined using the repeated measures ANOVA.



Chapter THREE

*Stimulation of Signal Transduction
Pathways in Osteoblasts by Mechanical
Strain Potentiated by Parathyroid Hormone*

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ABSTRACT

Second messenger systems have been implicated to transmit mechanical stimulation into cellular signals, however, there is no information on how mechanical stimulation is affected by systemic factors such as parathyroid hormone (PTH). Regulation of adenylyl cyclase and phosphatidylinositol pathways in rat dentoalveolar bone cells by mechanical strain and PTH was investigated. Two different cell populations were isolated after sequential enzyme digestions from dentoalveolar bone (group I and group II) in order to study potential differences in response. Mechanical strain was applied with 20 KPa of vacuum intermittently at 0.05 Hz for periods of 0.5, 1, 5, 10 and 30 minutes and 1, 3 and 7 days using the Flexercell System. Levels of cAMP, measured by RIA, and levels of inositol 1,4,5-triphosphate (IP₃) and protein kinase C activity (PKC), measured by assay systems, increased with mechanical strain. When PTH was added to the cells, there was a significant increase in levels of all the intracellular signals, which appeared to potentiate the response to mechanical strain. IP₃ levels (0.5 min) peaked before those of PKC activity (5 min) which in turn peaked before those of cAMP (10 min). Group II cells showed higher levels of cAMP and IP₃ than the group I cells. This suggests that the former may ultimately play the predominant roles in skeletal remodeling in response to strain. Immunolocalization of the cytoskeleton proteins vimentin and α -actinin, focal contact protein vinculin and PKC showed a marked difference between strained and non-strained cells. However, the addition of PTH did not cause any significant effect in cytoskeleton reorganization. Staining of PKC and vimentin, α -actinin and vinculin suggests that PKC participates actively in the transduction of mechanical signals to the cell through focal adhesions and the cytoskeleton, although only PKC seemed to change with short time periods of strain. In conclusion, dentoalveolar osteoblasts responded to mechanical strain initially through increase in levels of IP₃, PKC activity and later cAMP and this response was potentiated when PTH was applied together with mechanical strain.

INTRODUCTION

Mechanical strain or physical activity applied to the skeleton has been shown to alter the metabolism of bone cells (Lanyon, 1984; McLeod *et al.*, 1987). The mechanism controlling the adaptations of osteoprogenitor cells and osteoblasts to physical strain is poorly understood. It is known that bone cells respond to physical stimulation in a manner which is dependent on the periodicity or frequency of the applied stimulus (McLeod *et al.*, 1987). Osteogenic cellular synthesis of bone proteins appears to be activated preferentially by intermittent rather than continuous strain (Rubin, 1984) and frequency appears to be as important to remodeling as is the range of strain applied to bone (Rubin *et al.*, 1989). Studies have also reported that an increase in functional activity leads to an increase in bone mass (Lanyon, 1987; Rubin and Haussman, 1988). However, the intracellular mechanisms by which the cells sense the stimulation are not known. Several second messenger systems (Sandy, 1989; Brighton *et al.*, 1991; Sandy and Farndale, 1991; Watson, 1991; Brighton *et al.*, 1992) have been implicated in the role of transducing mechanical strain-derived signals in bone. However, variations in strain parameters and the interactions between the signaling pathways (Houslay, 1991) have complicated elucidation of force-induced signals.

Evidence indicates that the adenylyl cyclase pathway is involved in signal transduction in mechanically stimulated bone cells (Davidovitch *et al.*, 1984). Although increased cAMP levels are often correlated with cellular proliferation and matrix synthesis as a result of mechanical strain (Coprav *et al.*, 1985), this does not appear to be the primary response in mechanical cellular stimulation (Jones and Schlüßbers, 1987; Jones *et al.*, 1991). Alternative pathways, other than the adenylyl cyclase pathway, have been recently considered as the immediate signal transduction response in mechanically strained osteoblasts (Brighton *et al.*, 1991; Jones and Schlüßbers, 1987; Jones *et al.*, 1991).

Jones and Schlüßbers (1987) first suggested that the phosphoinositide pathway is the prime effector mechanism to transmit strain stimulation to bone cells. This is a dual

signaling pathway in which a membrane enzyme, phospholipase C (PLC) hydrolyses membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP₂) to inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DG). IP₃ and DG mobilize Ca²⁺-dependent and/or kinase-dependent (PKC) systems in bone. IP₃ appears to increase within 20 seconds of the application of mechanical strain and together with PKC, peaks after about 2 minutes of stimulation (Jones *et al.*, 1991). This is similar to the activation of phospholipase A₂, which seems to respond directly to mechanical strain (Binderman *et al.*, 1988). These changes occur earlier than changes in messengers such as prostaglandin E₂ (PGE₂) which are only detected after 10 minutes of strain (Jones *et al.*, 1991), even though PGE₂ may be derived from DG formation or phospholipase A₂ activity (Nollert *et al.*, 1990). Therefore, increase in prostaglandin levels may not be related to initial cellular activating messenger systems but occur as a secondary response (Jones *et al.*, 1991; Binderman *et al.*, 1988). However, it is important to keep in mind that PGE₂ synthesis may well be from multiple sources, and that there may be a dependency in signaling pathways as a result of strain. Nevertheless, it is suggested that there are different levels of response, and that the first response may not be dependent on the cAMP pathway (Jones *et al.*, 1991; Binderman *et al.*, 1988).

Hormonal changes may also determine the degree to which the bone cell populations respond to strain (Rubin and Haussman, 1988). This response may be dependent to a greater extent on autocrine and paracrine hormones. However, endocrine hormones will also play an important role in the process of strain stimulation. In particular parathyroid hormone (PTH) which regulates bone-related Ca²⁺ metabolism may be mediated by both adenylyl cyclase and phospholipase C-dependent hydrolysis of phosphatidylinositol in osteoblast cells (Fujimori *et al.*, 1992). In addition, even though strain-induced alteration of the cell shape may cause significant cytoskeleton rearrangement (Pender and McCulloch, 1991), the association of transduction signals with the cellular cytoskeleton are poorly understood. The present study examines the effects of mechanical

strain on two different alveolar bone cell populations. Specifically the effects of intermittent mechanical strain on cellular levels of cAMP, PKC and IP₃ in the presence or absence of PTH are studied shortly after the initiation of strain, since the mechanism of signal transduction seems to occur very quickly (Jones and Bingman, 1991; Jones *et al.*, 1991; Brighton *et al.*, 1992). The cell strain levels which induce changes in cAMP, PKC and IP₃ are used to determine if the cell response to strain can be correlated with changes in the intermediate filament proteins vimentin and α -actinin and the focal contact protein vinculin.

MATERIALS AND METHODS

Cell culture technique

Bone cells were isolated according to Bellows *et al.* (1986) from the alveolar processes of maxillae and mandibles removed aseptically from Sprague-Dawley rats. The maxillae and mandibles were carefully extracted, cleaned, washed four times in MEM, and the alveolar processes minced and placed in tubes with an enzyme mixture comprised of: clostridial collagenase (3 mg/ml), elastase (6.25 U/ml), chondroitin sulfate (6 mg/ml) and D-sorbitol (18.22 mg/ml) in Krebs buffered solution (pH 7.2) with agitation in a water bath at 37°C. The minced pieces of tissue were incubated for different time periods to yield two groups of bone cell populations (Bellows *et al.*, 1986). The supernatant after the first 20 minute incubation was discarded to avoid contamination by fibroblasts. The tissue was treated for two digestions of 20 and one of 30 minutes. The supernatants from these incubations were pooled and called group I cells. Three subsequent digestions of 30 minutes each, were also pooled and called group II cells. After harvesting, the cell suspension was mixed with an equal volume of fetal calf serum (Gibco, ON) and centrifuged at 250 g for 8 minutes at room temperature to collect the cells. Osteoblastic cells of both groups I and II were characterized by alkaline phosphatase activity, cAMP and their ability to form bone nodules *in vitro* as evidenced by Von Kossa silver nitrate stain (not shown) (Yen *et al.*, 1993). Isolated cells were resuspended in standard minimum essential medium (α -MEM) supplemented with 15 % FBS (Gibco, Burlington, ON), 0.3 μ g/ml Fungizone (Flow Laboratories, McLean, VA), 100 μ g/ml Penicillin G, 10 mM β -glycerophosphate, 50 μ g/ml ascorbic acid and 10^{-10} M dexamethasone (all from Sigma, Chemical Co., St. Louis, MO). Cells were plated at a density of 2×10^4 cells/cm² in 35 mm dishes and maintained in a humidified atmosphere consisting of 95 % O₂ and 5 % CO₂ at 37°C.

Mechanical strain method

Primary cultures were trypsinized after confluency at 1 week after initial extraction, collected by centrifugation, washed and plated into collagen-coated Flex type I dishes and grown for 1 week to confluency prior to being mechanically strained. These plates were strained using the Flexercell Unit (Flexcell Corp., McKeesport, PA). This computer-controlled apparatus is capable of applying mechanical deformation to the cells (Sumpio and Banes, 1988; Anderson *et al.*, 1992; Carvalho *et al.*, 1993).

Mechanical strain was applied using a pressure of 20 kPa at 3 cycles per minute (0.05 Hz) with 10 seconds of strain followed by 10 seconds of relaxation. The force applied resulted in <1 % to 24 % of strain to the dish bottom (Gilbert *et al.*, 1991). However most of the area of the dish fell within <1 % of strain (or 10,000 μ strains) with a frequency of the stimulus of 0.05 Hz.

Experimental dishes were strained for periods of 0.5, 1, 5, 10 or 30 min as well as longer periods of 1, 3 or 7 days. Control cultures were cultivated for the same time periods using rigid-bottomed collagen-coated Flex type II dishes. In addition, controls were used to test for the effect of the movement of medium in the diffusion boundary between medium and the cell as suggested by Stoker (1973). To this end, selected dishes from both cell groups I and II were carefully agitated to account for this fluid movement, approximately with the same frequency of that of the Flexercell apparatus. These experiments were repeated for assays of PKC and cAMP with similar time periods (described below) to determine if isolated fluid movement had any effect on cell signaling systems.

Alkaline phosphatase activity and cell number counts

Bone forming cells were identified by the alkaline phosphatase reaction (McCulloch *et al.*, 1990). The staining medium used was 0.1 % Fast Blue salt, 0.3 % Naphthol AS-MS phosphate alkaline solution, Mayer's hematoxylin solution and Citrate concentrated solution (all from Sigma, St. Louis, MO). Cells attached to the bottom of the wells were

incubated with a mixture of Fast Blue salt and naphthol for 40 min at room temperature, washed with deionized water and incubated with hematoxylin solution for 10 minutes prior to washing thoroughly in running water for 1 h. Alkaline phosphatase-positive cells were photographed using a Zeiss Optic photomicroscope (Zeiss, Germany).

Cell counts were taken after periods of 1, 2, 3, and 7 days in culture. Strained and unstrained wells for both cell populations, were washed 3 times in PBS and incubated with trypsin (Gibco, Burlington, ON) for 5 minutes at 37°C. Cells were washed and transferred to a tube containing fetal calf serum (Gibco, Burlington, ON) and centrifuged at 250 g for 8 minutes. Supernatant was discarded and the pellet resuspended, washed in Hank's buffer and aliquots were counted electronically in a Coulter Counter (Model ZBI, Hialeah, USA).

PTH studies

Confluent cell cultures were pre-incubated with 10^{-9} M PTH (Sigma) in MEM in the presence of 10 mM 3-isobutyl-1-methylxanthine (IBMX). The concentration of PTH was optimized in previous experiments in our laboratory for cAMP production in bone cells (data not shown). This concentration was comparable to other studies (Buckley *et al.*, 1988). Cells were exposed to the conditions for 30 min prior to mechanical strain in MEM with no FCS. Controls consisted of identical cultures in MEM with no FCS and 10 mM IBMX without the application of mechanical strain. The remaining samples were removed at the times indicated above according to the strain periods and the reaction was stopped with a 10 % solution of trichloroacetic acid (TCA), 20 % perchloric acid (PCA) or PKC buffer (50 mM Tris/HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 0.3% w/v β -mercaptoethanol, 10 mM benzamidine and 50 μ g/ml phenylmethylsulphonyl fluoride). PTH was used both in biochemical experiments and in immunolocalization of cytoskeletal proteins, focal contact protein and PKC described below. In some experiments, in addition to PTH, cells were incubated in complete medium with 14 μ M indomethacin (Sigma) for 30 min prior to the application of mechanical strain. Control unstrained cultures were also treated with 14 μ M indomethacin.

Immunofluorescence labeling

Cells grown in collagen-coated Flexercell dishes were rinsed with PBS containing 0.1 M glycine (to quench intrinsic fluorescence due to fixation) and fixed by incubation with 4 % paraformaldehyde in PBS for 10 minutes. After several washings with PBS, cells were permeabilized by incubation with 0.3 % Triton X-100 with 1 % normal goat serum (NGS) in PBS for 1 hour at 22°C (room temperature). Cells were washed again and incubated overnight at 4°C with primary antibodies for vimentin, vinculin, α -actinin and PKC. These were diluted in PBS with 1% NGS and 0.3 % Triton X-100. Thereafter, samples were extensively washed in PBS and incubated with FITC rabbit anti-mouse IgG, rabbit anti-mouse IgM or TRITC conjugated goat anti-rabbit IgG secondary antibodies for 2 hours at 22°C. For vimentin and α -actinin the cell cultures were fixed with methanol/acetone (1:1) for 5 minutes and nonspecific binding was blocked with 1% BSA in PBS. After the last rinse in PBS, the silastic bottoms of the dishes were removed and mounted with 90 % glycerol-10 % PBS, supplemented with 0.1 % phenylenediamine (Sigma, St. Louis, MO) to prevent rapid quenching of the fluorescence. Positive and negative controls for all the antibodies were performed (not shown). Cultures were photographed using a Zeiss epi-fluorescence microscope (filters of 450-490 nm excitation). Due to the gradient pattern of strain bi-axially in the silastic bottoms (1 to 24 % of strain), in order to study the effects of controlled levels of strain, all the photomicrographs were taken from the most central area (<1% strain) of the bottoms. Monoclonal antibodies anti-vimentin, anti-vinculin, FITC and TRITC-conjugated antibodies were from Sigma (St. Louis, MO) and monoclonal anti-PKC 1.3- γ was from Gibco (Burlington, ON).

Biochemical assays for cAMP, PKC and IP₃

To determine cAMP, 10 % TCA was added to the samples and these were vortexed and incubated overnight at 4°C. Samples were centrifuged (3,000 g for 15 min) and the supernatants were extracted with water-saturated ether, the lower phase was collected and dried under air. Samples were resuspended with 0.05 mM sodium acetate buffer (pH 6.2),

serially diluted and assayed for cAMP by radioimmunoassay as described by Steiner (1970) using the kit from New England Nuclear (Boston, MA). This RIA kit has been extensively tested for % of cross-reactivity with very low levels of (<0.01 %) for cGMP, GMP, ATP, ADP, AMP and theophylline. The protein content of bone cell cultures was measured by the method of Lowry *et al.* (1954) and the cellular cAMP concentration was calculated in pm/mg of protein. To determine PKC activity, experimental and control cultures were incubated with PKC buffer and centrifuged at 3,000 g for 15 min at 4°C. Pellets were resuspended in PKC buffer and sonicated for 5 min. PKC was measured in serial dilutions of the samples using the kit (Amersham, Oakville, ON) in which the transfer of [³²P] from [³²P]γ-ATP to a synthetic peptide is measured. Suitable blanks and controls, including the omission of the L-α-phosphatidylserine component to account for the non-protein kinase C-dependent phosphorylation were prepared. Specificity has been previously determined as in % of PKC activity with no cross-reactivity with other kinases. PKC measurements are expressed in pm/mg of cell protein/min of incubation time. To assay for IP₃, the experimental and control cultures were exposed to 20 % perchloric acid and placed on ice for 20 min. Samples were centrifuged at 2,000 g for 15 min at 4°C and supernatants were titrated to pH 7.5 with 10 N KOH and kept on ice. Suitable blanks and controls were prepared and IP₃ was measured by a competitive binding assay (Amersham, Oakville, ON). The specificity for this assay shows 100 % cross-reactivity with D-myo-Inositol 1,4,5-trisphosphate IP₃ and very low amounts (<0.01 %) to other related compounds such as IP, IP₁ and IP₂; <1 % to IP₄, IP₅, IP₆ and <0.001 % to cyclic IP, ATP, GTP and Inositol. The results are expressed in pm/mg of bone cell protein.

Statistical analysis

The values obtained for all the strained and unstrained cell populations were subjected to a two-way analysis of variance with Tukey's multiple comparison test. T-tests with the Bonferroni correction were also used to compare the strained and unstrained cells in the presence or absence of PTH.

RESULTS

Alkaline phosphatase activity in mechanically strained alveolar bone cells

Alkaline phosphatase activity in cells isolated from the mandibular and maxillary alveolar bones is shown in Fig. 1. Application of mechanical strain induced a progressive increase in alkaline phosphatase staining on days 1, 3 and 7 when compared with untreated cell cultures. Cell numbers also increased significantly with the application of strain for each time period when compared with unstrained controls (Table 1). Cultures subjected to mechanical strain did not show any significant degree of cell death as evidenced by trypan blue exclusion (not shown).

cAMP, PKC and IP_3 response to mechanical strain and PTH

Figure 2 shows the cAMP levels in strained and unstrained cells in the presence or absence of PTH. No significant effect of strain was detected in either cell population in the absence of PTH (Fig. 2a). On the other hand when PTH was added, significantly higher levels of cAMP ($p < 0.05$) were present in both the group I and group II cells strained for 10 minutes compared with unstrained cells (Fig. 2b). cAMP levels in the presence of PTH in group II cells strained for 1 minute and in both cell groups strained for 5 and 10 minutes were significantly greater ($p < 0.05$) when compared to their counterparts that were not exposed to PTH (Figs. 2a and 2b).

Figure 3 shows the IP_3 levels in strained and unstrained cells with or without PTH. In the absence of PTH, IP_3 levels were increased significantly ($p < 0.05$) in both groups of cells strained for 1 minute when compared with unstrained cells. When PTH was added to the cultures there was a similar pattern of response for both groups of cells, with a significant increase in IP_3 levels ($p < 0.01$) at 1 minute of strain. In addition, both PTH-treated group I and group II cells showed significantly greater ($p < 0.05$) IP_3 levels than those not treated with PTH after 1 minute of strain.

PKC activity (Fig. 4) in strained cells was significantly greater ($p < 0.05$) compared to control levels in PTH-treated cultures after 5 minutes of strain, and reverted to control levels at later times studied (Fig. 4b). Furthermore, strain for 5 minutes in the presence of PTH produced a significant increase ($p < 0.05$) in PKC activity compared to samples not exposed to PTH. In addition, PKC activity in unstrained groups treated with PTH was increased three times ($p < 0.05$) from the PKC activity levels of the groups not exposed to PTH. The levels of IP_3 , cAMP and PKC activity were measured in both strained and unstrained cell groups that were with the absence of FCS.

The time periods which showed increased PKC activity and cAMP levels with mechanical strain were selected to test for the possible effects of perturbation in the diffusion boundary between medium and cells. The effects of medium movement tested by agitating the cultures showed no significant change in PKC activity and cAMP levels compared to control values. Therefore, the effects of diffusion boundary perturbation in this system were not significant. Values for agitated and control cultures as well as strained and unstrained cultures are shown in Table 2.

In order to rule out the possibility that the activation of signal transduction pathways by PTH in this model was eicosanoid driven, strained and unstrained cultures were incubated with PTH in the presence of $14 \mu M$ indomethacin which inhibits PGE_2 production. The same time periods used for diffusion boundary perturbation experiments were used to study indomethacin response. The values for cAMP levels and PKC synthesis in Table 3 show that addition of the inhibitor did not alter cAMP or PKC levels in the presence of PTH.

Cytoskeleton organization in mechanically strained alveolar bone cells

In this study we examined the cytoskeletal organization in two groups of alveolar bone cells. Both cell groups showed no changes in cytoskeletal labeling for the different antibodies after periods of 1 and 10 minutes of mechanical strain. Furthermore there were no obvious differences between the two cell populations studied. The earliest change in

cytoskeletal organization was noted at 30 minutes after initiation of strain as shown by vimentin and vinculin staining (Fig. 5). The distribution of vimentin staining changed considerably in cells subjected to long periods of strain (1 to 7 days), showing a change in the orientation of the cells relative to the axis of the mechanical strain (Fig. 5c and 5d). The intensity of the stain in strained cells (Figs. 5b, 5c and 5d) was greater compared to unstrained cells (Fig. 5a). It is also noteworthy that in cell cultures strained for longer periods, there was a phenotypic change from cuboidal osteoblast-like cells to elongated fibroblast-like cells. To determine if strain also altered focal contact proteins, antibodies against vinculin were used. No noticeable changes were observed in the localization or the intensity of staining for vinculin until 30 minutes of stimulation (Fig. 5f). A difference in staining intensity was also noted between unstrained and strained cultures together with a change in the orientation pattern of response (Fig. 5e and 5f respectively).

Finally staining of α -actinin appeared to co-localize with vinculin and vimentin. Although, α -actinin is thought to be localized between focal contacts and the cytoskeleton, in this study we have noticed that its distribution did not change noticeably in strained (Fig. 5g) compared with unstrained cells (Fig. 5h). However, the distribution of α -actinin in unstrained cells was more concentrated perinuclearly. When mechanical strain was applied, the stain for α -actinin appeared to distribute in the periphery of the cell.

PKC- γ localization in strained alveolar bone cells

There have been no previous reports on the localization of PKC in strained cells. Immunolabeling of alveolar bone cells showed that the distribution of PKC changed as strain was applied (Fig. 6). No differences were detected in PKC staining at 1 minute of strain. However, by 5 minutes of strain application there was a change in the staining pattern in which discrete condensations of PKC were observed instead of continuous filamentous patterns (Fig. 6b). This observation seemed to correspond to the biochemical measurement of PKC observed for the same cell populations. After 10 or 30 minutes of

strain, PKC labeling was reduced which resembled the PKC localization in unstrained control cultures (Fig. 6c and 6d).

DISCUSSION

To date much is known about cellular membrane signaling and how information is transmitted through a cascade of intracellular components, although there are considerable gaps in the understanding of specific responses. This is still the case in the cell response to mechanical strain. In this study it is reported that: (1) bone cells respond to mechanical strain by an increase in both cell proliferation and alkaline phosphatase activity in cultures of 1, 3 and 7 days, (2) IP_3 is activated very quickly after the application of strain, (3) PTH added to the cell cultures causes a significant increase in cAMP, IP_3 and PKC after different periods of strain and (4) the distribution and expression of certain cytoskeletal proteins appears to change with mechanical strain when compared with unstrained cultures.

The application of mechanical strain *in vitro* is known to increase cell response in terms of proliferation (Sömjem *et al.*, 1990; Brighton *et al.*, 1991; Brighton *et al.*, 1992), DNA synthesis (Brunette, 1984; Hasegawa *et al.*, 1985; Sandy, 1989; Sömjem *et al.*, 1980; Sandy, 1993), collagen synthesis (Meikle *et al.*, 1982; Kollros *et al.*, 1987), cytoskeleton organization (Pender and McCulloch, 1991), cAMP levels (Davidovitch *et al.*, 1977; Davidovitch *et al.*, 1984) and more recently IP_3 levels and PKC activity (Bourne *et al.*, 1989; Brighton *et al.*, 1991). It can be assumed that all these factors are related in the cellular response to strain. However, this paper focused on effects of strain on signal transduction pathways in the presence or absence of stimulation by PTH.

The increase in alkaline phosphatase activity seen here has been used to show the ability of the system to form bone-like tissue (Fig. 1). Similarly, cell number increase has been observed in the cultures of 1, 3 and 7 days (Table 1), although this did not occur at the short time periods of 0.5, 1, 5, 10, and 30 minutes (not shown). This increase in staining for alkaline phosphatase suggests a stimulation of mineralized matrix formation. The effects of mechanical strain on signal transduction in relation to cell proliferation have not been studied here; however, it has been reported that cell proliferation as a result of strain is probably mediated by the phosphatidylinositol pathway through phospholipase C

and PKC activity (Sömjem *et al.*, 1990). This agrees with reports showing that the blocking of this pathway causes an inhibition of cell proliferation (Brighton *et al.*, 1992). Yamaguchi *et al.* (1988) have also shown that the cAMP signaling system is antiproliferative with respect to osteoblast function. Although, addition of PTH caused stimulation of DNA synthesis mediated by cAMP in the chick epiphyseal growth plate chondroprogenitor cells (Sömjem *et al.*, 1989), this was the only cell type reported to have PTH-dependent cAMP proliferative activity. On the other hand, non PTH-dependent cAMP may act as a mitogen in epidermal cells, epithelial cells and keratinocytes, but not in bone cells (Pines and Hurwitz, 1988). According to Sömjem *et al.* (1990) the latter provides evidence for a mechanism of PTH activation of both phosphatidylinositol and adenylyl cyclase signaling pathways. These diverse results may represent not only those of different cell types but also those of using different concentration of PTH in the cultures.

The inositol phosphate pathway has only recently been correlated with mechanical strain in bone cells *in vitro*. It has been reported that application of mechanical strain caused a rapid increase in this pathway almost immediately after the application of strain (Jones and Schlüßers, 1987; Brighton *et al.*, 1992). In fact cell response through an increase in IP₃ levels may be initiated in seconds (Brighton *et al.*, 1991). However, since phosphatidylinositol-4,5 bisphosphate hydrolysis depends on the activation of the membrane bound enzyme phospholipase C, the peak in IP₃ levels may be in the range of the one minute of cellular stimulation (unpublished results). Such response is also evidenced by the specific activity of PKC, itself a result of phospholipase C activation (Bourne *et al.*, 1989). In this study, IP₃ levels were significantly greater one minute after initiation of strain compared with later periods of 10 and 30 minutes (Figs. 3a and 3b), whereas PKC did not reach a peak before 5 minutes of strain, decreasing after that (Figs. 4a and 4b). It is important to mention that although the two arms of the phosphatidylinositol pathway have independent functions, IP₃ and PKC may act

synergistically to heighten cellular response, as has been shown in the induction of serotonin secretion in platelets (Morgan, 1989).

It has been shown that changes in cell response may be also caused by an increase in fluid movement perturbing the local concentration gradient and ultimately altering the growth of the cells (Stoker, 1973; Stoker and Piggot, 1974). In this study, fluid perturbation of the diffusion boundary layer to which the surface of the cell is subjected did not seem to cause the same effects observed by mechanical strain, for either cell counts, cAMP levels or PKC activity (Table 2). Although, any increase in medium velocity may reduce the diffusion boundary layer, therefore limiting the uptake of critical nutrients or growth factors from the medium (Stoker, 1973), the osteoblastic cells studied here appeared to adapt to this effect and still respond to mechanical strain. However, the changes in the microenvironment of cell boundary need more careful attention in further studies.

Strain induced stimulation of adenylyl cyclase has also been shown to occur in osteoblast cells, although in this study the level of cAMP only reached its peak at 10 minutes of strain. Jones and Schlüßbers (1987) found a similar response, which contradicts earlier studies that focused on cAMP as the primary signal in strained cells (Davidovitch *et al.*, 1977; Davidovitch *et al.*, 1984; Copray *et al.*, 1985). This relatively late response of the increase in cAMP levels to mechanical stimulation, suggests that cAMP is probably one of several secondary cell responses to strain, which may include ion changes across the membrane and possibly the involvement of the cytoskeleton (Sandy and Farndale, 1991). For instance, Ca²⁺ changes across the membrane are found within seconds of mechanical stimulation, however such changes may not constitute a separate pathway of response (unpublished results).

Additional drugs for the stimulation of the pathways reported (i.e. TPA for PKC and forskolin for cAMP) were not used in this study. It has been shown that the activation of cAMP with forskolin did not seem to affect the phosphatidylinositol pathway (Farndale

et al., 1988). Yet the pathways of adenylyl cyclase and phosphatidylinositol both responded to mechanical strain and to PTH. PTH has been shown to stimulate cell proliferation (Whitfield *et al.*, 1979), DNA synthesis in osteoblast cells in culture (Van der Plas *et al.*, 1985) and bone apposition rate when combined with Vitamin D (Slovik *et al.*, 1986; Sömjem *et al.*, 1990). Depending on its application PTH may also cause bone resorption in conjunction with its regulation of Ca^{2+} homeostasis (Sömjem *et al.*, 1990), and in high concentrations may inhibit cell proliferation (Farndale *et al.*, 1988). More recently, studies on signal transduction mechanisms and PTH action have been undertaken (Farndale *et al.*, 1988; Sömjem *et al.*, 1990; Fujimori *et al.*, 1992). However, to date there are no reports on the effects of mechanical strain together with PTH in signaling mechanisms. The effects of PTH in osteoblast cells in culture have been previously shown to cause an increase in inositol phosphates and cAMP (Farndale *et al.*, 1988). In this study we have demonstrated that PTH is capable of potentiating the activity of the cells as evidenced by an increase in the levels of cAMP (Figs. 2a and 2b), IP_3 (Figs. 3a and 3b) and in the activity of PKC (Figs. 4a and 4b) when strain is applied. Moreover, we have shown that these effects are not due to the eicosanoid production stimulated by mechanical strain (Table 3).

Farndale *et al.* (1988) showed that the addition of PGE_2 gave a similar response to PTH in both inositol and adenylyl cyclase pathways studied. In addition, the elevation of cAMP levels was dependent on the dosage used. However, the dosages of PTH that caused maximum stimulation of cAMP did not coincide with those that caused maximum response of IP_3 , even though the same dosages of PGE_2 produced maximal responses for both cAMP and IP_3 . It is interesting to note that the dosages did not coincide for PTH, since osteoblast cells have been shown to carry PTH receptors directly bound with the G-proteins of both cAMP and IP_3 pathways (Fujimori *et al.*, 1992). In addition, PGE_2 may itself be a derived product of the inositol pathway as a metabolite of arachidonic acid stimulation (Binderman, 1984; Brighton *et al.*, 1992). This dependency may constitute a

cross-linking of both pathways for PGE₂ stimulation as opposed to that of PTH. In this study, we have noticed that blocking of PGE₂ stimulation with indomethacin did not alter significantly the response of PTH with mechanical strain, suggesting that the effect observed for PTH stimulation was not eicosanoid driven (Table 3). This agrees with results previously reported, in which the activities of PTH and PGE₂ are comparable but not wholly coincident (Boland *et al.*, 1986). Ca²⁺ elevation has been shown to increase as a result of PTH but not PGE₂ stimulation (Boland *et al.*, 1986), while PTH stimulation may have been dependent on extracellular Ca²⁺ (Farndale *et al.*, 1988). In addition, strain is thought to stimulate PGE₂ production and to raise intracellular messenger levels (Steiner, 1970; Reich and Frangos, 1991), in accordance with our experiments with indomethacin, which showed a reduction of PKC activity and cAMP levels when no PTH was added (not shown). Further studies using PGE₂ stimulation in addition to mechanical strain to investigate the interaction of PTH and PGE₂ are warranted.

Changes in dosage of PTH may be comparable to changes in the magnitude of strain applied. To our knowledge, there are few studies that attempted to establish physiological values of strain applied to the cytoskeleton. *In vivo* studies were mostly done through the placement of strain gauges directly to the bones of animals to measure local strain. Brighton *et al.* (1991) reviewed some of these studies which attributed *in vivo* values of strain for the lowest strain range at 400 μ strains (O'Connor *et al.*, 1982) and the highest strain around 3,000 μ strains (Rubin, 1984; Rubin and Lanyon, 1985). On the other hand, only two studies attempted to investigate similar strain ranges to bone cells *in vitro*, with values ranging from 200 to 1,000 μ strains (Brighton *et al.*, 1991) and from 300 to 10,000 μ strains (Jones and Schlüßbers, 1987). In this study only the higher levels of strain were used, such as 10,000 μ strains. This was estimated from the increase of the cell circumference when compared before and after the strain application (Anderson *et al.*, 1992). In the system used here, for the mechanical bi-axial strain applied in the range of < 1 % strain, the amount of cell elongation was reported to be increased by approximately 3

% compared to non-strained controls (Anderson *et al.*, 1992). Although the levels of strain reported here are much higher than others published previously, the responses of signal transduction are fairly comparable, and the authors believe that such higher strain values are more indicative of force systems developed during tooth movement. Admittedly, one can not directly determine which strain may be experienced by bone cells either at the periosteal or the periodontal surface or within the calcified matrix, although attempts have been made using finite element models for periodontal ligament (Tanne *et al.*, 1988) and craniofacial skeleton (Tanne *et al.*, 1989). In the craniofacial study the maximum bone displacement was 3 μm . If one assumes an average of 10-15 μm for bone diameters (Marotti *et al.*, 1976) this would create about 10-15 % strain which is well beyond the elongation of bone cells in the system used in this study (Anderson *et al.*, 1992). In addition, the use of cyclical or intermittent loading is appropriate in this model when one considers that dentoalveolar bone is subjected to intermittent loading during mastication, swallowing and parafunction, all of which over extended periods (Ecarot-Charrier *et al.*, 1983). Orthodontic force is then superimposed upon this force system. Because orthodontic appliances have high load deflection ratios, high forces are created upon activation with rapid decay. This is then repeated with each activation (weakly to monthly) thus creating "intermittent" effect.

Until recently only fetal tissue derived cells had been used to test osteogenic differentiation *in vitro* (Ecarot-Charrier *et al.*, 1983; Bellows *et al.*, 1986). However, more differentiated tissue has been demonstrated to have the same potential as that of fetal tissue *in vitro* with the advantage of allowing the study of factors such as the effect of age of the donor on the expression of osteogenesis (Kasugai *et al.*, 1991).

Both osteoblastic cell populations in this study responded to mechanical strain with similar responses, and although the later digestions which were called group II cells seemed to have a higher response than that of the group I cells, these differences were not significant. These data disagree with previously published results in "periosteal" and

"haversian" cell populations isolated from long bones of cows (Jones *et al.*, 1991). According to Jones *et al.* (1991), the stimulation necessary to produce a response in the periosteal-type cells was minimal as evidenced by the changes in IP₃ when compared to haversian-type cells. These contradictions with our study may be due to differences in bone cell populations despite the use of similar classifications. The cell populations of Jones *et al.* (1991) were grown out from explants dissected from periosteal and haversian zones, while group I and group II cell populations in this study were digested sequentially and tested after one subculture.

No changes in cytoskeletal morphology of the cells studied here was noted for the strain levels of 0.5, 1, 5 and 10 minutes as evidenced by vinculin, vimentin and α -actinin staining, but some change in the alignment of the cytoskeleton seemed to be present at periods of 30 minutes and 1, 3 and 7 days (Fig. 5). The latter was in agreement with earlier studies which showed osteoblastic alignment perpendicular to the direction of strain (Buckley *et al.*, 1988). The preliminary information on the cytoskeleton rearrangement reported here due to mechanical strain has shown that subtle changes in the cellular environment are not sufficient for an immediate response in cellular architecture. However, changes in PKC staining were evident by 5 minutes of strain (Fig. 6b), which decreased in later periods of stimulation when compared with unstrained cells (Fig. 6a). This agrees with the biochemical data showing an increase in PKC activity as early as 5 minutes (Fig. 4).

PKC (Beckerle, 1990; Woods and Couchman, 1992) and other signal transduction derived messengers such as cAMP dependent kinases (Turner *et al.*, 1989; Woods and Couchman, 1992) are known to phosphorylate integral cell membrane and cytoskeletal proteins. The activation of PKC determines the formation of the focal adhesion (Burrige, 1986; Burrige *et al.*, 1988) changes in the aggregation of the cytoskeleton (Burrige and Connel, 1983; Burrige *et al.*, 1988) and membrane binding specificity to different substrates (Derventzi *et al.*, 1992). However, it has been shown that inactivation of PKC-

mediated phosphorylation caused an inhibition of focal contact organization, which did not occur when cAMP kinase phosphorylation was inhibited (Woods and Couchman, 1992). In this study we have shown that the localization of focal contact protein vinculin changed with periods of strain of 30 minutes (Fig. 5f) and 1 day (not shown), however, this occurred at a later time than those of PKC. It is conceivable that strain enhances the translocation of PKC to focal adhesion sites, although this direct effect was not measured. The exact role of PKC in the formation of focal adhesions is not yet known, however, it is thought that PKC may serve to stabilize the initial associations of the cytoskeleton with the focal contacts and with the membrane receptor proteins (Woods and Couchman, 1992).

The manner in which the mechanical strain applied here is transmitted to the osteoblast cells is open to question. Although in this study there were no significant differences between cell groups, the location of different osteogenic populations may determine the presence of the so-called "strain-sensitive cells". These cells would yield strain-induced, or related potentials to be measured as physiological signals (Basset *et al.*, 1964; Guharey and Sachs, 1984). Additional study is necessary to identify the cells which could ultimately play the primary roles in skeletal remodeling in response to strain.

Membrane or conformational changes in specialized membrane receptors linked to stimulatory G-proteins (Gs) may also characterize an integral part of this pathway (Brighton and McCluskey, 1988; Rubin and Hausman, 1988). Such is the case of the adenylyl cyclase, an enzyme stimulated by heterotrimeric G proteins to control the signal from the receptor to the effector (Taylor, 1990). There are currently two models for the kinetics of receptor-effector binding. These are the "precoupled" and the "collision coupling" models (Levitzki and Bar-Sinai, 1991). While the former predicts a proportional reduction of the extent but not the rate of activation, the latter predicts a reduction of the rate but not the extent of activation (Levitzki and Bar-Sinai, 1991). Previous studies have shown that regulation of adenylyl cyclase follows a monoexponential curve in which the catalytic subunit of the Gs protein can be regarded as a single effector unit (Gross and

Lohse, 1991). While catalytic activation by Gs follows the collision coupling, the precoupled model assumes a fixed coupling of effectors by receptors (Gross and Lohse, 1991). However, the interaction between receptor and effectors depends on their access, since mobility of receptor in the membrane is slow. This may contribute to a limitation of the catalytic effect of Gs. On the other hand, the application of mechanical strain in the presence of PTH in the cells studied as shown by the changes in cytoskeleton distribution, may increase the receptor diffusion process. Although, there may also be an increase in ligand-receptor occupancy with mechanical strain, this can not be accounted for in this study.

Signal transduction by structural deformation of the membrane components may be partly explained by the presence of the so-called membrane mechanotransducers (Guharey and Sachs, 1984; Sachs, 1988). Ion membrane channels may in fact behave as strain-sensitive channels (Guharey and Sachs, 1984; Lansman *et al.*, 1987; Jones *et al.*, 1991) and it has been suggested that in long periods of activity the only possible source of energy to open the channels is the stretch derived from the membrane strain (Guharey and Sachs, 1984; Sachs, 1988). However, these channels have been recently suggested as artifacts resulting from the patch-clamping technique (Jones *et al.*, 1991; Morris and Horn, 1991).

Although the mechanisms for recognition of mechanical stimulation by osteoblasts are not known, it has been shown that the short term changes in cAMP, PKC and IP₃ levels can play a role in cellular response. In addition, the monitoring of specific membrane-bound protein changes may be paramount for the elucidation of the missing link between stimulation and response. Therefore, further studies on the activity of the proteins that participate in this process and in the structure of the cellular cytoskeleton are indicated to better characterize the relationship between mechanical strain and cell response.

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REFERENCES

- Anderson J, Carvalho RS, Yen EHK and Scott JE 1992 Measurement of strain in cultured bone and fetal muscle and lung cells. *In Vitro Cell Dev Biol* **29**:183A-186A.
- Basset CAL, Pawluk RJ and Becker RO 1964 Effects of electric current on bone *in vivo*. *Nature* **204**:652-654.
- Beckerle MC 1990 The adhesion protein, talin, is phosphorylated *in vivo* in chicken embryo fibroblasts exposed to a tumor promoting phorbol ester. *Cell Reg* **1**:227-236.
- Bellows CG, Aubin JE, Heersche HNM and Antosz ME 1986 Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria cell populations. *Calcif Tissue Int* **38**:143-154.
- Binderman I 1984 Biochemical pathways involved in the translation of physical stimulus into biological message. *Calcif Tissue Int* **36**:82S-85S.
- Binderman I, Zor U, Kaye A, Shimshoni Z, Harell A and Sömjem D 1988 The transduction of mechanical force into biochemical events in bone cells may involve activation of phospholipase A₂. *Calcif Tissue Int* **42**:261-266.
- Boland CJ, Fried RM and Tashjian AH 1986 Measurement of free cytosolic Ca²⁺ concentrations in human and rat osteosarcoma cells: actions of bone resorption-stimulating hormones. *Endocrinology* **118**:980-989.
- Bourne H, Cohen P, Hardie G, Koshland D, Levitzki A, Mishel R, Mudge A and Schramm M, Sellinger Z and Vanaman T 1989 Cell Signaling. In: Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD, eds. *Molecular Biology of the Cell*, 2nd ed., Garland Publishing Inc., New York, pp. 681-726.
- Brighton CT and McCluskey WP 1988 Response of cultured bone cells to a capacitively coupled electric field: Inhibition of cAMP response to parathyroid hormone. *J Orthop Res* **6**:567-571.

- Brighton CT, Stratford B, Gross SB, Leatherwood DF, Williams JL and Pollack SR
1991 The proliferative and synthetic response of isolated calvarial bone cells of rats to cyclic biaxial mechanical strain. *J Bone Jt Surg* **73**:320-331.
- Brighton CT, Sennet BJ, Farmer JC, Iannotti JP, Hansen CA, Williams JL and Williamson J 1992 The inositol phosphate pathway as a mediator in the proliferative response of rat calvarial bone cells to cyclical biaxial mechanical strain. *J Orthop Res* **10**:385-393.
- Brunette DM 1984 Mechanical stretching increases the number of epithelial cells synthesizing DNA in culture. *J Cell Sci* **69**:35-45.
- Buckley MJ, Banes AJ, Levin LG, Sumpio BE, Sato M, Jordan R, Gilbert J, Link GW and Tay RTS 1988 Osteoblasts increase their rate of division and align in response to cyclic, mechanical tension *in vitro*. *Bone Min* **4**:225-236.
- Burridge K 1986 Substrate adhesions in normal and transformed fibroblasts: organization and regulation of cytoskeleton, membrane and extracellular matrix components at focal contacts. *Canc Rev* **4**:18-78.
- Burridge K and Connel L 1983 A new protein of adhesion plaques and ruffling membranes. *J Cell Biol* **97**:359-367.
- Burridge K, Fath K, Kelly T, Nuckolls G and Turner C 1988 Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol* **4**:487-525.
- Carvalho RS, Yen EHK and Suga DM 1993 Glycosaminoglycan synthesis due to growth and age in the rat articular disc. *Arch Oral Biol* **38**:457-466.
- Copray JCVM, Jansen HWB and Duterloo HS 1985 Effects of compressive forces on proliferation and matrix synthesis in mandibular condyle cartilage of the rat *in vitro*. *Arch Oral Biol* **30**:299-317.

- Davidovitch Z, Montgomery E and Shanfeld J 1977 Guanosine 3,5'-monophosphate in bone: microscopic visualization by an immuno-histochemical technique. *Calcif Tissue Res* **24**:73-79.
- Davidovitch Z, Shanfeld J, Montgomery E, Lally E, Laster L, Furst L and Korostoff E 1984 Biochemical mediators of the effect of mechanical forces and electric currents in mineralized tissues. *Calcif Tissue Int* **36**:86S-97S.
- Derventzi A, Rattan RIS and Clark BFC 1992 Phorbol ester-induced reorganization of the cytoskeleton in human fibroblasts during ageing *in vitro*. *Biochem Biophys Res Comm* **182**:1423-1428.
- Ecarot-Charrier B, Glorieux FH, van der Rest M and Pereira G 1983 Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. *J Cell Biol* **96**:639-643.
- Farndale RJ, Sandy JR, Atkinson SJ, Pennington SR, Meghji S and Meikle MC 1988 Parathyroid hormone and prostaglandin E₂ stimulate both inositol phosphates and cyclic AMP accumulation in mouse osteoblasts cultures. *Biochem J* **79**:230-239.
- Fujimori A, Cheng S, Avioli LV and Civitelli R 1992 Structure-function relationship of parathyroid hormone: activation of phospholipase-C, protein kinase-A and -C in osteosarcoma cells. *Endocrinology* **130**:29-36.
- Gilbert JA, Banes AJ, Link GW and Jones GL 1991 Video analysis of membrane strain: an application in cell stretching. *Exp Tech* **15**:43-45.
- Gross W and Lohse MJ 1991 Mechanism of activation of A₂ adenosine receptors. II. A restricted collision-coupling model of receptor-effector interaction. *Mol Pharmacol* **39**:524-530.
- Guharey F and Sachs F 1984 Stretch activated K⁺ channels in muscle cell membranes as mechanotransducers? *J Physiol (Lond)* **352**:685-701.

- Hasegawa S, Sato S, Saito S, Suzuki Y and Brunette DM 1985 Mechanical stretching increases the number of cultured bone cells synthesizing DNA alters their pattern of protein synthesis. *Calcif Tiss Int* **37**:431-436.
- Houslay MD 1991 'Crosstalk': a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. *Eur J Biochem* **195**:9-27.
- Jones DB and Bingman D 1991 How do osteoblasts respond to mechanical stimulation? *Cells Mat* **4**:1-12.
- Jones DB, Nolte H, Schlüßbers JG, Turner E and Veltel D 1991 Biochemical signal transduction of mechanical strain in osteoblast-like cells. *Biomaterials* **12**:101-110.
- Jones DB and Schlüßbers JG 1987 Evidence that phospholipase C mediates the mechanical strain response in bone. *Calcif Tissue Int* **41**:4-10.
- Kasugai S, Todescan RJr, Nagata T, Yao KL, Butler WT and Sodek J 1991 Expression of bone matrix proteins associated with mineralized tissue formation by adult bone marrow cells *in vitro*: inductive effects of dexamethazone on osteoblastic phenotype. *J Cell Physiol* **147**:111-120.
- Kollros PR, Bates SR, Matthews MB, Horwitz AL and Glagov S 1987 Cyclic AMP inhibits increased collagen production by cyclically stretched smooth muscle cells. *Lab Invest* **56**:410-417.
- Lansman JB, Hallam J and Rink TJ 1987 Single stretch activated ion channels in vascular endothelial cells as mechanotransducers? *Nature* **325**:811-813.
- Lanyon LE 1984 Functional strain as a determinant of bone remodeling. *Calcif Tissue Int* **36**:56S-61S.
- Lanyon LE 1987 Functional strain in bone tissue as an objective, and controlling stimulus for adaptive bone remodeling. *J Biomech* **20**:1083-1093.
- Levitzki A and Bar-Sinai A 1991 The regulation of adenylyl cyclase by receptor-operated G proteins. *Pharmacol Ther* **50**:271-283.

- Lowry OH, Roberts NR, Wu ML, Hixon WS and Crawford EJ 1954 The quantitative chemistry of the brain. II. Enzyme measurement. *J Biol Chem* **207**:19-27.
- Marotti G, Zallone AZ and Ledda M 1976 Number, size and arrangement of osteoblasts in osteons at different stages of formation. *Calcif Tiss Res* **21**:96S-101S.
- McCulloch CAG, Fair CA, Tenebaum HC, Limeback H and Homareau R 1990 Clonal distribution of osteoprogenitor cells in cultured chick periosteal: functional relationship to bone formation. *Dev Biol* **140**:352-361.
- McLeod KJ, Lee RC and Ehrlich HP 1987 Frequency dependence of electric field modulation of fibroblast protein synthesis. *Science* **236**:1465-1469.
- Meikle MC, Heath JK, Hembry RM and Reynolds JJ 1982 Rabbit cranial sutures fibroblasts under tension express a different collagen phenotype. *Arch Oral Biol* **27**:609-613.
- Morgan N 1989 Guanine-nucleotide binding proteins as signal transduction. In: Morgan N, ed. *Cell Signaling*. Guilford Press, London, pp.35-57.
- Morris CE and Horn R 1991 Failure to elicit neuronal macroscopic mechanosensitive currents anticipated by single-channel studies. *Science* **251**:1246-1249.
- Nollert MU, Eskin SE and McIntire LV 1990 Shear stresses increase inositol triphosphate levels in human endothelial cells. *Biochem Res Commun* **170**:281-287.
- O'Connor JA, Lanyon LE and McFie H 1982 The influence of strain rate on adaptive bone remodeling. *J Biochem* **15**:767-781.
- Pender N and McCulloch AG 1991 Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching. *J Cell Sci* **100**:187-193.
- Pines M and Hurwitz S 1988 The effect of parathyroid hormone and atrial natriuretic peptide on cyclic nucleotides production and proliferation of avian epiphyseal growth plate chondroprogenitor cells. *Endocrinology* **123**:360-365.
- Reich KM and Frangos JA 1991 Effect of flow on prostaglandin E₂ and inositol trisphosphate levels in osteoblasts. *Am J Physiol* **26**:428C-432C.

- Rubin CT 1984 Skeletal strain and the functional significance of bone architecture. *Calcif Tissue Int* **36**:11S-18S.
- Rubin CT and Hausman MR 1988 The cellular basis of Wölff's law. Transduction of physical stimuli to skeletal adaptation. *Rheum Dis Clin North Am* **14**:503-517.
- Rubin CT and Lanyon LE 1985 Regulation of bone mass by mechanical strain magnitude. *Calcif Tissue Int* **37**:411-417.
- Rubin CT, McLeod KJ and Lanyon LE 1989 Prevention of osteoporosis by pulse electromagnetic fields. *J Bone Jt Surg* **71**:411-417.
- Sachs F 1988 Mechanical transduction in biological systems. *Crit Rev Biomed Eng* **16**:141-169.
- Sandy J 1989 Dual elevation of cyclic AMP and inositol phosphate in response to mechanical deformation of murine osteoblasts. *Biochem Biophys Acta* **1010**:265-269.
- Sandy J 1993 DNA changes in mechanically deformed osteoblasts: a new hypothesis. *Br J Ortho* **20**:1-11.
- Sandy J and Farndale W 1991 Second messengers: regulators of mechanically-induced tissue remodeling. *Eur J Orthod* **13**:271-278.
- Slovik DM, Rosenthal DI, Doppelt SH, Potts JT, Daly AM, Campbell JA and Neer AM 1986 Restoration of spinal bone in osteoporotic men by treatment with human parathyroid hormone (1-34) and 1,25-dihydroxyvitamin D. *J Bone Min Res* **1**:377-382.
- Sömjem D, Binderman I, Berger E and Harell A 1980 Bone remodeling induced by physical strain is prostaglandin E₂ mediated. *Biochim Biophys Acta* **627**:931-934.
- Sömjem D, Binderman I, Schluter KD, Wingender E, Mayer H and Kaye AM 1990 Stimulation by defined parathyroid hormone fragments of cell proliferation in skeletal-derived cell cultures. *Biochem J* **272**:781-785.

- Steiner 1970 The measurement of cyclic nucleotides by radioimmunoassay. *Adv Biochem Psychopharmacol* **3**:89-111.
- Stoker MGP 1973 Role of diffusion boundary layer in contact inhibition of growth. *Nature* **246**:200-203.
- Stoker MGP and Piggot D 1974 Shaking 3T3 cells: further studies on diffusion boundary effects. *Cell* **3**:207-215.
- Sumpio BE and Banes AJ 1988 Prostacycline synthetic activity in cultured aortic endothelial cells undergoing cyclic mechanical deformation. *Surgery* **104**:383-389.
- Tanne K, Hiraga J, Kakiuchi K, Yamagata Y and Sakuda M 1989 Biomechanical effect of anteriorly directed extraoral forces on the craniofacial complex: A study using finite element method. *Am J Ortho Dentofac Orthop* **95**:200-207.
- Tanne K, Koenig HA and Burstone CJ 1988 Moment of force ratios and the center of rotation. *Am J Ortho Dentofac Orthop* **94**:426-431.
- Taylor CW 1990 The role of G proteins in transmembrane signaling. *Biochem J* **272**:1-13.
- Thomas NR and Peyton SC 1983 An electromyographic study of mastication in the freely-moving rat. *Arch Oral Biol* **28**:939-945.
- Turner CE, Pavalko FM and Burrige K 1989 The role of phosphorylation and limited proteolytic cleavage of talin and vinculin in the disruption of focal adhesion integrity. *J Biol Chem* **264**:938-944.
- Van der Plas A, Feyen JHM and Nijweide PJ 1985 Direct effect of parathyroid hormone on the proliferation of osteoblast-like cells; a possible involvement of cyclic AMP. *Biochem Biophys Res Commun* **129**:918-925.
- Watson PA 1991 Function follows form: generation of intracellular signals by cell deformation. *FASEB J* **5**:2013-2019.
- Whitfield JF, Boynton AL, MacManus P, Skorska M and Tsang BK 1979 The regulation of cell proliferation by calcium and cyclic AMP. *Mol Cell Biochem* **27**:155-179.

Woods A and Couchman JR 1992 Protein kinase C involvement in focal adhesion formation. *J Cell Sci* **101**:277-290.

Yamaguchi DT, Hahn TJ, Beeker TG, Kleeman CR and Muallen P 1988 Relationship of cAMP and calcium messenger systems in prostaglandin-stimulated UMR-106 cells. *J Biol Chem* **263**:10745-10753.

Yen EHK, Carvalho RS and Suga DM 1992 Intermittent strain levels and alveolar bone formation *in vitro*. *J Dent Res* **71**:613.

Figure 1. Photomicrographs of group II alveolar bone cells showing alkaline phosphatase positive staining. (a) Control unstrained cells show lower levels of alkaline phosphatase activity when compared to strained cells of 1 day (b), 3 days (c) and 7 days (d). There were no apparent differences between group I and group II cells for alkaline phosphatase activity. Cells were strained for 20 Kpa at 3 cycles/min. Arrow indicates the direction of strain. Magnifications (a-d) X 80.

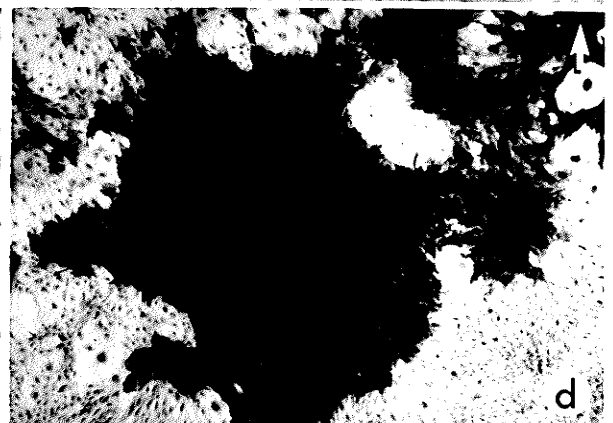
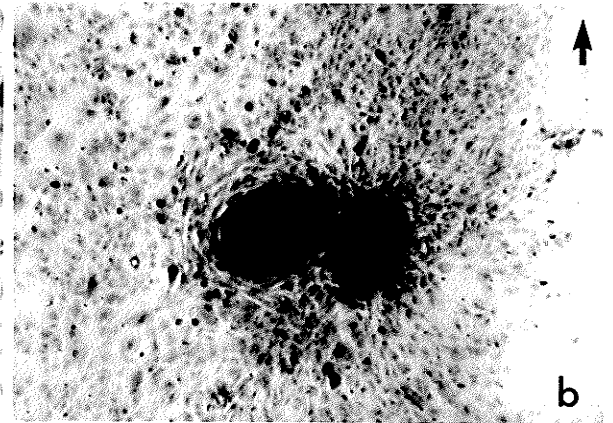
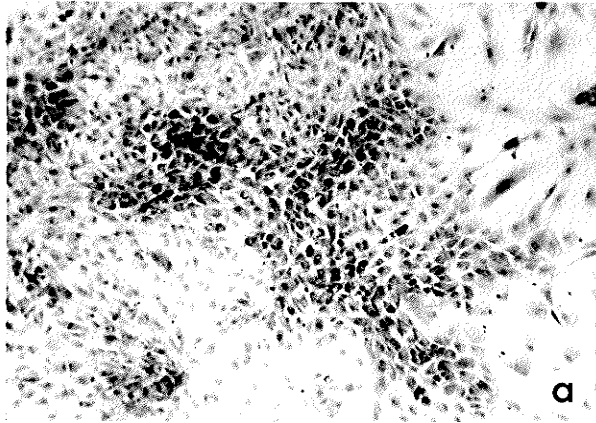
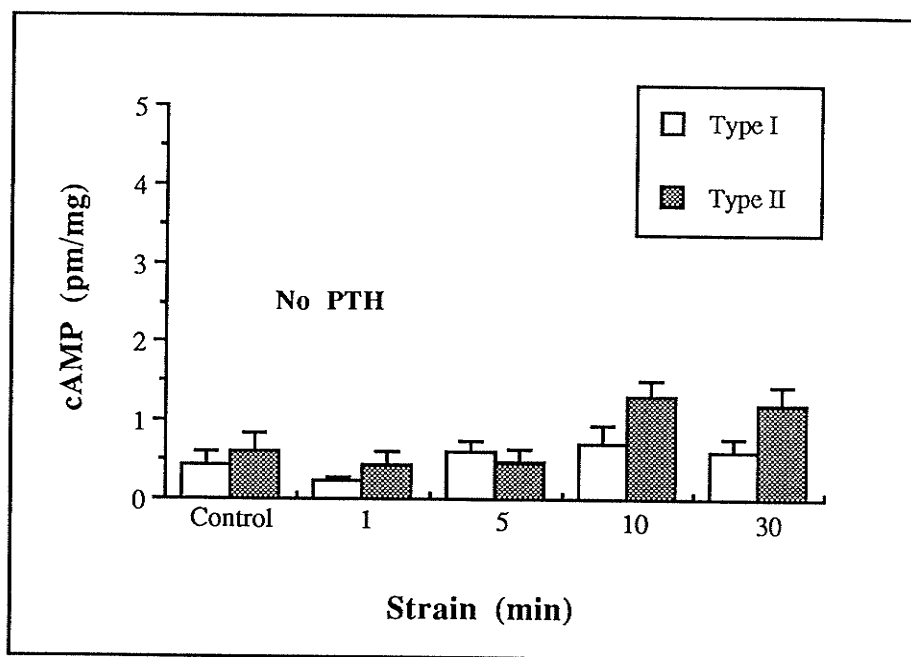
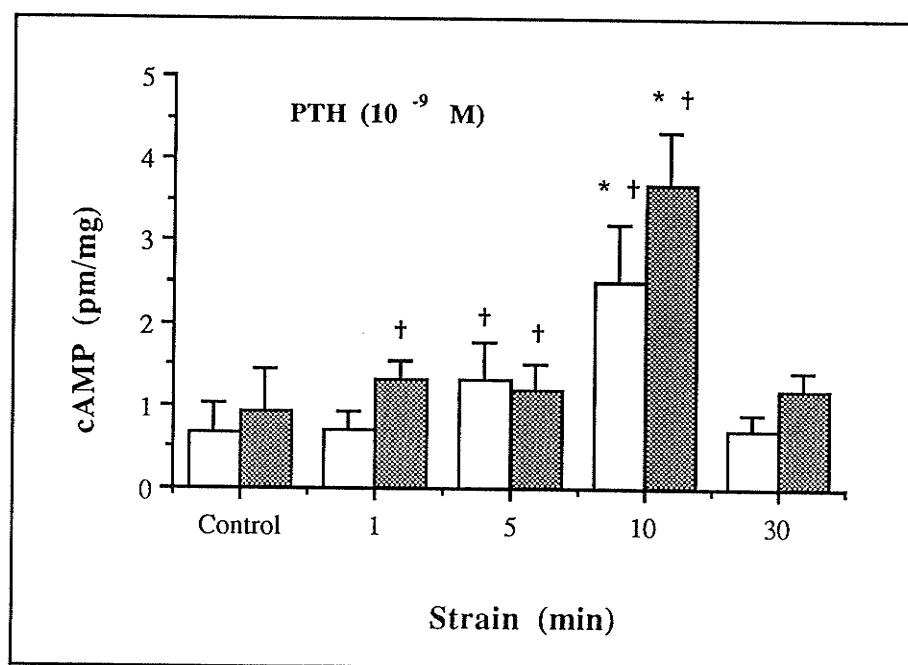


Figure 2. Effects of mechanical strain and parathyroid hormone on the cAMP levels in rat alveolar bone cells. Osteoblast cells attached to Flexercell dishes were grown to confluency for one week and strained (20 Kpa at 3 cycles/min) for time periods of 1, 5, 10 or 30 minutes. cAMP values were measured by RIA in cells strained in the absence (a) or presence of PTH (10^{-9} M) (b). IBMX (10 mM) was also added to the cultures. Values are means \pm standard deviation from at least 6 samples. * indicates significant difference ($p < 0.05$) between cells of different groups but same PTH treatment. † indicates significant difference ($p < 0.05$) between cells of same group but treated differently with PTH.

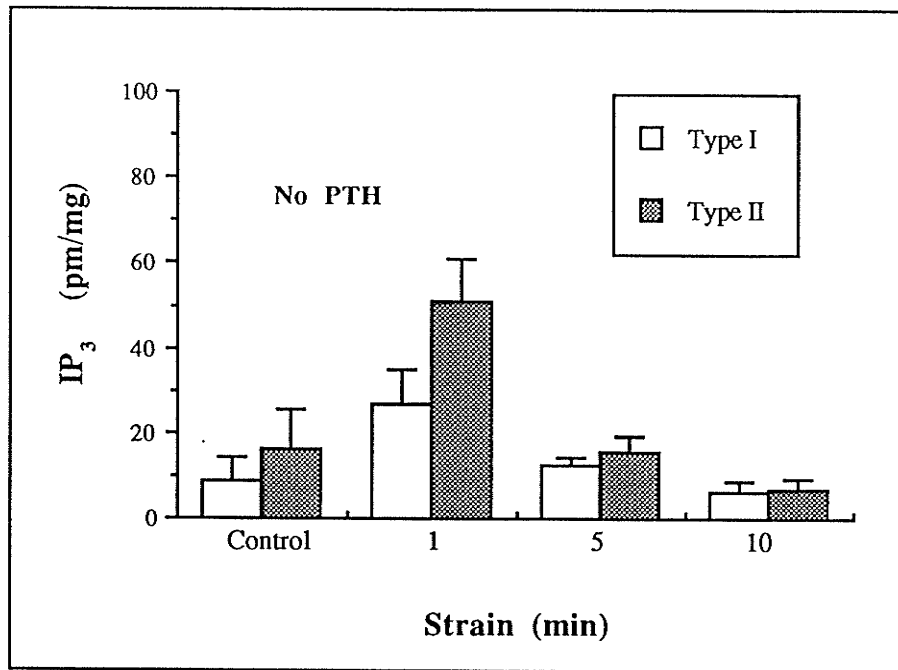


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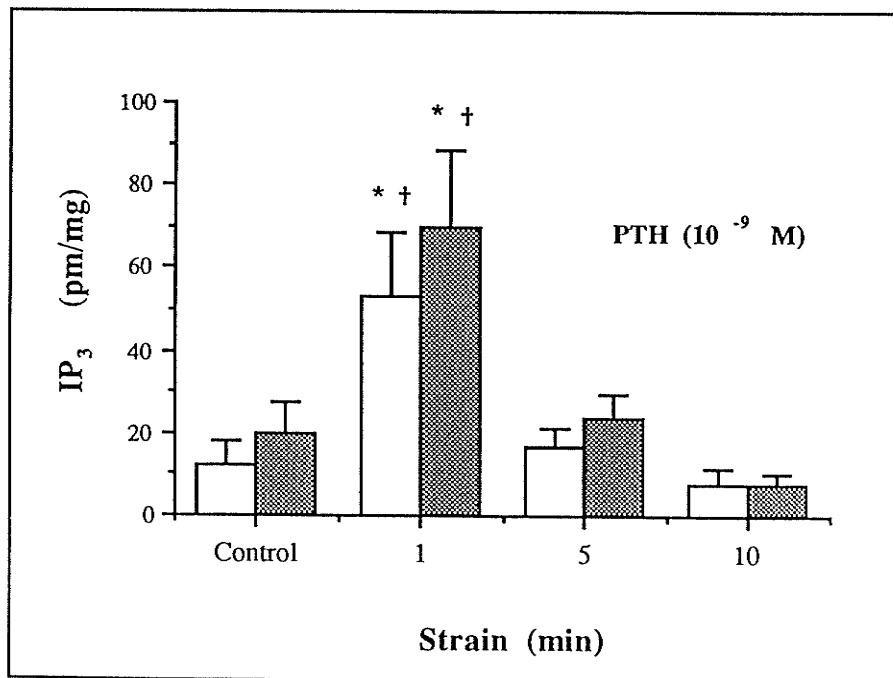


B

Figure 3. IP_3 levels in strained and unstrained cells in the absence (a) or presence (b) of PTH. (a) IP_3 increased very quickly with strain application (20 Kpa at 3 cycles/min) as shown at 1 minute without PTH (* $p < 0.05$), decreasing after that. (b) Addition of PTH potentiated the effect of strain that was significantly different ($p < 0.01$) after 1 minute of strain in PTH treated (10^{-9} M) compared to unstrained cultures. Values are means \pm standard deviation of at least 6 samples. * indicates significant difference ($p < 0.05$) between cells of different groups but same PTH treatment. † indicates significant difference ($p < 0.05$) between cells of same group but treated differently with PTH.

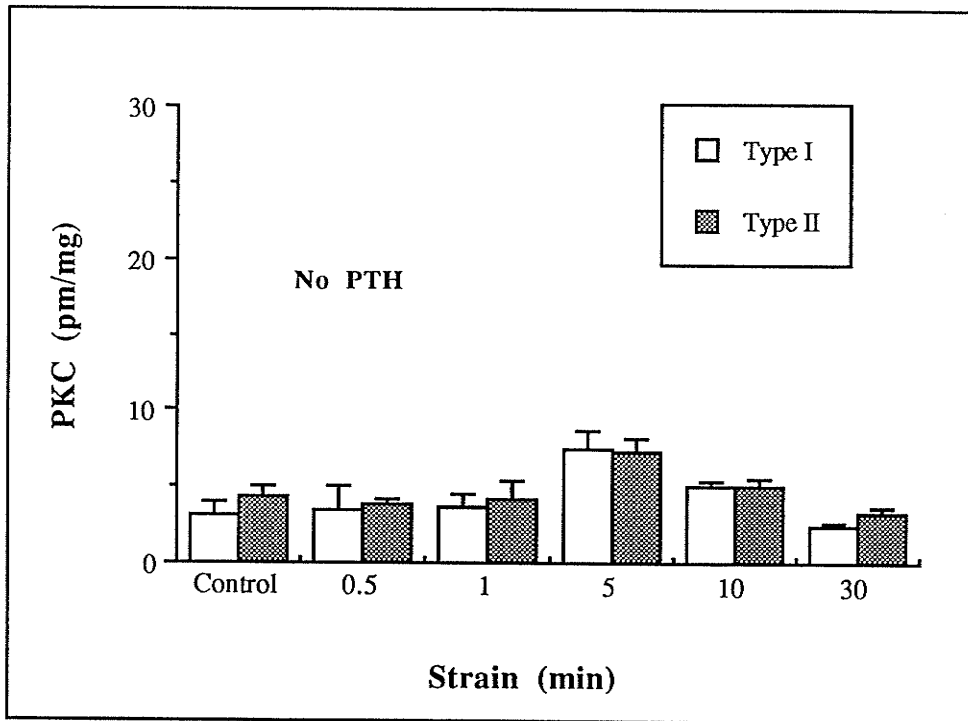


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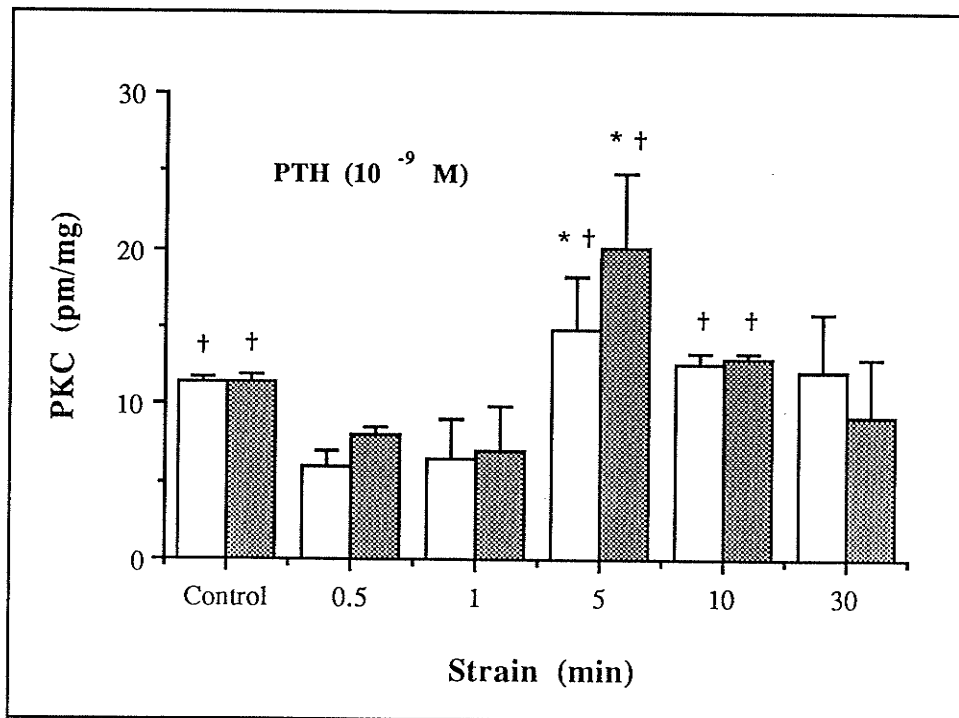


B

Figure 4. Effect of mechanical strain and PTH in PKC activity of rat alveolar bone cells. Cells were exposed to strain (20 Kpa at 3 cycles/min) with (b) or without (a) PTH (10^{-9} M). Strain did not increase significantly the activity of PKC in cultures not exposed to PTH (a). Addition of PTH caused an increase in response at 5 minutes of strain decreasing after that (b). Values are means \pm standard deviation from at least 6 samples. * indicates significant difference ($p < 0.05$) between cells of different groups but same PTH treatment. † indicates significant difference ($p < 0.05$) between cells of same group but treated differently with PTH.



A



B

Figure 5. Immunofluorescence staining of mechanically strained group I (20 Kpa at 3 cycles/min) and unstrained group II alveolar bone cells grown *in vitro*. Cells were fixed in 4 % paraformaldehyde, blocked and permeabilized with 1 % NGS/1 % BSA and 0.3 % Triton X-100. The cells were then incubated with 1° antibodies, washed and incubated with 2° antibodies FITC conjugated and/or with 2° antibodies TRITC conjugated. Cells were then washed and coverslipped with glycerol:PBS with phenylendiamine, and photographed in epifluorescent optics with a Zeiss microscope. Cytoskeletal staining of group I osteoblast-like cells with immunolocalization of vimentin (a-d), vinculin (e-f) and α -actinin (g-h). Non strained group I osteoblast-like cells are shown in (a, e and g) and mechanically strained cells in (b, c, d, f and h). Note that periods of time of 30 minutes (b) show different staining distribution of vimentin when compared with those of periods of 24 hours (c) and 7 days (d). Note that these cells orient themselves perpendicular (double white arrows) to the long axis of the applied mechanical strain (white arrow). Also note that there was change in the focal contact protein vinculin distribution in 30 minutes of mechanical strain (f) and that the distribution of α -actinin did change considerably with mechanical stimulation (h). White arrows indicate the direction of strain. Magnifications (a-d) X 500 and (e-h) X 1250.

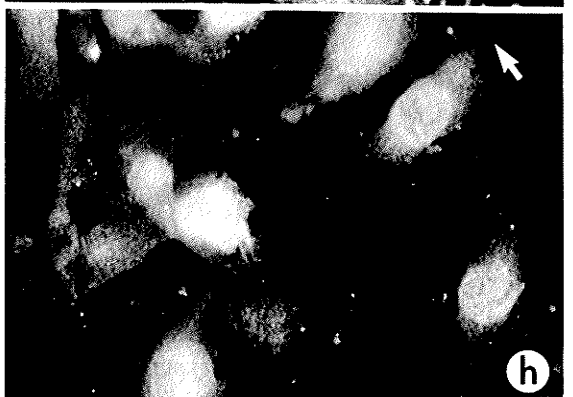
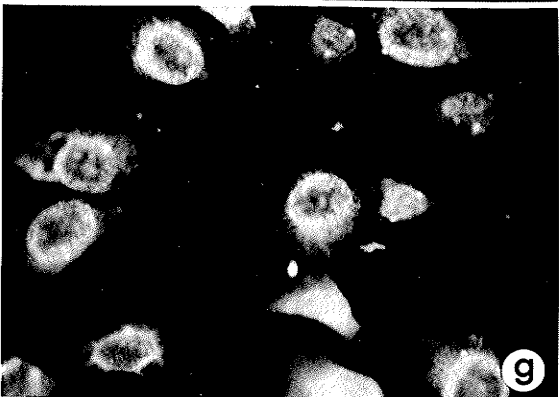
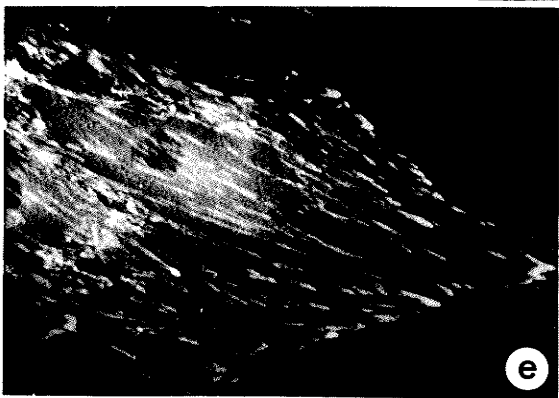
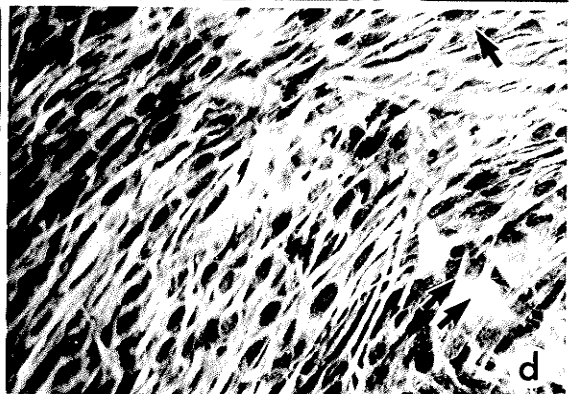
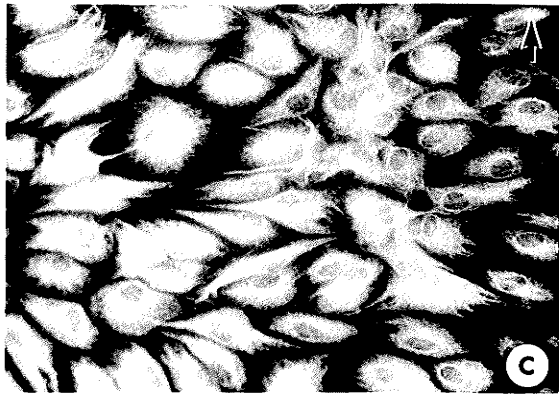
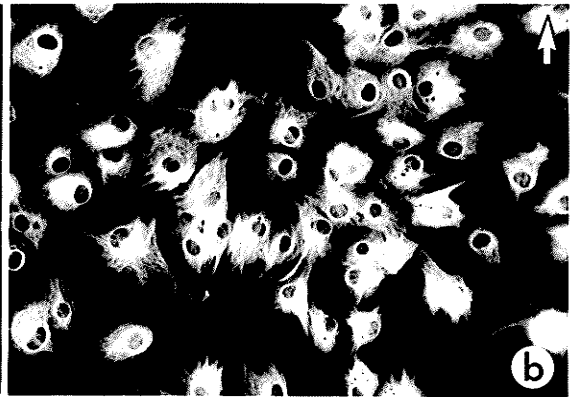
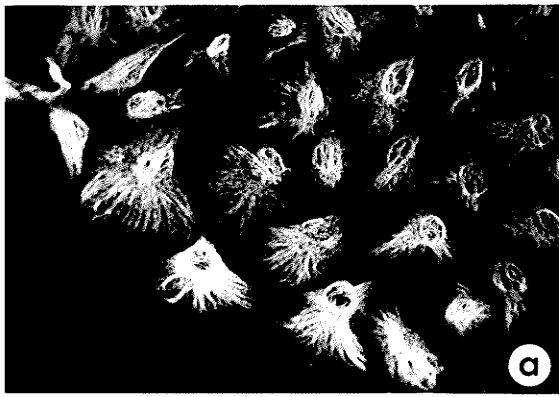


Figure 6. Immunofluorescent localization of PKC in unstrained (a) and mechanically strained (20 Kpa at 3 cycles/min) group II alveolar bone cells (b, c and d). Mechanical strain was applied for 5 minutes (b), 10 minutes (c) and 30 minutes (d). Notice the striated pattern (arrows) in cells mechanically stimulated for 5 minutes (b). Arrow indicates the direction of strain. Magnifications (a-d) X 1250.

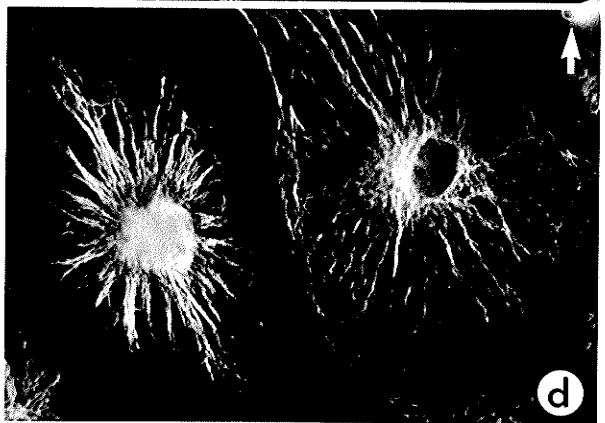
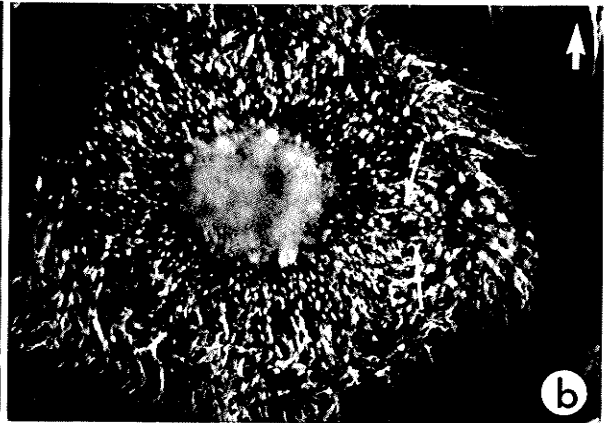
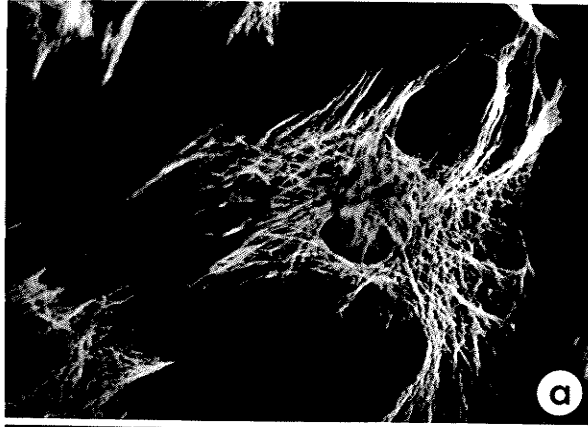


Table 1. Effect of mechanical strain on the mean cell number of two populations of osteoblast cells *in vitro*.^a

	Group I Cells			Group II Cells		
	<i>Strained</i>	<i>Unstrained</i>	<i>p</i>	<i>Strained</i>	<i>Unstrained</i>	<i>p</i>
1 Day	2.46 ± 0.57	1.87 ± 0.22	NS	2.63 ± 0.21	1.3 ± 0.12	0.05
2 Days	3.84 ± 0.21	3.35 ± 0.15	NS	3.31 ± 0.25	2.41 ± 0.90	NS
3 Days	5.79 ± 0.46	3.62 ± 0.36	0.05	5.90 ± 0.40	3.95 ± 0.43	0.01
7 Days	9.37 ± 0.62	6.62 ± 0.70	0.01	10.95 ± 0.14	6.65 ± 0.56	0.01

^aValues are expressed in mean ± standard deviation of at least 6 cultures for each time period studied. Osteoblast cell groups isolated from rat alveolar bone were subjected to mechanical strain intermittently at rates of 20 KPa of vacuum at 3 cycles/min and unstrained cells were used as controls. Cell count values are X 10⁴ for total cell number. NS, not significant.

Table 2. Effects of diffusion barrier perturbation in camp levels and PKC activity from two populations of osteoblast cells *in vitro*.^a

	Group I Cells	Group II Cells
<i>cAMP</i> *	<i>Fluid Motion</i>	
Control Value	0.93 ± 0.30	1.03 ± 0.15
1 Minute	1.1 ± 0.1	0.96 ± 0.37
5 Minutes	1.08 ± 0.24	1.33 ± 0.15
10 Minutes	1.26 ± 0.11	1.25 ± 0.27
<i>PKC</i> *	<i>Fluid Motion</i>	
Control Value	7.33 ± 1.98	6.32 ± 1.45
1 Minute	6.82 ± 2.04	6.65 ± 1.37
5 Minutes	6.47 ± 1.19	6.48 ± 1.32
10 Minutes	5.36 ± 1.27	5.76 ± 1.17

^aValues are expressed in mean ± standard deviation of at least 6 cultures for each time period studied. Osteoblast cell groups isolated from rat alveolar bone were grown in culture and subjected to agitation at rates similar to those used in the Flexercell system. cAMP levels and PKC activity are indicated in pm/mg of total cell protein. *Values are not significantly different.

Table 3. The effects of mechanical strain with the presence of PTH and indomethacin in Two populations of osteoblast cells *in vitro*.^a

		Group I Cells			Group II Cells		
<i>cAMP</i>		<i>PTH/</i>	<i>PTH</i>	<i>p</i>	<i>PTH/</i>	<i>PTH</i>	<i>p</i>
		<i>Indomethacin</i>			<i>Indomethacin</i>		
<i>Strained</i>	1 Minute	1.33 ± 0.15	1.27 ± 0.06	NS	1.83 ± 0.71	1.10 ± 0.26	NS
	5 Minutes	0.87 ± 0.15	0.61 ± 0.39	NS	0.76 ± 0.30	0.76 ± 0.12	NS
	10 Minutes	1.91 ± 0.60	2.17 ± 0.51	NS	3.90 ± 0.20	4.02 ± 0.41	NS
<i>Unstrained</i>	Control	1.26 ± 0.31	1.47 ± 0.15	NS	1.25 ± 0.07	1.21 ± 0.12	NS
		<i>PTH/</i>	<i>PTH</i>	<i>p</i>	<i>PTH/</i>	<i>PTH</i>	<i>p</i>
<i>PKC</i>		<i>Indomethacin</i>			<i>Indomethacin</i>		
<i>Strained</i>	1 Minute	7.66 ± 0.88	8.18 ± 1.88	NS	6.76 ± 2.01	9.53 ± 1.10	0.05
	5 Minutes	16.78 ± 3.95	20.46 ± 4.34	0.05	19.01 ± 1.99	19.01 ± 5.09	NS
	10 Minutes	6.24 ± 1.89	7.36 ± 3.59	NS	8.61 ± 2.21	6.24 ± 1.89	NS
<i>Unstrained</i>	Control	7.95 ± 2.88	9.16 ± 1.96	NS	7.01 ± 2.27	10.19 ± 2.04	0.05

^aValues are expressed in mean ± standard deviation of at least 6 cultures for each time period studied. Osteoblast cell groups isolated from rat alveolar bone were subjected to mechanical strain intermittently at rates of 20 KPa of vacuum at 3 cycles/min and unstrained cells were used as controls. NS, not significant. *cAMP* levels and *PKC* activity are indicated in pm/mg of total cell protein.



Chapter FOUR

*Cytoskeletal Changes in Human
Osteoblastic-like Cells Induced
by Mechanical Strain*

Carvalho RS, Scott JE and Yen EHK

In: Davidovitch Z, ed. *Biological Mechanisms
of Tooth Eruption, Resorption and Replacement
by Implants*. EBSCO Media, Harvard University
Press, Cambridge-MA, pp. 135-146, 1994.



ABSTRACT

The effects of mechanical strain on the cytoskeleton of osteoblasts are poorly understood. While changes in the cytoskeleton distribution of mechanically strained cells have been reported, little is known about the signal transduction pathways responsible for these changes. The aim of this work was to study the effects of strain on the synthesis and distribution of specific integrins and cytoskeleton proteins. These are possibly correlated with signal transduction mechanisms by mechanical strain in osteoblasts. HOS TE-85 were cultured in DMEM/F-12 and grown to confluency in Flexercell Type I dishes in a humidified incubator with 5 % CO₂ and 95 % air. Intermittent strain (3 cycles/min) was applied to the cells using the Flexercell Strain Unit System for periods of 6 and 24 hours and non-stressed cells were used as controls. Immunocytochemical localization for both experimental and control cultures was performed for the receptor proteins α_2 , α_3 , α_v and β_1 integrins, focal adhesion proteins talin and vinculin and intermediate protein vimentin. Quantitation for the synthesis of the same proteins was performed by immunoprecipitation. The results showed that mechanical strain significantly increased the synthesis of integrins α_2 , α_3 , α_v and β_1 , talin and vinculin. Strain also affected the distribution of all the proteins studied. The response of HOS cells to mechanical strain demonstrates that the cytoskeleton adapts to strain in association with increased synthesis of specific integrins, thus suggesting that integrins and related cytoskeleton proteins provide a possible pathway through which mechanical strain is transmitted to the osteoblastic-like cells.

INTRODUCTION

The processes of bone apposition and resorption in orthodontic patients are based on the principles that the mechanical environment of clinically applied forces are transferred to bone cells which translate these forces into a cell response. However, little is known about cell signaling in mechanically strained bone cells.

The mechanical stimulation of bone cells has been shown to increase the levels of intracellular second messengers cAMP, IP₃ and PKC (Davidovitch *et al.*, 1988; Brighton *et al.*, 1988, Jones *et al.*, 1991; Carvalho *et al.*, 1994). Although these messengers as well as eicosanoids (Sandy and Harris, 1984), phospholipid metabolites and Ca²⁺ (Brighton *et al.*, 1988, Jones *et al.*, 1991) can mediate parts of mechanical deformation in bone, the effects of strain can not be explained by any one of these pathways alone (Sandy *et al.*, 1993; Carvalho *et al.*, 1994). Rather, there seems to exist a synergistic and/or antagonistic cooperation between these pathways. For example, PTH is thought to mediate an increase in inositol phosphates as well as PGE₂ (Farndale *et al.*, 1988), however, more recent studies have shown that the roles of PTH and PGE₂ may be comparable but not wholly coincident in mechanically strained cells (Carvalho *et al.*, 1994). Inflammatory cell metabolites such as cytokines, interleukins and leukotrienes have also been thought to influence strained cell response (Davidovitch *et al.*, 1988; Sandy *et al.*, 1993). For mechanical strain to stimulate these responses a mechanotransduction pathway that would involve cell structural changes could be hypothesized.

Bone cells, like most other cell types, attach to the extracellular matrix through cell-surface receptors for specific matrix proteins. These "ligands" bridge the extracellular network to the intracellular cytoskeleton as an integral path to the signal transduction from matrix to cell function. Many of these receptors belong to the integrin superfamily (Virtanen *et al.*, 1990). All integrins are formed by two subunits, an α and a β , and to date at least 14 α and 8 or more β have been characterized (Virtanen *et al.*, 1990; Hynes, 1992; Juliano and Haskill, 1993). Different combinations of the subunits function as receptors

for attachment of different cell types to different extracellular proteins (Clover *et al.*, 1992). Each subunit is divided into a large extracellular domain, a transmembrane domain and a short cytoplasmic domain (Gailit *et al.*, 1993). The cytoplasmic region interacts with the actin network indirectly through other proteins such as talin, vinculin and tensin (Burrige *et al.*, 1988; Otey *et al.*, 1990) and is known to concentrate within attachment sites called focal contacts or focal adhesions (Burrige *et al.*, 1988). It has been shown that the integrins subunits that are expressed in bone cells are α_1 , α_2 , α_3 , α_v and β_1 (Clover *et al.*, 1992). These differ from those of osteoclasts (Horton and Davies, 1989; Horton *et al.*, 1991), which indicate that the combinations of these subunits within the extracellular matrix is likely to be critical in cell function. The integrin receptor $\alpha_1\beta_1$ binds to collagen and laminin while $\alpha_3\beta_1$ binds to collagen and laminin and also fibronectin (Clover *et al.*, 1992; Gailit *et al.*, 1993). The α_2 and α_v subunits also combine with β_1 to bind a wider range of ligands including vitronectin and von Willebrand's factor, osteopontin, bone sialoprotein and fibrinogen (Moyle *et al.*, 1991; Hynes, 1992).

The application of mechanical strain has been shown to cause an alteration of the cell shape (Buckley *et al.*, 1988; Pender and McCulloch, 1991) with significant actin rearrangement. In contrast, there is very little information on mechanical strain effects on integrin subunit changes (Virtanen *et al.*, 1990), although, oxidative stress has been shown to redistribute and to dysfunction integrins from renal cells *in vitro* (Gailit *et al.*, 1993).

Since strain causes the changes in intracellular messenger levels and in cytoskeleton rearrangement, it seems appropriate that a pathway be described to link these observed cellular phenomena. The aim of the present study was to examine the effects of mechanical strain on the change in distribution and synthesis of integrins, vinculin, talin and vimentin in human osteosarcoma cells in culture. The Flexercell system was used to provide strain cycles of the magnitude that would be similar to those found in orthodontically-strained periodontal tissues. A human osteosarcoma (HOS) cell line TE-85 provided a reproducible

supply of bone-like cells, in order to develop a cell strain model useful for cytoskeletal studies.

MATERIALS AND METHODS

Cell culture technique

HOS TE-85 were obtained from the American type culture collection (ATCC, Rockville, MD). Cells were maintained in DMEM/F-12 (Ham) medium (1:1) (Sigma, St. Louis, MO) supplemented with 5 % FBS (Gibco, Burlington, ON), 25 units/ml of Penicillin G, 25 µg/ml of streptomycin and 100 µg/ml ascorbic acid (all from Sigma). Cells were plated at a density of 2×10^4 cells/cm² in 35 mm dishes and maintained in a humidified atmosphere consisting of 5 % CO₂ and 95 % air. Cell cultures were subcultured every two days and were photographed using a phase-contrast Zeiss Optic photomicroscope (Zeiss, Germany).

Mechanical strain method

Cultures were plated into collagen-coated Flex type I dishes and grown for 2 days to confluency prior to being mechanically strained. These dishes were strained using the Flexercell Strain Unit system (Flexcell Corp., McKeesport, PA). This apparatus applies mechanical strain to cells as reported previously (Anderson *et al.*, 1992; Carvalho *et al.*, 1994). Mechanical strain was applied using a pressure of 20 kPa at 3 cycles per minute (0.05 Hz).

Experimental and control cultures were transferred to serum-free medium containing DMEM/F-12 (1:1) supplemented with 1 µg/ml insulin and 100 µg/ml BSA and 100 mg/ml ascorbic acid. Dishes were strained for periods of 6 and 24 hours. Cell cultures grown for periods of 1, 2, 7, 14, 21 and 28 days were also strained for 24 hours prior to harvesting. Control cultures were cultivated for the same time periods using rigid-bottomed (no strain) collagen-coated Flex type II dishes. All cell cultures were serum starved 24 hrs prior to the straining period or the equivalent time for the control cultures.

Von Kossa stain and cell number counts

HOS TE-85 cells were fixed in buffered formalin for 3 hours, and stained in silver nitrate for 30 minutes in the dark. The dishes were then washed three times with distilled water and the cells were incubated with 5 % sodium carbonate/25 % formaldehyde solution for 2 minutes, followed by a gentle wash in running water. The samples were dried and photographed.

Cell counts were taken at the same experimental and control periods as described above. Strained and unstrained dishes for both cell populations were washed 3 times in PBS and incubated with trypsin for 5 minutes at 37°C. Cells were washed and transferred to a tube containing FBS and centrifuged. The supernatant was discarded and the pellet resuspended, washed in Hank's buffer and aliquots were counted electronically in a Coulter Counter (Model ZBI, Hialeah, USA).

Immunofluorescence labeling

Strained and unstrained HOS TE-85 cells were rinsed with PBS and fixed by incubation with 4 % paraformaldehyde in PBS for 10 minutes. After several washings with PBS, cells were permeabilized by incubation with 0.5 % Triton X-100 in PBS for 20 minutes at room temperature. Cells were washed again in PBS and incubated for 1 hour with primary antibodies (diluted in PBS with 1 % BSA) for vimentin, vinculin, talin, tensin, α_v -integrin, α_2 -integrin, α_3 -integrin and β_1 -integrin. Thereafter, samples were extensively washed in PBS and incubated with goat Cy3 conjugated anti-mouse IgG or goat FITC conjugated anti-mouse IgG secondary antibodies for 40 minutes at 22°C in PBS/1 % BSA. Cultures were rinsed in PBS and after the last rinse, the silastic bottoms of the dishes were removed and mounted with mounting medium while recording strain and cell orientation. Controls for all the antibodies were performed. Cultures were photographed using a Zeiss epi-fluorescence microscope. Due to the gradient pattern of strain bi-axially in the silastic bottoms (1 to 24 % of strain), in order to study the effects of controlled levels of strain, all the photomicrographs were taken from the most central area

(<1 % strain) of the silastic membrane. Monoclonal antibodies anti-vimentin, anti-vinculin, anti-talin, FITC and Cy3-conjugated antibodies were from Sigma (St. Louis, MO) and monoclonal anti- α_v -integrin, anti- α_2 -integrin, anti- α_3 -integrin and anti- β_1 -integrin were from Gibco (Burlington, ON). Monoclonal antibody anti-tensin was a generous gift from Dr. L.B. Chen (Harvard Medical School, Boston, MA).

Immunoprecipitation assay

For immunoprecipitation, cells were washed in ice-cold PBS and incubated with lysis buffer containing 50 mM Tris, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1 % Nonidet P-40, 1 μ g/ml Aprotinin and 0,02 % Na Azide. Cells were microcentrifuged and the pellet discarded. The supernatant was pre-cleared with rabbit normal serum and formalin-fixed staphylococcus aureus (Sigma) for 2 hours on ice. Samples were again centrifuged and purified monoclonal antibodies for vinculin, vimentin or talin (Sigma, St. Louis, MO) or for α_v -integrin, α_2 -integrin, α_3 -integrin or β_1 -integrin (Gibco, Burlington, ON) were added to the samples of the lysate and incubated overnight at 4°C with rocking. The antibody-protein complexes were precipitated by addition of a 10 % solution of Protein A-sepharose beads 4 B (Sigma) in lysis buffer, and incubated for 1 hour at 4°C with rocking. The beads were then collected by centrifugation and washed extensively with lysis buffer to remove unbound proteins. Pellets were resuspended in SDS sample buffer (0.5 M Tris, pH 6.8, 90 % glycerol, 10 % SDS, 0.1 % bromophenol blue) and boiled for 5 minutes. The proteins were resolved in a 10 % SDS-PAGE under reducing (5 % vol/vol β -mercaptoethanol) and non-reducing conditions. Pre-stained molecular mass standards were from Amersham (Oakville, ON). The gels were then transferred to a nitrocellulose membrane (0.45 μ m pore diameter) which was probed with the same monoclonal antibodies, followed by horseradish peroxidase-labeled secondary antibodies (Amersham). This reaction was visualized using enhanced chemiluminescence (ECL) Western blotting method (Amersham), and the membranes exposed to Kodak XRP-1 X-ray films (Kodak Canada Inc., Toronto, ON). Individual tracks corresponding to one

sample were scanned at 550 nm with a spectrophotometer (Bio-Rad Laboratories Model 620 Video Densitometer, Matsushita Electric Industrial Co. Ltd., Japan). The proportions of cytoskeletal proteins and integrins subunits were measured relative to the total cell count at each time period.

Statistical analysis

The values obtained for all the strained and unstrained samples were subjected to a two way-analysis of variance with Tukey's multiple comparison test. T-tests with the Bonferroni correction were also used to compare the strained and unstrained samples within the time periods studied.

RESULTS

Von Kossa stain in mechanically strained bone cells

HOS TE-85 cells had a tendency to form small clusters both prior to and after confluency. The monolayer culture took approximately 36 to 48 hours to become confluent, however, bone nodules did not become evident until 72 hours of culture. Fig. 1 shows the different stages of cell growth, specifically the tendency of these cells to form clusters (Fig. 1c), which in later stages become the site for mineralized nodules (Fig. 1d). Cell shape also changed from slender to cuboidal, becoming more marked as the cells localized towards the center of the nodules. Initially nodules could be identified as white spots by the naked eye (Fig. 1c). However, as cultures aged, individual nodules could only be detected with von Kossa silver nitrate stain, suggesting the formation of bone-like nodules *in vitro* as identified by von Kossa stain (Fig. 2). Application of mechanical strain induced a progressive increase in von Kossa staining on days 2, 7, 21 and 28 when compared with unstrained cell cultures. The scattered von Kossa positive staining observed on days 2 and 7 (Figs. 2a, 2b, 2e and 2f) gradually increased in area to form large stained areas in the later strain periods of days 21 and 28 (Figs. 2c, 2d, 2g and 2h). Cell numbers also increased significantly with the application of strain at 2, 14 and 28 days when compared with the unstrained controls (Fig. 3). Cultures subjected to mechanical strain did not show any significant degree of cell death as evidenced by trypan blue exclusion (not shown). These osteoblastic-like cells also showed an increase in cAMP levels when PTH was added, as well as changes in alkaline phosphatase activity with mechanical strain (not shown).

Cytoskeleton organization in mechanically strained bone cells

In this study we also examined the cytoskeletal reorganization in TE-85 osteosarcoma bone-like cells. These cells showed changes in cytoskeletal labeling after periods of 6 and 24 hours of mechanical strain. Initially in order to determine if strain

altered focal contact proteins, antibodies against vinculin and talin were used. The labeling of talin and vinculin in Figures 4a to 4d shows that mechanical strain stimulated the formation of larger focal contact sites in strained cells compared to controls cells for the time periods studied. Strain also caused an increase in vimentin labeling, and although the latter did not appear to change at 6 hours of strain, vimentin staining in unstrained cultures (Fig. 4e) changed in cells subjected to strain for 24 hours (Fig. 4f). In addition, the cells showed a change in the orientation relative to the axis of the mechanical strain and in cultures strained for periods longer than 24 hours (not shown) there was a phenotypic change from cuboidal osteoblast-like cells to elongated fibroblast-like cells, although bone formation was still observed (Fig. 2). And finally labeling for tensin appeared to be similar to that of vimentin. Although tensin is not as abundant a protein as vimentin, cells also stained brightly for tensin (Fig. 4g). Application of strain to the same cells showed a more intense labeling (Fig. 4h). Because results of 6 and 24 hours of strain were similar, labeling at 6 hours is not shown.

Immunohistochemical analysis of the integrin subunits has indicated that these receptors are also influenced by mechanical strain. In fixed and permeabilized osteosarcoma cells antibodies anti- α_2 (Fig. 5a) labeled prominently clusters of integrins in the cell periphery. This antibody also revealed the presence of cellular "pseudopodia" or cellular extensions (Fig. 5b, double arrow). Labeling with antibodies anti- α_3 appeared similar to the one for anti- α_2 both in the unstrained (Fig. 5c) and strained cultures (Fig. 5d). However, the latter showed a smaller clustering of integrins at the cell periphery. This pattern was similar for the labeling of antibodies anti- α_v , and yet it was not as abundant as the previous ones (Fig. 5e and 5f). Similarly, labeling for antibody anti- β_1 was not coincident with α_2 or α_3 and did not show the formation of cellular extensions. On the other hand, β_1 staining from control unstrained cultures (Fig. 5g) appeared to increase in strained ones (Fig. 5h). Regardless of the integrin subunit, mechanical strain seemed to

have an effect on the integrin labeling in general. This occurred both at 6 and 24 hours of strain.

Synthesis of integrin subunits and cytoskeleton proteins

The effects of mechanical strain on the synthesis of the cytoskeletal proteins and integrins are shown in the immunoblots in Figures 6 and 7 and summarized in the graphs of Fig. 8. Fig. 6 shows the immunoblots from proteins talin, vinculin and vimentin at 24 hours of culture when strain appeared to cause its most marked effect. Talin, vinculin and vimentin were separated with SDS-PAGE under non-reducing conditions. Talin migrated as a major band of 215 kD similar to studies in chicken fibroblast cultures (Burrige *et al.*, 1988). A monoclonal anti-vinculin antibody precipitated a 130 kD band corresponding to the vinculin protein being expressed in culture. In addition vimentin migrated at 55 kD in both strained and unstrained cultures. Total protein content was shown to increase in strained compared with unstrained cultures (not shown). Synthesis of talin and vinculin seem to increase significantly ($p < 0.05$) with the application of mechanical strain both at 6 and 24 hours of strain (Fig. 8A). On the other hand vimentin only showed a significant increase ($p < 0.05$) at 24 hours of strain (Fig. 8A).

The different integrin subunits were separated by SDS-PAGE under reducing and non-reducing conditions. Immunoprecipitation with anti- β_1 subunit antibody without β -mercaptoethanol revealed a band of 130 kD, which co-migrated with α_2 (Fig. 7A, lanes B and C). In addition, under the same conditions, α_3 seemed to precipitate at around 140 kD (Fig. 7A, lanes D and E). This was similar for both strained and unstrained cells. Under reducing conditions the immunoblots with monoclonal antibody anti- β_1 subunit showed a band of 116 kD and another at approximately 130 kD (Fig. 7B, lanes D and E) in agreement with other authors (Uitto *et al.*, 1992; Tawil *et al.*, 1993). The latter band of 130 kD seems to correspond to α_2 under non-reducing conditions. Immunoprecipitation of the α_v integrin subunit under reducing conditions resulted in the migration of this band at approximately 140 kD (not shown), and both strained and unstrained cells were not

significantly different for α_v synthesis. Synthesis of β_1 and α_3 was increased significantly ($p < 0.05$) in strained cells both at 6 and 24 hours of culture as opposed to α_2 , which increased significantly ($p < 0.05$) only at 6 hours of strain compared with unstrained cultures (Fig. 8B). The α_v subunit did not change significantly from strained to control cultures at both time periods studied. In summary, the application of mechanical strain seemed to cause an increase in the synthesis of the integrin subunits in this study.

DISCUSSION

Bone remodeling occurs as a result of the environmental demands placed upon bone tissue (Burger *et al.*, 1992). As Sandy and collaborators (1993) have pointed out, perhaps no other specialty makes more use of induced bone remodeling than orthodontic therapy. Thus, a better knowledge of the mechanisms involved in orthodontically-induced bone remodeling is fundamentally important. The aim of this work was to study the effects of mechanical strain on changes in cytoskeleton and integrin subunit synthesis and distribution from bone cells grown *in vitro*.

The model used in this paper consisted of HOS TE-85 cells grown in culture which have been extensively characterized in previous studies. HOS TE-85 cells showing osteoblastic proliferation, differentiation, matrix maturation and long term histological characteristics have been described as mature osteocytes surrounded by mineralized matrix (Guo *et al.*, personal communication). In this study we have reported the formation of mineralized bone nodules *in vitro* (Fig. 2) as evidenced by silver nitrate stain.

Studies suggest that alterations in cell shape are mediated via alterations in cell cytoskeleton (Ben-Ze'ev, 1991; Gingell and Owens, 1992). This cytoskeletal restructuring probably involves integrin-mediated adhesion and the dynamic relationship of linkages between membrane receptors and focal contacts (Zetter and Brightman, 1990; Gingell and Owens, 1992). In this paper we have shown that mechanical strain increases the synthesis of integrins and focal contact proteins. Similarly, an increase in protein synthesis with mechanical strain has been shown in cardiac myocytes (Komuro *et al.*, 1990). We are interested, therefore, in the role of the cytoskeleton to determine cell shape through focal contacts and integrin receptors and their role in the transduction of strain-induced signals. It is conceivable that mechanical strain also stimulates adhesion mediated responses such as stimulation of mitosis (Folkman and Moscona, 1978), adhesion-dependent of targeting proteins (Kabat *et al.*, 1985) and the dependence of intracellular pH in the control of the cell cycle (Margolis *et al.*, 1989).

It has been reported that microfilaments are perhaps the best situated cytoskeleton component to detect environmental strains applied to bone cells (Sandy *et al.*, 1993). The major subunit protein in microfilaments is actin, which has indeed shown to change in fibroblasts subjected to mechanical stretch (Pender and McCulloch, 1991; Carvalho *et al.*, unpublished observations). However, microfilaments terminate at specialized sites of the cell membrane termed focal contact sites (Burrige, 1988; Beckerle and Yeh, 1990), in which talin and vinculin are the major proteins. The results reported here that these proteins changed their distribution (Fig. 4) and synthesis (Figs. 6 and 8A) when subjected to strain may represent an additional step in early strain-induced signal transduction, especially when one considers their relationship with the integrin family of extracellular matrix receptors (Hynes, 1992 for review).

Localization of integrin subunits appears to be either at point or focal contacts (Tawil *et al.*, 1993). Point contacts are characteristic of β_1 distribution in spread as opposed to focal contacts in flattened astrocytes (Tawil *et al.*, 1993). On the other hand, α subunits may appear in both forms, since these largely but not exclusively, determine ligand specificity and integrin localization (Vogel *et al.*, 1990). In this paper we observed that most of the integrin subunits formed focal contacts distributed at the periphery of the cells (Fig. 5) similarly to previous work by Virtanen *et al.* (1990) and by Ylänné *et al.* (1990). It has been reported that subunits α_1 , α_2 , α_3 , α_v and β_1 are primarily expressed in osteoblastic cells (Clover *et al.*, 1992). This study has confirmed the latter and shown that application of strain stimulated not only the distribution of integrins (Fig. 5), but also the synthesis of different subunits (Figs. 6, 7 and 8B).

The immunoprecipitation of integrin subunits has shown that α subunits were co-precipitated with β (Figs 7A and 7B). This would be expected, given the nature of the known $\alpha\beta$ structure of integrins. Marcantonio and Hynes (1988) have shown that in many cell types more than one α subunit may co-precipitate, which would then indicate the presence of two or more heterodimers. However, it is not possible to identify precisely

from this work which type of integrin heterodimers are expressed in HOS TE-85 cells. Ligand specificity of heterodimeric integrins is quite complex and different extracellular matrix proteins are known to bind to the same integrin subunits (Virtanen *et al.*, 1990). Therefore, cell-dependent regulation of integrin specificity and function may be given through tissue type and environmental demands. Distinct responses in different tissues have been observed when different forms of stress were shown to interfere with cell attachment in culture. Oxidative stress in epithelial cells was shown to cause a decrease in cell attachment through a disruption in focal contacts and a redistribution of integrin subunits (Gailit *et al.*, 1993). Chinese hamster ovary cells were inhibited to bind to vitronectin when heat shock stress was applied (Cress *et al.*, 1990). Although loss of cell attachment has not been seen in this study, this effect may not be ruled out as it could be due to the strain and cell type studied.

Thus, integrins are likely to transduce mechanical strain-induced signals into a morphogenetic cell response. Transduction of force seems to affect individual cells via ECM scaffoldings such as basement membranes, collagen matrix, cartilage and bone that link specialized tissues in the body (Lotz *et al.*, 1989; Ingber, 1991). Different systems such as the Ca²⁺ mobilization and surfactant release from lung epithelial cells (Wirtz and Dobbs, 1990), stimulation of adenylyl cyclase activity (Letsou *et al.*, 1990), PKC activity (De Groot *et al.*, 1990) and phosphoinositide metabolism (Nollert *et al.*, 1990) are affected by ECM strain. Evidence also suggests that additional mechanical strain is applied to the cells which already have a pre-existing force equilibrium determined in part by cell-surface receptors (Lotz *et al.*, 1989; Ingber, 1991). In osteoblasts, like most other cell types, integrins form the physical link from the associated cytoskeleton proteins to the extracellular matrix which contributes to a structural and biochemical combination of strain signals (Beckerle and Yeh, 1990; Otey *et al.*, 1990; Ingber, 1991; Hynes, 1992). However, although integrins are excellent candidates to function as mechanotransducers, the morphogenetic or biochemical response in the cells is still not clear.

Transduction of mechanical strain signals from the changes in cytoskeleton protein synthesis to alteration of gene expression is not yet known. Theories include the stimulation of ion flux (Sachs, 1988; Ingber, 1991), and the spacial tensegrity model described by Ingber (1993). Although, there may be a tensegrity structural component in cells that require structural stability, more likely, the transduction of signals is dependent on a intracellular signaling pathway. Transduction of the signals into cellular activity have been shown to take place partly through the PI pathway (Jones and Schlubbers, 1987; Carvalho *et al.*, 1994). Mechanical strain is thought to increase IP₃ levels followed by an increase in PKC activity shortly after strain stimulation (Jones *et al.*, 1991; Carvalho *et al.*, 1994). Inhibition of PKC has been shown to inhibit transcription of mechanical strain sensitive genes in myocyte stretching (Komuro *et al.*, 1991). These results are confirmed by studies indicating that PLC mediates several cellular strain-dependent events, such as the efflux of prostaglandins F₂ and E₂ as a result of muscle hypertrophy (Vandenburg *et al.*, 1990) and the production of prostaglandin I₂ and tissue plasminogen activator from endothelial cells (Komuro *et al.*, 1991). These suggest that PKC activity via PLC activation will transduce strain generated signals into various cellular events including gene expression (Komuro *et al.*, 1991). In other words, recent evidence indicates that PKC might be involved in the mechanical and also humoral factors involving gene expression of strained cells in culture. Further work is required to elucidate the molecular mechanisms derived from the translation of mechanical strain into PKC activity, however, this will not constitute by itself the answer in strained cell signaling. Different levels of response may trigger different pathways (Nollert *et al.*, 1990; Jones *et al.*, 1991), as has been shown for cAMP (Carvalho *et al.*, 1994) and cGMP (for a review see Meghji, 1992). Addition of PTH which regulates bone-related Ca²⁺ metabolism may be mediated by both adenylyl cyclase and PLC-dependent hydrolysis of PIP₂ in osteoblast cells (Fujimori *et al.*, 1992). Although later cell response involves the participation of the adenylyl cyclase pathway (Davidovitch *et al.*, 1988; Carvalho *et al.*, 1994) and a change in eicosanoid production

(Reich and Frangos, 1991), the early response to strain may be directly linked to the PI pathway mediated likely through IP₃ and PKC (Hsieh and Frangos, 1991; Komuro *et al.*, 1991; Carvalho *et al.*, 1994). This has also been shown for an increase in DNA synthesis with mechanical strain (Hasegawa *et al.*, 1985; Sandy *et al.*, 1993), in which cAMP had an inhibitory effect (Hakeda *et al.*, 1987). This response is not cAMP dependent as shown by Farndale *et al.* (1988); on the contrary, the effect is likely due to the phosphoinositide turnover (Hakeda *et al.*, 1986; Berridge, 1987; Sandy *et al.*, 1993).

Other molecules also have been associated with integrins and focal contacts such as pp60^{src} (sarcoma) (Nermut *et al.*, 1991) and intracellular Ca²⁺ (Jaconi *et al.*, 1991), as well as activation of the Na⁺/H⁺ antiporter with integrin binding to ECM (Ingber *et al.*, 1990), indicating that integrin may transduce signals in a similar fashion as growth factors (Ingber, 1991). Phosphorylation cascades in the cytoskeleton may also initiate through integrin-mediated response, as in the case of integrin and focal contact binding via talin (Burrige *et al.*, 1988), myosin (Omann *et al.*, 1987) and tensin (Davies *et al.*, 1991). In fact, phosphorylation has been suggested as the first identifiable step of the signaling process (Gingell and Owens, 1992). Stimulation of adhesion has caused an increase in tyrosine phosphorylation of a protein of 120 to 130 kD (Kornberg *et al.*, 1991) called pp125^{fak} (focal adhesion kinase) (Schaller *et al.*, 1992). It has been suggested that integrin signaling and activation of pp125^{fak} are related to control gene expression and differentiation (Juliano and Haskill, 1993).

Mechanical strain response at the molecular level has shown in cardiac myocytes that the early response is given by the immediate genes such as protooncogenes and heat shock protein genes (Izumo *et al.*, 1988). Similarly the late response in the same cells is induced by the fetal contractile protein genes and the atrial natriuretic peptide genes (Komuro *et al.*, 1991). It is known that stimulation of these genes may occur via adrenergic receptors such as α_1 (Bishopric *et al.*, 1987). In addition, gene regulation by mechanical strain/integrin-mediated response may resemble signaling through receptor

tyrosine kinases (RTKs). To this end proteins with SH2 domains, such as PLC and tensin, are stimulated upon effector-RTKs binding to subsequently elicit a response (Koch *et al.*, 1991). In case of PLC signaling, RTKs may directly stimulate PKC (for a review see Juliano and Haskill, 1993). The considerable amount of overlap between G-protein and RTKs mediated signaling added to the lack of understanding of the whole process of signaling cascades in gene regulation allows several questions to be raised concerning mechanical strain transduction. Further work is needed to clarify the role and dependency of integrin-mediated-RTK activation.

In conclusion, integrin subunits are excellent candidates to transmit strain generated signals into the cells. These signals are then converted into biochemical response leading to altered function. The resultant change in function as well as the biochemical signals and the forces applied need to be further clarified. Perhaps the biggest challenge is to elucidate the relationship of cytoskeleton adaptation with the various strain-induced signaling mechanisms discussed above, including various membrane receptors and biochemical pathways.

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REFERENCES

- Anderson J, Carvalho RS, Yen EHK and Scott JE 1992 Measurement of strain in cultured bone and fetal muscle and lung cells. *In Vitro Cell Dev Biol* **29**:183A-186A.
- Ben-Ze'ev A 1991 Animal shape changes and gene expression. *Bioessays* **13**:207-211.
- Beckerle MC and Yeh R 1990 Talin: role at sites of cell-substratum adhesion. *Cell Motil Cytoskeleton* **16**:7-13.
- Berridge MJ 1987 Inositol triphosphate and diacylglycerol: two interacting second messengers. *Ann Rev Biochem* **56**:159-193.
- Bishopric NH, Simpson PC and Ordahl CP 1987 Induction of the skeletal α -actin gene in α_1 -adrenoceptor-mediated hypertrophy of rat cardiac myocytes. *J Clin Invest* **80**:1194-1199.
- Brighton CT and McCluskey WP 1988 Response of cultured bone cells to a capacitively coupled electric field: Inhibition of cAMP response to parathyroid hormone. *J Orthop Res* **6**:567-571.
- Buckey MJ, Banes AJ, Levin LG and Sumpio P 1988 Osteoblasts increase their rate of division and align in response to cyclic, mechanical tension *in vitro*. *Bone Miner* **4**:225-236.
- Burrige K, Fath K, Kelly T, Nuckolls G and Turner C 1988 Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol* **4**:487-525.
- Burger EH, Klein-Nulend J and Veldhuijzen JP 1992 Mechanical stress and osteogenesis *in vitro*. *J Bone Min Res* **7**:397S-401S.
- Carvalho RS, Scott E, Suga DM and Yen EHK 1994 Stimulation of signal transduction pathways in osteoblasts by mechanical strain is potentiated by parathyroid hormone. *J Bone Min Res* **9**:999-1011.
- Clover J, Dodds RA and Gowen M 1992 Integrin subunit expression by human osteoblasts and osteoclasts *in situ* and in culture. *J Cell Sci* **103**:267-271.

- Cress AE, Majda JA, Glass JR, Stringer DE and Gerner EW 1990 Alteration of cellular adhesion by heat shock. *Exp Cell Res* **190**:40-46.
- Davidovitch Z, Nicolay OF, Ngan PW and Shanfeld GL 1988 Neurotransmitters, cytokines and the control of alveolar bone remodeling in orthodontics. *Dent Clin North Am* **32**:411-435.
- Davies S, Li ML, Lo SH, Lin S, Buttler JA, Drucker BJ, Roberts TM, An Q and Chen LB 1991 Presence of an SH2 domain in the actin-binding protein tensin. *Science* **252**:712-715.
- De Groot RP, Rijken PJ, Hertog GD, Boonstra J, Verkleij AJ, De Laat SW and Kruijer W 1990 Microgravity decreases *c-fos* induction and serum response element activity. *J Cell Sci* **97**:33-38.
- Farndale RJ, Sandy JR, Atkinson SJ, Pennington SR, Meghji S and Meikle MC 1988 Parathyroid hormone and prostaglandin E₂ stimulate both inositol phosphates and cyclic AMP accumulation in mouse osteoblasts cultures. *Biochem J* **79**:230-239.
- Folkman J and Moskona A 1978 Role of cell shape in growth control. *Nature* **273**:345-349.
- Fujimori A, Cheng S, Avioli LV and Civitelli R 1992 Structure-function relationship of parathyroid hormone: activation of phospholipase-C, protein kinase-A and -C in osteosarcoma cells. *Endocrinology* **130**:29-36.
- Gailit J, Colflesh D, Rabiner I, Simone J and Goligorsky MS 1993 Redistribution and dysfunction of integrins in cultured renal epithelial cells exposed to oxidative stress. *Am Physiol Soc* **263**:149F-157F.
- Gingell D and Owens N 1992 How do cells sense and respond to adhesive contacts? Diffusion-trapping of laterally mobile membrane proteins at maturing adhesions may initiate signals leading to local cytoskeletal assemble response and lamella formation. *J Cell Sci* **101**:255-266.

- Hakeda Y, Yoshino T, Natakani Y, Kurihara N, Maeda N and Kumegawa M 1986 Prostaglandin E₂ stimulates DNA synthesis by a cyclic AMP-independent pathway in osteoblastic clone MC3T3-E1 cells. *J Cell Physiol* **128**:155-161.
- Hakeda Y, Hotta T, Kurihara N, Ikeda F, Maeda N, Yagyu Y and Kumegawa M 1987 Prostaglandin E₁ and F₂ alpha stimulate differentiation and proliferation, respectively of clonal osteoblastic MC3T3-E1 cells by different second messengers *in vitro*. *Endocrinology* **121**:1986-1974.
- Hasegawa S, Sato S, Saito S, Suzuki Y and Brunette DM 1985 Mechanical stretching increases the number of cultured bone cells synthesizing DNA alters their pattern of protein synthesis. *Calcif Tiss Int* **37**:431-436.
- Horton MA and Davies J 1989 Perspectives: Adesion receptors in bone. *J Bone Min Res* **4**:803-807.
- Horton MA, Taylor ML, Arnett TR and Helfrich MH 1991 Arg-Gly-Asp (RGD) peptides and the anti vitronectin receptor antibody 23C6 inhibit dentine bone resorption and cell spreading by osteoclasts. *Exp Cell Res* **195**:368-375.
- Hsieh H, Li N and Frangos JA 1991 Shear stress increases endothelial platelet-derived growth factor mRNA levels. *Am Physiol Soc* **260**:642H-646H.
- Hynes RO 1992 Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**:11-25.
- Ingber D, Prusty D, Frangioni JV, Cragoe EJ, Lechene C and Schwartz M 1990 Control of intracellular pH and growth by fibronectin in capillary endothelial cells. *J Cell Biol* **110**:1803-1811.
- Ingber DE 1991 Integrins as mechanochemical transducers. *Curr Opin Cell Biol* **3**:841-848.
- Ingber DE 1993 Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* **104**:613-627.

- Izumo S, Nadal-Ginard B and Mahdavi V 1988 Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc Natl Acad Sci USA* **85**:339-343.
- Jaconi MEE, Theler JM, Schlegel W, Appel RD, Whright SD and Lew PD 1991 Multiple elevations of cytosolic free-Ca²⁺ in human neutrophils: initiation by adherence receptors of the integrin family. *J Cell Biol* **112**:1249-1257.
- Jones DB and Schlüßbers JG 1987 Evidence that phospholipase C mediates the mechanical strain response in bone. *Calcif Tissue Int* **41**: 4-10.
- Jones DB, Nolte H, Schlüßbers JG, Turner E and Veltel D 1991 Biochemical signal transduction of mechanical strain in osteoblast-like cells. *Biomaterials* **12**:101-110.
- Juliano RL and Haskill S 1993 Signal transduction from the extracellular matrix. *J Cell Biol* **120**:577-585.
- Kabat D, Gliniak B, Rohrschneider L and Polonoff E 1985 Cell anchorage determines whether mammary virus glycoproteins are processed for plasma membranes or secretion. *J Cell Biol* **101**:2274-2283.
- Koch CA, Anderson D, Moran MF, Ellis C and Pawson T 1991 SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science* **252**:668-674.
- Komuro I, Katoh Y, Kaida T, Shibazaki I, Kurabayashi M, Hoh E, Takaku F and Yazaki Y 1990 Stretching cardiac myocytes stimulates protooncogene expression. *J Biol Chem* **265**:3595-3598.
- Komuro I, Katoh Y, Kaida T, Shibazaki I, Kurabayashi M, Hoh E, Takaku F and Yazaki Y 1991 Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. *J Biol Chem* **266**:1265-1268.
- Kornberg L, Earp HS, Turner C, Procop C and Juliano RL 1991 Signal transduction by integrins: increased protein tyrosine kinase phosphorylation caused by clustering of beta 1 integrins. *Proc Natl Acad Sci USA* **88**:8392-8396.

- Letsou GV, Rosales O, Maitz S, Vogt A and Sumpio BE 1990 Stimulation of adenylate cyclase activity in cultured endothelial cells subjected to cyclic stretch. *J Cardiovasc Surg* **31**:634-639.
- Lotz MM, Burdsal CA, Erickson HP and McClay DR 1989 Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J Cell Biol* **109**:1795-1805.
- Marcantonio EE and Hynes RO 1988 Antibodies to the conserved cytoplasmic domain of the integrin β_1 subunit react with protein in vertebrates, invertebrates and fungi. *J Cell Biol* **106**:1765-1772.
- Margolis LB, Novikova IY, Rozovskaya IA and Skulachev VP 1989 K^+/H^+ -antiporter/nigericin arrest DNA synthesis in Ehrlich ascites carcinoma cells. *Proc Natl Acad Sci USA* **86**:6626-6629.
- Meghji S 1992 Bone remodeling. *Br Dent J* **172**:235-242.
- Moyle M, Napier NM and McLean JW 1991 Cloning and expression of a divergent integrin subunit β_8 . *J Biol Chem* **266**:19650-19658.
- Nermut MV, Eanon P, Hirst EMA and Kellje S 1991 Cell/substrate adhesions in RSV-transformed rat fibroblasts. *Exp Cell Res* **193**:382-397.
- Nollert MU, Eskin SE and McIntire LV 1990 Shear stresses increase inositol triphosphate levels in human endothelial cells. *Biochem Res Commun* **170**:281-287.
- Omman GH, Allen RA, Bokoch GM, Painter RG, Traynor AE and Sklar EA 1987 Signal transduction and cytoskeletal activation in the neutrophil. *Physiol Rev* **67**:285-322.
- Otey CA, Pavalko FM and Burridge K 1990 An interaction between α -actinin and the β_1 -subunit *in vitro*. *J Cell Biol* **111**:721-729.
- Pender N and McCulloch AG 1991 Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching. *J Cell Sci* **100**:187-193.
- Reich KM and Frangos JA 1991 Effect of flow on prostaglandin E_2 and inositol trisphosphate levels in osteoblasts. *Am J Physiol* **26**:428C-432C.

- Sachs F 1988 Mechanical transduction in biological systems. *Crit Rev Biomed Eng* **16**:141-169.
- Sandy JR and Harris M 1984 Prostaglandins and tooth movement. *Eur J Orthod* **6**:175-186.
- Sandy JR, Farndale RW and Meikle MC 1993 Recent advances in understanding mechanically induced bone remodeling and their relevance to orthodontic theory and practice. *Am J Ortho Dentofac Orthop* **103**:212-222.
- Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB and Parsons JT 1992 pp125 FAK, a structurally unique protein tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci USA* **89**:5192-5196.
- Tawil N, Wilson P and Carbonetto S 1993 Integrins in point contacts mediate cell spreading: factors that regulate integrin accumulation in point contacts vs focal contacts. *J Cell Biol* **120**:261-271.
- Uitto VJ, Larjava H, Peltonen J and Brunette DM 1992 Expression of fibronectin and integrins in cultured periodontal ligament epithelial cells. *J Dent Res* **71**:1203-1211.
- Vandenburg HH, Hatfaludy S, Sohar I and Shansky J 1990 Stretch-induced prostaglandins and protein turnover in cultured skeletal muscle. *Am J Physiol* **259**:232C-240C.
- Virtanen I, Korhonen M, Karineimi A, Gould VE, Laitinen L and Yläne J 1990 Integrins in human cells and tumors. *Cell Diff Dev* **32**:215-228.
- Vogel BE, Tarone G, Giancotti FG, Gailit J and Ruoslahti E 1990 A novel fibronectin receptor with an unexpected subunit composition ($\alpha_v\beta_1$). *J Biol Chem* **265**:5934-5937.
- Wirtz HRW and Dobbs LJ 1990 Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells. *Science* **250**:1266-1269.
- Yläne J, Cheresch DA and Virtanen I 1990 Localization of β_1 , α_5 , α_v and α_{Ib} subunits of the integrin family in spreading human erythroleukemia cells. *Blood* **76**:570-577.

Zetter BR and Brightman SE 1990 Cell motility and the extracellular matrix. *Curr Opin Cell Biol* 2:850-856.

Figure 1. Phase contrast micrograph of HOS TE-85 cells in culture. (a) Cells at low density prior to mechanical strain. (b) Cells reaching the confluency stage after 30 hrs in culture. Note the compact shape of the cells in the center. (c) Strained cells after 72 hours of initial plating. Single black arrows indicate the presence of bone nodule as a white spot. (d) One week after initial plating the cells are completely immersed by matrix. Note von Kossa stain of the mineralized nodule indicated by single black arrows. White arrows indicate the direction of strain. Magnifications (a-d) X 80.

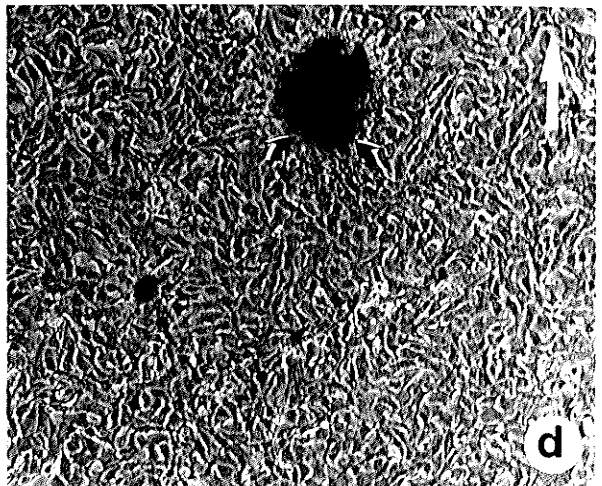
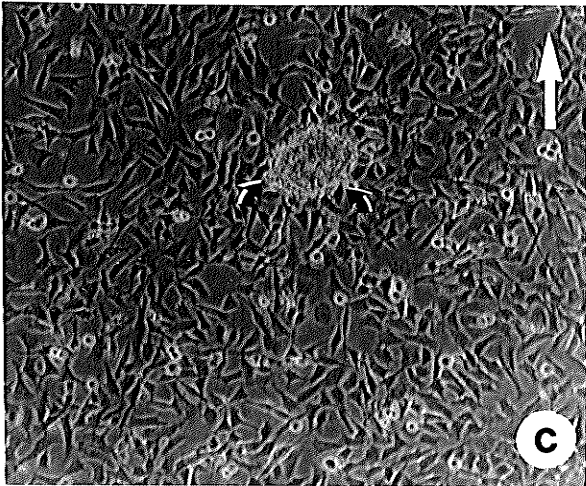
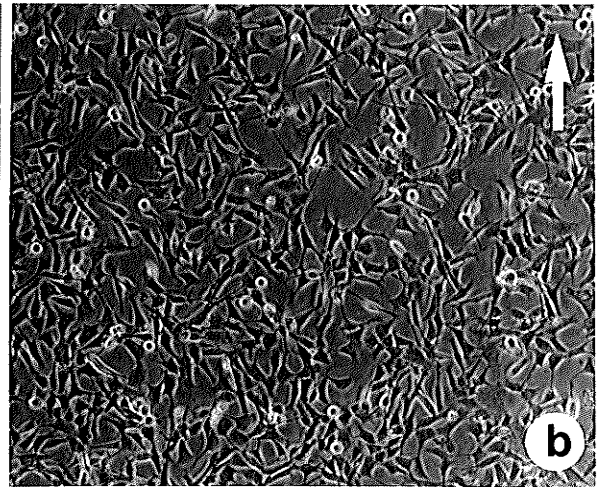


Figure 2. Effects of mechanical strain in bone nodule formation in HOS TE-85 cells. The sequence shows photomicrographs of strained and unstrained HOS cells. Strain was applied in (a), (b), (c) and (d), and unstrained controls were (e), (f), (g) and (h). The von Kossa stain indicated that individual nodules from days 2 (a), (e) and 7 (b), (f) gradually increased in area to form large black areas in the later strain and unstrained periods of days 21 (c), (g) and 28 (d), (h). At all time periods, there was an increase in nodule formation in strained when compared to unstrained cultures. Black arrow indicates direction of strain. Magnifications (a-h) X 10.

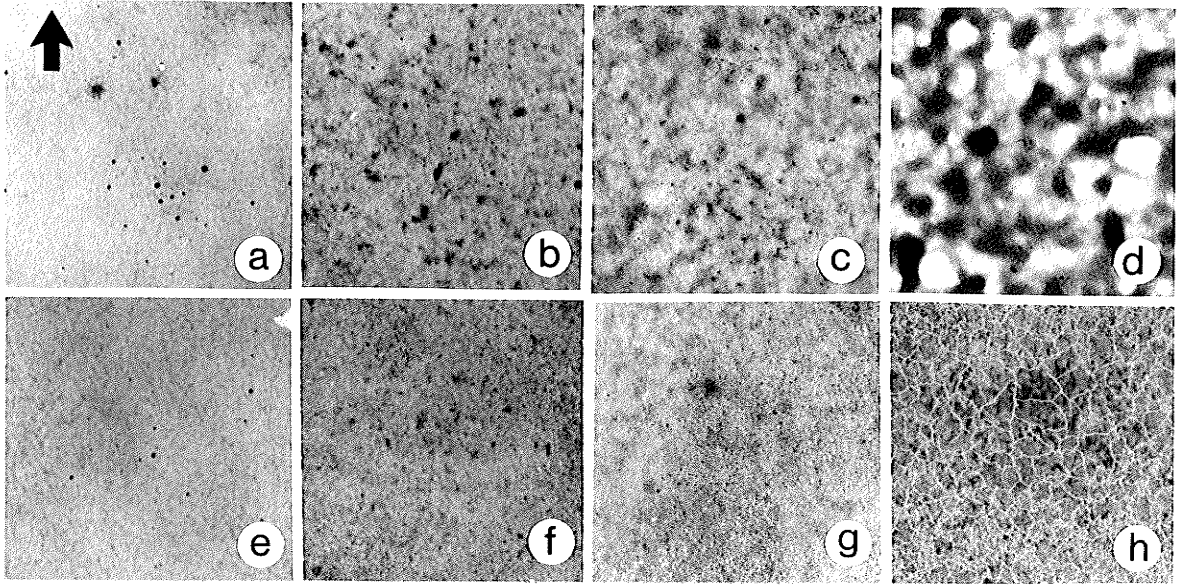


Figure 3. Effect of mechanical strain in the cell count number of HOS TE-85 cells. Note the increase in cell numbers through the time periods studied. At 2, 14 and 28 days of culture, the numbers of mechanically strained cells were significantly greater (* $p < 0.05$) when compared with those of unstrained controls.

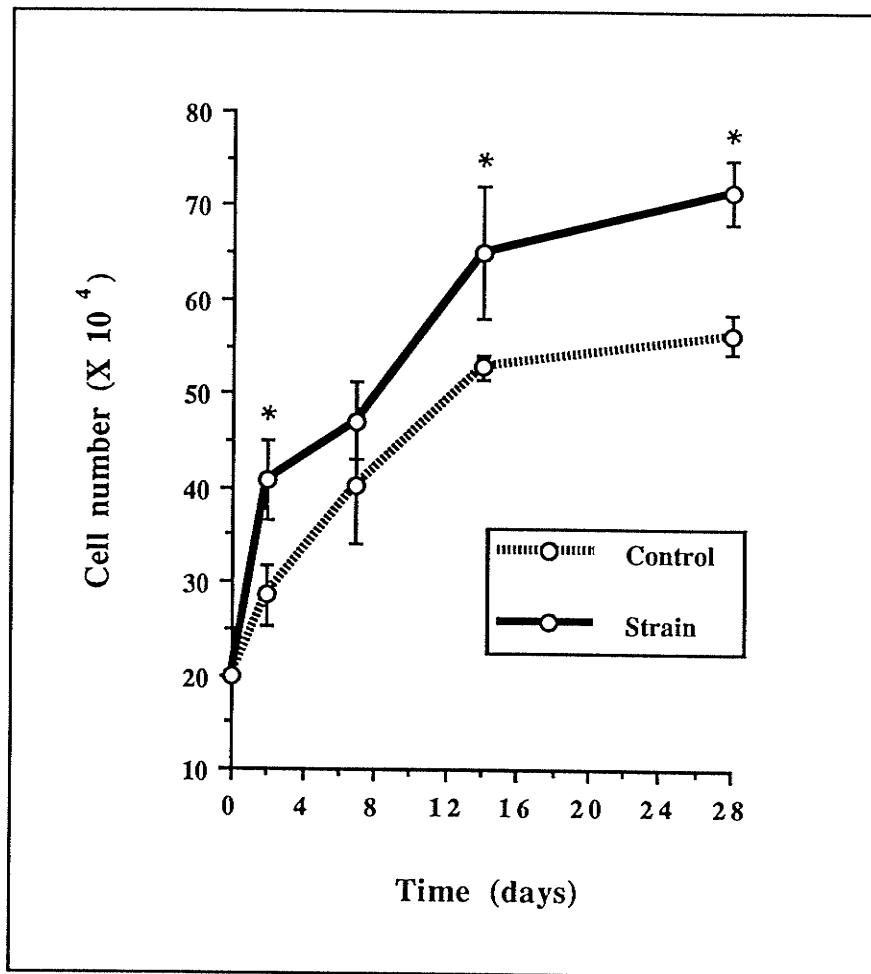


Figure 4. Immunolocalization studies of HOS TE-85 cells with antibodies anti-talin, anti-vinculin, anti-vimentin and anti-tensin. (a) Control unstrained cultures of talin, showed an increase in the focal contact areas when these cells were mechanically strained as evidenced by double white arrows, in (b). Similarly, this phenomenon appeared to take place for anti-vinculin labeling in control unstrained (c) and strained cells (d). Note the concentration of striations in the focal contact areas (double white arrows). (e) Labeling of antibodies anti-vimentin in control cultures showed less marked differences in labeling to the strained cultures (f). On the other hand tensin, appeared to increase in labeling when control cultures (g) were subjected to mechanical strain (h). Single white arrow indicates the direction of strain. Magnification (a-h) X 1250.

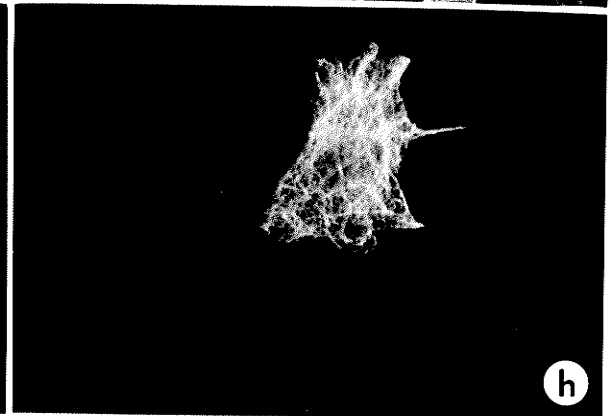
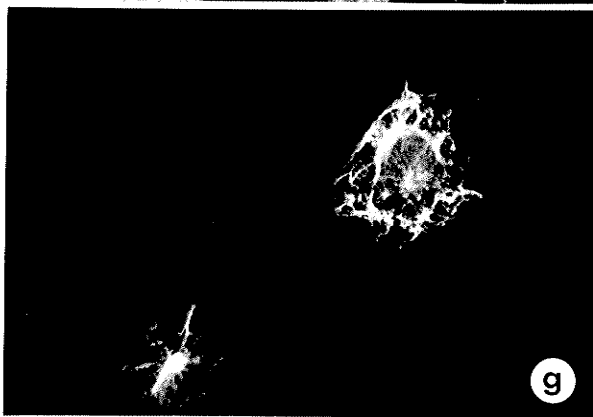
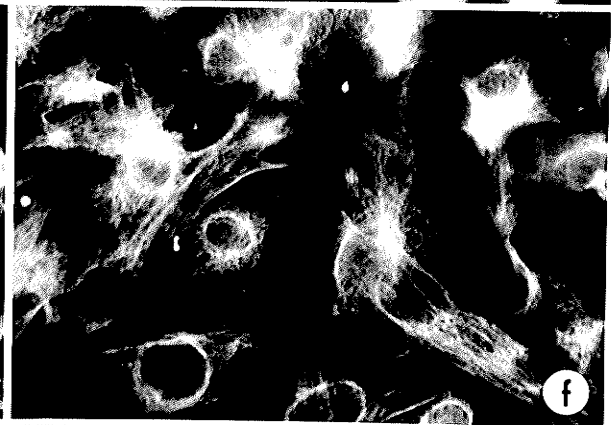
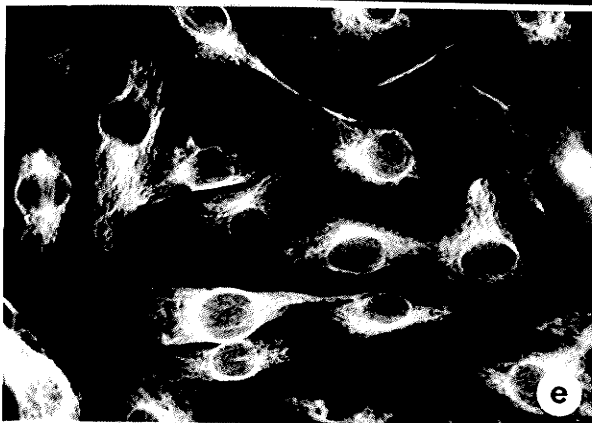
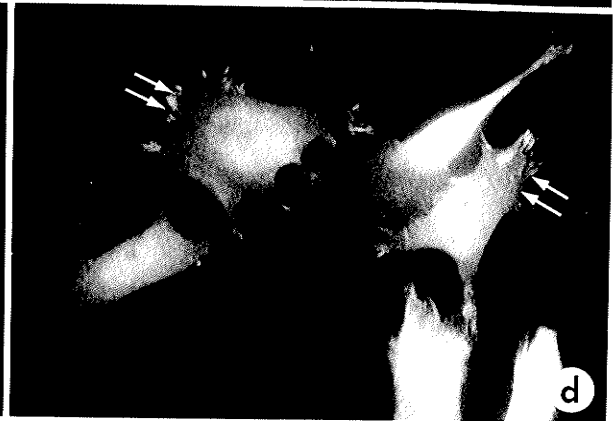
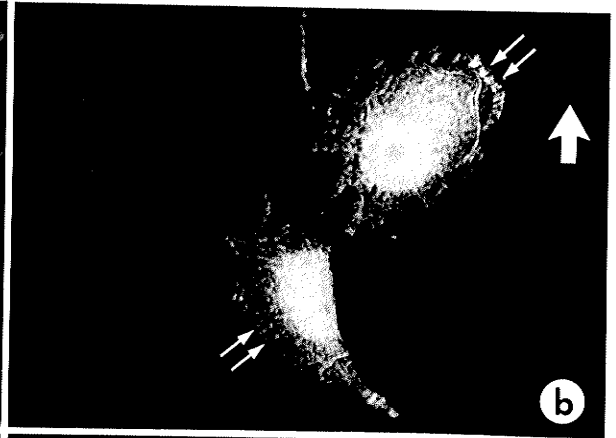
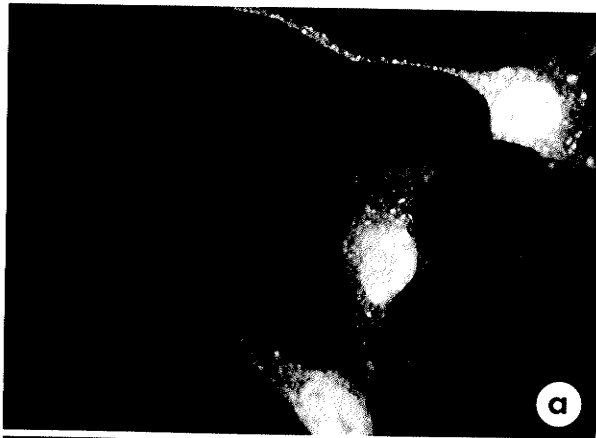


Figure 5. Immunofluorescence staining of mechanically strained and unstrained HOS TE-85 cells grown *in vitro*. Labeling of unstrained (a) and strained (b) cultures with α_2 -integrin was similar than those of unstrained (c) and strained (d) cultures labeled with α_3 -integrin. Note the presence of large clusters in the periphery of the cells (double white arrows). The labeling for α_v (e) and (f) and β_1 -integrin (g) and (h) appeared slightly different, and in β_1 -integrin labeling, no cellular extensions could be seen. However, mechanical strain appeared to change the distribution of α_v (f) and β_1 -integrin (h) compared with unstrained controls (e) and (g), respectively. Single white arrows indicates the direction of strain. Magnifications (a-h) X 1875.

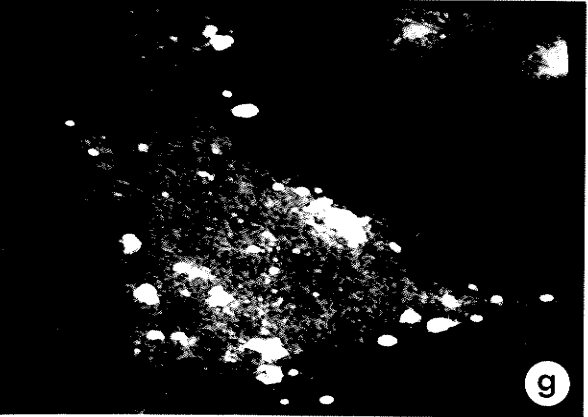
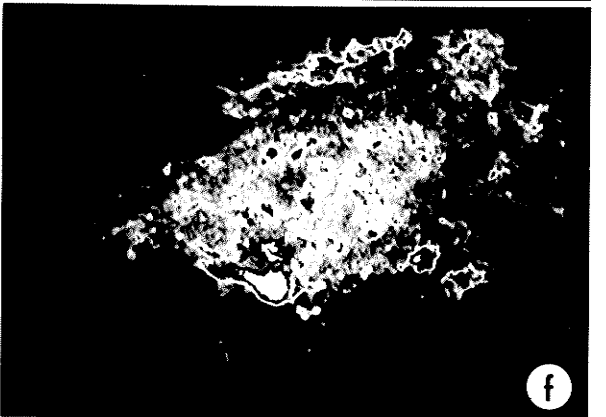
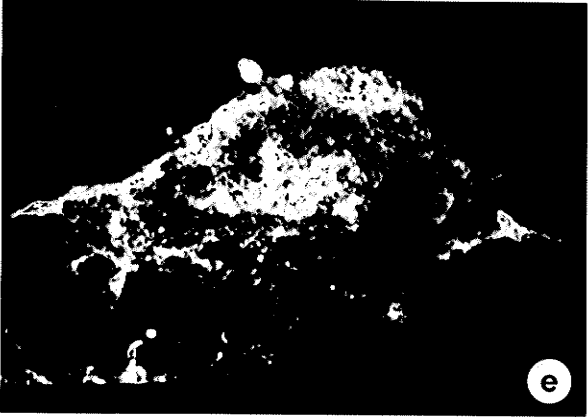
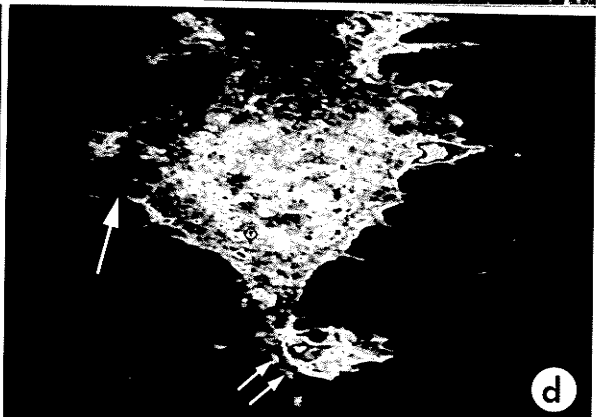
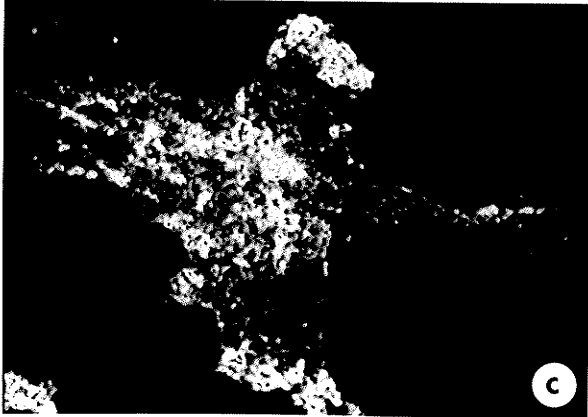
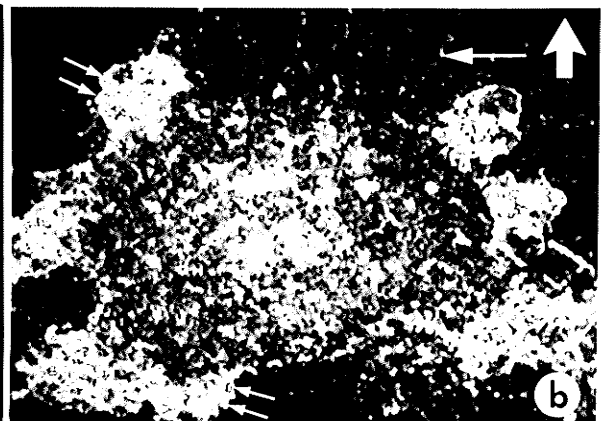
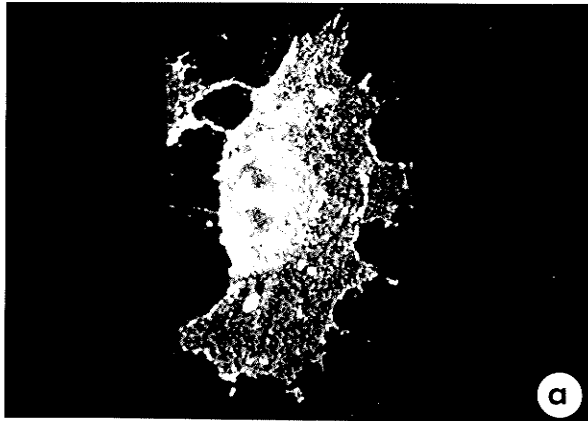
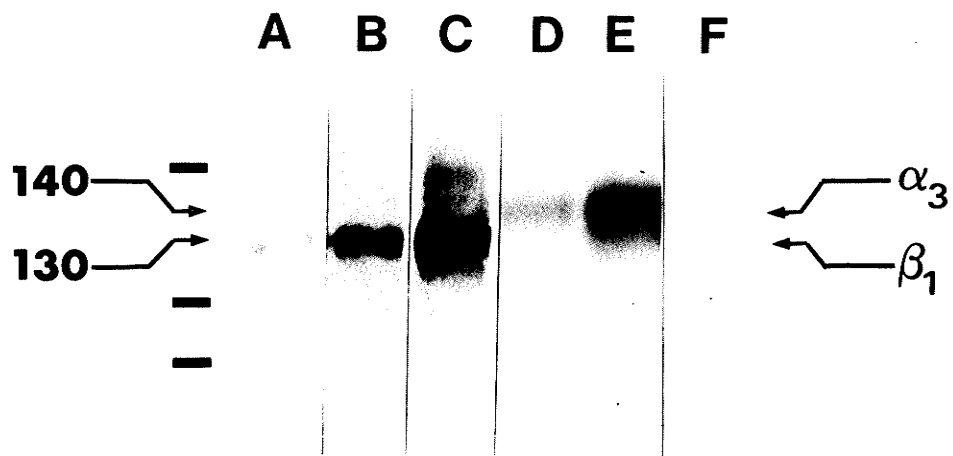
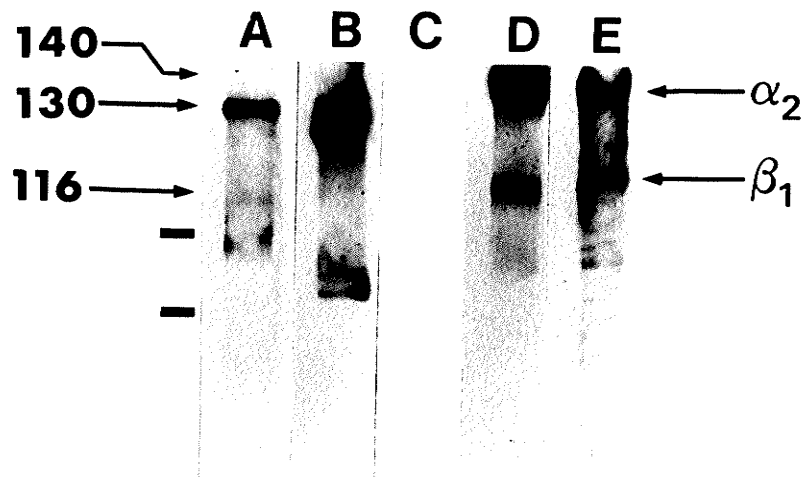


Figure 6. Immunoprecipitation analysis of talin, vinculin and vimentin in strained and unstrained HOS TE-85 cells grown in culture. The integrins were immunoprecipitated with antibodies against talin, vinculin and vimentin and resolved by SDS gel electrophoresis under non-reduced conditions. (Lane A) normal rabbit serum; talin immunoprecipitated from strained (Lane B) and unstrained cells (Lane C). (Lane D) normal rabbit serum; vinculin immunoprecipitated from strained (Lane E) and unstrained cells (Lane F). (Lane G) normal rabbit serum. Vimentin immunoprecipitated from strained (Lane H) and unstrained cells (Lane I). Molecular mass markers (200, 97.4, 67.5 and 44 KD) are indicated by bars on the left.

Figure 7. Immunoprecipitation analysis of integrin subunits in strained and unstrained HOS TE-85 cells grown in culture. These cells express the subunits α_2 , α_3 , α_v and β_1 in both strained and unstrained cultures. (A) Cells grown for 72 hours and strained for the last 24 hours of culture. Unstrained used as controls. (Lanes A and F) cells incubated with normal rabbit serum; (Lane B) unstrained and (Lane C) strained cells immunoprecipitated with anti- β_1 antibodies, non-reduced; (Lane D) unstrained and (Lane E) strained cells immunoprecipitated with anti- α_3 antibodies, non-reduced. Immunoprecipitation with anti- α_v and anti- α_2 antibodies in non-reduced conditions seemed to co-migrate with anti- β_1 . (B) (Lane A) unstrained and (Lane B) strained cells (6 hours) immunoprecipitated with anti- β_1 antibodies, non-reduced; (Lane C) cells immunoprecipitated with rabbit normal serum; (Lane D) strained and (Lane E) unstrained cells (6 hours) immunoprecipitated with anti- β_1 antibodies, reduced. Molecular mass markers (200, 97.4 and 67.5 KD) in (A) and (97.4 and 67.5 KD) in (B) are indicated by bars on the left.

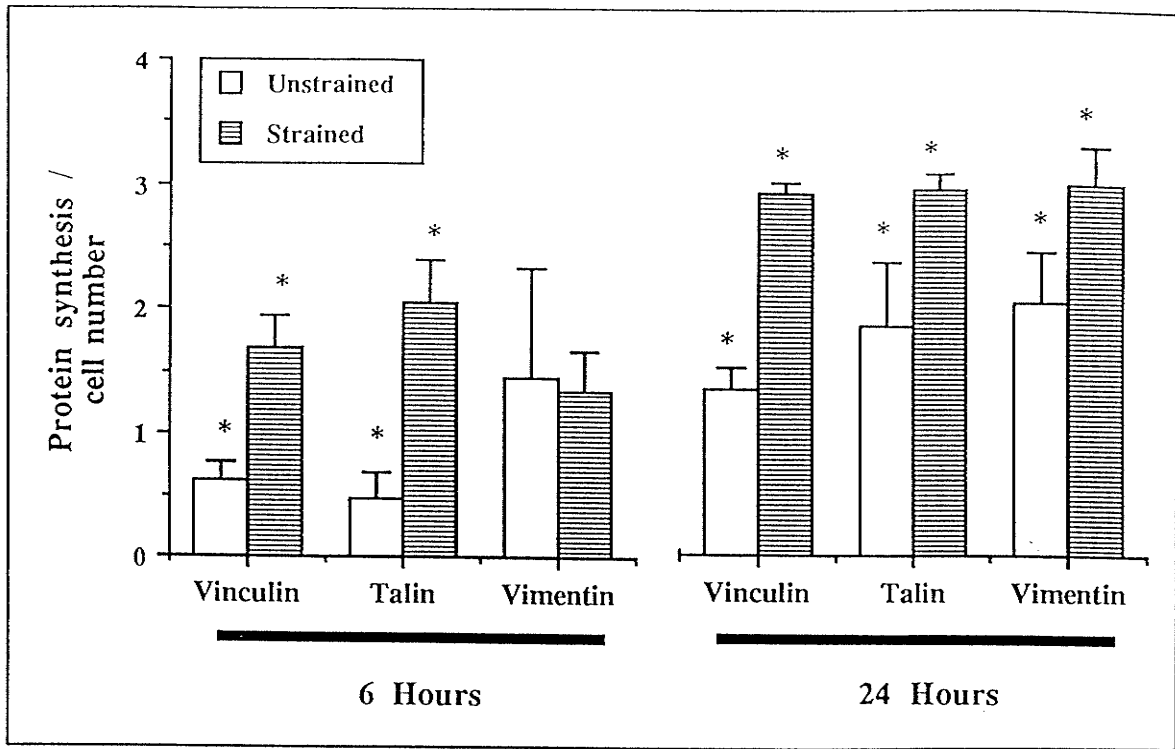


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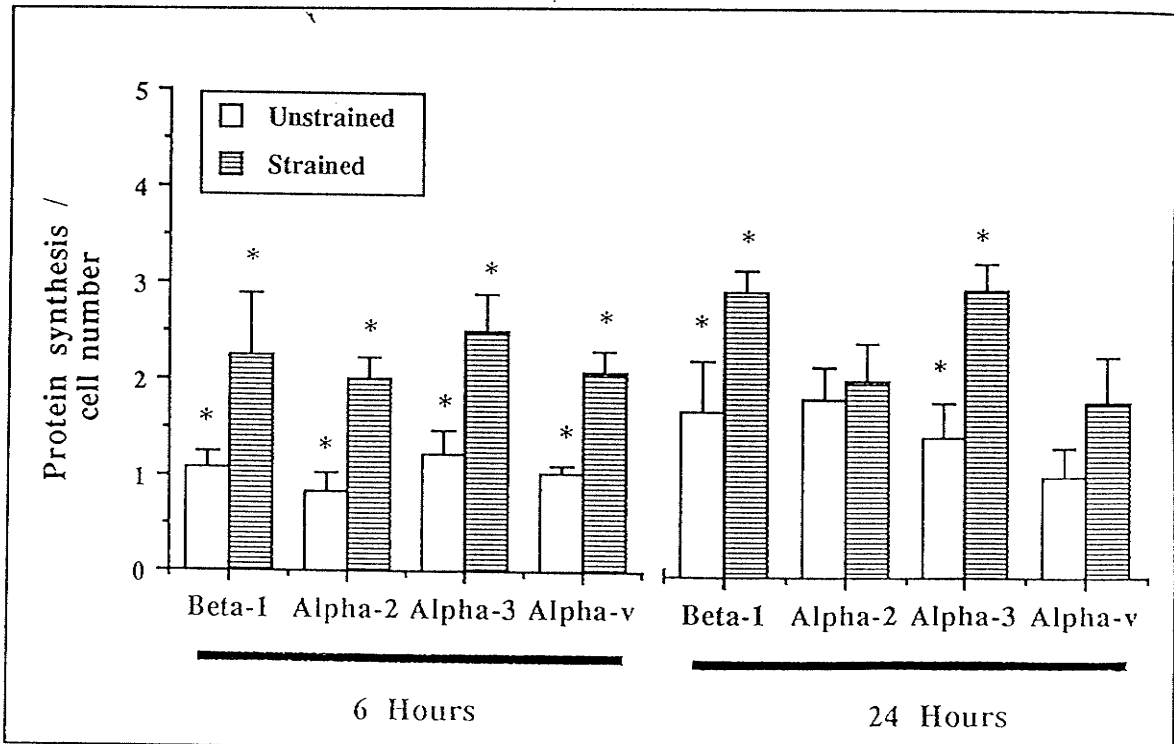


B

Figure 8. **(A)** Effect of mechanical strain in the synthesis of talin, vinculin and vimentin. (a) 6 hours and (b) 24 hours of culture. **(B)** Effect of mechanical strain in the synthesis of integrin subunits. (a) 6 hours and (b) 24 hours of culture. * indicates significantly greater ($p < 0.05$) in strained cultures when compared with unstrained controls.



A



B



Chapter FIVE

Connective Tissue Response to Mechanical Stimulation

Carvalho RS, Bumann A, Scott JE and Yen EHK

In: McNeil C, ed. Occlusion: Science and Practice,
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INTRODUCTION

The behavior of cells and tissues in the body is determined to a large extent by their surrounding biological environment. The immediate cellular environment, provided by the extracellular matrix (ECM) via specialized scaffoldings such as basement membranes, collagen matrix, cartilage and bone (Lotz *et al.*, 1989; Ingber, 1991) characterizes the cellular responses. From empirical observations into the effects of forces on bones (Wölff, 1892) to studies in weightlessness (Roberts *et al.*, 1982; Artner-Dworzak *et al.*, 1993; Mittelstaedt *et al.*, 1991) the effects of mechanical stimulation on connective tissues, especially bone tissue, are regarded as beneficial. It is a dynamic strain environment that creates the most appropriate conditions for connective tissue cells. Several studies *in vitro* have reported the effects of mechanical stimulation on different connective tissues and connective tissue cells such as fibroblasts (Yen *et al.*, 1990; Almekinders *et al.*, 1993), osteoclasts (Chiba, 1989; Burger *et al.*, 1991; Suda *et al.*, 1993), chondroblasts (Copravay *et al.*, 1985; Klein-Nulend *et al.*, 1986), osteoblasts (Sömjem *et al.*, 1980; Brunette, 1984; Hasegawa *et al.*, 1985; Sandy, 1989; Jones *et al.*, 1991; Brighton *et al.*, 1991; Brighton *et al.*, 1992; Sandy, 1993; Carvalho *et al.*, 1994a) and others. However, the question remains as to how much stimulation is considered beneficial to cells and tissues, or which parameters (i.e. periodicity, magnitude or frequency of the applied stimulus) are likely to characterize cell response. For instance, it is known that the osteogenic cellular synthesis of bone proteins appears to be activated preferentially by intermittent rather than continuous strain (Lanyon, 1984) and frequency appears to be as important to remodeling as is the range of stress which is applied to bone (Rubin *et al.*, 1989). And finally, how is the mechanical stimulation perceived by connective tissue cells and transduced into a recognizable cellular "language"?

To date, although mechanical stimulation has been shown to alter the metabolism of connective tissue cells (Lanyon, 1984; McLeod *et al.*, 1987), the mechanisms controlling the adaptations of these cells to mechanical stimuli are poorly understood. This paper

reports a summarized review from recent work in our and other laboratories. From our studies into the effects of mechanically stimulated organ and tissue cultures of osteoblasts, we have observed that mechanical stimulation both *in vivo* and *in vitro* results in biochemical modifications and adaptation in structure and function of the cells. This paper examines the present state of knowledge on the perception and transduction of mechanical stimulation into biochemical responses. In addition to different pathways of biochemically active mechanically-derived "second messengers" and structural proteins, the molecular basis of mechanical stimulation and cell response will also be reviewed. Finally, we attempt to present the clinical relevance of the basic information described here to the practitioner involved with the science and practice of occlusion and jaw mechanics.

MECHANOSENSITIVE MEMBRANE CHANNELS

The mechanisms by which the cells sense the mechanical environmental stimulation are open to question. Little is known about perception of the stimulation, however, some evidence exists of the presence of so-called "strain-sensitive cells". These cells would yield strain-induced, or related potentials to be measured as physiological signals (Basset *et al.*, 1964; Guharey and Sachs, 1984). Additional study is necessary to identify the cells which could ultimately play the primary roles in skeletal remodeling in response to strain.

Mechanically-activated cell response is more likely to take place through receptor-mediated cell signaling. Signal transduction may occur by structural deformation of the membrane components through the presence of the so-called membrane mechanotransducers (Guharey and Sachs, 1984; Sachs, 1988). These are ion membrane channels which may in fact behave as strain-sensitive channels (Guharey and Sachs, 1984; Lansman *et al.*, 1987; Sachs, 1988; Jones *et al.*, 1991). There are basically two categories for selectivity of stimulatory ion channels in mammalian cells: a) those nonselective for divalent cations and b) those with priority of specificity for selected ions (i.e. Ca^{2+} , K^{+}) (Watson, 1991). However, it appears that some ion channels are inactivated by strain or stretch. Speculation on the role of these channels varies from regulatory responses followed by cellular changes in osmolarity (Morris, 1990), and may include possible participation of cytoskeletal structures (Sachs, 1991; Sokabe *et al.*, 1991). These channels have been shown to exist in a variety of cell types, howbeit, not much is known about the biological processes that may be regulated or coupled to the increase of current in stretch-activated channels (Watson, 1991). In fact, it has been suggested recently that these channels are artifacts resulting from the patch-clamping technique (Jones *et al.*, 1991; Morris and Horn, 1991). The role of stimulation on receptor-mediated cell response will be dealt with in more detail in the following sections of this review.

CYTOKINES AND LEUKOTRIENES

Cytokines and leukotrienes may play an important role in the mediation of the specific response generated in mechanically-stimulated tissue. Cytokines are small molecules produced by cells which modify the behavior of other cells (Collet and Stewart, 1991). The cytokines that may influence mechanically-stimulated remodeling of connective tissue include all interleukins (ILs), tumor necrosis factors (TNFs), interferons (INFs), colony stimulating factors (CSFs) and several growth factors (GFs). These molecules were originally believed to be signal molecules produced by leukocytes, serving primarily for communication between immune system cells (Davidovitch, 1991).

Among the cytokines known to date, IL-1 (both α and β types) is the most prominent and is thought to be very important in mediating the effects of mechanical stimulation. This IL enhances various immune responses, such as B-lymphocyte differentiation, antibody secretion and T lymphocyte differentiation (Collet and Stewart, 1991; Meikle *et al.*, 1989). The effects of tensile stress on the production of the IL-1 by mechanically-stimulated fibroblasts, showed that IL-1 plays a regulatory response by enhancing the production of prostaglandin E (PGE) and has an additive effect on bone resorption activity (Davidovitch, 1991; Saito *et al.*, 1991). There appear to be two mechanisms whereby IL-1 stimulates PGE synthesis: hydrolysis of membrane phospholipids by activation of specific phospholipases and increased conversion of arachidonic acid into PGs by cyclooxygenase (Saito *et al.*, 1991; Korn *et al.*, 1989). Hence, simultaneous stimulation of both phospholipase and cyclooxygenase may explain the IL-1-enhanced PGE production (Davidovitch, 1989). Bone resorption activity may also be increased by other cytokines. TNF- α is a potent stimulator of bone resorption, stimulating secretion of IL-1 and increasing leukocyte adherence (Gamble *et al.*, 1985). Whereas INF- γ , has been shown to interfere with resorptive processes and to antagonize the action of a number of growth factors (Gowen and Mundy, 1986).

Leukotrienes (LTs) are also derived from inflammatory cells, through the transformation of arachidonic acid by the enzyme 5-lipoxygenase. LTs fall into two classes, differing in both their chemical and biological properties (Piper and Samhoun, 1987; Collet and Stewart, 1991): they are the cysteinyl-containing leukotrienes, LTC₄, LTD₄ and LTE₄ and the dihydroxy acid LTB₄. Each product has actions of vasoconstriction in some vascular beds, vasodilatation in the femoral circulation, increased vascular permeability as well as others. Some evidence of the action of leukotrienes in connective tissue remodeling comes from the work of Collins *et al.* (1987), who showed that application of a 5-lipoxygenase inhibitor, piroprost, enhances mechanically-induced bone formation. Other inhibitors applied in conjunction with mechanical stimulation of the teeth of experimental animals appears to cause a decrease in LT levels (Mohamed *et al.*, 1989).

The mechanisms of mechanically stimulated response seem to involve a series of interactions among cells of the nervous, immune and endocrine systems. Several neurotransmitters which may be implicated include substance P (SP), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and methionine enkephalin (ME) (Davidovitch, 1991). These neuropeptides are often found in sites of inflammation, some apparently stimulate bone resorption such as VIP, while others assume a mediatory role in the transmission of painful sensations in the primary afferent system, such as SP. It has been suggested (Davidovitch, 1991) that one pathway whereby mechanical stimulation causes cellular activation depends on a release of SP from sensory nerve endings adjacent to stimulated cells, followed by fluid movement distorting matrix and cells (Charles *et al.*, 1991). The vasodilatation caused by SP increases PG release, leukocyte migration and secretion of lymphokines, which together with "second messengers" (described below) will play important roles in mechanically-induced cell response. Although, there is growing evidence that selected mechanically-induced responses in connective tissue cells have an inflammatory component (i.e. orthodontic tooth movement), such responses are

not widespread in the mechanisms for maintenance of cellular phenotype. However, the influence of cytokines and leukotrienes in mechanically-stimulated tissues is very important and the corresponding regulatory process *in vivo* will certainly depend in part on a series of interactions among these factors.

ROLE OF SECOND MESSENGERS

The application of mechanical stimulation in connective tissues has been shown to stimulate the activation of a series of signaling molecules known as "second messengers". These messengers describe a limited number of intracellular molecules produced or activated to translate the responses of a wide range of primary external stimuli. These intracellular "transducers" are molecules such as Ca^{2+} (Charles *et al.*, 1991; Jones *et al.*, 1991; Watson, 1991) cyclic adenosine monophosphate (cAMP) from the adenylyl cyclase pathway (AC) (Davidovitch *et al.*, 1984; Saito *et al.*, 1991; Scott *et al.*, 1993; Scott *et al.*, 1994), cyclic guanosine monophosphate (cGMP) from the the guanylate cyclase pathway (GC) (Bourne *et al.*, 1989), inositol trisphosphate (IP_3) and protein kinase C (PKC) from the phosphatidylinositol pathway (PI) (Jones and Schlüßers, 1987; Bourne *et al.*, 1989; Brighton *et al.*, 1991), ions induced with the Na^+/H^+ exchanger (Ingber, 1991; Watson, 1991) and other combinations of membrane-associated receptors. Be that as it may, the two main second messenger pathways are the adenylyl cyclase and the phosphatidylinositol pathways. It is the combination of these pathways with subsequent enzyme cascades and protein phosphorylation events that specifically translate extracellular stimulation into some form of cellular activity.

A consensus exists that mechanical forces stimulate the levels of probably most of these messenger units corresponding to the activation of enzymes such as phospholipase C (PLC) and A_2 (PLA) (Jones *et al.*, 1991; Brighton *et al.*, 1992) and PKC (Jones and Schlüßers, 1987; Jones *et al.*, 1991). Although, mechanical stimuli appear to elicit a hierarchical sequence of responses as seen *in vitro*, the interrelationship of molecules and activities in different pathways has masked the activity of individual pathways. Therefore, most studies attempt to isolate specific pathway responses to mechanical stimulation. Classically, second messenger molecules for mechanical transduction have been attributed to the AC pathway. A schematic diagram of this pathway is seen in Fig. 1. It is now known that application of mechanical strain *in vitro* in osteoblastic cells stimulates cAMP

levels but only after 5 minutes and reaching its peak at 10 minutes of strain (Carvalho *et al.*, 1994a). Jones and Schlüßbers (1987) found a similar response, which contradicts earlier studies that focused on cAMP as the primary signal in strained cells (Coprey *et al.*, 1985; Binderman *et al.*, 1988; Davidovitch, 1991; Watson, 1991; Scott *et al.*, 1993). This relatively delayed response of an increase in cAMP levels to mechanical stimulation suggests that cAMP is probably a "late" cellular response to strain. On the other hand, it appears that application of mechanical strain causes a rapid increase in the phosphatidyl inositol phospholipid hydrolysis (PI pathway) almost immediately after the application of strain (Jones *et al.*, 1991; Brighton *et al.*, 1992). Only recently has this pathway been correlated with mechanical stimulation *in vitro* (Jones *et al.*, 1991; Sandy and Farndale, 1991), and a diagram of this pathway can be seen in Fig. 2. Cell response through an increase in IP₃ levels may be initiated in seconds (Jones *et al.*, 1991; Sandy and Farndale, 1991). Such response is also evidenced by the specific activity of PKC, itself a result of PLC activation (Sandy, 1989). While mechanical strain appears to increase IP₃ levels after one minute from initiation of strain compared with later periods of 10 and 30 minutes, PKC activity does not reach a peak until 5 minutes of strain (Jones *et al.*, 1991). The effects of mechanical stimulation in the levels of cAMP and IP₃ and in the activity of PKC in bone cells have been shown previously (Carvalho *et al.*, 1994a). It is important to mention that although the two arms of the phosphatidylinositol pathway have independent functions, IP₃ and PKC may act synergistically to heighten cellular response, as has been shown in the induction of serotonin secretion in platelets (Morgan, 1989). In addition to this rapid stimulation in IP₃ levels, the earliest change in connective tissue cells exposed to mechanical stimulation appears to be the Ca²⁺ influx across the plasma membrane. This influx occurs within seconds of mechanical stimulation, however such changes may not constitute a separate pathway of response. On the other hand, Ca²⁺ participates in a series of responses including IP₃-Ca²⁺ mobilization from intracellular stores during a number of

hormonally regulated metabolic events and/or mechanical stimuli (Yamaguchi *et al.*, 1988; Reich and Frangos, 1991; Arora and McCulloch, 1993).

The activation of both adenylyl cyclase and phosphatidylinositol pathways is tied to G protein-mediated receptor stimulation as shown diagrammatically in Figs. 1 and 2. These G proteins are heterotrimeric membrane-bound transducing proteins which bind guanosine triphosphate (GTP) in place of guanosine diphosphate (GDP) and can be stimulatory (Gs) or inhibitory (Gi). Down stream release of the messengers only takes place after a conformational change of G protein-associated receptors with effector binding (Rubin and Hausman, 1988; Brighton *et al.*, 1991). There are currently two models for the kinetics of receptor-effector binding. These are the "precoupled" and the "collision coupling" models (Levitzki and Bar-Sinai, 1991; Gross and Lohse, 1991). Incidentally, the application of mechanical strain in osteoblastic cells may increase the receptor diffusion process. The effects of mechanical stimulation in membrane-bound receptor signaling will be further discussed in "morphological alterations in cell structure".

Arachidonic acid metabolites, such as prostaglandins (PGs), and circulating hormones, such as parathyroid hormone (PTH) appear to play an important role in mechanically-stimulated second messenger response. Combinations of either PGE₂ or PTH with mechanical stimulation may enhance the effects of stimulation alone (Jones *et al.*, 1991; Kasugai *et al.*, 1991). However, their action may not be comparable. Farndale *et al.* (1988) showed that stimulation with PGE₂ gave a similar response to that of PTH in both the phosphatidylinositol and adenylyl cyclase pathways. However, cAMP and IP₃ were not stimulated to the same degree. This latter observation is interesting, since osteoblasts have been shown to carry PTH receptors directly bound with the G-proteins which initiate both cAMP and IP₃ pathways (Fujimori *et al.*, 1992). The activities of PTH and PGE₂ may therefore, be comparable but not wholly coincident (Boland *et al.*, 1986). Ca²⁺ elevation has also been shown to increase as a result of PTH but not PGE₂ stimulation (Boland *et al.*, 1986; Farndale *et al.*, 1988).

Functional involvement of the second messenger pathways are closely related to protein phosphorylation and the enzyme cascades indicated above. Enzymes such as PKC (Beckerle, 1990; Woods and Couchman, 1992) and other signal transduction-derived messengers such as cAMP-dependent kinases (Turner *et al.*, 1989; Woods and Couchman, 1992) are known to phosphorylate integral cell membrane and cytoskeletal proteins. Activation of PKC may determine the formation of focal adhesions (Burrige and Connel, 1983; Burrige, 1986; Burrige *et al.*, 1988), changes in cytoskeleton aggregation (Burrige and Connel, 1983; Burrige *et al.*, 1988; Carvalho *et al.*, 1994a) and membrane-binding specificity with regards to different substrates (Derventzi *et al.*, 1992). However, it has been shown that inactivation of PKC-mediated phosphorylation causes an inhibition of focal contact organization, which does not occur when cAMP kinase phosphorylation are inhibited (Woods and Couchman, 1992). It is conceivable that strain enhances the translocation of PKC to focal adhesion sites (Carvalho *et al.*, 1994a). Although the exact role of PKC in the formation of focal adhesions is not yet known, PKC may stabilize the initial association of the cytoskeleton with the focal contacts and with the membrane receptor proteins (Woods and Couchman, 1992).

MORPHOLOGICAL ALTERATIONS IN CELLULAR STRUCTURE

Stimulation of signaling mechanisms by mechanical strain involves various cellular components. Of special interest, the changes in the cellular ECM environment may cause "deformation", altering cell shape (Burrige *et al.*, 1988) with significant cytoskeleton rearrangement (Buckley *et al.*, 1988; Ben-Ze'ev, 1991; Pender and McCulloch, 1991; Gingel and Owens, 1992). However, the transduction of mechanical stimulation will most likely enter the cell through a cell membrane-associated response.

ECM-Cell Interactions

The monitoring of specific membrane-bound receptor changes is paramount for the elucidation of the missing link between ECM stimulation and cellular response. Bone cells, like most other cells, attach to ECM molecules including collagen, laminin, fibronectin and vitronectin. This interaction occurs through cell-surface receptors, which connect the extracellular protein network to the intracellular cytoskeleton as an integral component in signal transduction (Hynes, 1992). Many of these membrane receptors belong to the integrin superfamily (Hynes, 1992). All integrins are heterodimers formed by an α and a β subunit; 14 α and 8 β units have been characterized. Different subunit combinations function as receptors for attachment of different cell types to different extracellular proteins (Clover *et al.*, 1992). Each subunit possesses a large extracellular domain, a transmembrane domain and a short cytoplasmic domain (Gailit *et al.*, 1993). The cytoplasmic region interacts with several intracellular proteins (Burrige *et al.*, 1988; Hynes, 1992). The attachment properties of integrins are given by their affinity for the amino acid sequence arginine-glycine-aspartate (RGD) in their ECM protein ligand (Kabat *et al.*, 1985). It has been suggested that the manner by which these subunits bind the ECM is critical to cell function (Horton and Davies, 1989; Horton *et al.*, 1991). Thus, integrins are likely candidates for transducing mechanical strain-induced signals into a morphogenetic cell response.

The application of oxidative strain on integrin subunits from cells cultured *in vitro* appears to cause redistribution and dysfunction of integrins (Gailit *et al.*, 1993). Mechanical strain may also cause an increase in the synthesis and a change in the distribution of selected integrin subunits in bone cells (Carvalho *et al.*, 1994b). In our laboratory, isolated bone cells have been labeled with monoclonal antibodies against α and β integrin subunits. Application of mechanical stimulation causes a change in point and focal distribution of these subunits as seen in Figure 3. Studies in other cells also show an increase in protein synthesis with mechanical strain (Komuro *et al.*, 1990). As suggested by Milam *et al.* (1991) the changes in the relative abundance of integrins expressed on the cell surface may have a profound effect on the phenotype of a particular cell. Haskin and Cameron (1993) in a study using physiologic levels of hydrostatic pressure also showed increases in selected integrin subunits in bone-like cells. Quantitation of integrin subunits of bone cells *in vitro* from unstrained and strained cells was shown in Carvalho *et al.* (1994a). In addition, it appears that the environmental demands may not only determine protein synthesis and distribution, but also the regulation of integrin specificity and function. Cress *et al.* (1990) showed that cells are inhibited to bind to vitronectin when heat shock stress was applied. Loss of specificity may be due to a change in the combination of integrin $\alpha\beta$ heterodimers when the cells are subjected to mechanical stimulation.

Although integrin subunits appear to be affected by different types of stimulation, the mechanism responsible for integrin changes is not clear. While some investigators support a mechanical disruption of receptor subunits, others favor a biochemical pathway involving the second messenger systems discussed above. Nevertheless, a hypothesis combining mechanical activation of receptors to biochemical activation of G-proteins and phosphorylation cascades is perhaps more plausible.

In addition to integrins there are other less well characterized cellular membrane-associated receptors. These include cadherins, the binding of IgG to the high-affinity Fc

receptor, the glycoprotein complexes CD44 and CD45, Na⁺/K⁺ ATPases, the estrogen growth factor receptors, the glycoprotein 1b/9 and others (Stossel, 1993 for a review). However, to date there is very little information on any of these receptors mediating any mechanical stimulation-induced cellular signal transduction.

Cell Receptors-Cytoskeleton Interactions

The membrane-associated receptors, in particular integrin receptors, form the physical link from the ECM to the associated cytoskeleton proteins which most likely contribute to a structural and biochemical combination of strain signals (Beckerle and Yeh, 1990; Otey and Pavalko, 1990; Bodary and McLean, 1992; Hynes, 1992). These interactions of integrins-cytoskeleton may provide a mechanism for transduction of mechanical strain into cellular differentiation processes (Milam *et al.*, 1991; Carvalho *et al.*, 1994b).

It is believed that cytoskeletal proteins are perhaps the best situated cellular components to detect environmental strains applied to cells (Sandy *et al.*, 1993; Carvalho *et al.*, 1994a; Scott *et al.*, 1994). The major cytoskeletal protein is actin, which has indeed been shown to change in fibroblasts subjected to mechanical stretch (Pender and McCulloch, 1991). Microfilaments terminate at specialized sites of the cell membrane termed focal contact sites (Burrige *et al.*, 1988; Beckerle and Yeh, 1990), in which talin and vinculin are the major proteins. Figure 4 shows a hypothetical model for the pathway of mechanically-induced interactions among ECM, integrin-receptors, focal adhesion proteins and the cytoskeleton.

The relationship of actin filaments to focal contact proteins and integrin receptors is probably a dynamic one. All of the interactions depicted in Figure 4 do not exist simultaneously in mechanically responsive cells. This stimulation may parallel that of moving cells, in which constant assembly/disassembly of actin fibers and changes in focal contacts take place (Stossel, 1993). In our laboratory we have observed that focal contact proteins change their distribution (Figures 5 and 6) and synthesis (Carvalho *et al.*, 1994b)

when subjected to strain, which may represent an additional step in early strain-induced signal transduction. According to Haskin and Cameron (1993), cellular changes in actin and vimentin depolymerization with the application of pressure suggests a disintegration of integrin-cytoskeletal attachment complex. If mechanical stimulation disrupts this complex, the results from our work showing increases in synthesis of receptors and focal contact proteins (Carvalho *et al.*, 1994b) may be better explained as a partial compensation for the disruption of cytoskeletal components and/or focal contacts (Ingber, 1993).

Integrin receptors have been seen recently as biochemical signal transducers (Kornberg *et al.*, 1991; Bodary and McLean, 1992; Ingber, 1993; Juliano and Haskill, 1993). This refers to receptor modification activating specific membrane enzymes which in turn activate the phosphoinositide cycle. This results in propagation of the signal. An example of such modifications are the ligation of specific receptors leading to phosphorylation on tyrosines and tyrosine-kinases in the cytoplasmic tail of the receptors (RTKs). To this end proteins with SH2 domains, such as phospholipase C and tensin, may be stimulated upon effector-receptor tyrosine kinase binding to elicit a response (Koch *et al.*, 1991). In the case of PLC signaling, RTKs may directly stimulate PKC. This appears to be the case of integrin and focal contact binding via talin (Burrige and Connel, 1983; Burrige *et al.*, 1988) myosin (Omann *et al.*, 1987) and tensin (Davies *et al.*, 1991). In fact, phosphorylation has been suggested as the first identifiable step of the signaling process (Gingel and Owens, 1992). Stimulation of adhesion causes an increase in tyrosine phosphorylation of a protein of 120 to 130 kD (Kornberg *et al.*, 1991) called pp125^{FAK} (Schaller *et al.*, 1992). It has been suggested that integrin signaling and activation of pp125^{FAK} are related to control of gene expression and differentiation (Juliano and Haskill, 1993). For a review see Figure 4.

MECHANICAL STIMULATION AT THE MOLECULAR LEVEL

Mechanical stimulation response at the molecular level has been shown to be divided into early and late stages. The early response is associated with the activation of the immediate genes such as protooncogenes (i.e. *c-fos*, *c-jun* and *c-myc*) and heat shock protein genes (Izumo *et al.*, 1988; Hsieh *et al.*, 1991). The late response appears to be induced by the fetal contractile protein genes and the atrial natriuretic peptide genes (Komuro *et al.*, 1991). Thus, there seems to exist a hierarchy of gene activation characteristically associated with the mechanical strain-induced response. Several other pathophysiologically relevant genes have been expressed as a result of mechanical stimulation, including tissue plasminogen activator (tPA), transforming growth factor- β (TGF- β), platelet derived growth factor-A (PDGF-A) and PDGF-B chains (Resnick *et al.*, 1993). Consequently, mechanical stimulation could also be responsible for activating gene expression of integrin receptors, which appears to be the case. We have been able to demonstrate in our laboratory that the effects of mechanical stimulation on bone cells are: 1) upregulation of integrin gene expression through increases in mRNA levels of β_1 integrin subunit (Carvalho *et al.*, unpublished observations) and 2) specific changes in integrin mRNA levels for different subunits, since α_v integrin subunit mRNA levels did not appear to be affected by strain (Carvalho *et al.*, unpublished observations). Stimulation of integrin gene expression has also been demonstrated with certain differentiation-specific macromolecules such as IL-1 β , PGE₂, phorbol esters, Vitamin D₃ and retinoic acid (Sömjém *et al.*, 1990; Slovik *et al.*, 1986; Heino and Massagué, 1989; Milam *et al.*, 1991).

However, the question is how do mechanically-induced signaling molecules stimulate the transcription of genes? The diagram shown in Figure 7 describes a mechanically-specific nuclear receptor that binds to DNA molecules stimulating transcription. In order for this to occur, the promoter regions of selected genes must contain specific regions or sequences of DNA termed responsive elements (REs). These DNA sequences have the ability to bind protein, usually receptors called transcription

factors (TFs), which is the case for Vitamin D₃ and retinoic acid. Recently, Resnick *et al.* (1993) have identified a highly conserved RE in the PDGF-B chain gene from cells grown *in vitro* which may respond specifically to shear strain. However, to date very little is known about this mechanically-stimulated responsive element (MSRE) or the TFs. For instance, the distribution of these MSREs in other genes and/or the specificity of the TFs, as well as other characteristics have not been determined. Nevertheless, this is the first indication that the information contained in the cells responsive to mechanical stimulation appears to be at the genetic level. However, the question still remains; how is the information transmitted to the nucleus of the cells to stimulate the TFs? This is currently the topic of intense investigation. For instance, kinases such as PKC have been shown to mediate upregulation of gene expression through an increase in DNA binding affinities, changing the transactivation activities of TFs by phosphorylation (Bohman, 1990; Freed *et al.*, 1989; Hsieh *et al.*, 1991). In addition, PLC, Ca²⁺ and G proteins also may prove to be important in protooncogene expression. It is conceivable that mechanical strain stimulates integrin expression in a similar fashion. It is known that stimulation of these genes may occur via adrenergic receptors such as α_1 (Bishopric *et al.*, 1987). This may also mean that expression of the integrin receptors contributes to their auto-regulation, which seems to be the case when integrins are associated with other molecules such as pp60^{src} (Nermut *et al.*, 1991) and Ca²⁺ (Jaconi *et al.*, 1991) or phosphorylation cascades stimulated by mechanical strain (Kornberg *et al.*, 1991). Phosphorylation may also play a role by: 1) activation of TFs residing in the cytoplasm, stimulating translocation into the nucleus; 2) regulation of DNA binding, being either stimulatory or inhibitory; 3) regulation of transactivation of the gene, being either stimulatory or inhibitory, and also by 4) regulation of transrepression of the gene, stimulatory only (Freed *et al.*, 1989 for a review). However, in addition to the considerable overlap between G-proteins and kinase-mediated phosphorylation signaling, the lack of understanding of the whole process of signaling cascades in gene regulation allows several questions to be raised concerning mechanical strain transduction. Further

work is needed to clarify the role and dependency of mechanically-stimulated kinase activation in genetic regulatory mechanisms (Jones and Bingman, 1991).

Finally, cell shape may also determine the regulation of genes in mechanically-stimulated cells. This has been hypothesized in the tensegrity (tensional integrity) model which may explain not only nuclear responses to cell shape and morphogenetic changes, but also different cell shapes and control of cytoskeleton assembly (Wang *et al.*, 1993). An advantage of this system may be the "essentially instantaneous" signal transduction which could happen throughout the entire cell, presumably in a more efficient fashion than in diffusion-based signaling systems (Sims *et al.*, 1992; Ingber, 1993). Although, there may be a tensegrity component in cells that require structural stability, the signaling pathways described above must likely play a very important role in the process of mechanical-induced signal transduction, nonetheless.

CONCLUSIONS

The mechanically stimulated response *in vivo* appears to involve neural, vascular and physical phenomena which, probably combined, explain the extent of cellular activation by mechanical stimulation. However, keeping in mind that none of the systems described above is capable of solely explaining mechanically-induced cellular activity, the general conclusions are:

1. The effects of mechanical stimulation in connective tissues may cause a distortion of the ECM. In bone, such changes may be associated with the appearance of slow-dissipating streaming potentials with associated piezoelectric spikes. The resulting changes in the resting cellular membrane potential may activate the mechanosensitive ion channels.
2. Mechanical stimulation may also cause gradual fluid shifts in the ECM surrounding the cells resulting in nerve fiber distortion with a release of neuropeptides. These combined with leukocyte-secreted cytokines and leukotrienes may contribute to cellular stimulated activity.
3. Stimulation of membrane changes and distortion of ECM may activate membrane receptors associated with a series of second messenger systems that initiate mechanical-stimulated signal transduction.
4. The activated membrane channels and/or receptors will subsequently transduce this stimulation to the focal contacts and cytoskeleton proteins both physically and biochemically. Participation of tyrosine and other kinase proteins followed by a series of phosphorylation cascades will propagate the intracellular activation.
5. Following the activation of cytosolic proteins and second messengers, nuclear activation will take place. This may occur through some form of tensional integrity,

physical nuclear activation or the activation of a transcription factor. This factor would bind to DNA sequences and stimulate gene transcription.

A summary of the pathways and mechanisms described in this review is combined in our hypothetical mechanism of mechanically-induced signal transduction shown in Figure 8. From the studies described here, physiological mechanical stimulation of connective tissues has the capacity to affect cellular response culminating in the maintenance of tissue integrity. Furthermore, abnormal and excessive stimulation may lead to pathologies and tissue breakdown. Additional work is needed to elucidate the role of the different cellular components with the different intracellular responses.

CLINICAL RELEVANCE

The rate of mechanical loading in maintaining normal craniofacial skeleton form and function is a commonly accepted clinical concept, yet little direct evidence is available to correlate specific functional or parafunctional loading with such activities as temporomandibular joint remodeling, mandibular growth and dentoalveolar adaptation. Perturbations such as occlusal changes, occlusal function, orthodontics and periodontal support make drastic changes in the loading pattern, yet the mechanisms by which physiological load and physiological or pathological tissues respond to these loads are unclear.

Further elucidation to these mechanisms would greatly enhance our understanding of both normal development and pathogenesis of temporomandibular joint tissues and dentoalveolar and periodontal structures. Clinical management of the loading patterns could prevent or reverse tissue breakdown. Patients with tissues susceptible to breakdown because of their loading pattern could be identified at an early stage. Non-invasive techniques could be introduced to improve temporomandibular joint and occlusal function.

New breakthroughs in the field of mechanically-stimulated tissues are constantly being made. For instance, the environmental effect on biological tissues once believed to be isolated, has been recently shown to be genetically controlled (Resnick *et al.*, 1993). Different types of mechanical stimulation that clinicians observe in practice may be genetically determined. In other words, the information necessary for cells to respond either to compressive or tensional forces may be present in the form of DNA sequences, which may turn on or off a particular gene responsive to mechanical stimulation of any kind. This may be explained as the epigenetic control of mechanically-induced cellular responses; an area of intense investigation.

Indeed, much of the information derived from future studies of the signaling pathways between extracellular load in the matrix to the cellular response would have

tremendous implications in all aspects of clinical biology since loading and tissue response is a universal relationship.

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REFERENCES

- Almekinders LC, Banes AJ and Ballenger CA 1993 Effects of repetitive motion on human fibroblasts. *Med Sci Sports Exerc* **25**:603-607.
- Akiyama SK, Nagata K and Yamada KM 1989 Cell surface receptors for extracellular matrix components. *Biochim Biophys Acta* **1031**:91-110.
- Arora PD and McCulloch CAG 1994 Intracellular calcium responses of periodontal fibroblasts to mechanical stretch. *J Dent Res* **73**:1326.
- Artner-Dworzak E, Secnik P, Parrak V, Puschendorf B, Marosi M, Muigg A, Gerstenbrand F and Koller A 1993 Changes in muscular proteins during simulated microgravity. *J Neurol Sci* **119**:119-120.
- Basset CAL, Pawluk RJ and Becker RO 1964 Effects of electric current on bone *in vitro*. *Nature* **204**:652-654.
- Beckerle MC 1990 The adhesion protein, talin, is phosphorylated *in vivo* in chicken embryo fibroblasts exposed to a tumor promoting phorbol ester. *Cell Reg* **1**:227-236.
- Beckerle MC and Yeh RK 1990 Talin: role at sites of cell-substratum adhesion. *Cell Motil Cytoskeleton* **16**:7-13.
- Ben-Ze'ev A 1991 Animal shape changes and gene expression. *Bioessays* **13**:207-211.
- Binderman I 1984 Biochemical pathways involved in the translation of physical stimulus into biological message. *Calcif Tissue Int* **36**:82S-85S.
- Binderman I, Zor U, Kaye A, Shimshoni Z, Harell A and Sömjem D 1988 The transduction of mechanical force into biochemical events in bone cells may involve activation of phospholipase A₂. *Calcif Tissue Int* **42**:261-266.
- Bishopric NH, Simpson PC and Ordahl CP 1987 Induction of the skeletal α -actin gene in α_1 -adrenoceptor-mediated hypertrophy of rat cardiac myocytes. *J Clin Invest* **80**:1194-1199.

- Bodary SC and McLean JW 1992 The integrin beta 1 subunit associates with the vitronectin receptor alpha v to form a novel vitronectin receptor in a human embryonic cell line. *J Biol Chem* **265**:5938-5941.
- Bohman D 1990 Transcription factor phosphorylation: a link between signal transduction and the regulation of gene expression. *Cancer Cells* **2**:337-343.
- Boland CJ, Fried RM and Tashjian AH 1986 Measurement of free cytosolic Ca²⁺ concentrations in human and rat osteosarcoma cells: actions of bone resorption-stimulating hormones. *Endocrinology* **118**:980-989.
- Bourne H, Cohen P, Hardie G, Koshland D, Levitzki A, Mishel R, Mudge A, Schramm M, Sellinger Z and Vanaman T 1989 Cell Signaling. In: Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD, eds. *Molecular Biology of the Cell*, 2nd ed., New York, NY, Garland Publishing Inc.
- Brighton CT, Stratford B, Gross SB, Leatherwood DF, Williams JL and Pollack SR 1991 The proliferative and synthetic response of isolated calvarial bone cells of rats to cyclic biaxial mechanical strain. *J Bone Jt Surg* **73**:320-331.
- Brighton CT, Sennet BJ, Farmer JC, Iannotti JP, Hansen CA, Williams JL and Williamson J 1992 The inositol phosphate pathway as a mediator in the proliferative response of rat calvarial bone cells to cyclical biaxial mechanical strain. *J Orthop Res* **10**:385-393.
- Brunette DM 1984 Mechanical stretching increases the number of epithelial cells synthesizing DNA in culture. *J Cell Sci* **69**:35-45.
- Buckley MJ, Banes AJ, Levin LG, Sumpio BE, Sato M, Jordan R, Gilbert J, Link GW and Tay RTS 1988 Osteoblasts increase their rate of division and align in response to cyclic, mechanical tension *in vitro*. *Bone Min* **4**:225-236.
- Burger EH, Klein-Nulend J and Veldhuijzen JP 1992 Mechanical stress and osteogenesis *in vitro*. *J Bone Min Res* **7**:397S-401S.

- Burridge K and Connel L 1983 A new protein of adhesion plaques and ruffling membranes. *J Cell Biol* **97**:359-367.
- Burridge K 1986 Substrate adhesions in normal and transformed fibroblasts: organization and regulation of cytoskeleton, membrane and extracellular matrix components at focal contacts. *Canc Rev* **4**:18-78.
- Burridge K, Fath K, Kelly T, Nuckolls G and Turner C 1988 Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol* **4**:487-525.
- Carvalho RS, Scott E, Suga DM and Yen EHK 1994a Stimulation of signal transduction pathways in osteoblasts by mechanical strain is potentiated by parathyroid hormone. *J Bone Min Res* **7**:999-1011.
- Carvalho RS, Scott JE and Yen EHK 1994b Mechanical stimulation alters the synthesis and distribution of integrin subunits and cytoskeletal proteins in human osteosarcoma cells. In: Davidovitch Z, ed. *Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants*. ESBCO Media, Harvard University Press, Cambridge-MA, pp. 135-146.
- Charles AC, Merril JE, Dirksen ER and Sanderson MJ 1991 Intracellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* **6**:983-992.
- Chiba M 1989 Study of bone remodeling mechanism induced by mechanical stress. Differentiation of osteoclasts induced by compressive force in newborn rat cultured long bone. *Nippon Kyosei Shika Gakkai Zasshi* **48**:585-600.
- Clover J, Dodds RA and Gowen M. 1992 Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. *J Cell Sci* **103**:267-271.
- Collett ASR and Stewart AG 1991 Eicosanoids: physiology update and orthodontic implications. *Aust Orthod J* **12**:116-123.

- Collins JL, Daniel JW, Cederquist R. and Enlow DH 1987 The role of leukotrienes in force-induced bone development. *J Dent Res* **66**:328-333.
- Copray JCVM, Jansen HWB, Duterloo HS 1985 Effects of compressive forces on proliferation and matrix synthesis in mandibular condyle cartilage of the rat *in vitro*. *Arch Oral Biol* **30**:299-317.
- Cress AE, Majda JA, Glass JR, Stringer DE and Gerner EW 1990 Alteration of cellular adhesion by heat shock. *Exp Cell Res* **190**:40-46.
- Davidovitch Z, Shanfeld J, Montgomery E, Lally E, Laster L, Furst L and Korostoff E 1984 Biochemical mediators of the effect of mechanical forces and electric currents in mineralized tissues. *Calcif Tissue Int* **36**:86S-97S.
- Davidovitch Z 1991 Tooth Movement. *Crit Rev Oral Biol Oral Med* **2**:411-450.
- Davies S, Li ML, Lo SH, Lin S, Buttler JA, Drucker BJ, Roberts TM, An Q and Chen LB 1991 Presence of an SH2 domain in the actin-binding protein tensin. *Science* **252**:712-715.
- Derventzi A, Rattan RIS and Clark BFC 1992 Phorbol ester-induced reorganization of the cytoskeleton in human fibroblasts during ageing *in vitro*. *Biochem Biophys Res Comm* **182**:1423-1428.
- Farndale RJ, Sandy JR, Atkinson SJ, Pennington SR, Meghji S and Meikle MC 1988 Parathyroid hormone and prostaglandin E₂ stimulate both inositol phosphates and cyclic AMP accumulation in mouse osteoblasts cultures. *Biochem J* **79**:230-239.
- Freed E, Gailit J, van der Geer P, Ruoslahti E and Hunter T 1989 A novel integrin β subunit is associated with the vitronectin receptor α subunit (α_v) in a human osteosarcoma cell line and is a substrate for protein kinase C. *EMBO J* **8**:2955-2965.
- Fujimori A, Cheng S, Avioli LV and Civitelli R 1992 Structure-function relationship of parathyroid hormone: activation of phospholipase-C, protein kinase-A and -C in osteosarcoma cells. *Endocrinology* **130**:29-36.

- Gailit J, Colflesh D, Rabiner I, Simone J and Goligorsky MS 1993 Redistribution and disfunction of integrins in cultured renal epithelial cells exposed to oxidative stress. *Am Physiol Soc* **263**:149F-157F.
- Gamble JR, Harlam JM, Klebanoff SJ and Vadas MA 1985 Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc Natl Acad Sci USA* **82**:8667-8671.
- Gingell D and Owens N 1992 How do cells sense and respond to adhesive contacts? Diffusion-trapping of laterally mobile membrane proteins at maturing adhesions may initiate signals leading to local cytoskeletal assembly response and lamella formation. *J Cell Sci* **101**:255-266.
- Gowen M and Mundy GR 1986 Actions of recombinant interleukin 1, interleukin 2 and interferon-gamma on bone resorption *in vitro*. *J Immunol* **136**:2478-2482.
- Gross W and Lohse MJ 1991 Mechanism of activation of A₂ adenosine receptors. II. A restricted collision-coupling model of receptor-effector interaction. *Mol Pharmacol* **39**:524-530.
- Guharey F and Sachs F 1984 Stretch activated K⁺ channels in muscle cell membranes as mechanotransducers? *J Physiol* **352**:685-701.
- Hasegawa S, Sato S, Saito S, Suzuki Y and Brunette DM 1985 Mechanical stretching increases the number of cultured bone cells synthesizing DNA alters their pattern of protein synthesis. *Calcif Tiss Int* **37**:431-436.
- Haskin C and Cameron I 1993 Physiological levels of hydrostatic pressure alter morphology and organization of cytoskeletal and adhesion proteins in MG-63 osteosarcoma cells. *Biochem Cell Biol* **71**:27-35.
- Heino J and Massagué J 1989 Transforming growth factor-beta switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin. *J Biol Chem* **264**:21806-21811.

- Horton M A and Davies, J 1989 Perspectives: Adesion receptors in bone. *J Bone Min Res* **4**:803-807.
- Horton MA, Taylor ML, Arnett TR and Helfrich MH 1991 Arg-Gly-Asp (RGD) peptides and the anti vitronectin receptor antibody 23C6 inhibit dentine bone resorption and cell spreading by osteoclasts. *Exp Cell Res* **195**:368-375.
- Hsieh H, Li N and Frangos JA 1991 Shear stress increases endothelial platelet-derived growth factor mRNA levels. *Am Physiol Soc* **260**:642H-646H.
- Hynes RO 1992 Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**:11-25.
- Ingber D 1991 Integrins as mechanochemical transducers. *Curr Opin Cell Biol* **3**:841-848.
- Ingber DE 1993 Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* **104**:613-627.
- Izumo S, Nadal-Ginard B and Mahdavi V 1991 Protooncogene induction and reprograming of cardiac gene expression produced by pressure overload. *Proc Natl Acad Sci USA* **83**:339-343.
- Jaconi MEE, Theler JM, Schlegel W, Appel RD, Whright SD and Lew PD 1991 Multiple elevations of cytosolic free-Ca²⁺ in human neutrophils: initiation by adherence receptors of the integrin family. *J Cell Biol* **112**:1249-1257.
- Jones DB and Schlübbbers JG 1987 Evidence that phospholipase C mediates the mechanical strain response in bone. *Calcif Tissue Int* **41**:4-10.
- Jones DB and Bingman D 1991 How do osteoblasts respond to mechanical stimulation? *Cells Mat* **4**:1-12.
- Jones DB, Nolte H, Schlübbbers JG, Turner E and Veltel D 1991 Biochemical signal transduction of mechanical strain in osteoblast-like cells. *Biomaterials* **12**:101-110.
- Juliano RL and Haskill S 1993 Signal transduction from the extracellular matrix. *J Cell Biol* **120**:577-585.

- Kabat D, Gliniak B, Rohrschneider L and Polonoff E 1985 Cell anchorage determines whether mammary virus glycoproteins are processed for plasma membranes or secretion. *J Cell Biol* **101**:2274-2283.
- Klein-Nulend J, Veldhuijzen JP and Burger EH 1986 Increased calcification of growth plate cartilage as a result of compressive force *in vitro*. *Arthritis Rheum* **29**:1002-1009.
- Koch CA, Anderson D, Moran MF, Ellis C and Pawson T 1991 SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science* **252**:668-674.
- Komuro I, Katoh Y, Kaida T, Shibazaki I, Kurabayashi M, Hoh E 1990 Stretching cardiac myocytes stimulates protooncogene expression. *J Biol Chem* **265**:3595-3598.
- Komuro I, Katoh Y, Kaida T, Shibazaki I, Kurabayashi M, Hoh E, Takaku F and Yazaki Y 1991 Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. *J Biol Chem* **266**:1265-1268.
- Korn JH, Downie E, Roth GJ and Ho SY 1989 Synergy of interleukin (IL-1) with arachidonic acid and A23187 in stimulating PGE synthesis in human fibroblasts: IL-1 stimulates fibroblast cyclooxygenase. *Clin Immunol Immunopathol* **50**:196-204.
- Kornberg L, Earp HS, Turner C, Procop C and Juliano RL 1991 Signal transduction by integrins: increased protein tyrosine kinase phosphorylation caused by clustering of beta 1 integrins. *Proc Natl Acad Sci USA* **88**:8392-8396.
- Lansman JB, Hallam J and Rink TJ 1987 Single stretch activated ion channels in vascular endothelial cells as mechanotransducers? *Nature* **325**:811-813.
- Lanyon LE 1984 Functional strain as a determinant of bone remodeling. *Calcif Tissue Int* **36**:56S-61S.
- Levitzki A and Bar-Sinai A 1991 The regulation of adenylyl cyclase by receptor-operated G proteins. *Pharmacol Ther* **50**:271-283.

- Lotz MM, Bursal CA, Erickson HP and McClay DR 1989 Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J Cell Biol* **109**:1795-1805.
- McLeod KJ, Lee RC and Ehrlich HP 1987 Frequency dependence of electric field modulation of fibroblast protein synthesis. *Science* **236**:1465-1469.
- Meikle MC, Heath JK, Atkinson SJ, Hembry RM and Reynolds JJ 1989 Molecular biology of stressed connective tissues at sutures and hard tissues *in vitro*. In: Norton LA and Burnstone CJ, eds. *The biology of tooth movement*, Boca Raton, FL, CRC Press. pp. 71-86.
- Milam SB, Magnuson VL, Steffensen B, Chen D and Klebe RJ 1991 IL-1 β and prostaglandins regulate integrin mRNA expression. *J Cell Physiol* **149**:173-183.
- Mittelstaedt H and Glasauer S 1993 Illusions of verticality in weightlessness. *Clin Investig* **71**:732-739.
- Mohamed AH, Tatakis DN and Dziak R 1989 Leukotrienes in orthodontic tooth movement. *Am J Orthod Dentofac Orthop* **95**:231-237.
- Morgan N 1989 Guanine-nucleotide binding proteins as signal transduction. In: Morgan N, ed. *Cell Signaling*. Guilford Press, London, pp.35-57.
- Morris CE 1990 Mechanosensitive ion channels. *J Membr Biol* **112**:93-107.
- Morris CE and Horn R 1991 Failure to elicit neuronal macroscopic mechanosensitive currents anticipated by single-channel studies. *Science* **251**:1246-1249.
- Nermut MV, Eanon P, Hirst EMA and Kellje S 1991 Cell/substrate adhesions in RSV-transformed rat fibroblasts. *Exp Cell Res* **193**:382-397.
- Omann GH, Allen RA, Bokoch GM, Painter RG, Traynor AE and Sklar EA 1987 Signal transduction and cytoskeletal activation in the neutrophil. *Physiol Rev* **67**:285-322.
- Otey CA, Pavalko FM and Burridge K 1990 An interaction between α -actinin and the β_1 -subunit *in vitro*. *J Cell Biol* **111**:721-729.

- Pender N and McCulloch AG 1991 Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching. *J Cell Sci* **100**:187-193.
- Piper PJ and Samhoun MN 1987 Leukotrienes in Inflammation-mediators and mechanisms. *Br Med Bull* **43**:297-211.
- Reich KM and Frangos JA 1991 Effect of flow on prostaglandin E₂ and inositol trisphosphate levels in osteoblasts. *Am J Physiol* **26**:428C-432C.
- Resnick N, Collins T, Atkinson W, Bonthron DT, Dewey CF and Gimbrone MA 1993 Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. *Proc Natl Acad Sci USA* **90**:4591-4595.
- Roberts WE, Mozsary PG and Morey ER 1981 Suppression of osteoblast differentiation during weightlessness. *Physiologist* **24**:75S-76S.
- Rubin CT and Hausman MR 1988 The cellular basis of Wölf's law. Transduction of physical stimuli to skeletal adaptation. *Rheum Dis Clin North Am* **14**:503-517.
- Rubin CT, McLeod KJ and Lanyon LE 1989 Prevention of osteoporosis by pulse electromagnetic fields. *J Bone Jt Surg* **71**:411-417.
- Sachs F 1988 Mechanical transduction in biological systems. *Crit Rev Biomed Eng* **16**:141-169.
- Sachs F 1991 Mechanical transduction by membrane ion channels: a mini review. *Mol Cell Biochem* **104**:57-60.
- Saito M, Saito S, Ngan PW, Shanfeld J and Davidovitch Z 1991 Interlukin 1 beta and prostaglandin E are involved in the response of periodontal cells to mechanical stress *in vivo* and *in vitro*. *Am J Orthod Dentofac Orthop* **99**:226-40.
- Sandy J 1989 Dual elevation of cyclic AMP and inositol phosphate in response to mechanical deformation of murine osteoblasts. *Biochim Biophys Acta* **1010**:265-269.
- Sandy J and Farndale W 1991 Second messengers: regulators of mechanically-induced tissue remodeling. *Eur J Orthod* **13**:271-278.

- Sandy JR, Farndale RW and Meikle MC 1993 Recent advances in understanding mechanically induced bone remodeling and their relevance to orthodontic theory and practice. *Am J Ortho Dentofac Orthop* **103**:212-222.
- Sandy J 1993 DNA changes in mechanically deformed osteoblasts: a new hypothesis. *Br J Ortho* **20**:1-11.
- Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB and Parsons JT 1992 pp125 FAK, a structurally unique protein tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci USA* **89**:5192-5196.
- Scott JE, Yang SY, Stanik E and Anderson JE 1993 Influence of strain on [³H]thymidine incorporation, surfactant-related phospholipid synthesis, and cAMP levels in fetal type II alveolar cells. *Am J Respir Cell Mol Biol* **8**:258-265.
- Scott JE, Oulton R and Anderson JE 1994 Strain induces change in phospholipid and DNA synthesis, cAMP levels and cytoskeletal fibers in isolated fetal rabbit type II alveolar cells. In: Muller B and von Wichert P, eds. *Lung surfactant: basic research in the pathogenesis of lung disorders*. Prog Resp Res, Basel, Switzerland:Karger.
- Sims JR, Karp S and Ingber D 1992 Altering the cellular mechanical force balance results in integrated changes in cell, cytoskeleton and nuclear shape. *J Cell Sci* **103**:1215-1222.
- Slovik DM, Rosenthal DI, Doppelt SH, Potts JT, Daly AM, Campbell JA and Neer AM 1986 Restoration of spinal bone in osteoporotic men by treatment with human parathyroid hormone (1-34) and 1,25-dihydroxyvitamin D. *J Bone Min Res* **1**:377-382.
- Sokabe M, Sachs F and Jing Z 1991 Quantitative video microscopy of patch clamped membranes stress, strain, capacitance, and stretch channel activation. *Biophys J* **59**:722-728.
- Sömjem D, Binderman I, Berger E and Harell A 1980 Bone remodeling induced by physical strain is prostaglandin E₂ mediated. *Biochim Biophys Acta* **627**:931-934.

- Steiner P 1970 The measurement of cyclic nucleotides by radioimmunoassay. *Adv Biochem Psychopharmacol* **3**:89-111.
- Stossel TP 1993 On the crawling of animal cells. *Science* **260**:1086-1094.
- Suda N, Morita I, Kuroda T and Murota S 1993 Participation of oxidative stress in the process of osteoclast differentiation. *Biochim Biophys Acta* **1157**:318-323.
- Turner CE, Pavalko FM and Burrigge K 1989 The role of phosphorylation and limited proteolytic cleavage of talin and vinculin in the disruption of focal adhesion integrity. *J Biol Chem* **264**:938-944.
- Wang N, Butler JP and Ingber DE 1993 Mechanotransduction across the cell surface and through the cytoskeleton. *Science* **260**:1124-1127.
- Watson PA 1991 Function follows form: generation of intracellular signals by cell deformation. *FASEB J* **5**:2013-1029.
- Wölff JL 1892 The law of bone remodeling. In: Maquet P and Furlong R ed. (Translation, 1986) *Das Gesetz der Transformation der Knochen*. Berlin:Springer-Verlag.
- Woods A and Couchman JR 1992 Protein kinase C involvement in focal adhesion formation. *J Cell Sci* **101**:277-290.
- Yamaguchi DT, Hahn TJ, Beeker TG, Kleeman CR and Muallen F 1988 Relationship of cAMP and calcium messenger systems in prostaglandin-stimulated UMR-106 cells. *J Biol Chem* **263**:10745-10753.
- Yen EHK, Pollit DJ, Whyte WA and Suga DM 1990 Continuous stressing of mouse interparietal suture fibroblasts *in vitro*. *J Dent Res* **69**:26-30.

Figure 1. Diagram showing the adenylyl cyclase (AC) pathway. The stimulation, either chemical or mechanical, is thought to activate a cell receptor which binds to specific G proteins. These proteins are formed by three subunits (α, β, γ), of which the α is the active one. The AC pathway is believed to have two forms of G proteins which may bind to each specific receptor: one is stimulatory (G_s) and the other is inhibitory (G_i). Upon receptor-Gac coupling, GDP is displaced and GTP binds to the G protein causing the α subunit to dissociate from the β - γ complex and activate the adenylyl cyclase enzyme (Ac). This binding may be stimulatory or inhibitory, depending on the responsive G protein (G_s or G_i). Therefore, the nature of the binding will determine if the following conversion of adenosine thrisphosphate (ATP) into cyclic adenosine monophosphate (cAMP) will increase or decrease cAMP levels. The latter may modulate several cell mechanisms, including the activation of cAMP-protein kinase (cAMP-K*). The activated form of cAMP-K* goes on to phosphorylate a series of other cellular proteins.

Figure 2. Diagram showing the two branches of the phosphatidylinositol (PI) pathway. The stimulation, either chemical or mechanical, is thought to activate a cell receptor which binds to specific G proteins (G_q). These proteins are formed by three subunits (α, β, γ), of which the α is the active one. Upon receptor- G_q coupling, the α subunit is dissociated from the β - γ complex to activate the membrane bound enzyme phospholipase C (PLC). PLC cleaves 4,5-phosphatidylinositol bisphosphate (PIP_2) to generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). The IP_3 branch of the PI pathway continues with a release of Ca^{2+} from intracellular stores. On the other hand, DG, which remains in the cell membrane, activates protein kinase C (PKC). The activated form of PKC* phosphorylates a series of other cellular proteins.

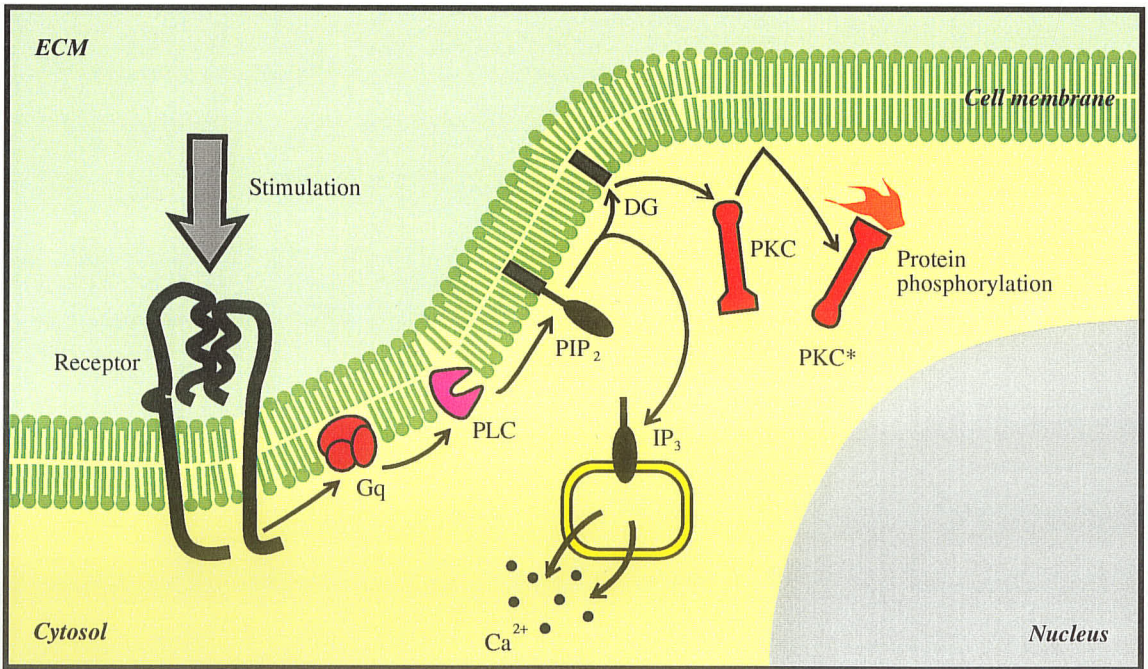
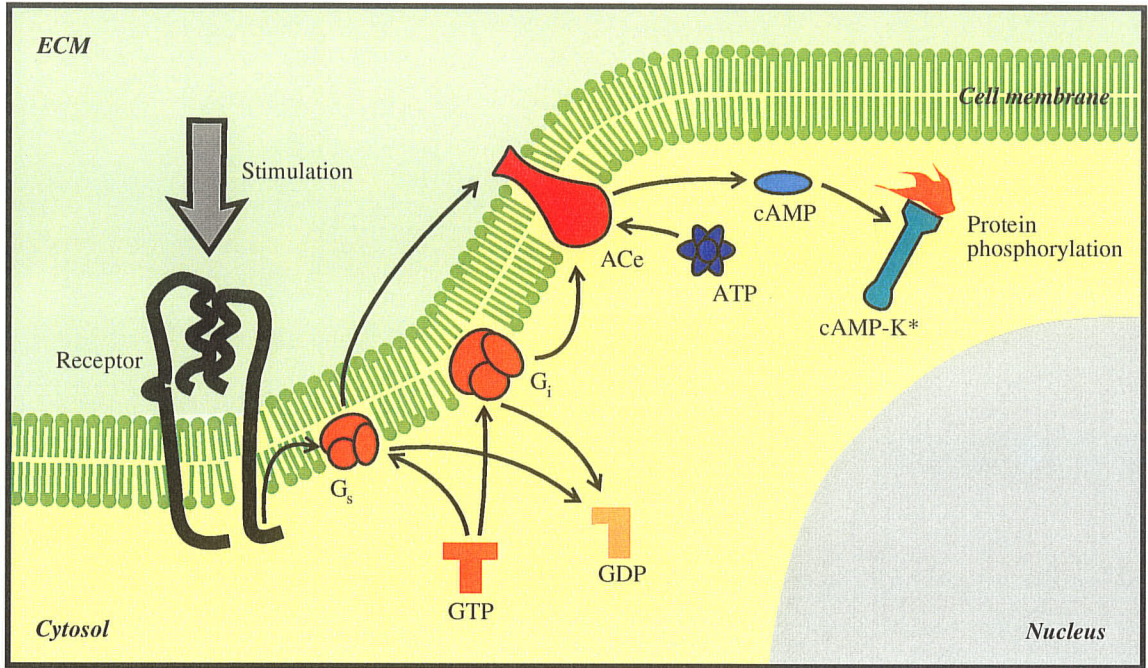


Figure 3. Immunofluorescent localization of integrin subunits in non-mechanically (a, c, e and g) and mechanically-stimulated (b, d, f and h) bone cells *in vitro*. Note that stimulation has changed the distribution pattern of the integrin subunits α_2 (a and b), α_3 (c and d), α_v (e and f) and β_1 (g and h). X 1875.

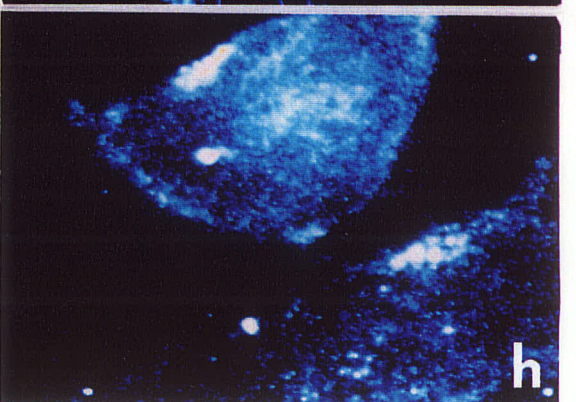
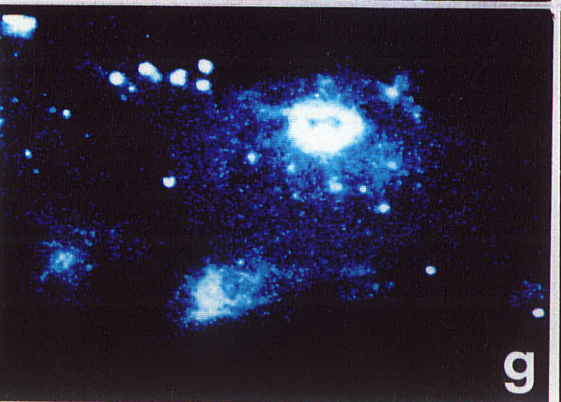
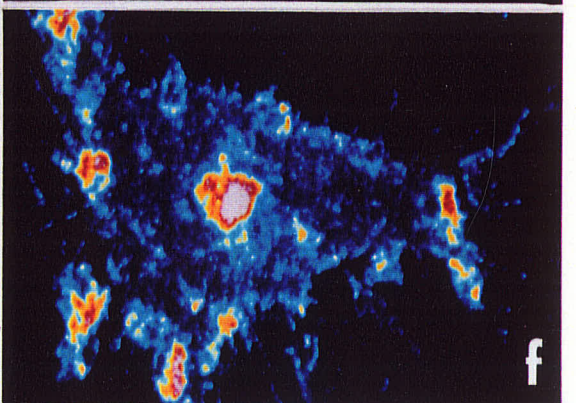
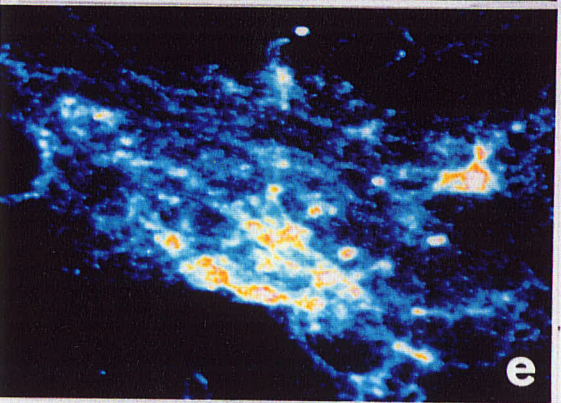
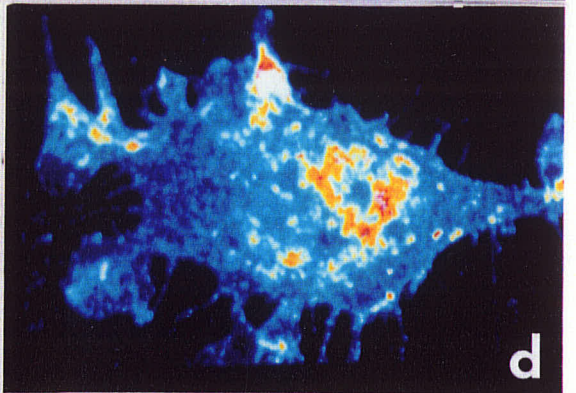
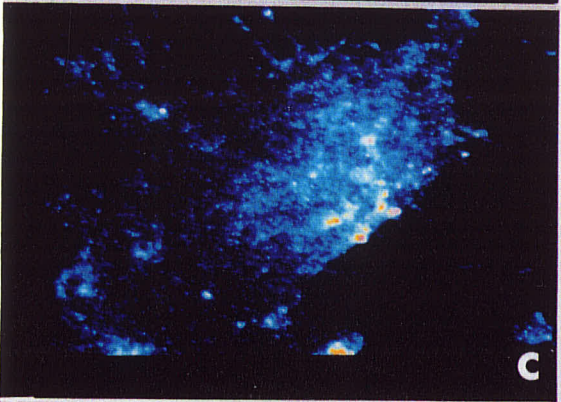
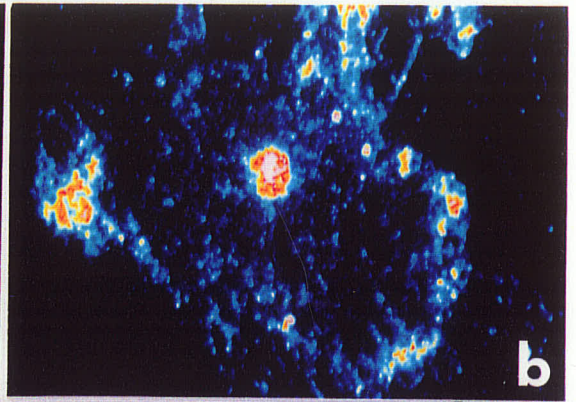
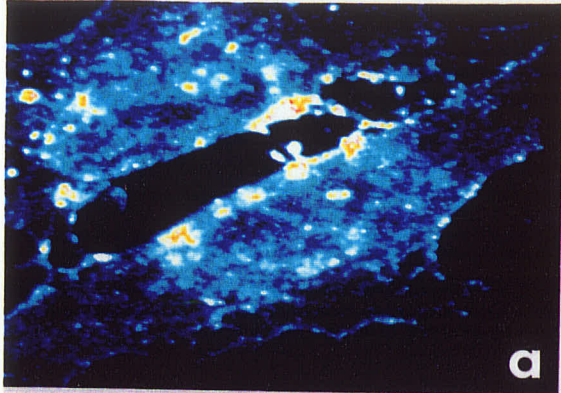


Figure 4. Diagram depicting a simplified model of the interactions of extracellular matrix (ECM) proteins, as characterized by fibronectin to the activated membrane receptors integrins (both α and β subunits) and the intracellular focal contact and cytoskeletal proteins. Focal contacts are shown as talin, vinculin and tensin and cytoskeletal are actin, α -actinin, filamin and vimentin. In addition, the important protein tyrosine kinase (PTK) in the membrane and proteins 125^{FAK} and 60^{src} are also shown. The application of mechanical stimulation causes a membrane deformation and an activation of integrin receptors. Subsequently, the transduction of mechanical stimulation may occur through a physical deformation of the focal and cytoskeletal proteins. In addition, activated PTK* and 125^{FAK} and 60^{src} may participate in the phosphorylation cascades of specific cellular proteins, such as tensin.

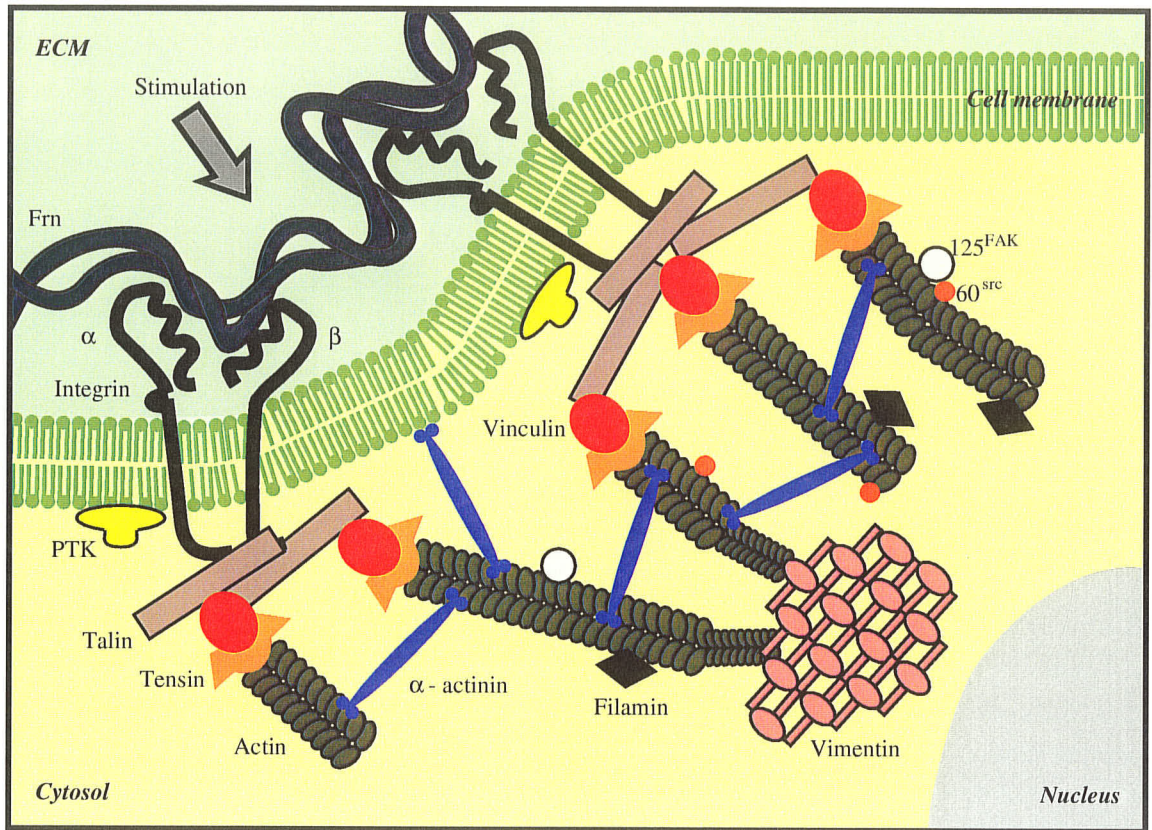


Figure 5. Immunofluorescent localization of proteins in non-mechanically (a, e and g) and mechanically-stimulated (b, c, d, f and h) bone cells *in vitro*. Note that stimulation has changed the distribution pattern of the intermediate cytoskeletal proteins vimentin (a, b, c and d) and the focal contact protein vinculin (e and f) and α -actinin (g and h). Note that periods of strain of 30 minutes (b) show different staining distribution of vimentin when compared with those of periods of 24 hours (c) and 7 days (d). Note that these cells orient themselves perpendicular (double white arrows) to the long axis of the applied mechanical strain (white arrow). Also note that there was change in the focal contact protein vinculin distribution in 30 minutes of mechanical strain (f) and that the distribution of α -actinin did change considerably with mechanical stimulation (h). White arrows indicate the direction of strain. Magnifications (a-d) X 500 and (e-h) X 1250.

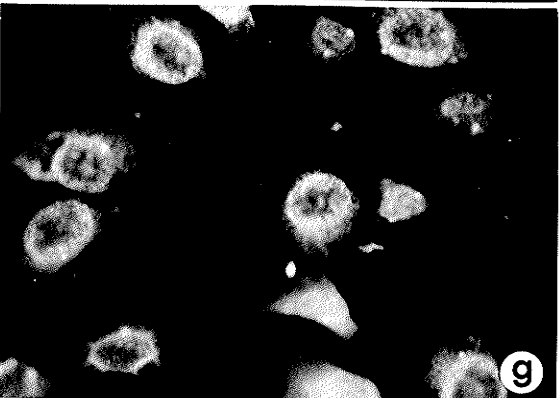
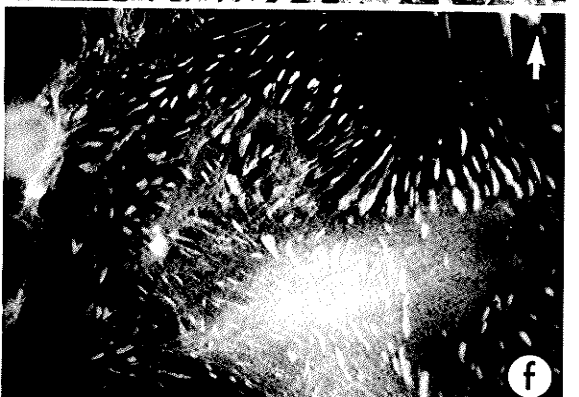
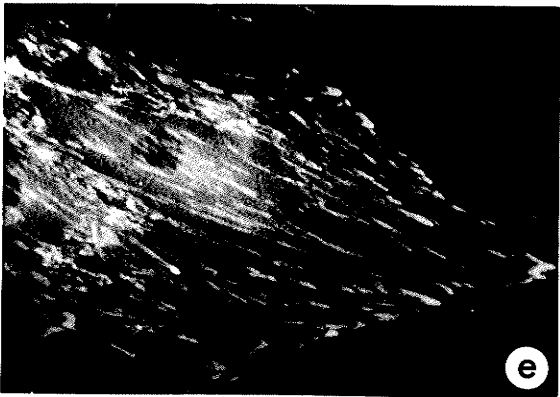
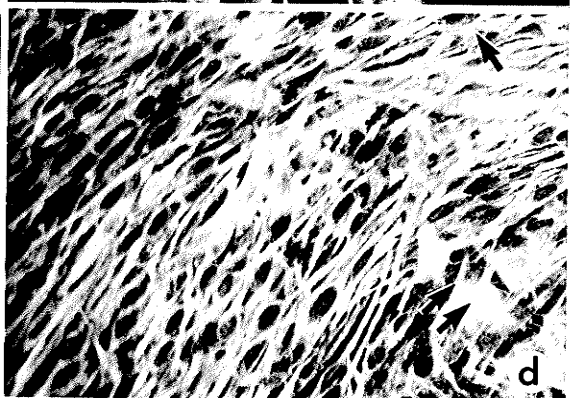
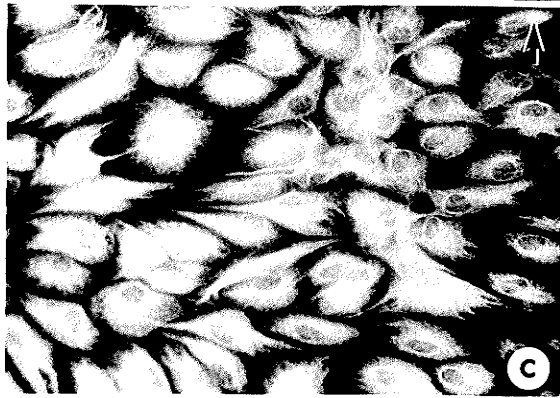
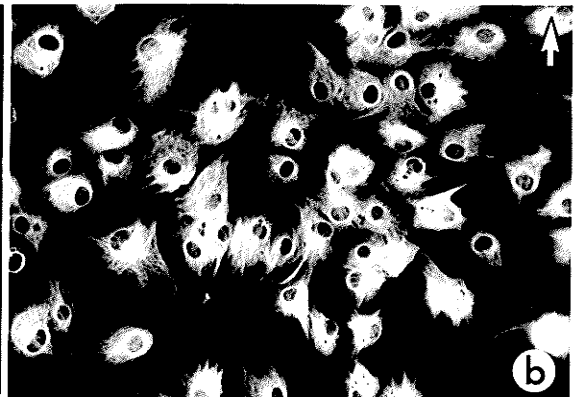
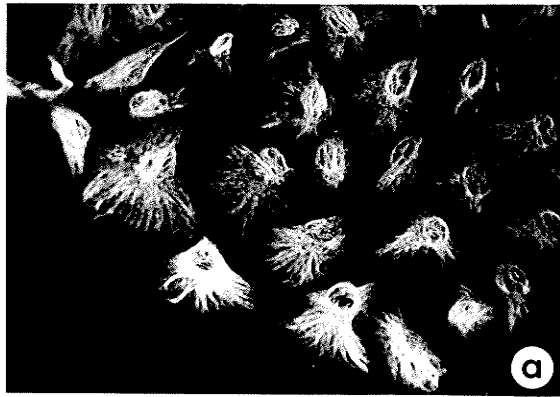


Figure 6. Immunofluorescent localization of focal contact proteins in non-stimulated (a and c) and mechanically-stimulated (b and d) bone cells *in vitro*. Note that stimulation has changed the distribution pattern of vinculin (a and b) and talin (c and d). X 1250.

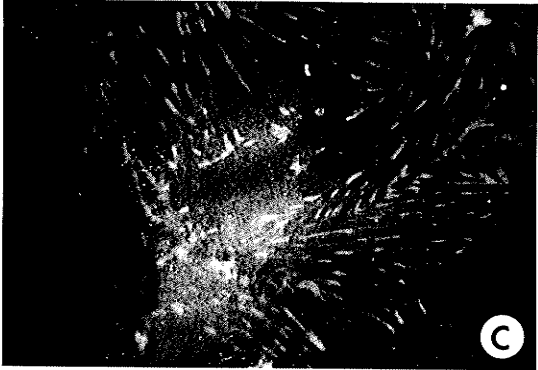
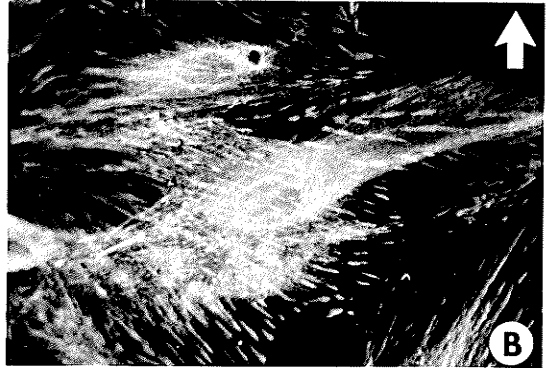


Figure 7. Diagram depicting a simplified model of the molecular interactions in mechanically-stimulated connective tissue cells. The application of mechanical strain causes a membrane deformation and an activation of integrin receptors. Following the participation of structural proteins and phosphorylation cascades in the transduction of mechanical stimulation into the cell cytoplasm, some of these events may transduce the stimulation into a nuclear response. Activated protein kinase C (PKC*) and protein tyrosine kinase (PTK*) may affect mechanical stimulation receptors (MSRs), which may occur in the cytoplasm or in the nucleus. If stimulated in the cytoplasm, the MSR will translocate into the nucleus to bind to mechanical stimulation responsive elements (MSRE) in the promoter regions of responsive genes. The binding of MSRs to MSREs may either enhance or repress transcription of a gene leading to control of cellular response by changing mRNA levels for the resulting protein. In addition, phosphorylation of PKC* may be also given by other proteins, which are not depicted in this diagram. The proteins 125^{FAK} and 60^{src} may also stimulate the MSRs. Finally, the tensegrity model may physically deform the nucleus resulting in the stimulation of the gene promoters similarly to the alternate stimulation by PTKs, bypassing the MSRs.

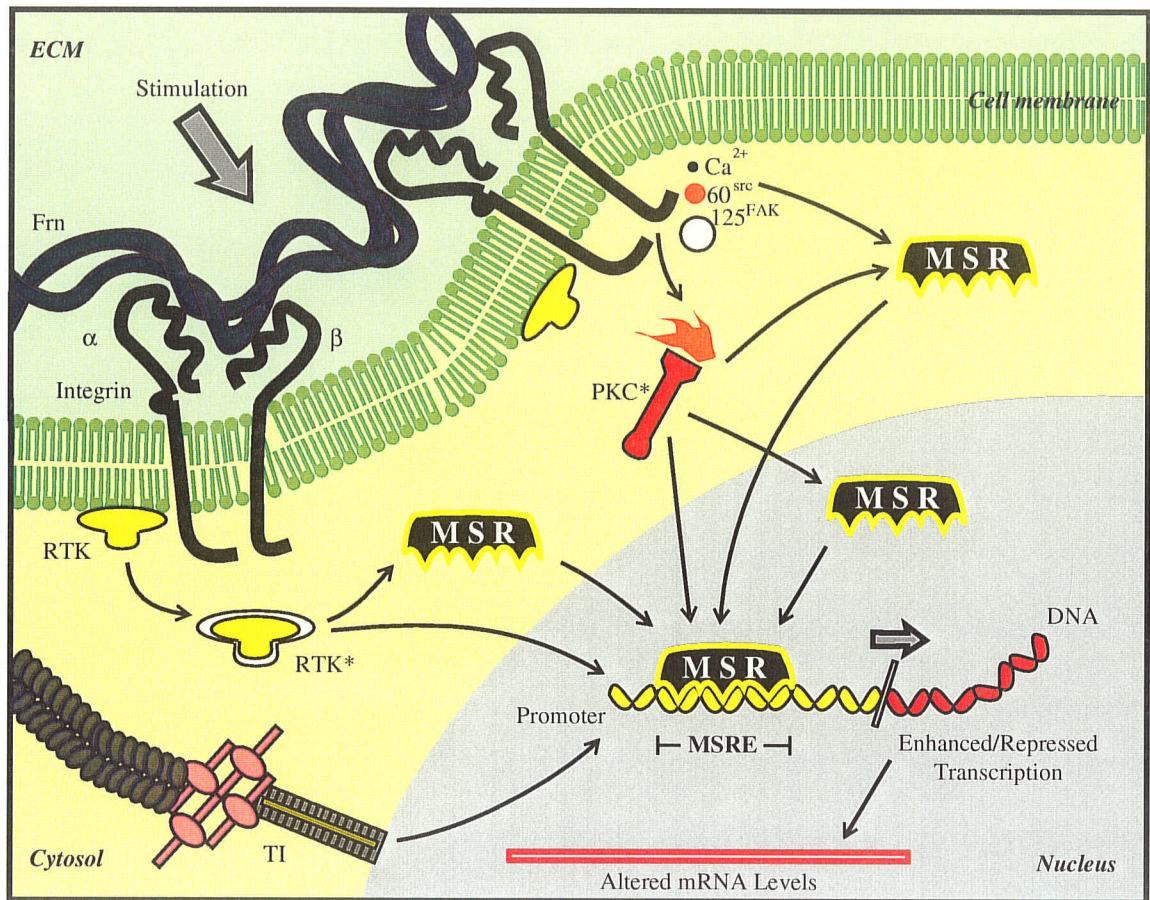


Figure 8. Diagram depicting our current model of mechanically-stimulated signal transduction mechanisms taking place in connective tissue cells. The interrelationships depicted are a simplification of the ones that may be occurring in cells *in vivo*, since it would not be possible to show all the interactions and pathways in mechanically stimulated cells. This diagram is a combination of previous figures which dealt with individual parts of the signal transduction mechanisms. Shown here are the extracellular matrix (ECM) protein fibronectin (Frn) binding to integrin receptors (α and β), transducing the stimulation to the cytosol to activate: 1) focal contact proteins talin (Ta), vinculin (V) and tensin (Te) which transduce the signals to actin (A), α -actinin (αA), filamin (F), vimentin (Vm) and 125^{FAK} and 60^{src}; 2) the phosphatidylinositol and adenylyl cylcase pathways (described in Figs. 1 and 2); 3) protein tyrosine kinases (PTKs) which when activated (PTK*) would stimulate other cytoskeleton proteins and the mechanical stimulation receptors (MSRs) and 4) the flux of Ca²⁺ into the cytosol causing a further series of responses. The subsequent molecular reactions in the nucleus have been already described in Figure 7.



Chapter SIX

*Distribution of beta₁-integrin and Expression
of beta₁-integrin mRNA in Mechanically
Strained Osteoblasts In Vitro*

Carvalho RS, Scott JE and Yen EHK

Archives of Oral Biology
(in press)



ABSTRACT

Mechanical stimulation applied to the skeleton has been shown to alter the metabolism of bone cells, however the effects of mechanical strain on the cytoskeleton of osteoblasts are poorly understood. While changes in the cytoskeleton distribution in mechanically strained cells have been reported, little is known about the pathways by which these changes are transduced into cell functions. HOS TE-85 cells were cultured in DMEM/F-12 and grown to confluency in Flexercell Type I dishes in a humidified incubator with 5 % CO₂ and 95 % air. Intermittent strain (3 cycles per min) was applied to the cells using the Flexercell Strain Unit System for periods of 15 and 30 minutes, 2, 4 and 24 hours and 3, 5, 7, 10, 14, 20 and 28 days. Unstrained cells were used as controls. Distribution of β_1 integrin was studied by immunocytochemical procedures. Total RNA was isolated at all the time periods and northern blots were performed to study the effects of strain on the levels of β_1 integrin expression in TE-85 cells. The results indicated that mechanical strain increased the synthesis of β_1 integrin. Northern blots showed that β_1 mRNA expression increased significantly ($p < 0.005$) at periods of 30 minutes and 3 days of strain application. In addition, strain also affected β_1 distribution markedly in periods of 24 hour cultures. The response of HOS cells to mechanical strain demonstrates that the osteoblast cytoskeleton adapts to strain through the stimulation of specific cytoskeletal and receptor proteins. These results suggest a pathway through which mechanical strain is transmitted to the osteoblastic-like cells.

INTRODUCTION

Orthodontic therapy requires the manipulation of dental and skeletal structures through the use of mechanical forces. Although, clinical treatment can be achieved by stimulating growth and tissue remodeling, the cellular mechanisms that control the tissue response to mechanical forces are poorly understood. The early histologic description of remodeling was associated with "tension" and "compression" in the periodontal ligament relative to bone apposition and bone resorption, respectively (Openheim, 1911). In later studies correlating the magnitude of applied force (Storey, 1973), to changes in matrix production (Martinez and Johnson, 1988) and in the levels of the so-called second messengers (Davidovitch *et al.*, 1988; Carvalho *et al.*, 1994) the effects of orthodontic forces in bone remodeling have been examined by direct mechanical straining of the cell structure (Davidovitch *et al.*, 1988; Brighton *et al.*, 1991; Vandeburgh, 1992), by using bioelectric fields to stimulate bone cells (Davidovitch *et al.*, 1988; Brighton and McCluskey, 1988) and by the production of biochemical signals (Harell *et al.*, 1977; Brighton *et al.*, 1991; Jones *et al.*, 1991; Vandeburgh, 1992). While the identification of these isolated strain-induced events has clarified important mechanisms taking place during orthodontic therapy, little is known about the sequence of events connecting these changes. In other words, the signal transduction pathways through which the orthodontic-induced response affects cell behavior requires further clarification.

The mechanisms by which the cells communicate may rely on actual physical cell to cell contacts through GAP junctions (Schirrmacher *et al.*, 1992). Although, small molecules can be exchanged directly between cells through the pores in the GAP (Morgan, 1989), this system suffers from a slow pace of information flow between cells. In contrast, the release of biochemical signals not only takes place quickly and efficiently, but also mediates the cell response at a most fundamental level (Morgan, 1989 for a review). This appears to be true in several systems of cellular stimulation, in particular mechanical strain stimulation (Davidovitch *et al.*, 1988; Brighton *et al.*, 1991; Vandeburgh, 1992;

Carvalho *et al.*, 1994). The release of intracellular messengers has been shown to affect crucial cellular events such as growth and proliferation (Burger *et al.*, 1992; Vandeburgh, 1992), differentiation (Kubota *et al.*, 1993) and gene regulation (Komuro *et al.*, 1990; Komuro *et al.*, 1991). Recent evidence suggests that there are different levels of response (Farndale *et al.*, 1988) and that there is considerable cross-talk between different intracellular signaling systems (Fujimori *et al.*, 1992). Nevertheless, messengers like cAMP, protein kinase C and Ca²⁺ which are some of the most ubiquitous, are known to be released through some form of membrane activation being either protein channels or protein receptors.

The studies describing changes in the levels of second messengers as a result of orthodontic forces *in vitro* have so far failed to establish the transduction pathway responsible for these changes. Ion flux (Sachs, 1992) and the tensegrity model (Ingber, 1991) are perhaps involved in strain-induced response. However, it has been suggested that receptor-mediated signaling through physical and biochemical systems is the most likely to respond to any direct stimulation such as orthodontically applied strain. The prime candidates for transduction of strain signals on cells are the members of the integrin receptor family (Hynes, 1992 for a review). These proteins attach to specific extracellular macromolecules through a high-affinity binding (Bodary and McLean, 1992), which when stimulated may biochemically transduce strain signals through G proteins (Sandy *et al.*, 1993) involving one or more group of prostaglandins and cAMP (Harell *et al.*, 1977; Sömjem *et al.*, 1980; Davidovitch *et al.*, 1988; Vandeburgh, 1992), phospholipases (Binderman *et al.*, 1988), inositol phosphates (Sandy *et al.*, 1993; Carvalho *et al.*, 1994), intracellular Ca²⁺ (Harell *et al.*, 1977; Jones *et al.*, 1991) and protein kinase C (Jones *et al.*, 1991; Carvalho *et al.*, 1994). Integrins may also physically stimulate cytoskeleton rearrangement through focal contacts (Gailit *et al.*, 1993) and/or through stimulation of protein phosphorylation cascades (Juliano and Haskill, 1993).

Furthermore, integrins are heterodimeric formed by two (α and β) subunits (Virtanen *et al.*, 1990; Hynes, 1992). Many different subunits have been characterized and different combinations of α and β subunits may function as receptors for a variety of extracellular proteins (Clover *et al.*, 1992; Hynes, 1992). Among this large family, the β_1 -integrin subunit is highly expressed in bone cells both *in vitro* as well as *in vivo* (Clover *et al.*, 1992). The aim of this paper is to study the possible role of *in vitro* mechanical strain in the mRNA expression and distribution of the β_1 -integrin subunit. These results in combination with previous studies relating strain-induced biochemical changes to cytoskeletal rearrangement are directed to defining an orthodontic signal transduction pathway.

MATERIALS AND METHODS

Human osteosarcoma TE-85 cells were obtained from ATCC (ATCC, Rockville, MD). Isolated cells were maintained in DMEM/F-12 Ham medium (1:1) (Sigma, St. Louis, MO) supplemented with 5 % FBS (Gibco, Burlington, ON), 25 units/ml of penicillin G, 25 µg/ml of streptomycin and 100 µg/ml ascorbic acid (all from Sigma, Chemical Co., St. Louis, MO). Cells were plated at a density of 2×10^4 cells/cm² in collagen-coated Flexercell Type I and Type II dishes (Flexcell Corp. McKeesport, PA) and maintained in a humidified atmosphere consisting of 5 % CO₂ and 95 % air at 37°C. Cell cultures were grown for 2 days to confluency prior to being mechanically strained and were photographed using a phase-contrast Zeiss Optic photomicroscope (Zeiss, Germany).

Mechanical strain was applied by using the Flexercell Strain Unit (Flexcell Corp., McKeesport, PA). This apparatus has been extensively studied as reported previously (Anderson *et al.*, 1992; Carvalho *et al.*, 1994). Mechanical strain was applied in cells grown in Type I dishes (flexible bottoms) using a pressure of 20 kPa at 3 cycles per minute with 10 seconds of strain followed by 10 seconds of relaxation. The force applied resulted in <1 % to 24 % of strain to the dish bottom (Gilbert *et al.*, 1991; Gilbert *et al.*, 1994). However most of the area of the dish fell within <1 % of strain (or 10,000 µstrains).

Experimental dishes were strained for periods of 15 and 30 minutes, 2, 4 and 24 hours. Cell cultures of 1, 3, 5, 7, 10, 14, 20 and 28 days were also strained for the 24 hours prior to the ending of each time period. Control cultures were cultivated for the same time periods using rigid-bottomed (no strain) collagen-coated Flex type II dishes. All cell cultures were serum starved 24 hrs prior to the straining period or the equivalent time for the control cultures. Aliquots from strained and unstrained dishes of all the time periods were counted electronically in a Coulter Counter (Model ZBI, Hialeah, USA).

Immunohistochemistry was performed in strained and unstrained TE-85 cells. Samples were rinsed with PBS containing 0.1 M glycine (to quench intrinsic fluorescence due to fixation) and fixed by incubation with 4 % paraformaldehyde (pure grade) in PBS

for 10 minutes. Cells were washed in PBS, permeabilized in 0.5 % Triton X-100 in PBS for 20 minutes at room temperature and washed again in PBS. Primary IgG antibody (diluted in PBS with 1 % BSA (Sigma, St. Louis, MO)) mouse anti-human β_1 -integrin (Gibco, Burlington, ON) was added for 1 hour also at room temperature. Samples were extensively washed in PBS and incubated with goat Cy3 conjugated anti-mouse IgG (50:1) secondary antibody for 40 minutes at room temperature in PBS/1 % BSA. After rinsing in PBS the silastic bottoms of the dishes were removed and mounted with fluoromount. Positive and negative controls for all the antibodies were performed. Cultures were photographed using a Zeiss epi-fluorescence microscope (filters of 450-490 nm excitation). Due to the gradient pattern of strain bi-axially in the silastic bottoms (1 to 24 % of strain), to account for controlled levels of strain, all the photomicrographs were taken from the most central area (<1 % strain) of the bottoms.

In order to study the effect of mechanical strain on gene expression, total RNA was extracted from strained and unstrained TE-85 cells grown in culture as previously described by Chomzynski and Sacchi (1987). Cells were rapidly rinsed in ice-cold PBS, incubated in a denaturing solution (4 M guanidinium thiocyanate, 25 mM NaCl, pH 7.0, 0.5 % sarcosyl and 0.1 % β -mercaptoethanol), homogenized and incubated with water-saturated phenol, 2 M Na acetate and chloroform:isoamyl alcohol (49:1). Samples were centrifuged (10,000 g for 20 minutes) and the water phase was incubated with isopropanol at -70°C. The samples were again centrifuged and the pellet was resuspended in the denaturing solution. Once again the samples were incubated with isopropanol, centrifuged and the pellet washed in 70 % ethanol and dried at room temperature. Samples were resuspended in distilled RNase-free water. RNA was quantified by spectrophotometry (Bio-Rad Laboratories Model 620 Video Densitometer, Matsushita Electric Industrial Co. Ltd., Japan). 20 μ g of RNA per sample was fractionated by electrophoresis on a 1.2 % agarose gel, and northern blots were performed at least 4 times according to Kubota *et al.* (1993). RNA was transferred to a nitrocellulose membrane, pore size 0.45 μ m (MSI, Fisher Sci.,

Winnipeg, MB) overnight at room temperature. A cDNA probe for β_1 integrin of 1.2 kb was obtained from Gibco (Burlington, ON). The probe was labeled with [32 P]- γ CTP with a random prime kit (Amersham, Oakville, ON), and the membranes were hybridized overnight at 42°C. In addition, a probe for cyclophilin of 750 bases was used for control (generous donation of Dr. J. Richman). The excess probe was washed by subsequent changes of SSPE buffer (3 M NaCl, 23 mM NaH₂PO₄, 2 mM EDTA, pH 7.4) with varying degrees of stringency, and the blots were exposed to Kodak XRP-1X-ray films (Kodak Canada Inc., Toronto, ON). Data were analyzed statistically by a two-way analysis of variance with Tukey's multiple comparison test. T-tests with the Bonferroni correction were also used to compare mRNA expression from strained and unstrained cells. All data represent the average of 4 experiments performed under identical conditions.

RESULTS

The TE-85 cells had a tendency to form small clusters both prior to and after confluency. The monolayer culture took approximately 36 to 48 hours to become confluent. However, bone nodule formation did not become evident until 72 hours of culture. Fig. 1 shows the different stages of cell growth, specifically the tendency of these cells to form nodules, first as a white spot (Fig. 1c) and in later stages mineralized nodules (Fig. 1d). Cell shape also changed from slender to cuboidal in the center of the cultures, increasing as the cells localized towards the center of the nodules (not shown). The force applied in strained samples resulted in <1 % to 24 % of strain to the dish bottom. However most of the area of the dish fell within <1 % of strain (or 10,000 μ strains) as reported previously (Gilbert *et al.*, 1991; Anderson *et al.*, 1992; Gilbert *et al.*, 1994). TE-85 cells have been extensively characterized with von Kossa silver nitrate stain (Carvalho *et al.*, unpublished observations). Cell numbers also increased significantly ($p < 0.005$) with the application of strain for all the time periods starting at 3 days when compared with unstrained controls (Fig. 2). Cultures subjected to mechanical strain did not show any significant degree of cell death as evidenced by trypan blue exclusion (not shown). Osteoblastic-like cells also showed significant increase in cAMP levels in the presence of parathyroid hormone and significant changes in alkaline phosphatase activity were earlier detected following mechanical strain (Carvalho *et al.*, 1994).

Mechanical strain-induced changes in β_1 integrin reorganization demonstrated that this integrin subunit is influenced by strain application as early as 4 hours from the onset of stimulation. In fixed and permeabilized TE-85 cells anti β_1 -integrin subunit antibodies labeled prominent clusters of integrins in the cell periphery (Fig. 3). The concentrations of these clusters in unstrained controls (Fig. 3b) appeared to increase in volume when these cells were mechanically strained, redistributing towards the center of the cells (Fig. 3c and 3d). The distribution of the integrin clusters showed a focal contact format. From these data we cannot determine if the cells changed significantly their size. However, when the

strained cells were incubated with Hoechst nuclear blue stain (No. 2338), the nuclear volume was not significantly affected by strain (Fig. 3e). This pattern was not the same as for the labeling of other integrin subunits seen previously (Hayashi *et al.*, 1990). Mechanical strain *in vitro* also caused a significant increase in β_1 integrin synthesis (data not shown).

In order to determine if the change in shape observed for β_1 integrin reflected an increase in the expression of β_1 -integrin subunit mRNA, total RNA was extracted from strained and unstrained cells at different times and northern hybridization analyses were done as shown in Fig. 4. Northern blots of the RNAs derived from cells exposed to different strain periods showed a single band of 3.8 kb, corresponding to the β_1 integrin subunit. This is the same band size as seen for chicken NIH 3T3 cells (Hayashi *et al.*, 1990). The same amount of RNA was used in this study for both strained and unstrained cultures, and the level of expression of human β_1 integrin subunit mRNA depended on the particular time period studied. Significant hybridization was observed at 30 minutes and 3 days of strain stimulation in TE-85 cells (Fig. 4). Therefore, application of strain caused a significant increase ($p < 0.005$) in the integrin mRNA expression (Fig. 5).

DISCUSSION

The application of orthodontic forces in order to facilitate the movement of teeth generates above all a series of undesired reactions in the underlying supporting structures of the alveolar bone, periodontal ligament and the teeth themselves. Although, modern clinical techniques have minimized to a large extent this nonprecise nature of orthodontic therapy, the biological mechanisms "transducing" external forces into cell responses are still elusive. In this study we have tried to correlate the expression of a membrane-associated receptor to this so-called transduction of force, helping to fill the gaps between orthodontic force and desired/undesired cell responses.

To test the response of bone-like cells, we have used a transformed osteosarcoma cells line (TE-85). It is well accepted that the osteoblast is the cell that regulates both the formative and the resorptive phases of the remodeling process (Sandy *et al.*, 1993). Consequently, it was appropriate to use a homogeneous cell model that would easily reproduce an osteoblastic-like phenotype with only a small degree of variability as reported previously (Carvalho *et al.*, unpublished observations). In addition, the high levels of strain used in this study are above the estimated "physiological" levels of strain applied to bone cells *in vitro* (Rubin and Hausman, 1988; Brighton *et al.*, 1991). However, these values were used firstly because they generated fairly comparable biochemical response with previous more physiological studies (Rubin *et al.*, 1989; Jones *et al.*, 1991) and secondly because the authors believe that such higher strain values are more indicative of force systems developed during orthodontic tooth movement. The mechanical stretching of the TE cells *in vitro* induced a significant change in distribution and expression of the β_1 -integrin subunit. However, changes in integrin expression shown in this study are probably not specific to bone-like cells, and may rather represent a more generalized cell response to mechanical stimulation. For the bi-axial strain applied in this study the amount of cell elongation reportedly increased by 3 to 5 % compared with unstrained controls (Anderson *et al.*, 1992). Thus, the changes observed here could be attributed to direct

mechanical strain application to connective tissue cells, such as those induced during tooth movement (Storey, 1973).

The expression of integrins in bone has not been widely studied. Nevertheless, it was reported that β_1 -integrin together with α_1 , α_2 , α_3 and α_v are primarily expressed in bone cells (Clover *et al.*, 1992). Each of these subunits is divided into a large extracellular domain, a transmembrane domain and a short cytoplasmic domain (Gailit *et al.*, 1993). Through its cytoplasmic region, integrins physically link extracellular matrix proteins with the intracellular actin network via binding interactions with different actin-associated proteins such as talin, vinculin and tensin, located near the inner surface of the membrane (Burrige *et al.*, 1988; Hynes, 1992). In addition, both extracellular matrix proteins and the extracellular domain of the β chain contain a RGD peptide which is largely responsible for their high affinity binding (Sims *et al.*, 1992). Studies have shown that when cells were incubated with a retraction buffer which induced cell rounding by dislodging integrin receptors from their RGD binding sites (Sims *et al.*, 1992), the transmission of mechanical forces to the cell cytoskeleton was prevented (Ingber, 1993). Hence it was suggested that integrins may not only regulate cell shape by a chemical mechanism but also through a biochemical component (Ingber, 1991; Ingber, 1993). Such biomechanical component may be central in mechanical strain-induced cell response (Beckerle and Yeh, 1990).

While the concept that "form alters function" has been accepted since the early studies by Wölff (1892) it was not until recently that the application of mechanical strain was shown to cause an alteration of the cell shape with significant cytoskeleton rearrangement (Buckley *et al.*, 1988; Pender and McCulloch, 1991) or integrin expression (Gailit *et al.*, 1993). By the same token, the little information on signal transduction pathways of mechanically strained cells with regard to the cytoskeleton, integrin receptors or biochemical changes is often over simplified. Previous studies have shown that second messenger response to orthodontic forces demonstrably far exceeds the role of cAMP alone (Farndale *et al.*, 1988; Sandy *et al.*, 1993). In particular, the phosphatidylinositol pathway

through protein kinase C and inositol trisphosphate as well as cytokine and prostaglandin production (Davidovitch *et al.*, 1988), has been shown to be the mediator for a number of events in mechanically strained tissues (Sandy *et al.*, 1989; Jones *et al.*, 1991; Carvalho *et al.*, 1994). Furthermore, the changes in cell shape through cytoskeletal matrix interactions may trigger a series of cell responses relevant in transduction of strain to bone (Rubin and Hausman, 1988; Ingber, 1991). However, these findings fail to explain the mechanism responsible for initiating these changes, notably second messengers and shape changes through cytoskeletal rearrangement. In this paper we discuss the hypothesis that mechanical strain stimulates signal transduction through the action of integrin receptors and especially the ubiquitous β_1 -integrin subunit.

In bone, integrin expression for specific ligands is likely to be critical in controlling cell function (Clover *et al.*, 1992). This cell membrane receptor determines the local environment of cells, which combined with the extracellular matrix can exert direct effects on cell function. In this paper we have reported that mechanical strain has increased mRNA expression. By contrast, it is not clear how mechanical strain affects cell response through the expression of β_1 -integrin mRNA at the molecular level. Although, application of mechanical stimulation have increased the cell number, changes in β_1 -integrin mRNA are not likely to be explained by this, as control mRNA (cyclophilin) was used to compare all the lanes. Izumo *et al.* (1988) showed that strain caused an increase in "immediate" gene expression (such as proto-oncogenes and heat shock protein genes) in cardiac myocytes. Similarly the "late" response in the same cells was induced by the fetal contractile protein genes and the atrial natriuretic peptide genes (Komuro *et al.*, 1990). Thus, whether these genes are also expressed in bone cells remains to be established, however, there seems to exist a hierarchy of gene activation characteristically associated with the mechanical strain-induced response. This may partially explain the magnitude of differences at the various time periods shown in Figure 5. In addition, the expression of the β_1 -integrin receptor may contribute to its auto regulation. This appears to be the case when integrins are associated

with other molecules such as pp60^{src} (Nermut *et al.*, 1991) and intracellular Ca²⁺ (Jaconi *et al.*, 1991) or phosphorylation cascades stimulated by mechanical strain (Kornberg *et al.*, 1991). Recently, a regulatory, cis-acting, fluid shear-stress-responsive element was identified in the promoter region of the platelet-derived growth factor B gene (Resnick *et al.*, 1993). We hypothesize that the β_1 -integrin receptor responds to strain by a similar mechanism. Further work is necessary to clarify the mechanical stimulation at the molecular level.

In conclusion, stimulation of integrin expression by mechanical strain in bone cells may indicate a possible signal transduction pathway in orthodontic applied forces. These receptors, by either chemical and/or biomechanical stimulation, may mediate the strain signals into a vast range of cell functions. Further work is needed to elucidate the role of the different membrane receptors with the different intracellular responses. Ultimately, clinical orthodontic therapy may be better controlled because of our understanding of how appliance force systems are transduced to intracellular cell response. This would lead to more effective tooth and bone movement and more stable orthodontic results.

REFERENCES

- Anderson J, Carvalho RS, Yen EHK and Scott JE 1992 Measurement of strain in cultured bone and fetal muscle and lung cells. *In Vitro Cell Dev Biol* **29**:183A-186A.
- Beckerle MC and Yeh RK 1990 Talin: role at sites of cell-substratum adhesion. *Cell Motil Cytoskeleton* **16**:7-13.
- Binderman I, Zor U, Kaye A, Shimshoni Z, Harell A and Sömjem D 1988 The transduction of mechanical force into biochemical events in bone cells may involve activation of phospholipase A₂. *Calcif Tissue Int* **42**:261-266.
- Bodary SC and McLean JW 1992 The integrin beta 1 subunit associates with the vitronectin receptor alpha v to form a novel vitronectin receptor in a human embryonic cell line. *J Biol Chem* **265**:5938-5941.
- Brighton CT and McCluskey WP 1988 Response of cultured bone cells to a capacitively coupled electric field: Inhibition of cAMP response to parathyroid hormone. *J Orthop Res* **6**:567-571.
- Brighton CT, Stratford B, Gross SB, Leatherwood DF, Williams JL and Pollack SR 1991 The proliferative and synthetic response of isolated calvarial bone cells of rats to cyclic biaxial mechanical strain. *J Bone Jt Surg* **73**:320-331.
- Buckley MJ, Banes AJ, Levin LG, Sumpio BE, Sato M, Jordan R, Gilbert J, Link GW and Tay RTS 1988 Osteoblasts increase their rate of division and align in response to cyclic, mechanical tension *in vitro*. *Bone Min* **4**:225-236.
- Burger EH, Klein-Nulend J and Veldhuijzen JP 1992 Mechanical stress and osteogenesis *in vitro*. *J Bone Min Res* **7**:397S-401S.
- Burridge K, Fath K, Kelly T, Nuckolls G and Turner C 1988 Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Ann Rev Cell Biol* **4**:487-525.

- Carvalho RS, Scott E, Suga DM and Yen EHK 1994 Stimulation of signal transduction pathways in osteoblasts by mechanical strain is potentiated by parathyroid hormone. *J Bone Min Res* 7:999-1011.
- Chomzynski P and Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159.
- Clover J, Dodds RA and Gowen M 1992 Integrin subunit expression by human osteoblasts and osteoclasts *in situ* and in culture. *J Cell Sci* 103:267-271.
- Davidovitch Z, Nicolay OF, Ngan PW and Shanfeld GL 1988 Neurotransmitters, cytokines and the control of alveolar bone remodeling in orthodontics. *Dent Clin North Am* 32:411-435.
- Farndale RJ, Sandy JR, Atkinson SJ, Pennington SR, Meghji S and Meikle MC 1988 Parathyroid hormone and prostaglandin E₂ stimulate both inositol phosphates and cyclic AMP accumulation in mouse osteoblasts cultures. *Biochem J* 79:230-239.
- Fujimori A, Cheng S, Avioli LV and Civitelli R 1992 Structure-function relationship of parathyroid hormone: activation of phospholipase-C, protein kinase-A and -C in osteosarcoma cells. *Endocrinology* 130:29-36.
- Gailit J, Colflesh D, Rabiner I, Simone J and Goligorsky MS 1993 Redistribution and dysfunction of integrins in cultured renal epithelial cells exposed to oxidative stress. *Am Physiol Soc* 263:149F-157F.
- Gilbert JA, Banes AJ, Link GW and Jones GL 1991 Video analysis of membrane strain: an application in cell stretching. *Exp Tech* 15:43-45.
- Gilbert JA, Weinhold PS, Banes AJ, Link GW and Jones GL 1994 Strain profiles for circular cell culture plates containing flexible surfaces employed to mechanically deform cells *in vitro*. *J Biomech* 27:1169-1177.
- Harell D, Dekel S and Binderman I 1977 Biochemical effect of mechanical stress on cultured bone cells. *Calcif Tissue Res* 22:202S-207S.

- Hayashi Y, Haimovich B, Reszka A, Boettinger D and Horwitz A 1990 Expression and function of chicken integrin β_1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. *J Cell Biol* **110**:175-184.
- Hynes RO 1992 Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**:11-25.
- Ingber DE 1991 Integrins as mechanochemical transducers. *Curr Opin Cell Biol* **3**:841-848.
- Ingber DE 1993 Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* **104**:613-627.
- Izumo S, Nadal-Ginard B and Mahdavi V 1991 Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc Natl Acad Sci USA* **83**:339-343.
- Jaconi MEE, Theler JM, Schlegel W, Appel RD, Whright SD and Lew PD 1991 Multiple elevations of cytosolic free- Ca^{2+} in human neutrophils: initiation by adherence receptors of the integrin family. *J Cell Biol* **112**:1249-1257.
- Jones DB, Nolte H, Schlüßbers JG, Turner E and Veltel D 1991 Biochemical signal transduction of mechanical strain in osteoblast-like cells. *Biomaterials* **12**:101-110.
- Juliano RL and Haskill S 1993 Signal transduction from the extracellular matrix. *J Cell Biol* **120**:577-585.
- Komuro I, Katoh Y, Kaida T, Shibasaki I, Kurabayashi M and Hoh E 1990 Stretching cardiac myocytes stimulates protooncogene expression. *J Biol Chem* **265**:3595-3598.
- Komuro I, Katoh Y, Kaida T, Shibasaki I, Kurabayashi M, Hoh E, Takaku F and Yazaki Y 1991 Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. *J Biol Chem* **266**:1265-1268.

- Kornberg L, Earp HS, Turner C, Procop C and Juliano RL 1991 Signal transduction by integrins: increased protein tyrosine kinase phosphorylation caused by clustering of beta 1 integrins. *Proc Natl Acad Sci USA* **88**:8392-8396.
- Kubota T, Yamauchi M, Onozaki J, Sato S, Susuki Y and Sodek J 1993 Influence of an intermittent compressive force on matrix protein expression by ROS 17/2.8 cells, with selective stimulation of osteopontin. *Archs Oral Biol* **38**:23-30.
- Martinez RH and Johnson RB 1988 Effects of orthodontic tooth movement on the alcian blue staining pattern of rat alveolar bone: an histochemical study. *Histol Histopathol* **3**:39-47.
- Morgan N 1989 Guanine-nucleotide binding proteins as signal transduction. In: Morgan N, ed. *Cell Signaling*. Guilford Press, London, pp.35-57.
- Nermut MV, Eanon P, Hirst EMA and Kellje S 1991 Cell/substrate adhesions in RSV-transformed rat fibroblasts. *Exp Cell Res* **193**:382-397.
- Oppenheim A 1911 Tissue changes particularly of the bone incident to tooth movement. *Angle Orthod* **3**:57-67.
- Pender N and McCulloch AG 1991 Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching. *J Cell Sci* **100**:187-193.
- Resnick N, Collins T, Atkinson W, Bonthron DT, Dewey Jr CF and Gimbrone Jr MA 1993 Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. *Proc Natl Acad Sci USA* **90**:4591-4595.
- Rubin CT and Hausman MR 1988 The cellular basis of Wölff's law. Transduction of physical stimuli to skeletal adaptation. *Rheum Dis Clin North Am* **14**:503-517.
- Rubin CT, McLeod KJ and Lanyon LE 1989 Prevention of osteoporosis by pulse electromagnetic fields. *J Bone Jt Surg* **71**:411-417.
- Sachs F 1992 Mechanical transduction in biological systems. *Crit Rev Biomed Eng* **16**:141-169.

- Sandy JR, Meghji S, Farndale RW and Meikle MC 1989 Dual elevation of cyclic AMP and inositol phosphates in response to mechanical deformation of murine osteoblasts. *Biochem Biophys Acta* **1010**:265-269.
- Sandy JR, Farndale RW and Meikle MC 1993 Recent advances in understanding mechanically induced bone remodeling and their relevance to orthodontic theory and practice. *Am J Ortho Dentofac Orthop* **103**:212-222.
- Schirrmacher K, Scmitz I, Winterhager E, Traub O, Brümmer F, Jones D and Bingmann D 1992 Characterization of Gap junctions between osteoblast-like cells in culture. *Calcif Tiss Int* **51**:285-290.
- Sims JR, Karp S and Ingber D 1992 Altering the cellular mechanical force balance results in integrated changes in cell, cytoskeleton and nuclear shape. *J Cell Sci* **103**:1215-1222.
- Sömjen D, Binderman I, Berger E and Harell A 1980 Bone remodeling induced by physical strain is prostaglandin E₂ mediated. *Biochim Biophys Acta* **627**:931-934.
- Storey E 1973 The nature of tooth movement. *Am J Orthod* **63**:292-314.
- Vandenburgh HH 1992 Mechanical forces and their second messengers in stimulating cell growth *in vitro*. *Am J Physiol* **262**:350R-355R.
- Virtanen I, Korhonen M, Karineimi A, Gould VE, Laitinen L and Yläne J 1990 Integrins in human cells and tumors. *Cell Diff Dev* **32**:215-228.
- Wölff JL 1892 The law of bone remodeling. In: Maquet P and Furlong R, eds. (Translation, 1986) *Das Gesetz der Transformation der Knochen*, Springer-Verlag, Berlin.

Figure 1. Phase contrast photomicrograph of TE-85 cells in culture. (a) Cells at low density prior to mechanical straining. (b) Cells reaching confluency after 30 hrs in culture. (c) Strained cells after 72 hours of initial plating. Single black arrows indicate the presence of bone nodule as a white spot. (d) One week after initial plating the cells are completely immersed by matrix. Note mineralized nodule indicated by single black arrows. White arrow indicates the direction of strain. Magnifications (a-d) X 80.

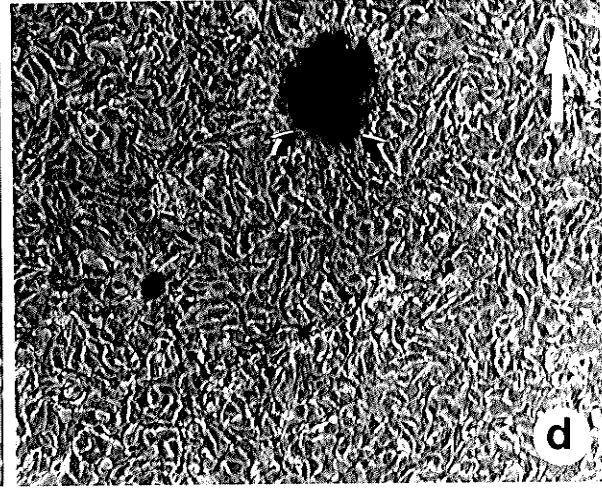
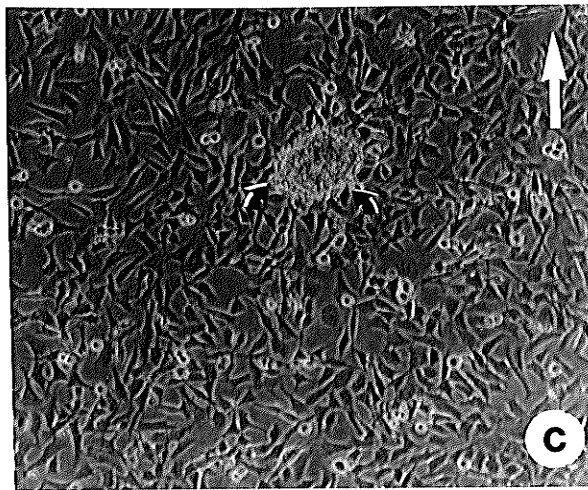
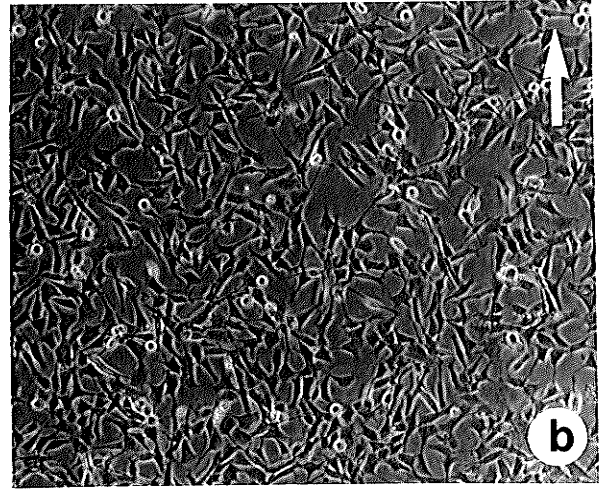
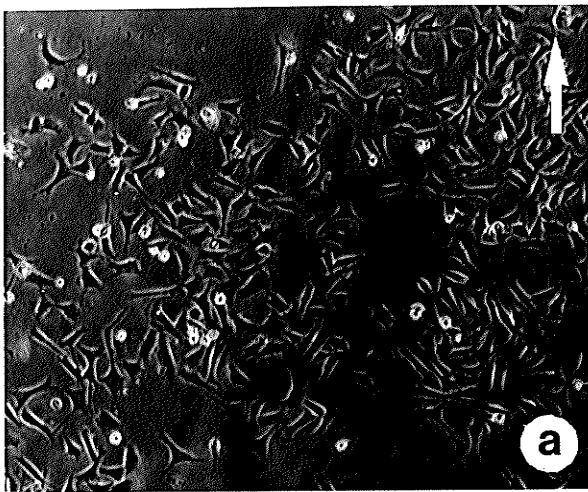


Figure 2. Effect of mechanical strain in the cell count number of TE-85 cells. Note the increase in cell numbers through the time periods studied.

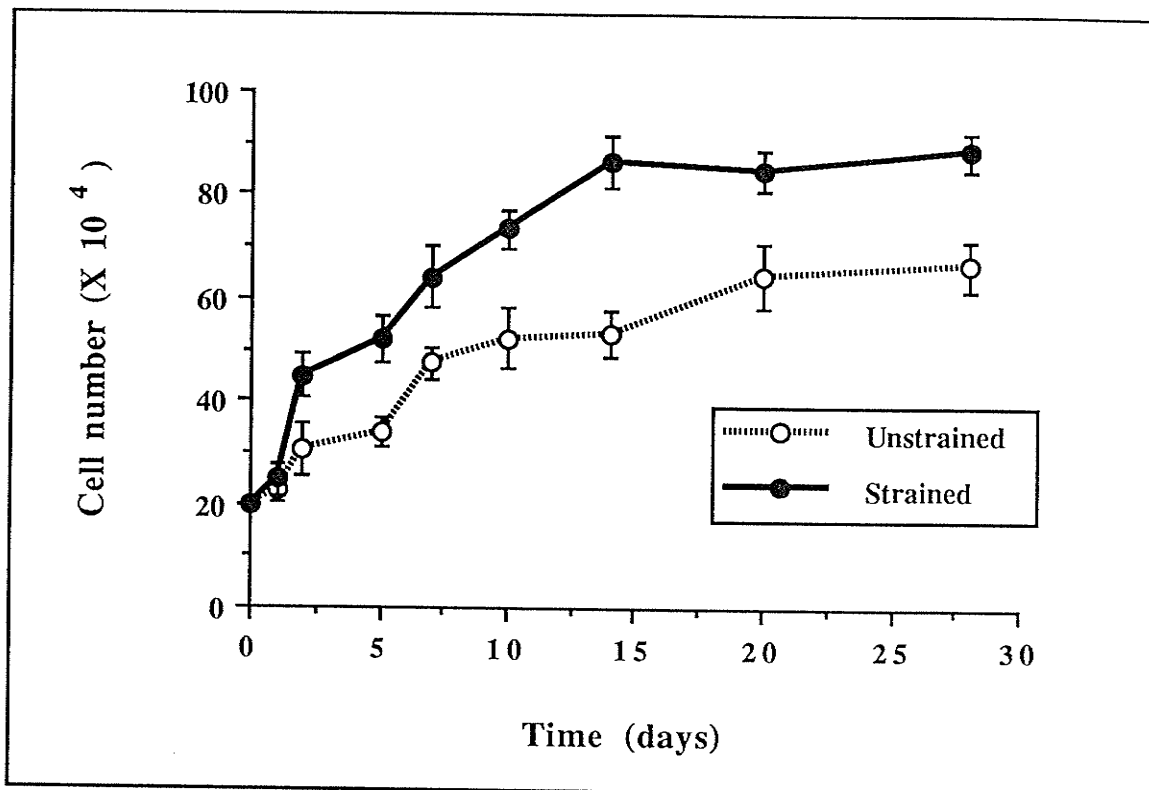
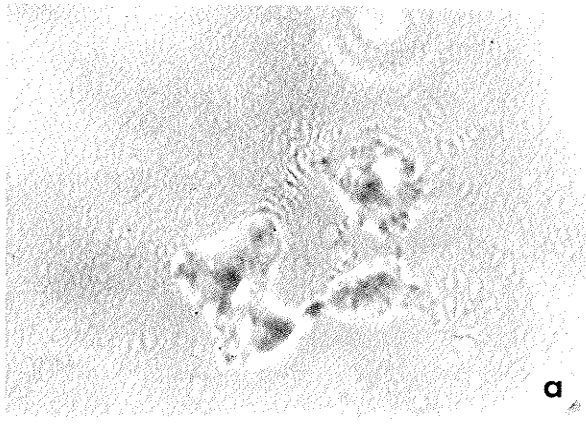
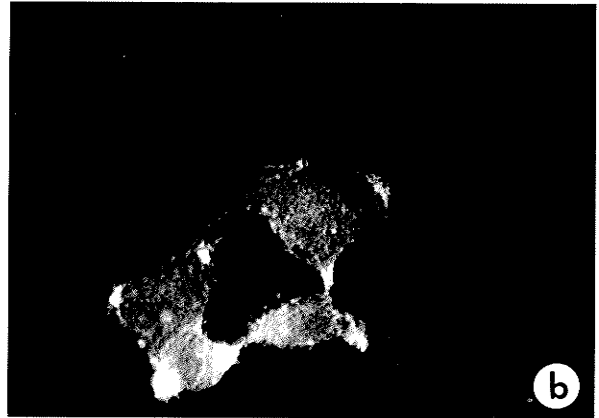


Figure 3. Immunolocalization studies of TE-85 cells. (a) phase contrast photomicrograph of unstrained TE-85 HOS cells. (b) control unstrained cultures incubated with β_1 -integrin antibodies showed an increase in the labeling when these cells were mechanically strained as evidenced by double white arrows in 30 minutes (c) and 3 days of strain (d). (e) cell nuclear stain from the same cells shown in (d). (f) negative control, in which the primary antibody was omitted. White arrows indicate the direction of strain. Magnification (a-f) X 1250.



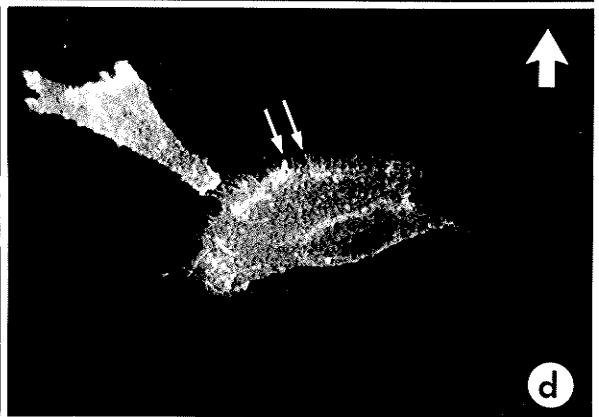
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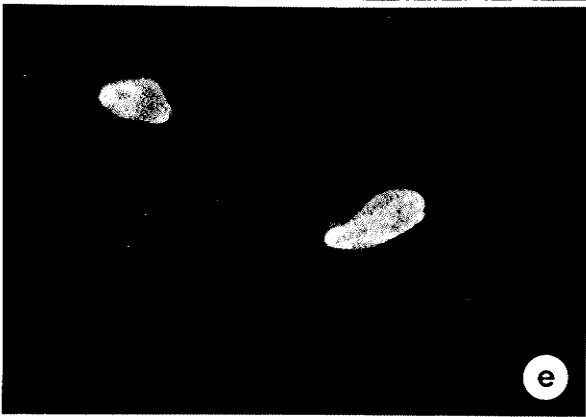
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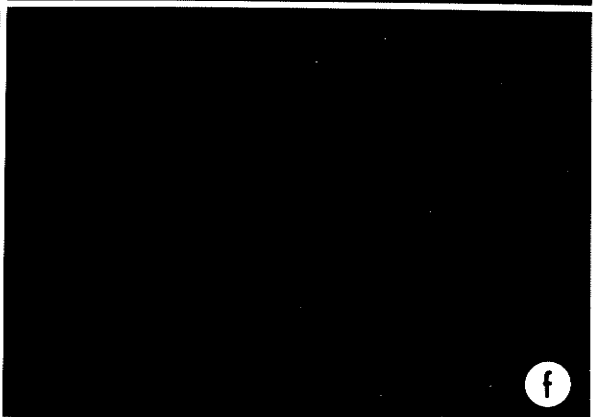
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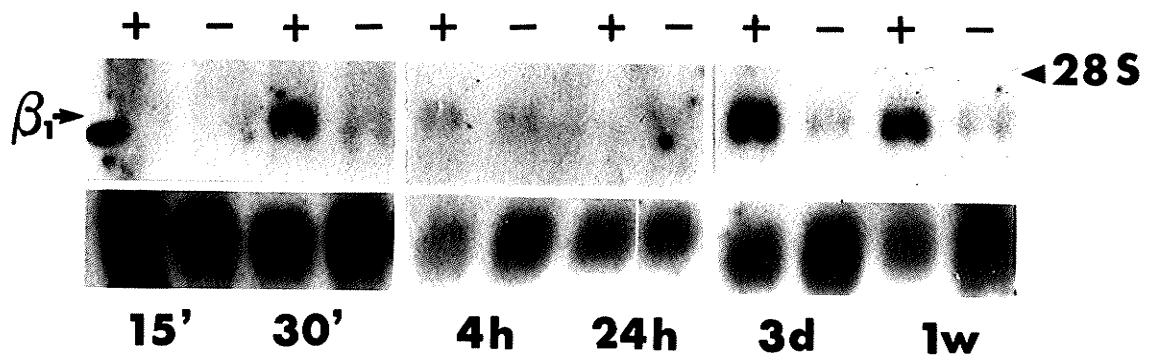


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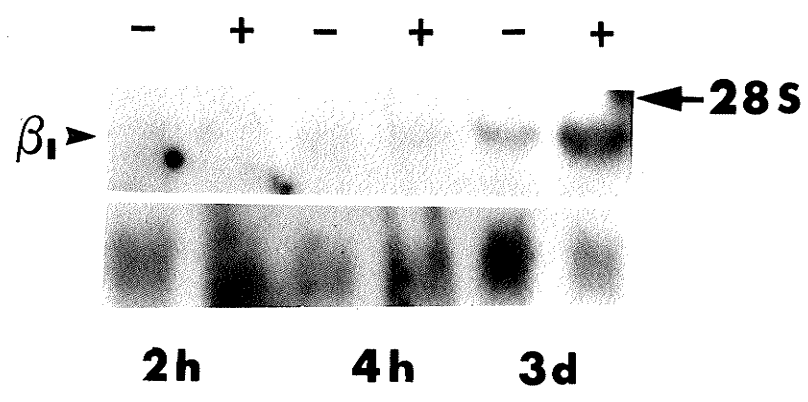


f

Figure 4. Photomicrographs of northern blots showing mRNA of β_1 -integrin subunit. (a) effects of orthodontic strain in β_1 -integrin subunit mRNA expression. Note that strain (+) causes an increase of β_1 mRNA at 30 minutes and again at 3 days and 1 week when compared with unstrained controls (-). Control mRNA (probe for cyclophilin) indicating comparable amounts of RNA per lane are shown by double arrows. Legends are: 15' (15 minutes), 30' (30 minutes), 2 h (2 hours), 4 h (4 hours), 24 h (24 hours), 3 d (3 days) and 1 w (1 week). (b) same as that shown in (a). Note the increase in β_1 mRNA at 3 days of strain application. Legends are: 2 h (2 hours), 4 h (4 hours) and 3 d (3 days).

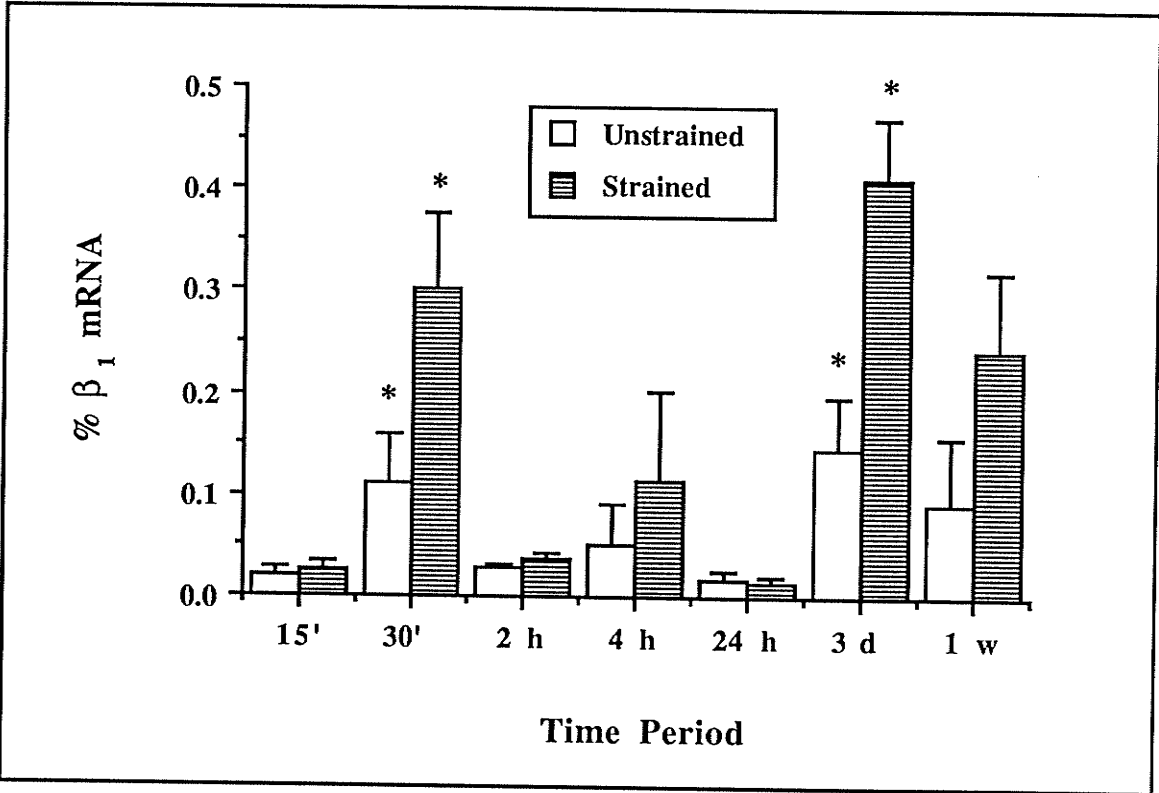


A



B

Figure 5. Effect of mechanical strain in the expression of β_1 -integrin subunit mRNA. Values indicate at least 4 cultures for each time period studied. β_1 -integrin mRNA expression is represented by its ratio (%) to the internal control (cyclophilin expression) from densitometric scans of x-ray films. * indicates significantly greater ($p < 0.005$) in strained cultures when compared with unstrained controls. Legends are: 15' (15 minutes), 30' (30 minutes), 2 h (2 hours), 4 h (4 hours), 24 h (24 hours), 3 d (3 days) and 1 w (1 week).





Chapter SEVEN

*Selective Expression of α_v and β_1 -integrin
Subunits and Their Role in the Signal Transduction
Pathways of Mechanically Strained Human
Osteosarcoma TE-85 Cells*

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(submitted)



ABSTRACT

The relationship between different types of orthodontic force systems and periodontal/alveolar tissue response at the cellular level is not clear. Elucidation of the signal pathways affecting cell response could facilitate biological response required for desired tooth movement and skeletal remodeling. Mechanical strain in HOS cells were correlated with changes in distribution and mRNA levels of selected integrins. HOS TE-85 cells were cultured in DMEM/F-12 and grown to confluency in Flexercell Type I dishes in a humidified incubator with 5 % CO₂ and 95 % air. Intermittent strain (0.05 Hz) was applied to the cells using the Flexercell Strain Unit System for periods of 15 and 30 minutes, 2 and 24 hours and 3, 5 and 7 days. Unstrained cells were used as controls. The distribution of α_v and β_1 integrin was studied by immunocytochemical procedures while the mRNA levels for the same subunits was studied by northern blot analysis. Total RNA was isolated at all time periods and northern blots were performed to study the effects of strain on levels of α_v and β_1 integrin expression in HOS TE-85 cells. The results indicated that, although the expression of β_1 mRNA increased significantly ($p < 0.005$) at periods of 30 minutes and 3 days of strain application, the expression of α_v mRNA did not appear to change. However, strain not only markedly affected the distribution of β_1 integrin but also that of α_v integrin in periods of 24 hour cultures. Osteoblast-like cells appear to respond to mechanical strain through the stimulation of selected integrin subunits. This together with our earlier results suggests that integrins and the cytoskeleton may provide a strain induced signal transduction pathway in osteoblast-like cells.

INTRODUCTION

The mechanical properties of bone cells stem from the load bearing function of bone tissue (Lanyon, 1992). Independent of strain types applied to bone cells or bone-like material, studies suggest that mechanical stimulation alter the metabolism of bone cells in culture (Hasegawa *et al.*, 1985; Rubin *et al.*, 1989; Burger *et al.*, 1992; Anderson *et al.*, 1992; Carvalho *et al.*, 1994; Carvalho *et al.*, 1995a). While the mechanisms of strain-related signal transduction are not clear, it is well known that extracellular matrix (ECM)-cell associations through transmembrane receptors may initiate bone cell response in both short and long term modeling/remodeling (Lanyon, 1992; Hynes, 1992; Sandy *et al.*, 1993; Carvalho *et al.*, 1994). In other words, cells respond to ECM-mediated signals through matrix-specific transmembrane cellular receptors, which have a primarily structural or mechanical role (Damsky and Werb, 1992).

Integrins are a large family of transmembrane receptors glycoproteins that attach to classical ECM molecules such as collagen, fibronectin, laminin and vitronectin (Hynes, 1992; Hormia and K n nen, 1994). This variability of integrin binding properties is due to a wide array of structurally diverse subunits. These may interact with different ECM ligands according to their function and cell type-expression (Hormia and K n nen, 1994). All integrins appear to be heterodimers formed by 14 α and 8 β subunits, for a total of 20 $\alpha\beta$ receptor combinations (Hynes, 1992; Clover *et al.*, 1992; Gailit *et al.*, 1993; Hormia and K n nen, 1994). The attachment properties of integrins are given by their affinity for the amino acid sequence "Arg-Gly-Asp" (RGD) present in the cytoplasmic region of their domains (Hynes, 1992). Among RGD-binding integrin receptors, fibronectin ($\alpha_5\beta_1$) and vitronectin ($\alpha_v\beta_3$) appear to play a very important role in the attachment of bone cells (Clover *et al.*, 1992; Gailit *et al.*, 1993).

Since integrins were so named because they integrate ECM with intracellular cytoskeleton (Tooney *et al.*, 1993), signaling may be considered a secondary function of these receptors. Be that as it may, integrins were shown to transduce signals involved in

the regulation of cytosolic Ca^{2+} (Ng-Sikorski *et al.*, 1991), activation of T lymphocytes (Nojima *et al.*, 1990; Shimizu *et al.*, 1990) and control of tumorigenesis (Schreiner *et al.*, 1991; Kornberg and Juliano, 1992; Juliano and Haskill, 1993). However, these receptors appear to lack the appropriate structural characteristics for a receptor involved in signal transduction, such as the presence of an associated enzymatic activity in its cytoplasmic domain (Morgan, 1989). Thus, since integrins do not resemble any classical signal transduction receptor, it is intriguing as to how they transduce these signals. It appears that the role of integrin-dependent signal transduction may be both structural and biochemical (Juliano and Haskill, 1993; Kornberg *et al.*, 1993; Sastry and Horwitz, 1993). In this investigation we have studied the role of mechanical strain in the expression and distribution of integrin subunits for both fibronectin (β_1) and vitronectin (α_v). We also attempted to correlate any strain-induced changes in integrin distribution and mRNA levels with the signal transduction mechanisms of integrin subunits.

MATERIALS AND METHODS

HOS TE-85 were obtained from ATCC (ATCC, Rockville, MD). Isolated cells were maintained in DMEM/F-12 Ham medium (1:1) (Sigma, St. Louis, MO) supplemented with 5 % FBS (Gibco, Burlington, ON), 25 units/ml of penicillin G, 25 μ g/ml of streptomycin and 100 μ g/ml ascorbic acid (all from Sigma, Chemical Co., St. Louis, MO). Cells were plated at a density of 2×10^4 cells/cm² in collagen-coated Flexercell Type I and Type II dishes (Flexcell Corp. McKeesport, PA) and maintained in a humidified atmosphere consisting of 5 % CO₂ and 95 % air at 37°C. Cell cultures were grown for 2 days to confluency prior to being mechanically strained and were photographed using a phase-contrast Zeiss Optic photomicroscope (Zeiss, Germany). Mechanical strain was applied by the Flexercell Strain Unit (Flexcell Corp., McKeesport, PA) as described previously (Carvalho *et al.*, 1994). Mechanical strain was applied in cells grown in Type I dishes (flexible bottoms) using a pressure of 20 kPa at 0.05 Hz. Experimental dishes were strained for periods of 15 and 30 minutes, 2 and 24 hours. Cell cultures of 1, 3, 5 and 7 days were also strained for 24 hours prior to the ending of each time period. Control cultures were cultivated for the same time periods using rigid-bottomed (no strain) collagen-coated Flex type II dishes. All cell cultures were serum starved 24 hrs prior to the straining period or equivalent time for control cultures.

Immunohistochemistry, also described previously (Carvalho *et al.*, 1995a), was performed in strained and unstrained HOS TE-85 cells. Samples were rinsed, fixed with 4 % paraformaldehyde for 10 minutes at room temperature, permeabilized in 0.5 % Triton X-100 for 20 minutes and washed again. Primary IgG antibody mouse anti-human β_1 -integrin (Gibco, Burlington, ON) or mouse anti-human α_v -integrin (Gibco, Burlington, ON) were added for 1 hour followed by the incubation with goat Cy3 conjugated anti-mouse IgG secondary antibody for 40 minutes. Positive and negative controls for all the antibodies were performed. Cultures were photographed using a Zeiss epi-fluorescence microscope (filters of 450-490 nm).

For gene expression studies, total RNA was extracted from HOS TE-85 cells as previously described (Chomzynski and Sacchi, 1987; Carvalho *et al.*, 1995a). Cells were rinsed, incubated in a denaturing solution (4 M guanidinium thiocyanate, 25 mM NaCl, pH 7.0, 0.5 % sarcosyl and 0.1 % β -mercaptoethanol), homogenized, incubated with water-saturated phenol, 2 M Na acetate and chloroform:isoamyl alcohol and centrifuged. The water phase was incubated with isopropanol at -70°C , centrifuged and the pellet was resuspended in the denaturing solution. The pellet was finally washed in 70 % ethanol and dried. Samples were resuspended in distilled RNase-free water. RNA was quantified by spectrophotometry (Bio-Rad Laboratories Model 620 Video Densitometer, Matsushita Electric Industrial Co. Ltd., Japan). 20 μg of RNA per sample was fractionated by electrophoresis on a 1.2 % agarose gel, and northern blots were performed at least 4 times according to Kubota *et al.* (1993). RNA was transferred to a nitrocellulose membrane and cDNA probes for β_1 and for α , integrins were obtained from Gibco (Burlington, ON). The probes were labeled with [^{32}P]- γCTP by random prime kit (Amersham, Oakville, ON), and hybridized to the membrane overnight at 42°C . Controls were performed using a probe for cyclophylin (generously donated of Dr. J. Richman). Data were analyzed statistically by a two-way analysis of variance with Tukey's multiple comparison test. T-tests with the Bonferroni correction were also used to compare mRNA expression from strained and unstrained cells. All data represent the average of 4 experiments performed under identical conditions.

RESULTS

Immunohistochemical fluorescence microscopy showed that the HOS TE-85 cells adhered to Flexercell dishes without any significant degree of cell death both prior to and after application of mechanical strain (not shown). Subsequently, the distributions of β_1 and α_v integrin subunits were studied in strained and unstrained HOS TE-85 cells. Initial experiments showed that integrin distribution appeared to change significantly when mechanical strain was applied (Fig. 1). Figures 1a and 1c show control micrographs of unstrained cells stained for β_1 and α_v respectively. Following 24 hours of strain (Figs. 1b), β_1 has reorganized in the center of the cells forming clusters, contrasting with the diffuse distribution of β_1 seen in unstrained cells (Fig. 1a). Although, mechanical strain also appeared to have changed the distribution of α_v subunit at 24 hours of stimulation (Fig. 1d), when compared to its unstrained control (Fig. 1c), it did to a lesser extent than to that of β_1 (Fig. 1b). Similarly to β_1 , α_v distribution showed fine streaks in the cell periphery in unstrained control samples, which showed to be redistributed in strained samples (white arrow) (Fig. 1d). In unstrained cells, as spreading occurred, the labelling for both integrin subunits appeared increasingly similar. However, redistribution after mechanical strain was distinct for each subunit. In some cases, little staining is seen in the periphery of strained cells labeled with α_v as shown in Figure 1d. For the most part in α_v distribution, labeling can still be seen at the cell periphery, contrary to those of β_1 . Although, from these data we have observed a change in the integrin distribution, we can not determine if the cells changed significantly their size after the application of strain. In order to determine whether such subunit distribution corresponded with changes in mRNA levels, both strained and unstrained cell cultures were labeled with β_1 and α_v monoclonal antibodies for periods of 30 minutes, 24 hours or 3 days. Figure 2 shows the distribution of β_1 (a, b, c and d) and α_v (e, f, g and h) respectively, in unstrained cells (Figs. 2a and 2e) and cells strained for 30 minutes (Figs. 2b and 2f), 24 hours (Figs. 2c and 2g) and cells strained for 24 hours prior to the end of 3 day culture periods (Figs. 2d and 2h). Strain periods of 30

minutes did not appear to show any integrin reorganization, in contrast to 24 hour and 3 day cultures. The redistribution at 24 hours and 3 days of culture was more marked in cultures labelled with β_1 than those of labelled with α_v . Previous studies (Carvalho *et al.*, 1995a) have indicated that β_1 mRNA expression increased at that 30 minutes and 3 days from the onset of mechanical stimulation. Thus, the changes in integrin distribution were not consistent with those of mRNA expression.

For the expression of both β_1 and α_v subunits mRNA, total RNA was extracted from strained and unstrained cells at different time periods and northern hybridization analyses were performed. Figures 3A and 3B show a single band of 3.8 kb, correspondent to the β_1 integrin subunit. This subunit has been demonstrated previously for chicken NIH 3T3 cells (Hayashi *et al.*, 1990). The application of mechanical strain caused a significant increase in hybridization ($p < 0.005$) of β_1 integrin mRNA probe at 30 minutes and 3 days of culture in HOS TE-85 cells as seen previously (Carvalho *et al.*, 1995a). However, the expression levels for this subunit in strained and unstrained cells of other time periods studied were very low (not shown). On the other hand the message for α_v integrin appeared highly expressed in both strained and unstrained cells at all time periods (Figs. 4A and 4B). However no significant differences were detected between strained and control cultures for α_v integrin mRNA expression. α_v integrin mRNA was hybridized as a single band of approximately 7 kb as previously shown by Suzuki *et al.* (1987). The same amount of total RNA was used in this study for both strained and unstrained cultures of α_v and β_1 integrin subunits as shown in Figure 5.

DISCUSSION

ECM-cell interaction is a complex process. Ligand-receptor binding, such as the one which occurs between integrins and ECM proteins may be influenced by a variety of factors. For instance, the presence of different isoforms of the same ECM protein, the unavailability of the entire ECM protein ligand or the presence of other ECM ligands that modify ECM-cell interactions may alter binding (Damsky and Werb, 1992). Nevertheless, cell-ECM and cell-cell interactions take place in a mechanically active environment which may influence the specificity of certain protein-protein bindings. In this study we observed that application of mechanical strain *in vitro* in osteoblast-like cells causes an increase in the mRNA levels of β_1 but not α_v integrin subunit. However, the same levels of stimulation do alter distribution of these integrin subunits. Such a response may be explained by the structure-functional differences of each subunit.

Integrins are effective signal transducing molecules (Nojima *et al.*, 1990; Shimizu *et al.*, 1990; Schreiner *et al.*, 1991; Kornberg and Juliano, 1992; Juliano and Haskill, 1993). The signaling by these molecules may influence cell differentiation, proliferation, differential gene expression, cytoskeletal assembly and migration (Hynes, 1992; Damsky and Werb, 1992; Kornberg and Juliano, 1992; Juliano and Haskill, 1993; Kornberg *et al.*, 1993; Sastry and Horwitz, 1993; Schwartz, 1993). There are at least two channels by which the information may be transduced through integrins: 1) a structural signal initiated by integrin ligation with the ECM which appears to modulate differentiation and gene expression (Damsky and Werb, 1992; Kornberg and Juliano, 1992; Ginsberg *et al.*, 1992; Yoruchko *et al.*, 1992; Juliano and Haskill, 1993; Kornberg *et al.*, 1993; Sastry and Horwitz, 1993); and 2) a biochemical signal initiated by the release of intracellular messengers including changes in the affinity and specificity of external cellular ligands (Sastry and Horwitz, 1993; Carvalho *et al.*, 1994; Carvalho *et al.*, 1995a). In both cases it has been shown that the active subunit of the receptor is the cytoplasmic domain of β_1 integrin subunit (Damsky and Werb, 1992; Reszka *et al.*, 1992). The presence of tyrosine

phosphorylation following β_1 integrin ligation to this subunit suggests the organization and stabilization of the cytoskeletal interactions as a function of the β_1 cytoplasmic domain (Sastry and Horwitz, 1993). Conversely, the function of the α subunit appears to be ligand specific (Panetti and McKeown-Longo, 1993). This subunit may also participate indirectly in the process of maintenance and organization of focal contacts (La Flamme *et al.*, 1992) by possibly regulating integrin localization (Fath *et al.*, 1989), β integrin binding and/or inhibition of the localization of unoccupied integrins (Ylänné, 1993). The α subunits may therefore, determine when and which integrins localize at focal contacts. The distribution of both subunits seen here resemble those of focal contact proteins (Figures 1 and 2). Since different combinations of integrins possess different α subunit cytoplasmic domains, it is possible that the different combinations could promote the formation of distinct cytoplasmic protein networks (Sastry and Horwitz, 1993). The latter would therefore, initiate a different cellular response according to the ECM binding protein and/or tissue demands. Upon the application of mechanical stimulation, such a response may be taking place as evidenced by the changes in α_v and β_1 distribution (Figures 1 and 2) and those of focal contact proteins (Carvalho *et al.*, 1994).

When one considers integrin-associated signal transduction, the first signaling response that comes to mind is the typical cellular outside-inside reaction, by which ECM signals are transmitted to the cell. However, another type of integrin-mediated signal transduction termed inside-outside signaling involves effectors in the cytoplasm, which modulate affinity and/or specificity of the integrin for its ECM ligands (Damsky and Werb, 1992; Hynes, 1992; Sastry and Horwitz, 1993). The interactions of cytoskeletal network is a result of both outside-in and inside-out signaling mechanisms, starting with the specific interactions between both α and β subunits to cytoplasmic proteins (Damsky and Werb, 1992). Our results indicate that activation of integrin subunit gene expression by mechanical stimulation in osteoblast-like cells may take place through regulation of specific integrin subunits. Similarly, there is evidence to demonstrate that induction of integrin-

dependent gene expression depends on the activation of a specific integrin subunit. This is also true for the stimulation of Ca^{2+} flux (Nojima *et al.*, 1990; Damsky and Werb, 1992). Confirmation of these observations corroborates the properties of outside-in and inside-out signaling of integrin subunits in mechanically-stimulated osteoblasts. Similar mechanisms take place in integrin signaling in cooperation with growth factor receptors in order to integrate ECM responses to specific signals (Damsky and Werb, 1992; Sastry and Horwitz, 1993). However, integrins unlike growth factor receptors, do not possess either kinase or phosphatase activity in their cytoplasmic domains (Morgan, 1989; Hynes, 1992; Sastry and Horwitz, 1993). Thus, the nature of integrin-effector interaction may take place through direct or indirect binding. Sastry and Horwitz (1993) proposed that upon ligation, integrins would interact directly with tyrosine kinases and thus become functionally similar to growth factors, such as shown in the activation of T lymphocytes (Shimizu *et al.*, 1990; Schreiner *et al.*, 1991). The latter may also take place through the binding with tensin, a major substrate of tyrosine kinase through its SH2 domain.

One may assume that the mechanism of integrin signaling is dependent on the critical assembly of cytoskeletal components to effectively interact with the components of the intracellular signaling system (Carvalho *et al.*, 1995a, Carvalho *et al.*, 1995b). Phosphorylation cascades may be tied to the association and dissociation of integrin-cytoskeleton interactions (Sandy *et al.*, 1993; Gailit *et al.*, 1993; Tooney *et al.*, 1993), which in turn stimulate enzymatic regulatory proteins, such as focal adhesion kinase¹²⁵ and protein kinase C (Jaken *et al.*, 1989; Schaller *et al.*, 1992; Carvalho *et al.*, 1995a). Integrin receptors may also communicate with each other or with different receptors to modulate distinct responses. For instance, in platelets the interaction of integrin $\alpha_v\beta_3$ with integrin $\alpha_2\beta_1$ causes an increase in intracellular free Ca^{2+} (Leavesley *et al.*, 1992). The interaction of integrin $\alpha_v\beta_1$ with the heparin-binding/assembly domain has also been shown to suppress ECM remodeling enzymes and assembly of focal contacts, whereas binding of

$\alpha_v\beta_1$ alone does not have any suppression activity (Crossin, 1988; Damsky and Werb, 1992; Sastry and Horwitz, 1993).

Two observations from this study are noteworthy: 1) the redistribution of integrin subunits with strain did not follow changes in mRNA levels as shown in Figure 2. This was particularly evident for β_1 integrin. 2) the magnitude of β_1 integrin mRNA expression fluctuated in different time periods studied. The specificity of response among integrin subunits combined with an hierarchy of gene expression as a result of mechanical stimulation (Komuro *et al.*, 1991) may partially explain the differences in magnitude of β_1 integrin expression in various time periods studied (not shown). In addition, such differences may indicate an interruption on the mechanical stimulation of the HOS cells at the genetic level (Haskin and Cameron, 1993). Whether such "interruption" translates into cell function through β_1 integrins is not known, however, recruitment of integrin following mechanical strain-induced integrin rearrangements may not be due solely to integrin synthesis (Milam *et al.*, 1991). Changes in mRNA levels of β_1 integrin may be accounted by the differences in kinetics of the maturation process of integrins. It has been found that most of the β_1 integrin remains as immature precursors in the endoplasmic reticulum, prior to transferring to the cell membrane upon dimer formation with the α subunits (Heino *et al.*, 1989; Santala *et al.*, 1994). Consequently, in many cell lines, the β_1 subunit is produced in large excess to α subunits (Akiyama and Yamada, 1987; Heino and Massagué, 1989). Furthermore, studies suggest that the size of the precursor pool is not stable and can be regulated by growth and differentiation factors (Koivisto *et al.*, 1994). Integrin β_1 molecules that do not dimerize with α molecules will be directed to the intracellular pathway (Santala *et al.*, 1994). Therefore, it is conceivable that in our study mechanical stimulation may enhance the mRNA expression of β_1 integrin to a given requirement of the precursor pool at different time periods. Reduction of this precursor pool has been observed in other transformed cell models (Akiyama *et al.*, 1990), however, further study is required to

elucidate the role of integrin maturation changes in receptor function and mechanically-stimulated cell responses.

According to Damsky and Werb (1992), responses of normal cells to extracellular stimuli are tightly regulated and include integrated changes in gene expression, cytoskeleton and ECM regulation. Direct stimulation of β_1 integrin subunits has been shown to cause an increase in both the steady-state of mRNA levels and protein synthesis. Mechanically stimulated tissues, particularly those which show an increase in collagen and/or fibronectin levels (Hasegawa *et al.*, 1985; Damsky and Werb, 1992; Sandy *et al.*, 1993), demonstrate that increased amounts of these ECM components contribute to an increase in integrin-associated signaling. This regulation of ECM-cell signaling response explains the exacerbation of a pathological state and/or inflammatory response (Yoruchko *et al.*, 1992). Changes in mRNA levels of β_1 in our study may also represent an expression of cellular differentiation in bone. The absence of stimulation of α_v mRNA would be compatible with this hypothesis, since cell differentiation may be promoted without changing the expression levels of selected integrin subunits (Felding-Habermann and Cheresch, 1993).

In summary, integrins appear to selectively regulate adhesion to different substrates and have been implicated as potential signal transduction molecules. In this investigation, we have reported the effect of mechanical stimulation on expression of integrin subunits β_1 and α_v . While mechanical stimulation appears to regulate β_1 expression, this did not occur for α_v . The mechanisms of integrin-cytoskeleton interaction appear to select for the expression of each subunit in response to strain. In the near future, determination of the regulatory mechanisms in mechanically stimulated tissues will clarify both the cytoplasmic and nuclear signaling cascades. We anticipate the presence of DNA regulatory proteins and mechanical stimulation-specific cellular receptors acting perhaps, as transcription factors.

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REFERENCES

- Akiyama S. and Yamada KM 1987 Biosynthesis and acquisition of biological activity of the fibronectin receptor. *J Biol Chem* **262**:17536-17542.
- Akiyama SK, Larjava H and Yamada KM 1990 Differences in the biosynthesis and localization of the fibronectin receptor in normal and transformed cultured human cells. *Cancer Res* **50**:1601-1607.
- Anderson J, Carvalho RS, Yen EHK and Scott JE 1992 Measurement of strain in cultured bone and fetal muscle and lung cells. *In Vitro Cell Dev Biol* **29**:183A-186A.
- Burger EH, Klein-Nulend J and Veldhuijzen JP 1992 Mechanical stress and osteogenesis *in vitro*. *J Bone Min Res* **7**:397S-401S.
- Carvalho RS, Scott JE, Suga DM and Yen EHK 1994 Stimulation of signal transduction pathways in osteoblasts by mechanical strain is potentiated by parathyroid hormone. *J Bone Min Res* **9**:999-1011.
- Carvalho RS, Scott JE and Yen EHK 1995a The effects of mechanical stimulation on the distribution of β_1 -integrin and expression of β_1 -integrin mRNA in TE-85 human osteosarcoma cells. *Arch Oral Biol* In press.
- Carvalho RS, Scott JE, Bumann A and Yen EHK 1995b Connective Tissue Responses to Mechanical Stimulation. In: McNeil C, ed. *Occlusion: Science and Practice*, Quintessence Publishing Co In press.
- Chomzynski P and Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**:156-159.
- Clover J, Dodds RA and Gowen M 1992 Integrin subunit expression by human osteoblasts and osteoclasts *in situ* and in culture. *J Cell Sci* **103**:267-271.
- Crossin KL 1991 Cytotaktin binding: inhibition of stimulated proliferation and intracellular alkalization in fibroblasts. *Proc Natl Acad Sci USA* **88**:11403-11407.

- Damsky CH and Werb Z 1992 Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* 4:772-781.
- Fath KR, Edgell CS and Burridge K 1989 The distribution of distinct integrins in focal contacts is determined by the substratum composition. *J Cell Sci* 92:62-75.
- Felding-Habermann B and Cheresch DA 1993 Vitronectin and its receptors. *Curr Opin Cell Biol* 5:864-868.
- Gailit J, Colflesh D, Rabiner I, Simone J and Goligorsky MS 1993 Redistribution and dysfunction of integrins in cultured renal epithelial cells exposed to oxidative stress. *Am Physiol Soc* 263:149F-157F.
- Ginsberg MH, Du X and Plow EF 1992 Inside-out integrin signaling. *Curr Opin Cell Biol* 4:766-771.
- Hasegawa S, Sato S, Saito S, Suzuki Y and Brunette DM 1985 Mechanical stretching increases the number of cultured bone cells synthesizing DNA alters their pattern of protein synthesis. *Calcif Tiss Int* 37:431-436.
- Hayashi Y, Haimovich B, Reszka A, Boettinger D and Horwitz A 1990 Expression and function of chicken integrin β_1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. *J Cell Biol* 110:175-184.
- Heino J, Ignatz RA, Hemler ME, Crouse C and Massagué J 1989 Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. *J Biol Chem* 264:380-388.
- Heino J and Massagué J 1989 Transforming growth factor-beta switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin. *J Biol Chem* 264:21806-21811.
- Hormia M and Könönen M 1994 Immunolocalization of fibronectin and vitronectin receptors in human gingival fibroblasts spreading on titanium surfaces. *J Periodont Res* 29:146-152.

- Hynes RO 1992 Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**:11-25.
- Jaken S, Leach K and Klauck T 1989 Association of type 3 protein kinase C with focal contacts with rat embryo fibroblasts. *J Cell Biol* **109**:697-704.
- Juliano RL and Haskill S 1993 Signal transduction from the extracellular matrix. *J Cell Biol* **120**:577-585.
- Koivisto L, Heino J, Häkinen L and Larjava H 1994 The size of intracellular β_1 -integrin precursor pool regulates maturation of β_1 -integrin subunit and associated α -subunits. *Biochem J* **300**:771-779.
- Komuro I, Katoh Y, Kaida T, Shibasaki I, Kurabayashi M, Hoh E, Takaku F and Yazaki Y 1991 Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. *J Biol Chem* **266**:1265-1268.
- Kornberg L and Juliano RL 1992 Signal transduction from the extracellular matrix: the integrin-tyrosine kinase connection. *TIPS* **13**:93-95.
- Kornberg L, Earp HS, Turner C, Procop C and Juliano RL 1993 Signal transduction by integrins: increased protein tyrosine kinase phosphorylation caused by clustering of beta 1 integrins. *Proc Natl Acad Sci USA* **88**:8392-8396.
- Kubota T, Yamauchi M, Onozaki J, Sato S, Susuki Y and Sodek J 1993 Influence of an intermittent compressive force on matrix protein expression by ROS 17/2.8 cells, with selective stimulation of osteopontin. *Archs Oral Biol* **38**:23-30.
- La Flamme SE, Akiyama SK and Yamada KM 1992 Regulation of fibronectin receptor distribution. *J Cell Biol* **117**:437-447.
- Lanyon LE 1992 Control of bone architecture by functional load bearing. *J Bone Min Res* **7**:369S-375S.
- Leavesley DI, Schwartz MA, Rosenfeld M and Cheresch DA 1992 Integrin β_1 and β_3 mediated endothelial cell migration is triggered through distinct cellular mechanisms. *J Cell Biol* **121**:163-170.

- Morgan N 1989 Receptors and their regulation. In: Morgan N. Cell Signaling, Guilford Press, London.
- Ng-Sikorski J, Anderson R, Patarroyo M and Anderson T 1991 Calcium signaling capacity of the CD 11b/CD 18 integrin on human neutrophils. *Exp Cell Res* **195**:504-508.
- Nojima Y, Humphries MJ, Mould AP, Komoriya A, Yamada KM, Schlossman SF and Morimoto C 1990 VLA-4 mediates CD 3-dependent CD 4 T cell activation via the CS 1 alternatively spliced domain of fibronectin. *J Exp Med* **172**:1185-1192.
- Panetti TS and McKeown-Longo PJ 1993 The $\alpha_v\beta_5$ integrin receptor regulates receptor-mediated endocytosis of vitronectin. *J Biol Chem* **268**:11492-11495.
- Reszka AA, Hayashi Y and Horwitz AF 1992 Identification of amino acid sequences in the integrin β_1 cytoplasmic domain implicated in cytoskeleton association. *J Cell Biol* **117**:1321-1330.
- Rubin CT, McLeod KJ and Lanyon LE 1989 Prevention of osteoporosis by pulse electromagnetic fields. *J Bone Jt Surg* **71**:411-417.
- Sandy JR, Farndale RW and Meikle MC 1993 Recent advances in understanding mechanically induced bone remodeling and their relevance to orthodontic theory and practice. *Am J Ortho Dentofac Orthop* **103**:212-222.
- Santala P, Larjava H, Nissinen L, Riiikonen T, Määttä A and Heino J 1994 Suppressed collagen gene expression and induction of $\alpha_2\beta_1$ integrin-type collagen receptor in tumorigenic derivatives of human osteogenic sarcoma (HOS) cell line. *J Biol Chem* **269**:1276-1283.
- Sastry SK and Horwitz AF 1993 Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr Opin Cell Biol* **5**:819-831.

- Schaller MD, Borgman CA, Cobb S, Vines RR, Reynolds AB and Parsons JT 1992 FAK¹²⁵, a structurally distinctive protein tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci USA* **89**:5192-5196.
- Schreiner CL, Hussein S, Fisher M and Juliano RL 1991 Increased tumorigenicity of fibronectin receptor deficient chinese hamster ovary cell variants. *Cancer Res* **51**:1738-1740.
- Schwartz MA 1993 Signaling by integrins - implications for tumorigenesis. *Cancer Res* **53**:1503-1506.
- Shimizu Y, Van Seventer GA, Horgan KJ and Shaw S 1990 Costimulation of proliferative responses of resting CD 4⁺ T cells by the interaction of VLA-4 and VLA-5 with fibronectin or VLA-6 with laminin. *J Immunol* **145**:59-67.
- Suzuki S, Argraves W, Arai H, Languino L, Pierschbacher M and Ruoslahti E 1987 Amino acid sequence of the vitronectin α subunit and comparative expression of adhesion receptor mRNAs. *J Biol Chem* **262**:14080-14085.
- Tooney PA, Agrez MV and Burns G 1993 A re-examination of the molecular basis of cell movement. *Immunol Cell Biol* **71**:131-139.
- Ylänne J, Chen Y, O'Toole TE, Takada Y and Ginsberg M.H 1993 Distinct functions of α and β subunit cytoplasmic domains in cell spreading and formation of focal adhesions. *J Cell Biol* **122**:223-233.
- Yoruchko AD, Du DY, Bierman D and Haskill S 1992 Integrins as a primary signal transduction molecule regulating monocyte immediate-early gene induction. *Proc Natl Acad Sci USA* **89**:9034-9038.

Figure 1. (a and c) photomicrographs of control unstrained HOS TE-85 cells. (b and d) photomicrographs of strained HOS TE-85 cells for 24 hours. HOS TE-85 cells were labeled with antibodies anti- β_1 (a and b) and anti- α_v integrins (c and d). Note the marked change in β_1 distribution when unstrained cultures (a) are mechanically strained (b). This can also be seen in unstrained (c) and strained cultures (d) labeled with α_v . Note that both subunits appear distributed in the cell periphery prior to mechanical strain application. Following strain, both subunits redistribute towards the center of the cells, although staining in the periphery of the cells could still be seen for those labeled with α_v . However, some cells labeled with α_v did not show any staining at the cell periphery with mechanical strain (small white arrow) (d). White arrow indicates the direction of strain. Magnifications (a-d) X 1250.

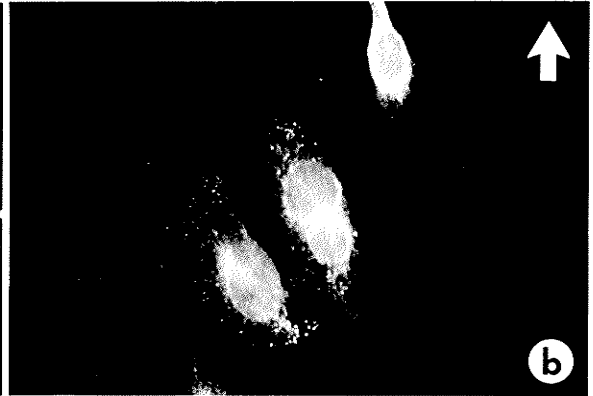
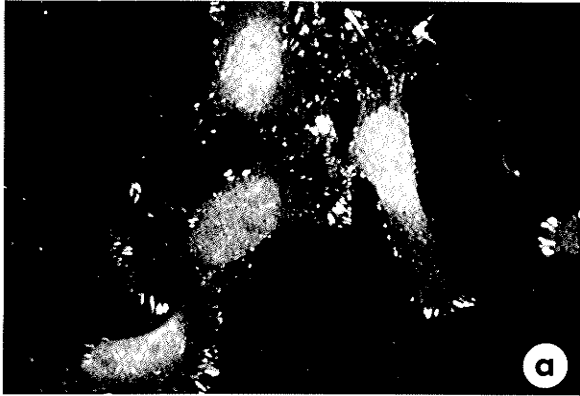


Figure 2. Immunofluorescent localization of β_1 and α_v integrin subunits in strained and unstrained HOS TE-85 cells. (a, b, c and d) HOS TE-85 cells labeled with monoclonal antibodies against β_1 integrin subunit. Similarly, (e, f, g and h) show HOS TE-85 cells labeled with monoclonal antibodies against α_v integrin subunit. Unstrained HOS cells (a and e) were strained for 30 minutes (b and f), 24 hours (c and g) and 24 hours prior to the end of a 3 day culture period (d and h). Note that application of mechanical strain at 24 hour (c) and 3 day culture periods (d) has changed significantly the distribution of β_1 subunit, when compared with those of unstrained (a) and strained cells for 30 minutes (b). Similarly, strain also stimulated a change in α_v subunit distribution in later periods of time (g and h) when compared with unstrained (e) or cells strained for 30 minutes (f). White arrow indicates the direction of strain. Magnifications (a-h) X 1250.

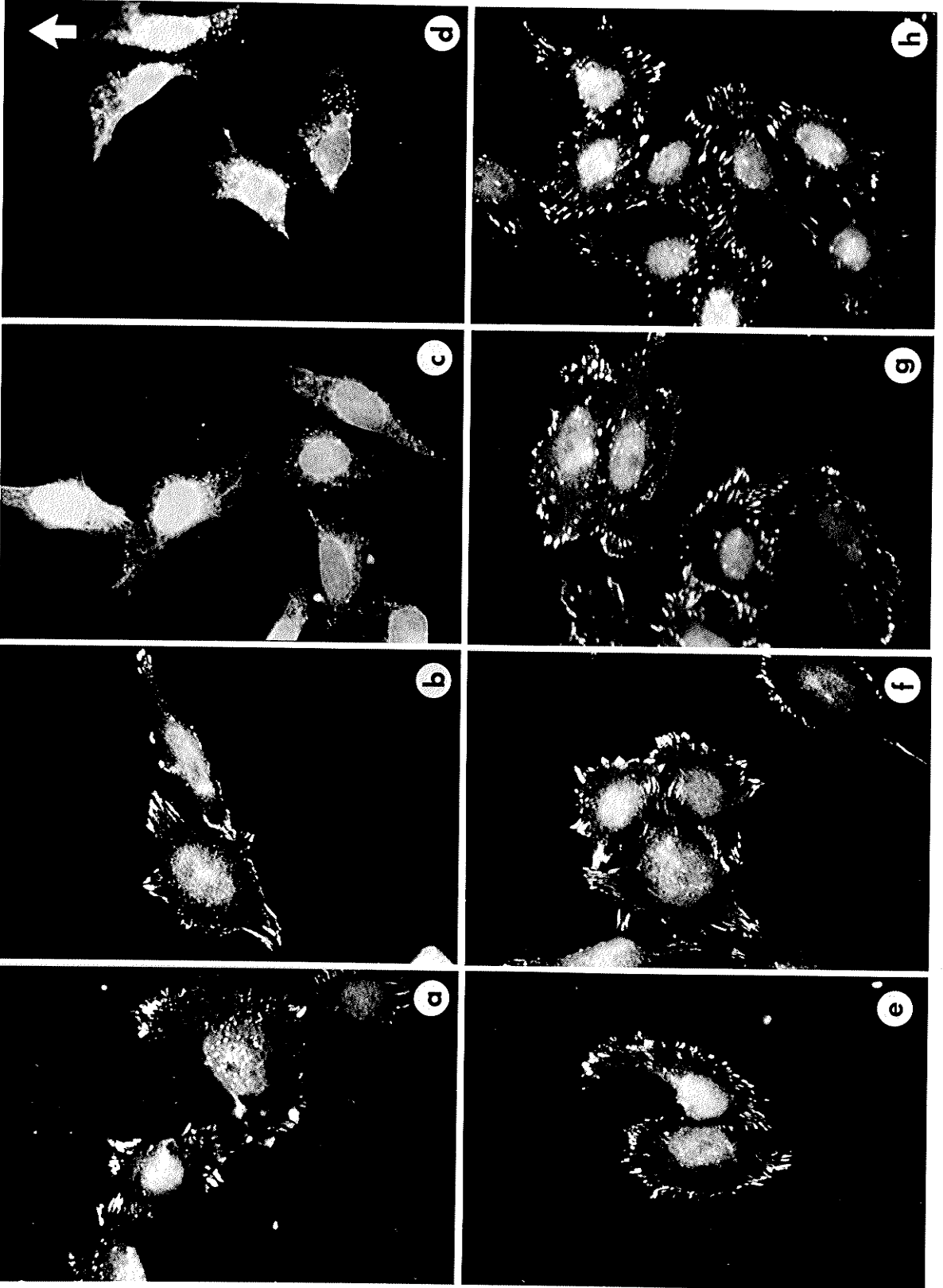


Figure 3. Photomicrographs of northern blots showing mRNA of β_1 -integrin subunit. (A and B) effects of mechanical strain in β_1 -integrin subunit mRNA expression. Note that strain (+) causes an increase of β_1 mRNA at 30 minutes and again at 3 days when compared with unstrained controls (-). In addition, there is very low expression of β_1 -integrin from cells cultured beyond 1 week. Control mRNA (probe for cyclophilin) indicating comparable amounts of RNA per lane is also shown in (b). Legends are: 15' (15 minutes) and 30' (30 minutes).

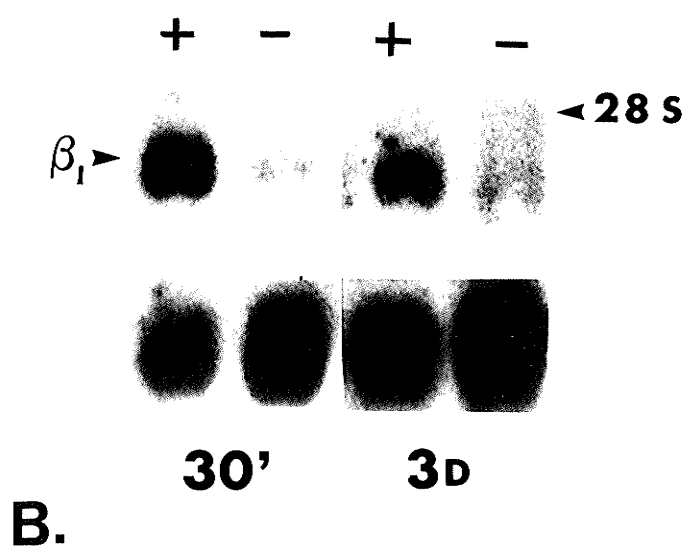
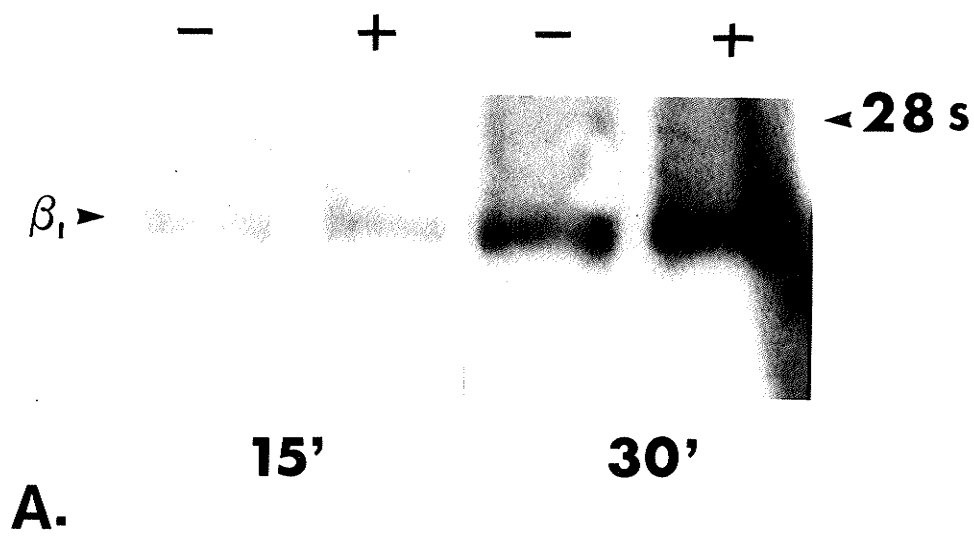


Figure 4. Photomicrographs of northern blots showing mRNA of α_v -integrin subunit (A and B). Note that the effects of mechanical strain in α_v -integrin subunit mRNA expression (+) did not appear to change when compared with unstrained cultures (-). Control mRNA (probe for cyclophilin) indicating comparable amounts of RNA per lane is also shown. Legends are: 15' (15 minutes), 30' (30 minutes), 2 h (2 hours), 24 h (24 hours), 3 d (3 days) and 1 w (1 week).

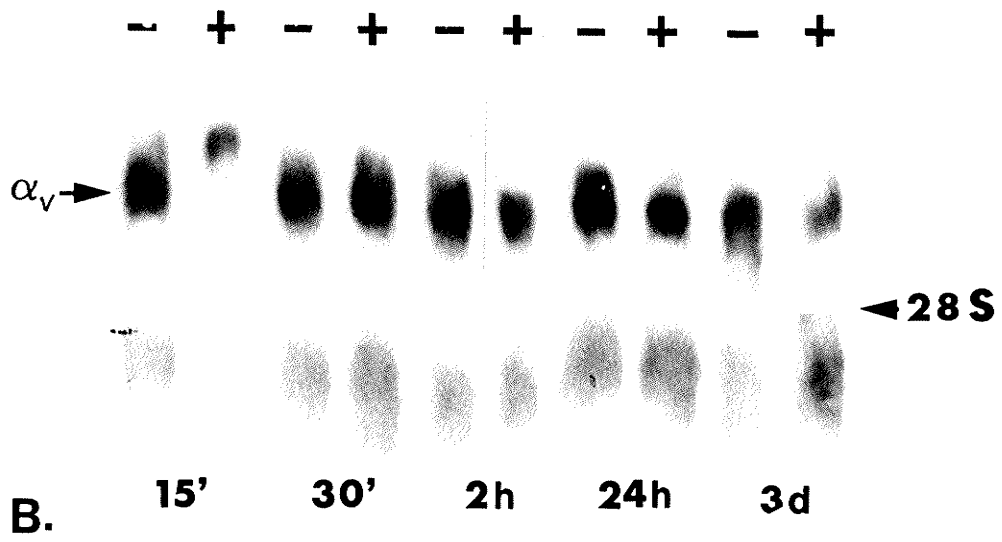
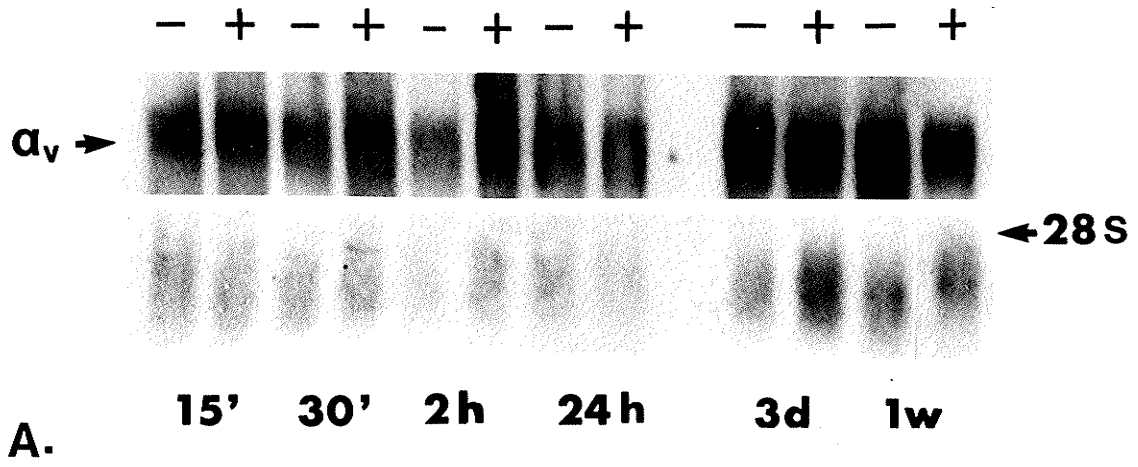
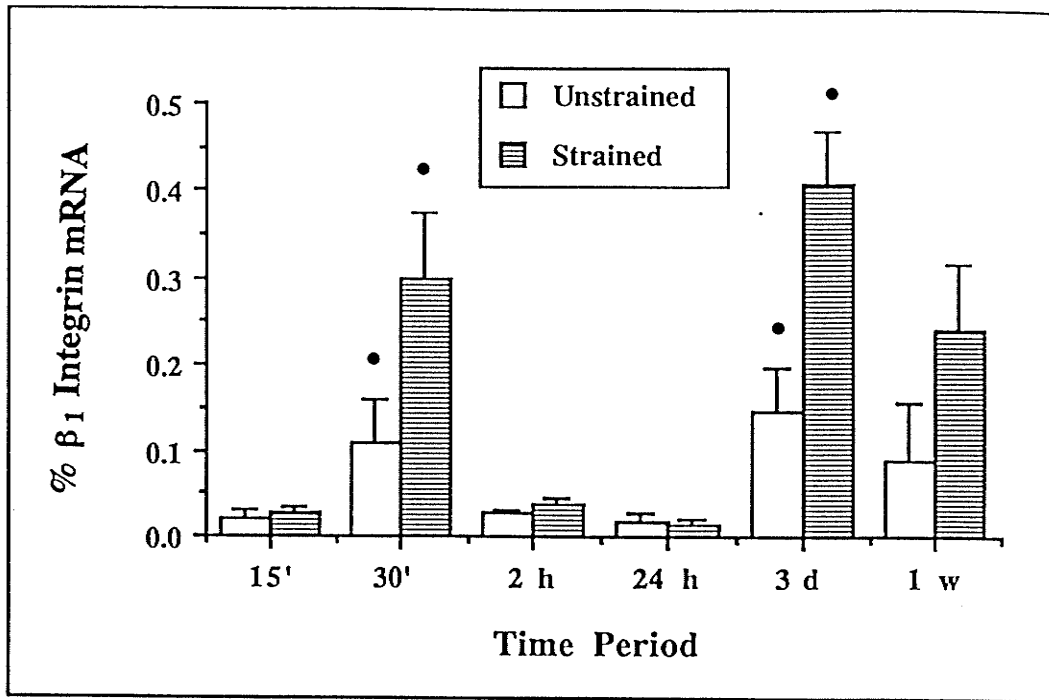
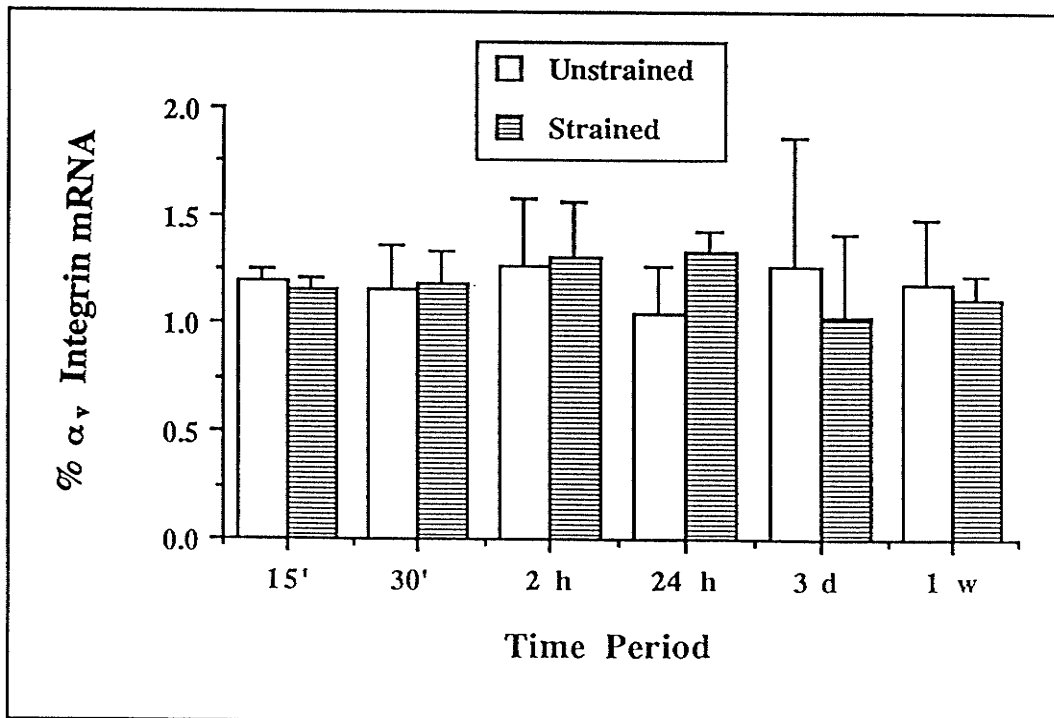


Figure 5. Effect of mechanical strain in the expression of β_1 and α_v -integrin subunits mRNA. Values indicate at least 4 cultures for each time period studied. β_1 and α_v -integrin mRNA expression is represented by its ratio (%) of to the internal control (cyclophilin expression) from densitometric scans of x-ray films. • indicates significantly greater ($p < 0.005$) in strained cultures when compared with unstrained controls. Legends are: 15' (15 minutes), 30' (30 minutes), 2 h (2 hours), 24 h (24 hours), 3 d (3 days) and 1 w (1 week).



A



B



Chapter EIGHT

*Mechanical Strain Stimulates Cytoskeleton
Adaptation in Human Osteosarcoma
TE-85 Cells In Vitro*

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ABSTRACT

The cytoskeleton of osteoblasts actively participates in cellular changes in size and shape upon the application of mechanical stimulation. Three main classes of proteins encompass the cytoskeleton of osteoblasts: microfilaments containing actin, microtubules containing tubulin and intermediate filaments containing vimentin. The purpose of this study was to investigate the effects of strain on the distribution of actin, vimentin and focal contact proteins vinculin, talin and tensin in osteoblast-like cells. Human osteosarcoma cells TE-85 were cultured in DMEM/F-12 and grown to confluency in Flexercell Type I dishes. Intermittent strain was applied to the cells using the Flexercell Strain Unit System for periods of 2, 6 and 24 hours and unstrained cells were used as controls. Immunocytochemical localization for actin, vimentin, vinculin, talin and tensin for both experimental and control cultures was undertaken. Negative controls were also performed. The results showed that mechanical strain altered the distribution of all proteins studied. Although strain appeared to alter the distribution of tensin at 2 hours of strain, the distribution of other proteins studied did not change significantly until 6 hours. The changes in distribution of both talin and vinculin indicated an increase of focal contacts after strain application. However, any changes in cell orientation due to strain in this study were inconclusive. The response of HOS cells to mechanical strain demonstrates that the cytoskeleton adapts to strain through the stimulation of specific cytoskeleton and receptor proteins. These data suggest that the cells may increase their substratum anchorage properties as a result of strain.

INTRODUCTION

Regulation of bone metabolism is influenced by a variety of biochemical and biomechanical signals. The transduction of such signals into bone cellular functions allows for continuous bone remodeling. Therefore, the study of underlying mechanisms of bone cellular response can potentially determine the basic mechanisms of bone biology. In particular, understanding changes in osteoblasts under loading may provide clues as to the capacity of bone to withstand and respond to mechanical stimulation. Initially the mechanical signal appears to be perceived and transduced from the extracellular matrix (ECM) into cellular messages by a strain-sensitive cell population (Rubin, 1984). The loading signal can be determined by an increased perfusion or cell membrane deformation. This is followed by the "signal message", dependent on the transduction of force into specific cellular strain (Brighton and McCluskey, 1988; Rubin and Hausman, 1988). Although the concept of the manner by which osteoblasts perceive mechanical stimulation is poorly understood, there is strong evidence to suggest that from the eligible biological "strain transducers", the cellular cytoskeleton stands as a prime candidate.

The cytoskeleton network of most eukaryotic cells is formed by three main classes of structural proteins: actin, tubulin and vimentin (Janmey *et al.*, 1991). These proteins constitute the microfilaments; microtubules and intermediate filaments, respectively (Ingber, 1993). The attachment of this network in the cytoplasm to the plasma membrane in connective tissue cells is provided by specialized areas of contacts called focal adhesions (FAs) (Fath *et al.*, 1989; Pavalko *et al.*, 1989; Burridge *et al.*, 1990; Dowrick *et al.*, 1991). The proteins in these areas belong to a class of actin binding proteins, or connecting linker proteins, that allow for a cascade of extracellular stimuli to be transmitted into the cell. The best known linker proteins are talin and vinculin (Burridge *et al.*, 1988; Pavalko *et al.*, 1989). These not only appear evenly distributed over the FAs but also may be used as molecular markers for these areas (Geiger *et al.*, 1985; Opas, 1989). Talin appears to be always present in association with vinculin, thus linking vinculin to actin filaments (Rees *et*

al., 1990; Lankes and Furthmayr, 1991). It has been postulated that these two proteins form a complex of increased stability in cell-cell and cell-ECM binding (Burrige *et al.*, 1988). The bridge of FAs and plasma membrane appears to be formed by binding of vinculin to actin and talin to membrane receptors such as integrins (Burrige *et al.*, 1988; Burrige *et al.*, 1990; Hynes, 1992). According to their name, integrin "ligands" integrate the extracellular network to the intracellular cytoskeleton as an integral path in signal transduction mechanisms (Hynes, 1992). Thus, the wide range of applied stimuli likely enters the cell through a receptor-mediated response (Brighton *et al.*, 1991; Carvalho *et al.*, 1995a). A detailed description of the effects of mechanical stimulation on integrins can be found elsewhere (Milam *et al.*, 1991; Haskin and Cameron, 1993; Carvalho *et al.*, 1995a; Carvalho *et al.*, 1995b; Carvalho *et al.*, 1995c).

Actin filaments appear to associate with other FA proteins such as tensin (Davies *et al.*, 1991; Weigt *et al.*, 1992) and radixin (Stossel, 1993). Tensin also binds to talin and vinculin (Burrige *et al.*, 1988; Hynes, 1992) and has shown to mediate important phosphorylation reactions in the cytoskeleton through protein kinase binding (Davies *et al.*, 1991). Phosphorylation cascades and a variety of second messenger systems take place through a series of cytoskeleton-associated interactions (Hynes, 1992; Carvalho *et al.*, 1995a; Carvalho *et al.*, 1995b). These properties in control of the signal message render the cytoskeleton with a central role in such processes as the maintenance of bone homeostasis and bone remodeling.

Several indications exist that mechanical stimulation is fundamental in maintaining physiologic bone metabolism (Rubin, 1984; Rubin and Hausman, 1988; Carter and Orr, 1992). It is also known that application of mechanical strain may cause an alteration of cell shape with significant cytoskeleton rearrangements (Buckley *et al.*, 1988; Pender and McCulloch, 1991; Haskin and Cameron, 1993). In addition, the behavior of the cytoskeleton appears to be dependent essentially on architectural distribution of proteins within it (Carvalho *et al.*, 1994). Thus, it becomes very important to address the potential

role of mechanical stimulation on bone cells as it influences the formation and distribution of cytoskeletal linking maps through FAs in the membrane and the overall increase in cellular activity due to cytoskeletal mechanics.

In view of the relationship of stimulation and cell response, we have hypothesized that mechanical stimulation causes a distinct reorganization of the cytoskeleton and proteins in the FAs. This study was designed to study the role of the mechanical stimulation on the rearrangement of the cytoskeleton of osteoblast-like cells. Specifically we have analyzed strain-induced changes in distribution of actin filaments, FA proteins vinculin, talin and tensin and the intermediate filamentous protein vimentin in a selected population of human osteosarcoma cells in culture. Our experimental model is capable of investigating the correlation of high mechanical strain to more likely "pathological" conditions associated with clinically applied forces.

MATERIALS AND METHODS

Cell culture techniques

Osteoblast-like cells derived from rat bone and human osteosarcoma were used in this study. Rat cells were isolated according to Carvalho *et al.* (1994) from the alveolar processes of maxillae and mandibles removed aseptically from Sprague-Dawley rats. Cells were isolated with an enzyme mixture comprised of: clostridial collagenase (3 mg/ml), elastase (6.25 U/ml), chondroitin sulfate (6 mg/ml) and D-sorbitol (18.22 mg/ml) in Krebs buffered solution (pH 7.2) with agitation in a water bath at 37°C. A total of 5 digestions were performed and cells were pooled, followed by resuspension in standard minimum essential medium (α -MEM) supplemented with 15 % FBS (Gibco, Burlington, ON), 0.3 μ g/ml Fungizone (Flow Laboratories, McLean, VA), 100 μ g/ml Penicillin G, 10 mM β -glycerophosphate, 50 μ g/ml ascorbic acid and 10^{-10} M dexamethazone (all from Sigma, Chemical Co., St. Louis, MO). Primary cultures were trypsinized after confluence at 1 week following initial extraction, collected by centrifugation, washed and plated in experimental dishes. Human osteosarcoma cells (HOS) TE-85 were obtained from ATCC (American Type Culture Collection, Rockville, MD). Isolated cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 Ham medium (1:1) (Sigma, St. Louis, MO) supplemented with 5 % FBS (Gibco, Burlington, ON), 25 units/ml of Penicillin G, 25 μ g/ml of streptomycin and 100 μ g/ml ascorbic acid (all from Sigma). HOS cells, originally at passage 27th were grown to passage 31th prior to the plating the experimental dishes. Both rat and human cell types were plated at a density of 2×10^4 cells/cm² in 35 mm dishes in collagen-coated Flex type I dishes (Flexcell Corp., McKeesport, PA) and maintained in a humidified atmosphere consisting of 95 % O₂ and 5 % CO₂ at 37°C.

Mechanical strain method

The experimental cell cultures were mechanically strained using the Flexercell Unit (Flexcell Corp., McKessport, PA). This apparatus is capable of applying mechanical strain to the cells as reported previously (Anderson *et al.*, 1992; Carvalho *et al.*, 1994). Mechanical strain was applied using a pressure of 20 kPa at 3 cycles per minute with 10 seconds of strain followed by 10 seconds of relaxation. The force applied resulted in <1 % to 24 % of strain to the dish bottom, however, most of the area of the dish fell within <1 % of strain (or 10,000 μ strains) (Gilbert *et al.*, 1991; Gilbert *et al.*, 1994). Dishes were strained for periods of 2, 6 or 24 h. Control cultures were cultivated for the same time periods using rigid-bottomed (no strain) collagen-coated Flex type II dishes (Flexcell Corp.). All cell cultures were serum-starved 24 h prior to the straining period. Cell counts were taken at the same experimental and control periods as described above. Strained and unstrained dishes for both rat and human cell populations were washed in Hank's buffer and aliquots were counted electronically in a Coulter Counter (Model ZBI, Hialeah, USA).

Alkaline phosphatase activity

Bone forming cells were identified by the alkaline phosphatase reaction (McCulloch *et al.*, 1990). The staining medium used was 0.1 % Fast Blue salt, 0.3 % Naphthol AS-MS phosphate alkaline solution, Mayer's hematoxylin solution and Citrate concentrated solution (all from Sigma, St. Louis, MO). Cells attached to the bottom of the wells were incubated with a mixture of Fast Blue salt and naphthol for 40 min at room temperature, washed with deionized water and incubated with hematoxylin solution for 10 minutes prior to washing thoroughly in running water for 1 h. Alkaline phosphatase-positive cells were photographed using a Zeiss Optic photomicroscope (Zeiss, Germany).

Immunofluorescence

Immunofluorescence was done in strained and unstrained HOS TE-85 cells. These were rinsed with PBS and fixed by incubation with 4 % paraformaldehyde in PBS for 10

minutes. After several washings, cells were permeabilized with 0.5 % Triton X-100 (Sigma) in PBS for 10 minutes at room temperature. Cells were washed and incubated for 1 hour with primary mouse antibodies (in PBS with 1 % bovine serum albumin (BSA)) for vimentin, vinculin, talin, tensin also at room temperature. For actin labeling, cells were incubated with FITC-phalloidin. Thereafter, samples were extensively washed in PBS and incubated with goat Cy3 conjugated anti-mouse IgG or goat FITC conjugated anti-mouse IgG secondary antibodies for 40 minutes. Cultures were rinsed and silastic membranes of the dishes were removed and fixed to histologic slides (Fisher Sci., Winnipeg, MB) with 90 % glycerol and 10 % PBS, supplemented with 0.1 % phenylenediamine (Sigma, St. Louis, MO). Negative controls for all antibodies were performed. Cultures were photographed using a Zeiss epifluorescence microscope (filters of 450-490 nm excitation). Some cultures were photographed by using a Molecular Dynamics Confocal Scanning Laser Microscope (CSLM). Resolution in the CSLM at the x/y-axis approaches 0.1 μm and is approximately 0.5 μm in the z-axis. The CSLM used the Voxel/View/Ultra software from Vital Images running on a Silicon Graphics Indigo XS24 workstation (generously made available by Dr. D. Nance).

Due to the gradient pattern of strain bi-axially in the silastic membranes (1 to 24 % of strain) all photomicrographs were taken from the most central area (<1 % strain) of membranes and fields were selected randomly. FITC-phalloidin and monoclonal antibodies anti-vimentin, anti-vinculin, anti-talin and Cy3-conjugated were from Sigma and monoclonal anti-tensin was generously donated by Dr. Wong (Dana-Farber Institute, Boston-MA).

Statistical analysis

The values for cell number obtained for all the strained and unstrained cell populations for both rat and HOS cell types were subjected to a t-test with the Bonferroni correction.

RESULTS

Cell cultures

Figure 1 shows phase contrast photomicrographs of HOS TE-85 cells grown in culture. Both HOS cells and the rat alveolar bone cells demonstrated the same growth pattern. In Figure 1b, confluent cells started to form defined structures or clusters, which have been shown to mature into bone nodules. Under the strain periods used in this study, application of mechanical stimulation did not alter nodule formation (not shown), and either rat bone cells or HOS TE-85 cells in confluent cultures did not align in the strain direction. The polygonal shape of unstrained cells (Fig. 1a) became more evident as the cells reached a confluent stage (Fig. 1c).

Growth curves for the HOS TE-85 cells demonstrated that these cells proliferated faster than rat cells (Fig. 2). Similarly for cell morphology, mechanical strain appeared to increase cell number for both cell types. However, while application of strain resulted in a significant increase ($p < 0.05$) of cell number at 24 h when compared with the unstrained controls (Fig. 2), changes in rat cell number were not significant. Cultures subjected to mechanical strain did not show any significant degree of cell death as evidenced by trypan blue exclusion (not shown).

Alkaline phosphatase

In order to determine if the HOS-TE 85 transformed cells did possess osteoblastic potential, the activity of alkaline phosphatase was measured upon mechanical stimulation. Strain resulted in an increase on alkaline phosphatase activity at 24 hours from the onset of stimulation in TE-85 cells (Fig. 3). Changes in staining (Fig. 3B) did not appear to be correlated with the direction of applied strain when compared with unstrained controls (Fig. 3A).

Localization of cytoskeleton proteins

In this study we have localized different cytoskeletal protein labeling as a result of mechanical stimulation in HOS TE-85 cells. No significant changes were observed in actin staining in these cells until periods of 6 to 24 hours of strain. Figure 4 shows HOS control unstrained cells (Fig. 4a) and strained cells at 2 hours (Fig. 4b), 6 hours (Fig. 4c) and 24 hours of stimulation (Fig. 4d). Strain appeared to increase actin microfilament bundles at 6 hours (Fig. 4b). Although, at 24 hours of intermittent stimulation actin fluorescence labeling seemed to be intense (Fig. 4d) when compared to previous times, any changes in labeling were negligible after 6 hours (Fig. 4c). This was also observed for almost all the cell cultures and it may represent the presence of a recovery period of cytoskeletal labeling. However, this was not measured here. In addition, most of the cell cultures were composed of isolated cells at the time of actin labeling, so that no conclusive changes in cell orientation could be made.

Vimentin labeling also showed a different distribution after mechanical stimulation as seen in Figure 5. HOS TE-85 cells labeled intensely for vimentin in both strained and unstrained cells, however, the label varied according to confluency stage (Fig. 6A and 6B). Figure 5A shows unstrained TE-85 cells labeled for vimentin with less intermediate filaments in a random distribution, when compared to strained cells (Fig. 5B). Although, such assessment is rather qualitative, preliminary study with a 3-dimensional reconstruction using confocal microscopy on these cells show a marked difference between attached (Fig. 5C) and upper side of these cells (Fig. 5D). Small white arrows indicate distinct ridges of cytoskeletal attachment to substratum. Mechanical stimulation appeared to have increased filament distribution and fluorescence intensity (Fig. 5B), while keeping the same pattern of 3 D-distribution (not shown). In figure 6B, vimentin distribution with strain is also clear when compared with that of unstrained control (Fig. 6A). However, only subtle changes are observed, particularly in slender extensions of filaments from strained cells, seen here at 6 hours (Fig. 6B).

Cultured rat alveolar bone cells and human TE-85 cells were subsequently identified by their labeling of FA proteins vinculin, talin and tensin. Most of the cell cultures (80 %) demonstrated distinct FAs. The only difference in strained and unstrained cells for the labeling of talin (Figs. 6C and 6D) and vinculin (Figs. 6E, 6F, 7A and 7B) was in the size of the FAs. When control cultures (Figs. 6C, 6E and 7A) were strained for 6 hours (Figs. 6D to 6F and 7B), both talin and vinculin appeared to form larger FAs. Talin and vinculin labeling are seen with small double arrows and tensin by small single arrow. HOS cells showed a concentration of FAs in the periphery of the cells, partly due to a non-specific staining at the center of the cells. Specifically, increase of FAs is clearly seen for vinculin staining after mechanical strain in Figs. 6F and 7B. However, changes in FA orientation were not evident in HOS cells, contrary to rat cells (not shown). On the other hand, although mechanical strain stimulated tensin distribution at 2 (Fig. 7D) and 24 hours (Fig. 7F), it did not seem to change its distribution at 6 hours (Fig. 7E). This maybe due to a partly recovering period, similarly to that of seen for actin labeling (Fig. 4). Finally, Figure 8 shows a hypothetical diagram for the cytoskeleton interactions taking place in bone cells before and after the application of mechanical stimulation.

DISCUSSION

The importance of mechanical stimulation on bone metabolism has long been recognized. Although, mechanically derived signals are essential in processes such as bone remodeling (Burger *et al.*, 1992; Sandy *et al.*, 1993) the mechanisms involving in strain-transduction into cell function and to the corresponding changes in cell shape are poorly understood. Such changes were investigated here by studying the cytoskeletal behavior of osteoblasts upon mechanical stimulation. We used the well characterized osteoblast cell line HOS TE-85.

Studies have shown that osteoblastic cell populations derived from normal human or animal bone tissue contain heterogeneous cell populations comprised of cells from various maturation stages (Rodan *et al.*, 1988; Clover and Gowen, 1994). This does not appear in transformed cell lines, which are clonal cells capable of forming a homogeneous cell population when cultured *in vitro*. However, the main disadvantage of transformed cells is their growth rate. A comparison of the growth rate of the HOS TE-85 cell line used here to a well characterized osteoblast cell line from rats (Carvalho *et al.*, 1994) showed a significant increase of the former with an approximate doubling time of 24 hours when mechanical strain was applied (Fig. 2). However, the growth rate from unstrained HOS cells was not significantly different to that of rat cells (Fig. 2). In general, although transformed cells possess abnormal growth characteristics, they offer several advantages for the study of specific biological functions (Haskin and Cameron, 1993). HOS TE-85 cells, derived from a sarcoma of a 13-year old female (McAllister *et al.*, 1971), have been used to study the effects of estrogen, localization of estrogen receptors and purification of insulin-like growth factor binding proteins (Clover and Gowen, 1994 for a review). In addition, we have previously shown that HOS TE-85 cells constitute a good model for localization of cytoskeleton proteins and integrins (Carvalho *et al.*, 1995a) and for the study of integrin subunit mRNA expression (Carvalho *et al.*, 1995b). Further characterization of this cell line in this study demonstrates that these cells resemble the polygonal morphology

of normal bone cells (Fig. 1) and that they make bone-like tissue *in vitro* (not shown). We have also demonstrated that mechanical strain stimulates the activity levels of the enzyme alkaline phosphatase (Fig. 3). The role of this enzyme is not yet known, however, it has long been thought as a phenotype marker for bone formation through its role in bone mineralization (Buckley *et al.*, 1990). The ability of these cells to secrete the bone matrix protein osteocalcin has also been shown by Clover and Gowen (1994).

Alterations in cell shape are thought to be mediated via alterations in the cell cytoskeleton (Ben-Ze'ev, 1991; Pender and McCulloch, 1991; Gingell and Owens, 1992; Oakley and Brunette, 1993). This cytoskeletal restructuring likely involves receptor-mediated adhesion and the dynamic relationship of membrane receptors and FAs (Zetter and Brightman, 1990; Gingell and Owens, 1992). Thus, the main aim of this study was to compare the distribution of selected cytoskeletal proteins in mechanically-stimulated osteosarcoma cells to those of unstrained controls.

The effects of mechanical stimulation in HOS TE-85 cells showed a change in actin distribution (Figs. 4 and 8). Although they are consistent with previous observations on osteoblastic (Buckley *et al.*, 1988; Buckley *et al.*, 1990), endothelial (Sumpio *et al.*, 1988; Iba and Sumpio, 1991) and fibroblastic cells (Pender and McCulloch, 1991), the changes described here are morphological and can not account for changes attributed to regulatory mechanisms of actin polymerization (Stossel, 1993). However, effects of mechanical stimulation on the actin polymerization/depolymerization mechanisms appears to take place remarkably fast independently of cell type (Pender and McCulloch, 1991). Providing that the most obvious function of the cytoskeleton appears to be maintenance of cell shape (Bretscher, 1986), it is not surprising that cytoskeleton orientation (Buckley *et al.*, 1988; Sumpio *et al.*, 1988; Buckley, 1990; Iba and Sumpio, 1991) and cell polarity (Buck, 1980) are affected by mechanical strain. Moreover, substrate composition (Otey *et al.*, 1990; Hynes, 1992) and timing of mechanical stimulation (Pender and McCulloch, 1991; Janmey *et al.*, 1991) are critical factors in determining cytoskeleton distribution.

The direction of cell alignment in accordance with characteristics of substrate composition and topography has been termed "contact guidance" (Curtis and Clark, 1990; Oakley and Brunette, 1993). While the mechanism for contact guidance has been based on the role of actin filament bundles (Dunn and Brown, 1986), Oakley and Brunette (1993) have recently found that the microtubular system is a primary cytoskeleton candidate in determining cell polarity. According to research in plant systems by Williamson (1991) and Hush and Overall (1991), microtubules change their orientation in response to environmental demands such as mechanical strain. In fact additional studies suggest that mechanical stimulation may determine cell behavior, such as cell polarity, through dynamics on the environment of the substratum (Schulze and Kirscher, 1988; Sammak and Borisy, 1988; Oakley and Brunette, 1993). Therefore, it is likely that mechanical strain system studied here will also modulate microtubule distribution.

Patterns of contact guidance have shown that the first intracellular elements to align according to substratum topography were microtubules, followed by actin bundles and then FAs (Oakley and Brunette, 1993). By contrast, strain applied to endothelial cells showed that 24 hours of cyclic strain did not alter the distribution of tubulin or vimentin, while actin fibers aligned perpendicular to the force vector after 15 minutes from the onset of the stimulation (Iba and Sumpio, 1991). The progressive actin alignment (Sumpio *et al.*, 1988) followed by cell alignment as a result of strain appears to initiate at 4 hours of stimulation (Buckley *et al.*, 1988). Although we are not able to evaluate the timing of cell cytoskeleton adaptation from this study, we have previously shown that under mechanical strain, osteoblastic cells change FA distribution very early (Carvalho *et al.*, 1994). We demonstrated that changes in both FA and actin filaments were not evident until 2 and 6 hours of stimulation respectively (Figs. 4C, 6D and 6F) even though any changes in talin, vinculin and tensin reported here may not indicate changes in cell alignment.

Stimulation of FA proteins was noticed mostly as the size of FAs appeared to have increased with the application of mechanical strain (Figs. 6 and 7). Unfortunately, there is

little information available from other studies on effects of strain in FA proteins. Previously, we have demonstrated that mechanical stimulation appears to change the orientation of vinculin at 30 minutes from the onset of strain (Carvalho *et al.*, 1994). This was similar for the results in this study (not shown), however, such orientation change was not evident here and perhaps may account for some cell type differences. We speculate that strain appears to increase FA distribution in order to provide further anchorage of cell-substratum. An increase in membrane ligands from mechanically-stimulated cells supports this idea (Haskin and Cameron, 1993; Carvalho *et al.*, 1994). Nevertheless, tensin appeared distinct from both talin and vinculin. This protein has been so called due to its putative function in maintaining tension in actin fibers at their point of anchorage (Davies *et al.*, 1991) as seen in Figure 8. Although, strain stimulated tensin rearrangement at 2 and 24 hours, it did not at 6 hours. This could be the result of a recovery period. Such effect seems to be evident in distribution changes of actin (Fig. 4) and that of microtubules following stimulation by mechanical strain (Janmey *et al.*, 1991; Haskin and Cameron, 1993).

The question of microtubule versus actin-dependent mechanical response is an interesting one. While microtubules appear to respond to the substratum environment before any change in actin distribution (Oakley and Brunette, 1993), previous studies have shown that microtubules do not appear to prevent attachment and spreading in many cell types (Vasiliev, 1987; Middleton *et al.*, 1988; Ingber, 1993). Thus, is actin alone capable of supporting most changes in cell form and cell shape? This is possible but maybe not certain. Many studies observed the establishment of a mechanically balanced relationship between microtubules and actin microfilaments for structural stability of cells and tissues (Ingber, 1993 for a review). Microtubules not only potentiate the formation of actin nucleation assembly sites (Tooney *et al.*, 1993), but also help to stabilize the other cytoskeleton networks (Janmey *et al.*, 1991). In addition, the other main class of structural proteins, the intermediate filaments, also participates in the maintenance of structural

stability (Torpey *et al.*, 1992) and is involved in the hormone-dependent integration of cell shaping through mechanical forces (Aumuller *et al.*, 1992). However, conflicting changes in cytoskeleton behavior maybe due to the magnitude of the strain applied and to cell type (Pender and McCulloch, 1991).

Osteoblastic cells under physiological levels of strain experienced significant depolymerization and repolymerization of vimentin and α -tubulin, prior to any changes in actin (Haskin and Cameron, 1993). Mechanical strain has shown to collapse the appearance of actin filaments, giving them a punctate format. On the other hand, high strains such as the strain regimen described here, revealed that vimentin networks resisted breakage, while causing high rigid actin networks to fluidize (Janmey *et al.*, 1991). Similar changes on a decrease of actin polymerization were observed very early from the onset of the stimulation, even though F-actin appeared to increase with continuous strain (Pender and McCulloch, 1991). In this study we did observe a subtle change in distribution of actin (Fig. 4) as well as vimentin (Fig. 5) at high strains. However, according to Haskin and Cameron (1993) the changes in cytoskeleton proteins with strain maybe correlated with cell-cell adhesion and confluency stage. The cytoskeleton from confluent cells appears to reorganize sooner than that of isolated cells. This is consistent with our data (unpublished observations), although the effects of isolated versus confluent cells with mechanical strain were not reported herein.

The problem of how mechanical strain is translated into cellular functions still remains. For instance, do bone cells exhibit specific receptors for mechanical stimulation? There are suggestions that cytoskeleton interactions with the integrin family of proteins may function to transmit force-generated signals (Burrige *et al.*, 1988; Lotz *et al.*, 1989; Wang *et al.*, 1992; Ingber, 1993; Tooney *et al.*, 1993; Carvalho *et al.*, 1995b). The relationship of ECM-integrins to FA proteins supports this hypothesis (Burrige *et al.*, 1988; Beckerle and Yeh, 1990). In addition, there is strong evidence that mechanical stimulation participates on several chemical signals (Watson, 1991; Vandeburgh, 1992; Carvalho *et*

al., 1995a for a review). According to Ingber (1993) the structural stability of the cells may follow a "tensegrity model". This model, based on the concepts of tension and structural integrity, may explain the mechanical coordination of the cell as a whole in which the cytoskeleton plays a central role (Ingber, 1991; Ingber, 1993; Wang *et al.*, 1993). The strained actin network appears to be supported by microtubule stimulation (Heideman, 1993) through an increased interaction with intermediate filaments (Aumuller *et al.*, 1992). Thus, coordination of response among these main structural proteins, FA proteins and integrins will contribute to structural and biochemical combinations of strain signals (Beckerle and Yeh, 1990; Otey *et al.*, 1990; Ingber, 1991; Hynes, 1992). A diagram of ECM-integrins and the cytoskeleton is shown in Figure 8.

Although, it is clear that mechanically stimulated changes in the individual cytoskeleton components are interconnected (Oakley and Brunette, 1993; Ingber, 1993) care must be taken in the interpretation of specific strain sensitive proteins. Not all proteins that mediate cytoskeleton attachment to integrin receptors appear to change with strain. The actin and integrin-binding proteins filamin and src (Burrige *et al.*, 1988; Burrige *et al.*, 1990) did not appear to change their staining patterns after mechanical stimulation in osteoblast cells (Haskin and Cameron, 1993).

Finally, as strain magnitude levels are critical in understanding mechanically-stimulated cytoskeletal changes, it is important to briefly discuss the strain system used here. The Flexercell system for mechanical stimulation has been widely used in a variety of cells including fibroblasts (Buckley *et al.*, 1988; Almekinders *et al.*, 1993), endothelial cells (Sumpio *et al.*, 1990; Iba and Sumpio, 1991), lung cells (Scott *et al.*, 1991; Anderson *et al.*, 1992), muscle cells (Anderson *et al.*, 1992) and bone cells (Buckley *et al.*, 1990; Vadiakas and Banes, 1992; Carvalho *et al.*, 1994; Carvalho *et al.*, 1995c). The parameters for strain environment are based on the finite element modeling studies of the substratum deformation (Gilbert *et al.*, 1991; Gilbert *et al.*, 1994). However, the membrane stretching may vary from 1 to 24 % of the culture dish, and although care was taken to study the area

correspondent to 1 % deformation, some variation due to strain levels can not be excluded. On the other hand, other reports using systems with a more homogeneous strain distribution have shown similar results, supporting those from our laboratory (Jones *et al.*, 1991; Wang *et al.*, 1993; Neidlinger-Wilke *et al.*, 1994; Schaffer *et al.*, 1994). By contrast to the intermittent levels of strain described here, Tooney *et al.* (1993) observed that single static stretch increased protein synthesis and substantial skeletal remodeling. Additional work on more defined systems is needed to address the important questions of strain magnitude and cellular activity on the mechanically-stimulated cell response of osteoblasts.

The relationship of strain stimulation and cell function is a dynamic one. It has also been suggested that with changes in subcellular localization, cytoskeletal proteins assume different functions (Pavalko *et al.*, 1989). Whether mechanically-stimulated cytoskeleton protein distribution determines distinct cell function is not known. This is, however, an intriguing hypothesis and further study is warranted. Constant investigation on both chemical and biomechanical signals are continuing to shed new light in this important area of bone biology.

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REFERENCES

- Almekinders LC, Banes AJ and Ballenger CA 1993 Effects of repetitive motion on human fibroblasts. *Med Sci Sports Exerc* **25**:603-607.
- Anderson J, Carvalho RS, Yen EHK and Scott JE 1992 Measurement of strain in cultured bone and fetal muscle and lung cells. *In Vitro Cell Dev Biol* **29**:183A-186A.
- Aumuller G, Schulze C and Viebahn C 1992 Intermediate filaments in Sertoli cells. *Microsc Res Tech* **20**:50-72.
- Beckerle MC and Yeh RK 1990 Talin: role at sites of cell-substratum adhesion. *Cell Motil Cytoskeleton* **16**:7-13.
- Ben-Ze'ev A 1991 Animal shape changes and gene expression. *Bioessays* **13**:207-211.
- Bretscher A 1986 Microfilament structure and function in the cortical cytoskeleton. *Annu Rev Cell Biol* **7**:337-374.
- Brighton CT and McCluskey WP 1988 Response of cultured bone cells to a capacitively coupled electric field: Inhibition of cAMP response to parathyroid hormone. *J Orthop Res* **6**:567-571.
- Brighton CT, Stratford B, Gross SB, Leatherwood DF, Williams JL and Pollack SR 1991 The proliferative and synthetic response of isolated calvarial bone cells of rats to cyclic biaxial mechanical strain. *J Bone Jt Surg* **73**:320-331.
- Buck RC 1980 Reorientation response of cells to repeated stretch and recoil of the substratum. *Exp Cell Res* **127**:470-474.
- Buckley MJ, Banes AJ, Levin LG, Sumpio BE, Sato M, Jordan R, Gilbert J, Link GW and Tay RTS 1988 Osteoblasts increase their rate of division and align in response to cyclic, mechanical tension *in vitro*. *Bone Min* **4**:225-236.
- Buckley MJ, Banes AJ and Jordan RD 1990 The effects of mechanical strain on osteoblasts *in vitro*. *J Oral Maxillofac Surg* **48**:276-282.
- Burger EH, Klein-Nulend J and Veldhuijzen JP 1992 Mechanical stress and osteogenesis *in vitro*. *J Bone Min Res* **7**:397S-401S.

- Burridge K, Fath K, Kelly T, Nuckolls G and Turner C 1988 Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol* 4:487-525.
- Burridge K, Nuckolls G, Otey C, Pavalko F, Simon K and Turner C 1990 Actin-membrane interactions in focal adhesions. *Cell Differ Dev* 32:337-42.
- Carter DE and Orr TE 1992 Skeletal development and bone functional adaptation. *J Bone Min Res* 7:389S-395S.
- Carvalho RS, Scott JE, Suga DM and Yen EHK 1994 Stimulation of signal transduction pathways in osteoblasts by mechanical strain is potentiated by parathyroid hormone. *J Bone Min Res* 9:999-1011.
- Carvalho RS, Scott JE, Bumann A, Schwarzer C and Yen EHK 1995a The effects of mechanical stimulation on the distribution of β_1 -integrin and expression of β_1 -integrin mRNA in TE-85 human osteosarcoma cells. *Arch Oral Biol* In press.
- Carvalho S, Bumann A, Scott JE and Yen EHK 1995b Connective tissue response to mechanical stimulation. In: McNeil C, ed. *Occlusion: Science and Practice*, Quintessence Publishing Co In press.
- Carvalho RS, Bumann A, Schwarzer C, Scott JE and Yen EHK 1995c Selective expression of α_v and β_1 -integrin subunits mRNA and their role in the signal transduction pathways of mechanically strained human osteosarcoma TE-85 cells. *Arch Oral Biol* Submitted.
- Clover J and Gowen M 1994 Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of osteoblastic phenotype? *Bone* 15:585-591.
- Curtis ASG and Clark P 1990 The effects of topographic and mechanical properties of materials on cell behavior. *Crit Rev Biocompatib* 5:343-362.
- Davies S, Li ML, Lo SH, Lin S, Buttler JA, Drucker BJ, Roberts TM, An Q and Chen LB 1991 Presence of an SH2 domain in the actin-binding protein tensin. *Science* 252:712-715.

- Dowrick PG, Prescott AR and Warn RM 1991 Catter factor affects changes in the cytoskeleton organization of epithelial cells. *Cytokine* **3**:299-310.
- Dunn GA and Brown AF 1986 Alignment of fibroblasts on grooved surfaces described by a single geometric transformation. *J Cell Sci* **83**:313-340.
- Fath KR, Edgell CS and Burridge K 1989 The distribution of distinct integrins in focal contacts is determined by the substratum composition. *J Cell Sci* **92**:62-75.
- Geiger B, Avnur Z, Volberg T and Volk T 1985 Molecular domains of adherens junctions. In: Edelman GM, ed. *Cell in Contact*. NY: Wiley, pp. 461-89.
- Gilbert JA, Banes AJ, Link GW and Jones GL 1991 Video analysis of membrane strain: an application in cell stretching. *Exp Tech* **15**:43-45.
- Gilbert JA, Weinhold PS, Banes AJ, Link GW and Jones GL 1994 Strain profiles for circular cell culture plates containing flexible surfaces employed to mechanically deform cells *in vitro*. *J Biomech* **27**:1169-1177.
- Gingell D and Owens N 1992 How do cells sense and respond to adhesive contacts? Diffusion-trapping of laterally mobile membrane proteins at maturing adhesions may initiate signals leading to local cytoskeletal assembly response and lamella formation. *J Cell Sci* **101**:255-266.
- Haskin C and Cameron I 1993 Physiological levels of hydrostatic pressure alter morphology and organization of cytoskeletal and adhesion proteins in MG-63 osteosarcoma cells. *Biochem Cell Biol* **71**:27-35.
- Heideman SR 1993 A new twist on integrins and the cytoskeleton. *Science* **260**:1080-1081.
- Hush JM and Overall RL 1991 Electrical and mechanical fields orient cortical microtubules in higher plant tissues. *Cell Biol Int Rep* **15**:551-560.
- Hynes RO 1992 Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**:11-25.

- Iba T and Sumpio BE 1991 Morphological response of human endothelial cells subjected to cyclic strain *in vitro*. *Microvasc Res* **42**:245-254.
- Ingber D 1991 Integrins as mechanochemical transducers. *Curr Opin Cell Biol* **3**:841-848.
- Ingber DE 1993 Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* **104**:613-627.
- Janmey PA, Euteneuer U, Traub P and Schliwa M 1991 Viscoelastic properties of vimentin compared with other filamentous biopolymer networks. *J Cell Biol* **113**:155-160.
- Jones DB, Nolte H, Schlüßers JG, Turner E and Veltel D 1991 Biochemical signal transduction of mechanical strain in osteoblast-like cells. *Biomaterials* **12**:101-110.
- Lankes WT and Furthmayr H 1991 Moesin: A member of the protein 4.1-talin-ezrin family of proteins. *Proc Natl Acad Sci USA* **88**:8297-301.
- Lotz MM, Bursal CA, Erickson HP and McClay DR 1989 Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J Cell Biol* **109**:1795-1805.
- McAllister RM, Gardner MB, Greene A, Bradt C, Nichols WW and Landing BH 1971 Cultivation *in vitro* of cells from a human osteosarcoma. *Cancer* **27**:397-402.
- McCulloch CAG, Fair CA, Tenebaum HC, Limeback H and Homareau R 1990 Clonal distribution of osteoprogenitor cells in cultured chick periosteum: functional relationship to bone formation. *Dev Biol* **140**:352-361.
- Middleton CA, Brown AF, Brown RM and Roberts DJH 1988 The shape of cultured epithelial cells does not depend on the integrity of their microtubules. *J Cell Sci* **91**:337-345.
- Milam SB, Haskin C, Zardeneta G, Chen D, Magnuson VL and Klebe RJ 1991 Cell adhesion proteins in oral biology. *Crit Rev Oral Biol Med* **2**:451-491.

- Neidlinger-Wilke C, Wilke HJ and Claes L 1994 Cyclic stretching of human osteoblasts affects proliferation and metabolism: A new experimental method and its application. *J Orthop Res* **12**:70-78.
- Oakley C and Brunette DM 1993 The sequence of alignment of microtubules, focal contacts and actin filaments in fibroblasts spreading on smooth and grooved titanium substrata. *J Cell Sci* **106**:343-354.
- Opas M 1989 Expression of the differentiated phenotype by epithelial cells *in vitro* is regulated by both biochemistry and mechanics of the substratum. *Devel Biol* **131**:281-93.
- Otey CA, Pavalko FM and Burridge K 1990 An interaction between α -actinin and the β_1 -subunit *in vitro*. *J Cell Biol* **111**:721-729.
- Pavalko FM, Otey CA and Burridge K 1989 Identification of a filamin isoform enriched at the ends of stress fibers in chicken embryo fibroblasts. *J Cell Sci* **94**:109-18.
- Pender N and McCulloch AG 1991 Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching. *J Cell Sci* **100**:187-193.
- Rees DJG, Ades SE, Singer SJ and Hynes RO 1990 Sequence and domain structure of talin. *Nature* **347**:685-689.
- Rodan GA, Heath JK, Yoon K, Noda M and Rodan SB 1988 Diversity of the osteoblast phenotype. Cell and molecular biology of the vertebrate hard tissues. *Ciba Found Symp* **136**:78-91.
- Rubin CT and Hausman MR 1988 The cellular basis of Wölff's law. Transduction of physical stimuli to skeletal adaptation. *Orth Sur Deg Arth* **14**:503-517.
- Rubin CT 1984 Skeletal strain and the functional significance of bone architecture. *Calc Tiss Intl* **36**:11S-18S.
- Sammak PJ and Borisy GG 1988 Detection of single fluorescent microtubules and methods for determining their dynamics in living cells. *Cell Motil Cytoskel* **10**:237-245.

- Sandy JR, Farndale RW and Meikle MC 1993 Recent advances in understanding mechanically induced bone remodeling and their relevance to orthodontic theory and practice. *Am J Ortho Dentofac Orthop* **103**:212-222.
- Schaffer JL, Rizen M, L'Italien GJ, Benbrahim A, Megarnan J, Gerstenfeld LC and Gray ML 1994 Device for the application of a dynamic biaxially uniform and anisotropic strain to a flexible cell culture membrane. *J Orthop Res* **12**:709-719.
- Schulze E and Kirschner M 1988 New features of microtubule behavior. *Nature* **334**:356-359.
- Scott JE, Yang SY, Stanik E and Anderson JE 1993 Influence of strain on [³H]thymidine incorporation, surfactant-related phospholipid synthesis, and cAMP levels in fetal type II alveolar cells. *Am J Respir Cell Mol Biol* **8**:258-265.
- Stossel TP 1993 On crawling of animal cells. *Science* **260**:1086-1094.
- Sumpio BE, Banes AJ, Buckley M and Johnson G 1988 Alterations in aortic endothelial cell morphology and cytoskeletal protein synthesis during cyclic tensional deformation. *J Vasc Surg* **7**:130-138.
- Sumpio DE, Banes AJ, Link GW and Iba T 1990 Modulation of endothelial cell phenotype by cyclic stretch: inhibition of collagen production. *J Surg Res* **48**:415-420.
- Tooney PA, Agrez MV and Burns GF 1993 A re-examination of the molecular basis of cell movement. *Immunol Cell Biol* **71**:131-139.
- Torpey N, Wylie CC and Heasman J 1992 Function of maternal cytokeratin in *Xenopus* development. *Nature* **357**:413-415.
- Vadiakas GP and Banes AJ 1992 Verapamil decreases cyclic load-induced calcium incorporation in ROS 17/2.8 osteosarcoma cell cultures. *Matrix* **12**:439-447.
- Vandenburg HH, Hatfaludy S, Sohar I and Shansky J 1990 Stretch-induced prostaglandins and protein turnover in cultured skeletal muscle. *Am J Physiol* **259**:232C-240C.

- Vasiliev JM 1987 Actin cortex and microtubular system in morphogenesis: cooperation and competition. *J Cell Sci* **8**:1S-8S.
- Wang N, Butler JP and Ingber DE 1993 Mechanotransduction across the cell surface and through the cytoskeleton. *Science* **260**:1124-1127.
- Watson PA 1991 Function follows form: generation of intracellular signals by cell deformation. *FASEB J* **5**:2013-2019.
- Weigt C, Gaertner A, Wegner A, Korte H and Meyer HE 1992 Occurrence of an actin-inserting domain in tensin. *J Mol Biol* **227**:593-595.
- Williamson RE 1991 Orientation of cortical microtubules in interphase plant cells. *Int Rev Cytol* **129**:135-206.
- Zetter BR and Brightman SE 1990 Cell motility and the extracellular matrix. *Curr Opin Cell Biol* **2**:850-856.

Figure 1. Phase contrast photomicrograph of HOS TE-85 cells in culture. (a) Cells at low density prior to mechanical strain. (b) Mechanically strained confluent cells forming defined structures or clusters. Arrows indicate clustering of the cells with strain. (c) Strained cells after 72 hours of initial plating. Note the compact shape of the cells in the center of the clusters. Single black arrows indicate the cuboidal characteristics of the cells. Magnifications (a and c) X 80 and (b) X 40.

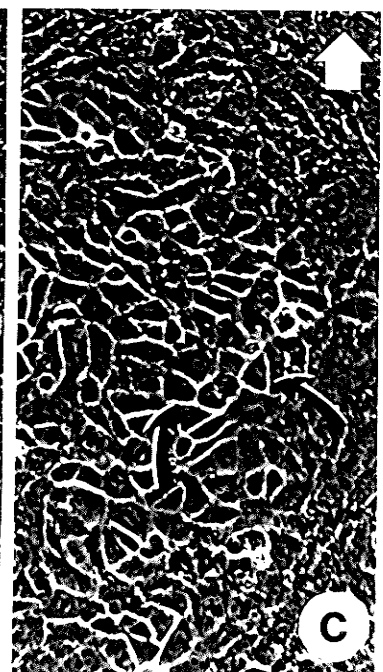
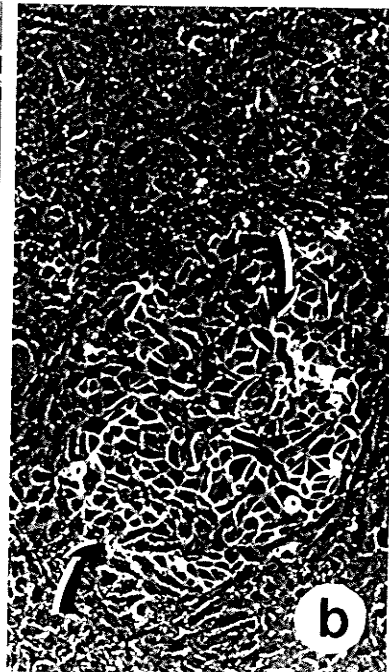
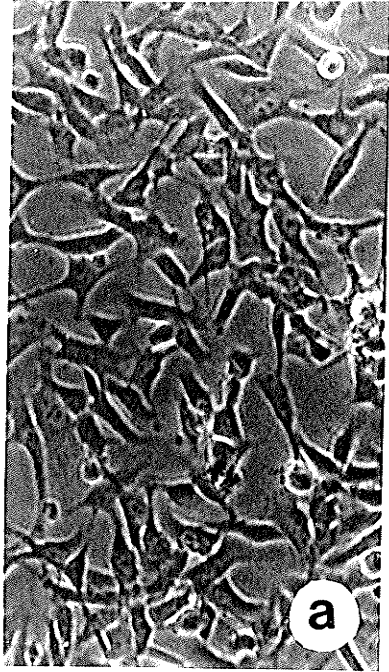


Figure 2. Effects of mechanical strain in the cell counts of HOS TE-85 and rat alveolar bone cells. While changes in rat cell numbers with strain did not appear to change, HOS cells increased their number significantly at 24 hours of applied strain (* $p < 0.05$). However, unstrained HOS cells compared to strained and unstrained rat cells were not significantly different.

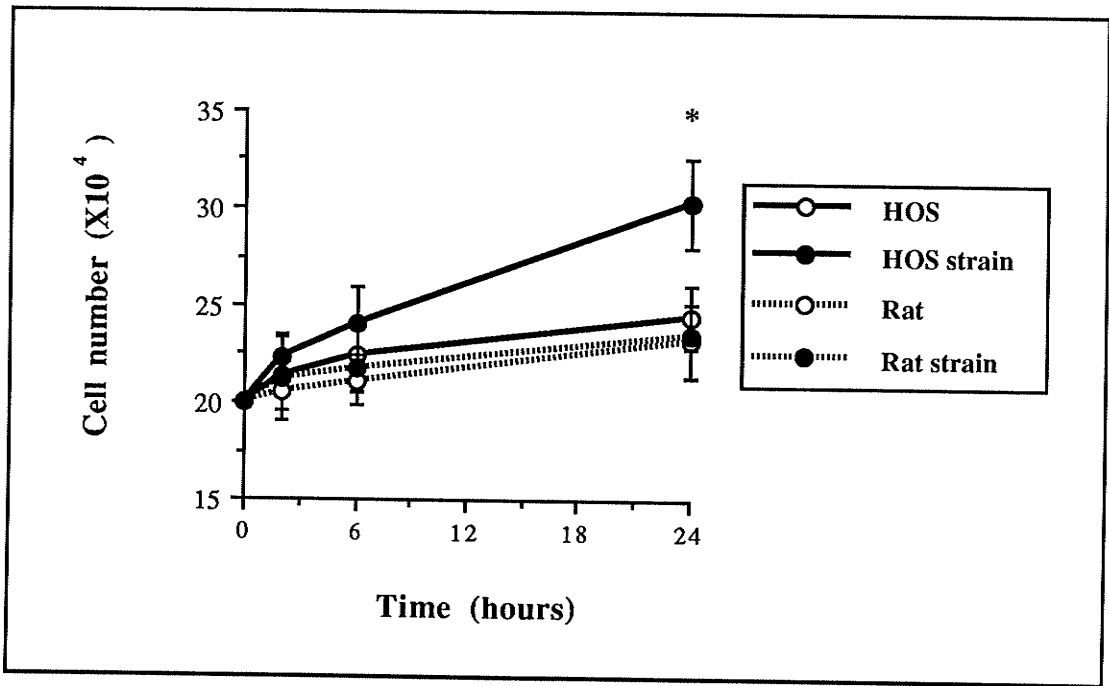


Figure 3. Photomicrographs showing alkaline phosphatase positive staining for HOS TE-85 cells. (A) Control unstrained cells show lower levels of alkaline phosphatase activity when compared to strained cells at 24 h (B). Arrow indicates the direction of the strain. X 80.

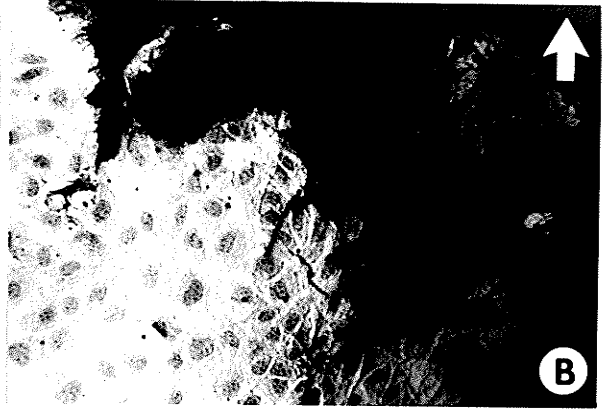
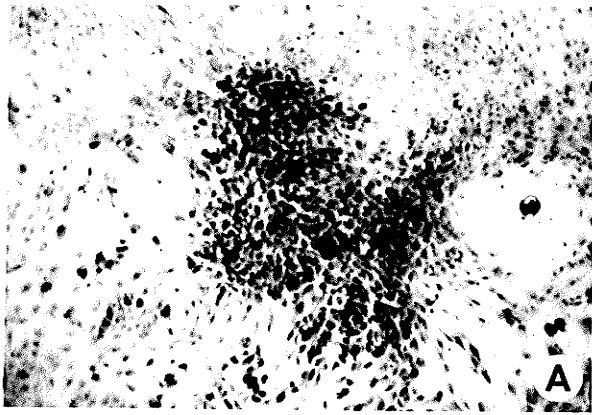


Figure 4. Immunofluorescence of actin filaments in strained and unstrained HOS TE-85 cells grown *in vitro*. (a) Control unstrained cultures. HOS cultures strained for 2 h (b), 6 h (c) and 24 h (d). Note the increase in actin filament bundles at 6 h. Changes in 24 h stimulation are negligible compared to those of 6 h. At 24 h, there is a suggestion of a gradual change in cell orientation perpendicular to the direction of strain. White arrow indicates the direction of strain. X 1250.

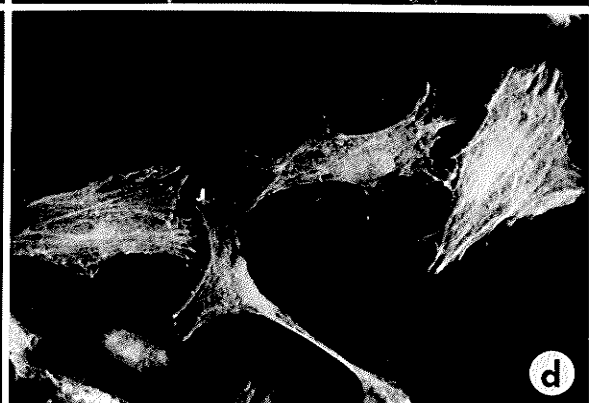
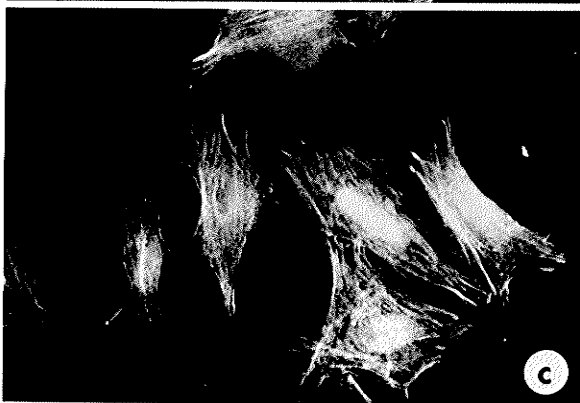
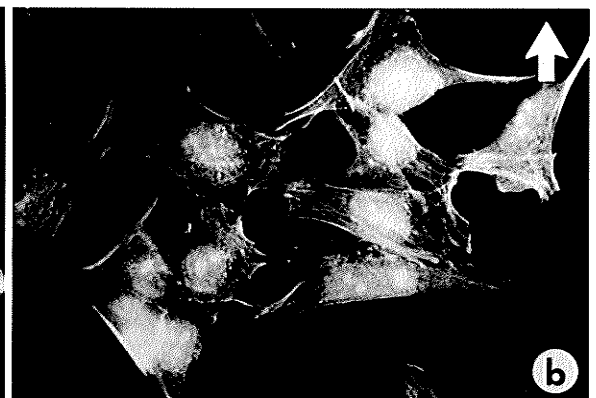


Figure 5. Immunolocalization of vimentin in strained and unstrained HOS TE-85 cells grown *in vitro*. Unstrained (A) and strained for 24 h (B) HOS cells labeled with antibodies anti-vimentin. Note that there is an increased number of cells in (B), and some changes may be due to the confluency stage of the cells. Confocal photomicrographs show a marked difference between the substratum attached (lower) side (C) and the non attached (upper) side of the cells (D). Note the three-dimensional distribution of vimentin. Double-arrows indicate distinct ridges of cell-substratum attachment. White arrow indicates the direction of strain. X 1250.

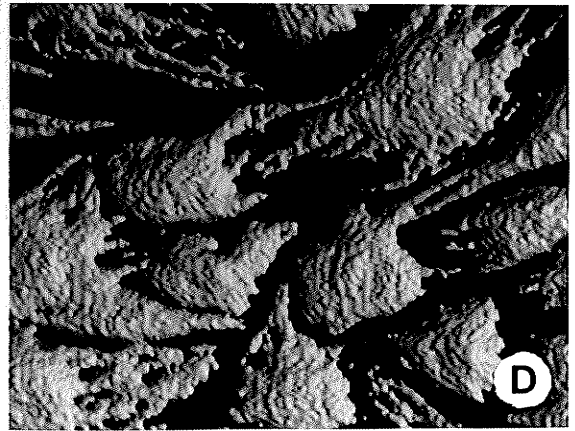
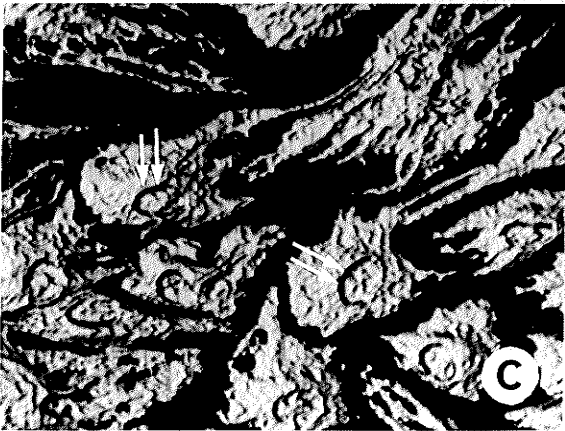
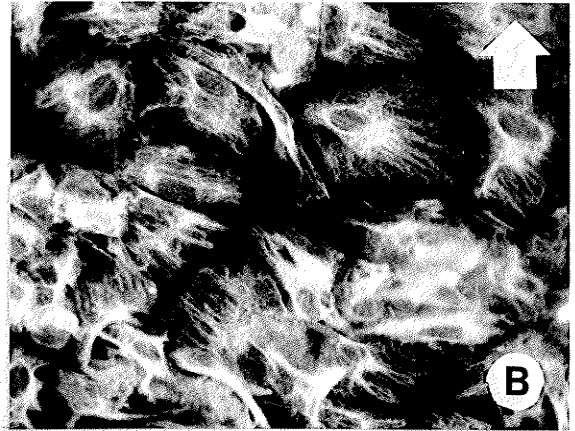


Figure 6. Immunolocalization of vimentin, talin and vinculin in strained and unstrained HOS TE-85 cells grown *in vitro*. (A) Control unstrained cultures of vimentin show an increase in intermediate filaments extensions, when mechanical strain is applied (B). Similarly, mechanical stimulation appeared to increase focal contacts when control cultures (C and E) were compared with strained ones (D and F). Cells were labeled with antibodies anti-talin (C and D) and anti-vinculin (E and F). Note the concentration of striations in the focal contact areas (double white arrows). White arrow indicates the direction of strain. Mechanical stimulation was applied for 6 h in all the photomicrographs. X 1250.

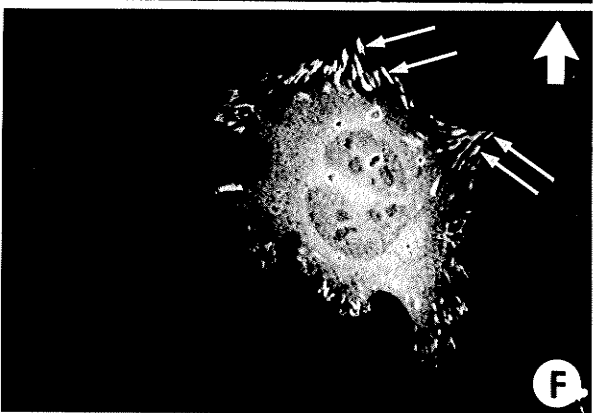
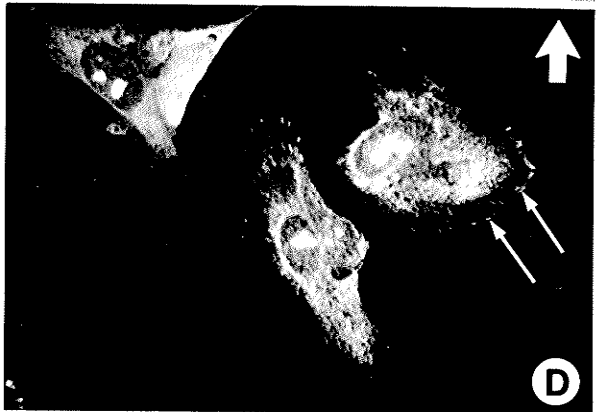
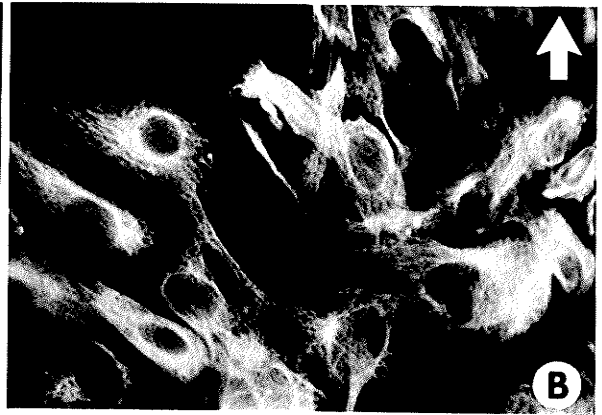
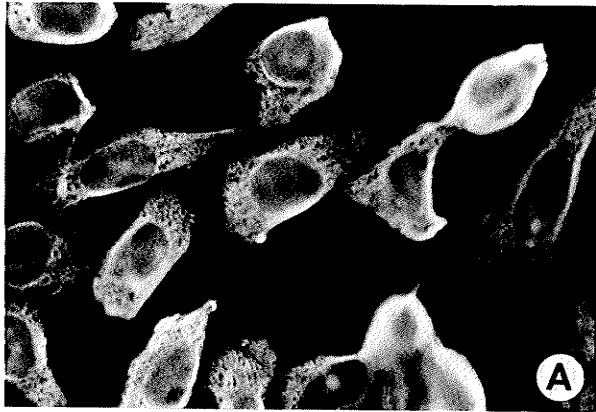


Figure 7. Immunolocalization of vinculin and tensin in strained and unstrained HOS TE-85 cells grown *in vitro*. Vinculin staining in control (a) and 24 h strained cultures (b). Note the concentration of striations in the focal contact areas (double white arrows). Focal contact protein tensin also appear to change its distribution with mechanical strain. Although, controls cultures (c) changed tensin distribution at 2 h (d) and 24 h (f) of strain, they did not at 6 h of strain (e). The similarities in tensin labeling in both (d) and (f) are shown by the small arrow. White arrow indicates the direction of strain. X 1250.

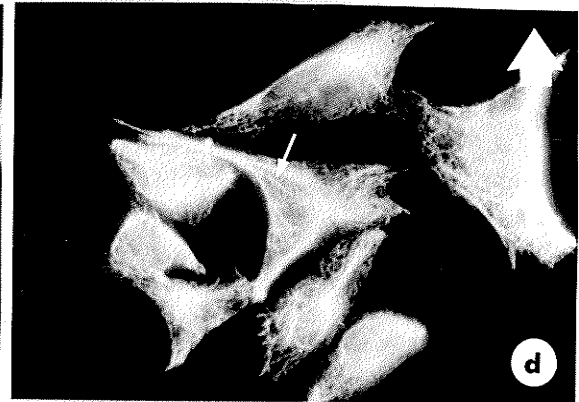
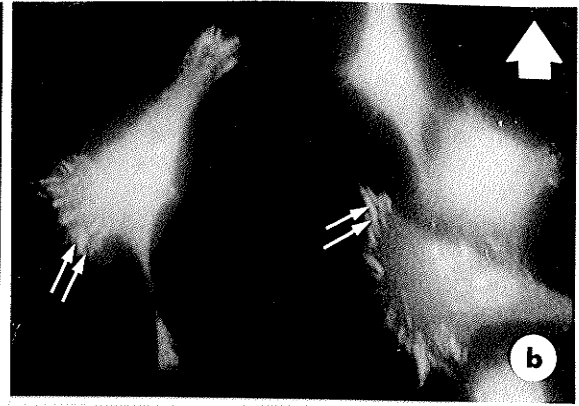
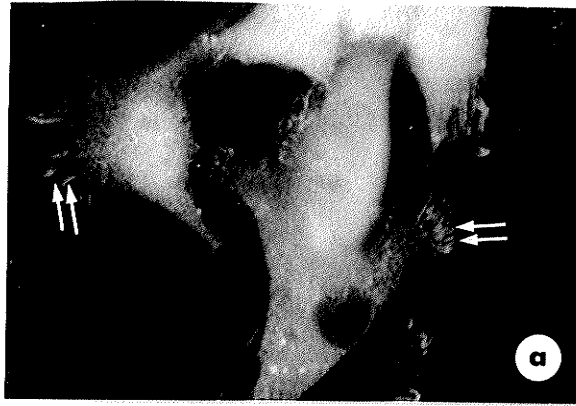
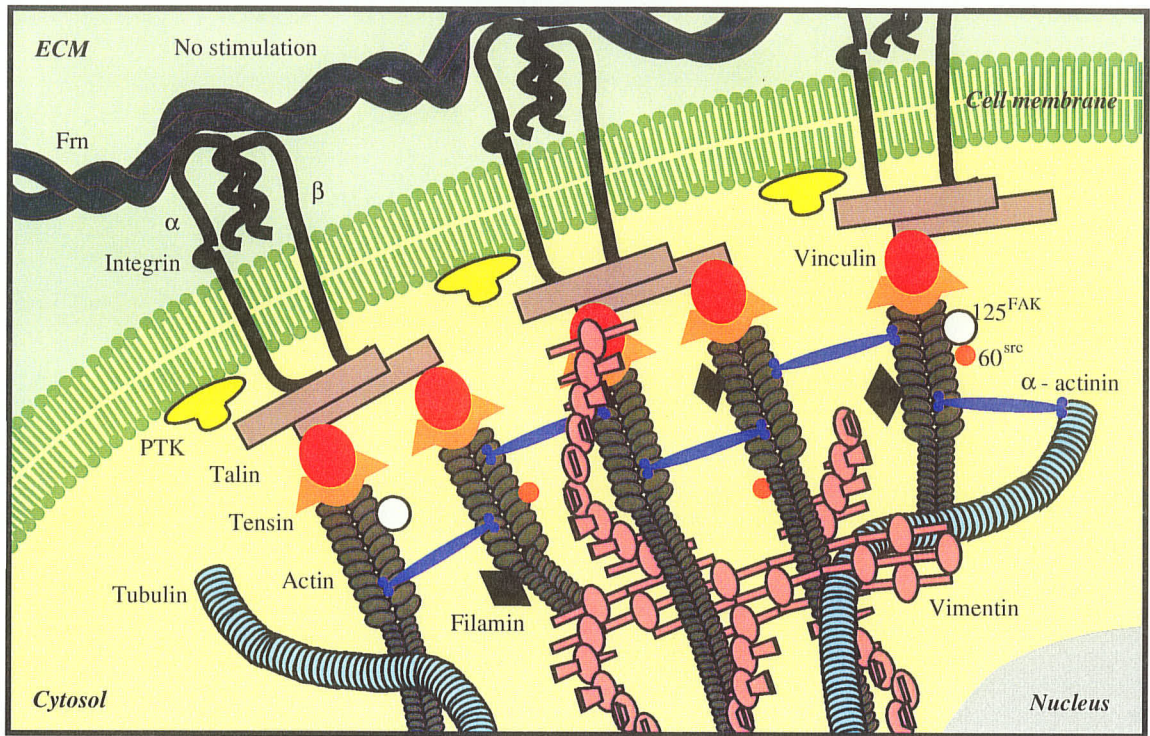
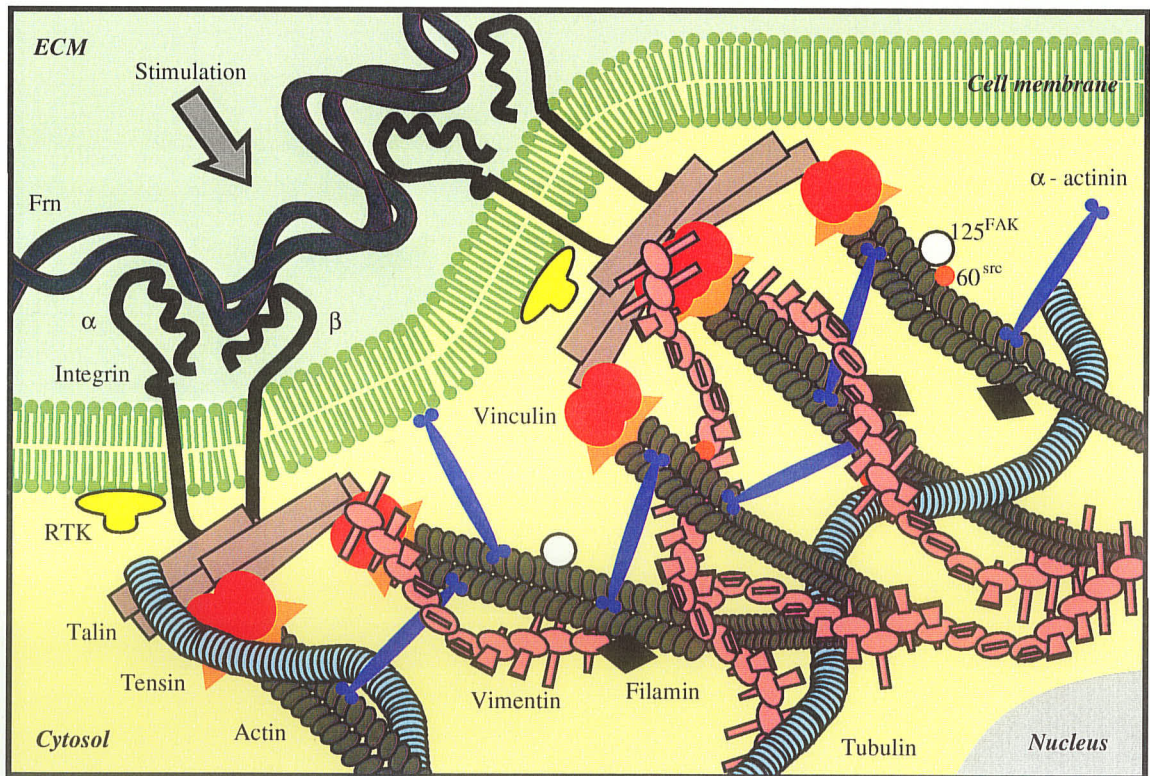


Figure 8. Diagram depicting a simplified model of cell-extracellular matrix (ECM) interactions. (A) The ECM protein fibronectin (Frn) is shown binding to membrane receptor integrins (both α and β subunits). Subsequently, this integrin heterodimer binds the intracellular focal contact and cytoskeletal protein complex. (B) Application of mechanical stimulation causes a membrane deformation and an activation of integrin receptors (conformational change). The strain signals may be transduced to the cell through Frn-integrin binding. Strain also appears to cause a physical deformation of the focal and cytoskeletal proteins. Focal contacts proteins talin, vinculin and tensin appear to increase as well as the volume of FAs. Similarly, the cytoskeleton of actin, vimentin and tubulin redistributes significantly, when compared to that of control cells (A). In addition, α -actinin, filamin, protein tyrosine kinases (PTK) and the proteins 125^{FAK} and 60^{src} are also shown. PTK*, 125^{FAK} and 60^{src} may participate in transduction of strain signals through a series of phosphorylation cascades of specific cellular proteins.



A



B



Chapter NINE

A Molecular Model of Mechanically-stimulated Cell Response

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ABSTRACT

The purpose of this paper is to discuss a molecular mechanism in signal transduction pathways of the regulation of integrin genes taking place in bone cells as a result of the orthodontic or mechanical stimulation. Human osteosarcoma (HOS) TE-85 cells were cultured in DMEM/F-12 and grown to confluency in Flexercell Type I dishes in a humidified incubator with 5 % CO₂ and 95 % air. Orthodontic forces were applied to bone cells via an intermittent strain of 3 cycles/minute using the Flexercell Strain Unit System for periods of 15 and 30 minutes, 2 and 24 hours and 3 days. Antibodies against β_1 and α_v integrins were immunolocalized in strained and unstrained cultures. Total RNA was extracted from time period and cDNA probes were used to measure mRNA expression of β_1 (1.2 kb) and α_v (1.1 kb) integrins. A cDNA probe for cyclophilin (750b) was used for controls of gene expression. Results showed that mechanical stimulation caused a reorganization of integrin distribution as compared with non stimulated controls. mRNA for β_1 expression showed a marked increase at 30 minutes and 3 days, while mRNA levels for α_v did not change with strain. Selective expression of integrins mRNA is indicative of a specific gene regulation by mechanical stimulation. It has been proposed that such selectivity follows an activation of DNA binding regions (REs) by receptors or transcription factors (TFs) specific to strain. Presence of such receptors (TFs) characterizes the cell response to mechanical stimulation. The model described here helps to explain why seemingly clinical "controlled" orthodontic forces may develop undesirable biological effects.

INTRODUCTION

The application of mechanically generated forces is central in prevention and correction of dentofacial discrepancies and dentoalveolar malocclusions. During clinical treatment, these forces are highly effective in determining tooth position. However, they may also generate a wide range of undesired biological reactions associated with the force-induced response. While mechanical forces may be regarded as beneficial in connective tissues such as bone (Wölff, 1892; Lanyon, 1987), the cellular mechanisms involved in the translation of clinical forces into biological responses are poorly understood. The macroscopic studies of orthodontically treated teeth have shown the remarkable potential for connective tissue remodeling both *in vivo* (Yen and Chiang, 1984; Yen *et al.*, 1989a, 1989b) and *in vitro* (Reitan and Kvam, 1971; Yen *et al.*, 1990). Partly due to the effectiveness of clinical treatment, this remodeling potential has been largely based on the type, duration and magnitude of applied forces (Storey, 1973), specifically to those descriptions of tissue tension and compression associated with bone apposition and bone resorption, respectively. These concepts stem mainly from early histological observations, including a variety of morphological and histochemical studies undertaken to clarify orthodontic tissue response (Reitan and Kvam, 1971; Storey, 1973; Rygh, 1976; Lilja *et al.*, 1983; Martinez and Jonhson, 1987; Davidovitch, 1991). Meanwhile, the clinically applied force may appear highly "uncontrolled" at the cellular level, possibly explaining why seemingly "light" and "controlled" orthodontic forces may, in addition to acceptable tissue remodeling, generate undesired cellular responses on occasion.

Rygh observed considerable tissue degradation in the periodontal ligament following orthodontic treatment (Rygh, 1976; 1977). Kvam (1972) found that root resorption was present in all orthodontically-stressed teeth, while Sims and co-workers (Langford and Sims, 1982; Sims and Weekes, 1985) determined that root resorption was directly related to the magnitude of orthodontic force applied. A possible explanation for this phenomena may be associated with injury to the periodontal ligament, since it has been

reported that intentional ligament trauma could initiate patterns of root resorption (Nakane and Kameyama, 1987). As yet, theories of trauma and/or magnitude of forces can not explain the changes induced in cell behavior as a result of orthodontic and especially physiologic stimulation. Better understanding of mechanisms of cellular signal transduction in mechanically-stimulated connective tissues may help to explain unexpected cell reactions observed as sequelae in the majority of orthodontic-treated clinical cases.

It has been suggested that the cellular environment of specialized matrix scaffoldings characterizes the cellular responses (Ingber, 1991). Lanyon (1987) has concluded that the matrix provides a source of "strain memory" which can continue the process of cell stimulation even some time after removal of the initial force. However, physiological and mechanically-induced processes depend on critical recognition and binding of selected cell ligands thereby ensuring appropriate functioning of certain cell properties such as cell adhesion and cell migration. These interactions are manifested by changes in cell morphology, proliferation and gene expression (Ginsberg *et al.*, 1992). A prime candidate for cell-matrix and cell-cell interaction is the family of adhesion molecules called integrins.

Integrin receptors are cell-surface heterodimers formed by an α and a β subunit associated non-covalently (Ingber, 1991; Hynes, 1992; Yamada *et al.*, 1992). In addition to their potential for transducing signals from the extracellular to the intracellular environment (Damsky and Werb, 1992; Sastry and Horwitz, 1993), integrin gene expression in the nucleus and protein post-translational modifications in the cytoplasm also appear to regulate this "outside-in" mechanism of cellular messages, establishing an "inside-out" signaling pathway (Damsky and Werb, 1992; Sastry and Horwitz, 1993). This concept is very important in the meaning of orthodontic-stimulated tissue response, since mechanical stimulation has recently been shown to modulate mRNA expression of selected integrin subunits in bone cells *in vitro* (Carvalho *et al.*, 1995a). Therefore, the aim of this paper is to discuss a molecular model for the mechanisms of mechanical signal

transduction of integrin gene regulation which takes place in bone cells as a result of orthodontic forces. Such information may be crucial in understanding the epigenetic control of tissue remodeling, in which both environment and genetic mechanisms may predetermine the optimal cell and tissue response.

MATERIALS AND METHODS

Human osteosarcoma TE-85 cells from ATCC (Rockville, MD) were grown in DMEM/F-12 (Sigma, St. Louis, MO) with 5 % FBS, 25 units/ml penicillin G, 25 μ g/ml streptomycin and 100 μ g/ml ascorbic acid (all from Sigma, St. Louis, MO). Mechanical strain was applied by using the Flexercell Strain Unit (Flexcell Corp., McKeesport, PA) with cells grown in Type I dishes (flexible bottoms) at 20 kPa at 3 cycles/min (10 seconds of strain/10 seconds of relaxation). Cells were strained for periods of 15 and 30 minutes, 2 and 24 hours and 3 days. Control cultures were cultivated for the same time periods using rigid-bottomed dishes. Immunohistochemistry was performed on strained and unstrained HOS TE-85 cells. Samples were rinsed with PBS fixed with 4 % paraformaldehyde for 10 min, washed in PBS, permeabilized in 0.5 % Triton X-100 for 20 min and washed again in PBS. Monoclonal mouse anti-human β_1 -integrin antibodies or anti-human α_v -integrin (both from Gibco, Burlington, ON) were added for 1 h at room temperature. Samples were washed and incubated with goat Cy3 conjugated anti-mouse IgG for 40 min. Negative controls for all antibodies were performed. To study the effect of mechanical strain on gene expression, total RNA was extracted from TE-85 cells as described previously (Chomzynski and Sacchi, 1987). Cells were rinsed in ice-cold PBS, incubated in a denaturing solution (4 M guanidinium thiocyanate, 25 mM NaCl, pH 7.0, 0.5 % sarcosyl and 0.1 % β -mercaptoethanol), incubated with water-saturated phenol, 2 M Na acetate and chloroform:isoamyl alcohol (49:1). Samples were centrifuged (10,000 g for 20 min) and the water phase and transferred to isopropanol at -70°C, centrifuged, resuspended in the denaturing solution, incubated with isopropanol, centrifuged, washed in 70 % ethanol and dried at room temperature. Samples were resuspended in distilled RNase-free water. RNA was quantified by spectrophotometry (Bio-Rad Laboratories Model 620 Video Densitometer, Matsushita Electric Industrial Co. Ltd., Japan). Twenty μ g of RNA per sample was fractionated on a 1.2 % agarose gel and RNA was transferred to a nitrocellulose membrane, pore size 0.45 μ m (MSI, Fisher Sci., Winnipeg, MB) overnight

at room temperature. cDNA probes for β_1 integrin of 1.2 kb or α_v integrin of 1.1 kb (Gibco, Burlington, ON) were labeled with [32 P]- γ CTP by a random prime kit (Amersham, Oakville, ON) and the membranes were hybridized overnight at 42°C. In addition, a probe for cyclophilin of 750 b was used as a control (generous donation of Dr. J. Richman). The excess probe was removed by subsequent changes of SSPE buffer (3 M NaCl, 23 mM NaH₂PO₄, 2 mM EDTA, pH 7.4) with varying degrees of stringency, and the blots were exposed to Kodak XRP-1X-ray films (Kodak Canada Inc., Toronto, ON). Data were analyzed statistically by a two-way analysis of variance with Tukey's multiple comparison test. In addition, T-tests with the Bonferroni correction were used to compare mRNA expression from strained and unstrained cells. All data represent the average of 4 experiments performed under identical conditions.

RESULTS

Confluent TE-85 cell cultures formed bone-like material as evidenced by bone nodules shown in Figure 1. Mineralized matrix was characterized by alkaline phosphatase activity and Von Kossa stain (not shown). Immunohistochemical fluorescence microscopy showed that the application of mechanical strain for 24 hours appeared to induce a reorganization of integrin distribution. β_1 integrin from untreated cultures (Fig. 2a) appeared to cluster in the center of cells after mechanical strain (Fig. 2b). The intensity of staining also seemed to increase. α_v integrin subunit staining was redistributed with strain when untreated cultures (Fig. 2c) were mechanically strained (Fig. 2d). Labeling of α_v integrin showed that clusters were more intense at the periphery of the cells in unstrained cultures (Fig. 2d). Following the application of strain, some cells did not show labeling for α_v at their periphery (not shown), but in the majority of cells α_v redistributed as indicated by white arrows (Fig. 2d). Northern blots revealed that the application of mechanical strain caused a significant increase in hybridization ($p < 0.005$) of β_1 integrin mRNA probe at 30 minutes and 3 days of culture in HOS TE-85 cells when compared with other time periods studied as shown in Figures 3A and 3B. On the other hand, the application of mechanical stimulation did not change the expression of α_v integrin mRNA in experimental cultures compared with untreated controls (Figs. 3C and 3D). The same amount of total RNA was used in this study for both strained and unstrained cultures of α_v and β_1 integrin subunits.

DISCUSSION

Therapeutic corrections in clinical orthodontics and dentofacial orthopedics attempt to stimulate cell and tissue remodeling by manipulation of mechanical forces. Since early studies showed that these forces produced a series of undesirable tissue reactions (Kvam, 1972; Rygh, 1977; Langford and Sims, 1982; Sims and Weekes, 1985; Nakane and Kameyama, 1987), the lack of knowledge between physiological and clinical force systems has been evident. Of special interest in orthodontics is the response of cells from supporting structures of teeth, such as periodontal ligament and bone to mechanical stimulation. Our understanding in this field has increased rapidly in the past few years, however, as this area expands more questions arise. In this paper we discuss signal transduction and the regulation of mechanical stimulation in bone cells at the molecular level.

Even though, the pathway or pathways which are responsible for translation of extracellularly applied mechanical forces into intracellular signals are still not clear, it is beyond the scope of this paper to discuss this question in detail. These concepts have been reviewed elsewhere (Carvalho *et al.*, 1995b). The rationale for this study is based on the assumption that cellular changes in morphology, proliferation and synthetic activity are correlated directly or indirectly to gene expression. By understanding mechanisms of mechanically-stimulated gene regulation, we believe that ultimately, the clinical application of forces will be delivered in such a manner as to communicate with the cells in their "language". This scenario will allow the most appropriate results with a minimum of biological tissue damage.

Role of the cytoskeleton, integrins and nuclear matrix in mechanically-stimulated signaling

The cellular cytoskeleton serves not only to provide a positional information for a variety of intracellular organelles including the nucleus, but also it has been shown to bind directly and regulate the expression of various mRNAs (Biegel and Patcher, 1992;

Simpson *et al.*, 1994). Nuclear positioning within the cell has also been shown to be essential in regulation of protein synthesis and gene expression (Russel and Dix, 1992). This positioning is partly regulated by binding of the cytoskeleton to the nuclear membrane (Bissel *et al.*, 1982; Berezny, 1991) which allows transduction of signals to the nucleus (Ingber, 1991; Simpson *et al.*, 1994). Therefore, association of integrin receptor proteins with the underlying cellular cytoskeleton (Ingber, 1991; Lotz *et al.*, 1989; Hynes, 1992; Yamada *et al.*, 1992) partly explains their regulation of gene expression (Ng-Sikorski *et al.*, 1991).

In addition to stimulating gene expression, integrin receptors have functions as signal transduction channels (Kornberg *et al.*, 1991; Hynes, 1992) in a series of critical recognition events of cell-substratum and cell-cell adhesion. These events are key in a variety of biological processes such as embryonic development, proliferation, differentiation, wound healing, tumor cell metastasis, cell polarity, cell migration, organ function, tissue organization and immunological recognition (Ingber, 1991; Kornberg *et al.*, 1991; Milam *et al.*, 1991; Clover *et al.*, 1992; Ginsberg *et al.*, 1992; Hynes, 1992; Yamada *et al.*, 1992; Majda *et al.*, 1994).

In mechanically-induced signal transduction, signals begin in the extracellular matrix (ECM). The predominantly physical stimulus is translated by integrins to some form of chemical signals which are propagated through the cytoskeleton and by a variety of second messenger molecules. These signals are progressively enhanced or repressed to finally reach the nucleus in which they regulate gene expression. It has been recently hypothesized that integrins translate mechanical stimulation from outside to inside the cells ("outside-in") (Sastry and Horwitz, 1993; Carvalho *et al.*, 1995b). On the other hand, the regulation of gene expression by integrins (Damsky and Werb, 1992) has revealed the potential for the "inside-out" regulation of mechanically-induced signal transduction (Sastry and Horwitz, 1993). The latter involves effectors in the cytoplasm which modulate affinity and/or specificity for its ECM ligand (Damsky and Werb, 1992). Thus, changes in integrin

mRNAs could regulate the cellular protein synthetic machinery through mRNA-cytoskeleton binding upon mRNA translation (Bissel *et al.*, 1982).

In addition to nuclear positioning, arrangement of the ECM also appears to provide positional information for transmission of mechanical forces. Moreover, the three-dimensional arrangement of DNA, a major part of the nuclear matrix, is believed to play a significant role in gene regulation (Simpson *et al.*, 1994). Thus, regulation of "inside-out" signaling by integrins may take place by a mechanism of interaction of nuclear factors in the nuclear matrix and cytoplasmic proteins. Pinkel *et al.* (1989) observed that nuclear functions are localized to specific regions in association with the nuclear matrix. It has been suggested that matrix-associated regions may act as regulatory sequences in mechanically responsive genes (Berezny, 1991). This is consistent with the idea that arrangement of chromatin loops of DNA may have a strong role in regulation of gene expression (Simpson *et al.*, 1994). Since the distribution of DNA appears tightly connected to the nuclear envelope, application of mechanical stimulation may determine DNA-protein binding, either by exposure or masking of key sequences, thus acting as a gene regulatory component. This is accomplished by controlling the interaction of various transcription factors with DNA sequences (Berezny, 1991; Getzenberg *et al.*, 1991).

Mechanical stimulation at the molecular level

It is important to understand the mechanisms by which cells process mechanical signals and control the message levels of various affected genes. Regulation of gene expression by mechanical stimulation appears to follow an established order, with early and late responses (Izumo *et al.*, 1988; Hsieh *et al.*, 1991; Komuro *et al.*, 1991; Resnick *et al.*, 1993). Izumo *et al.* (1988) and Komuro *et al.* (1991) have described the immediate early response in mechanically stimulated cardiac skeletal cells as the induction of proto-oncogenes and the accumulation of their protein products. Heat shock proteins also appear to be increased in early stages of mechanical stimulation (Simpson *et al.*, 1994). Although function of these proteins remains rather obscure, they could represent components of a

secondary signal transduction system (Simpson *et al.*, 1994). Such an intermediate system may enhance or inhibit specific signals from the cytoplasm to the nucleus (Distel *et al.*, 1987).

Molecular mechanisms of response have contributed to reveal the recognition events in the interactions of integrins and the cytoskeleton as discussed above (Hsieh *et al.*, 1991; Simpson *et al.*, 1994). The expression of integrin genes may be regulated by differentiation-specific macromolecules such as Vitamin D₃ and retinoic acid (Haussler, 1986; Slovik *et al.*, 1986; Heino and Massague, 1989). Such regulation is known to be dependent on the group of DNA-binding proteins called transcription factors (TFs) (Haussler, 1986; Getzenberg *et al.*, 1991). Interestingly, this may represent a very similar form of stimulation compared to that of mechanical strain. In this study we have found expression of β_1 integrin as early as 30 minutes (Fig. 3). However, the mechanically-stimulated response for the distribution of the integrin subunits studied here does not appear to follow the same pattern. The latter may be expected since such distribution is taking place after gene regulation. Even though there may be interactions with other systems including changes in membrane fluidity (Bissel *et al.*, 1982) causing integrin redistribution as a result of mechanical stimulation, mRNA changes for β_1 integrin seen here are remarkably similar to studies for other genes such as platelet derived growth factor-B (PDGF-B) (Hsieh *et al.*, 1991; Resnick *et al.*, 1993). Recently, studies on the PDGF-B gene have caused a breakthrough towards the understanding of the mechanically-induced molecular response. This will be discussed in more detail below.

Transcription factors are regulatory molecules of gene expression

The primary objective of gene expression is the synthesis of messenger RNA (Mitchell and Tjian, 1989). This phenomenon depends largely on the TFs, nuclear proteins also called "receptors" that bind to specific DNA regions of gene promoters or sites for transcriptional control of a gene. These regions are called responsive elements (REs) and depending on the message encoded in the RE sequence, the TFs may enhance or inhibit the

transcription of a given gene. However, how do mechanically-induced signaling molecules stimulate the transcription of genes?

As discussed above, Resnick *et al.* (1993) studied the effect of mechanical stimulation on the PDGF-B gene. These authors have identified a region of DNA in the PDGF-B gene promoter, which appears to be required in order to confer responsiveness to this gene as a result of mechanical stimulation. In other words, a mechanical stimulation responsive element (MSRE) has been localized. MSREs are highly conserved regions and appear in other genes that have been found to be up-regulated by mechanical stimulation, such as transforming growth factor- β_1 (Heino and Massagué, 1989) and tissue plasminogen activator (Diamond *et al.*, 1990). Thus, the presence of MSREs also indicate the activity of a TF which is likely to be specific to mechanical stimulation. TFs have been extensively studied for such biological regulatory molecules, such as vitamins, hormones, neurotransmitters and other biological modifiers (Haussler, 1986). Such is the case for Vitamin D₃ and retinoic acid (Haussler, 1986; Slovik *et al.*, 1986; Heino and Massagué, 1989). Studies on these molecules have demonstrated their regulation of integrin expression (Sömjem *et al.*, 1980; Slovik *et al.*, 1986; Heino and Massague, 1989), which likely, takes place through a TF-RE regulated mechanism.

Metabolism of mechanical stimulation specific receptor (MSSR)

The expression, regulated post-translational modifications, binding affinities and interactions with nuclear proteins play an important role in the specificity of TFs to REs. This appears to be the case for a hypothetical TF specific to mechanical stimulation or MSSR (Fig. 4). Nuclear protein extracts or MSSRs that have the ability to bind specifically to MSRE, have also been shown to form multiple complexes with other nuclear proteins (Resnick *et al.*, 1993). Although further study is necessary to characterize interactions of the MSREs, interaction of the TFs (MSSR) with other nuclear proteins appears to be required for gene activation. TF oncogenes such as *c-fos* form heterodimers and *c-jun* form homodimers in order to initiate gene transcription at their specific REs

(Kouzarides and Ziff, 1988). In fact, dimerization properties may be essential for RE recognition, since sequence specificity of the REs apparently is not the only consideration to determine RE selectivity of TF binding (Kouzarides and Ziff, 1988; Mitchell and Tjian, 1989). The latter is seen in the transformation of steroid receptors by hormone binding (Beato, 1989) such as in Vitamin D₃ and retinoic acid (Haussler, 1985; Heino and Massagué, 1989). However, at this point it is difficult to determine the exact binding of MSSRs to MSREs since the regulation of TFs appears to be very complex. TF-TF or TF-protein interactions may play a role in a variety of gene regulation mechanisms. Cytoplasm of nuclear TFs prior to being stimulated may be associated with other proteins, to which they are dissociated upon stimulation (Yamamoto, 1988). Yet intermediate cytoplasmic proteins may play a role in mediating TF-RE binding (Nevins, 1987). In addition, one must keep in mind that regulation of TFs and gene regulation by TFs are cascade processes and rate-limiting steps are likely to be present. In the case of mechanical stimulation of endothelial cells by flow, expression of the TFs *c-jun* and *c-fos* may also represent an active binding of their respective REs (Hsieh *et al.*, 1991). However, it is not clear if such binding is limiting for the activation of other TFs.

To date, little is known about MSREs and the proposed MSSR. Yet, the significance of these findings to the clinical orthodontic practice is indicative that a mechanical stimulatory-response on biological tissues once believed to be isolated, has been shown to be genetically controlled. Our data on integrin regulation is consistent with this idea. Figures 4 and 5 depict a model for integrin regulation of MSSRs to MSREs binding, showing the role of the cytoskeleton and of nuclear proteins in mechanically-stimulated gene transcription. By contrast, it can not be overlooked that although mechanical stimulation is very important in maintenance of physiological and orthodontic-induced tissue metabolism, there is also evidence that other factors may be involved. According to Banes (unpublished observations), it seems to be just as important for some "selected" cells to ignore mechanical activity. This appears to take place in endothelial and

smooth muscle cells in some refractory periods of cellular activity primarily as a result of shear stress. This selectivity within similar cell populations may be explained by a lack of MSSRs or MSREs or their modification, thus inhibiting gene activation. In this study we have shown that mechanical stimulation is responsible for a selective expression of integrins (Fig. 3) corroborating earlier findings which show differences in stimulation and/or inhibition of expression of certain genes by strain (Komuro *et al.*, 1991).

Although, information on binding proteins and matrix-associated regions with other components of the nuclear matrix is still speculative, undoubtedly, the DNA sequences of MSREs and the hypothetical MSSRs have a great potential of mediating mechanical signal transduction. We have discussed a missing link in binding of matrix-associated regions and/or REs. However, it is important to remember that mechanical stimulation is not an isolated phenomenon. Stimulation by mechanical signals at the cell level is also regulated by a variety of other morphogens, such as growth factors, hormones and morphoregulatory molecules (Simpson *et al.*, 1994). In addition, a single system probably does not solely explain the complexity of the mechanically-induced cellular response.

New studies on the effects of mechanical stimulation upon these basic cellular mechanisms are starting to clarify the principles of cellular behavior that are frequently taken for granted at the clinical level. Future experimentation both at the basic and clinical levels will greatly enhance our understanding of both physiological and therapeutic application of mechanical forces. Nevertheless the main goals remain to deliver clinical management through controlled loading patterns which could prevent and/or reverse tissue breakdown and yet provide the desired functional and aesthetics results.

ACKNOWLEDGEMENTS

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REFERENCES

- Beato M 1989 Gene regulation by steroid hormones. *Cell* **56**:335-344.
- Berezny R 1991 The nuclear matrix: a heuristic model for investigating genomic organization and function in the cell nucleus. *J Cell Biochem* **47**:109-123.
- Biegel D and Patcher JS 1992 mRNA association with the cytoskeleton framework likely represents a physiological binding event. *J Cell Biochem* **48**:98-106.
- Bissel MJ, Hall HG and Parry G 1982 How does extracellular matrix direct gene expression? *J Theor Biol* **99**:31-68.
- Carvalho RS, Scott JE, Bumann A, Schwarzer C and Yen EHK 1995a Selective expression of β_1 and α_v integrin subunits mRNA and their role in the signal transduction pathways of mechanically strained human osteosarcoma TE-85 cells. *Archs Oral Biol* Submitted.
- Carvalho RS, Bumann A, Scott EJ and Yen EHK 1995b Connective tissue response to mechanical stimulation. In: McNeil C, ed. *Occlusion: Science and Practice*, Quintessence Publishing Co. In press.
- Chomzynski P and Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**:156-159
- Clover J, Dodds RA and Gowen M 1992 Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. *J Cell Sci* **103**:267-271.
- Damsky CH and Werb Z 1992 Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* **4**:772-781.
- Davidovitch Z 1991 Tooth Movement. *Crit Rev Oral Biol Oral Med* **2**:411-450.
- Diamond SL, Sharefkin JB, Diffenbach C, Scott KF, McIntire LV and Eskin SG 1990 Tissue plasminogen activator messenger RNA levels increase in cultured human endothelial cells exposed to laminar shear stress. *J Cell Physiol* **143**:364-371.

- Distel RJ, Ro H, Rosen BS, Groves DL and Spiegelman BM 1987 Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct participation of c-Fos. *Cell* 49:835-844.
- Getzenberg RH, Pienta K, Ward WS and Coffey DS 1991 Nuclear structure and the three-dimensional organization of DNA. *J Cell Biochem* 47:289-299.
- Ginsberg MH, O'Toole TE, Loftus JC and Plow EF 1992 Ligand binding to integrins: Dynamic regulation and common mechanisms. In: *Cold Harbor Symposia on Quantitative Biology*. Volume 57, Cold Spring Harbor Laboratory Press, pp. 221-231.
- Haussler MR 1986 Vitamin D Receptors: nature and function. *Ann Rev Nutr* 6: 527-562.
- Heino J and Massagué J 1989 Transforming growth factor-beta switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin. *J Biol Chem* 264:21806-21811.
- Hsieh H, Li N and Frangos J A 1991 Shear stress increases endothelial platelet-derived growth factor mRNA levels. *Am Physiol Soc* 260:642H-646H.
- Hynes RO 1992 Integrins: versatility, modulation and signaling in cell adhesion. *Cell* 69:11-25.
- Ingber DE 1991 Integrins as mechanochemical transducers. *Curr Opin Cell Biol* 3:841-848.
- Izumo S, Nadal-Ginard B and Mahdavi V 1988 Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc Natl Acad Sci USA* 83:339-343.
- Komuro I, Katoh Y, Kaida T, Shibazaki I, Kurabayashi M, Hoh E, Takaku F and Yazaki Y 1991 Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. *J Biol Chem* 266:1265-1266.

- Kornberg L, Earp HS, Turner C, Procop C and Juliano RL 1991 Signal transduction by integrins: increased protein tyrosine kinase phosphorylation caused by clustering of beta 1 integrins. *Proc Natl Acad Sci USA* **88**:8392-8396.
- Kouzarides T and Ziff E 1988 The role of leucine zipper in the fos-jun interaction. *Nature* **336**:646-551.
- Kvam E 1972 Cellular dynamics on the pressure side of the rat periodontium following experimental tooth movement. *Scand J Dent Res* **80**:357-383.
- Langford SR and Sims MR 1982 Root surface resorption repair, and periodontal attachment following rapid maxillary expansion in man. *Am J Ortho* **81**:108-115.
- Lanyon LE 1987 Functional strain in bone tissue as an objective, and controlling stimulus for adaptive bone remodeling. *J Biomech* **20**:1083-1093.
- Lilja E, Lindskog S and Hammaström L 1983 Histochemistry of enzymes associated with tissue degradation incident to orthodontic tooth movement. *Am J Orthod* **83**:62-75.
- Lotz MM, Bursal CA, Erickson HP and McClay DR 1989 Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J Cell Biol* **109**:1795-1805.
- Majda JA, Gerner EW, Vanlandingham B, Gehlsen KR and Cress AE 1994 Heat shock-induced shedding of cell surface integrins in A549 human lung tumor cells in culture. *Exp Cell Res* **210**:46-51.
- Martinez RH and Jonhson RB 1987 Effects of orthodontic forces on the morphology and diameter of Sharpey fibers of the rat. *Anat Rec* **219**:10-20.
- Milam SB, Magnuson V, Steffensen B, Chen D and Klebe RJ 1991 IL-1 β and prostaglandins regulate integrin mRNA expression. *J Cell Physiol* **149**:173-183.
- Mitchell PJ and Tjian R 1989 Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**:371-378.
- Nakane S and Kameyama Y 1987 Root resorption caused by mechanical injury of the periodontal soft tissue in rats. *Journal of Periodontal Research* **22**:390-395.

- Nevins JR 1987 Regulation of early adenovirus gene expression. *Microbiol Rev* 51:419-430.
- Ng-Sikorski J, Andersson R, Patarroyo R and Andersson M 1991 Calcium signaling capacity of the CD11b/CD18 integrin on human neutrophils. *Exp Cell Res* 195:504-508.
- Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, Lucas J and Gray J 1989 Fluorescence *in situ* hybridization with human chromosome specific libraries: detection of trisomy 21 and translocation of chromosome 4. *Proc Natl Acad Sci USA* 85:9138-9142.
- Reitan K and Kvam E 1971 Comparative behavior of human and animal tissue during experimental tooth movement. *Angle Orthod* 11:1-8.
- Resnick N, Collins T, Atkinson W, Bonthron DT, Dewey CF and Gimbrone MA 1993 Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. *Proc Natl Acad Sci USA* 90:4591-4595.
- Russel B and Dix DJ 1992 Mechanisms for intracellular distribution of mRNA: *in situ* hybridization studies in muscle. *Am J Physiol* 262:1-8.
- Rygh P 1976 Ultrastructural changes in tension zones of rat molar periodontium incident to orthodontic tooth movement. *Am J Orthod* 70:269-281.
- Rygh P 1977 Orthodontic root resorption studied by electron microscopy. *Angle Orthod* 47:1-16.
- Sastry SK and Horwitz AF 1993 Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr Opin Cell Biol* 5:819-831.
- Simpson DG, Carver W, Borg TK and Terracio L 1994 Role of mechanical stimulation in the establishment and maintenance of muscle differentiation. *Int Rev Cyto* 150:69-94.

- Sims MR and Weekes P 1985 Resorption related to orthodontics and some morphological features of the periodontal microvascular bed. *Int End J* 18:140-145.
- Slovik DM, Rosenthal DI, Doppelt SH, Potts JT, Daly AM, Campbell JA and Neer AM 1986 Restoration of spinal bone in osteoporotic men by treatment with human parathyroid hormone (1-34) and 1,25-dihydroxyvitamin D. *J Bone and Min Res* 1:377-382.
- Sömjem D, Binderman I, Berger E and Harell A 1980 Bone remodeling induced by physical strain is prostaglandin E₂ mediated. *Biochim Biophys Acta* 627:931-934.
- Storey E 1973 The nature of tooth movement. *Am J Orthod* 63:292-314.
- Wölff JL 1892 The law of bone remodeling. In: Maquet P and Furlong R, eds. (Translation, 1986) *Das Gesetz der Transformation der Knochen*, Springer-Verlag, Berlin.
- Yamada KM, Aota S, Akiyama SK and LaFlamme SE 1992 Mechanisms of fibronectin and integrin function during cell adhesion and migration. In: *Cold Harbor Symposia on Quantitative Biology*. Volume 57, Cold Spring Harbor Laboratory Press, pp. 203-212.
- Yamamoto KR 1988 A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. *Cell* 54:1073-1080.
- Yen EHK and Chiang K 1984 A radioatographic study of the effect of age on the protein-synthetic and bone-deposition activity in interparietal sutures of male white mice. *Archs Oral Biol* 29:1041-1047.
- Yen EHK, Yue CS and Suga DM 1989a Effect of force level on synthesis of type III and type I collagen in mouse interparietal suture. *J Dent Res* 68:1746-1751.
- Yen EHK, Yue CS and Suga DM 1989b The effect of sutural growth rate on collagen phenotype synthesis. *J Dent Res* 68:1058-1063.
- Yen EHK, Pollit DJ, Whyte WA and Suga DM 1990 Continuous stressing of mouse interparietal suture fibroblasts *in vitro*. *J Dent Res* 69:26-30.

Figure 1. Phase contrast micrographs of HOS-TE 85 cells in culture. (A) 24 hours after seeding of the cells, there is rapid growth and the cells start forming clusters (arrows). (B) Cell clusters from (A), grow and coalesce with other clusters until complete confluency is reached. (C) At 48 to 72 hours of the initiation of the cultures, increased cell proliferation takes place at localized sites (arrows) in which mineral is deposited and bone nodules are formed (arrow) in (D).

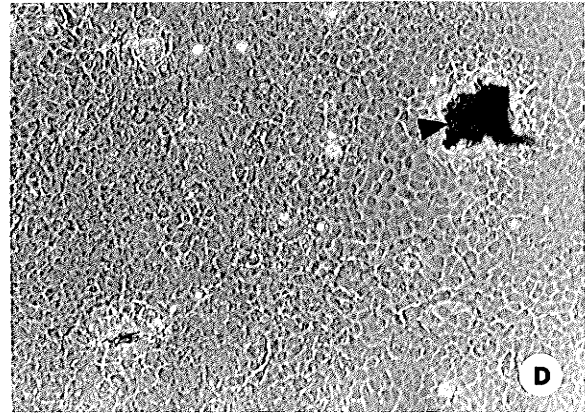
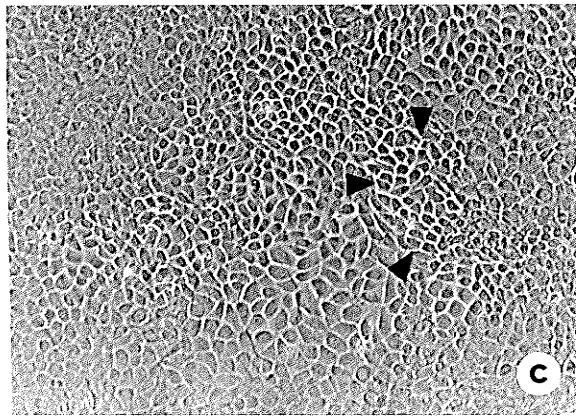
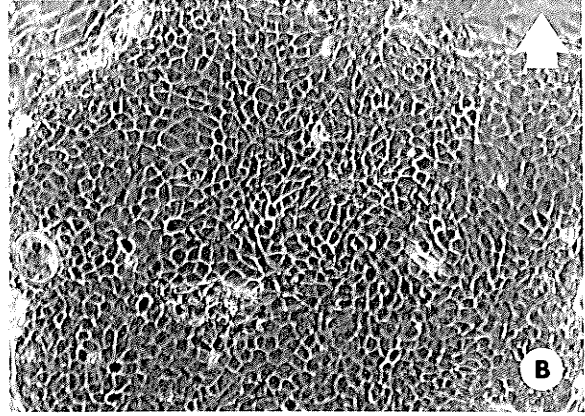
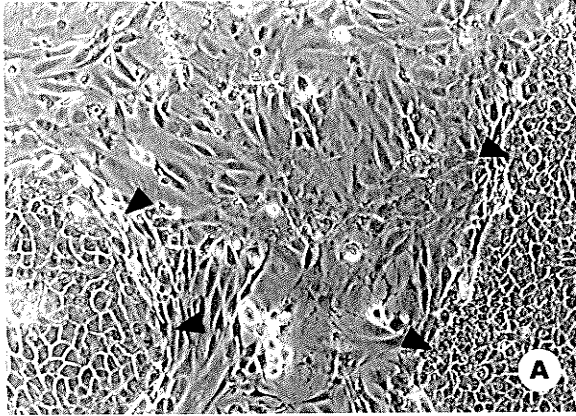


Figure 2. Immunolocalization studies of HOS TE-85 cells. Photomicrographs of unstrained (a and c) and strained (b and d) TE-85 HOS cells. (a) unstrained cultures incubated with β_1 integrin antibodies showed a change in the labeling distribution when these cells were mechanically strained, as integrin subunits appeared to redistribute towards the cell center (b). Similarly, control unstrained cultures incubated with α_v integrin antibodies (c) also showed a change in labeling after mechanical stimulation (d), however, α_v labeling was still evident at the cell periphery in both unstrained and strained cells. Magnification (a-f) X 1250.

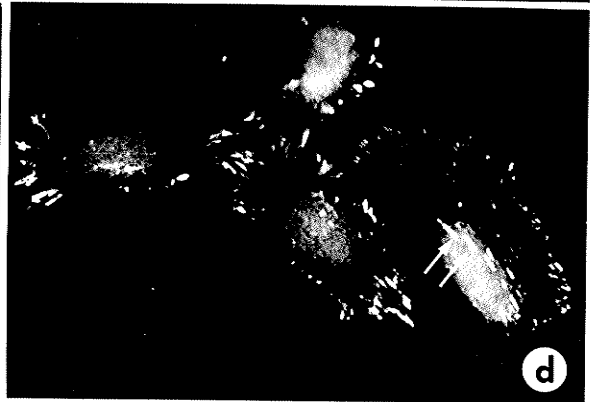
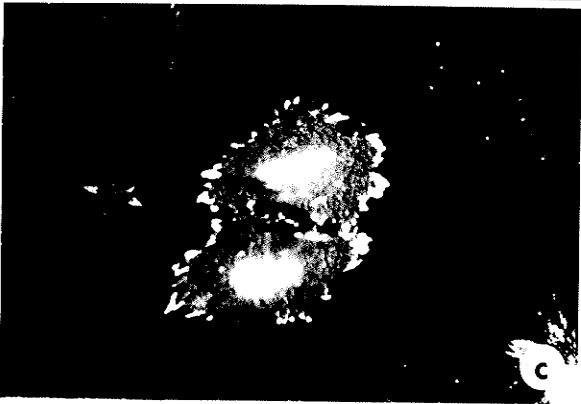
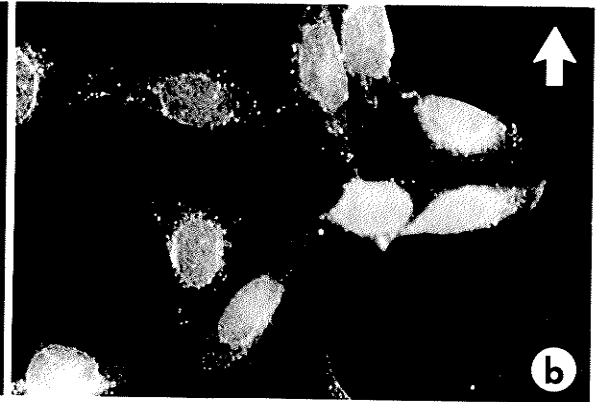
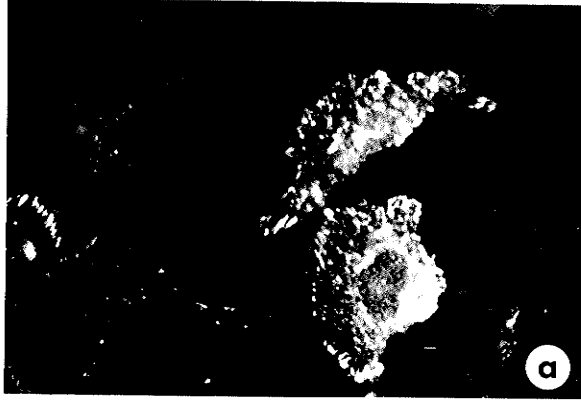


Figure 3. Photomicrographs of northern blots showing mRNA of β_1 and α_v integrin subunits. (A) effects of mechanical strain in β_1 -integrin subunit mRNA expression. Note that strain (+) causes an increase of β_1 mRNA at 30 minutes and again at 3 days and 1 week, when compared with unstrained controls (-). (B) same as that shown in (A). Note the increase in β_1 mRNA at 3 days of strain application. (C) effects of mechanical strain in α_v integrin subunit mRNA expression. Note that strain (+) does not change the mRNA expression at any given time period when compared with unstrained controls (-). (D) same as that shown in (C). Note that no changes are seen with the application of strain. Control mRNA (probe for cyclophylin) indicates comparable amounts of RNA per lane and 28 S indicates the position of the 28 srRNA. Legends are: (A) and (C) 15' (15 minutes), 30' (30 minutes), 2 h (2 hours), 24 h (24 hours), 3 d (3 days) and 1 w (1 week); and (B) and (D) 2 h (2 hours), 24 h (24 hours) and 3 d (3 days).

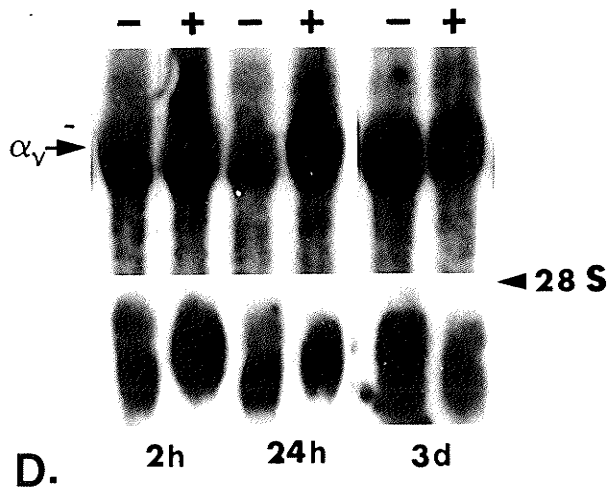
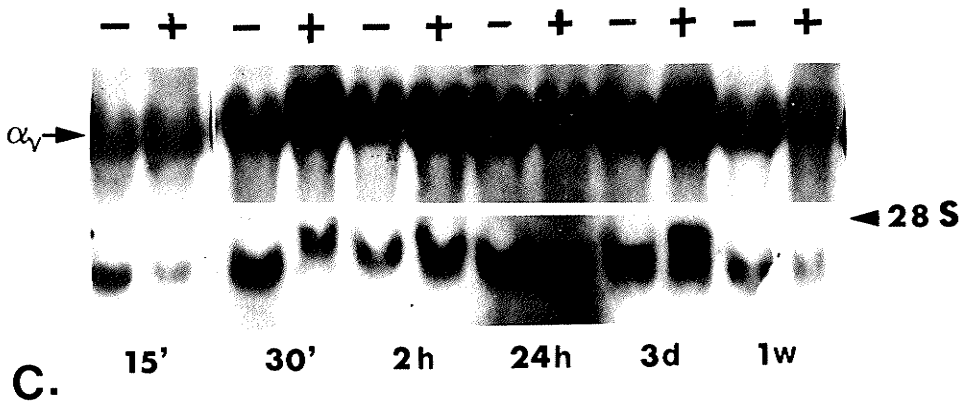
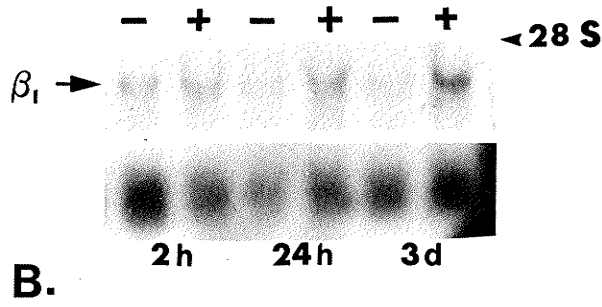
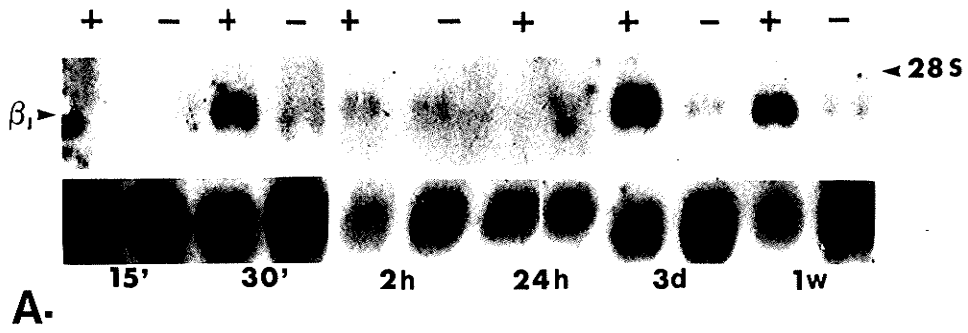


Figure 4. Diagram depicting a simplified model of integrin-regulated mechanical stimulation-induced response in bone cells. Although, the interactions of integrins-cytoskeleton and cytoskeleton-nucleus have not been described here, they play a very important role in the transducing of mechanically-induced signals. However, the focus of this work is to describe a molecular mechanism for this response. The mechanical stimulation specific receptor (MSSR) may be located at the cytosol or directly into the nucleus as shown here. Upon mechanical stimulation the MSSR could be translocated from the cytosol to the nucleus or get activated in the nucleus by a cascade process, terminating with its binding to the mechanical stimulation responsive element (MSRE). This binding carries the stimulation or inhibition of expression in selected genes. In the nucleus the activity of MSSR may be regulated by: 1) 3-D arrangement of DNA, 2) a dimerization of the MSSR itself, 3) binding of other nuclear factors or proteins (NP) and 4) interaction of MSSR with nuclear matrix proteins (#s 1 and 4 are further discussed in Fig. 6). All these regulatory events modulate the activity of MSSRs, however, it may be highly speculative to suggest which relationship these factors may have, if any.

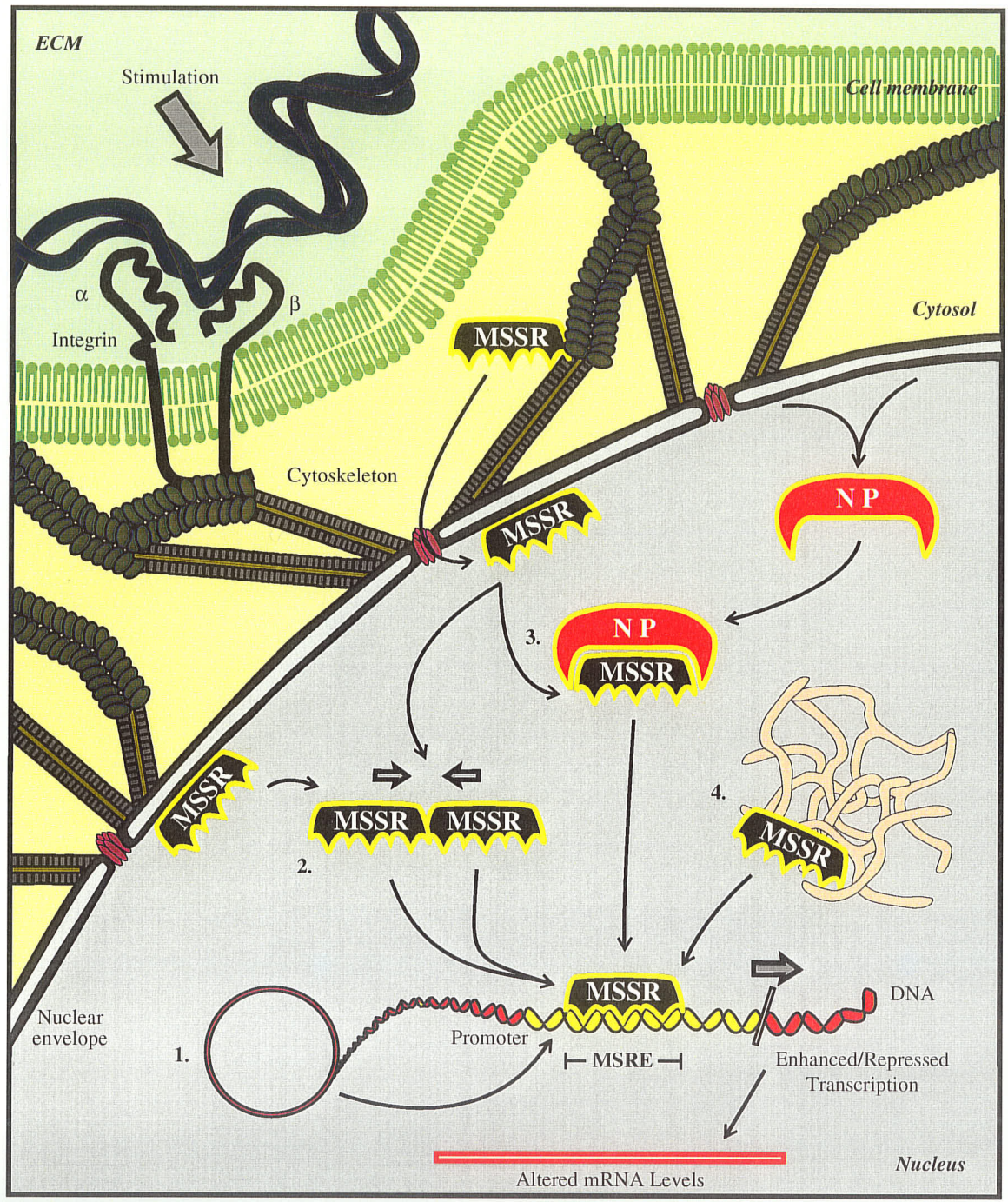
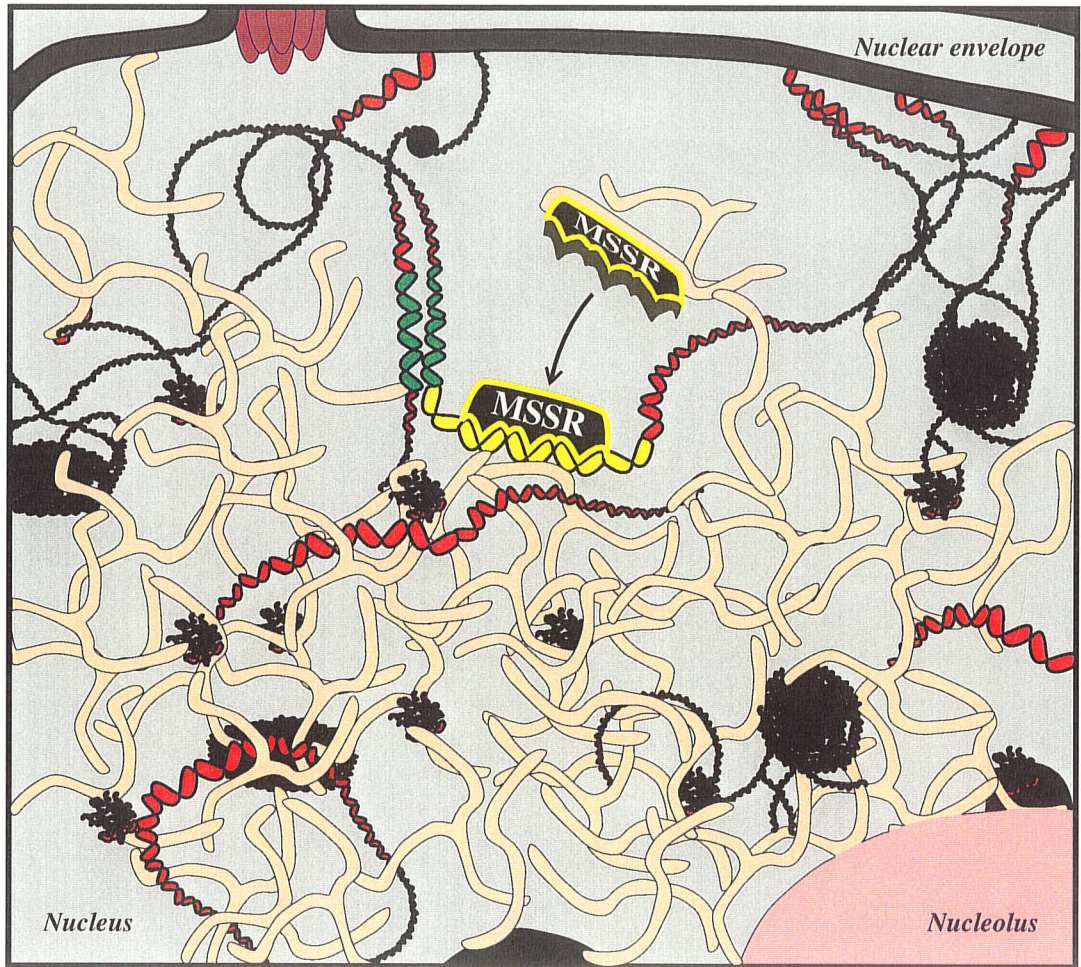


Figure 5. Diagram describing a simplified model for the association of MSSR with nuclear matrix proteins. Upon mechanical stimulation the nuclear matrix may distribute itself so as to allow for MSSR-MSRE binding as indicated, since active genes have been shown to be associated with the nuclear matrix. Similarly the three-dimensional distribution of DNA, also given by the nuclear matrix and modulated by mechanical stimulation, confers tissue specificity (green areas) for the MSSRs binding as shown here.





Chapter TEN

General Discussion



GENERAL DISCUSSION

During the course of this thesis, the importance of mechanical stimulation in the function and maintenance of bone has been emphasized many times. This statement often came followed by a recognition that an insufficient body of literature is available to explain underlying cellular mechanisms responsible for such dynamic cell stimulation. Although, the main purpose of this investigation is to clarify the complexity of strain-stimulated cell responses, few direct answers may have resulted from the present studies, in fact more questions were raised during the studies described herein. Since each chapter includes pertinent discussions, an attempt will be made to avoid unnecessary repetitions. In this general discussion, initially the main conclusions will be drawn from chapters two to nine. Subsequently, our new data will be correlated with existing knowledge in the area of strain-induced signal transduction mechanisms.

Initially, a reproducible and accurate system for mechanical stimulation of bone cells was needed. Such interest stemmed from observations in which cells subjected to stimulation might respond in a similar fashion to their counterparts *in vivo*, functioning constantly under some form of load. Preliminary information suggested that the Flexercell Apparatus would be a useful method for studying strain in many cell types, including bone. The properties of this system focused on stretching a deformable substrate on which the cells attach. However, there was no proof that attached cells would indeed be strained and thereby stimulated under these circumstances. Consequently, the Flexercell apparatus was redesigned to allow for direct observation of cell shape changes following strain. In chapter two, we concluded that strain was effectively applied, producing an increase in peripheral area of three different cell types without producing any visible degree of cell damage. These observations substantiate the potential for this apparatus in the study of strain-induced cell responses. The advantages and disadvantages of this and other systems currently under use to study similar mechanisms were discussed in chapters two, three and eight and have been reviewed by Gilbert *et al.* (1994).

After the necessary characterization of the culture conditions, further experiments were designed in a logical sequence. In chapter three, experiments were designed to study the signal transduction pathways in mechanically-stimulated bone cells. The response of bone cells to strain showed that stimulation of IP₃ levels and PKC activity take place sooner than any changes in cAMP levels. The relationship of strain-stimulated responses with parathyroid hormone (PTH) has indicated that strain induction of PKC, IP₃ and cAMP were potentiated by PTH, and that these were not eicosanoid driven. From these studies, we hypothesized that signal transduction was closely related to the cell cytoskeleton. The tight binding of phosphatidylinositol lipids to the cytoskeleton has been known for some time in intracellular signaling mechanisms (Janmey, 1994 for a review). In chapter three, the changes in the immunolocalization of PKC confirmed the latter. Moreover, also in this chapter, strain-induced changes in cytoskeleton were evidenced by immunolocalization of vimentin, vinculin and α -actinin. Chapter four explored further strain-induced cytoskeleton stimulation. The synthesis and distribution of both the focal adhesion proteins vinculin and talin and the intermediate protein vimentin were increased following strain during the time periods studied. This was also true for selected subunits of the integrin family of receptor proteins. From this we concluded that the mechanisms of cell response to strain would likely involve integrin-mediated signaling, through focal contact areas. Chapter five illustrated many hypothetical aspects of connective tissue response to strain in a mini-review format and, therefore, will not be discussed further. Once the potential for integrin-mediated signaling was established, based on these and other studies in the literature, interest arose concerning the mechanism by which molecular expression of these receptors could be regulated by strain. Thus, experiments were designed to study the expression of the ubiquitous integrin subunit β_1 at various time periods as observed in chapter six. It was found that β_1 mRNA levels increased sharply at 30 minutes and 3 days from the onset of stimulation. In chapter seven, study of additional α_v integrin subunit revealed that molecular response was not coincident for both subunits studied. Strain did not change α_v

integrin mRNA expression at any time period. The conclusions drawn from chapters six and seven indicated that strain effects appeared to be quite specific and revealed a distinct strain response at the nuclear level. Chapter eight further describes the cytoskeletal adaptations upon mechanical strain stimulation. It was concluded that stimulated cells appear to increase cell attachment through an increase in focal contacts. Finally, chapter nine postulates a model for a molecular response of strain centered on integrin mRNA expression. Molecular mechanisms may include a mechanical stimulation specific receptor (MSSR) and a series of nuclear modulated interactions.

Implications of strain-induced response in signal transduction mechanisms

It is very important to determine the underlying mechanisms by which bone and other cell types modulate their structure in response to physical stimulation (Simpson *et al.*, 1994). Hence, original questions in this study were formulated to determine: to what extent strain induced specific signaling mechanisms; how these mechanisms translated into cell functions; how changes in membrane signals correlated with cytoskeletal and receptor rearrangement; and how strain stimulation could determine nuclear cell response. In chapter three changes in well known signaling pathways have been shown as well as a PTH-induced potentiation of bone strain response. PTH-induced response in bone cells comprises several mechanisms that have been described elsewhere. However, it is relevant for this discussion to indicate how selected PTH action may regulate strain response, specially as PTH associates with Ca^{2+} . For instance, two Ca^{2+} channels have been identified as PTH-activated, these are cAMP-regulated and PKC-activated channels (Yamaguchi *et al.*, 1987; Vadiakas and Banes, 1992). Although, Ca^{2+} level changes with strain were not studied here, the activation of a Ca^{2+} -induced pathway as a result of strain can not be ruled out. As discussed in chapter one, there are many studies suggesting the role of Ca^{2+} channels as mechanotransducers. A plausible hypothesis has been suggested by Alcon and Rasmussen (1988); strain appears to cause an increase in the cycling between influx/efflux of Ca^{2+} across the plasma membrane, even though the concentration of Ca^{2+}

remains low after transient increases following mechanical stimulation. However, we are still somewhat reluctant to consider Ca^{2+} channels as true mechanotransducers. Watson (1991) suggested that a mechanotransducer must contain two basic characteristics: a functional association with the underlying cytoskeleton and a secondary or tertiary structure capable of transducing signals via interactions with signaling pathways and the cytoskeleton. By contrast, although Ca^{2+} as a second messenger participates in numerous intracellular mechanisms, ion permeability is a rather general response and a transient membrane puncture cannot be ruled out in mechanically strained cells (Charles *et al.*, 1991). However, preliminary studies using Ca^{2+} channel blockers failed to elicit a response in our experiments. Currently, it is still not clear what role extracellular Ca^{2+} may play in strain-induction of Ca^{2+} as opposed to that of strain-induced Ca^{2+} from intracellular stores. Additional studies focusing on this problem are warranted.

Eventhough Ca^{2+} may not constitute a separate signaling pathway, its action as a messenger is closely related to other pathways. Increases in membrane phospholipids with mechanical strain are associated with transient increases in Ca^{2+} levels, due to the interaction between Ca^{2+} and phospholipids. In chapter three, it has been shown that strain caused an increase in inositolphospholipid activation prior to any other pathway. Additional studies in the literature have reported a very rapid increase in intracellular Ca^{2+} levels after a single membrane stimulation (see Jones *et al.*, 1991 for a review), suggesting that in our studies this Ca^{2+} -phospholipid interaction may also be taking place. Such a relationship has long indicated the importance of Ca^{2+} -protein and Ca^{2+} -lipid-binding as shown by transitions between gel/sol states of the actin cytoskeleton altering the mechanical state of the cell (Janmey, 1994). There are many actin-binding proteins regulated by Ca^{2+} . These can be divided into filament-severing, filament end-blocking, F-actin side-binding or cross-linking, proteins that link F-actin to other structures and motor proteins (see Stossel, 1993 and Janmey, 1994 for a review). According to Janmey (1994), Ca^{2+} and phospholipids have opposite effects in the cell cytoskeleton. Ca^{2+} appears to

solate the cell cytoskeleton through specific protein binding, while phospholipids appear to block Ca^{2+} effects enabling a reorganization of the cytoskeleton. Similarly, PTH-induced Ca^{2+} increase has also been implicated in cytoskeletal disassembly (Egan *et al.*, 1991). Cytoskeleton assembly/disassembly is vital in processes such as cell migration and chemotaxis (Stossel, 1989; Stossel, 1993 for a review); processes closely related with changes induced by activation of Ca^{2+} channels. One may argue that Ca^{2+} channels are indeed mechanotransducers! However, as pointed out by Ingber (1993), simply because a biological mechanism can be explained by a single theory does not mean that such a mechanism is correct.

From chapters four to nine we have identified the integrin family of membrane receptors as prime candidates for the role of mechanotransducers. It must not be overlooked, however, that such a role may be by no means exclusive to integrins or to Ca^{2+} channels or to any other pathway that has not been examined here. As an example, the vitronectin receptor α subunit appears to contain multiple short sequence elements that are homologous to the Ca^{2+} binding elements of other proteins (Suzuki *et al.*, 1987). It has been shown that the α subunit of the platelet receptor gp IIb-IIIa is able to bind Ca^{2+} (Fitzgerald and Phillips, 1985). Moreover, it appears that the presence of Ca^{2+} is necessary for assembly of the receptor intracellularly (Fitzgerald and Phillips, 1985; Suzuki *et al.*, 1987), although these cations have not yet been shown to possess any regulatory role in integrin activity. This interaction is very interesting and rather complex, nevertheless, at this point integrins appear to stand out as strain transducers according to the classic definition by Watson (1991).

Implications of strain-induced response in integrin receptors and cytoskeleton

Many reports have shown an increase in cell growth with the application of mechanical forces (discussed in chapters three to nine). In fact, strain may stimulate cell growth by either an independent and/or a synergistic response to growth factors (Vandenburgh, 1992 for a review). Growth factors, as well as polypeptide hormones such

as PTH, modulate cell activity through specific receptor-mediated responses (Moolenaar *et al.*, 1986). Activated receptors propagate signals via an integration of cascade reactions of biochemical and physiological changes (Abdel-Latif, 1986). The mechanoreceptor hypothesis corresponds closely to the activation of specific receptors in growth factor-activated mechanisms (Ingber, 1991 for a review). Not only integrins behave as mechanotransducers by both biochemical signaling and structural stimulation of the cytoskeleton, but also it has been shown here that integrins respond selectively to strain. Therefore, it is not farfetched to hypothesize that integrins may perceive strain-stimulation in a fashion similar to growth factors. However, one may find it difficult to consider mechanical stimulation and integrins as having a true agonist-receptor relationship. Indeed, a wide range of mechanical stimulations affecting cell behavior and specificity of response may not always be possible from the extracellular environment. By contrast, nothing prevents intracellular signals from modulating membrane receptor activity (Ginsberg *et al.*, 1992). Such inside-out signaling is precisely what appears to take place in integrin-mediated cell signaling (Damsky and Werb, 1992; Ginsberg *et al.*, 1992; Sastry and Horwitz, 1993), as discussed in chapter seven.

Accessory protein binding play an important role in integrin subunit signaling. Examples have been described for both membrane-bound and cytoplasmic proteins. Brown *et al.* (44) sequenced a 50 kDa integrin-associated membrane protein required for enhancement of neutrophil phagocytosis and Hermanowsky-Vosatka *et al.* (1992) described a cytosolic low molecular weight lipid, called integrin modulating factor (IMF-1), which rises as a consequence of leukocyte activation to control cell adhesiveness (Damsky and Werb, 1992). Ridley and Hall (1992) have reported that the GTP-binding protein *rho* appears to regulate the assembly of stress fibers in focal contact sites. Actin polymerization changes induced by agonists such as insulin also takes place through *rho* or *rac* (Ridley *et al.*, 1992). A similar mechanism appears to be present in the activation of platelets through surface integrin-associated glycoproteins (Damsky and Werb, 1992; Clark and Brugge,

1993). However, the interaction of inositolphosphates with the cytoskeleton (Divecha and Irvine, 1995) demonstrates that signaling mechanisms can be directly associated with membrane receptors (Stephens *et al.*, 1993). Furthermore, integrin association with insulin receptor substrate (Vuori and Ruoslahti, 1994) clearly demonstrates that a cytoskeletal-mediated signaling has a synergistic growth factor effect. Integrins may represent a form of anchoring site for proteins and small enzymes, allowing their interaction with the respective substrates, thereby stimulating signaling cascades.

In chapters four to seven and nine, strain-induced integrin rearrangement has been examined. Although, we did not study dimerization properties of different subunits, we observed that application of strain may cause a change in spatial distribution of integrin receptors and/or a reorganization of different subunits, thus possibly creating a chimeric receptor. This so-called "new" receptor may only last during the onset of the stimulatory period. By comparison, cell stimulation in the form of heat shock has been shown to cause loss of immunoreactive integrins at the cell surface (Majda *et al.*, 1994). This phenomenon may also be explained by a change in the conformational shape of the receptor or by receptor shedding from the membrane, similar to that during remodeling and cell locomotion (Bretscher, 1986; Ginsberg *et al.*, 1992; Majda *et al.*, 1994). A temporary rearrangement of integrin subunits as a result of mechanical stimulation appears to reflect an increase in protein synthesis and/or metabolism. Although, in chapter four, we have found an increase in synthesis of most integrin subunits studied following strain application, it is puzzling why similar stimulation fails to increase mRNA expression in both β_1 and α_v in a manner similar to that of protein synthesis. Chapter seven described in detail the differences in mRNA expression for both subunits. A simple explanation may reside in the existence of a subcellular pool for α_v , similar to the one seen for β_1 subunit, even though experimental problems in protein synthesis studies cannot be discarded. In any case, it has been reported here for the first time, that mechanical stimulation caused an increase in the mRNA expression of specific integrin subunits. Such stimulation appears to be highly

selective and time-dependent. It is disappointing, however, the lack of information available on stimulation of integrin mRNAs from other studies, even though some comparisons can be drawn from reports showing a decrease in integrin mRNA levels in heart myocytes of normotensive rats following an increase in blood pressure (Mamuya *et al.*, 1992). Unfortunately, it was not possible to expand the observations found here with further experiments using additional integrin subunits. It may be anticipated that, similar to substrate-adhesion experiments, mRNA expression for different integrin subunits will respond in a strain-type and time dependent manner.

Each integrin heterodimer promotes somewhat different ligand binding specificities (Cheresh *et al.*, 1989). Nonetheless, the interaction of integrin subunits with the ECM modulates cell behavior and cytoskeleton organization (Romer *et al.*, 1992). Such interaction is central in integrin-mediated signal transduction following strain. Studies have shown that the cytoplasmic domain of β_1 integrin can bind to focal contact proteins like talin (Tapley *et al.*, 1989) and α -actinin (Otey *et al.*, 1990). In addition, the same domain promotes identification of integrins in focal contact areas co-localizing with proteins such as vinculin and tensin (Burrige *et al.*, 1988; Burrige *et al.*, 1992). This ability of the cytoplasmic domain of β_1 , and recently of β_3 integrins, to direct receptor localization has been further identified by using chimeric proteins lacking the presence of the extracellular ligand binding region of the receptor (La Flamme *et al.*, 1992; Yamada *et al.*, 1992). As a result the α subunit appears not to have sufficient information to characterize integrin distribution (Yamada *et al.*, 1992). This intracellular mechanism involving receptor distribution which is possibly controlled by agonist-receptor occupancy corroborates the hypothesis of strain-induced inside-out signaling (Ginsberg *et al.*, 1992). Characterization of integrin binding dynamics will not only clarify processes of signal transduction but also cell adhesion, cell migration and matrix assembly.

The areas of focal contacts are considered very important, especially as sites of signal transduction (Burrige *et al.*, 1988; Burrige *et al.*, 1992 for a review). In which

case, it was logical for us to study the effects of strain in these areas in addition to the underlying cytoskeleton. Application of mechanical strain in bone cells appears to cause an increase in focal contacts as well as a change in focal contact orientation. Strain changed the distribution (chapters three to five and eight) and synthesis (chapter four) of focal contact proteins. This was not surprising, since strain is capable of promoting a substantial increase in total protein synthesis (Komuro *et al.*, 1990; Sumpio *et al.*, 1990, Komuro *et al.*, 1991) and a considerable degree of cytoskeleton remodeling (Terracio *et al.*, 1989; Pender and McCulloch, 1991; Simpson *et al.*, 1994). In muscle cells, both synthesis and degradation of total protein appear to be elevated (Simpson *et al.*, 1994). However, the result is a net growth since protein synthesis is elevated to a greater degree (Laurent and Millward, 1980; Simpson *et al.*, 1994). There is little information on protein synthetic activity in strain-induced bone cells. Buckley *et al.* (1990) showed that strain increased both collageneous and non-collageneous proteins, including vinculin, in osteoblasts. This agrees with our findings. Focal contact and actin-binding proteins, such as α -actinin, filamin and talin, have been shown to bind to phosphorylated phosphatidylinositol pospholipids (Janmey, 1994). Consequently, it can be suggested that an increase in cellular focal contact areas (as observed from studies in chapters three, five and eight) will potentially translate into a more efficient signaling pathway from membrane receptors to cell cytoskeleton, both through chemical and mechanical signals.

Regulation of cytoskeletal proteins is fundamental in determining the shape and function of cells (Ben Ze'ev, 1991 for a review). Changes in cell shape have been considered as potential regulators of transduction signals (Hong and Brunette, 1987; Watson, 1991; Oakley and Brunnete, 1993; Sandy *et al.*, 1993). Application of strain has clearly been shown to change bone cell shape in this study. However, a potential pitfall of cytoskeletal staining here has been the study of isolated cells, as opposed to confluent cultures. In addition, such an approach does not account for the presence of a strain-stimulated cellular response being transient or too short to detect. Furthermore, interactions

of nucleus-cytoskeleton may be continuous with cytoskeletal-ECM and to neighboring cells *in vivo* (Pienta and Coffey, 1991 for a review). Such relationships potentially play an important role in cell orientation and cell polarity. Nevertheless, as structural interactions determine cell shape, we believe that the combination of stimulated cytoskeletal components, focal adhesion proteins and membrane signaling pathways systematically work together to translate mechanical strain into strain-induced cell response. The concept of "tensegrity" has been suggested to explain the interaction of all these mechanisms. Components of the cytoskeleton network may be divided into contractile, elastic or noncompressible proteins (Watson, 1991). Tensegrity is but the balance between contraction, resistance of contractile microfilaments and compression-resistant microtubules, respectively (Pienta and Coffey, 1991; Watson, 1991; Ingber, 1993). This model provides some attractive features to explain the equilibrium amongst cytoskeletal components, cell shape and cellular signaling. Currently, more work is needed to fully characterize this or other models of strain-directed cellular function and response.

The cellular cytoskeleton is a remarkable and complex three-dimensional structure participating in the regulation of many vital cellular processes. A complete description of cytoskeleton involvement in cellular functions is beyond the scope of this discussion, nonetheless, this system determines structural positionings of many intracellular components, including that of the nucleus. Nuclear positioning has been shown to possess essential functions in the regulation of protein synthesis and gene expression (Russel and Dix, 1992). This positioning is partly regulated by the binding of the cytoskeleton to the nuclear membrane (Berezny, 1991; Bissel *et al.*, 1992). In chapter nine we have discussed a novel approach to nuclear signal transduction following mechanical stimulation. This is an active area of study, and future investigations will clarify the role of strain and those of the cytoskeletal scaffolding in the modulation of regulatory proteins in gene expression.

In conclusion, combinations of signaling pathways, receptors and cytoskeletal components involve a series of chemomechanical reactions within a mechanically

stimulated cell environment. Models have been suggested to explain the coordination of these chemomechanical responses. However, many questions in strain-induced cell response remain, and new information will undoubtedly stimulate innovative ideas for the transfer of information, cell activity and cell functions in cell biology of mechanically strained cell systems.

REFERENCES

- Abdel-Latif AA 1986 Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacol Rev* 38:227-272.
- Alcon D and Rasmussen H 1988 A spatial-temporal model of cell activation. *Science* 239:998-1004.
- Ben Ze'ev A 1991 Animal cell shape changes and gene expression. *BioEssays* 13:207-211.
- Berezny R 1991 The nuclear matrix: a heuristic model for investigating genomic organization and function in the cell nucleus. *J Cell Biochem* 47:109-123.
- Bissel MJ, Hall HG and Parry G 1982 How does extracellular matrix direct gene expression? *J Theor Biol* 99:31-68.
- Bretscher A 1986 Microfilament structure and function in the cortical cytoskeleton. *Annu Rev Cell Biol* 7:337-374.
- Brown E, Hooper L, Ho T and Gresham H 1992 Integrin associated protein: a 50 kD plasma membrane antigen physically and functionally associated with integrins. *J Cell Biol* 111:2785-2794.
- Buckley MJ, Banes AJ and Jordan RD 1990 The effect of mechanical strain on osteoblasts *in vitro*. *J Oral Maxillofac Surg* 48:276-282.
- Burridge K, Fath K, Kelly T, Nuckolls G and Turner C 1988 Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Ann Rev Cell Biol* 4:487-525.
- Burridge K, Nuckolls G, Otey C, Pavalko F, Simon K and Turner C 1990 Actin-membrane interactions in focal adhesions. *Cell Differ Dev* 32:337-42.
- Charles AC, Merrill JE, Dirksen ER and Sanderson MJ 1991 Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* 6:983-992.

- Cheresh DA, Smith JW, Cooper HM and Quaranta V 1989 A novel vitronectin receptor integrin ($\alpha v \beta x$) is responsible for distinct adhesive properties of carcinoma cells. *Cell* **57**:59-69.
- Clark EA and Brugge JS 1993 Redistribution of activated pp60c-src to integrin-dependent cytoskeletal complexes in thrombin-stimulated platelets. *Mol Cell Biol* **13**:1863-1871.
- Damsky C H and Werb Z 1992 Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* **4**:772-781.
- Divecha N and Irvine RF 1995 Phospholipid signaling. *Cell* **80**:269-278.
- Egan JJ, Gronowicz G and Rodan GA 1991 Parathyroid hormone promotes the disassembly of cytoskeletal actin and myosin in cultured osteoblastic cells: mediation by cyclic AMP. *J Cell Biochem* **45**:101-111.
- Fitzgerald LA and Phillips DR 1985 Calcium regulation of the platelet membrane glycoprotein IIb-IIIa complex. *J Biol Chem* **260**:11366-11374.
- Gilbert JA, Weinhold PS, Banes AJ, Link GW and Jones GL 1994 Strain profiles for circular cell culture plates containing flexible surfaces employed to mechanically deform cells *in vitro*. *J Biomech* **27**:1169-1177.
- Ginsberg MH, Du X and Plow EF 1992 Inside-out integrin signaling. *Curr Opin Cell Biol* **4**:766-771.
- Heramnowsky-Vosatka A, Van Strijp JAG, Swiggard WJ and Wright SD 1991 Integrin modulating factor-1: a lipid that alters function and leukocyte integrins. *Cell* **68**:341-352.
- Hong HL and Brunette DM 1987 Effect of cell shape on proteinase secretion by epithelial cells. *J Cell Sci* **87**:259-267.
- Ingber DE 1991 Integrins as mechanochemical transducers. *Curr Opin Cell Biol* **3**:841-848.

- Ingber DE 1993 Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* **104**:613-624.
- Janmey PA 1994 Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu Rev Physiol* **56**:169-191.
- Jones DB, Nolte H, Scholübbbers JG, Truner E and Veltel D 1991 Biochemical signal transduction of mechanical strain in osteoblast-like cells. *Biomaterials* **12**:101-110.
- Komuro I, Katoh Y, Kaida T, Shibazaki I, Kurabayashi M, Hoh E, Takaku F and Yazaki Y 1990 Stretching cardiac myocytes stimulates protooncogene expression. *J Biol Chem* **265**:3595-3598.
- Komuro I, Katoh Y, Kaida T, Shibazaki I, Kurabayashi M, Hoh E, Takaku F and Yazaki Y 1991 Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. *J Biol Chem* **266**:1265-126.
- La Flamme SE, Akiyama SK and Yamada KM 1992 Regulation of fibronectin receptor distribution. *J Cell Biol* **117**:437-447.
- Laurent GJ and Millward DJ 1980 Protein turnover during skeletal muscle hypertrophy. *Fed Proc* **39**:42-47.
- Majda JA, Gerner EW, Vanlandingham B, Gehlsen KR and Cress AE 1994 Heat shock-induced shedding of cell surface integrins in A549 human lung tumor cells in culture. *Exp Cell Res* **210**:46-51.
- Mamuya W, Chobanian A and Brecher P 1992 Age-related changes in fibronectin expression in spontaneously hypertensive, Wistar-Kyoto, and Wistar rat hearts. *Circ Res* **71**:1341-1350.
- Moolenaar WH, Defize LHK and Laat SW 1986 Calcium in the action of growth factors. *Ciba Found Symp* **122**:21-231.
- Oakley C and Brunette DM 1993 The sequence of alignment of microtubules, focal contacts and actin filaments in fibroblasts spreading on smooth and grooved titanium substrata. *J Cell Sci* **106**:343-354.

- Otey CA, Pavalko FM and Burrige K 1990 An interaction between α -actinin and the β_1 -subunit *in vitro*. *J Cell Biol* **111**:721-729.
- Pender N and McCulloch AG 1991 Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching. *J Cell Sci* **100**:187-193.
- Pienta KJ and Coffey DS 1991 Cellular harmonic information transfer through a tissue tensegrity-matrix system. *Med Hypoth* **34**:88-95.
- Ridley AJ and Hall A 1992 The small GTP-binding protein rho regulates the assembly of focal contact adhesions and actin stress fibers in response to growth factors. *Cell* **70**:389-399.
- Ridley AJ, Paterson HF, Johnston CL, Diekmann D and Hall A 1992 The small GTP-binding protein rac regulates growth-factor induced membrane ruffling. *Cell* **70**:401-410.
- Romer LH, Burrige K and Turner CE 1992 Signaling between the extracellular matrix and the cytoskeleton: tyrosine phosphorylation and focal adhesion assembly. In: Cold Harbor Symposia on Quantitative Biology. Volume 57, Cold Spring Harbor Laboratory Press, pp. 193-202.
- Russel B and Dix DJ 1992 Mechanisms for intracellular distribution of mRNA: *in situ* hybridization studies in muscle. *Am J Physiol* **262**:1-8.
- Sastry SK and Horwitz F 1993 Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr Opin Cell Biol* **5**:819-831.
- Simpson DG, Carver W, Borg TK and Terracio L 1994 Role of mechanical stimulation in the establishment and maintenance of muscle differentiation. *Int Rev Cyto* **150**:69-94.
- Sandy JR, Farndale RW and Meikle MC 1993 Recent advances in understanding mechanically induced bone remodeling and their relevance to orthodontic theory and practice. *Am J Orthod Dentofac Orthop* **103**:212-222.

- Stephens LR, Jackson TR and Hawkins PT 1993 Activation of phosphatidylinositol 4,5-bisphosphate supply by agonists and non-hydrolysable GTP analogs. *Biochem J* 296:481-488.
- Stossel TP 1989 From signal to pseudopod: how cells control cytoplasmic actin assembly. *J Biol Chem* 264:18261-18264.
- Stossel TP 1993 On the crawling of animal cells. *Science* 260:1086-1094.
- Sumpio DE, Banes AJ, Link GW and Iba T 1990 Modulation of endothelial cell phenotype by cyclic stretch: inhibition of collagen production. *J Surg Res* 48:415-420.
- Suzuki S, Argraves W, Arai H, Languino L, Pierschbacher M and Ruoslahti E 1987 Amino acid sequence of the vitronectin α subunit and comparative expression of adhesion receptor mRNAs. *J Biol Chem* 262:14080-14085.
- Tapley P, Horwitz A, Buck C, Duggan K and Rohrschneider L 1989 Integrins isolated from Rous sarcoma virus-transformed chicken embryo fibroblasts. *Oncogene* 4:325-333.
- Terracio L, Gullberg D, Rubin K, Craig S and Borg TK 1989 Expression of collagen adhesion proteins and their association with the cytoskeleton in cardiac myocytes. *Anat Rec* 223:62-71.
- Vadiakas GP and Banes AJ 1992 Verapamil decreases cyclic load-induced calcium incorporation in ROS 17/2.8 osteosarcoma cell cultures. *Matrix* 12:439-447.
- Vandeburgh HH 1992 Mechanical forces and their second messengers in stimulating cell growth *in vitro*. *Am J Physiol* 262:350R-355R.
- Vuori K and Ruoslahti E 1994 Association of insulin receptor substrate-1 with integrins. *Science* 266:1576-1578.
- Watson PA 1991 Function follows form: generation of intracellular signals by cell deformation. *FASEB J* 5:2013-2019.

- Yamada KM, Aota S, Akiyama SK and LaFlamme SE 1992 Mechanisms of fibronectin and integrin function during cell adhesion and migration. In: Cold Harbor Symposia on Quantitative Biology. Volume 57, Cold Spring Harbor Laboratory Press, pp. 203-212.
- Yamaguchi D, Hahn T, Lida-Klein A, Kleeman C and Mualem S 1987 Parathyroid hormone activated calcium channels in an osteoblast-like clonal osteosarcoma cell line UMR-106. J Biol Chem 262:7711-7718.



Chapter ELEVEN

Future Directions



FUTURE DIRECTIONS

Many are the biological processes that appear to mediate and be modulated by mechanical stimulation-induced cell response. Therefore, only a sample of possible future research can be included here. A logical priority would be an attempt to study some of the concepts described herein with another system capable of reproducing a more favorable physiological type of strain. In addition, it would be beneficial to study a system that took into account the micro environment of cell boundary stimulation. The processes of bone formation can also be further characterized with an in depth histological analysis of bone nodules formed *in vitro*. This approach would put to rest many criticisms on strain levels and on the significance of cell culture models to study strain-induced cell response.

Due to a vast array of messengers and signaling pathways, it has been impractical to include more pathways in our investigations. However, many mechanisms are still largely unknown in bone cells stimulated by strain. Additional studies on mechanically-stimulated protein synthesis are also warranted. Specifically, different stimulation periods could be used to investigate the timing of focal contacts and cytoskeletal rearrangement. The preliminary information reported here in strain-induced protein reorganization is somewhat qualitative. The use of more innovative techniques will determine the interaction of microfilament, intermediate filaments and microtubules with mechanical strain and greatly enhance the precise determination of important factors such as protein distribution, cell polarity, cell orientation, protein spatial and temporal organization, nuclear matrix distribution and others. To that end techniques such as fluorescence activated cell sorting could be used. Furthermore, it is necessary to examine the role of Ca^{2+} in actin-dependent cytoskeleton assembly. Individual Ca^{2+} dependent proteins, such as gelsolin and filamin, can be studied to determine their role in strain-induced cytoskeletal rearrangement. For instance, by inhibiting the activity of these and other cytoskeletal proteins that specialize in actin-filament severing, one may gain valuable insights on how strain may determine the

process of cytoskeletal rearrangement. Similarly in studies of protein synthesis, timing can also be improved for mRNA analysis in strained cells.

The importance of integrin modulated signaling transduction pathways in strained cells has been extensively discussed. Thus, it would be valuable to look into the role of strain in phosphorylation studies of specific integrins, actin-binding and membrane-binding proteins. In particular, how these proteins may enhance signal transduction mechanisms of bone response. Molecular analysis could be used to study the cytoskeletal protein described here, integrin subunits not discussed here and other cellular receptors such as cadherins, selectins and members of the immunoglobulin superfamily which likely play a role in the processes of mechanically stimulated responses. Our and other models on the molecular strain-induced cellular response could be further investigated by using gel shift, footprinting and run on assays. Finally, the use of transgenic animals could be used to study the role of defined molecular cellular components in regulating strain-induced signal transduction.