THE DEVELOPMENT OF AN INTERMITTENT AND CONTINUOUS LOADING MODEL FOR SUTURAL CELLS IN VITRO; COLLAGEN PHENOTYPE RESPONSE

BY

WILLIAM ALEXANDER WHYTE

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ABSTRACT

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The macroscopic and microscopic changes associated with orthodontic tooth movement have been extensively investigated and reported (Reitan, 1985; Storey, 1973; Rygh, 1986), unlike the underlying biologic processes and control mechanisms that produce the remodelling which is required for orthodontic tooth movement and craniofacial orthopaedic change. The purpose of the present investigation was to develop an in vitro model that would enable intermittent or continuous loading of sutural cells and allow the subsequent metabolic remodelling response to be investigated.

Collagen is the primary structural protein within the sutural connective tissues, that remodel during orthodontic tooth movement, and changes in the ratio of collagen phenotypes have been demonstrated to fluctuate with the remodeling activity of these tissues (Meikle et al., 1982; Yen et al., 1989b). This is especially true with respect to type III collagen, thus the proportion of type III collagen synthesized can be used as a biologic indicator of the remodelling response of these tissues.

A model for producing intermittent and continuous stress was developed. Using this model experiments were performed in which 5 week old male Sprague-Dawley rat interparietal sutural fibroblasts were subcultured into flexible bottomed 50 mm round petriperm dishes and when confluent the cell layer was stressed by centering the dishes upon convex glass domes and then resting a 670 gm weight on top of the petriperm culture plate. Three groups were identified based on differing patterns of stress application: a nonstressed control group, as well as continually and intermittently (1 hour on/1 hour off) stressed experimental groups.

A photographic investigation of the model, using low power phase contrast microscopy, suggested that the tissue layer undergoing stress application, was not torn or detached from the petriperm dish bottom. Further, using this model a preliminary study of the influence of stress on the collagenous and noncollagenous protein synthesis revealed no significant changes in the ratio of collagenous protein synthesis to noncollagenous protein synthesis, total collagenous protein synthesis or noncollagenous protein synthesis due to varying stress patterns over a 12 hour period.

In an investigation of the effect of mechanical stress on the collagen phenotype synthesis. Data was collected at 0, 2, 4, 8 and 12 hours following stress application. It was observed that the ratio of type III collagen to total collagen synthesis was significantly increased in the experimental groups during the 2 to 8 hour interval. At 12 hours only the intermittently stressed group maintained an increased proportion of type III collagen synthesis.

It was concluded that tissue stretching signals the in vitro fibroblast cultures to remodel, and that intermittent stress maintains the remodelling response longer than continuous stress. The effects of intermittent or continuous loading on the tissue culture, and the overall collagenous and noncollagenous protein synthesis, require further investigation.

This Work Is Lovingly Dedicated To My Family

My wife and parents for their love, support and prayers

My wife and children for their patience

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INTRODUCTION

INTRODUCTION

The clinical practise of orthodontics is largely based on empirical knowledge. For the most part, research efforts have been clinically based - improved appliances, "ideal" force levels or systems delivered by appliances or superior diagnostic techniques. Traditionally the biology of orthodontics has been speculative or directed at the gross and histologic morphologic changes of the periodontium associated with the remodelling and iatrogenic processes brought about by orthodontic and facial orthopaedic therapy. To a lesser degree orthodontic research has attempted to uncover the biologic mechanisms of tooth movement (or more generally the process of fibrous suture remodelling).

Remodelling of the fibrous sutures, periodontal ligament (PDL) and craniofacial sutures, facilitates orthodontic tooth movement and facial orthopaedic change. In clinical practise, mechanically generated force systems are directly applied to the teeth, and to a limited degree the surrounding periodontium, via orthodontic appliances. The forces on the teeth are transmitted to a fibrous joint(s), the periodontal ligament, where the mechanical signal is transduced into a biologic signal that conveys information to the cells. This information results in intracellular changes that instruct the cells to begin the remodelling process. In situations where the force is of sufficient magnitude to cause ischemic destruction of the PDL, this message is also directed to the

marrow space cells along the endosteal surface of the alveolar bone that borders this fibrous joint.

The histologic remodelling changes that are present in the PDL during orthodontic force application (for review, see Reitan, 1985; Storey, 1973; Rygh, 1986; and Roberts et al., 1981) and with respect to the sutural response to orthopaedic stress (Cleall et at., 1965; Murray and Cleall, 1971; Moffett, 1971, 1973; Droschl, 1975; Linge, 1972, 1976) have been well documented. In addition, remodelling in craniofacial sutures at a distance from the maxillary dentition, to which traction has been applied, has been demonstrated histologically (Droschl, 1973).

The biologic pathways and cellular control mechanisms for remodelling of the PDL and suture are still obscure. The mechanisms converting an external mechanical stimulus into extracellular, and subsequently intracellular, biologically active compounds are unknown. Undoubtedly the remodelling process involves a multifaceted cascade of events that are initiated, directed and coordinated by biologic signals that possibly involve cell surface receptor proteins, membrane-bound enzymes, physical perturbation or cell shape change, altered ion concentrations, feedback pathways and cellularly released agents (eg. chemotactic agents), as well as, many other biologically active compounds. These signals indirectly produce the necessary changes required to initiate and successfully complete the tissue remodelling (eg. cell

migration and recruitment, cell differention, altered cellular metabolism, tissue removal and tissue production) and restore homeostasis.

The predominant component of all fibrous sutures is the structural protein collagen. Central to a greater understanding of sutural remodelling (and thus the biology of orthodontics) is the elucidation of collagen metabolism during remodelling.

The subsequent review will discuss the major soft tissue components of the fibrous suture, collagen metabolism, theories of tooth movement and the biologic sequelae resulting from force application to sutural joints (and similar tissue).

REVIEW OF THE LITERATURE

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COLLAGEN

Connective tissues are characterized by a production of an extracellular matrix containing fibrous and amorphous substances, of which collagen is the main component (Kivirikko and Risteli, 1976; Bornstein and Traub, 1979). Collagen, is a fibrous material synthesized by connective tissue cells (fibroblasts, chondroblasts, osteoblasts and odontoblasts) and to a limited extent by the epithelial cells involved in secreting the basement membrane protein (Barbanell et al., 1978). Collagen, the most abundant structural protein of the body, is a family of proteins which comprise about one quarter of the total body weight (Parry and Craig, 1988).

The relative proportion of cells to its matrix reflects the specialized function of a particular connective tissue (Hall, 1978a, 1978b). In addition, it has been observed that the fiber patterns may vary within the sutural connective tissue depending on the localized demands of that tissue (Persson, 1973; Koskinen et al., 1976). This demonstrates a single suture's ability to respond to mechanical stresses, of differing magnitude and vector, in different areas (Nanda, 1978).

Collagen is responsible for structural stability and compartmentalization of the major organ systems. Secondly, it is believed to play a vital role in cell-matrix interactions

leading to tissue differentiation (Shekhter, 1986; Amenta et al., 1986).

COLLAGEN STRUCTURE

The tropocollagen molecule is the simplest unit of the extracellular collagen fiber. It is approximately 1.5nm in diameter and 280 to 300nm in length with a molecular weight of 300,000 (Nimni, 1980; Miller, 1988; Alberts et al., 1989). This molecule consists of three polypeptide alpha-chains, each coiled in a left-handed minor helix due to the frequent presence of the cyclic imino acids, proline and hydroxyproline. The alpha-chains are intertwined into a single right-handed rod-like super helix (Gay and Miller, 1978).

The alpha-chains are comprised of approximately 1052 sequential amino acid residues in a repeating triplet, $(gly\text{-}X\text{-}Y)_n$, which consists of glycine, the smallest amino acid, and two other amino acids (Barbanell et al., 1978; Bansal et al., 1975). The Y position is usually occupied by proline and hydroxyproline, the latter is unique to collagen (Cheah, 1985). Glycine represents one third of the amino acid residues in collagen while proline and hydroxyproline make up about one quarter of the remaining amino acid residues. (Due to their ring structure, prolyl and hydroxy prolyl residues, stabilize the left-handed helical arrangement of the polypeptide chain with three residues per turn.) Nine percent

of the collagen residues are alanine, the most common amino acid found in the X position, while lysine and hydroxylysine contribute 1.3% of the amino acids in collagen and are important in the formation of intermolecular linkages.

Changes in hydroxyproline content in urine is an indication of the status of collagen metabolism (Prockop, 1979a). This amino acid stabilizes the collagen triple helix (Jimenez et al., 1973; Pinnell et al., 1987) and it's absence produces a structurally unstable collagen (Berg and Prockop, 1973) with an altered rate of cellular secretion.

COLLAGEN BIOSYNTHESIS

In the extracellular matrix, collagen is continuously being turned over and being replaced by newly secreted collagen. The amount of collagen secreted is the net result of intracellular collagen synthesis and intracellular collagen degradation. It has been reported that 20 to 40% of newly synthesized procollagen is degraded intracellularly (Bienkowski et al., 1978a, 1978b). Although it would appear to be inefficient to degrade newly synthesized collagen molecules, this may be a way to allow for rapid adjustments in collagen production. In such a system the activation or inhibition of existing metabolic pathways modulates the amount and type of collagen production without requiring a significant lag phase during which these pathways are formed de novo. Additionally the degradation may be a method of quality control since it has been shown to recognize and

selectively destroy structurally aberrant procollagen molecules (Rennard et al., 1982).

The biosynthesis of a collagen molecule involves at least 8 distinct enzymes, 5 intracellularly and 3 extracellularly. In general, collagen synthesis involves a process similar to other secretory proteins (for review, see Palade, 1975; Farquhar and Palade, 1981), however it is unique in its post-translational modifications of the primary molecule (Barbanell et al., 1978; Minor, 1980). It is synthesized by membrane-bound ribosomes and the resultant polypeptide has a signal sequence at its amino terminus.

Initially, in the cell nucleus, as a result of some biologic signal, heterogeneous nuclear RNA (hnRNA) is transcribed from the appropriate complimentary DNA. Small nuclear RNAase removes the introns to produce messenger RNA (mRNA) which is further modified by complexing with two proteins. The processed mRNA exits the nucleus through a nuclear pore.

Once in the cytoplasm, the mRNA is translated by polysomes (multiple ribosome complexes) located on the external surface of the endoplasmic reticulum (McGilvery, 1983). The result is a transcribed polypeptide chain which is transported within the lumen of the rough endoplasmic reticulum (RER) to the Golgi body where it is packaged for secretion into condensing granules and secretory vesicles (Fessler and Fessler, 1978; Bornstein and Traub, 1979;

Weinstock and Leblond, 1974; Karim et al., 1979). During this phase the polypeptide undergoes post-translational modification (Barbanell et al., 1978; Minor, 1980) involving the removal of the signal polypeptide, hydroxylation of specific proline and lysine residues, glycosylation of specific hydroxylysine residues, pro-alpha chain association, intra- and inter-chain disulphide bond formation, as well as the formation of an overall triple helical structure. This is now a procollagen molecule, also referred to as soluble or transport collagen, which is secreted into the extracellular space.

The hydroxylation of proline and lysine is dependent on the presence of ascorbic acid (Levene et al., 1972; Murad et al., 1981; Ten Cate et al., 1976). (In vitro even in the absence of ascorbic acid, collagen is still produced and secreted by fibroblasts, however there is a significant decrease in hydroxylation (Peterkofsky, 1972).

Procollagen precursors, of collagen phenotypes I, II and III, are converted to insoluble tropocollagen by procollagen peptidase mediated cleavage of the non-helical propeptides at the amino termini (Berger et al., 1985; Tanzawa et al., 1985). This enzymatic removal of the procollagen peptides is thought to occur in a series of stages (Hall, 1976) and that partial retention of the procollagen sequence temporarily distinguishes the immature collagen by preventing it from

fully aligning in a manner typical of mature collagen. The conversion to mature collagen is time dependent.

It appears as though helical portions of the new tropocollagen molecules bind to the cell membrane (Goldberg and Burgeson, 1982) and aggregate by means of a cell-mediated multi-step process (Trelstad, 1982) to form collagen fibrils. The fibril formation results in physiologically useful collagen. Type I collagen efficiently converts from procollagen to tropocollagen while type III fibrillar structures retain portions of the procollagen sequences for longer periods of time. This may render type III collagen more susceptible to extracellular degradation (Miller et al., 1976).

Collagen fibrils are about 10 to 300nm in diameter and have the characteristic crossbanding with a major periodicity of 66.8nm, and finer bands between the major bands visible with electron microscopy. Over time inter- and intra-molecular cross-links form resulting in increased tensile strength. Fibrils continue to aggregate producing a collagen fiber (Prockop et al., 1979a,b).

Collagen molecule cross-links form extracellularly after initial fibrillar organization (Tanzer, 1973; Siegel, 1979). The conversion of select amino groups to aldehydes is important in the process of cross-link formation.

As the tropocollagen molecules mature, covalent linkages form and serve to augment the early non-covalent and hydrogen

bond cross-linkages (Bailey et al., 1974). The covalent cross-links become more stable as collagen matures (Bentley, 1979). The cross-linking is important for optimal functioning and may also serve to regulate the rate of in vivo catabolism (Harris and Farrell, 1972; Yamauchi et al., 1988).

Selective gene expression, control over the process of polypeptide cleavage, varying the amount and nature of the noncollagen matrix molecules secreted and alterations in the amount of collagen cross-linking have all been proposed as mechanism of cellular control over collagen fibril size and structure (Alberts et al., 1989). Further, in vitro, fibroblasts have been demonstrated to physically move and compact collagen into cables and sheets (Stopak and Harris, 1982).

COLLAGEN DEGRADATION

Although the body's total amount of collagen is stable throughout life (Lovell et al., 1987), collagen can be rapidly synthesized or degraded depending on the tissue requirements of external stimuli. This is essential for rapidly remodelling tissues.

Collagen is degraded by several different mechanisms. Shortly after translation 20 to 40% of newly synthesized procollagen is degraded within the cell (Bienkowski et al., 1978b). There are two major modes of intracellular degradation - basal and enhanced (Bienkowski, 1983, 1984; Berg et al., 1980). Basal degradation is believed to occur between

the endoplasmic reticulum and the Golgi apparatus, whereas enhanced degradation involves the lysosomal system (Berg, 1986).

The degradation of de novo collagen is presumed to be a mechanism by which the cell destroys abnormal molecules (Goldberg and St. John, 1976), selects for collagen types by controlling the ratio of interstitial collagen chains available (Bienkowski, et al., 1978a) and rapidly modulates the amount of collagen being produced.

Once extracellular collagen is cross-linked it is largely resistant to degradation by proteolytic enzymes. The peptide bonds of the collagen are protected from enzymatic attack, and subsequent denaturation, by the cross-linkages of the fibrils and triple helical arrangement of the collagen molecule.

Collagenase, a metalloproteninase, is responsible for extracellular collagen homeostatic and remodelling turnover (Gross, 1976; Gross and Nagar, 1965; Woolley and Evanson, 1980). Collagenases are produced by fibroblasts, PMN leukocytes and macrophages (Strickling et al., 1977) and is produced by cells of the periodontal ligament (Christner, 1980). It is a second method of collagen degradation.

This family of enzymes attacks collagen at specific sites on the molecule, thus splitting it into segments which denature (Harris, 1978; Jeffrey, 1986). The denatured chains are digested by proteolytic enzymes (Weiss, 1976).

Collagenase degradation's selectivity for specific collagen types has been questioned (McCroskery et al., 1975; Miller et al., 1976). Despite this, Horwitz et al. (1977) reported that during the inflammatory process type I collagen degradation is proportionally greater than type III degradation. This is suggested to be due to polymorphonuclear leukocyte collagenase selectively degrading type I collagen and the result is an increase in the relative amount of type III collagen.

Trypsin has been demonstrated to favour type III collagen degradation (Miller et al., 1976) however, since trypsin is not found in the extracellular matrix in vivo it is not a physiologic consideration. It may indicate type III collagen's preferential susceptibility to other proteases.

The third method of collagen degradation is via phagocytosis (Ten Cate and Deporter, 1975). It has been observed that fibroblasts can phagocytose enzymatically cleaved collagen fiber segments and subsequently degrade collagen within phagolysosomes (Melcher and Chan, 1981; Rose et al., 1980). This intracellular degradation has been seen in the PDL of mice (Deporter and Ten Cate, 1973; Beertsen, 1987), rats (Garant, 1976) and monkeys (Svoboda et al., 1979); the gingiva of mice (Ten Cate and Syrbu, 1974) and man (Yajima and Rose, 1977); and cranial sutures in mice (Ten Cate et al., 1976).

Beertsen (1987) believes that the collagen phagocytosis is related to the rate of collagen turnover. During hypofunction, there is a net loss of extracellular collagen fibrils due to two-fold increase in its cellular ingestion. It appears that during orthodontic tooth movement that PDL fibroblasts can be stimulated to become phagocytic (Ten Cate et al., 1976). This may reveal a method by which an external stimulus may control collagen metabolism.

COLLAGEN PHENOTYPE

Identification

Collagens are a family of structural proteins with at least twelve structurally and genetically distinct phenotypes identifiable by the arrangement of the differing alpha-chains (Tanzer, 1988). This diversity reflects the varying functional requirements of collagen.

Roman numerals are used to designate the collagen phenotypes, eg. type I collagen (Cheah, 1985). There are various methods for identifying collagen types and their proportion within a tissue.

One method utilized cyanogen bromide to breakdown the polypeptide chains at the methionine residues or at protease specific sites along the collagen molecule, followed by sodium dodecylsulphate-polyacrylamide gel (SDS-Page) electrophoresis (Bonner and Laskey, 1974; Dunbar, 1987). Each polypeptide chain is separated according to its molecular weight and net charge. Particular significance is given to the delayed

addition of beta-mercaptoethanol to reduce the disulfide bonds present in type III collagen (Chung and Miller, 1974). Sykes et al. (1976) described this method as a way to separate the $alpha_1$ (III) chains from the $alpha_1$ (I) chains during electrophoresis and this results in the typical 'peptide maps' that are specific for each chain (Bornstein and Traub, 1979).

A second method involves separating the collagen types based on size by chromatographic and electrophoretic techniques (Miller, 1976). Additional methods make use of protease digestion (Sage and Bornstein, 1979), comparison of molecular weights (Dunbar, 1987) and characterization of noncollagenous sequences (Anesey et al., 1975).

Distribution and Physiology

It appears as if none of the collagen types are exclusive to any one tissue, however the proportion of the types does appear to reflect differing functional requirements of specific anatomical locations (Bornstein and Sage, 1980).

Type I collagen represents about 90% of human body collagen and is found in skin, tendon, bone, cornea, placenta, intervertebral disc, dentine, gingiva and the PDL. It is the only type identified in mineralized tissue. It has low levels of hydroxylysine and carbohydrate content.

The primary structure of this phenotype is consistent in all tissues, however the collagen fibers may differ in mechanical properties based on varying post-translational modifications (Barbanell et al., 1978). In bony tissue there

is a higher hydroxylysine content and therefore more cross-linking leading to tightly packed collagen fibrils (Stoltz et al., 1973) giving this tissue greater tensile strength and stability.

The type I collagen three polypeptide chains are comprised of two identical [alpha$_1$ (I)] chains and one [alpha$_2$ (I)] chain. A variant, type I trimer has three identical chains ([alpha$_1$ (I)]$_3$) and is found in diseased gingiva (Narayanan and Page, 1983a), as well as skin, cartilage, aged chondrocytes and fibroblasts cultured in vitro (Minor, 1980).

In mouse periodontal explants, in organ culture, under conditions of low PO_2, the ratio of alpha$_1$ (I) to alpha$_2$ (I) was as high as 6:1, indicating the synthesis of inordinate amounts of type I trimer (Yen and Melcher, 1978). It seems that type I trimer synthesis may be indicative of abnormal conditions (Mayne et al., 1976; Minor, 1980; Nimni, 1974; Narayanan et al., 1980), however it has also been detected in healthy tissue. The synthesis of type I trimer persists through several generations of cultured fibroblasts, possibly suggesting a genetically stable clonal cell population (Narayanan and Page, 1983a and 1983b).

Three identical alpha$_1$ (II) chains make up type II collagen and it is written as [alpha$_1$ (II)]$_3$ (Miller, 1972; Bornstein and Sage, 1980; Minor, 1980). Its alpha chains differ considerably in amino acid composition and sequence

from $alpha_1$ (I). It is high in hydroxylysine and carbohydrates (Alberts et al., 1989).

Type III collagen is comprised of three identical alpha chains ($[alpha_1 (III)]_3$) and contains the highest levels of glycine; it is high in hydroxyproline; it is low in hydroxylysine and carbohydrates (Alberts et al., 1989). This collagen type has interchain disulphide bonds near the carboxyl terminals of the alpha chains that are absent in type I and III (Bornstein and Traub, 1979). Type I and III collagen co-exist in a variety of tissues including skin, lung, heart, uterus, nerve, liver, placenta, umbilical cord, spleen, intestine, kidney, lymph nodes, the dental papilla and pulp, and the periodontal ligament (Bornstein and Sage, 1980).

The proportion of type I is usually much greater than type III (Epstein and Munderloch, 1975), however the exact ratio varies with tissue type (Butler et al., 1975a,b; Sodek and Limeback, 1979), age (Epstein, 1974), altered metabolic states (Narayanan et al., 1980, 1983; Narayanan and Page, 1983a), granulation tissues during wound healing (Miller, 1976; Gay et al., 1978; Gomez et al., 1989), and during periodontal and sutural remodelling subsequent to the application of mechanical force (Yen et al., 1980; Chiang, 1981; Yue, 1984).

Although type III collagen represented about 15% of the in vivo collagen in the PDL, it accounted for 20% of that produced by PDL fibroblasts in vitro (Limeback and Sodek,

1979). These authors also reported that type III collagen was slower than type I collagen in its conversion from procollagen to collagen.

The ratio of type I to type III collagen is significant with respect to the functional requirements of a tissue in that it is a major factor in determining its mechanical properties (Horowitz et al., 1977). Distensible tissues (skin, blood vessels, lung) tend to have elevated type III collagen while rigid tissues (bone, cartilage, tendon) usually only have type I.

Rapid collagen synthesis is correlated with increased proportion of type III collagen. The ability of a tissue to respond to functional and physiologic demands may be reflected in its ability to alter its type III collagen ratio (Pinnell et al., 1987).

Types IV and V are basement membrane collagens and generally contain more residues with bulky hydrophobic side chains and significantly fewer alanyl and arginyl residues than the other types of collagen (Sage and Bornstein, 1979). These collagens are synthesized by epithelial cells of adjoining cell linings (Minor et al., 1976a,b).

Type IV collagen has the molecular formula of $[alpha_1(IV)]_3$. It is nonfibrillar and is the major collagen of basement membranes. It is high in hydroxylysine and carbohydrates (Alberts et al., 1989).

Type V collagen has three different chain associations that have been delineated [$alpha_1$ $(V)_2$, $alpha_2$ (V)], [$alpha_1$ $(V)]_3$ and [$alpha_1$ (V), $alpha_2$ (V), $alpha_3$ (V)] (Cheah, 1985). This collagen was originally observed in fetal membranes (Burgeson et al., 1976; Narayanan and Page 1983b) but is also a minor constituent in most connective tissues (Rhodes and Miller, 1978). It is unique in its localization in pericellular spaces and near basement membranes (Madri and Furthmayri, 1979; Roll et al., 1980).

Collagen types I, II and III comprise the primary interstitial collagens while collagens type IV and V are the basement membrane collagens. The remaining collagens are not well categorized and are present only in small amounts (Cheah, 1985). The major tissue distribution for type VI is the intima of blood vessels (Furthmayr et al., 1983); type VII is in chorioamniotic membranes (Bentz et al., 1983); type VIII is in endothelium (Sage et al., 1983); and types IX, X and XI are in cartilage (Van der Rest et al., 1985; Kielty et al., 1985; Cheah, 1985).

Periodontal Collagen Phenotype Distribution and Turnover Rate

The periodontium consists of the epithelial and connective tissues of the gingiva, PDL, root cementum surface and alveolar bone. The connective tissues are highly collagenous with functionally specific orientation and distribution of the collagen fibers.

The PDL is composed of mostly type I collagen, trace amounts of type V collagen and as much as 15 to 20% type III collagen (Butler et al., 1975a and 1975b; Sodek and Limeback, 1979). The large percentage of type III may reflect the rapid collagen turnover associated with the PDL. PDL collagen turnover may be more rapid than any other tissue of the body (Sodek, 1978).

Gingiva has the greatest representation of the different phenotypes. Its collagen population includes 95% type I, 9% type III and trace amounts of type II, IV and V (Sodek and Limeback, 1979; Hammouda et al., 1980; Narayanan et al., 1981). The collagen turnover in the gingiva is rapid relative to other connective tissues but at a much slower rate than PDL collagen turnover (Kameyama, 1973; Skougaard et al., 1970; Sodek, 1976). By means of tritiated proline labelled mouse collagen, it was observed that the collagen half-life in gingiva was 25 days and 5.7 days in PDL (Bernick, 1960; Sodek, 1977).

The collagen of alveolar bone has been shown to be type I phenotype (Narayanan and Page, 1983a). The cementum collagen is primarily type I collagen with up to 5% type III. The type III collagen in cementum may be due to embedded periodontal fibers (Butler et al., 1975b; Birkedal-Hanson et al., 1977; Christner et al., 1977).

Collagen Phenotype as a Biologic Signal

Collagen has been shown histologically (Stallard, 1963; Carniero and Leblond, 1966) and biochemically (Sodek, 1977; Sodek et al., 1977) to be metabolically active. Collagen synthesis responds to physiologic and functional demands on the tissue and this response often produces changes in the proportion of collagen phenotypes. This is most notable for the types I and III interstitial collagens.

It appears as though the proportion of type III collagen is increased in tissues requiring synthesis, eg. fetal skin (Epstein, 1974; Chung and Miller, 1974; Miller, 1976); rapid turnover, eg. PDL (Butler et al., 1975a, 1975b); during inflammation (Weiss et al., 1975); and during early phases of wound healing (Gay et al., 1978; Reddi et al., 1977; Merkel et al., 1988).

Biochemical investigations have demonstrated the PDL's rapid collagen turnover (Page and Ammons, 1974; Sodek, 1976; Sodek and Limeback, 1979). The need for rapid turnover of collagen in the PDL could be due to chronic physical stress produced with occlusal function or chronic irritation from oral bacteria producing a perpetual state of wound healing and repair (Claycomb et al., 1967; Sodek, 1976). This situation may explain the PDL's ability to rapidly respond with remodelling following orthodontic force application. The reasonably high proportion of type III collagen relative to

other tissues supports its use as a biologic indicator of enhanced remodelling.

There are three possible routes by which a tissue may alter the amount and proportion of its collagen phenotypes. Clonal selection of cells producing a particular collagen phenotype would produce a long-term shift in collagen phenotype production by changing the ratio of fibroblast subpopulations and would involve stimulation of progenitor cells to proliferate and eventually fully differentiate (Bordin et al., 1984). A change in the present cell population's synthetic pattern would produce a more rapid response and a more short-term resolution (Rosenbloom et al., 1984). The third method would be to alter the susceptibility of various collagen phenotypes to degradation. For example, in certain situations extracellular collagenase has been observed to be relatively selective in its digestion of collagen phenotypes (Horowitz et al., 1977).

The ability to modulate the overall synthetic pattern of various collagen phenotypes may be essential for normal tissue development and repair. Therefore changes in the proportion of collagen phenotypes (especially that of type I and type III) may be a biologic indicator of the metabolic state of a connective tissue, the dynamic functional demands being experienced and the changing cellular activity.

COLLAGEN METABOLISM DURING WOUND HEALING

The term wound usually refers to a lesion that involves tissue loss and tearing (eg. an incision). It can also apply to tissue destruction following burns, ischemia or hypersensitivity reactions. Wound healing is the body's response to this injury, and it ends in the restoration of tissue continuity (for review, see Hunt and Goodson, 1988; Shoshan, 1981).

Using dermal wound healing as a model, adult mammalian tissue wound repair involves a complex sequence of biochemical events that ultimately leads to scar formation (Hunt and Goodson, 1988). The stages of wound healing include hemostasis, inflammation, proliferation and remodelling. Hemostasis involves vasoconstriction, platelet aggregation and degranulation, blood clotting and fibrin formation. This produces a passive barrier to the entry of foreign material.

Inflammation represents the cascade of invading blood borne cells, beginning with the polymorphpnuclear leukocytes (PMNs) and followed by macrophages and lymphocytes. This stage is responsible for actively defending against bacterial infection and the secretion of numerous growth factors, cytokines and extracellular matrix components. The macrophage is the primary inflammatory cell responsible for the coordination of the repair process at this stage.

Once an effective defense reaction has been organized, the reparative phenomena begins with the synthesis of new

connective tissue (Gabbiani and Montandon, 1977). Initially this involves granulation tissue formation which rapidly becomes highly vascularized. Granulation tissue allows for the synthesis of a new type of connective tissue and contraction of the wound. The modified connective tissue has a greater proportion of type III collagen and large amounts of hyaluronic acid.

The prolferative phase involves multiplication of fibroblasts, endothelial cells and epithelial cells (Hunt and Goodson, 1988). The initial proteoglycan rich fibrin matrix is replaced by collagen. In the final remodelling stage, collagen is cross-linked to form a mature scar. As the fibroblasts proliferate and synthesis proceeds, the fibers begin to occupy an increasingly greater proportion of the volume of the healing tissue. Eventually the capillaries are compressed and a largely acellular blanched scar with a high tensile strength results.

Collagen is the major component of the mature scar, comprising about half of its volume (Alvarez and Gilbreath, 1982). The collagen is responsble for the strength of the scar. The scar collagen fibrils are primarily composed of type I collagen molecules with a small amount of type III collagen molecules.

In abnormal scar healing sometimes hypertrophic scars or keloids may form. It has been suggested that hypertrophic scars are characterized by an increase in proteoglycan content

(Shetlar et al., 1978). A possible link between an increased collagen-proteoglycan interaction and excess scar formation has been demonstrated (Linares, 1983). Interestingly, fetal wound healing involves regeneration and not scarring until late in gestation when the adult wound healing processes develop (Longaker and Adzick, 1991). It is postulated that differences in the deposition of hyaluronic acid may be responsible for the lack of fetal scar formation.

It has also been observed that keloid and hypertrophic scars have elevated levels of type III collagen relative to mature scar tissue or normal dermis (Bailey et al., 1975). Perhaps the excess scar tissue formation associated with these disorders is the result of prolonged collagen synthesis in a manner normally associated with earlier stages of wound healing.

The connective tissue reaction to an injury may be described as an inflammatory-reparative response (Gay and Miller, 1978). The inflammatory response includes the clot formation and the appearance of the phagocytic cells. The reparative component involves the connective tissue cell proliferation and the subsequent deposition of a fine reticular network of collagen fibrils initially comprised of mostly type III collagen molecules. In later stages the type I collagen molecule predominates.

The stages of wound healing are not distinct in time but rather overlap during the course of repair, and collagen is

involved in all stages of wound repair. In the initial phase of wound healing fibrillar collagens (types I and III) are believed to be responsible for platelet aggregation following vascular injury (Shoshan, 1981). The binding of the platelets to the fibrillar collagen of the adjacent connective tissue leads to the release of two large glycoproteins, fibronectin and thrombospondin. Fibronectin and collagen fragments , which may be generated by macrophage released collagenases, are chemotactic for fibroblasts (Seppa et al., 1982). Thrombospondin is involved in platelet aggregation.

Fibroplasia is the term used to describe the process of fibroblast invasion and deposition of a collagen-based extracellular matrix (McPherson and Piez, 1990). If this is accompanied by neovascularization then granualtion tissue forms. This phase typically begins 3 to 5 days following wounding and lasts about 10 days. There is rapid synthesis of type I and III collagen and a concommitant increase in the tensile strength of the wound.

Both platelets and macrophages have been demonstrated to release substances that increase collagen synthesis during wound healing. Platelet-derived growth factor is believed to recruit fibroblasts into the wound area via its chemotactic activity (Knighton et al., 1982). Another platelet factor, transforming growth factor-beta (TGF-beta), has been observed to increase collagen synthesis (Lawrence et al., 1986; Quaglino et al., 1990).

The macrophage derived factors are more important in the control of wound healing collagen synthesis than the platelet derived factors. They produce a greater response and are present in significant quantities during fibroplasia while the platelet derived factors are not (McPherson and Piez, 1990).

In addition epidermal growth factor (EGF) has been demostrated to increase the rate of collagen deposition in wounds (Hennessey et al., 1991). This is brought about by increasing the rate of early type I collagen synthesis, thus decreasing the ratio of type III collagen to total wound collagen (Hennessey et al., 1990).

In experiments involving rat dermal wound healing granulation tissue a high proportion of type III collagen (up to 30% of the total collagen) was observed, in contrast to the usually much lower proportion found in normal rat skin (approximately 10%) (Bailey et al., 1975; Gabbiani et al., 1976; Clore et al., 1977). This increase in type III collagen synthesis was observed as early as 10 hours after wounding while by 24 hours after wounding the synthesis of type III collagen was significantly decreased.

Healing transected canine medial collateral ligaments were found to have increased amounts of de novo type III collagen and reduced amounts of de novo type I collagen (Gomez et al., 1989). Similarly, it has been shown that human children have an increase in type III collagen production in the early stages of wound healing (Viljanto, 1976). Gay and

coworkers (1978) found this increase to occur during the first 3 to 4 days of wound healing.

It has been speculated that wound contracture is produced by fibroblasts and collagen fibrils (Ehrlich, 1988). The contraction can be modelled in vitro by placing fibroblasts in collagen gels (Bellows et al., 1983, Farsi and Aubin, 1984). It is suggested that contractile cells, fibroblasts, attach to the extracellular matrix collagen fibers that are anchored in the tissue. The cells apply traction to the collagen fibers and contract the healing tissue.

The composition of the extracellular matrix may be the factor controlling the contractile activity of the fibroblasts. A matrix rich in type III collagen contracts more quickly and to a greater extent than does a matrix rich in type I collagen (Ehrlich, 1988).

Wound contracture is not the same process as scar contracture. The former occurs relatively early in wound healing while the latter may occur over several years and follows scar formation. Muscle activity has been proposed as the force responsible for scar contracture, this in association with longterm remodelling of the collagen matrix is proposed as being the underlying cause of scar contracture (McPherson and Piez, 1990). Others have suggested that the forces generated by the fibroblasts are responsible for both wound and scar contracture (Ehrlich, 1988).

NON-FIBROUS COMPONENTS OF THE EXTRACELLULAR MATRIX (GROUND SUBSTANCE)

The extracellular matrix (EC) is secreted by cells and is a loosely organized gel consisting of glycoproteins, proteoglycans and glycosamminoglycans (Hay, 1981). It represents a much smaller proportion of the EC matrix than does the fibrous fraction, but it is important in the hydration, transport and sieving of EC molecules (Laurent, 1966).

Hay (1978, 1981) has described a proteoglycan carrier system that transports tropocollagen molecules to remodelling fibers. Further, it has been proposed that glycosaminoglycan side chains, of a collagen molecule, determine the timing, location, orientation and dimension of deposited collagen fibrils (Gross, 1969; Toole and Lowther, 1968; Breen et al., 1972).

The proteoglycans are able to form complexes by means of cross-linking with other components of the EC matrix, due to a highly anionic charge resulting from the presence of the glycosaminoglycans (Bartold and Page, 1985). In this way collagen is intimately linked to the ground substance. Roberts and co-workers (1981) postulated that the displacement of the PDL during orthodontic tooth movement allows the negatively charged ground substance to migrate toward the positively charged collagen molecules in the localized areas of microscopic widening. This produces a high ground

substance to collagen ratio and a net negative charge which has been implicated as being a stimulus for osteogenesis.

Direct alterations of the ground substance and its involvement in the remodelling of other tissue components in response to orthodontic force are likely. The ground substance may play an important role in orthodontic tooth movement.

FIBROBLASTS

Many different cell types are found within the periodontal ligament: osteoclasts, thought to be of hemopoietic origin; cementoblasts; vascularly derived macrophages and mast cells; nerve cells; endothelial cells; pericytes; and the localized epithelial cells of the rests of Malassez; as well as the locally derived blast cells (ie. osteoblasts lining the alveolar bone, fibroblasts within the fibrous soft tissue and cementoblasts associated with the cementum). The most common cell of PDL is the fibroblast.

Traditionally fibroblasts are ascribed anabolic roles and macrophages the catabolic functions in the loose connective tissue. Macrophages are known to be very active in phagocytic degradation, however they are usually only observed in the PDL during inflammatory reactions, in which case they migrate into the tissue from the blood vessels.

Fibroblasts not only synthesize collagen, they also have been demonstrated to simultaneously degrade extracellular (EC) collagen fibers (Ten Cate, 1972; Ten Cate et al., 1976).

Profiles of mature collagen fibers with the characteristic banding pattern have been observed in various stages of breakdown in fibroblast phagosomes while no such profiles have been noted in the osteoblasts or cementoblasts. This has led to the hypothesis that in both anabolism and catabolism, fibroblasts play a vital role in collagen remodelling (Deporter and Ten Cate, 1973, 1980; Ten Cate and Freeman, 1974).

Fibroblasts can also generate forces that are capable of organizing the surrounding connective tissue as seen in wound contracture or collagen fiber alignment (Ehrlich, 1988). The regulation of the cellular contractile forces may involve the composition of the EC matrix, since the greater the proportion of type III collagen present, the more rapid is the contraction of the tissue. The fibroblast microfilaments are the primary structures involved in generating the forces required to align and compact collagen while the microtubules may play a secondary role or none at all (Farsi and Aubin, 1984).

Once a tissue is formed the synthetic activity of the fibroblasts may be controlled or altered in several ways. The protein synthesis of the tissue is closely controlled by the cells (Breul et al., 1980) likely through feedback mechanisms that influence the cell's synthetic activities (Wiestner et al., 1979; Paglia et al., 1981). The synthetic activity can be influenced by environmental factors or ligands acting on

the cell. During dramatic environmental changes (eg. repair) the process of clonal selection can be reactivated in order to alter the synthetic activities of the synthetic cells of the connective tissue.

FEEDBACK

The primary control over collagen synthesis is believed to be via a feedback loop (Wiestner et al., 1979). It is known that the amino terminal peptides cleaved from secreted procollagen molecules modulate collagen synthesis patterns (Paglia et al., 1981; Perlish et al., 1985). The concentration of these polypeptide chains is inversely related to collagen synthesis. It has been observed that the carboxyl terminus polypeptides also inhibit collagen synthesis but to a lesser extent than aminoterminus polypeptides. It appears as though the negative feedback loop acts pretranslationally by altering the amount of mRNA available (Uitto et al., 1985; Wu et al., 1986). Carboxyl terminal polypeptides may act to inhibit nuclear transcription of hnRNA.

CLONAL SELECTION

It has been illustrated that the fibroblasts of a connective tissue are not a homogeneous population but rather a heterogeneous group of various fibroblast subpoplulations (Kaufman et al., 1975; Ko et al., 1977; Bordin et al., 1984; Korn, 1985). The progenitor cell population is comprised of

cells with varying phenotypic potentials (ie. the cells are committed to expressing a specific phenotype).

In a stimulated tissue, progenitor cells divide and after several generations become terminally differentiated daughter cells that synthesize proteins characteristic of their phenotype. This could be a random process (Angello and Prothero, 1985) but this would not explain a tissue's ability to modify its response to reflect specific environmental changes. A better theory is that ligands act on the level of the progenitor cells and selectively activate those whose progeny will become the cell population required to appropriately adapt the tissue to the environmental stimulus.

The progenitor cells proliferate through several cell divisions and then become fully differentiated, while a few daughter cells replenish the progenitor cell population. In this way the process is self-limiting and is able to customize its response to the stimulus (Martin et al., 1974; Hassell and Stanek, 1983).

This selection of a specific subpopulation of fibroblasts is termed clonal selection and it allows for long-term shifts in tissue collagen phenotype production. An alternate explanation is that all fibroblasts are capable of producing all the collagen phenotypes and thus changes in a tissues production of various phenotypes reflects a shift in a previously differentiated cell's protein synthesis. This would involve altered gene transcription to produce mRNA's for

the translation of the alpha-chains required for the new collagen phenotype. This would provide a more rapid response.

It is conceivable that both clonal selection and a shift in cellular gene expression are possible, but it is a contentious issue nonetheless.

CELL SHAPE

It is possible that physical perturbations of the cell leading to alternations in its shape could modify cell function. Folkman and co-workers (1975, 1978) were able to demonstrate a change in fibroblast DNA synthesis was associated with a change in cell shape. Shape has been observed to be a controlling factor in cell growth (Kulesh and Greene, 1986; O'Neill et al., 1986), differentiation (Shannon and Pitelka, 1981; Senechal et al., 1984) and protein production (Ben-ze'ev et al., 1980; Ben-ze'ev, 1984; Aggler et al., 1984; Quinones et al., 1986; Benecke et al., 1980; Unger et al., 1986; Reiter et al., 1985; Glowacki et al., 1983).

Although some authors (Tucker et al., 1981) have attributed the modification in cell function with differing cell shapes, to an alteration in the sensitivity or number of cell surface receptors available to bind, Vandenburgh (1983) believed the cellular shape change directly activated the cell's second messenger system and bypassed the membrane receptors. Others suggest cell shape leads to modification in the mRNA and thus alters protein synthesis (Farmer et al.,

1983). The cell shape changes are believed to be transduced by the cytoskeleton (Ben-ze'ev, 1983; 1985).

Researchers have developed techniques to stretch cells in tissue culture (Hasegawa et al., 1985; Somjen et al., 1980; Harell et al., 1977; Meikle et al., 1979) and it is possible in these experiments cell shape changes could be involved in the tissue response to mechanical stress. If the ability to alter cell shape by physical means results in the alteration of a cell's activity then this may be important in understanding how orthodontic forces are transduced into biologic signals.

ORTHODONTIC TOOTH MOVEMENT

Tooth movement is a physiologic process that occurs continuously throughout life. During our growing years the movement is dramatic and reflects the changing oral environment, as the face grows and teeth erupt, as well as the forces that act on the teeth during oral function. In adulthood tooth movement does not cease, although its rate is much slower. Indeed the oral hard and soft tissue age related changes and altered function require the dentition to adapt by means of tooth movement in order to bring the forces impacting on the teeth into equilibrium.

The orthodontist takes advantage of this natural phenomenon by imposing artificial force systems on the dentition and thereby moving teeth. Ideally these should

operate within the physiologic limits of the PDL's ability to respond by noniatrogenic remodelling.

Orthodontic tooth moving forces disturb the equilibrium of the PDL and exceed the normal homeostatic mechanism's ability to restore the equilibrium (eg. PDL width). This results in changes in the normal histologic architecture, altered metabolism, changes in vascularization, as well as cell proliferation and migration.

Generally, different areas of the PDL are either compressed or stretched by a displacement of the tooth root within the alveolar socket. This sets up a series of microenvironments where the tooth is either pulled away from (tension) or pushed toward the alveolar bone (compression). Areas experiencing alveolar compression respond with bony resorption and areas of tension exhibit bony apposition, thus shifting the tooth socket until homeostasis is re-established with a PDL of normal width, free of areas of compression or tension.

Frequently orthodontic tooth movement involves forces exceeding the ability of the PDL's vasculature to remain patent in the areas of compression. This results in areas of ischemia producing hyalinization or tissue necrosis. The necrosis is followed by resorption of adjacent bone (frontal resorption), or if the surrounding PDL is obliterated the resorption could begin from the endosteal surface of the alveolar bone (undermining resorption).

Osteoclast progenitor cells are delivered to areas of hyalinization via the increased vascularity associated with wound healing. Osteoclasts also differentiate and migrate from adjacent marrow spaces into the PDL in cases of undermining resorption (Gianelly, 1969; Khouw and Goldhaber, 1970). These cells resorb bone and digest debris as they eliminate the hyalinized tissue. It has been suggested that invading macrophages, rather than the osteoclasts, are responsible for the degradation of the collagen fibrils (Rygh, 1984).

As the areas of tissue destruction are removed, fibroblasts of adjacent tissue profilerate and restore the PDL to its normal width and architecture thereby completing the repair process (Rygh, 1973). New layers of cementum and alveolar bone are deposited thus providing newly synthesized collagen fibers the opportunity to embed in the new matrices, in order to become Sharpey's fibers, anchoring the fibers in hard tissue upon mineralization of the matrices.

Hyalinization and tissue disruption occur in areas of tension with high force levels, however it is usually less extensive relative to areas of compression. Lower force levels bring about remodelling that reorients the collagen fibers parallel to the vector of the resultant force. In either scenario, tissue repair mechanisms remove necrotic material, replace damaged tissue, realign the collagen fibers and remodel the alveolar bone.

In addition to the changes in the PDL, the gingival tissues are also remodelled during orthodontic tooth movement. It has been demonstrated that the principle fibers of the gingival tissue are more refractory to realignment, than the PDL collagen fibers, even with long-term retention of the new tooth positions (Edwards, 1968; 1970; 1988). This may be a reflection of a much less efficient collagen turnover in the gingival tissues relative to the PDL (Skougaard et al., 1970; Sodek, 1976).

Although the histologic events during tooth movement have been well documented, the dynamic mechanism transducing the external stimulus (ie. orthodontic force application) into cellular activity has not been ascertained. It is likely that extracellular and intracellular events initiate and direct the tissue's remodelling. Several theories regarding these biologic control mechanisms have been proposed.

PRESSURE-TENSION THEORY

Schwarz (1932) reported that Sandstedt, in 1904, was the first investigator to report histologic evidence, of bone resorption in areas of pressure and bone apposition in areas of tension, associated with tooth movement. Based on this work, Schwarz (1932) formulated the pressure-tension theory, which has become the classic hypothesis explaining tissue changes seen in orthodontic tooth movement.

It was proposed that tooth movement required light forces so that the pressure in the PDL would be no greater than the

PDL capillary blood pressure, in order to produce the histologic changes required for tooth movement and not occlude the PDL capillaries. Larger forces produced cell free zones, later known as hyalinization, due to ischemia following the collapse of the PDL blood vessels. It was felt that the cellular activity was in response to the areas of pressure and tension produced within the PDL.

Gianelly (1969) modified this theory to include the effect of the force on the vasculature. He felt that the histologic findings were in response to partial vascular occlusion. It was believed that in order to have efficient tooth movement it was essential to use light forces and not prevent blood flow thus preventing the slower tooth movement following hyalinization and undermining resorption.

Kardos and Simpson (1980) felt that the PDL was thixotropic. Under pressure the matrix became less viscous and thus allowed rapid cell migration to areas of lower pressure. They suggested this explained the loss of tissue architecture associated with cell free zones and not tissue necrosis. This variation of the theory has found little support.

Epker and Frost (1965) and Baumrind (1969) proposed that the histologic changes associated with areas of pressure and tension were due to differing cellular responses to the different types of bony deformation produced in each area. Baumrind suggested cell proliferation decreases in areas of

pressure due to vascular occlusion and it increases in areas of tension due to the stretching of the collagen fibers. This hypothesis has not been supported by later research.

Overall, the pressure-tension model attempts to explain the changes in the tissue that bring about the remodelling that produces tooth movement. It does little to elucidate the control mechanisms on the molecular or cellular level.

PIEZO-ELECTRIC THEORY

An electric potential is generated within the tissue as an applied force deflects the alveolar bone (Fukada and Yusada, 1957; Bassett and Becker, 1962). It has been suggested that the microporosity of the bone, which is composed of fluid filled channels in and around the mineral encrusted collagen matrix, enables the production of these stress-generated potentials (Salzstein et al., 1987; Salzstein and Pollack, 1987).

Histologically the electronegative regions are associated with evidence of bone production and electropositive regions with evidence of bone resorption (Bassett et al., 1964; Bassett, 1968; Zengo et al., 1973, 1974, 1976). Davidovitch and co-workers (1978a, 1978b, 1979, 1980a, 1980b) have demonstrated an increase in cell numbers and activity in ligaments in which an electric current is passed. This has also been implicated in accelerated tooth movement.

This theory does attempt to explain the method by which the mechanical stress is transduced into a signal to which the

cells respond. Possible candidates for an intracellular message, that would translate the signal into cellular activity, may involve electron migration across biologic membranes (Jahn, 1962), proton charge transport through the cytoplasm (Eigen and DeMaeyer, 1958), and amphoteric ion exchange (Jahn, 1968).

OXYGEN-TENSION THEORY

It is theorized that the tissue strain, resulting from orthodontic force application, produces stenosis of the PDL capillaries in areas of compression. This leads to the formation of a series of cirsoid or enlarged aneurysms (Bien, 1966). The blood pressure is lowered in areas after the stenosis thus permitting minute oxygen bubbles to come out of solution, cross the blood vessel walls and aggregate adjacent to the alveolar bone surface. This is believed to initiate osseous resorption in these areas.

Oxygen tension has been demonstrated to be related to bone resorption *in vitro* (Goldhaber, 1958, 1961, 1966; Stern *et al.*, 1966) suggesting it could be involved in its regulation *in vivo*. Vascularity is increased during orthodontically induced frontal bone resorption (Gianelly, 1969; Khouw and Goldhaber, 1970) and Rygh (1984) suggested that activation of the vasculature is directly related to the efficiency of tooth movement. The alterations in blood flow and blood volume could be the way in which oxygen tension is controlled, however it must be noted that the increased blood

flow may be secondary to the events regulating remodelling and may not be a primary or causative factor.

Blood flow interruption does result in tissue damage and alterations that could directly or indirectly provide the signal for tissue remodelling. Subsequently, increased vascularity leading to more rapid remodelling is integral to the process. The manner in which alternations in the vascularity may serve to regulate the cellular activities leading to orthodontic tooth movement have yet to be established.

This theory does propose a molecular basis for the control but it is highly speculative. It does not delineate the manner in which the proposed molecular signal interacts with the cells. Experimental support for this model is lacking.

SECOND MESSENGER THEORY

A recent hypothesis, explaining the way in which orthodontic tooth movements act to produce the described histologic changes, is the second messenger theory. The theory of a second messenger postulates that upon application of orthodontic force on a tooth the PDL is physically altered. This alteration produces an extracellular first messenger (eg. a ligand). It may involve inorganic molecules (eg. O_2), an electrical charge, physical perturbations or organic compounds (eg. a hormone) which interact with specific cell surface

receptors leading to enzymatic activities that produce an intracellular second messenger (eg. cAMP). The second messengers serve to modify cellular activity in such a way that the remodelling process involved in orthodontic tooth movement occurs.

First Messenger

Many possible first messengers have been proposed: systemically acting hormones such as calcitonin (Murad et al., 1981; Rodan and Rodan, 1974; Nagata et al., 1975) or parathyroid hormone (Chase and Auerbach, 1970; Vaes, 1970; Murad et al., 1970; Peck et al., 1973); prostaglandins (Goldring et al., 1979); calcium ion concentrations (Whitfield et al., 1979); growth factors (Redini et al., 1988) electrical stimulation (Davidovitch et al., 1978a, 1979, 1980a, 1980b; Rodan et al., 1978); interleukin I (Loato and Hemo, 1988); and the mechanical deformation of cells (Binderman and Cox, 1977; Harell et al., 1977; Somjen et al., 1980).

Transforming growth factor beta has been shown to stimulate collagen and glycosaminoglycan synthesis in connective tissue cell cultures (Redini et al., 1988). Granulation tissue cultures exposed to interleukin I were observed to decrease collagen production yet maintain cellular proliferation (Loato and Hemo, 1988). Parathyroid hormone (PTH) has been demonstrated to enhance the clinical and histologic evidence of orthodontic tooth movement (Gianelly and Schmur, 1969; Kamata, 1972; Davidovitch et al., 1972).

Based on studies involving animals and humans, Yamasaki et al. (1980, 1988) reported that tooth movement is more efficient if prostaglandins (PG) are administered in conjunction with orthodontic force application. It was felt that this was due to PG mediated enhanced bone remodelling.

Rennard et al. (1982) produced an in vitro decrease in lung fibroblast collagen synthesis associated with an increase in cellular cAMP upon addition of PGE_1, to tissue culture. The type I collagen production was decreased 58% and type III collagen production was decreased only 9% thus leading to an increase in the proportion of type III collagen. This was assumed to be due to altered cellular metabolism since there was no collagenase in the tissue culture.

Baum et al. (1980) reported a decrease in collagen production due to an increase in the degradation of newly synthesized collagen following an administration of PGE_1. There was an increase in intracellular cAMP levels. However the administration of a NSAID, indomethacin, acts to inhibit cyclo-oxygenase mediated production of PG and associated with this was a decrease in collagen synthesis during wound healing (Carlstedt et al., 1986).

In an attempt to study PGE_1, as a possible regulator of orthodontic remodelling, it was added to the organ cultures of a fibrous sutures under mechanical stress (Yen and Suga, 1986; Yen et al., 1985). There was an observed increase in osteoclasts and protein synthesis while PGE_1, had no effect on

the ratio of collagen types being synthesized (Yen and Suga, 1986).

As of yet the first messenger is unknown, however it has been suggested that this agent interacts with cell surface receptors to create a second messenger. One idea is that cell membrane-bound enzymes, adenylate cyclase and guanylate cyclase, convert adenosine triphosphate (ATP) and guanosine triphosphate (GTP) into proposed second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) respectively (Sutherland et al., 1962; Hardman and Sutherland, 1969; White et al., 1969). This will be discussed.

Second Messenger

The second messenger is an intracellular biologic signal generated in response to the first messenger. This signal influences the cell to alter it's metabolic activity.

In this discussion, the intracellular cyclic nucleotides will be used as a potential example of a second messenger. The cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) have long been considered to be intracellular second messengers because they have been associated with converting membrane effects into cellular activity (Sutherland and Rall, 1960; Sutherland et al., 1962, 1965; Hardman and Sutherland, 1969).

Initiation of bone progenitor cell proliferation has been related to decreased cAMP within the cell (Norton et al.,

1977). Fluctuations in cyclic nucleotide levels has been demonstrated in areas of tension and compression within the PDL of cats undergoing orthodontic tooth movement (Davidovitch et al., 1975, 1976a, 1976b, 1977, 1984).

A selective decrease in collagen production has been associated with elevated levels of cAMP (Baum et al., 1980). Cyclically stretched tissue cultures of smooth muscle cells grown on elastin membranes increase their rate of collagen and non collagen protein synthesis unless cAMP was added to the culture (Kollros et al., 1987). Interestingly cAMP did not effect the synthetic activity of non stressed tissue cultures.

It is postulated that the first messenger causes cell membrane-bound enzymes, adenylate cyclase and guanylate cyclase to convert adenosine triphosphate (ATP) and guanosine triphosphate (GTP) into the second messengers cAMP and cGMP respectively (Sutherland et al., 1962; Hardman and Sutherland, 1969; White et al., 1969).

It is possible that these metabolites influence cellular activity by acting as co-factors in enzymatic phosphorylation reactions.

This theory does address the intracellular and extracellular molecular events that could direct the metabolic activity of the PDL cells. A major stumbling block to accepting the fluctuating levels of cyclic nucleotides as second messengers is the ubiquitous and indiscriminate nature of this finding. Altered levels of cAMP and cGMP have been

associated with a plethora of cellular metabolic responses to a variety of primary stimuli, and the metabolic changes attributed to the increase or decrease of the intracellular cyclic nucleotides have been contradictory in various published studies.

If, however, a cell's synthetic activity is limited to a few specific metabolic pathways upon terminal differentiation, then the cyclic nucleotides could act as a toggle mechanism. In this case a first messenger's influence on the cell produces a response involving stimulation or inhibition of its limited number of metabolic functions. In such a scenario a simplistic second messenger response (eg. altered cAMP or cGMP levels) could signal the cell to alter its metabolic activity. The specificity and appropriateness of the metabolic changes thus produced could be regulated by the first messenger's ability to target the appropriate cells or progenitor cells.

CELLULAR RESPONSE TO PHYSICAL STRESS

As early as 1926 it had been reported that the application of tension to fresh wounds influences the fibroblasts to align and divide on axes parallel to the line of action of the force (Bunting and Eades, 1926). The response was believed to involve new and old fibroblasts.

Using an <u>in vivo</u> model, Arem and Madden (1976) demonstrated the ability of the normally random fibers of scar collagen, in a subcutaneous wound, to align along the lines of an applied force. In this case proplast bars connected by

scar collagen were implanted in rats. The bars under the influence of a magnetic field caused cyclical stretching of the tissue. Similarly, in vivo, Buck (1980) was able to intermittently stress fibroblasts by stretching the silicone rubber surface on which they were cultured. Mirroring the in vivo results the tissue cultures demonstrated a reorientation of the fibroblasts and their mitotic activity to reflect the line of force application.

An increase in bone mass has been associated with the application of forces within a physiologic range, and loss of bone mass has been associated with a lack of force application (Rubin and Lanyon, 1984). In adaptive bone remodelling, the rate at which strain is generated in the tissue is directly related to the quantity of new bone formation (O'Connor et al., 1982).

Bassett and Becker (1962) found that the flexing of feline long bones generated a negative electropotential with a magnitude that was directly related to the degree and rate of the bony deformation. By applying a weak direct electrical current, to long bones of dogs, they were able to align the collagen fibrils and promote new bone formation in areas of electronegativity (Bassett and Becker, 1964). This work may indicate that the generation of the electropotential within the tissue was the biologic mechanism whereby stress, on bone, signals the tissue to remodel by osteogenesis.

Similar results were reported by Zengo and co-workers (1973, 1974 and 1975) upon studying the deformation of alveolar bone following orthodontic tipping of canine teeth. It was hypothesized that electronegative areas were osteoblastic and that electropositive were osteoclastic. These responses were suggested to involve the areas of the PDL under tension and compression respectively.

By implanting electrodes in the periodontium of cats, Davidovitch and co-workers (1980a, 1980b) demonstrated that the application of direct electrical currents accelerated tooth movement. There was an increase in cAMP and cGMP within the osteoblasts and fibroblasts. Increased bone resorption near the positive electrode and osteogenesis near the negative electrode were reported. This is in agreement with the hypothesis of Zengo et al. (1976) regarding the relationship of bone metabolism and electropotentials.

Oscillating electrical fields have been demonstrated to increase cAMP levels and DNA synthesis in cartilage cells (Rodan et al., 1978; Norton et al., 1977). These and other studies show that electric currents can alter a tissue's metabolic activities but they do not prove that under normal conditions electropotentials function in this role.

Several authors have used polystyrene bottomed petri dishes fitted with an orthodontic expansion screw to stretch osteoblast tissue cultures. An initial increase in prostaglandin-E_2 and intracellular cAMP levels was followed by

an increase in DNA synthesis (Harrell et al., 1977; Somjen et al., 1980). It is possible that in this situation that prostaglandin-E_2 could be a first messenger and cAMP the second messenger causing the tissue to respond with cellular proliferation.

Intermittent elongation of elastin membranes, on which muscle cells were grown, produced increased rates of synthesis of proteins and, in particular collagen (Kollros et al., 1987). The addition of cAMP to the culture prevented this synthetic response to cyclic stretching but did not alter the synthetic activities of non stressed cultures. It appears as though cAMP is able to modulate the tissue response to mechanical stress.

A model, the Flexercell Strain Unit®, has been developed to use a vacuum generating instrument to produce compressive or tensile strain of variable magnitude and duration in cell tissue cultures (Banes et al., 1985). Computer software has been developed to regulate the timing and rate of deformation produced by air evacuation beneath a series of flexible bottom culture plates (Banes et al., 1988). The amplitude and the frequency of the imposed deformation can be reproducibly regulated.

Cyclic strain of tendon fibroblast cultures (Banes et al., 1988), as well as human dermal and scar fibroblast cultures (Henderson et al., 1988) was found to produce fibroblast alignment directly proportional to the rate and

degree of strain delivered. The fibroblast alignment was first observed 3 days following the onset of experimental stress and was dependent upon intact microfilaments, since it was inhibited by cytochalasin B which prevents actin polymerization. (Actin is a functional subunit of microfilaments.)

In addition Buckley and coworkers (1988a,b; 1990) demonstrated avian calvarial osteoblast alignment in response to cyclic stress over 3 days. Sumpio and Banes (1988a,b,c) observed that porcine smooth muscle cells align perpendicular to the direction of the imposed strain vector.

Not all cell lines respond similarly to the intermittent strain generated in vitro. Bovine endothelial cells were noted not to change their alignment pattern during experimentally produced intermittent stress, in contrast to other cell types studied using the flexercell strain unit.

With respect to cell division the in vitro responses to the Flexercell® Strain were variable and appear to be related to the donor tissue and cell type. Aortic endothelial cells and osteoblasts were seen to increase their rate of cell division (Sumpio et al., 1987; Buckley et al., 1988a); while aortic smooth muscle cells and dental pulpal fibroblasts were retarded (Sumpio et al., 1988a; Levin et al., 1988); still others, eg. internal tendon fibroblasts, were refractory (Banes et al., 1988) to the applied strain.

The Flexercell Strain Unit® has been shown to produce variable results with respect to protein synthesis in differing tissue cultures undergoing cyclic deformation. Human pulpal fibroblasts and porcine aortic smooth muscle cells exhibited an overall increase in both collagen and noncollagen protein synthesis (Levin et al., 1988; Sumpio et al., 1988b) while chick calvarial osteoblasts exhibited a general decrease in protein synthesis (Buckley et al., 1990).

A pattern of mechanical stress involving 10 second intervals of stress followed by 10 seconds of relaxation applied to 4-week-old chick calvaria osteoblast cultures not only resulted in altered cellular alignment, increased DNA synthesis and increased growth rate but also stimulated bone matrix mineralization (Buckley et al., 1988a, 1988b, 1990). The pulsatile stretch of the Flexercell Strain Unit® stimulated bovine endothelial cells to alter their morphology by becoming more rounded, enlarged and having increased peripheral vacoulization. These cells demonstrated increased organization of intracellular stress fibers, possibly due to an increased need for adhesion to the growth substrate of the Flexcell® tissue plates.

In recent work with the Flexercell® model, osteoblasts were shown to alter their mineralization pattern to reflect the different degree of strain present in localized areas of the Flexcell® dish. Increased numbers of bone nodules were found in areas of less than 1% strain while fewer bone nodules

were associated with areas of greater strain, however the areas of large strain appeared to generate larger nodules (Visconti et al., 1991).

Rat gingival fibroblast Flexcell® cultures subjected to intermittent strain produced an increased percentage of type II collagen, while the continuously strained and unstrained cultures did not (Anderson, 1991). The enhanced type III collagen synthesis of the intermittently stressed group peaked 4 to 8 hours following initial stress application and then decreased to levels no different from nonstressed cultures by the end of the experiment (24 hours). The modulation of the type III collagen synthesis was not dependent on the frequency or the magnitude of the intermittent stress application.

This research tool, the Flexercell Strain Unit®, provides the opportunity to not only observe tissue changes associated with mechanical stress but to also observe the response in relation to differing types and magnitudes of stress. It is hoped that such a device will dramatically aid the research efforts by enabling more and better experimentation.

Meikel et al. (1979) were able to double collagen synthesis by placing newborn rabbit interparietal sutures under tensile loading. There were increases in the enzyme activity of collagenase, gelatinase and NMP III without a change in the overall degradation of structural proteins (Meikle et al., 1980). Further, the cells in the stressed

fibrous suture synthesized significant amounts of type III collagen (Meikle et al., 1982).

In vivo expansion of mouse interparietal suture resulted in an increased proportion of type III collagen synthesis relative to the total collagen synthesis (Chaing, 1981) in response to the imposed strain. This was observed after four hours of tensile stress and peaked by one week. There was dramatic sutural expansion but by 4 weeks remodelling had restored the original sutural width.

Expansion of mouse interparietal sutures led to an increase in the ratio of type III collagen synthesis to the total type I and type III collagen synthesis that was directly proportional to the force level applied and the rate of sutural expansion (Yen et al., 1989b). It was found that light expansion forces resulted in a response more resembling physiologic response levels than did heavier forces (Yen et al., 1989b, Yue, 1984).

Using mechanically stressed mouse molar periodontal ligament organ cultures it was found that there was an increase in the proportion of type III collagen synthesized but no significant change in the prostaglandin synthesis (Duncan et al., 1984; Duncan, 1982).

It has been shown that rat molar PDL epithelial cell rests of Malassez proliferate in areas of tension and are lost in areas of compression during orthodontic tooth movement (Gilhouse - Moe, 1972). If epithelial cells are grown on

petriperm culture plates and if the flexible plastic membrane bottom of the dish is elongated, there is a significant increase in the number of cells synthesizing DNA, an increase in the intracellular filamentous structures and an increase in the number of desmosomes per unit length (Brunette, 1984).

In vitro stretching of cranial sutural fibroblast tissue cultures grown in petriperm dishes has been shown to increase the ratio of newly synthesized type III collagen to newly synthesized total collagen (Yen and Suga, 1988).

An ingenious in vitro model that attempted to simulate the 3-dimensional arrangement of cells and fibers between two hard substrates as seen in craniofacial sutures in vivo has been developed (Pollit et al., 1988). This model involves placing a collagen coated titanium disk upon a small glass slide on which sutural fibroblasts have been cultured. Once the culture has formed an attachment to the disk, the slides is tilted so that gravity acts on the disk and thereby stresses the tissue culture. The observed result was an increase in the proportion of newly synthesized type III collagen relative to the total newly synthesized collagen following continuous stress application using this model.

It would seem that the biologic response to physical stress is not uniform. The response varies with the loading pattern; the rate and magnitude of loading; as well as other parameters. It is also influenced by the cell type and the donor tissue from which the cells were obtained. Further

complicating this are the technique artefacts inherent in the research models discussed.

IN VITRO MODELS

The results obtained from experiments utilizing various *in vivo* and *in vitro* models were presented in the previous section. The biologic investgestion of any physiologic process is dependent upon the use of models. The advantages and limitations of the various models must be understood in order to put the observations into proper perspective. Since no cell culture or animal model fully replicates the human responses to a given stimulus; care must be taken when applying results from an animal or cell culture model to the human context.

In vitro models permit the observation of the behaviour of a single tissue or cell type under very controlled conditions. The influencing factors can be carefully regulated, in contrast to the *in vivo* situation where the influence of the adjacent tissues or systemic factors cannot be easily separated from the influence of the variable being studied. However, the elimination of the systemic and local influences, as well as the altered tissue architecture, achieved in tissue culture models introduces significant artefacts. The *in vitro* behaviour of the tissue may be quite different that that observed *in vivo*.

Organ culture has been used to study the influence of mechanical stress on the remodelling of the interparietal sutures of rodents (Meikle *et al.*, 1979, 1980, 1982; Yen *et*

al., 1980, 1989a; Miyawaki and Forbes, 1987). This in vitro model maintains the local tissue architecture, unlike tissue culture models. However due to the need to perfuse the entire organ culture with growth media, in order to prevent tissue necrosis, the explant must be small and can only be kept in organ culture for several days.

Compared to an in vivo model, the organ culture can be quite cost effective in that any drugs or radioactively labelled substances, used in the study, can be administered in much smaller dosages. Organ culture preserves the tissue architecture while eliminating the systemic influences.

Organ culture does not eliminate locally derived factors that may influence the behaviour of the tissue being investigated. Further, artefacts are introduced by the surgery used to obtain the explant and the difficulty in adequately perfusing the entire explant with growth media.

In the model reviewed, stainless steel springs were used to expand the interparietal sutures. This produced continuous stress application until the spring became deactivated. It would be difficult to produce intermittent loading with this model.

Leung and coworkers (1976) developed a model that allowed cyclic stretching or agitation of cells cultured on elastin membranes. The membranes were obtained by slicing .5 to 1 mm thick sections from frozen bovine aorta blood vessel walls into approximately 3cm by 5cm sheets. Each elastin membrane

was stretched and attached to a frame within an airtight chamber. The frame was mounted on tracks so that it could move as a unit (agitation) or allow only one side to be moved along the track (stretching). In some cases the frame was stationary.

Stretching or agitation of the elastin membrane was produced by the back and forth movement of a plunger which passes through the chamber wall via an airtight seal. One end of this plunger was attached to a variable speed motor outside the chamber and the other end to the frame within the chamber.

This model has been used by several authors (Leung et al., 1976, 1977; Sottiurai et al., 1983). This model allows for different frequencies of cyclic stretching to be applied to the tissue culture and it allows for agitation of the tissue culture. It has not been used to produce continuous stress application. It is cumbersome and quite large, thus limiting the number of samples that can be observed at any one time.

The growth substrate is arguably the biggest weakness in this model. The use of bovine elastin membranes introduces the possibility of an interaction, between the cells and the growth substrate, which may result in significant artefacts. This model has only been used with smooth muscle cells and may not be amenable to other cell types. The elastin membranes may be identical and thus may be a source of variability in the experimental results, this is especially critical if the

sample size is small. No attempts have been made to demonstrate that the applied tension is distributed throughout the elastin membrane.

Kollros et al., 1987, modified the above model so that the number of samples could be doubled. They simply attached two sheets of elastin membrane to each frame. This enabled an increase in the sample size.

Buck (1980) grew cells on rectangular silicone rubber sheets and then cyclically stretched the substrate. One end of the sheet was attached to a growth flask bottom and the other to one end of a nylon cord which passed out of the growth flask. The other end of the cord attached to an eccentric disk mounted on the shaft of a small electric motor. As the disk turned the growth substrate was cyclically stretched and relaxed.

This model is very similar to the previous model, however it uses a significantly different growth substrate. The author claims that this substrate is smooth, elastic, inert and nontoxic. These claims have not been verified.

Due to the awkward nature of this model there is a problem with simultaneously investigating a large number of samples with this model. The ability to use this model with a number of different cell types has not been demonstrated. No attempt has been made to use this model to produce continuous stress within a cell culture. The ability of this model to

produce tension throughout the whole growth surface has not been studied.

Binderman and Cox (1977) described a model that could produce continuous stress in vitro. Each half of an orthodontic expansion screw was embedded in one of two pieces of self-curing acrylic. Each piece of acrylic was attached to the bottom a polystyrene petri dish by means of an epoxy glue. As the expansion screw was activated it generated tension within the growth surface of the tissue culture dish.

Brunette (1984) modified this model slightly. He attached the orthodontic expansion screw apparatus to the bottom of a Petriperm® dish. The Petriperm® dish has a flexible bottom.

This model is less cumbersome than the two tissue culture models already reviewed, and therefore, with this model it should be possible to use a reasonably large sample size. Although the investigations employing this model have studied bone cells (Binderman and Cox, 1977; Harrell et al., 1977; Somjen et al., 1980), a modification of this model has been used to study epithelial cells (Brunette, 1984). It should be possible to study a variety of cell types with this model.

This model only allows continuous stress application and only within a limited range. The polystyrene dish is stiff and brittle, excess force has been reported to cause the polystyrene dish bottom to fail (Harrell et al., 1977). The strain generated by activating the expansion screw is not well distributed over the plastic dish bottom, rather it is

concentrated around the areas where the screw appliance was glued to the dish.

The use of Petriperm® growth plates in a model to continuously or intermittently stress tissue cultures was first described by Hasegawa and coworkers (1985). In this model the growth substrate is deformed by centering the dish on a glass dome and then resting a weight on the top of the dish.

The Petriperm® model combines the advantages of a flexible growth substrate model with the benefits of a petri dish model. That is, this model can be used to intermittently or continuously stress the tissue culture with a simple and easily managed stress mechanism. The sample size can be reasonably large and it is expected that a variety of cell types can be used in this model.

The stress is distributed over the entire growth surface, although not uniformly. The magnitude of the stress applied as well as the frequency of stress application can be varied.

The stress is manually applied or removed, thus the ability to use this model for short intervals of stress application and relaxation is limited. It is difficult for a single investigator to intermittently stress the dishes for long periods of time (eg. days) or to simultaneously run a large number of samples. Therefore experiments of long duration or involving large sample sizes would involve considersable manpower needs.

A powerful model that overcomes the manpower restrictions of the Petriperm® model while still using a flexible bottomed growth plate is the Flexercell Strain Unit®. This automated model for producing in vitro stress has already been described in the preceding section. It combines all the advantages of the petriperm model with the benefits of using a computer to regulate the stress application. This model allows for continuous or intermittent stress application. The strain rate, magnitude, frequency and duration can be carefully controlled.

This model, like the Petriperm® model, does not produce uniform strain throughout the growth substrate, however, the developers of the Flexercell Strain Unit® have provided information on the strain distribution (Banes et al., 1985). The downward deformation of the Flexcell® dish bottom results in tension in most areas with compression in the center of the dish. It is suspected, although not verified, that the upward deformation of the Petriperm® dish bottom results in tension in all regions.

An interesting model has been developed by Pollit (1987). This model was described in the previous section and it will be discussed in the next section.

THE DEVELOPMENT OF A CONTINUOUS AND INTERMITTENTLY LOADING MODEL FOR SUTURAL CELLS

THE DEVELOPMENT OF A CONTINUOUS AND INTERMITTENTLY LOADING MODEL FOR SUTURAL CELLS

In collaboration with Daniel Pollit (1987) an in vitro model that permitted the continuous stressing of mouse interparietal suture fibroblasts was developed. Cells from mouse interparietal suture explant cultures were subcultured onto sterile glass coverslips. Each glass slide was placed in a petri dish with growth media and incubated.

After three days the cells on the glass slides had achieved confluence and on the center of each glass slide a collagen coated sintered titanium disk was placed. The cells formed an attachment to the disk during an eighteen day incubation period. Once the cell cultures on the glass slides had attached to the disks the experimental slides were maintained at a angle of 75 degrees. This was achieved by securing each glass slide to a vertical divider in the culture dish and elevating the opposite side of the dish so that the bottom of the dish was 25 degrees from horizontal. The glass slide was held in position with a spring clip fabricated from TMA orthodontic wire (77.8% titanium, 11.3% molybdenum, 6.6% zirconium, and 4.3% tin)(Goldberg and Burstone, 1982). The control slides were allowed to remain horizontal.

Scanning electron microscopy revealed that a multilayered ligament-like structure had formed a collar around the periphery of each disk (Whyte et al., 1988). This collar was

continuous with the tissue culture on the glass slide and it was believed to be responsible for the attachment of the disk to the glass slide.

After 48 hours, in contrast to the nonstressed samples, the experimentally stressed samples exhibited a statistically significant increase in the ratio of newly synthesized type III collagen to the total newly synthesized type I and type III collagen (Yen et al.; 1990). The continuous stress application produced changes in the collagen phenotype synthesis consistent with the results obtained from in vivo and organ culture models used to stress sutural tissues (Duncan et al., 1984; Yen et al., 1989b). The increase in the proportion of de novo type III collagen appeared to peak at 36 hours and return to nonstressed levels by 72 hours following the onset of stress application (Pollit, 1987).

This model attempts to produce a ligament-like structure, between two hard surfaces, analogous to craniofacial sutures. This model applies a continuous shear force to the tissue culture. The force is concentrated in the ligament-like attachment and the cells adjacent to the disk.

The force could be altered by changing the weight of the disk or changing the angle to which the glass slide, with the attached disk, is tipped. Although intermittent force application had not been performed with this model, it was believed that it was possible. Intermittent force application would involve repeatedly tipping the glass slide, with the

attached disk, to a prescribed angle followed by returning the glass slide to a horizontal position.

In order to produce tension within the tissue culture, with this model, the disk attached to the superior surface of the glass slide would have to be inverted. TMA wire was used to fabricate frames that would allow the glass slides with the attached disks to be held in an inverted position. The disks, of two different weights, were left on the glass slides between 15 to 45 days. In all cases, inversion of the glass slide caused the disk to fall off the glass slide, although in one case this did not occur until after the first thirty minutes. It is very difficult to use this model to create tension in vitro.

A major problem with this model was the high rate of attrition of the cell cultures, during the time between subculturing and the onset of force application. Cell cultures were lost due to infection and tissue death. This results in a reduction in the sample size and the possible need to alter the design of an experiment.

The high rate of the loss of samples was attributed to the inordinate time the cells were maintained in tissue culture. Typically the explant cultures were 14 to 21 days old prior to subculturing onto glass slides. The disks were placed on the glass slides 3 to 5 days following subculturing and then the attachment to the disk was allowed to form over the

next 15 to 18 days. The experimental application of stress lasted as long as 5 days.

In order to overcome the problem of cell culture attrition, alterations in the model were instituted. The time in explant culture was limited to 10 to 14 days. In an attempt to shorten the time between subculturing and the onset of force application, a greater number of cells were subcultured onto the glass slides and the glass slides with the disk were tipped to 45 and 75 degrees over a period of 8 to 15 days following placement of the titanium disks.

It was discovered that the attachment of the cell culture to the disk required 15 days despite the increased concentration of cells subcultured unto the glass slide. Attempts to tip the glass slides to either 45 degrees or 75 degrees prior to 15 days resulted in virtually all of the disks falling off the glass slides.

An attempt was made to use this model to intermittently stress mouse interparietal fibroblast cultures. The disks were placed on the glass slides and allowed to form an attachment over a period of 15 to 18 days. The culture dishes were placed on a rack that permitted the glass slides to be repeatedly tipped to 45 degrees from a horizontal position. The slides were maintained at the 45 degree angle, or in the horizontal position, for thirty minute intervals.

After the first hour, 6 of 22 disks had dislodged from their attachment to the glass slide. An additional 12 disks

fell off the glass slides over the next two hours of intermittent force application and by 5 hours no disks were left attached to the glass slides.

A variety of cell types were investigated in order to determine which was best able to survive for long periods of time in tissue culture. It was also hoped that a different cell type may be able to form a stronger attachment between the glass slide and the disk. If such a cell line could be found, it would make it easier to maintain a reasonable sample size and it may enable intermittent stress application with this model.

Periodontal ligament tissue was harvested from humans, pigs and rats. Attempts to grow periodontal ligament cells in vitro provided cultures with only a limited number of cells. These cultures tended to be slow growing and none reached confluence.

Gingival tissue from humans, pigs, rats and mice was grown in tissue culture. All of these tissues grew well, with the exception of the porcine gingiva which grew moderately well. Although dentally significant, these tissues were not sutural and the present investigation was primarily interested in sutural tissues. Further the sources of the human and porcine gingiva ceased to be able to supply these tissues.

Rat and mouse interparietal tissue cultures grew well. Of all the tissues studied the rat interparietal tissue was best able to survive for long periods of time in vitro. Although

even with rat sutural tissue there was a significant decrease in the number of viable samples if they were maintained in tissue culture for more than 40 days.

To minimize the sources of infection many modifications to the tissue culture technique were instituted. However, the loss of in vitro samples seemed to be related to the length of time the tissues were maintained in the culture plates rather than the hygiene protocol.

The rat tissues (interparietal sutural and gingival fibroblasts) were observed to survive better in tissue culture compared to the other cell types studied. The tissue donor, a 5 week old Sprague-Dawley male rat, was chosen because the Sprague-Dawley rat has a peak in its relative amount of type III collagen synthesis, in the interparietal suture, at 5 to 6 weeks of age. This increase corresponds to the adolescent growth spurt (Loo et al., 1988). It is expected that by using these rapidly growing sutural tissues that any changes in the proportion of type III collagen synthesis in response to mechanical stimulation would be more dramatic, easier to identify and provide a greater opportunity to appreciate any subtle changes.

Unfortunately, the rat fibroblasts when subcultured onto the glass slides tended to migrate off the glass substrate and spread out over the plastic growth plate bottom. After about 5 days very few fibroblasts remained on the glass coverslip.

Small plastic slides made of culture plate material were substituted for the glass slides. Rat interparietal sutural fibroblasts were subcultured unto these slides. Several slides were placed in each 30mm petri dish used in this study. The fibroblasts grew on the slides and readily migrated off the slides and grew over the entire growth dish bottom. The disks were placed on the glass slides upon confluence and left in place for 18 days. The slides were separated from the dish bottom by means of a scalpel. It was found that any attempt to cut the plastic slides free from the bottom of the dish resulted in loss of the disk attachment and significant damage to the tissue layer.

In order to encourage the rat fibroblasts to grow on the glass slides, the glass slides were coated with collagen. This modification resulted in the rat fibroblasts growing to confluence on the glass slides with very little migration off the glass slide.

The disks were placed on the glass slides for a period of 15 days and then 40 samples were continuously stressed by placing 20 slides at a 45 degree angle and 20 slides at a 75 degree angle. All 20 slides tipped to 45 degrees retained their attached disks in a stable position over a three week period. The continuous stress applied by placing glass slides at a 75 degree angle caused two disks to become dislodged immediately and 5 more over a period of three weeks.

An additional 20 glass slides with attached disks were intermittently stressed by placing the slides at an angle of 45 degrees for one hour followed by one hour of being left flat. Over a period of 12 hours 12 of the disks had become dislodged. For 6 different glass slides with attached disks the interval of being stressed and relaxed was reduced to 10 seconds. In this case, after 30 cycles only 2 of the disks had fallen off the glass slides, however this still represents 1/3 of the total sample.

This model appears to provide a means of applying continuous stress to a tissue culture but it is not a feasible method of applying intermittent stress to a tissue culture. The loss of disk attachment is too great during intermittent loading of the disks, resulting in a significant reduction in the overall sample size. Even the glass slides that still have the disks attached are of questionable value since the attachment is undoubtedly damaged by the intermittent force application.

Interestingly, when disks were left on glass slides coated with collagen, but without the addition of any cells, they could be tipped to 45 or 75 degrees without significant movement of the disks. Only 2 of 20 disks fell off the glass slides when the glass slides were placed at a 45 degree angle for 32 days. One of 10 disks became dislodged upon initially tilting the glass slides to 75 degrees and 4 more slipped off the glass slides over a 3 week period. From these results it

appears as though the disk attachment to the collagen coated glass slides is largely due to the collagen coating and the addition of fibroblasts only marginally improved the disks ability to avoid being dislodged during continuous stress application. Therefore it is suspected that the addition of a collagen coating to the glass slides significantly reduces the stress that is being applied to the tissue culture during continuous stress application, thus reducing the effectiveness of this model as a means of applying stress in vitro.

While the previous model was being modified, another model was also being investigated. The Petriperm® model had been shown to be capable of producing continuous and intermittent stress in bone cell cultures (Hasegawa et al., 1985). This model overcame several of the problems encountered with the previous model. It permitted both continuous and intermittent loading; it required the cells under investigation to be maintained for reasonably short periods of time in tissue culture; the applied stress generated strain throughout the tissue layer; a variety of different cell types could be grown in the Petriperm® dishes; and the magnitude of the force could be altered as well as the frequency with which the force is applied. In this model the cells are grown in a monlayer and do not form the multilayered ligament-like attachment between two hard surfaces, an advantage of the previous model.

In the Petriperm® model the stress is manually applied or removed, thus the ability to use this model for short intervals of stress application and relaxation is limited. It is also difficult to simultaneously stress a large number of tissue culture samples. Therefore experiments of long duration or involving large sample sizes would involve considerable manpower needs.

The Petriperm model was used in two pilot studies that involved rat interparietal and gingival fibroblasts respectively. In each study the ratio of de novo type III collagen to the total de novo type I and type III collagen following continuous and intermittent mechanical stretching was investigated. Adapting the method of Hasegawa and coworkers (1985) rat sutural fibroblasts were subcultured into 50 mm round elastic bottom (Petriperm) plates and mechanically stressed following confluence. The cell layer was placed under tensile stress by centering each dish on a glass dome and then resting a 670g weight on the top of the dish. The tension was either applied continuously or intermittently (1 hour stress/1 hour relaxation) for up to 12 hours. Two hours prior to sample collection (at 0, 2, 6 and 12 hours), 3 dishes in each of the two experimental groups and the unstressed control group were labelled with ^{14}C-Glycine. The cells and mediums were collected for collagen extraction followed by SDS-Page electrophoresis. The gels were dried, impregnated with fluor and exposed to x-ray films which were subsequently

developed and densitometrically scanned for collagen types I and III. Although the cells grew well on the Petriperm® growth substrate, the developed x-ray films were not able to be read due a lack of separation of the type I and type III alpha chains during SDS-Page electrophoresis. This was believed to be due to a lack of familiarity with recently acquired hardware which allowed the use of smaller gels. These gels could be run in significantly shorter periods of time than the more familiar larger gels. It could also have been due to aberrant collagen synthesis by the tissue cultures or inappropriate handling of the samples during the collagen extraction procedures.

This model is studied in greater depth in the following section.

THE EFFECTS OF CONTINUOUS AND INTERMITTENT STRESS ON RAT INTERPARIETAL FIBROBLASTS IN VITRO

THE EFFECTS OF CONTINUOUS AND INTERMITTENT STRESS ON RAT INTERPARIETAL FIBROBLASTS IN VITRO

INTRODUCTION

Fibrous sutural joints undergo physiologic remodelling during growth and development changes in order to maintain the relationship between the tooth and its periodontium and between the articulating bones of the craniofacial skeleton. This remodelling does not cease, but continues at a decreased rate into adulthood in order to allow the stomatognathic system to adapt to alterations in function, the occlusion and the status of the tooth supporting structures. It has been demonstrated histologically that the remodelling process, which involves the hard and soft connective tissues of the craniofacial sutures, is required for orthodontic tooth movement and facial orthopaedic change (Roberts et al., 1981; Reitan, 1985; Rygh, 1986). Although the gross and histologic changes in morphology associated with this process have been extensively reported, there is little information regarding the dynamic cellular and molecular mechanisms underlying these changes. A better understanding of the biologic events in connective tissue remodelling will aid in improving clinical techniques.

Various models that stretch the substrate upon which cells have been cultured in vitro have been used to observe the effect of tensile strain on cellular activities. Harrell

et al., (1977) attached an orthodontic expansion screw to the polystyrene bottom of tissue culture dishes, in order to generate continuous tension within osteoblast tissue cultures. The stress resulted in increased proliferation of the osteoblasts. In another model, Leung and coworkers (1976) were able to intermittently stretch the elastic membrane upon which rabbit aortic smooth muscle cells were grown and observe an increase in protein synthesis, but not an increase in the proportion of type III collagen synthesis relative to the total collagen synthesis (Leung et al., 1976, 1977). Buck (1980) developed a model that intermittently stretched fibroblasts in vitro. He was able to demonstrate that the fibroblasts alter their orientation to reflect the line of force application. None of these models was capable of producing both intermittent and continuous stress.

Hasegawa et al., (1985) described a model that produced both intermittent and continuous stretching, of calvarial bone cells grown in petriperm dishes. The bone cells increased their DNA and overall protein synthesis in response to the applied stress. In the present research this model has been modified to permit the investigation of the effects of intermittent and continuous loading on sutural fibroblasts grown in tissue culture.

The effect of the induced stress on the cranial sutural fibroblasts in tissue culture was studied by light microscopic techniques. It is important to know whether the model destroys

tissue architecture or if it operates within the bioelastic limits of the tissue culture in order to better understand the results of the biochemical investigations.

In general, cyclic stressing of connective tissue in vitro has been shown to increase protein synthesis. Rabbit aortic smooth muscle cells are stimulated to increase protein synthesis (Leung et al., 1976, 1977; Sottiurai et al., 1983) following intermittent stretching. An increase in collagenous and noncollageneous protein synthesis was produced by intermittent stretching of bovine aortic smooth muscle cells (Sumpio et al., 1988b), porcine aortic smooth muscle cells (Sumpio and Banes, 1988a) and human pulpal fibroblasts (Levin et al., 1988). The cyclic stressing of osteoblasts from chick calvaria was observed to result in a decrease in both collagen and noncollagen protein synthesis (Buckley et al., 1990).

Following cyclic stretching of bone cells, grown in Petriperm® dishes, there was no change in the amount of total collagen synthesis although the amount of noncollagenous protein synthesis increased (Hasegawa et al., 1985). Using a model similar to that reported by Hasegawa and coworkers (1985) and by measuring radioactively labelled proline incorporation, an attempt was made to determine the effects of continuous and intermittent stress on in vitro rat interparietal fibroblast collagenous and noncollagenous protein synthesis.

Collagen is the main extracellular component of the connective tissues (Kivirikko and Risteli, 1976; Bornstein and Traub, 1979) including fibrous sutures (Barbanell et al., 1978). Collagen is not a homogeneous protein, rather it is a family of structural proteins consisting of several different phenotypes, whose distribution and relative proportions are tissue specific (Tanzer, 1988).

The relative proportion of collagen phenotypes being synthesized changes during growth and development, during tissue repair and with changes in the functional demands on the tissue (Bornstein and Sage, 1980; Gomez et al., 1989; Pinnell et al., 1987). In fibrous sutural joints, an increase in type III synthesis is observed during the rapid remodelling associated with growth and development and during tissue response to an applied mechanical stress (Yen et al., 1980, 1989a, 1989b; Duncan et al., 1984). The modulation of the relative proportions of newly synthesized collagen phenotypes, especially that of type I and type III, serves as an important parameter of the metabolic state of the connective tissue (Chung and Miller, 1974; Epstein, 1974; Butler et al., 1975a, 1975b; Gay and Miller, 1978). Thus, the change in the ratio of type III collagen synthesis to type I collagen synthesis may be used to demonstrate changing cellular activities associated with remodelling.

Collagen phenotype synthesis fluctuates, with an increase in type III collagen synthesis, following the application of

stress to sutural joints of the adolescent mouse (Yen et al., 1980; Duncan et al., 1984) and neonatal rabbit (Meikle et al., 1982). Similar findings were observed in sutural fibroblast tissue cultures undergoing mechanical stress (Pollit, 1987; Yen and Suga, 1988).

The investigations involving the influence of mechanical stress on fibrous sutural joint remodelling have used models involving continuous force application. The present investigation will attempt to observe the in vitro remodelling response of rat interparietal fibroblasts, in terms of collagen phenotype synthesis, during both continuous and intermittent loading patterns.

MATERIALS AND METHODS

DISSECTION AND PREPARATION OF IN VITRO CULTURES

Sprague Dawley male rats aged 5 weeks, inbred in our facility, had surgery performed under ether anaesthesia (Mallinckrodt, Inc., Paris, Kentucky). Following cervical section of the vertebral column, a midsagittal incision was made through the scalp to expose the calvaria. Overlying periosteum was removed and interparietal sutures were excised with about 1mm of adjacent bone, and placed in medium, prewarmed to 37°C.

The growth medium consisted of DMEM (Dulbecco's minimum essential medium with L-glutamine, without ribonucleosides and deoxyribonucleosides)(Gibco/BRL, Burlington, Ont.), penicillin G (400 units per ml) and streptomycin sulphate (0.56mg per ml)

(Gibco, Grand Island, N.Y.), ascorbic acid (0.2mg per ml) (Sigma Chemical Co., St. Louis, MO), and sodium bicarbonate (2.2mg per ml) (Fisher Scientific, Fair Lawn, NJ), and fungizone (10 μL/ml)(Flow Laboratories, Inc., Mississauga, Ont.), pH 7.4. The medium was filter-sterilized using a Millipore 0.22-μm filter (Millipore Corp., Mississauga, Ont.). To this was added sterile fetal calf serum (Boknek, Rexdale, Ont.) so that it made up 10% of the volume of the growth medium.

Following surgery, subsequent manipulations of the sutural tissues were conducted in a laminar flow hood (Nuaire, Minneapolis, Minnesota). Any adherent extraneous soft tissue was removed from the sutures. The sutures were washed three times in growth medium to remove blood and any soft tissue debris. The explants were cut into 1.5mm square segments and randomly placed into 60mm petri dishes previously coated with a thin layer of 100% fetal calf serum (to help anchor the explants) and prewarmed for one hour at 35°C. Each dish contained about eight evenly distributed explant segments. To these dishes was added 4ml of growth medium.

The dishes were incubated (National incubator model 4200, Portland, Oregon) in a humidified atmosphere of 5% CO_2 at 37°C. Three ml of the growth medium was replaced every two days and the cell growth was monitored with an inverted phase contrast microscope (Nikon, Japan) until confluence.

SUBCULTURING

Upon confluence, usually within two weeks, the fibroblasts were subcultured into 50mm culture plates with a flexible plastic growth surface (Petriperm, Tekmar, Cincinnati, OH). The explant dishes were washed three times with 3ml of Hanks Buffered Saline. For 30 seconds, 2ml of recently thawed trypsin (Coopes Biomedical, Malvern, PA) was left in the petri dishes. The majority of the trypsin was decanted off and the dishes were incubated at 37°C for 3 minutes until most of the fibroblast cells had rounded up and detached from the substrate. The cells were resuspended in 2ml of growth medium and a 100μl aliquot was counted using a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). Appropriate volumes of the cell suspension were added to 3 ml of prewarmed medium in Petriperm® dishes so that each dish received 500,000 cells. The volume of medium in each dish was made up to 4ml. After 10 days all the dishes had reached confluence.

STRESS MECHANISM

The model for stretching the tissue culture has been previously described by Hasegawa et al., (1985). The elastic nature of the Petriperm dish growth surface enables reversible deformation thus permitting alternating periods of mechanical loading and relaxation (ie. intermittent force application).

In this experiment, confluent cell layers were placed under tensile stress by placing a 670g weight on the top of

petriperm dishes that had been centered on the convex surface of inverted watch glasses. The uniformly curved glass surface should produce strain within the total surface of the dish bottom. Following loading the change in surface area of the growth substrate was less than 4%. This strain is consistent with that produced by Hasegawa and coworkers (1985).

LIGHT MICROSCOPY

The three control dishes were unstressed throughout the experiment while there were two dishes assigned to each of the experimental groups. The continuously stressed dishes were stretched from time 0 to time 12 hours (with the exception of the time required for photography). The intermittent force was applied for one hour followed by one hour of relaxation.

Photographs of the unstressed controls were recorded at time zero (0 hours) and at the termination of the experiment (12 hours). The continuously stressed dishes were photographed at time zero (0 hours) as well as at 8 and 12 hours following initial force application. Photographs of the intermittently stretched cultures were obtained at time zero (0 hours), 10 minutes, 1 hour, 4 hours, 8 hours and 12 hours following initial force application.

Photographs were recorded by means of a Nikon camera body attached to a Nikon Diaphot-TMD inverted phase contrast microscope (Nikon, Nippon Kogaku K.K., Japan) using Panatomic X 32 150 32/16° black and white film (Kodak Canada Inc.,

Toronto, Ont.). The photographs were obtained at magnifications of 40X, 100X, 200X and 400X.

A COLLAGENOUS AND NONCOLLAGENOUS PROTEIN SYNTHESIS STUDY

The intermittent force was applied for one hour followed by one hour of relaxation to 4 dishes. Another 4 dishes received continuous stretching and 5 dishes were unstressed controls. Dishes of each experimental group were harvested at 4 and 12 hours after initiating the mechanical stressing. The total number of dishes used was 13.

One dish was harvested at the beginning of the experiment (time 0 hours). One unstressed control dish was collected at 4 hours and three control dishes were collected at 12 hours following the beginning of the experiment.

One continuously stretched dish was collected at 4 hours following initial force application while three continuously stretched dishes were collected at the termination of the experiment (time 12 hours). Similarly, an intermittently stretched dish (one hour of force application/one hour of relaxation) was collected at 4 hours and three were collected at 12 hours.

Radioactive Labelling

Two hours prior to termination, to each sample was added 10 uCi/ml of H^3-proline (CFA.30, Amersham Corp., Oakville, Ont., specific activity 56.0 mCi/mol). The medium and cell layers were collected separately and dialyzed against 500 ml

of cold medium followed by two changes of 50 mM Tris/HCL buffer pH 7.6 with 5 mM $CaCl_2$. In microcentrifuge tubes, 250 ml samples were digested with 16 μl of 10 μg/ml of Clostridium histolyticum collagenase in 0.05 M Tris and 0.01 μM Ca acetate for 1 hour at 37°C and then centrifuged. This supernatant which contains collagenase digestible protein, was set aside while the pellet was resuspended in 300 ul of 7% trichloroacetic acid (TCA). The resuspended pellet was centrifuged and this supernatant contained residual collagen polypeptides dissolved in the TCA. This supernatant was combined with the previously collected supernatant to become sample A.

To the second precipitate was added fresh 7% TCA. This combination was left in a hot water (80°C) shaker bath for 1 hour and then centrifuged. The resultant supernatant contained residual collagen and was labelled sample B.

The resultant pellet was twice incubated overnight with 50 μl of 70% formic acid at 37°C and the solubilized noncollagenous protein was collected as samples C and D respectively.

The amount of radioactivity in each sample was determined by dissolving appropriate aliquots in 5 ml of ecolume and counting for 10 minutes on a liquid scintillation counter (Beckman LS 5801). The scintillation counts were adjusted to account for the background. To accomplish this blank samples of 5 ml ecolume were analyzed in the scintillation counter, as

well as standard samples of A, B, C, and D. Aliquots of the blank and standard samples were added to 5 ml of ecolume. The amount of proline incorporated into collagen was recorded as the total adjusted scintillation counts obtained for samples A and B while the amount of proline incorporated into noncollagenous proteins was recorded as the sum of the adjusted scintillation counts obtained for samples C and D. A one-way analysis of variance was performed on the data collected at the termination of the experiment (12 hour time period).

A COLLAGEN PHENOTYPE STUDY

The intermittent force was applied for one hour followed by one hour of relaxation to 16 dishes. Another 16 dishes received continuous stretching and 20 dishes were unstressed controls. Four dishes of each experimental group were harvested at 2, 4, 8, and 12 hours after initiating the mechanical stressing. In addition four control (unstressed) dishes were collected at time 0 hours. The total number of dishes used was 52.

Two hours prior to termination, to each sample was added 10μCi/ml of ^{14}C-glycine (CFA.30, Amersham Corp., Oakville, Ont., specific activity 56.0mCi/ml). The cultures were terminated by placing the medium in a collection tube and then washing the dish with 1ml of medium, which was later added to the collected medium. The cells were harvested with two 1ml

volumes of 1% acetic acid solution, consecutively added to the dish, combined with vigorous washing using a pasteur pipette.

PEPSIN-SOLUBILIZATION OF COLLAGEN

The acid cell extracts and medium samples were frozen and lyophilized. This was followed by pepsin (0.1mg/ml in 0.5 N acetic acid; Sigma Chemical Co., St. Louis, MO) digestion for 4 hours at 16°C. The samples were then dialysed against 1% HAc for 5 days at 4°C. The samples were once again frozen and lyophilized.

ELECTROPHORESIS

Collagen alpha-chains and procollagens were separated by sodium dodecyl sulphate-polyacrylamide gel on 20-cm slab gels. The procedure followed was a modification of that described by Laemmli (1970). It utilized a 7.5% cross-linked separating gel, a 2.5% stacking gel, and Tris/glycine buffers (Bio-Rad Laboratories, Richmond, CA). Type I and type III collagen alpha-chains were separated by means of interrupted electrophoresis (Sykes et al., 1976).

Freeze-dried samples were dissolved in 70μl of reservoir buffer containing 2M urea, 2% sodium dodecyl sulphate and 0.1% bromophenol blue, and were heated to 60°C for 30 minutes to denature the collagen. Samples were placed in the sample wells and electrophoresis was performed for 1 hour at 160 V. Electrophoresis was halted and the samples reduced by adding 20% mercaptoethanal to the sample wells. This allows the type

III alpha-chains to penetrate the gel. After being allowed to stand for 1 hour, the electrophoresis was resumed at 24mA/gel until the tracking dye reached the base of the gel.

To obtain fluorographic visualization of separated radiolabelled collagen bands, gels were washed twice in dimethyl sulfoxide (Fisher Scientific, Fair Lawn, NJ) and impregnated with 2,5-diphenyloxazole (New England Nuclear, Boston, MA), as described by Bonner and Laskey (1974). The gels were placed on filter paper and dried on a slab drier (Bio-Rad, Richmond, CA) and exposed to Kodak XRP-1 x-ray film (Kodak Canada, Inc., Toronto, Ont.) at -60°C for various periods of time, as indicated by scintillation counts of the samples.

Once developed, individual sample tracks of the fluorograph were scanned at 550nm and the proportion of type III alpha-chains relative to the total of type I and type III alpha-chains calculated on a spectrophotometer (Bio-Rad model 620; Matsushita Electric Industrial Co. Ltd., Japan). Each track was read in at least three different locations and the average value of each track was tabulated. The values obtained were subjected to statistical analysis using a one way analysis of variance and a Steffe's procedure.

RESULTS

A schematic representation of the model used to generate tissue stretching by tensing the petriperm base is illustrated in Fig. 1. **Light Microscopy**

The results are represented by selected photographs. As representative samples, photographs of unstressed and intermittently stressed cultures at time zero and 12 hours following stress application are presented.

The assessment of the morphologic changes observed is quite subjective. In general the stretching of the growth substrate did not tear the cell layer nor did it detach the cell layer from the petriperm dish bottom.

Anecdotally, it was difficult to focus immediately following removal of stress application due to time dependent changes in the growth substrate or tissue layer or both. After 2 to 3 minutes the tissue layer and plate bottom were stable enough to allow observation with only infrequent focus adjustments.

There was no appreciable change in the appearance of the unstressed tissue layer between time zero and termination of the experiment at 12 hours (Figures 2 to 5). At low magnification (40X) the cell layer was reasonably confluent and there were localized areas of a loosely swirled arrangement of cells. At higher magnifications there were a few spaces between cells, and most of the cells were stellate and a few were spindle shaped.

The influence of stress on the cell layer was subtle and any changes in the overall tissue architecture were not readily observed using the inverted phase contrast microscope (Figures 6 to 9). It must be added that the present

investigation was more interested in identifying dramatic changes such as large tissue tears or detachment of the tissue layer from the growth substrate.

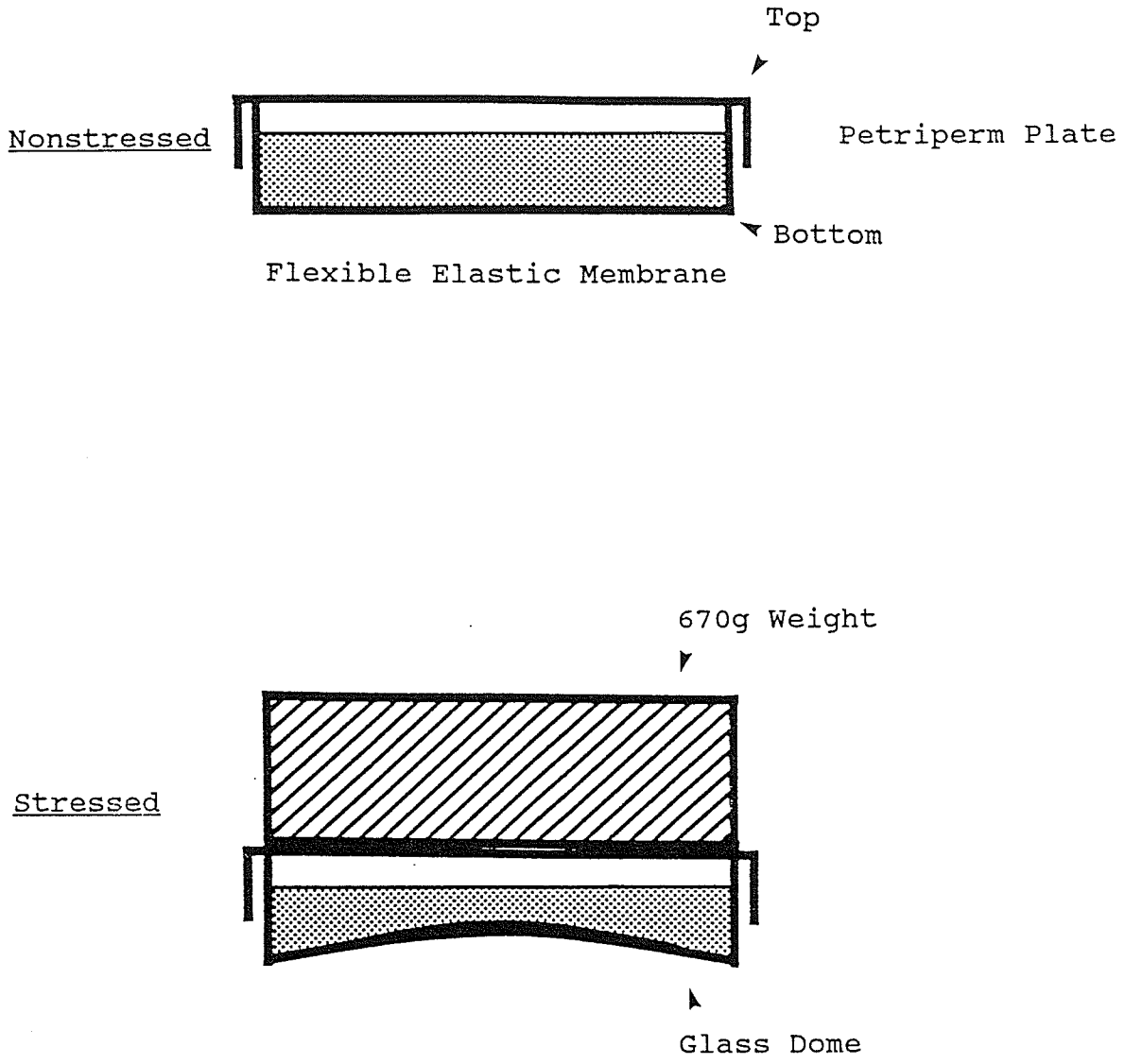


Fig. 1 A schematic representation of the nonstressed and stressed flexible membrane of the petriperm dish.

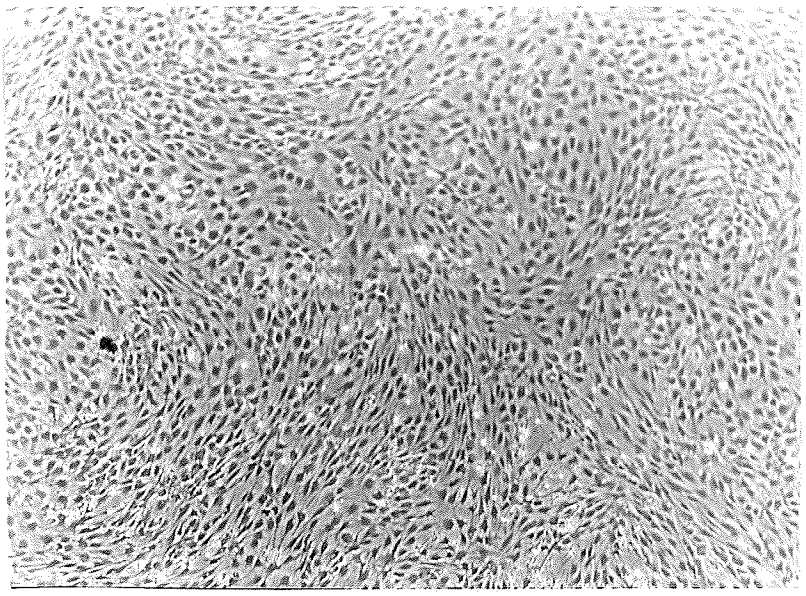


Fig. 2 Photograph (40X) of unstressed control fibroblast culture at time zero.

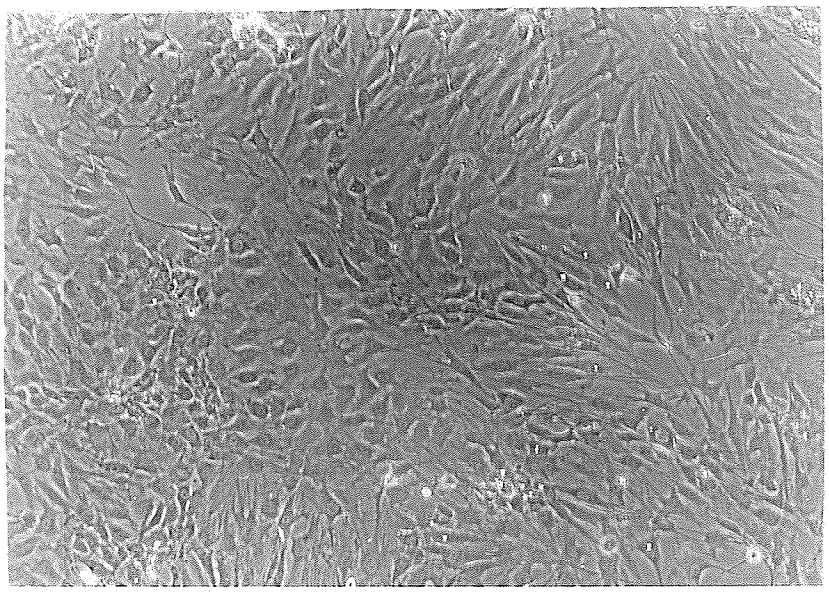


Fig. 3 Photograph (100X) of unstressed control fibroblast culture at time zero.

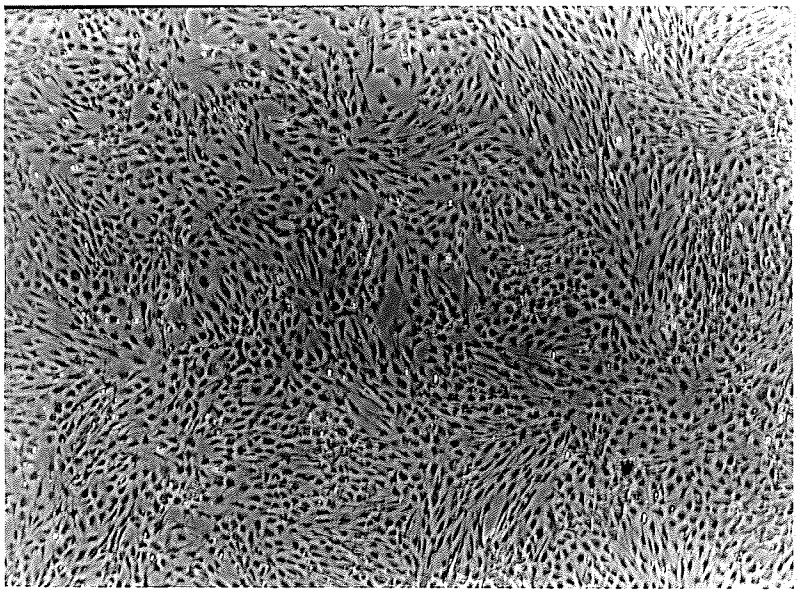


Fig. 4 Photograph (40X) of unstressed control fibroblast culture at 12 hours.

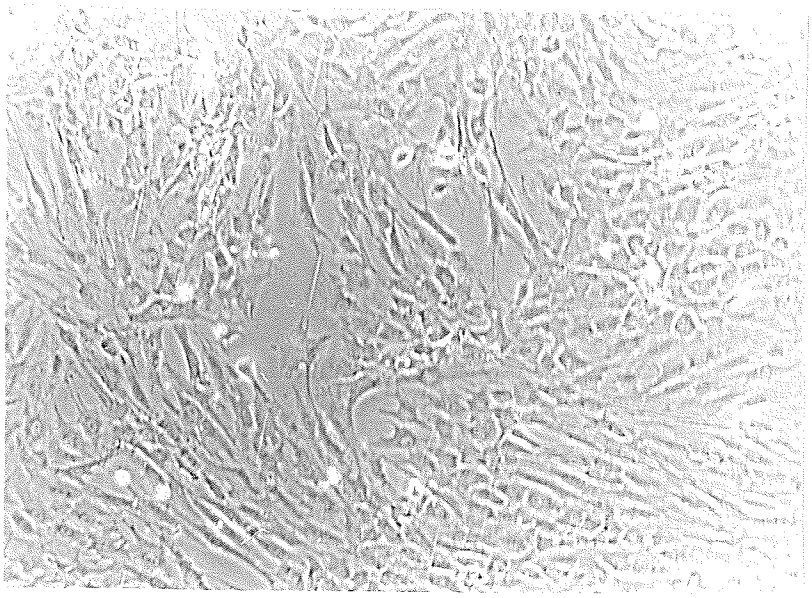


Fig. 5 Photograph (100X) of unstressed control fibroblast culture at 12 hours.

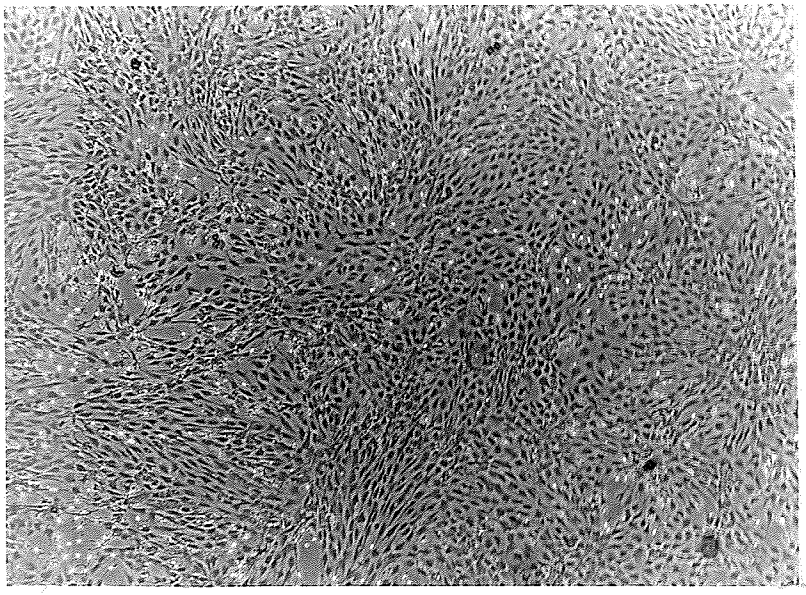


Fig. 6 Photograph (40X) of intermittently stressed fibroblast culture at time zero.

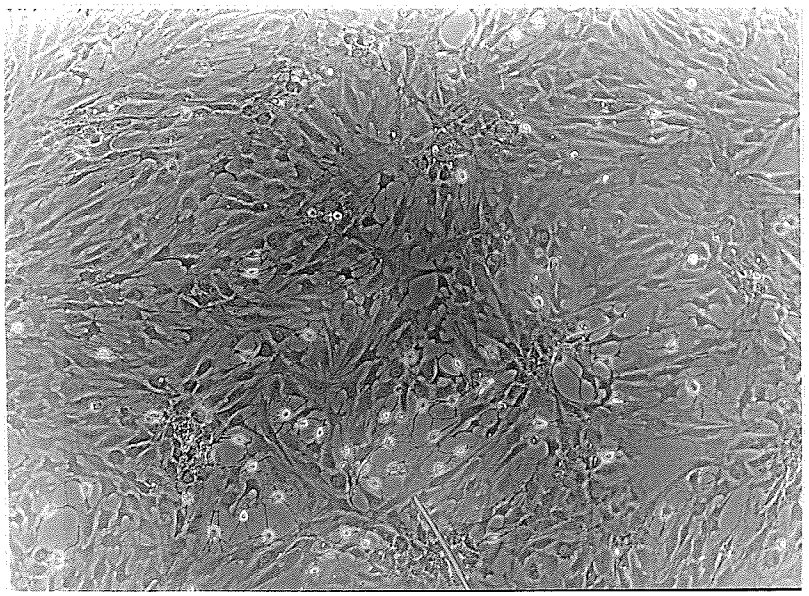


Fig. 7 Photograph (100X) of intermittently stressed fibroblast culture at time zero.

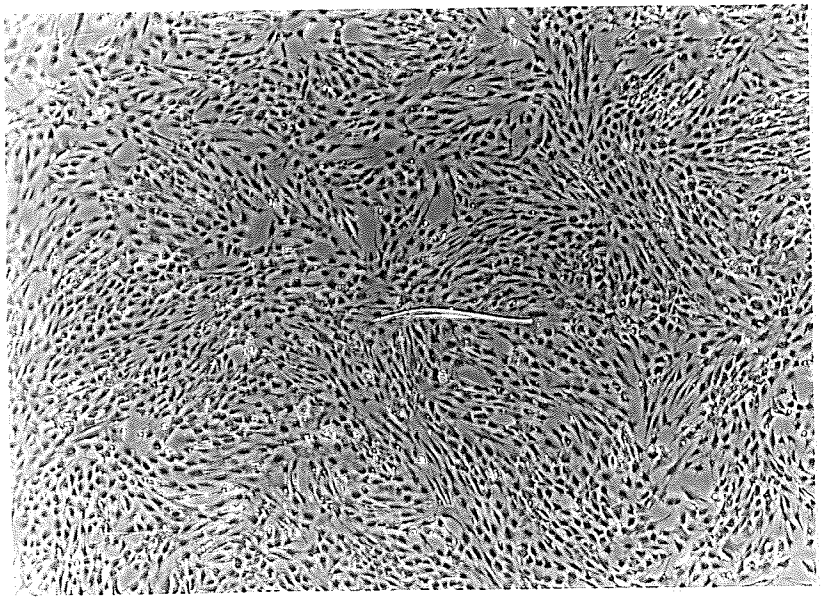


Fig. 8 Photograph (40X) of intermittently stressed fibroblast culture at 12 hours.

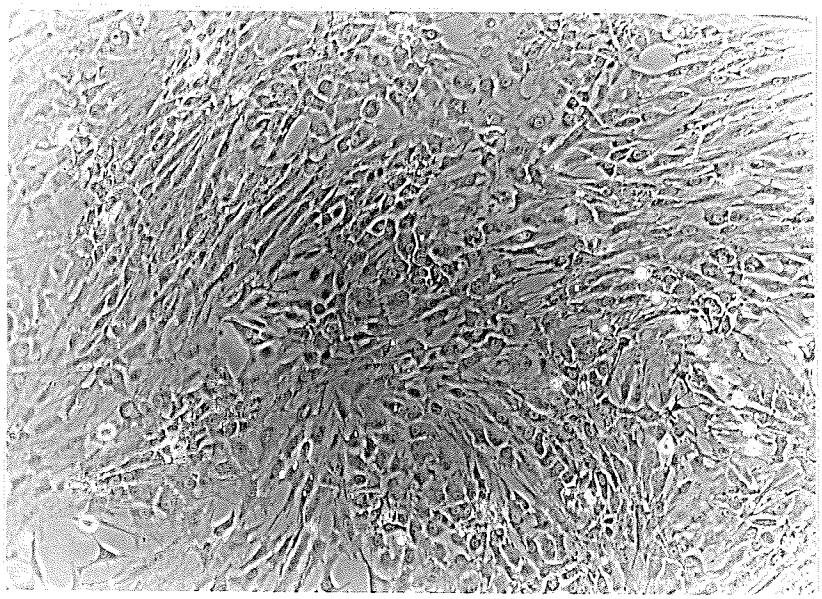


Fig. 9 Photograph (100X) of intermittently stressed fibroblast culture at 12 hours.

COLLAGENOUS AND NONCOLLAGENOUS PROTEIN SYNTHESIS

The results have been tabulated (Table I, II, III and IV) and graphically represented (Figures 10 to 12). The untreated data for samples obtained at 12 hours following the onset of force application are found in appendix A.

The ratio of newly synthesized collagen to total newly synthesized protein (as measured by ^{3}H-proline incorporation) did not change with differing force applications or over time (Tables I and II; Figure 10) for either the cell or medium samples. At the 4 hour interval, the collagenous polypeptide synthesis to the total polypeptide synthesis ratio showed the greatest differences with the intermittently stressed experimental values increasing about only 9% over the control values.

In the cell samples there was an appreciable increase in the collagenous protein synthesis over time for the intermittent force application, this increase was most intense at 4 hours measuring over 20% for the intermittently stressed group and only 8% for the continuously stressed group. The increase in the intermittent group was maintained at the 12 hour interval but to a lesser degree (12%). The changes in the noncollagenous protein synthesis mirrored the increases in the collagenous protein synthesis with the continuously stressed group changing very little from the control group and the intermittently stressed group having its greatest increase at 4 hours (23%) and a minor increase at 12 hours (12%). Thus,

despite observable changes in the amount of protein synthesized with stress application, the ratio of collagenous to noncollagenous protein being synthesized remained the same. However, there was no significant difference in the collagenous or noncollagenous protein synthesis between the differing force applications and the controls at the termination of the experiment.

In the medium samples there was an appreciable increase in the collagenous and noncollagenous protein synthesis with both types of force application. The response of increased collagenous protein synthesis was greatest for intermittent force (peaking at 4 hours) while the response for increased noncollagenous protein was slightly greater with continuous force. For collagenous polypeptides the intermittently stressed group expressed roughly 30% increases over nonstressed values at the 4 and 12 hour time periods while the continuously stressed group had 12 to 20% increases. The noncollagenous polypeptide synthetic activity of the experimental goups was only marginally higher than the nonstressed controls at 4 hours, 11% and 3% for the continuous and intermittent groups respectively. However the 12 hour experimental values for noncollagenous protein synthesis were 30% and 22% higher than the control values, with the continuous group representing the greatest increase. The difference in noncollagenous protein synthesis between the experimental and control groups increased over the 4 to 12

hour interval. It should be noted that the experimentally induced changes in the protein synthesis were not significantly different from the control values.

TABLE I

The Ratio of Collagen Synthesis (CP) to Total Protein Synthesis (CP + NCP) Observed at Various Times in the Cell Samples

[CP/(CP + NCP)]

Time (hours)	0	4	12*
<u>Force Application</u>			
Unstressed Controls	0.340 (n = 1)	0.364 (n = 1)	0.351 (0.034) (n = 3)
Continuous Stress		0.385 (n = 1)	0.336 (0.035) (n = 3)
Intermitten Stress (1 hour intervals)		0.356 (n = 1)	0.358 (0.006) (n = 3)

*For hour 12 the results are presented as X (std.dev.)

n = sample size

TABLE II

The Ratio of Collagen Synthesis (CP) to the Total Collagen and Noncollagen Protein Synthesis (NCP + CP) at Various Times in the Medium Samples

[CP/(CP + NCP)]

Time (hours)	0	4	12*
Force Application			
Unstressed Controls	0.571 (n = 1)	0.590 (n = 1)	0.589 (0.018) (n = 3)
Continous Stress		0.596 (n = 1)	0.576 (0.098) (n = 3)
Intermitten Stress		0.646 (n = 1)	0.606 (0.016) (n = 3)

* For hour 12 the results are presented as X (std.dev.)

n = sample size

Figure 10 The Ratio of Collagen Synthesis (CP) to Total Protein Synthesis (CP + NCP) Observed at Various Times, in the Cell and Medium Samples Following Force Application.

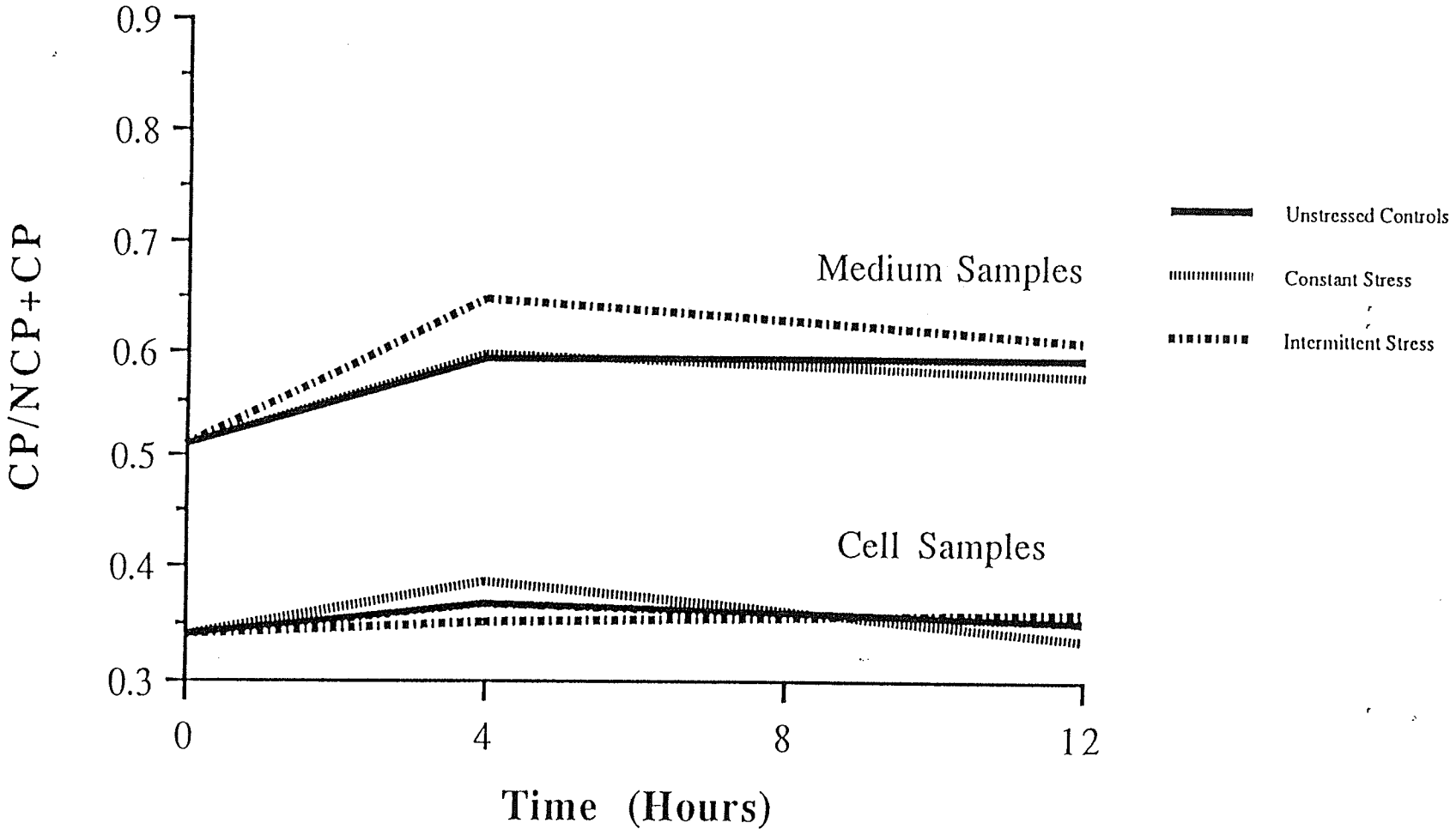


TABLE III

The Medium and Cell Sample Scintillation Counts (dpm) of ^{3}H-Proline Incorporation Into the Collagenous Protein (CP) Fraction of Newly Synthesized Protein Over Time

(CP in dpm)

Time (hours)	0	4	12*
<u>Mediums</u>			
Unstressed Controls	3489.70	3551.80	3296.03 (447.78) (n = 3)
Continuous Stress		4054.70	3948.70 (301.93) (n = 3)
Intermittent Stress		4633.65	4255.95 (25.63) (n = 3)
<u>Cells</u>			
Unstressed Controls	1545.50	1608.50	1552.03 (153.48) (n = 3)
Continuous Stress		1732.70	1522.07 (267.54) (n = 3)
Intermittent Stress		1918.45	1744.80 (270.31) (n = 3)

* 12 hour results reported as X (std.dev.)
n = 1 unless otherwise specified

TABLE IV

The Medium and Cell Sample Scintillation Counts (dpm) of ^{3}H-Proline Incorporation Into the Noncollageneous Protein (NCP) Fraction of Newly Synthesized Proteins at Various Times

(NCP in dpm)

Time (hours)	0	4	12*
Mediums			
Unstressed Controls	2624.70	2473.10	2299.60 (306.81) (n = 3)
Continuous Stress		2742.80	2997.83 (1068.72) (n = 3)
Intermittent Stress		2542.40	2803.3 (173.68) (n = 3)
Cells			
Unstressed Controls	3001.30	2813.00	2877.83 (307.28) (n = 3)
Continuous Stress		2768.86	2986.43 (247.96) (n = 3)
Intermittent Stress		3473.00	3131.70 (442.42) (n = 3)

n = 1 unless othersie specified
* 12 hour results reported as X (std.dev.)

Figure 11 The Cell Sample Scintillation Counts (dpm) of ^{3}H-Proline Incorporation into the Collagenous (CP) and Noncollagenous (NCP) Protein Fractions of Newly Synthesized Proteins at Various Times and with Differing Force Applications

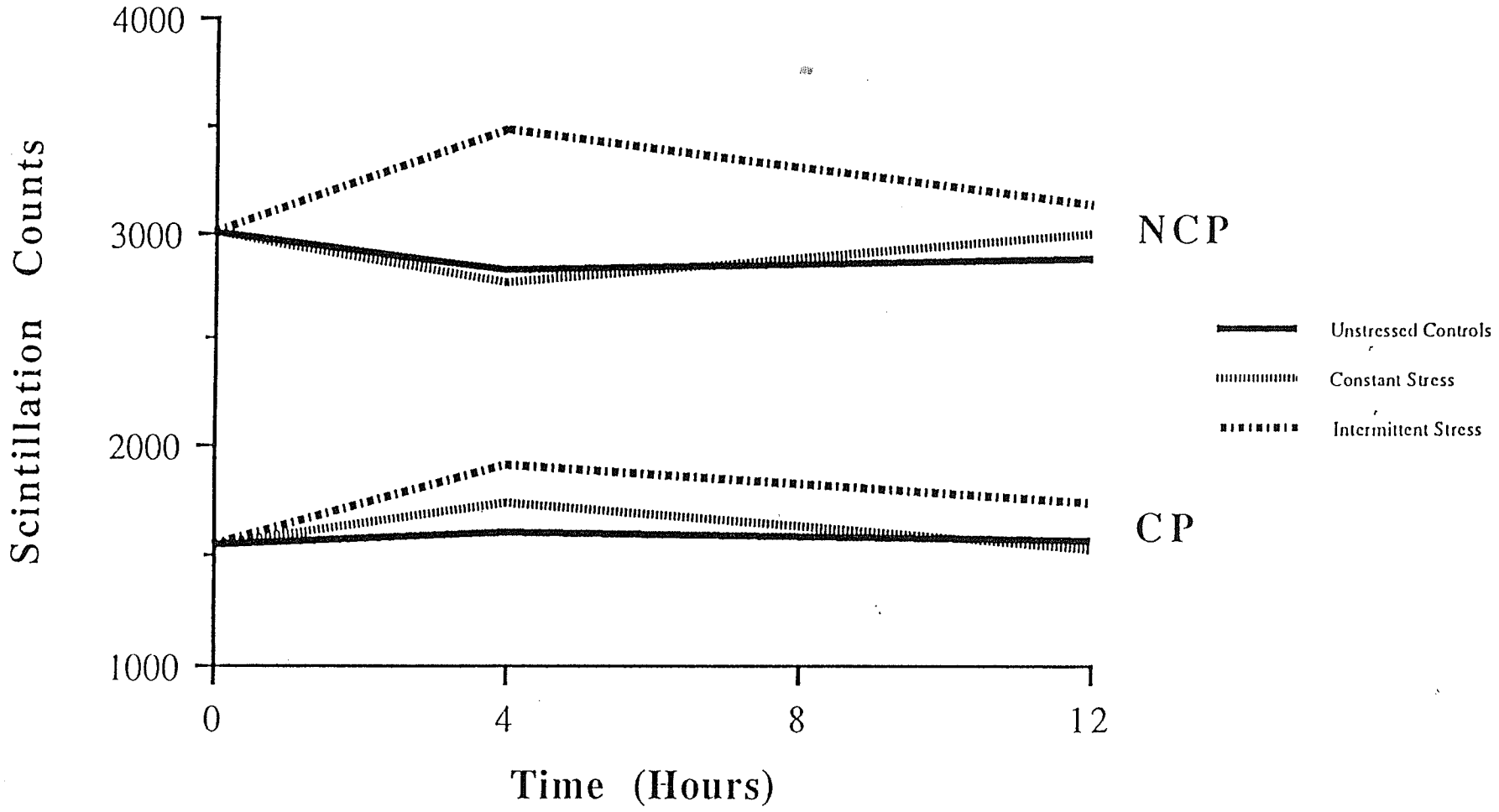
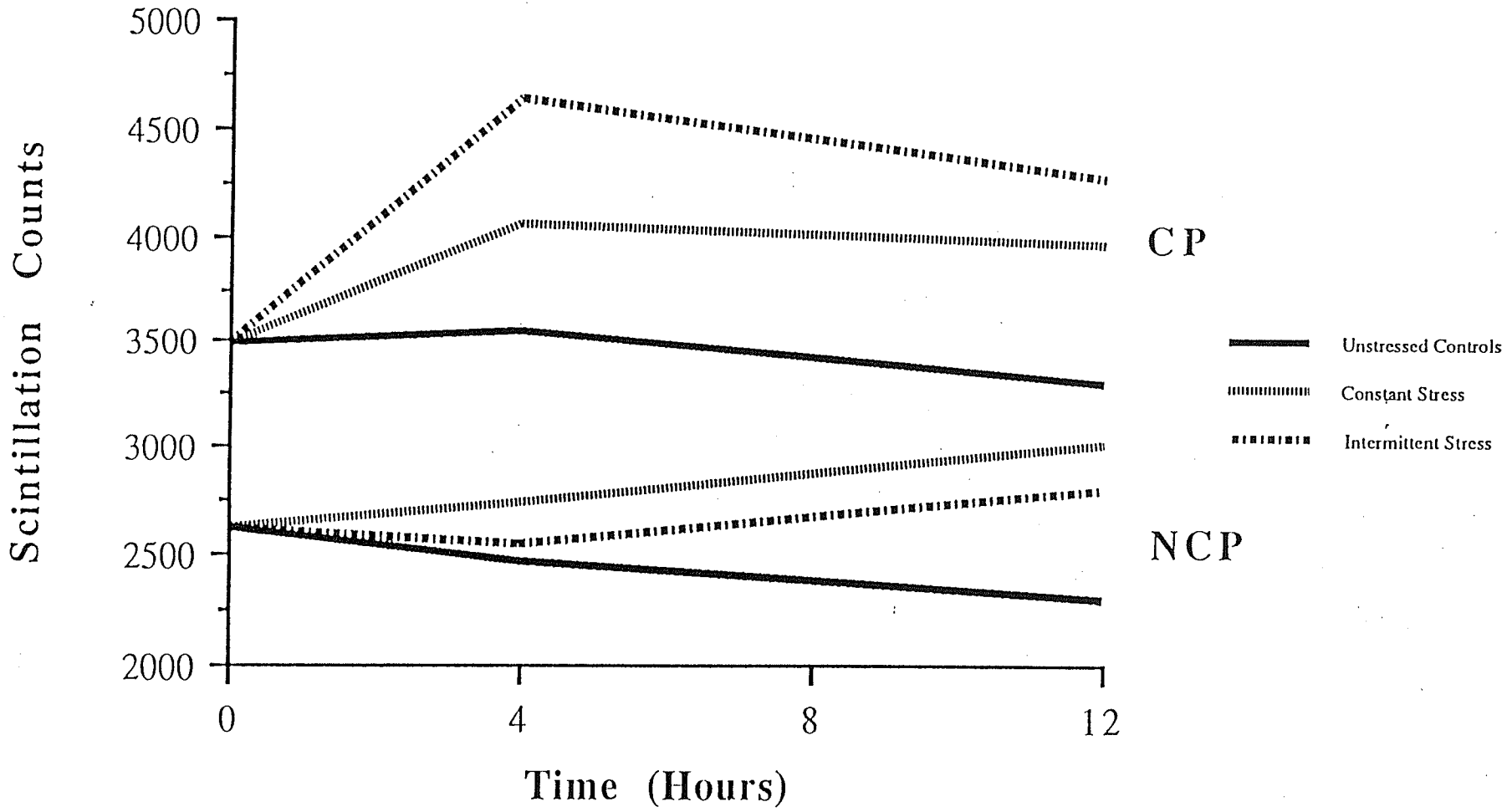


Figure 12 The Medium Sample Scintillation Counts (dpm) of ^{3}H-Proline Incorporation into the Collagenous (CP) and Noncollagenous (NCP) Protein Fractions of Newly Synthesized Proteins at Various times and with Differing Force Applications



COLLAGEN PHENOTYPE

The newly synthesized collagen alpha-chains from the medium and cell samples harvested, after two hours of [^{14}C]-glycine labelling, were extracted and separated by SDS-page gel electrophoresis. The separated radioactive alpha-chain bands of the dried floor impregnated gels were exposed to x-ray films. Following this, the developed photoradiographs (Fig 13) were scanned densitometrically for the relative amounts of collagen alpha-chains (alpha$_1$ (I), alpha$_2$ (I) and alpha$_1$ (III)). A typical spectrophotometer analysis is shown as a line tracing in Fig. 14.

Although medium gels were run for all samples, the photoradiographs did not reveal well separated alpha-chain bands. A densitometric analysis of the relative amounts of the type I and type III alpha-chains was impossible.

The results of spectrophotometric analysis of the fluorographs obtained from the cell samples are found in appendix B. The means and standard deviations of the percentage of newly synthesized collagen type III alpha-chains relative to total collagen synthesis of the controls and experimental treatments (ie, force applications) for the various time periods tested have been tabulated (Table V) and graphed (Fig. 15) for easy reference.

Qualitatively, the stressed cultures increased their proportion of type III collagen synthesis. This peaked at 2 hours for both intermittent and continuous stretching. The

continuous force maintained a reasonably consistent percentage of type III collagen until the 8 to 12 hour interval. In the last time period

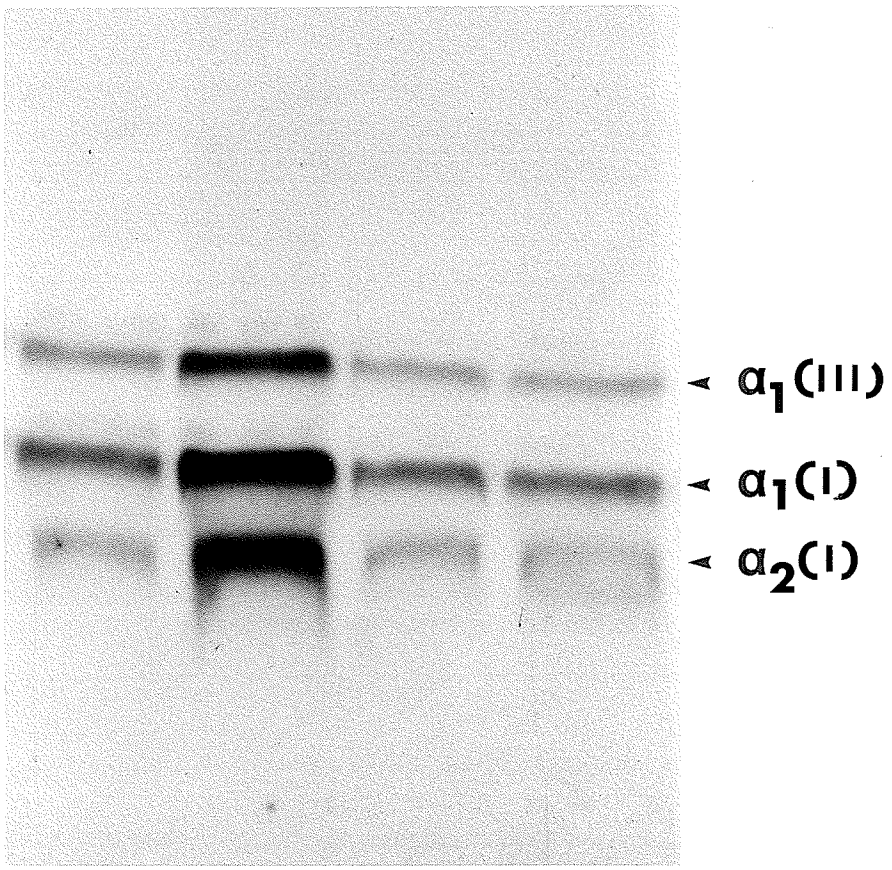


Fig. 13 Photoradiograph of radiolabelled collagen alpha-chains synthesized in tissue culture. The positions expected for the band representing type III alpha-chains and both of the type I alpha-chains are identified.

RUN # 356 AUG/11/89 17:31:58
WORKFILE ID: B
WORKFILE NAME:

AREA%

RT	AREA	TYPE	AR/HT	AREA%
0.50	3.9636E+07	VP	0.354	18.856
1.33	1.2466E+08	PY	0.367	59.307
2.03	4.5902E+07	VP	0.337	21.837

TOTAL AREA= 2.1020E+08
MUL FACTOR= 1.0000E+00

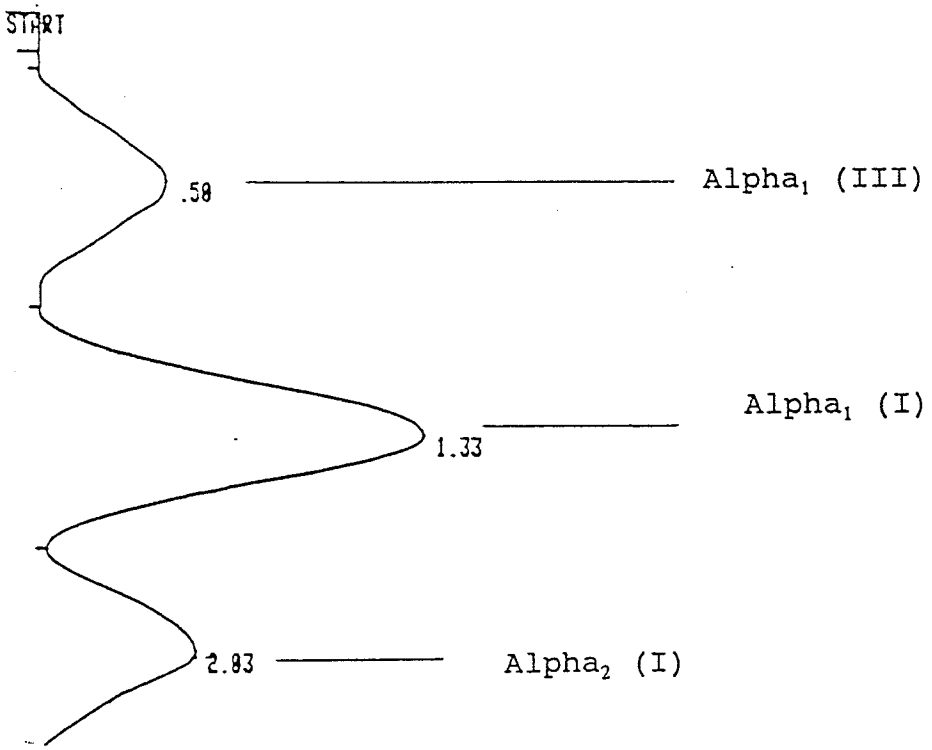


Fig. 14 A tracing depicting the spectrophotometer analysis of the proportion of the type I and type III alpha-chains in the radiolabelled collagen bands visualized in a sample track from a photo-radiograph.

(12 hours) reported the proportion of type III collagen was very similar to the values for nonstressed cultures.

The intermittent force values peaked early (2h) followed by a moderate drop in the proportion of collagen synthesis over the next two observations (4 hours and 8 hours). There was only a minor decrease in the percentage of type III collagen production over the 8 to 12 hour time period. At 12 hours the type III collagen synthesis was still considerably higher than the values recorded for the nonstressed cultures.

The statistical comparisons of the collagen synthesis with respect to stress application and the time periods studied will be discussed in terms of each force design and each time period.

Control (No Force)

There was no significant difference in the relative amount of type III collagen synthesis between any of the times reported.

Intermittent Force

Although there were minor fluctuations in the relative amount of type III collagen synthesis over the 2 to 12 hour time period studied, no time period had a proportion of type III collagen synthesis that was significantly different from any other time period.

Continuous Force

A one way analysis of variance noted a significant difference ($F=6.01$, $p<0.01$) between the various times, with

respect to the proportion of type III collagen synthesis. The 12 hour observation was significantly different from all the other time periods.

Table V

The Proportion Of Newly Synthesized Type III Collagen Alpha-Chains as a Percentage of The Total Newly Synthesized Type I and Type III Collagen Alpha-Chains From The Cell Layer.

	Time (in hours)				
Treatment	0	2	4	8	12
Control (No Force)	10.051 (0.214)	9.652 (1.479)	8.845 (0.743)	9.363 (1.477)	10.378 (1.312)
Intermittent Force		19.720 (3.497)	18.745 (3.514)	16.869 (2.225)	16.497 (2.72)
Continuous * Force		18.106 (3.193)	17.321 (3.059)	17.535 (1.916)	11.231 (2.128)

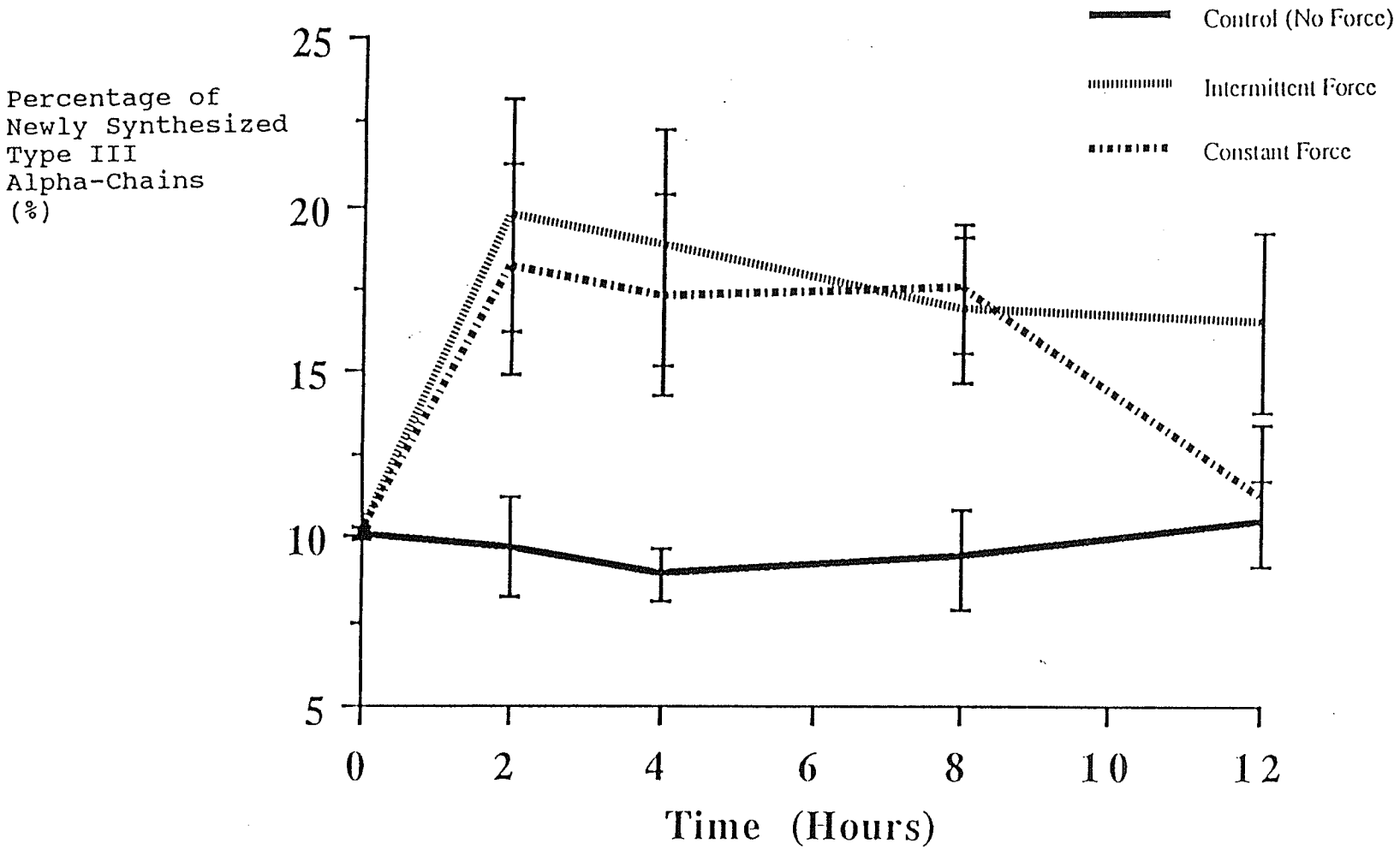
The data presented for each time period and force application is the mean value of the percentage that newly synthesized type III collagen comprises of the total newly synthesized collagen. The corresponding standard deviations are reported in parentheses immediately below the mean values.

The individual sample data is in Appendix B. In this table n=4 for each mean (and standard deviation) with N=52 for total subjects reported.

* One Way ANOVA of rows only found one significant difference between the various time periods for any of the force treatments. Following continuous force application the percentage of type III collagen being synthesized at 12 hours was significantly less than that being synthesized at 2, 4 or 8 hours ($F=6.011$, $p<.01$).

* One Way ANOVA of columns noted that at all time periods, except for the 12 hour continuous force, the experimental treatments were significantly different form the control values.

Fig. 15 The In Vitro Proportion Of Newly Synthesized Type III Collagen Alpha-Chains As A Percentage Of The Total Collagen Alpha-Chains Synthesized By Rat Interparietal Fibroblasts Following Different Force Treatments For Varying Time Periods.



studied. The 12 hour proportion of type III collagen synthesis was lower than the 2, 4 and 8 hour observations.

Time Zero

Only control samples were obtained for this time since it is expected that prior to force application that all the tissue cultures behave similarly. The time zero values were considered to be the starting values for both experimental groups (intermittent and continuous force application) as well as for the control group.

Time 2 Hours

A one way analysis of variance noted a significant difference in the collagen synthesis between the various force designs ($F=14.26$, $p<0.001$). This difference occurred between the control and experimental means. The stressed cultures produced a significantly greater percentage of type III collagen, however, there was no significant difference between the two types of force application.

Time 4 Hours

The one way analysis of variance reported a significant difference ($F=15.45$, $p<.001$) in collagen synthetic activity between the various force applications. The experimental groups were producing a significantly higher proportion of type III collagen than the control groups. The two experimental groups were not significantly different.

Time 8 Hours

The greatest significant difference occurred at this time interval (One way analysis $F=22.88$, $p<.0003$). As for the two previous time intervals the stressed groups were significantly higher than the unstressed group with respect to the relative amount of type III collagen being synthesized. Once again there was no significant difference between the two experimental groups.

Time 12 Hours

This time period revealed the least statistical difference ($F=9.65$, $p<.006$) between the groups separated by force application. The difference was still quite statistically significant and the intermittent force application maintained a significantly greater proportion of type III collagen synthesis over that of the control group (no force). The results of the intermittent group were higher than that of the continuous force group at 12 hours while the continuous force application did not produce significantly different results compared to the unstressed controls.

DISCUSSION

DISCUSSION

It is the capacity of fibrous sutures, periodontal ligament (PDL) and craniofacial sutures, to remodel that enables the successful practise of orthodontics and craniofacial orthopaedics. Presently mechanical force systems are applied to the teeth and craniofacial sutures in order to produce the desired clinical results. In this process, the external mechanical stress is converted to a biologic signal(s), in the tissue, that stimulates the connective tissue cells to begin remodelling. Through greater knowledge of the biologic events involved in orthodontically produced tissue remodelling it is hoped orthodontists will be able to improve their clinical treatment modalities.

The elucidation of the biologic events responsible for orthodontic tooth movement should improve the clinical orthodontic techniques. It is expected that such knowledge will enable the development of treatment modalities that produce efficient tooth movement with minimal untoward sequelae.

Orthodontic tooth movement has been described as inflammatory wound healing (Storey, 1973; Ten Cate et al., 1976) and physiologic tooth movement in response to force application (Roberts et al., 1981). In either case, there is rapid collagen synthesis and remodelling. Since collagen is the primary structural protein of all craniofacial sutures, understanding its remodelling is central to our understanding

of the remodelling of these sutures. Therefore, the remodelling of collagen is an important aspect of the biology of orthodontic tooth movement.

LIGHT MICROSCOPY

The main objective of the light microscopic investigation was to study the Petriperm® model with respect to the overall effect of stress application on the physical status of the tissue layer. It was hoped that we could determine whether or not the tissue layer was torn or detached from the Petriperm® plate bottom. There was no attempt to use this study to investigate more subtle changes (eg. microscopic tears or cell shape changes).

The application of either intermittent or continuous stress did not significantly alter the morphology of the sutural fibroblast tissue layer grown in Petriperm® culture plates. Initially the general cell arrangement consisted of confluent cell layers loosely arranged in localized circular patterns probably reflecting the manner in which the dishes grew out from individual cells following subculturing. The cells were elongated and steliate. Although at higher magnifications there were some areas that appear to be vacant spaces between cells, these areas undoubtedly are covered with extracellular matrix.

There was no detachment of the cell layer coincident to force application. Therefore, any cellular response to Petriperm® growth membrane deformation is due to tissue

stretching. In addition there no evidence of inordinate cellular damage or destruction.

The strain generated in the flexible Petriperm® dish bottom and the cell layer was not instantaneously reversed following relaxation of the external stress. This could be due to a delayed elastic recoil of the dish bottom or cell layer. This observation could also be due to adaptive changes of the cell layer to the external stress.

Upon application of the tensile stress the motile fibroblasts may have spread out over the increased growth surface area. This would minimize the tensile strain experienced by the cells. Following removal of the external stress the growth substrate decreases in area and the redistributed fibroblasts must now adapt to this new environment. If this is the case, removal of the tensile stress would place the cell layer under compression.

Past studies have reported increased motility of stretched fibroblasts as they attempt to align in a way that would minimize an imposed strain. Many investigators have reported the ability of fibroblasts to align in response to the application of stress to a tissue (Bunting and Eades, 1926; Arem and Madden, 1976; Buck, 1980; Banes et al., 1988; Henderson et al., 1988).

No definite changes in the overall alignment of the tissue were observed in response to force application in the present experiment. However, Banes and coworkers (1988) did

not report changes in cellular alignment using the Flexercell Strain Unit® until three days following the onset of stress application, therefore cellular alignment in the petriperm model may require more than 12 hours.

Tension has been shown to alter cellular activity. The mechanism through which tensile stress influences cellular metabolism is unclear, but in the petriperm model it appears not to be via disruption of the in vitro cell layer.

COLLAGENOUS AND NONCOLLAGENOUS PROTEIN SYNTHESIS

The results of the collagenous (CP) and noncollagenous protein (NCP) synthesis study must be interpreted with caution due to the small sample size and the highly variable results observed within each experimental group. The patterns of force application (nonstressed, continuously stressed, intermittently stressed) were represented by one sample at the 4 hour time interval and only one dish was collected at time zero. Only at the termination (time 12 hours) of the investigation were multiple samples (n=3) used for data collection. Thus only the 12 hour results could be subjected to statistical analysis. Despite these serious shortcomings, the discussion will highlight the suggested trends observed.

The inability to easily interpret the findings, of this part of the present experiment, reflect the variability of the CP and NCP synthesis results. The relatively high variability is due to the small sample size employed and possibly other factors as well. For example, the strain level may not be

inappropriate, if it is pathologic it may produce variable cellular responses.

The strain level imposed on the tissue layer was arbitrarily chosen, although it was based on previous research using the Petriperm® model (Hasegawa et al., 1985) and was believed to be within the strain levels generated in the periodontal ligament following the application of tooth moving forces. The Petriperm® model produced less than 4% strain and in vivo the width of the periodontal ligament has been shown to be virtually obliterated with large orthodontic forces and significantly greater than 4% width reduction is expected with even light orthodontic forces (Storey, 1973). It is possible that the in vivo orthodontic tissue strain is pathologic.

It has been reported that the typical physiologic strain levels required to maintain bone mineralization are on the order of 0.2% strain, while 0.2 to 0.6% result in increased bone mineralization (Rubin, 1990). Greater strain levels would be pathologic and could produce aberrant or highly variable tissue response.

In soft tissue in situ, it would not be atypical to see strain values of 4 to 25% (eg. blood vessel walls, lungs, skin, bladder) similar to strain levels produced in vitro with the Petriperm® model. These soft tissues are comprised of cells embedded in shock absorbing matrix that significantly reduces the strain levels experienced on the cellular level. In the abscence of significant matrix support, as in unilayer

in vitro tissue cultures, the strain imposed on the growth substrate is probably almost fully realized on the cellular level. Even 4% in vitro strain may exceed the range of physiologic strain experienced on the cellular level in soft tissues in vivo. If the strain was pathologic this may result in variable tissue response.

In the CP and NCP synthesis study, it appears as though the application of neither continuous nor intermittent stress had any effect on the ratio of de novo collagen to total de novo polypeptides. This is consistent with the report of Miyawaki and Forbes (1987) who used stainless steel expansion springs to stretch rat interparietal sutures and noted no change in the percentage of de novo collagen relative to the total amount of de novo sutural proteins. They did, however, notice an increase in the rate of sutural protein synthesis with stress application. Yen and Suga (1986) studied the expansion of rat interparietal sutures in organ culture and reported an increase in sutural protein synthesis. In another study, the expansion of rabbit interparietal sutures was observed to produce a doubling of the amount of collagen synthesis (Meikle et al., 1979).

These findings could be reconciled if the rate of synthesis for both CP and NCP was increased to the same degree. Thus the CP:NCP ratio would be constant while the amount of protein synthesis increased.

This would be consistent with the anticipated demands of a remodelling tissue. Not only does collagen need to be replaced, but there is also a need for the synthesis of other matrix sutural proteins, extracellular enzymes and intracellular enzymes.

In this study, there is a suggested trend toward an increase in the CP and NCP synthesis at time 4 and 12 hours for both intermittent and continuous force regimens in the medium data and for the intermittent force application in the cell data. The response observed in the cell layer for the intermittently stressed samples was greatest at 4 hours for CP and NCP synthesis and it declined over the 4 to 12 hour interval. The CP and NCP synthetic patterns paralleled each other and thus maintained a continuous CP:(NCP + CP) ratio in the cell samples.

The cell and medium results produced similar trends with respect to collagen synthesis in the intermittently stressed group; a rise in synthesis by 4 hours followed by a subsequent decrease following 12 hours (but the collagen synthesis of the experimental groups tended to be greater than that of the nonstressed controls at 12 hours). The continuously stressed group medium data exhibited similar results for collagenous polypeptide synthesis as derived for the intermittent group but to a lesser degree. (It should be noted that the no statistically significant differences were observed, in this

investigation, between protein synthesis rates of the experimental groups and the control group.)

The NCP synthesis observed in the medium of the stressed groups increased over time and was greatest at 12 hours. This was more pronounced with continuous stress application. It is possible this suggested trend could signal a remodelling response that if measured over longer time periods could reduce the CP:CP + NCP ratio. This is highly speculative, but would be consistent with the findings of Hasegawa et al., (1985). It was observed that rat calvarial osteoblast cultures stretched using the Petriperm model increased their NCP synthesis over time while there was no increase in CP synthesis. This led to a decrease in the CP:(CP + NCP) ratio. Interestingly, these authors also noted, but did not explain, an increase in NCP synthesis by the control samples over time. The increase in the control groups noncollagenous polypeptide synthesis was not as pronounced as that of the experimental group such that by 12 hours following initial force application the NCP synthesis of the experimental group was significantly greater than that of the control group.

In general, cyclic stressing of connective tissues in vitro has been shown to increase protein synthesis. Rabbit aortic smooth muscle tissue cultures are stimulated to increase protein synthesis following intermittent stretching (Leung et al., 1976, 1977; Sottiurai et al., 1983). Other in vitro studies have reported increases in collagenous and

noncollagenous protein synthesis following intermittent stretching of bovine aortic smooth muscle cells (Sumpio et al., 1988b), porcine aortic smooth muscle cells (Sumpio and Banes, 1988a) and human pulpal fibroblasts (Levin et al., 1988). However, not all tissues respond to intermittent stress with an increase in protein synthesis. For example cyclic stretching of chick chondrocytes led to no change in collagen synthesis, although a significant increase in the rate of proteoglycan synthesis was detected (De Witt et al., 1984). The cyclic stretching of osteoblasts from chick calvaria was observed to result in a decrease in both CP and NCP synthesis (Buckley et al., 1990). The synthetic response elicited reflects the cell type being studied. These synthetic activities in response to stress reflect the normal synthetic differences between these tissues.

The results of the present study were too variable to draw any conclusions. This variability is likely due to the small sample size. The present research suggests that there may be an increase in synthetic activity of the cells with stress application; however, this requires further investigation.

COLLAGEN PHENOTYPE

In the present work, the mechanical stress applied to rat interparietal fibroblast tissue cultures produced a fluctuation in the ratio of the synthesis of collagen phenotypes. The results were consistent with in vivo and in

vitro studies that demonstrated an increase in the ratio between newly synthesized type III collagen and total newly synthesized collagen from fibroblasts, derived from fibrous sutures, under the influence of continuous tension (Chaing, 1981; Miekle et al., 1982; Duncan et al., 1984; Yen and Suga, 1986; Pollit, 1987; Yen et al., 1989b) and intermittent tension (Yen and Suga, 1988). For the most part these studies involved continuous force application and none compared the effect of intermittent and continuous loading on the proportion of collagen phenotypes being produced.

Anderson (1991) used the Flexercell Strain Unit® to study the effect of intermittent and continuous stress on the proportion of collagen phenotypes being synthesized by rat gingival fibroblasts. He noted a short lag phase prior to an increase in the proportion of type III collagen synthesis at 4 hours, the increase peaked between 4 to 8 hours and decreased to levels comparable to the nonstressed controls by the end of the experiment (24 hours). This increase in the relative amount of type III collagen being synthesized following intermittent loading was similar to the results observed using rat sutural fibroblasts in the Petriperm® model.

In contrast, the continuously stressed group did not demonstrate a significantly different ratio of type III collagen synthesis to total collagen synthesis compared to the nonstressed control group (Anderson, 1991). The differences in

the results obtained following continuous loading using the Flexercell Strain Unit® and Petriperm® model could reflect differences in the tissue strain generated by the two models. The Flexercell® produces tension in most areas of the dish bottom while it also produces compression in some areas. The Petriperm® produces only tension. The Flexercell® produces a reproducible pattern of tensile strain, while the petriperm dishes are more variable.

Other differences between the two models could be used to explain the contrasting results following continuous loading of rat fibroblasts . The cell types employed in each model were similar but not identical; the magnitude of the maximum strain was much greater in the Flexercell® model than the Petriperm® model; and the Flexercell® model produced a downward deformation of the dish bottom while the Petriperm® model produced an upward deformation of the dish bottom.

In this discussion, all of the studies examining collagen phenotype response have used ^{14}C-glycine to label the collagen alpha-chains. The radiolabelled glycine will be incorporated into a variety of recently synthesized noncollagenous proteins and polypeptide chains. These entities are separated from the radiolabelled collagen alpha-chains during the pepsin-solubilization of collagen and subsequent sodium dodecyl sulphate-polyacrylamide gel electrophoresis. However, the collagen cores of recently synthesized proteoglycans represent a possible source of contamination. These collagen molecules

will not be separated from the collagen alpha-chains, of similar phenotype, derived from fibrillar collagen.

Proteoglycan synthesis occurs early in wound healing (Hunt and Goodson, 1988). This possible source of contamination may significantly effect the results of initial collagen synthesis during remodelling. To determine the possible extent of such contamination the proteoglycan synthesis could be monitored by using an appropriate label (eg. ^{35}sulfur).

In the present study it appears as though the *in vitro* response to tension, within the tissue layer, was rapid as suggested by the increased proportion of type III collagen synthesized, compared to non-stressed controls, seen at the earliest time period (2 hrs) following force application. The percentage of *de novo* type III collagen peaked for both force patterns at 2 hours and the response to intermittent force was slightly higher than that of the continuous force; however, there was no statistically significant difference between the response to intermittent or continuous force at 2, 4 or 8 hours. Furthermore, there was no significant differences in the type III collagen production from 2 to 12 hours of intermittent force nor from 2 to 8 hours of continuous force. Thus, the type III collagen synthetic response to either force design was comparable for the first 8 hours of force application. This suggests that the initial remodelling response did not vary with the pattern of force application.

By 12 hours the tissue cultures experiencing a continuous load had returned to a level of type III collagen synthesis no different than that of the nonstressed controls. The intermittent loading maintained a level of type III collagen synthesis significantly elevated above that of control tissue populations throughout the experiment. This may be due to the remodelling signal being produced upon initial tissue stretching, in the continuous force group and the signal's effects may have dissipated by 12 hours while the intermittently stretched tissue was repeatedly signalled to undergo remodelling.

For example, this signal could be a substance released with tissue tearing. Alternately the continuously stretched cells could have adapted to the altered substrate during the 12 hour period in order to dissipate the tensile strain, whereas the intermittently stretched cultures were never similarly able to adapt due to the frequent changes in their environment. If the change in synthetic activity was due to cell shape change, then repeated stretching would continually signal the cells to alter their collagen phenotype synthesis while in a continuously maintained deformation, of the Petriperm® plate bottom, the cells would only be initially signaled to alter their collagen phenotype synthesis since the motile fibroblasts could return to their original cell shape and dimensions following elongation.

The difference in collagen phenotype response generated by the two different force applications may reflect the adaptive ability of the fibroblasts. As was mentioned previously, upon application of the tensile stress the motile fibroblasts may have spread out over the increased growth surface area. This would minimize the tensile strain experienced by the cells. Following removal of the external stress the growth substrate decreases in area and the redistributed fibroblasts must now adapt to this new environment. If this is the case, removal of the tensile stress would place the cell layer under compression. Therefore, the intermittently loaded cell cultures are experiencing a different force system than the continuously loaded cell cultures. This may be the cause of the prolonged increase in the proportion of type III collagen synthesis observed following intermittent force application.

The specific mechanisms translating force application to changes in cell synthetic activity are unknown; however, it could be postulated that in the tissue studied, it is related to the initial change in the environment and in order to maintain the change in synthetic activity, the local environment must continue to change. Such a hypothesis would explain why the intermittently stressed tissue cultures sustained the remodelling activity while the continuous application of force, which did not continue to change the growth substrate, did not. (In this scenario it is assumed

that the constant deformation of the substrate was not producing a sustained strain in the tissue and that the changes were sufficient to alter collagen phenotype synthesis from 2 to 8 hours following the onset of the force application.) This would also explain why an in vitro model with a truly continuous force would note an elevated type III collagen ratio after 36 hours (Pollit, 1987). In this model the force of gravity acted to strain a ligamentous tissue culture of mouse interparietal suture fibroblasts grown between a superiorly positioned titanium disk and an inferiorly positioned glass slide when the culture was placed on an angle. The shearing force thus produced was acting to continuously change the local tissue layer environment.

In vivo expansion of interparietal sutures demonstrated an increase in protein synthesis up to 7 days (Chiang, 1981), and expansion of interparietal sutures in organ culture produced an increase in the type III collagen ratio up to 7 days (Yen et al, 1989b). The continuous force in these experiments was possibly producing continuous tension in the suture due to continuous expansion over time, unlike the limited deformation of the Petriperm® dish, which was fully achieved almost instantaneously following force application. Thus the aforementioned hypotheses, regarding the change in the environment, could explain the differences in remodelling activity of continuously applied loads in the Petriperm® model and other in vivo and in vitro models discussed.

The differences between stressed *in vitro* sutural fibroblast tissue cultures and stressed *in vivo* or *in vitro* organ culture sutural fibroblast tissue, could be due to exogenous factors not available in tissue culture (eg. blood borne factors or compounds from adjacent tissue). Prostaglandin-E_1 has been shown to produce a increase in type III collagen synthesis relative to total collagen synthesis (Rennard *et al*, 1982) and parathyroid hormone has been shown to enhance the histologic changes associated with sutural joint remodelling during tooth movement (Davidovitch *et al*, 1972). It could be that non fibroblast derived substances serve to prolong the remodelling response, and more specifically the alteration in collagen phenotype metabolism, in organ culture and *in vivo* research.

In the present study there was no statistically significant change in the elevated percentage of type III collagen synthesis relative to total collagen synthesis between 2 and 12 hours in the intermittently stressed group. Qualitatively, the highest ratio of type III to total collagen synthesis was observed at 2 hours followed by a moderate decrease to 8 hours and a continued, but slower, rate of decrease to 12 hours. Despite the lack of statistical significance, the consistent trend of decreasing type III collagen ratio over time suggests the intermittently stressed cultures will not maintain this remodelling response indefinitely. Further, this would be consistent with the

results obtained following intermittent loading of rat gingival fibroblasts using the Flexercell® model (Anderson, 1991). In this in vitro study intermittent loading generated an increase in the ratio of de novo type III collagen and total de novo collagen at 4 hours, it peaked at 4 to 8 hours and subsided to nonstressed levels by 24 hours. In the Flexercell® and Petriperm® experiments, intermittent stress produced a rapid increase in the proportion of de novo type III collagen followed by a gradual decrease over the following time periods studied.

Extrapolating from the Flexercell® results reported by Anderson (1991), if the Petriperm® experiment had been prolonged, a decrease in the ratio of newly synthesized type III collagen to the total newly synthesized collagen may have been observed. This decrease may have resulted in a return of the de novo collagen phenotype ratio to nonstressed levels. Such results could be explained by incomplete relaxation of the induced deformation of Petriperm® base (ie. progressive plastic deformation of the Petriperm® dish bottom so that further stretching is not reproducing strain in the tissue layer or at least to a lesser degree) or an adaptation of the tissue culture to the deformation being reproduced.

The de novo collagen phenotype response to intermittent stress longer than 12 hours requires further investigation. Based on the lack of a statistically significant change in the type III collagen synthesis over the 2 to 12 hour interval of

intermittent stress using the Petriperm® model, it is possible that the level of the proportion of total collagen being comprised of type III collagen could plateau at a level significantly different from the nonstressed cultures. This may be due to the continued reproducibility of strain within the tissue layer over an indefinite period of time following intermittent loading using this model. This would demonstrate a truly elastic response of the tissue and growth substrate. It is further possible that the type III collagen ratio could increase at a later time due to recruitment of a fibroblast subpopulation clonally selected for type III collagen production. This late process would involve a lag phase in order to allow progenitor cell proliferation, differentiation and subsequent formation of the necessary intracellular metabolic pathways. Such a lag phase associated with the fluctuation of the collagen phenotype ratio following the application of in vitro stress to sutural fibroblasts has been observed by Pollit (1987).

The intermittent loading resulted in a greater, overall, remodelling response relative to the continuous loading, as observed in the present research. This was due to a prolonged increase in the ratio of type III to total collagen being synthesized and not a substantially quicker rate of initial response or attainment of dramatically higher proportions of the type III collagen phenotype. The present work does not

indicate the duration of this response; this warrants further study.

The fluctuation in the ratio of the synthesis of type III collagen to total collagen appears to play an important role in the initial remodelling events observed in fibrous sutural tissue. Yen et al. (1989b) noted that the increase in the rate of type III collagen synthesis in mouse interparietal sutures under tension was directly related to the magnitude of the force employed and the rate of sutural expansion. Taken together with the present observations, it may be suggested that the remodelling response may be modulated by the force level and the nature of the force application (eg. intermittent versus continuous force application).

FUTURE CONSIDERATIONS

The ability of this model to produce cyclic or continuous strain within the tissue was supported by the observations of this experiment. The dish bottom and attached cell layer exhibited a relaxation of the elastic deformation upon cessation of the force application. Therefore, the Petriperm® model should be able to be repeatedly deformed in order to produce true cyclic stretching of the tissue culture.

The physical perturbation of the cell layer produced during force application could easily alter cell shape as well as cell to cell or matrix to cell relationships. It is also conceivable that the physical changes in the tissue layer may elicit the release or synthesis of biologically active

compounds. The exact control mechanisms initiating and regulating the remodelling response need to be investigated.

In addition, the mechanisms by which the remodelling response is produced requires further study. The altered synthetic pattern, of the in vitro tissue, could be the result of a modulation of cellular metabolism through selective intra or extracellular degradation of specific collagen phenotypes; or feedback inhibition or stimulation of specific synthetic pathways. Alternatively the existing metabolic pathways of the cells, already present in the tissue culture, could be changed; cells could be stimulated to change their metabolic products by altering their gene expression. This may involve dedifferentiation and/or development of new synthetic pathways de novo. Finally, progenitor cells of specific fibroblast subpopulations could be stimulated to produce cloned fibroblasts selected for the collagen phenotype they produce.

The change in the cellular metabolism was reasonably quick and, therefore, likely involved a modulation of the metabolic activities of the fibroblasts. The response was too quick to be the result of a clonal selection process although this process may be occurring, the results of which may show up in altered fibroblast synthetic activities beyond the 12 hour limit of the present study. It is also possible that clonal selection could account for the sustained increase in type III collagen synthesis seen in the intermittently stressed cultures. The results, however, are easily

understood as being due to a modulation of the fibroblasts metabolic activities following initial deformation, and then reverting back to the nonstressed metabolic activities if the deformation is not repeatedly performed. This would explain why only the intermittently stressed tissue layers maintained the elevated type III to total collagen synthesis pattern.

It is highly controversial as to whether or not a fully committed cell can dedifferentiate to express an altered phenotype. It may be that fully differentiated fibroblasts can produce more than one type of collagen phenotype and it is simply a matter of selective gene expression or regulation of existing synthetic and degradative pathways. The phenomenon of degradation of newly synthesized procollagen has been proposed as a manner in which the ratio of collagen phenotypes being produced can be altered (Bienkowski et al., 1978a).

The exact mechanisms responsible for the results observed in the experiments involving collagen and protein synthesis are undetermined and require investigation.

The Petriperm® model could be used in future investigations of the connective tissue response to mechanical perturbation. Investigations could attempt to identify the biologic signals involved in regulating the remodelling process or the mechanisms by which these changes are produced. In this model, physical and chemical factors can be manipulated, in addition to being able to vary the design of the force system. The pattern of force application could

involve continuous stress, intermittent stress of differing rates of application and frequencies, differing magnitudes of stress and various durations of stress application. The concentrations and time of administration of exogenous chemical factors could be controlled *in vitro*, and their influence on remodelling could be observed either independently or in combination with force application.

In the future it may be possible to use pharmacological means to produce more predictable and more efficient responses to the biomechanical treatment strategies presently employed in orthodontic tooth movement. Regardless, it is expected that an improved understanding of the biology of tooth movement will lead to improvements in the clinical practise of orthodontics.

CONCLUSIONS

CONCLUSIONS

1. The application of continuous and intermittent stress to rat interparietal sutural fibroblasts grown in Petriperm® dishes causes minimal tissue architecture changes within 12 hours. The tensile stress did not tear the tissue layer, nor did it detach it from the growth substrate even after 12 hours of continuous or intermittent application.
2. The Petriperm® model of *in vitro* stress application produced reversible tissue deformation, at least in part. The tensile deformation generated was largely within the elastic limits of the Petriperm® plate elastic growth membrane and the attached fibroblast cell layer thus permitting intermittent stress application. However, the Petriperm® dish bottom and fibroblast tissue layer do not immediately return to prestressed conditions following the removal of the applied stress. This may be due to a delay in the recovery of the elastic strain generated in the plate bottom or tissue layer. It could also be explained by adaptive changes in the tissue layer that occurred during the period of external tensile stress application. Following the removal of the external stress, the adaptive changes may result in the tissue layer experiencing placed under compressive strain.
3. Stretching of the rat interparietal fibroblast cultures stimulated tissue remodelling as noted by a significant

increase in the ratio of newly synthesized type III collagen to total newly synthesized collagen over that of unstressed controls. This remodelling response occurred at the earliest time period studied (2 hours) and was similar in magnitude for both force systems (continuous and intermittent) for all time periods studied prior to termination of the experiment (12 hours). At 12 hours following the onset of force application only the intermittent force application maintained an elevated percentage of type III collagen synthesis. It may be that intermittent stress signals the tissue fibroblasts to sustain their remodelling activities for longer periods of time, than does continuous stress.

4. It is guardedly stated that the application of stress tended to produce an increase in the protein synthetic activities of the fibroblast tissue cultures. Although there was no change in the ratio of collagen polypeptide synthesis to the total collagenous and noncollagenous polypeptide synthesis, the experimentally stressed groups appeared to produce greater amounts of collagenous and noncollagenous proteins than did nonstressed control groups. This was not shown to be statistically significant possibly due to the small sample size. This response requires further investigation.

5. The model of <u>in vitro</u> stress application using Petriperm® tissue cultures can be used to study the effect of

tension on cellular activities. This model permits modification of the force application with respect to pattern (continuous versus intermittent), magnitude, rate and frequency of cyclic force application, and duration. This model also permits controlled administration of exogenous substances (eg. prostaglandin) in order to study their influence on the cellular activities alone or in concert with force application.

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APPENDICES

APPENDIX A

TABLE I

Scintillation Counts (dpm) of ^{3}H-Proline Incorporation Into Collagenous and Noncollagenous Protein Fractions Obtained from Cell and Medium Samples at Termination (Time 12 Hours) of the Experiment Involving Various In Vitro Stress Applications Using Rat Sutural Fibroblasts in the Petriperm Model

Scintillation Counts (dpm)

	Collagenous Protein (CP)	Noncollagenous Protein (NCP)	CP / [NCP+CP]
Cells			
Unstressed Controls	1557.90	3231.40	0.325
	1395.70	2726.80	0.339
	1702.40	2675.30	0.389
Continuous Stress	1268.20	2739.80	0.316
	1801.45	2983.80	0.376
	1496.55	3235.70	0.316
Intermittent Stress	2051.45	3614.30	0.362
	1541.05	2745.30	0.360
	1641.90	3036.50	0.351
Mediums			
Unstressed Controls	2805.80	1957.00	0.589
	3398.80	2549.00	0.571
	3683.50	2392.80	0.606
Continuous Stress	3854.70	2529.30	0.604
	4286.45	2243.40	0.656
	3704.95	4220.80	0.467
Intermittent Stress	4254.00	2868.50	0.606
	4282.50	2606.40	0.622
	4231.35	2935.00	0.590

Tables II, III and IV report the 12 hour CP, NCP and CP/(CP + NCP) data in terms of mean (standard deviation). The data for the 0 and 4 hour observations are also presented.

TABLE II

The Medium and Cell Sample Scintillation Counts (dpm) of ^{3}H-Proline Incorporation Into the Collagenous Protein (CP) Fraction of Newly Synthesized Protein in the Experiment Involving Differing <u>In Vitro</u> Stress Applications in the Petriperm® Model Over Time (0 to 12 Hours)

(CP in dpm)

Time (hours)	0	4	12+
<u>Mediums</u>			
Unstressed Controls	3489.70	3551.80	3296.03 (447.78) (n = 3)
Continuous Stress		4054.70	3948.70 (301.93) (n = 3)
Intermittent Stress		4633.65	4255.95 (25.63) (n = 3)
<u>Cells</u>			
Unstressed Controls	1545.50	1608.50	1552.03 (153.48) (n = 3)
Continuous Stress		1732.70	1522.07 (267.54) (n = 3)
Intermittent Stress		1918.45	1744.80 (270.31) (n = 3)

+ 12 hour results reported as X (std.dev.)

n = 1 unless otherwise specified

TABLE III

The Medium and Cell Sample Scintillation Counts (dpm) of ^{3}H-Proline Incorporation into the Noncollagenous Protean in the Experiment Involving Differing <u>In Vitro</u> Stress Applications in the Petriperm® Model at Various Times (0 to 12 Hours)

(NCP in dpm)

Time (hours)	0	4	12+
<u>Mediums</u>			
Unstressed Controls	2624.70	2473.10	2299.60 (306.81) (n = 3)
Continuous Stress		2742.80	2997.83 (1068.72) (n = 3)
Intermittent Stress		2542.40	2803.3 (173.68) (n = 3)
<u>Cells</u>			
Unstressed Controls	3001.30	2813.00	2877.43 (307.28) (n = 3)
Continuous Stress		2768.86	2986.43 (247.96) (n = 3)
Intermittent Stress		3473.00	3131.70 (442.42) (n = 3)

+ 12 hour results reported as X (std.dev.)
n = 1 unless otherwise specified

TABLE IV

The Medium and Cell Sample Ratio of Collagen Synthesis (CP) to Total Protein Synthesis (CP + NCP) Observed in the Experiement Involving Differing <u>In Vitro</u> Stress Applications in the Petriperm Model at Various Times (0 to 12 Hours)

[CP/(CP + NCP)]

Time (hours)	0	4	12*
<u>Cell Samples</u>			
Unstressed Controls	0.340 (n = 1)	0.364 (n = 1)	0.351 (0.034) (n = 3)
Continuous Stress		0.385 (n = 1)	0.336 (0.035) (n = 3)
Intermittent Stress (1 hour intervals)		0.356 (n = 1)	0.358 (0.006) (n = 3)
<u>Medium Samples</u>			
Unstressed Controls	0.571 (n = 1)	0.590 (n = 1)	0.589 (0.018) (n = 3)
Continuous Stress		0.596 (n = 1)	0.576 (0.098) (n = 3)
Intermittent Stress		0.646 (n = 1)	0.606 (0.016) (n = 3)

* For hour 12 the results are presented as X (std.dev.)

n = sample size

Appendix B

The Proportion (in Percent) Of Newly Synthesized Collagen Type III Alpha-Chains To The Total Of Type I and Type III Alpha-Chains From The Cell Tissue Layer Of Fibroblasts, Derived From Rat Interparietal Sutures Grown In Petriperm® Dishes, Undergoing Differing Stress Applications

Time (hours)	0	2	4	8	12
Treatment					
	9.887	11.682	9.183	10.379	8.417
Control	10.109	8.596	8.157	7.460	10.981
(No Stress)	10.331	8.511	9.730	10.671	10.935
	9.879	9.817	8.310	8.941	11.179
Intermittent		20.514	22.234	16.397	15.632
Stress		24.263	21.204	14.786	13.504
(1 hour		17.814	15.021	16.272	20.016
intervals)		16.288	16.521	20.020	16.835
		15.456	16.840	16.277	14.207
Continuous		19.013	19.831	20.330	9.206
Stress		22.235	13.186	17.232	11.055
		15.722	19.427	16.301	10.456

N = 52, n = 4

The corresponding mean (and standard deviation) values for the data of Table I is reported in Table II.

Table II

The Percentage of Newly Synthesized Type III Collagen Alpha-Chains Relative to the Total of Newly Synthesized Type I and Type III Collagen Alpha-Chains from the Cell Layer in the Petriperm® Model Following Differing Stress Applications at Various Time Periods. (The data is reported as Mean (Standard Deviation).)

Time (hours)	0	2	4	8	12
Treatment					
Control (No Force)	10.051 (0.214)	9.652 (1.479)	8.845 (0.743)	9.363 (1.477)	10.378 (1.312)
Intermittent Force		19.720 (3.497)	18.745 (3.514)	16.869 (2.225)	16.497 (2.72)
Continuous Force		18.106 (3.193)	17.321 (3.059)	17.535 (1.916)	11.231 (2.128)

N = 52, n = 4