THE STRESSING OF MOUSE INTERPARIETAL SUTURE FIBROBLASTS *IN VITRO*

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ABSTRACT

Clinical orthodontics is practised today with many problems that can be ascribed to poor understanding of its biological basis. It is our goal to conduct studies that will allow these problems to eventually be solved.

Although orthodontics has been practised for over 100 years, solutions to problems such as slow movement of teeth and root resorption have not been found. The reason for this can be partially attributed to the limited scope of the mechanical principles used in therapy. More effective clinical treatment can be expected when it is appreciated that orthodontics is the manipulation of craniofacial fibrous articulations such as sutures and the periodontal ligament. The understanding of the role that these tissues play is our area of research. Our specific task is to better understand the role of fibroblasts, found in both these tissues, in tooth movement.

In breaking down our problem to its simplest form, we are left with the question, what is the cellular response of fibroblasts to mechanical force? This work will lead into questions addressing the mechanism of external force transduction and the interaction of other external ligands on this system.

We are not the first people to appreciate the significance of this question. Other workers have developed systems both in vivo and in vitro that have addressed this area. It is our feeling that work still needs to be done as the models used to date have not successfully created force systems that closely resemble those which occur when teeth are moved.

Our model is unique in that it establishes a continuous force on cells between two hard surfaces. We feel that this is analogous to the in vivo situation. Another strength of our system is that the force system is based on gravity and requires no mechanisms that require calibration.

Our model utilizes fibroblasts grown out from the interparietal suture of 7-9 week old, male white mice. Cells from the first subculture are seeded onto sterile glass slides which measure 12.5 x 7 mm. At confluency titanium disks with a sintered surface and which have been covered with collagen are placed in the center of the glass slide. After 18 days the glass slides are tipped at an angle of approximately 75 degrees. The disks are acted upon by gravity but are held to the glass slide by the multilayer of cells around and beneath the disks. At this point in time the model is

ready for experimentation.

The first goal was to better understand what this system is doing to the cells. This was achieved visually utilizing a scanning electron microscope. The data indicated that the cells were growing between the glass slide and the titanium disk in a three dimensional ligament like structure.

The next goal was to establish if the force applied to these cells had an affect. We had chosen to look at type III collagen synthesis as the variable in question since it has been shown to reflect the remodelling state of tissues. Specifically, does the proportion of type III synthesized collagen increase with force application as compared to no force. The design of this experiment compared collagen synthesis between slides that were at 75 degrees and slides that have been left flat. In addition, we will also looked at the effect of the disk being present by including a sample of glass slides with cells but no disks. The results for the cell and media data both showed a significant difference between the control and experimental samples but not between the two different control samples.

Following this experiment, we looked in more depth at this shift in collagen synthesis. Our goal was to determine the length of time following force application

at which the shift was maximal. This was conducted using time periods of 6, 12, 24, and 36 hours and 3 and 5 days. There was no significant difference between the time experimental samples and the controls. The time period where the maximum shift in type III synthesis occurred was at 36 hours.

In addition two pilot studies are discussed in appendices 5 and 6.

This new system overcomes the shortcomings of previous in vitro force systems in that it generates a continuous force on cells precisely and easily. In addition, the cells acted upon are in a three dimensional ligament-like structure, not flattened out.

DEDICATION

TO HENRY AND FAY

For their continuous support and encouragement
in all my undertakings.

TO JANET

A true friend.

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(A) METHODOLOGY AND FINDINGS OF CHANGES OF FIBROUS JOINTS INCIDENTAL TO MECHANICAL STRESS

The capability of fibrous joints to remodel in response to mechanical stress is central to the successful practise of orthodontics and facial orthopaedics. The proof to this statement has been provided time and time again in histological studies (for review, see Reitan, 1975) of the periodontal ligament during orthodontic tooth movement and of sutural response to orthopaedic stress (Cleall et al., 1965; Murray and Cleall, 1971; Moffett, 1971, 1973; Droschl, 1975; Linge, 1972, 1976). In addition, remodelling of sutures quite distant from the dento-alveolar complex resulting from posteriorly (Droschl, 1973; Elder and Tuenge, 1974) and anteriorly (Nanda, 1978) directed traction to the maxillary dentition has also been studied histologically and cephalometrically.

The biological pathways and the cellular control mechanisms for remodelling of the periodontal ligament and suture are still largely unknown. The extent of our present understanding has resulted from much experimental work done in the past. It is essential that this work be examined in order to illustrate how present ideas evolved and more importantly, to clarify what are the most important questions that still remain to be

answered. This section will conclude with a look at the current trends in research in this area.

(1) Pressure-Tension Theory

This theory suggests that tooth movement came about as a result of bone resorption in areas of pressure and bone apposition in areas of tension. The first individual to histologically identify areas of bone resorption and apposition in teeth that were orthodontically moved was Sandstedt between 1904 and 1905 (Schwarz, 1932). Later, Schwarz (1932) formulated the pressure-tension theory based on Sandstedt's work. He speculated that the effect was limited to the periodontal ligament space and required very low force levels so as to not obstruct capillary blood flow and cause tissue necrosis or hyalinization. Gianelly (1969), also believed that the vasculature played an important role in the pressure-tension theory. He suggested that it was essential in bringing O_2, nutrients and osteoclasts into the periodontal ligament in order for frontal resorption to occur. Excessive force would close down the capillaries and thus destroy this tissue. The resulting tissue destruction was called hyalinization. Bone resorption following hyalinization of the

periodontal ligament occurred on the marrow side of the alveolar bone and was called undermining resorption.

Kardos and Simpson (1980) have put forward an alternate interpretation for the pressure-tension theory. They proposed that the ligament possesses thixotropic properties. Under pressure, the matrix becomes more fluid and facilitates the migration by cells out from this area. They suggested that hyalinized areas were thus not necrotic. To date this theory has received no substantiating support and remains speculation.

In work by Epker and Frost (1965) and Baumrind (1969), it was concluded that the effect of the applied force was not limited to the ligament. They suggested that the alveolar bone was deformed by this force and that the resulting cellular activity depended on the type of deformation. Epker and Frost made brief mention of bone deformation leading to voltages being generated.

The level at which this theory attempts to explain the mechanism for tooth movement does not clarify which cellular mechanisms are involved. Thus future work will be aimed at attempting to find these mechanisms.

(2) Piezo-Electric Theory

It has been observed that an electric

potential is generated by the application of a force to bone (Fukada and Yusada, 1957) including alveolar bone (Zengo et al., 1973, 1974). An increase in electrical activity has been associated with an increase in osteogenic activity, as demonstrated by elevations in osteoblast number and quantity of bone formation (Bassett et al., 1964; Bassett, 1968; Zengo et al., 1976), as well as a corresponding increase in intracellular activity (Davidovitch et al., 1978a, 1978b, 1979, 1980a, 1980b).

The method for transducing electric current into the observed cellular behavior has not been delineated, although hypotheses involving the migration of electrons through biologic membranes (Jahn, 1962), proton charge transport through the cytoplasm (Eigen and DeMayer, 1958) and amphoteric ion exchanges (Jahn, 1968) have been advanced.

(3) Oxygen-Tension Theory

Bien (1966) hypothesized that capillaries constricted by compression of periodontal ligament fibres form a series of cirsoid or enlarged aneurysms. Below each site of stenosis, decreased blood pressure results in formation of minute oxygen bubbles which can

diffuse through the vessel wall and lodge against bone surfaces leading to osseous resorption.

Relationship between oxygen tension and bone resorption has been demonstrated in vitro (Goldhaber, 1958, 1961, 1966; Stern et al., 1966), but no attempts have been made to either measure fluctuations in oxygen tension or to manipulate oxygen tension within the periodontal ligament and observe the effects upon bone resorption. Although increased vascularity of the periodontal ligament has been associated with frontal bone resorption occurring with the application of orthodontic force (Gianelly, 1969; Khouw and Goldhaber, 1970), the question of whether the increased vascularity found in areas of active bone resorption is primarily or secondary to the resorption has yet to be resolved.

Even after the resolution of whether the pressure-tension theory is due to vascular occlusion, bone deformation or thixotropic behavior of the periodontal ligament or maybe a combination of all three, it still does not offer an explanation on the cellular mechanisms involved. Similarly, an increase in osteogenic cell number and intracellular activity have been associated with piezo-electric potentials as a result of mechanical deformation of the dento-alveolar complex. Thus, the

inadequacies of all the three classical theories for orthodontic tooth movement are evident.

(4) Present Day Perspectives

Orthodontics and facial orthopaedics as practised today continues to be rather instinctive and empirically based. The shift from being an art to a science will require answering some fundamental questions. These tend to fall into two categories; those asked by clinicians and those by researchers. Some questions that seem to be paramount in the minds of clinicians are:

(a) What is the force level which results in the most efficient remodelling, the shortest treatment time and the least treatment sequelae?
(b) What is the ideal reactivation schedule?
(c) Are the different optimal force levels and different ideal reactivation schedules dependent upon the type of movement required and the particular fibrous joint(s) needed to be remodelled?
(d) Is continuous activation always better than intermittent activation?

(e) How do we define intermittent activation?

(f) Are there treatment modalities other than mechanical?

(g) What is the potential for pharmacological agents in the future practice?

(h) What effects would combined mechanical and pharmacological agents have on treatment response?

There is no doubt that these questions are important but they pre-suppose that we understand the mechanism by which tooth movement occurs. It is in this direction that researchers are presently aiming their questions.

The knowledge would also give us a better understanding of the disease states when fibrous joints behave abnormally, e.g. in Apert's, Crouzon and Treacher Collins Syndromes, in cleidocranial dysostosis and in tooth ankylosis. In addition, the etiology of malocclusion as well as dysplastic craniofacial skeletal relationship of fibrous joints could be better understood which may lead to their prevention and/or treatment.

Recently, researchers have focused on cellular and molecular aspects of orthodontic tooth movement. Hormones and other physiological mediators with known

effects on the metabolism of osseous tissues have been studied in relation to orthodontic tooth movement. Also the effect of mechanical force on cellular activity has been studied.

The ability of parathyroid hormone to enhance orthodontic tooth movement, both micro- and macroscopically, has been recorded by Gianelly and Schmur (1969), Kamata (1972) and Davidovitch and co-workers (1972).

Davidovitch and co-workers (1975, 1976a, 1976b, 1977,1984) have also associated fluctuations in cyclic nucleotide levels with areas of tension and compression in periodontal ligament of cats following tooth movement in vivo.

In addition, the possible role of prostaglandins as a mediator of bone resorption during orthodontic tooth movement was first investigated by Yamasaki and co-workers (1980) in cats.

However, the lack of localized specificity of the parathyroid hormone presents technical difficulties in the study of its possible involvement in the regulatory mechanism of orthodontic tooth movement. Similarly, it is difficult to assess the specific role of cAMP which functions as a secondary messenger in almost all aspects of physiology of both high and low forms of life

(Rasmussen, 1970). Although prostaglandins have been implicated in both physiological and pathological bone and collagen metabolism (for review, see Duncan, 1982), and also have been shown to be produced in response to mechanical stretching of cells in vitro (Harell et al., 1977; Binderman and Cox, 1977; Somjen et al., 1980), their role in fibrous joint remodelling is still quite controversial (Duncan, 1982).

In addition to increased prostaglandin production, other effects have been shown to occur as a result of mechanical stress being applied to cells. Meikle et al. (1984) found that protein, and DNA synthesis were increased in sutures after 6 hours. He specifically noted that the increase in protein production was not limited to collagen and included metalloproteinases. Hasegawa and co-workers (1985) showed that mechanical force increased DNA synthesis 64% in suture cells. They speculated that not all cells were capable of responding and recommended experiments with cloned subpopulations.

Thus, findings from the above studies are usually inconclusive and generally, the methodology employed is not amenable to expansion and development for further investigation.

Moreover, these "fishing expedition" approaches tend to generate quantities of data that are often

difficult, if not impossible, to relate with one another and generally only reveal phenomena of association rather than provide cause and effect information, and are thus of limited value.

A more rational and potentially fruitful approach would be first to identify the cellular and extracellular components that are actively involved in fibrous joint remodelling during orthodontics and facial orthopaedics, followed by the development of a model system which can be precisely defined and also readily reproduced. The elucidation of the sequence of events on the cellular and molecular levels of the biochemical aspects of fibrous joint remodelling in response to mechanical stress can then be approached systematically using the model system developed and may eventually lead to the understanding of the regulatory and control mechanisms.

Collagen is the most abundant structural protein of fibrous joints (Barbanell et al., 1978) and could be most significant in light of its contribution to the unique architecture and function of these tissues (Page and Schroeder, 1982). The most notable ultra-structural characteristics of fibroblasts and osteoblasts in tension zones of rat molar periodontium incidental to orthodontic tooth movement is a highly developed system

of rough endoplasmic reticulum, suggesting a high level of matrix synthesis by these cells of which collagen is the main component (Rygh, 1976). Electron microscopic studies also indicated that remodelling of the periodontal ligament during normal physiologic tooth movement and when cranial sutures are subjected to tensile mechanical stress involves rapid turnover of collagen macromolecules (Ten Cate et al., 1976, 1977).

Diaz (1978) indicated a decrease in collagen degradation in his study of the effect of applied force in the periodontal ligament of the rat using [^{3}OH]-proline radioautography, while Meikle and co-workers (1979) reported a two-fold increase in protein and collagen synthesis in expanded cranial sutures of neonatal rabbits in vitro.

Thus, not only is collagen the major structural component of fibrous joints, but its metabolism is also central to their remodelling activity. The study of collagen biochemistry in fibrous joints during normal growth and development, as well as when subjected to therapeutic mechanical manipulation, may provide insight into the regulators and control mechanisms of the remodelling process.

(B) BIOCHEMISTRY OF COLLAGEN

(1) Structure and Biosynthesis

Collagen is the main extracellular component within all connective tissues (Bailey and Robins, 1976; Kivirikko and Risteli, 1976; Bornstein and Traub, 1979). It is the most abundant structural protein and represents about one-quarter to one-third by weight of the total body protein. The initial role for this protein was thought to be limited to forming and maintaining tissue structure. A second and newly emerging role is in cell-matrix interactions which are thought to be key factors in tissue differentiation. (Shekhter, 1986; Amenta et al., 1986).

A single collagen molecule is approximately 15 Å in diameter and 2800 Å in length with a molecular weight of about 300,000 (Gay and Miller, 1978; Nimni, 1980). This is called a tropocollagen molecule and consists of three polypeptide chains termed alpha-chains. Each alpha-chain is coiled in a left handed helix due to the frequent presence of the cyclic imino acids proline and hydroxyproline. The three chains together are intertwined into a right handed, rod-like triple helix (Gay and Miller, 1978).

Each alpha-chain consists of about 1052 amino-acid residues. The primary structure for each is composed of the repeating triplet $(gly\text{-}X\text{-}Y)_n$ which consists of glycine and two other amino acids (Prockop et al., 1979). The X position is occupied usually by proline and the Y position by hydroxyproline (Cheah, 1985). Thus, one-third of the amino acids in collagen are glycine. One-quarter of the remaining amino acids are proline and hydroxyproline. Hydroxyproline is found almost exclusively in collagen. As a result, hydroxyproline content in urine can be assayed when investigating problems which cause changes in collagen metabolism (Prockop, 1979a). The other constituent amino acids most commonly occupy the X position. Alanine, a neutral amino acid, makes up about 9%. Lysine and hydroxylysine, which are important in the formation of intermolecular linkages, constitute only 1.3% of the total amino acids in the molecule.

Collagen production is the net result of synthesis and intracellular degradation. It has been shown in growing cultures that 20 to 40 percent of newly synthesized procollagen is degraded intracellularly shortly after it has been translated (Bienkowski et al., 1978). In terms of energy efficiency this system is wasteful but it allows production to rapidly adjust to

changes by activating or inhibiting existing pathways rather than having to form them de novo. It has been shown that this system is able to recognize and selectively destroy procollagen with abnormal structure. Perhaps this system may function as the cells' quality regulation system (Rennard et al., 1982).

Cells derived from mesenchymal cells such as fibroblasts, chondroblasts, osteoblasts and odontoblasts synthesize collagens. Basement membrane collagen is uniquely produced by epithelial cells (Barbanell et al., 1978).

Collagen formation follows the normal path for protein synthesis. Following an appropriate stimulus heterogeneous nuclear RNA (hnRNA) is transcribed for collagen. While still in the nucleus, the introns are removed with the aid of small nuclear RNAse (snRNAse). The resulting messenger RNA (mRNA) is further modified by the addition of two proteins. At this point the completed mRNA moves throught the nuclear pore and into the cytoplasm. The mRNA is then translated by polysomes located on the endoplasmic reticulum (McGilvery, 1983). Once the transcribed polypeptide chain enters the endoplasmic reticulum it is transported to the golgi apparatus where it is packaged for secretion. During this time the molecule undergoes unique post-

translational modifications to its structure. (Kivirikko and Risteli, 1976; Barbanell et al., 1978; Minor, 1980). These include removing the signal peptide, 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 a triple helical structure. The hydroxylation of proline and lysine is dependent on ascorbic acid being present (Levene et al., 1972; Murad et al., 1981). The molecule is secreted as procollagen, also referred to as soluble or transport collagen. Procollagen is transformed to tropocollagen through the enzymatic extracellular cleavage of the non-helical propeptides called the amino and carboxyl terminals. Tropocollagen is insoluble and represents the simplest building block unit of collagen in the extracellular space.

The extracellular aggregation of individual collagen molecules is not a spontaneous process (Trelstad, 1982). It is thought to occur via multiple steps under cellular control. Evidence suggests that the helical portions of procollagen molecules bind to cell membranes (Goldberg and Burgeson, 1982). It is through this close participation by cells in fibril assembly that functional adaptation of the fibres is possible.

Collagen fibrils display a typical banding pattern that is duplicated every 66.8 nm. Over time, the tensile strength of the fibril increases due to the formation of both intra- and inter-molecular cross-links (Tanzer, 1973). Further collagen fibril aggregation gives rise to collagen fibres (Barbanell et al., 1978; Prockop et al., 1979a, 1979b).

The breakdown of collagen fibres is essential for rapidly remodelling tissues. It is only by specific collagenases that these molecules can be cleaved. Collagenase, a metalloproteinase, attacks collagen at a specific site and splits it into segments which denature in vivo. These denatured chains are vulnerable to proteases under physiologic conditions. This enzyme is produced by fibroblasts which suggests that these cells are capable of remodelling fibrous tissues.

It has also been demonstated that fibroblasts are able to phagocytose enzymatically cleaved collagen fiber segments and degrade them intracellularly in phagolysosomes (Melcher and Chan, 1981; Yajima and Rose, 1977; Rose et al., 1980).

Collagens are a heterogenous group of proteins. The purpose for this diversity is presumably the variation in functional requirements for the tissues in which collagens are located.

The collagens represent a group of at least eleven closely related but genetically distinct structural proteins designated as type I, I trimer, II, III, IV , V, VI, VII, VIII, IX, X, and XI (Cheah, 1985). Identification and comparison of different collagen phenotypes can be carried out by at least two methods. The first involves treating the samples with cyanogen bromide, to break down the polypeptide chains at the methionine residues, followed by SDS-polyacrylamide gel electrophoresis. This results in a typical pattern for each collagen type (Bornstein and Sage, 1980). A second method involves separating the differerent collagen types by chromatography and electrophoresis. This separates the collagens based on their size.

It appears that almost all tissues contain more than one type of collagen. The types present and their proportions are tissue specific. Also very few, if any, collagen types have a unique distribution and it is likely that as methods for detection become more sensitive, a broader distribution will be found for each of the collagens (Bornstein and Sage, 1980).

(2) Distribution and Physiology

Type I collagen is the most abundant species

and is located predominantly in skin, tendon, bone, placenta, dentine, gingiva and the periodontal ligament. It is the only kind found so far in mineralized connective tissues It is composed of two structurally different alpha-chains, alpha 1(I) and alpha 2(I). The molecular formula for type I collagen is [alpha 1(I)]$_2$ alpha 2(I).

Type I trimer is represented by the molecular formula [alpha 1(I)]$_3$. It can be found in diseased gingiva (Narayanan and Page, 1983a) as well as skin, cartilage, aged chondrocytes and fibroblasts cultured *in vitro* (Minor, 1980). It is also synthesized in organ cultures of rat incisor odontoblasts (Munksgaard *et al.*, 1978). Within *in vitro* cultures of mouse periodontium explants with low PO_2 (100-120 mm Hg), the ratio between alpha 1(I) and alpha 2(I) chains was found to be as high as 6:1 suggesting that large amounts of type I trimers were synthesized (Yen, 1978). Although it seems that type I trimers are synthesized under abnormal or altered physiological and, or biochemical conditions only, it has also been found in chick embryo tendons and calvaria *in vivo* (Jiminez *et al.*, 1977).

Type II collagen seems to be cartilage specific (Miller, 1972; Bornstein and Sage, 1980; Minor, 1980). It is composed of three identical alpha-chains with the

molecular formula, [alpha $1(II)_3$] .

Type III collagen is composed of three similar chains, [alpha $1(III)]_3$. It often co-exists with type I, in various proportions, which together are called interstitial collagens. Moreover, the relative amounts can change with age, e.g. skin (Epstein, 1974); with tissue type, e.g. gingiva and the periodontal ligament (Butler et al., 1975; Sodek and Limeback, 1979); with altered metabolic states, e.g. diseased gingiva (Narayanan et al., 1980, 1983; Narayanan and Page, 1983a), granulation tissues during wound healing (Miller, 1976; Gay et al., 1978) and during periodontal and sutural remodelling in response to mechanical forces (Yen et al., 1980). Type III is also present in blood vessels (Bornstein and Sage, 1980).

Type IV collagen has the molecular formula, [alpha $1(IV)]_3$. Unlike the other types, described above, which are fibrillar, type IV is non-fibrillar and is located in basement membranes. (Bornstein and Sage, 1980).

Type V collagen was first isolated from fetal membranes (Burgeson et al., 1976). However, later studies revealed that it is present, as a minor component, along with type I and III or type II (Rhodes and Miller, 1978) in most connective tissues, and that it has a unique ultrastructural localization in

pericellular spaces and near basement membranes (Madri and Furthmayr, 1979; Roll et al., 1980).

The alpha-chain composition for type V collagen is [alpha 1(V)]$_2$ alpha 2(V) (Rhodes and Miller, 1978). Alpha 1(V), alpha 2(V) and alpha 3(V) chains represent B, A and C chains from the old nomenclature respectively (Bornstein and Sage, 1980). Though alpha 3(V) chain described in the gingival tissue by Narayanan and co-workers in 1980 is a contaminant and not a constituent of type V (Narayanan and Page, 1983b), type V can also exist in the molecular form, [alpha 1(V)]$_3$ (Rhodes and Miller, 1978).

Not only is collagen the main structural protein in connective tissues, histological (Stallard, 1963; Carneiro and Leblond, 1966) and biochemical (Sodek, 1977; Sodek et al., 1977) studies have demonstrated that collagen is active metabolically as well. This collagen synthesis is in response to physiological and functional demands from the tissue and frequently results in a change in the relative amounts of the different collagens present. The interstitial collagens, types I and III, frequently behave in this manner.

It has been suggested that when rapid collagen synthesis is required, a higher than normal proportion of type III collagen is produced. Thus, while type I is

predominant in adult skin, type III accounts for over 60% of the total collagen in fetal skin (Epstein, 1974; Chung and Miller, 1974; Miller, 1976). Also type III accounts for 15% of the total collagen in the rat periodontal ligament (Sodek and Limeback, 1979) and 20% in the bovine periodontal ligament (Butler et al., 1975). This could be a product of the constant physical and bacterial stresses placed on the periodontium which have created a continuous state of wound healing and repair (Claycomb et al., 1967). Biochemical studies (Page and Ammons, 1974; Sodek, 1976; Sodek and Limeback, 1979) have also shown that in comparison with other connective tissues, collagen turnover in the periodontal ligament is rapid. The reasons for this are not clear, but it has been suggested that intermittent occlusal forces acting on the collagen fibres, particularly during mastication, may be partially responsible (Sodek, 1976).

The proportions of type I and III collagen are also affected by various abnormal and pathological states. The amount of type III increases during inflammation (Weiss et al., 1975), and during early phases of wound healing in skin (Gay et al., 1978; Reddi et al., 1977). Type III collagen persists in the dentine from patients with osteogenesis imperfecta (Penttinnen et al., 1975;

Sauk et al., 1980) and is synthesized by synovial cells from patients with rheumatoid arthritis (Eyre and Muir, 1975), while it is deficient in patients with hereditary Ehlers-Danlos Syndrome type IV (Pope et al., 1975; Aumailley et al., 1980).

Three mechanisms exist that produce changes in amounts and proportions of collagens present in tissues (Narayanan and Page, 1985). Clonal selection is a long term method for shifting collagen production (Bordin et al., 1984). More short term is shifting the cells' synthetic pattern (Rosenbloom et al., 1984). The third is differential susceptibility to extracellular collagenases produced under different conditions (Horwitz et al., 1977).

Thus, the proportions for various collagen types are different from one anatomic site to another. Clearly this is related to the functional requirements of each tissue. The ability to change the synthesis pattern of several collagens may also be crucial for the normal development and tissue repair (Bornstein and Sage, 1980). Failure of the mechanism often results in various disease states.

(3) Collagen Phenotype Ratio as a Biological Signal

Collagen is the principal structural protein of fibrous joints. Its integrity is essential for normal development and function of this connective tissue entity. One would expect that under similar conditions, the metabolic behavior of collagen in fibrous joints would be the same as that in other connective tissues. Orthodontic tooth movement has been likened to an inflammatory wound healing response (Storey, 1973; Ten Cate et al., 1976) similar to other situations with rapid collagen synthesis and remodelling and where the proportion of type III/type I + III is significantly affected. Thus, collagen phenotype ratio can serve as an important parameter, indicating the metabolic state within the fibrous joints during the remodelling process in response to orthopaedic and orthodontic stress. Any change in collagen phenotype ratio may serve as an indicator for changing cellular activities and thus facilitate studying the regulation of collagen synthesis. With insight into this process we come closer to understanding fibrous joint remodelling during growth, development and therapeutic manipulation.

(C) BIOCHEMICAL CONTROL OF FIBROBLASTS

(1) Overview

The diversity in form and function of connective tissues is indicative of the many different roles played by fibroblasts. In general, the specific part an individual cell plays within a tissue is determined at the time of, and subsequent to, the tissue formation. At the start of tissue formation, many progenitor cells are present, each possessing unique phenotypic qualities. During tissue formation, factors present determine which progenitor cells rapidly proliferate and go on to make up the tissue. This process is termed clonal selection and it is in this way that tissues are formed with a specific phenotypic behavior. After tissue formation, protein synthesis is tightly controlled by the cells (Breul et al., 1980). It is most likely through feedback mechanisms that cells are able to control their synthesis patterns (Wiestner et al., 1979; Paglia et al., 1981). Within a limited range this pattern can be modulated by environmental factors or ligands acting on the cell. Under conditions where dramatic environmental changes are occurring, such as wound healing, the process of clonal selection again

comes into play. These three approaches together allow tissues to modify their functions.

(2) Clonal Selection

The role of connective tissue is astonishingly dynamic. During growth, development and wound healing, connective tissues are called upon to change their functional roles in terms of PGE_2 synthesis (Korn, 1985) and amount and proportions of proteins produced. For large shifts that are to persist for long periods of time, the mechanism for changing is through variation in the composition of the parts that make up the tissue as a whole. These parts, fibroblasts, collagen and proteoglycans are usually thought of as homogeneous (Kondo et al., 1985). The situation that exists is quite the reverse. As stated previously, as many as eleven types of collagen exist and the types and proportions are known to change as demands change. It has been shown that alteration in extracellular substance produces changes in the physiologic functions of cells (Bartold et al., 1986). Several studies have demonstrated that connective tissues are populated by heterogeneous cell populations (Kaufman et al., 1975; Ko et al., 1977; Hassell and Stanek, 1983). In order to understand this last concept, the role of progenitor

cells must be made clear. These cells make up a heterogenous group of cells with unique phenotypic qualities. In a stimulated tissue these cells are able to continually divide and produce many cells. At some point in time, these cells no longer can divide and become terminally differentiated daughter cells. A major role of these cells is to synthesize proteins. One theory states that under controlled conditions, progenitor cells randomly become terminally differentiated (Angello and Prothero, 1985). This idea is too simplistic for a system that is able to respond to its changing environment. A second theory is that ligands act on the progenitor cells and selectively activate cells that will produce the correct cell type to carry out the task required. This process is termed clonal selection. Following several divisions during which the number of progenitor cells increases, all the cells divide and become differentiated. In this way the process is self-limiting and continually able to respond to new environmental changes (Martin et al., 1974; Hassell and Stanek, 1983). It is through the continual selection of various subpopulations, a process termed clonal selection that long-term shifts of tissue collagen production occur.

Support for the second theory has been provided by

work showing that connective tissues are made up of fibroblast subpopulations (Bordin et al., 1984; Korn et al., 1985). This heterogeneity has been shown to also exist in dental tissues (Hassell and Stanek, 1983). Additional support comes from work showing that these subpopulations are differentially sensitive to factors such as PGE_2. Subpopulations which respond to this soluble ligand decrease their rate of replication allowing insensitive subpopulations to repopulate the tissue.

(3) Feedback

Not surprisingly, collagen synthesis is a tightly controlled process. It is believed that the nature of this control is primarily through a feedback loop (Wiestner et al., 1979). Although it has not yet been shown if the collagen molecules themselves have an effect on synthesis, it has been shown that NH_2 terminal polypeptides that have been cleaved off of procollagen molecules extracellularly modulate collagen synthesis patterns (Wiestner et al., 1979; Paglia et al., 1981; Perlish et al., 1985). As the concentration of these polypeptide chains increases, collagen synthesis decreases. Work by Uitto et al. (1985) which

showed a relationship between mRNA and collagen synthesis lead Wu et al. (1986) to investigate if propeptides acted at the translational level. In fact what they found was that the amino terminus appeared to act pretranslationally, possibly by increasing the rate of mRNA breakdown. In addition, they made the observation that the carboxyl terminus inhibited synthesis. The degree of inhibition by this polypeptide was less than the amino terminus and appeared to act at the level of transcription (Wu et al., 1986). The exact place in the synthetic chain where this negative feedback takes place has not, as of yet, been found but it was speculated by Goldenberg and Fine (1985) to act directly at the level of translation and by Horlein et al. (1981) at the level of transcription.

(4) Environmental Ligands

Much work has been done to investigate the types of factors that have a direct effect on fibroblast synthesis. They range from the very precise cell-cell direct contact interactions, to the generalised control of all target cells in the organism, as occurs with the central release of hormones. In between are interactions in which ligands or matrix components influence cells regionally. The results are that some changes are fast

acting and very localised while others are slower to take place and affect a much larger population of cells.

Turkewicz and Rodan (1983) and Murray and Farndale (1985) suggested that pulsed magnetic fields were able to increase protein synthesis. It is noteworthy that Murray and Farndale (1985) were only able to show this affect in confluent cell cultures. They suggested that the changing field modified intracellular cAMP metabolism and thus exerted a physiologic effect.

The role of cell to cell contact has been investigated by Bard and Elsdale (1986). They found that cell division was inhibited by this contact. They were also able to show how isolated proteins from cell membranes were able to mimic this effect. They postulated that these proteins reversibly reduced the membranes' permeability to ions that were necessary intracellularly to allow conditions suitable for division to develop. One controversial finding they made was that inhibition of cell division did not seem to be due to soluble mediators released locally. They also introduced the idea that collagen production by cells leads to growth inhibition at a lower cell density than in cultures without collagen. The mechanism was assumed to be via reduced cell mobility.

Another method of conveying information to cells is through ligands acting on cell surface receptors. Examples of soluble ligands are C1q, PGs, mononuclear cell supernates (Korn, 1985 ; Kahaleh et al., 1986), epithelial growth factors and interleukin 1. Another group of ligands are the insoluble factors, such as hyaluronic acid. Unlike the soluble mediators which are only effective for a short period of time, a factor like hyaluronic acid influences the cells within it until it has been remodelled.

It has been shown that fibroblasts respond to ligands with changes in protein synthesis, secretion, and DNA synthesis. In a heterogeneous population, not all cells will respond similarly to a specific ligand. Thus in the short term, protein synthesis will shift as some cells increase and others decrease their production of proteins. An example of this in cell culture is the effect of foetal calf serum. It is usually used at a concentration of 10% and contains many ligands. The effect of the liguands contained in the foetal calf serum on cells is to increase their production of collagen type I (Narayanan and Page, 1977).

In orthodontics we may be using forces to bring about these three changes to the cells in the ligament. It is necessary to test this hypothesis in order to

better understand the role of force in orthodontics and allow us to rationally change our clinical methods. Thus it is necessary to investigate the effect of force on cell populations. Our goal is to clarify how forces directly or indirectly effect cell functions. More specifically, we would like to see if short term protein synthesis patterns are changed via forces and if long term protein synthesis patterns change via clonal selection due to forces.

(D) FIBROBLAST PHYSIOLOGY AND CELL SHAPE

Cell shape has traditionally been discussed in terms of the cytoskeleton. This is presently thought to be made up of 3 types of structures: microfilaments, microtubules and intermediate filaments (Wang et al. 1986)

Microfilaments are composed of actin and myosin molecules. They are present throughout the cytoplasm and are responsible for generating force.

Microtubules are composed of tubulin and are also present throughout the cytoplasm. They are thought to be responsible for intracellular transport of organelles and vesicles (Weiss and Gross, 1983).

The intermediate filaments are composed of the protein vimentin in connective tissue fibroblasts (Wang et al., 1986). It has been shown to extend from the cell membrane to the nuclear pores. The function of these filaments may be to maintain cell shape. In addition, DNA, RNA and protein synthesis have been shown to occur in association with these structures (Reiter et al., 1985).

One implication from the preceding section is that cell shape can additionally be directly dictated by the extracellular matrix. Folkman and Greenspan (1975)

suspended fibroblasts in soft agar and found that they assumed a spheroid shape. Later work by many workers showed how various substates modified the amount that cells spread (Folkman and Moscona, 1978). As an example, cell attachment to collagen matrix results in parallel elongated morphology. This is explained by the regularly arranged fibronectin binding sites on collagen. This was shown by Yamada et al. (1977) and Spiegelman and Ginty (1983) using cells that had lost the ability to produce fibronectin. By adding fibronectin to these cells grown on collagen matrices they produced elongation and parallel cell arrangement. The same result was not produced by repeating the experiment on a plastic or nonadherent substrate.

The importance of shape to cell physiology has been well established. Folkman and co-workers (1975, 1978) first showed how changing fibroblast cell shape effected DNA synthesis. Other work followed showing how shape was a factor in controlling cell growth (Vandenburgh, 1983; 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). Reiter et

al. (1985) found that suspension of 3T3 swiss mouse fibroblasts in methylcellulose reduced protein synthesis by 80%.

Many mechanisms have been speculated on for how shape acts to modify cell functions. One set of ideas given by Tucker et al. (1981) was 1) that the cell surface area was changed and resulted in a substantially different number of receptors being available to bind serum growth factors and 2) that the cytoskeletal link between receptors, binding growth factors, and the nucleus, is inoperative with certain cell shapes. Vandenburgh (1983) showed how the shape change effect was not by a modification of membrane receptor sensitivity. His work suggested that shape change directly activated the cells second messenger system, bypassing the membrane receptors. Farmer et al. (1983) suggested that mRNA was reversibly modified during experimentally induced shape changes which resulted in a change in protein production. Ben-ze'ev (1983, 1985) suggested that in the cytoskeleton, either the microfilaments or the intermediate filaments, were responsible for transducing shape changes. Strong arguments were made for the intermediate filaments as they have been shown to extend from the cell membrane to the nuclear pores. Bissell et al. (1982) suggested a

"structuring" hypothesis. It stated that when the organelles were correctly related, protein synthesis proceeded smoothly. When physical or chemical stimuli from the extracellular matrix exert a change on the cell via receptors, it affects the cytoskeleton which then changes the relationship of the mRNA and organelles to the cytoskeleton. Thus with a shape change to the cell, the organelles become disorganized resulting in vacuoles containing newly synthesised proteins not being emptied into the extracellular matrix. Instead they are broken down and negatively feed back on the synthetic system. Evidence in support of this theory was given by Lawrence and Singer (1986). They illustrated how mRNA for specific proteins had definite patterns of distribution within the cell.

In the orthodontic literature, much interest has been focused on the impact of stress on cells associated with sutures and the periodontal ligament. Various authors have developed techniques to stretch cells (Hasegawa et al., 1985; Somjen et al., 1980; Harell et al., 1977; Meikle et al., 1979). The basic assumption in these techniques has been that substrate deformation would produce cell shape change and lead to physiologic changes.

(E) FIBROBLASTS IN VITRO

No variation for collagen synthesis in vitro was found between confluent cell cultures and growing cultures (Tolstoshev et al., 1981; Tajima and Pinnell, 1981; Voss and Bornstein, 1986). This is not to say that the rate of synthesis was constant. Tolstoshev et al. (1981) found that synthesis rates were higher in log phase cells but this was balanced by an increased degradation rate.

(1) Fibroblast Shedding

In cell culture, specimens observed under scanning electron microscope often appear as flat sheets. In addition, spheroids or cells that have rounded up are present (Skehan et al.μ, 1986). These cells form immediately following mitosis during which cells are undergoing a change in shape (Laudry et al., 1982). Often if fluid movement is sufficient these cells will become dislodged and suspended in the medium (Terasima, 1962). It has been shown that if removed and allowed to settle in a new dish, these cells will attach and resemble the original cell culture. If areas exist where they are more numerous, it is probably indicative

of cells that are rapidly dividing.

(2) Fibroblast Traction

Fibroblast mobility is an important requirement for the correct formation and functioning of most tissues. Investigation into this property by workers such as Harris, Stopak and Wild (1981) and Stopak and Harris (1982) demonstrated how it may also be responsible for determining cell and tissue morphology. They showed how cells on silicone surfaces created wrinkles in the surface indicating the exertion of strong, localised traction forces associated with cell elongation (as opposed to contraction forces associated with cell shrinkage). Further evidence of how this property could have an impact on tissue morphology was given by Bell et al. (1979). Their work showed how fibroblasts became aligned and then contracted into a collagen lattice.

Work by Bellows and co-workers (1980, 1981, 1982a, 1982b, 1983) also looked at the traction property of fibroblasts as it applied to dental tissues. Initially they demonstrated fibroblast attachment to partially demineralized dentinal explants in vitro and how surrounding cells were arranged radially around these explants with their long axis parallel to the direction

of tension. The same work showed how cells aligned between two dentinal explants and eventually formed a bridge. This was followed by work with collagen lattices showing how fibroblasts became uniformly oriented over a 24 hour period and eventually contracted the lattice once one end was cut free. The latest work demonstrated how the traction force generated within a collagen lattice was capable of raising a root slice and leads to a theory for cell mediated tooth eruption.

The ability of fibroblasts to exert traction forces has been well examined. Many workers have shed some light on the intracellular mechanism by which this process occurs.

(3) Actin and Myosin

Actin and myosin are present in muscle cells and are responsible for generating contractile forces. These proteins are also present in fibroblasts in the form of fibers called microfilaments or stress fibers. It has been suggested (Ehrlich et al., 1986; Borland et al., 1986) that fibroblast traction forces are generated by these fibers. They have shown that traction forces can be eliminated if the myosin head ATPase enzyme is not activated and if actin is inhibited by cytochalasin B. Evidence has also been provided through visualization

of the actin and myosin molecules (Sanger et al., 1986; Lawson, 1986). Although the actin filaments formed a polygonal network, myosin was found to be associated only with actin filaments aligned with the cells' long axis. In addition, the myosin was distributed in patches, which would explain the cells ability to make small localized contractions.

Additional studies have shown that the intracellular actin and myosin fibrils extend to and attach to cell membrane. At these same membrane loci, collagen has been shown to attach. These points of attachment are called fibronexis (Singer, 1979). In order to have attachment to extracellular collagen, endogenously produced proteins called fibronectins are required.

From this work it can be seen how fibroblasts generate traction forces. The myosin is distributed so as to only activate parallel actin filaments. Thus the traction force is linear and in the same direction as the long axis of the cell. The distribution of myosin into small patches allows for cell elongation to occur while individual patches are contracting. The application of force to the extracellular matrix is facilitated by the actin fibrils terminating at membrane fibronexis which are the attachment sites for collagen

and other extracelluar compounds.

CHAPTER II

STATEMENT OF THE PROBLEM

Collagen metabolism, while central to fibrous joint remodelling during growth, development and physical stress, is just beginning to be understood. The role of type I and type III collagen appear to be related to the remodelling state of the tissue. Further understanding of how collagen metabolism is controlled will require a model system in which the cell subpopulations are better defined than is possible using tissue culture. Thus it is necessary to work with cell cultures with the ultimate aim of using cloned populations. In this way the environment can be controlled and the effect of individual ligands can be investigated. In order to understand the impact of mechanical stress on collagen metabolism, a standardized, force system in vitro is necessary.

The first experiment will attempt to produce an in vitro system for culturing fibroblasts which will incorporate a mechanism utilizing gravity to provide a standardized force to the cells in culture.

The second experiment will attempt to determine the ratio of type III collagen produced during progressively lengthened periods of force application. This is essential for determining the optimal time period at which to test the effect of ligands on stressed fibroblasts.

Knowledge of this behaviour would be the first step in identifying effects of control mechanisms and ultimately the control mechanisms themselves which regulate fibrous joint remodelling. The eventual clinical manipulation of such control mechanisms would facilitate more efficient and less traumatic anatomical remodelling as required in orthodontic therapy.

CHAPTER III

CONTINUOUS STRESSING OF MOUSE INTERPARIETAL SUTURE FIBROBLASTS *IN VITRO*

SUMMARY

The morphological and biochemical response of fibroblasts *in vitro* to continual force was examined. Cells from mouse interparietal sutures were grown out and subcultured onto glass slides. Titanium disks coated with collagen were allowed to attach to the cellular multilayers. Five glass slides were then placed at an angle of 75 degrees for a period of 3 days while five others were left flat. Also five glass slides were left flat with no disk. Following the incubation period the dishes were labelled with ^{14}C-Glycine for 15 hours. The cells and medium were then collected for collagen extraction followed by SDS-polyacrylamide gel electrophoresis. Dried gels impregnated with fluor were exposed to x-ray films which were then scanned densitometrically for collagen types I and III. It was found that the proportion of type III collagen increased with the application of continuous stress. A second set of experimental and control glass slides were fixed in glutaraldehyde and post fixed in osmium tetroxide. Following critical drying and coating, the glass slides were examined under a scanning microscope. The scanning images showed the formation of a ligament-like structure

between the disk and the glass slide. We feel that this system offers a more standardized force system and stresses cells in a ligament-like structure and thus provides a model more analogous to clinical orthodontic and orthopaedic stress.

INTRODUCTION

Several authors have reported the stressing of fibroblasts in cell culture (Brunette, 1984 ; Hasegawa et al., 1985; Binderman and Cox, 1977). The methods utilized to stress these cells have principally been through stretching the substrate upon which the confluent layer of cells had been grown. Although these studies were successful in demonstrating changes in cell physiology due to stress, the experimental techniques had shortcomings. In the systems used, force level standardization was not possible. In addition it was not possible to precisely test different force levels. These systems were not able to test continuous forces. Finally, cell shape was not considered. It has been shown that cell shape plays a large part in determining cell function. Many studies (O'Neill et al., 1986; Senechal et al., 1984; Quinones et al., 1986) have shown how cell shape effects cell growth as measured by DNA

synthesis, cell differentiation and, mRNA and protein synthesis. With this insight it becomes important during in vitro experiments to work with cells that are morphologically similar to in vivo cells, and whose shapes are monitored during stress.

Work has been done to generate fibroblast cell cultures that are suspended in artifical ligaments. Bellows et al., (1983) grew cells in collagen lattices that were used to demonstrate tooth eruption in vitro. Aukhil and Fernyhough (1986) were able to grow cells in a space between pieces of tooth root and bone, quite analogous to the periodontal ligament space.

In this paper, we report on a standardized method for continually stressing cells in a ligament-like structure between two hard surfaces. This stress was shown to increase the proportion of type III collagen synthesized after acting on the cells for 3 days. This system has several advantages over systems used in the past and should provide a more analogous model to orthopaedical and orthodontical stress on cell populations in vivo.

METHODS AND MATERIALS

Culture Technique

Fibroblast cells were derived from interparietal sutures from 7 to 9 week old white Swiss male mice, randomly outbred in our facility. Surgery was performed under ether anesthesia (Mallinckrodt, Inc., Paris, Kentucky). A midsagittal incision was made through the scalp exposing the calvaria. The periosteum was removed and the interparietal suture was dissected free. All further manipulation of tissues and cells was conducted in a laminar flow hood (Nuaire, Minnesota). After 2 washings in growth medium (see below) to remove the blood and the dissection of any adherent soft tissue, the sutures were cut into 1 mm x 1 mm segments and placed into 60 mm petri dishes (Falcon, Oxnard, CA)(16 segments per dish placed evenly thoughout the dish)(dishes were incubated with 100% fetal calf serum for 1 hour prior to the addition of the explants to facilitate adhesion; fetal calf serum decanted prior to addition of growth medium) containing growth medium at 5 ml per 25 cm^2. Growth medium consisted of DMEM, penicillin G (400 units per ml) and streptomycin sulphate (.56 mg per ml)(Gibco, Grand Island, NY),

ascorbic acid (0.2 mg per ml)(Sigma Chemical Co., St. Louis, MO), sodium bicarbonate (2.2 mg per ml)(Fisher Laboratory Chemical, Fair Lawn, NJ), and fungizone (10 µl per ml)(Flow Laboratories, Inc., Mississauga, Ont.), pH 7.4. Media was filter-sterilized using a Millipore 0.22 µm filter (Millipore Corp., Mississauga, Ont.). Sterile fetal calf serum (Boknek, Rexdale, Ontario) was added to 10%. The dishes were incubated (National incubator model 4200, Portland, OR) in a humidified atmosphere of 5% CO_2 at 37 degrees C. The growth medium was replaced every 2-3 days and the cells were monitored with a phase contrast microscope (Nikon, Japan) for confluence.

Subculturing

When confluence was reached after 15 to 18 days, the cells were subcultured onto sterile glass coverslips (Fisher Scientific Co., Winnipeg, Man.) measuring 12.5 mm x 7 mm. The dishes were washed 3 times in Hanks Buffered Saline followed by 2 ml of trypsin (Cooper Biomedical, Malvern, PA) at 4 degrees C for 30 seconds. The trypsin was decanted off and the dishes were incubated for 5 minutes in a humidified atmosphere at 37 degrees C to allow the cells to detach from the dish. The cells were then resuspended in 1 ml of growth

medium. A 100 μl sample was counted on a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) and then appropriate volumes of the cell suspension were placed onto each glass slide to provide 50,000 cells. After 3 hours, all the cells had attached to the glass slide cell and 3 ml of medium were added to the dishes (no evidence of significant cell growth occurring off the glass slide during the experiment was observed).

Stress Mechanism

After a period of 3 days, the cells had achieved confluency on the glass slides. Titanium disks (custom fabricated, Metallurgy Lab., University of Toronto)(Lowenberg et al., 1987) were coated two times with a collagen suspension (Vitrogen 100, Collagen Corporation, Palo Alto, CA). Following rehydration of the collagen with medium, the disks were then placed in the center of the glass slides such that their sintered surface was in contact with the cell multilayer. An incubation period of 18 days followed to allow the cells to attach to the disk. The experimental slides (Figure III-1) were then maintained at an angle of 75 degrees with a spring fabricated out of TMA orthodontic wire (77.8% titanium, 11.3% molybdenum, 6.6% zirconium, and

4.3% tin)(Ormco-Division of Sybron Corp., Glendora, CA)(Goldberg and Burstone, 1979). The control slides were allowed to remain horizontal (Figure III-2).

Scanning Electron Microscope

Specimens were obtained after 3 and 12 days of cell attachment to the disks. In addition, a control for the 3 day specimen was obtained. The specimens were first washed 3 times in Hanks Buffered Saline. They were then fixed in 2.5% glutaraldehyde (Fisher Scientific, Winnipeg, Man.) for 1 hour. This was followed by a post fixation in 1% osmium tetroxide (Sigma Chemical Co., St. Louis, MO) for 1 hour. The specimens were then dehydrated in a graded series of acetones followed by critical drying (Critical Point Dryer, The Bomar Co., Tacoma, WA), mounting on metal stubs (custom fabricated, metal shop, University of Manitoba) with high purity silver paste (JBEM Services, Montreal, Que.), coated with 150-200 nm of palladium-gold (60:40)(Sputter Coater, Technics, Springfield, Virginia), and examined in a JEOL-35C scanning electron microscope (JOEL Ltd., Tokyo, Japan) utilizing 20 KV acceleration voltage.

Radioautography

The cells were grown on glass slides for 21 days prior to being stressed. The experimental samples were stressed for 48 hours prior to labelling. 10 μCi/mL ^{14}C-glycine (CFA.30, Amersham Corp. Oakville, Ont., specific activity 56.0 mCi/mmol) was added to the medium of 6 control and 3 experimental dishes and the incubation continued for 15 hours. The cultures were terminated by removing the medium and harvesting the cells in 1% acetic acid.

Pepsin-Extraction of Collagen

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

Electrophoresis

Collagen alpha-chains and procollagens were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on a 20 cm slab gel. The method followed was a modification of that used by Laemmli (1970) and

utilized a 7.5% cross-linked separating gel, a 2.5% stacking gel, and tris/glycine buffers. The interruption method of Sykes et al. (1976) was used to achieve a separation of type I and type III collagen alpha-chains.

Freeze-dried samples were dissolved in 70 μL of reservoir buffer containing 2 M urea, 2% sodium dodecyl sulphate, and 0.1% bromophenol blue, and were heated at 60 degrees C for 30 minutes to denature the collagen. Samples were introduced to the sample wells and electrophoresis was performed for 1 hour at 160 V. Electrophoresis was stopped and samples were then reduced by the addition of 20% mercaptoethanol to the sample wells to allow the type III alpha-chains to penetrate the gel. After standing for 60 minutes, the electrophoresis was continued at 24 mA/gel until the tracking dye reached the base of the gel.

For fluorographic visualization of separated radio-labelled 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 films (Kodak Canada, Inc., Toronto,

Ont.) at -60 degrees C for varying periods of time as indicated by the scintillation counts of the samples.

Individual sample tracks from the developed fluorographs were scanned at 550 nm and proportions 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). Values obtained for collagen ratios were subjected to statistical analysis using a one way analysis of variance (Steel and Torrie, 1960). In addition, the Tukey Multiple Comparison was done to establish if the control means differed significantly from each other and if the experimental mean differed each control mean.

RESULTS

Scanning

Control

The cells on the glass slide (Figure III-3) appeared to be slightly stressed. This was apparent from the uniform orientation of the cells axially around the titanium disk and the spaces present between the cells. When observed from above, no areas of attachment were

apparent in contrast to the findings of Aukhil and Fernyhough (1986). There were very few spheroid cells present on the glass slide or disk (Figure III-4) and their distribution appeared to be nonspecific. Direct observation in the area of attachment revealed two types of cell attachment: 1) cells present where the disk contacts the cell multilayer, 2) multicell sheets connecting the disk to the multilayer (specimen was damaged by electron beam before these features could be photographed). There were few processes extending up to the disk.

Experimental Day 3

The cells on the glass slide tipped to provide mechanical stress (Figure III-5) appeared similar to those in the control sample except that the spaces between the cells were notably larger. Another difference was the presence of spheroid cells in two areas when the sample was viewed from above. They were where the metal clip had been placed on the glass slide and in the immediate vicinity of the upper third of the disk (Figure III-5, III-6 and III-7). Looking at the disk/slide interface (Figure III-8 and III-9), the two types of attachment were still present. They differed from the controls in that the cells are highly oriented

and had many processes. The cells appeared to be highly stressed. It was also apparent that spheroid cells were present on the disk adjacent to the attachment sites.

Experimental Day 12

At this point in time, the major difference in the attachment was in the development of a cell sheet that extended from the slide to the disk (Figure III-10, III-11 and III-12). It appeared to contain many cells. The morphology of the cells adjacent to the membrane had also changed, appearing spindle-like, not flattened out.

Biochemistry

The nature of the collagens synthesized by the samples labelled for 15 hours during either stressed or unstressed conditions are demonstrated in the following photoradiograph (Figure III-13).

The ratios of type III alpha-chains relative to the total type I and III alpha-chains under the various experimental conditions are presented in Tables III-1 and III-2 and in Figure III-14. One way analysis of variance showed a significant difference between the mean values for cell and medium data. The Tukey analysis of the cell data revealed that the experimental mean

was significantly different from each of the control means ($p<.01$ for the glass slide; $p<.05$ for the glass slide and disk) while analysis of the control means did not yeild a significant difference. The Tukey analysis of the medium data also showed the experimental mean to be significantly different from each of the control means ($p<.025$ for the glass slide: $p<.05$ for the glass slide and disk). The two medium control means were also not found to be significantly different.

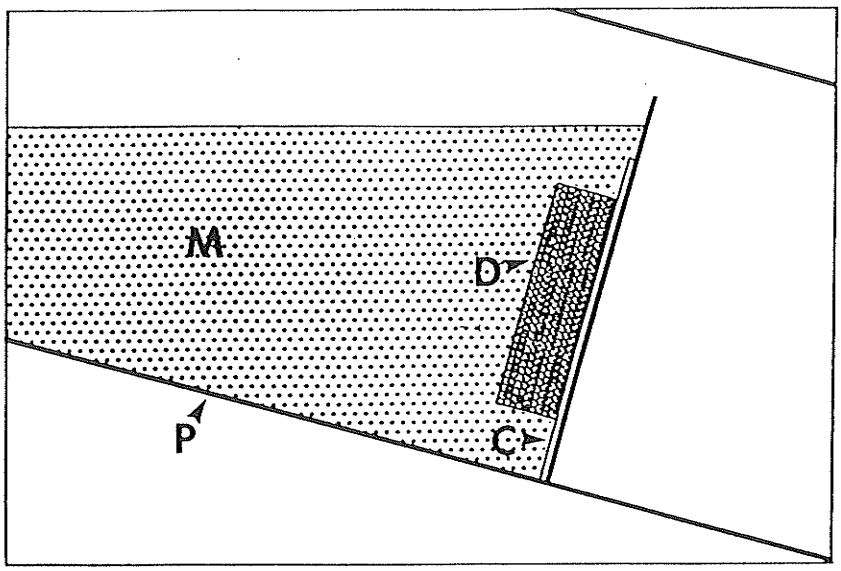

FIG. III-1. Diagram of disk and slide at 75 degrees in a petri dish. M-Medium, P-Petridish D-Titanium Disk, C-Coverslip

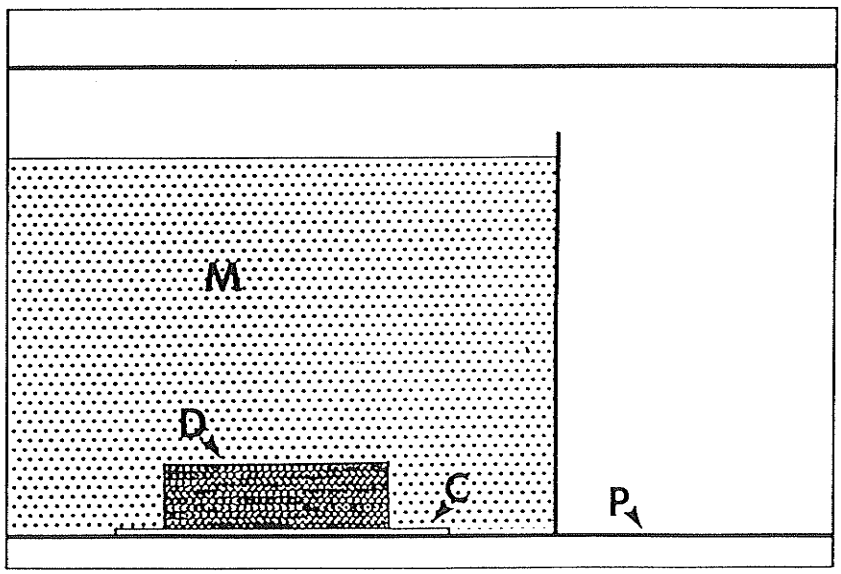

FIG. III-2. Diagram of control sample. Slide and disk are sitting on the base of the petri dish. M-Medium, P-Petriperm, D-Titanium Disk C-Coverslip

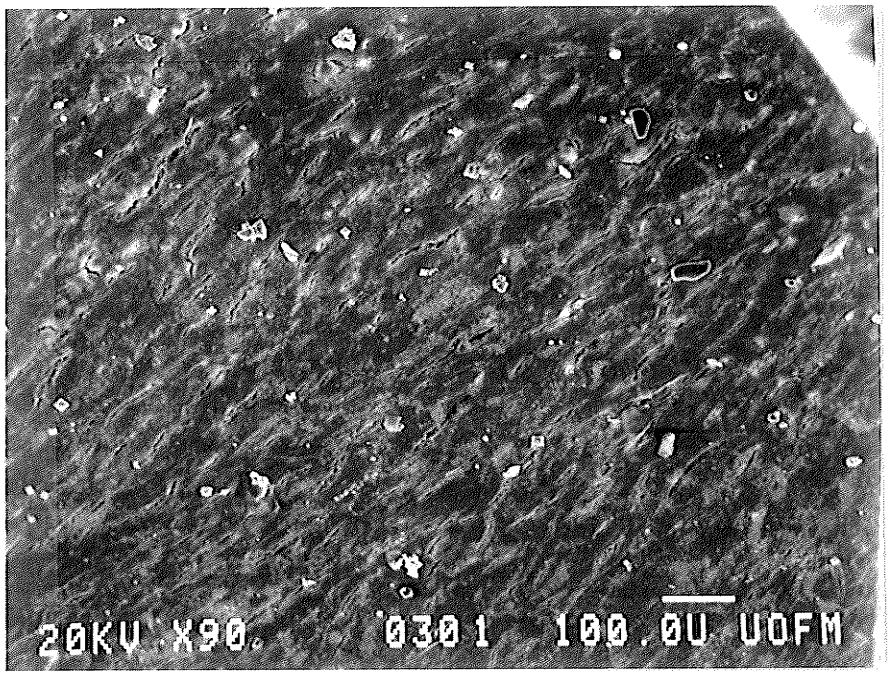

FIG. III-3. Scanning electron micrograph (SEM) of cells on control glass slide. Orientation of cells is radially around the disk (top right).

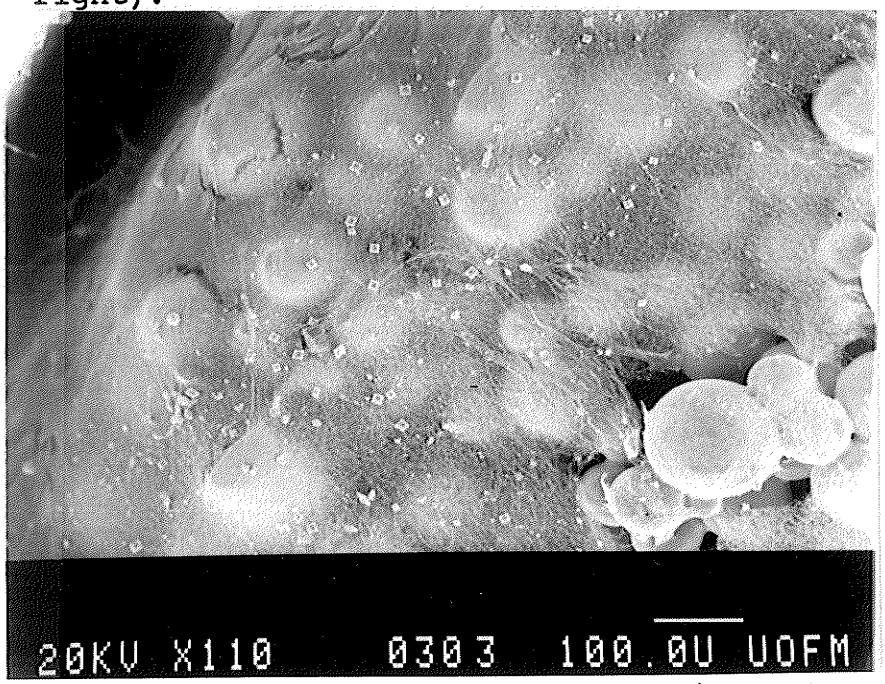

FIG. III-4. SEM of control disk and glass slide (top left). Collagen coating and scattered cells present. No attachment shown.

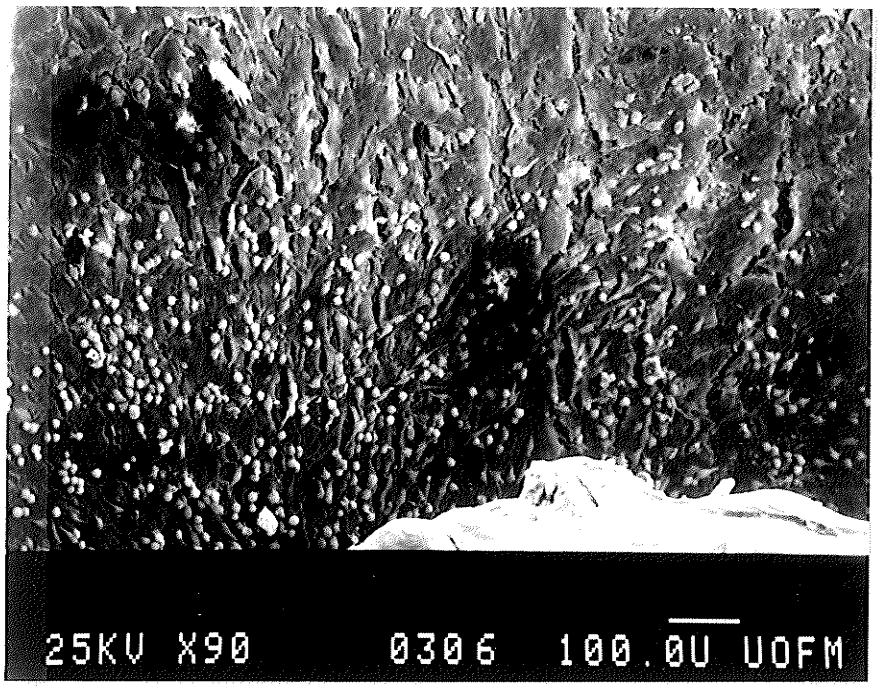

FIG. III-5. SEM of cells on experimental glass slide above disk. Cells oriented radially around disk. Large spaces between cells revealing multilayering.

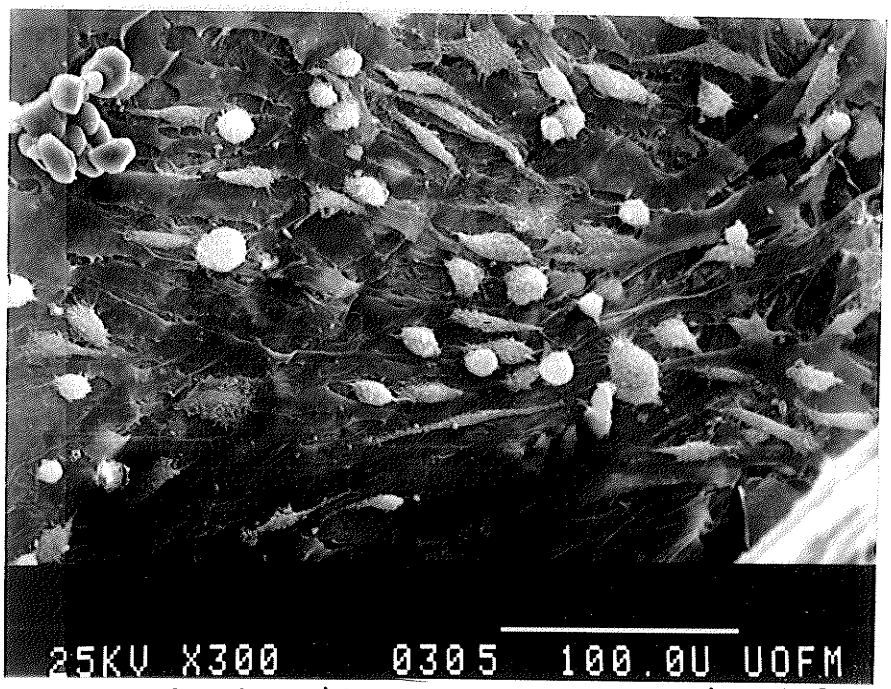

FIG. III-6. SEM of spheroid cells on experimental slide. Gravity pulling disk to right.

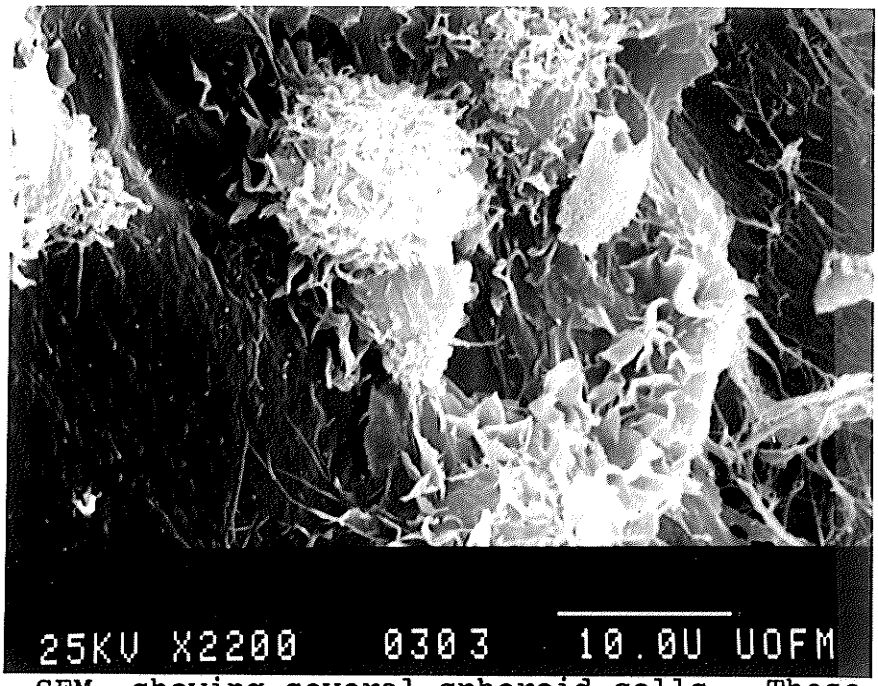

FIG. III-7. SEM showing several spheroid cells. These cells are undergoing mitosis and are easily dislodged from surface.

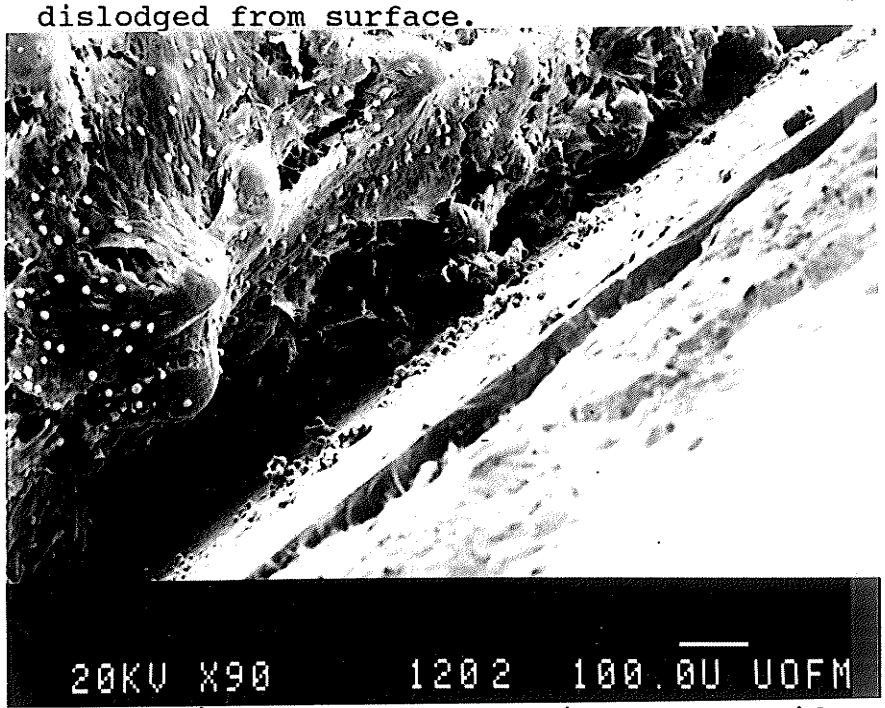

FIG. III-8. SEM of interface between disk and slide. Spheroid cells present adjacent to and on disk. Attachment present at edge of interface.

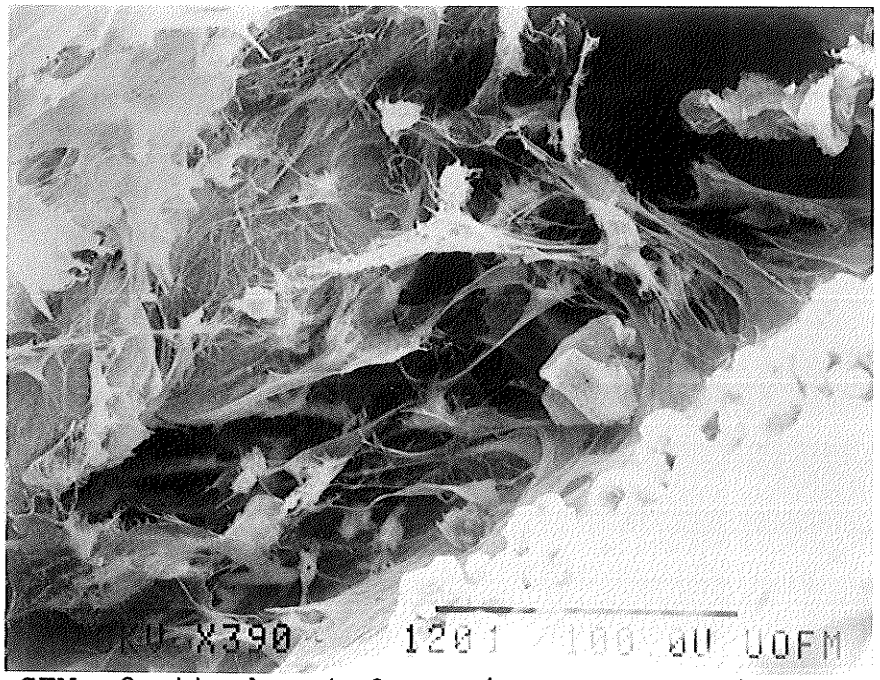

FIG. III-9. SEM of attachment from Fig. III-8. Evidence of cells attaching to disk (top left) and glass slide (bottom right). Spheroid cells in foreground.

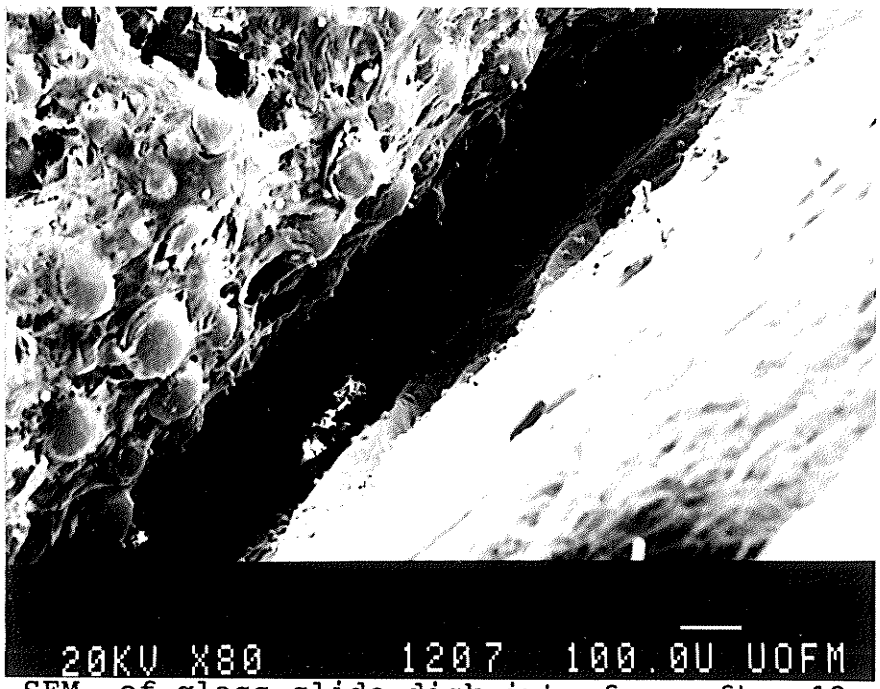

FIG. III-10. SEM of glass slide disk interface after 12 days. Attachment is present beneath disk.

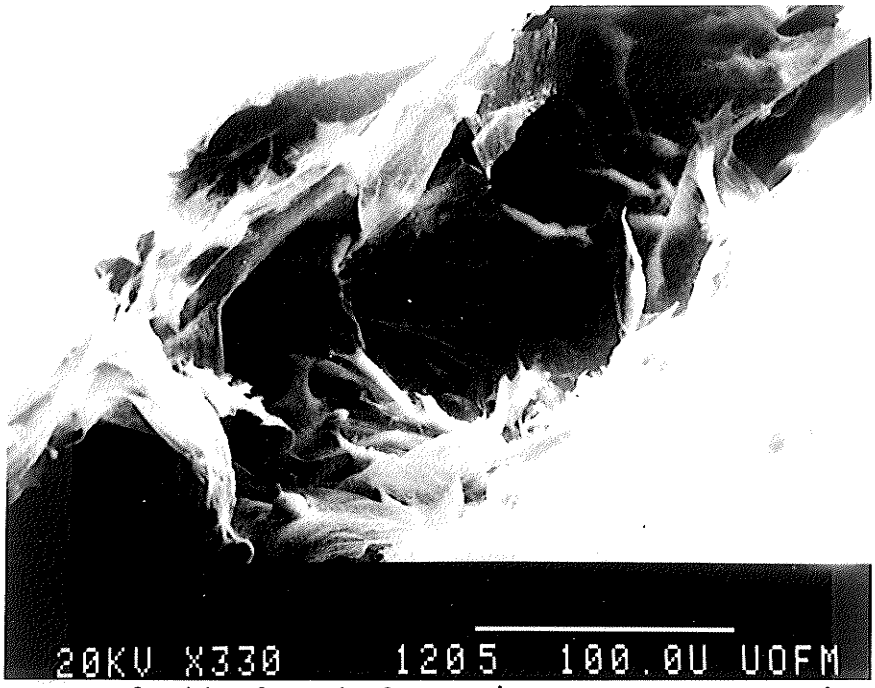

FIG. III-11. SEM of attachment from Fig.III-10. Cells in foreground have become spindle shaped. In background, ligament like tissue has formed.

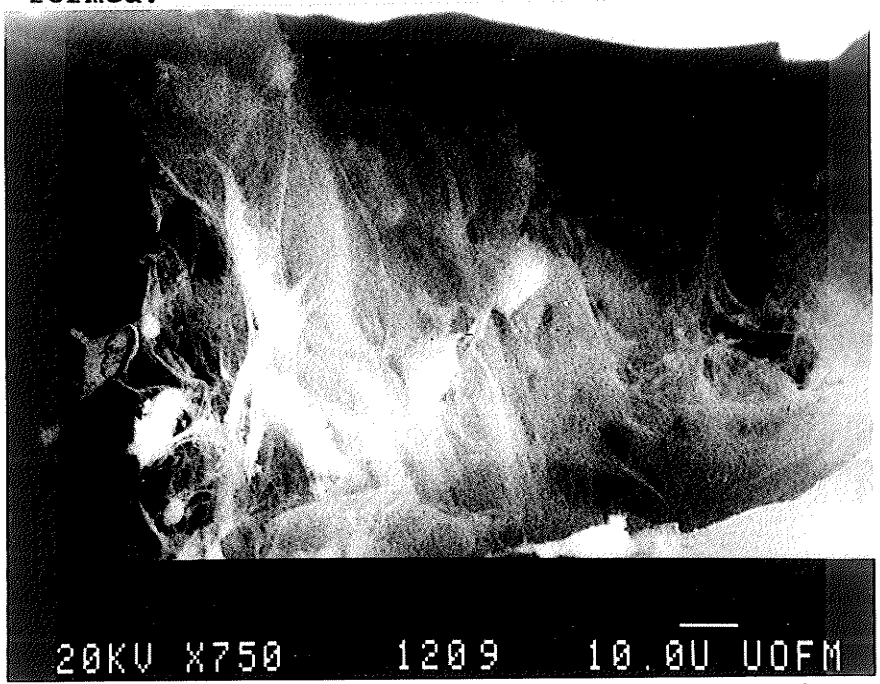

FIG. III-12. Second SEM from 12 day sample showing tissue formation between disk (top) and glass slide (bottom).

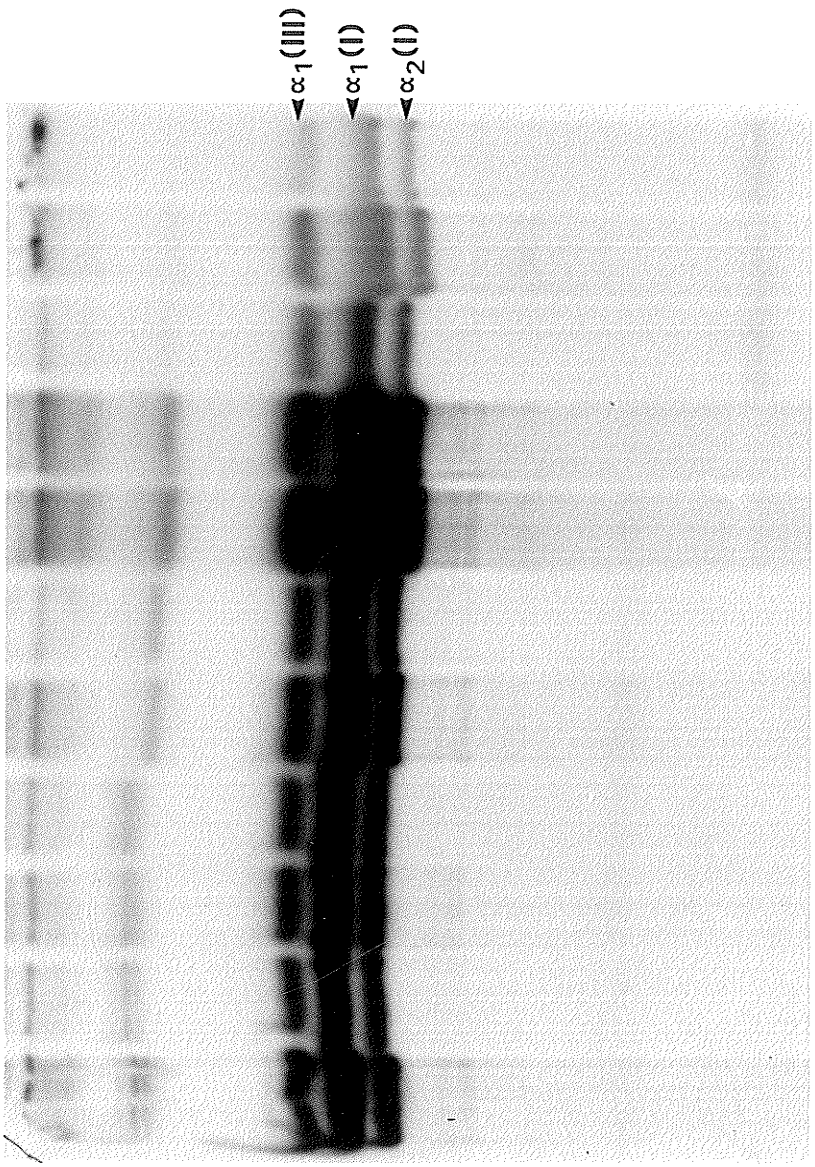

FIG. III-13. Photoradiograph of radiolabelled collagen alpha-chains synthesized in tissue culture. The positions expected for type I and type III alpha-chains are shown.

TABLE III-1

PROPORTION OF TYPE III ALPHA-CHAINS TO THE TOTAL OF TYPE I & III ALPHA-CHAINS FROM CELLS

TREATMENT	N	MEAN	STANDARD DEVIATION
CONTROL	4	25.021	1.65
CONTROL and DISK	2	26.541	1.07
EXPERIMENTAL	4	31.578	1.34

For One Way ANOVA $F_{2,7}$ =15.66, p<0.01
Individual sample data in Appendix-2

TABLE III-2

PROPORTION OF TYPE III ALPHA-CHAINS TO THE TOTAL OF TYPE I & III ALPHA-CHAINS FROM MEDIUM

TREATMENT	N	MEAN	STANDARD DEVIATION
CONTROL	3	17.933	3.78
CONTROL and DISK	2	21.521	0.72
EXPERIMENTAL	2	41.226	7.46

For One Way ANOVA $F_{2,4}$ =9.0, p<0.05
Individual sample data in Appendix-2

FIGURE III-14

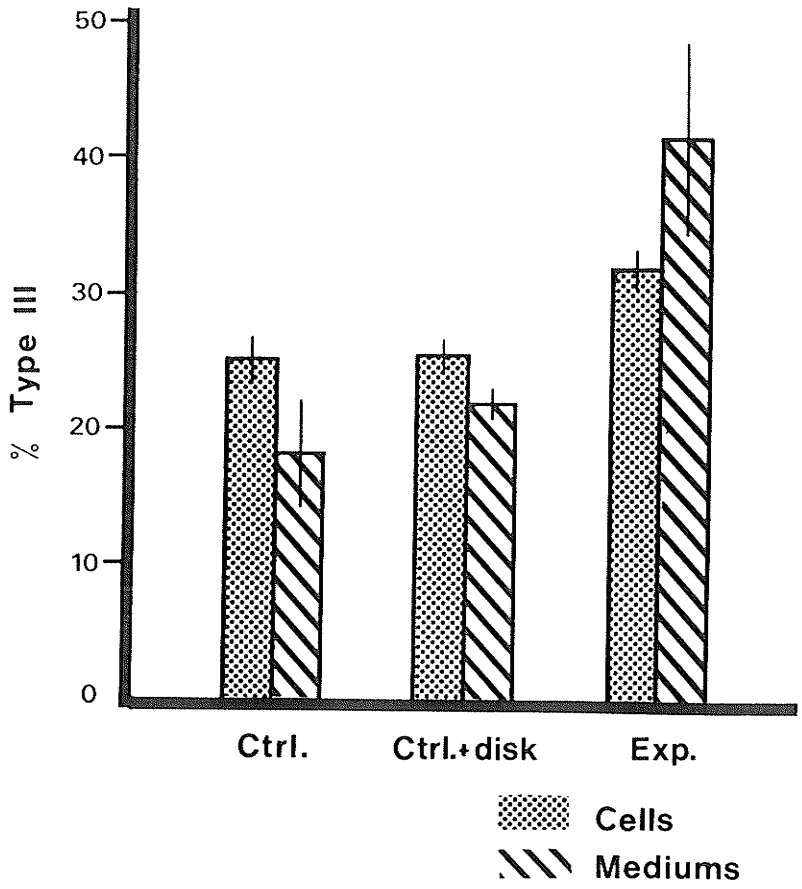

Fig.III-14 PROPORTION OF TYPE III ALPHA-CHAINS TO THE TOTAL TYPE I & III ALPHA-CHAINS FROM CELLS

DISCUSSION

The results indicate that this system has produced changes in protein synthesis similar to work by Duncan et al., 1984 and Yue, 1984. Unlike their work, this system utilized a standardized force system. In addition this system was partially composed of cells that were incorporated in a ligament-like structure. Unfortunately, the biochemical results could not be totally attributed to these cells.

In order to correctly interpret the biochemical results, it is important to understand what appears to be occurring when the disk/slide combination is tilted 75 degrees. Most important is the observation that the disk remains attached to the slide. This can be explained by the scanning electron micrographic evidence showing a sheet of cells extending from the glass slide to the disk. It is likely that this tissue anchors the disk to the glass slide and is thus under stress in the experimental samples. A second observation is that the cells present on the glass slide are also affected by forces. This was observed by the population of spheroid cells around the superior aspect of the disk. As has been suggested in the literature (Laudry, 1982), this

cell morphology appears in mitotic cells. It thus appears that the cells in this region are being stimulated to multiply, as found by others such as Brunette, 1984. The most probable cause for this tension in the cell sheet, is due to the disk being pulled down. This would explain the pattern of distribution of these cells. The appearance of spheroid cells adjacent to the clip attachment was due to movement of the clip during placement that cleared away part of the slide. During the subsequent 3 days cells grew into this area. This localized increase in growth was apparent by the increase in numbers of mitotic cells. The significance of this observation is that these areas of stressed cells on the glass slide provided new cells that may contribute to the observed shift in type III collagen synthesis along with the cells in the ligament between the disk and the slide.

What is not known is what is occurring beneath the cell surface. It would be important to identify the mitotic state of these cells to fully understand the extent of the force stimulated mitosis. This could be done by using thymidine labelling followed by autoradiography of the intact specimen. This would locate dividing cells throughout the thickness of the specimen. Thus evidence is present identifying two

populations of cells that are contributing to the observed collagen synthesis shift. The first is represented by the flat cells on the glass slide and the second is the cells making up the ligament-like tissue between the disk and the slide. We are most interested in the cells in the ligament-like tissue as they have been shown not to be flat morphologically. In fact they appear to be spindle shaped.

The shift in type III collagen that occurred was 6% which was similar to that found by Duncan et al., (1984). Hasegawa et al.(1985) found no difference in the amount of total collagen produced but they did not look for differences in the types produced. This may be due to the time period selected to sample the cells. In order to better understand this system, it will be necessary to carry out a study to determine the nature of the collagen shift over time. A direct result from this work will be the selection of the optimal time to test the effect of ligands on stressed cells.

What remains to be answered is how the force applied to these cells is interpreted to produce the changes in protein synthesis and cell proliferation. One possible explanation is the change in cell shape that occurs when these cells are acted on by forces. This may rearrange the organelles present within the cells thus

changing the synthetic pattern. Another theory is based on the idea that the cytoskeleton was affected by the cell shape change resulting in a direct message being given to the protein production mechanism and nucleus. These questions can be addressed using this system by examining the impact of various ligands on this standardized force system.

(For further discussion, see Chapter 5)

CONCLUSIONS

In this study, a system has been presented which utilizes a standardized force system to apply shear-forces to cells. The effect is not felt by all the cells present but is limited to two groups. One is composed of the flattened cells just superior to the disk. The second group comprises ligamentous-like cells making up the attachment apparatus between the disk and the slide. This system should be most advantageous for investigating the effect of force magnitude, force duration and the effects of ligands on cells.

CHAPTER IV

TIME-COURSE STUDY OF COLLAGEN PHENOTYPE SYNTHESIS IN MOUSE INTERPARIETAL SUTURE FIBROBLASTS UNDER SHEAR-LIKE STRESS *IN VITRO*

SUMMARY

The biochemical response at various times of fibroblasts *in vitro* to continual force was examined. Cells from mouse interparietal sutures were grown out and subcultured onto glass slides. Titanium disks coated with collagen were allowed to attach to the cellular multilayers. Samples were selected for six time periods: 6, 12, 24, and 36 hours and 3 and 5 days. Each sample consisted of four controls and four experimental cultures. Following the incubation period the dishes were labelled with ^{14}C-Glycine for 6 hours. The cells and medium were then collected for collagen extraction followed by SDS-polyacrylamide gel electrophoresis. Dried gels were exposed to x-ray films which were then scanned for collagen types. It was found that the synthesis of type III collagen did not shift significantly during the experimental period.

INTRODUCTION

The study of fibrous joint remodelling is important in developing an understanding of orthodontic tooth movement. Collagen metabolism in response to forces has been studied in attempting to clarify the role of fibroblasts (Yen et al., 1980; Meikle et al., 1984; Yue, 1984). The approach to this area has been diverse involving in vivo experiments, cell culture and organ culture. The advantage of in vivo studies has been the preservation of the three dimensional structure in which the cells function. Unfortunately, the cellular environment is complex in ways that make precisely contolled studies difficult. This provided the incentive to isolate the cells of interest in a uniform, controlled environment. The major shortcoming with these studies (Brunette, 1984; Hasegawa et al., 1985; Binderman and Cox, 1977) has been the loss of the tissue architecture which is known to be important in modifying cell function (O'Neill et al., 1986; Senechal et al., 1984; Quinones et al., 1986). Thus any attempt to recreate the original tissue architecture in cell culture would be important (Bellows and co-workers, 1981, 1982a, 1982b, 1983; Aukhil and Fernyhough, 1986).

A cell culture system has been developed which attempts to recreate the periodontal ligament structure. It is necessary to better understand this system before it can be effectively utilized. It is with this goal that the current work was undertaken. In this chapter, we report on a cross sectional study where cells were continually stressed in a ligament like structure between two hard surfaces. In this study we were unable to detect a significant shift in type III collagen synthesis as a result of the application of stress to the cells in culture.

METHODS AND MATERIALS

Culture Technique

Fibroblast cells were derived from interparietal sutures from 7 to 9 week old Swiss male white mice, randomly outbred in our facility. Surgery was performed under ether anesthesia (Mallinckrodt, Inc., Paris, Kentucky). A midsagittal incision was made through the scalp exposing the calvaria. The periosteum was removed and the interparietal suture was dissected free. All further manipulation of tissues and cells was conducted in a laminar flow hood (Nuaire, Minnesota). After 2 washings in growth medium (see below) to remove the blood and any adherent soft tissue the sutures were cut into 1 mm x 1 mm segments and placed into 60 mm petri dishes (Falcon, Oxnard, CA.)(16 segments per dish evenly distributed)(dishes were incubated with 100% fetal calf serum for 1 hour prior to the addition of the explants to facilitate adhesion; fetal calf serum decanted prior to addition of growth medium) containing growth medium at 5 ml per 25 cm^2. Growth medium consisted of DMEM, penicillin G (400 units per ml) and streptomycin sulphate (.56 mg per ml)(Gibco, Grand Island, NY), ascorbic acid (0.2 mg per ml)(Sigma Chemical Co., St.

Louis, MO), sodium bicarbonate (2.2 mg per ml)(Fisher Laboratory Chemical, Fair Lawn, NJ), and fungizone (10 μl per ml)(Flow Laboratories, Inc., Mississauga, Ontario), pH 7.4. It was filter-sterilized using a Millipore 0.22 μm filter (Millipore Corp., Mississauga, Ont.). Sterile fetal calf serum (Boknek, Rexdale, Ontario) was added to 10%. The dishes were incubated (National incubator model 4200, Portland, OR) in a humidified atmosphere of 5% CO_2 at 37 degrees C. The growth medium was replaced every 2-3 days and the cells were monitored with a phase contrast microscope (Nikon, Japan) for confluence.

Subculturing

When confluence was reached after 15-18 days, the cells were subcultured onto sterile glass coverslips (Fisher Scientific Co., Winnipeg, Man.) measuring 12.5 mm x 7 mm. The dishes were washed 3 times in Hanks Buffered Saline followed by 2 ml of trypsin (Cooper Biomedical, Malvern, PA) at 4 degrees C were added for 30 seconds. The trypsin was decanted off and the dishes were incubated for 5 minutes in a humidified atmosphere at 37 degrees C to allow the cells to detach from the dish. The cells were then resuspended in 1 ml of growth

medium. A 100 μl sample was counted on a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) and then appropriate volumes of the cell suspension were placed onto each glass slide to provide 50,000 cells. After 3 hours all the cells had attached to the glass slide and 3 ml of medium was added to the dishes.

Stress Mechanism

After a period of 3 days, during which time the cells have achieved confluency on the glass slides, titanium disks (custom fabricated, Metallurgy Lab., University of Toronto) were coated two times with a collagen suspension (Vitrogen 100, Collagen Corporation, Palo Alto, CA). Following rehydration of the collagen with medium, the disks were then placed in the center of the glass slides such that their sintered surface was in contact with the cell multilayer. An incubation period of 18 days followed to allow the cells to attach to the disk. The slides were then maintained at an angle of 30 degrees.

Radioautography

The cells were grown on glass slides 21 days prior to being stressed. Six samples of eight dishes were

randomly selected for each of the following time periods: 6, 12, 24, 36 hours and 3 and 5 days. Four dishes from each time period were used as controls and the remaining four were designated as experimental. At the beginning of the experiment, all the experimental samples were placed at 30 degrees and the controls were left flat. Six hours prior to the termination of each sample they were labelled with 10 μCi/mL ^{14}C-glycine (CFA.30, Amersham Corp. Oakville, Ont., specific activity 56.0 mCi/mmol). The cultures were terminated by removing the medium and harvesting the cells in 1% acetic acid.

Pepsin-Extraction of Collagen

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

Electrophoresis

Collagen alpha-chains and procollagens were separated by sodium dodecyl sulphate-polyacrylamide gel

electrophoresis on a 20 cm slab gel. The method followed was a modification of that used by Laemmli (1970) and utilized a 7.5% cross-linked separating gel, a 2.5% stacking gel, and tris/glycine buffers. The interruption method of Sykes et al. (1976) was used to achieve a separation of type I and type III collagen alpha-chains.

Freeze-dried samples were dissolved in 70 μL of reservoir buffer containing 2 M urea, 2% sodium dodecyl sulphate, and 0.1% bromophenol blue, and were heated at 60 degrees C for 30 minutes to denature the collagen. Samples were introduced to the sample wells and electrophoresis was performed for 1 hour at 160 V. Electrophoresis was stopped and samples were then reduced by the addition of 20% mercaptoethanol to the sample wells to allow the type III alpha-chains to penetrate the gel. After standing for 60 minutes, the electrophoresis was continued at 24 mA/gel until the tracking dye reached the base of the gel.

For fluorographic visualization of separated radio-labelled 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 films (Kodak Canada, Inc., Toronto, Ont.) at -60 degrees C for varying periods of time as indicated by the scintillation counts of the samples.

Individual sample tracks from the developed fluorographs were scanned at 550 nm and proportions 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). Values obtained for collagen ratios were subjected to statistical analysis using a two way analysis of variance (Steel and Torrie, 1960).

RESULTS

The results of this experiment are presented in table IV-1 and figure IV-1. This represents the data from the cells. The medium data was highly inconsistent (Appendix-4) due to technical problems.

TABLE IV-1

PROPORTION OF TYPE III ALPHA-CHAINS TO THE TOTAL OF TYPE I & III ALPHA-CHAINS FROM CELLS

TIME	CTRL	ST.DEV	EXP.	ST.DEV
6 Hours	22.18	3.13	22.6	4.06
12 Hours	26.7	4.21	28.28	2.73
24 Hours	27.7	3.48	24.1	5.4
36 Hours	28.33	3.91	34.78	7.8
3 Days	27.45	2.09	26.1	2.38
5 Days	19.2	8.2	18.6	2.59

Individual sample data in Appendix-3

FIGURE IV-1

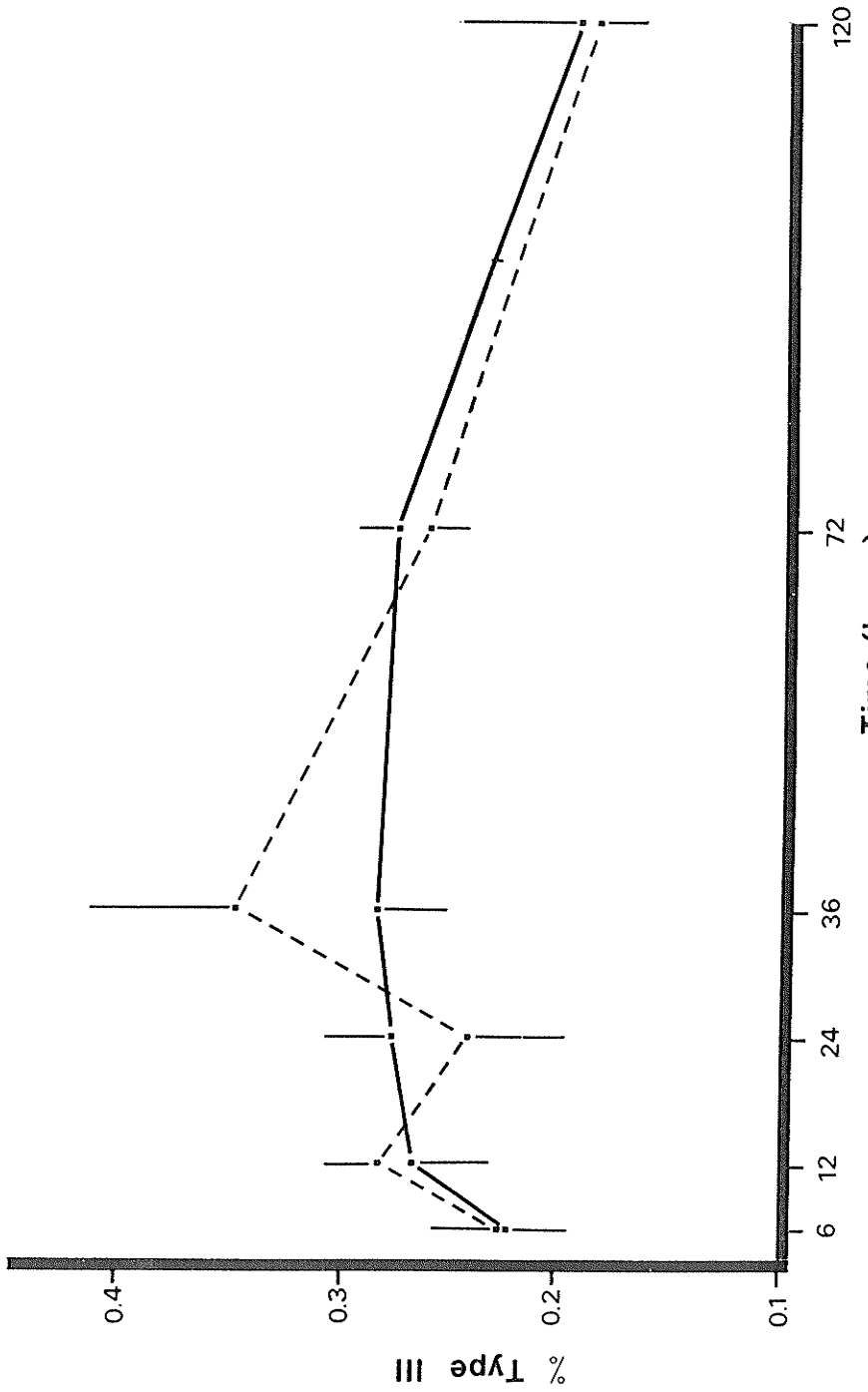

Fig. IV-1 Proportion of Type III Collagen During Stress

DISCUSSION

This work has demonstrated a method of generating an *in vitro* ligament-like structure that can be continually stressed. It has also been shown how this structure responds to stress over a 5 day time period. Unlike previous studies in this area, we were unable to find a statistically different pattern of type III collagen synthesis over the time period that we studied. There are several possible explanations for this finding. The most promising direction to investigate is that the force generated in the samples at 30 degrees is too small to have a measurable impact on cell activity. If this were true, additional studies using various force magnitudes would go a long way in helping orthodontists to understand how their mechanics work biologically.

Another possible explanation is that the cell layer on the glass slide hadn't formed a ligament as substantial as in the previous experiment. This is supported by the observation that we were unable to tip these samples more than 40 degrees without failure (unlike the previous experiment where they stayed attached at 75 degrees). One possible way to improve the

attachment would be to lightly sand the sintered surface of the disk. This would serve to increase the number of metal points contacting the glass and thus the number of areas which initiate the formation of the ligament. In this way the strength of the attachment could be increased and allow not only vertical sample orientation but in addition inverted sample orientation.

Unfortunatly, we were unable to produce good quality data from our medium samples. There appeared to be a wide range of results within samples treated similarly. It appeared that the technique for processing these samples is not yet perfected.

From this work, it is possible to test more questions relating to the practice of clinical orthodontics. Of paramount importance to orthodontists has been what is an ideal force level with which teeth should be moved? This model will provide insight into this question since it can easily be set up using different forces on the cells.

Another important direction that this work can take is to use human periodontal ligament cells. This would remove the step of extrapolating animal results to human cells. In addition, differences or similarities between the various cell lines under experimental conditions, as with our attempt to use rat cells, would be useful.

The six hour labelling period that was used in this work was necessary in order to allow the small number of cells present to produce a detectable amount of collagen. With the success of this work, it would be important to try and reduce this labelling period in future experiments to minimize the destruction of labelled collagen that would be occurring in vitro. With this modification, a better picture of the collagen synthesis pattern would be produced.

The rationale for this new model system has been to better simulate the periodontal ligament in vitro. It would be appropriate to compare the effect of forces on cells between this system and others such as with petriperm. The results of this comparison would indicate if the cells response to forces varies between cells in a sheet and those in a ligament.

Another direction for study that this technique presents is in applying different types of forces on cells. We successfully generated tensile forces on cells using tooth slices but were unable to repeat this work using the titanium disks. The problem appears to be with the mass of the metal disks tearing the attachment within 30 minutes of activation. Success in this direction will most likely come about with the use of thinner and thus lighter disks or by improving the

attachment with the present disks by providing more contact points as previously mentioned.

The success of this work opens up many possible directions for future investigations using this model system.

CONCLUSIONS

In this study, a system has been presented which utilizes a standardized force system to apply shear-forces to cells. No physiologic effect due to this force was detected when the proportion of type III collagen that was being produced was measured. This lack of effect may have been due to insufficient force magnitude as the slide was elevated only 30 degrees. This system should be most advantageous for investigating the effect of force magnitude, force duration and various ligands on cells.

CHAPTER V

DISCUSSION

Discussion

The goal of this line of research is to better understand the biological component of tooth movement as it applies to clinical orthodontics. In simplifying the questions to a level where meaningful answers would be possible, we have found the need to look at individual cell types *in vitro*. Thus efforts were directed at developing an experimental model that would allow us to answer questions relating to forces and cellular responses of cloned cell populations *in vitro*.

The results of the first experiment have shown that this model system simulates a ligament-like structure *in vitro* which can be stimulated by shear-like forces. The results of the second experiment have shown how the forces applied have to be above a minimum value in order to significantly effect the cell activity.

The results from these two experiments suggest that this system may provide a new approach to examining the effect of forces on cells. At this point it would be beneficial to discuss several important observations that would aid in the application of this technique.

Two questions that have yet to be answered concern the effect of different types and different magnitudes

of forces on cells. More specifically, do cells differentiate shear forces from tensile and do cells differentiate different force magnitudes. This line of questioning can be pursued with this system by simply orienting the coverslip with the cell-attached disks "upside down" to produce tensile stress on the cell populations.

This study has demonstrated that cells do respond to shear-like forces. It would be relatively simple to take the glass-disk sample and suspend it inverted to create a tensile force on the supporting cells. This has in fact been attempted in a pilot study. The disks were successfully suspended but for unknown reasons detached after 30 minutes. In a previous pilot study (Appendix 6) we successfully suspended root slices and demonstrated that this idea was in fact fesable. Once this has been successfully completed, it will be valuable to see the cell responses to the different forces. If in fact there is a differing response, it will change the presently held concept that all clinically applied forces on teeth result in a uniform cellular response.

The design of this model can be readily modified to compare the cell responses to different force magnitudes. This would be done by changing the angle at which the glass slide is tipped. The force level would

be limited, on the low side, by static friction which would eventually be greater than the force of gravity pulling along the glass. On the high side, it is limited by the strength of the attachment which was found to fail at about 90 degrees.

The adaptation of the tensile force system to measure the effect of force magnitude would be expensive but possible. It would involve the assembly of several sets of disks, each of a different weight. Thus it should be apparent that this system is flexible in its examination of cell response to forces.

The utilization of this system would be most informative when used with cloned populations of cells. It would then be possible to gain insight into the manner by which different cell populations respond to forces. It is not known whether a specific cell would shift its synthesis pattern in proportion to the force applied or completely once a threshold force level is applied.

One apparent shortcoming of this model system is that it will not provide insight into the effect of intermittent forces versus continuous forces.

In summary, this thesis is the start of work that could reveal the character of cell responses to forces. It will be from this information that more specific

questions can be raised as to the mechanisms of cellular force transduction. The eventual success of this line of questioning may soon lead to improved clinical techniques that will enhance the face of orthodontics.

CHAPTER VI

SUMMARY AND CONCLUSIONS

(1) A model system was developed that utilized the acceleration of gravity and standardized masses of titanium to apply continuous forces to cells in culture.

(2) Scanning electron microscopic images were used to illustrate the character of the attachment achieved by the cells. It appeared to be in the form of a ligament-like structure that formed a ring beneath the disk. Evidence of an increased mitotic rate for surface cells away from which the disk moved provided further information as to the extent of the force system.

(3) The first study indicated that the application of a constant force generated an increase in the proportion of type III collagen synthesis.

(4) The second study indicated that the force system doesn't generate enough force to stimulate the cells at a statistically significant level when the samples are placed at an angle of 30 degrees.

(5) This system can be easily modified to allow the testing of the effect of various force magnitudes and force types (shear and tensile). This facility is essential if the nature of the response to forces is to be characterized.

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APPENDIX

Appendix-1

TITANIUM DISKS

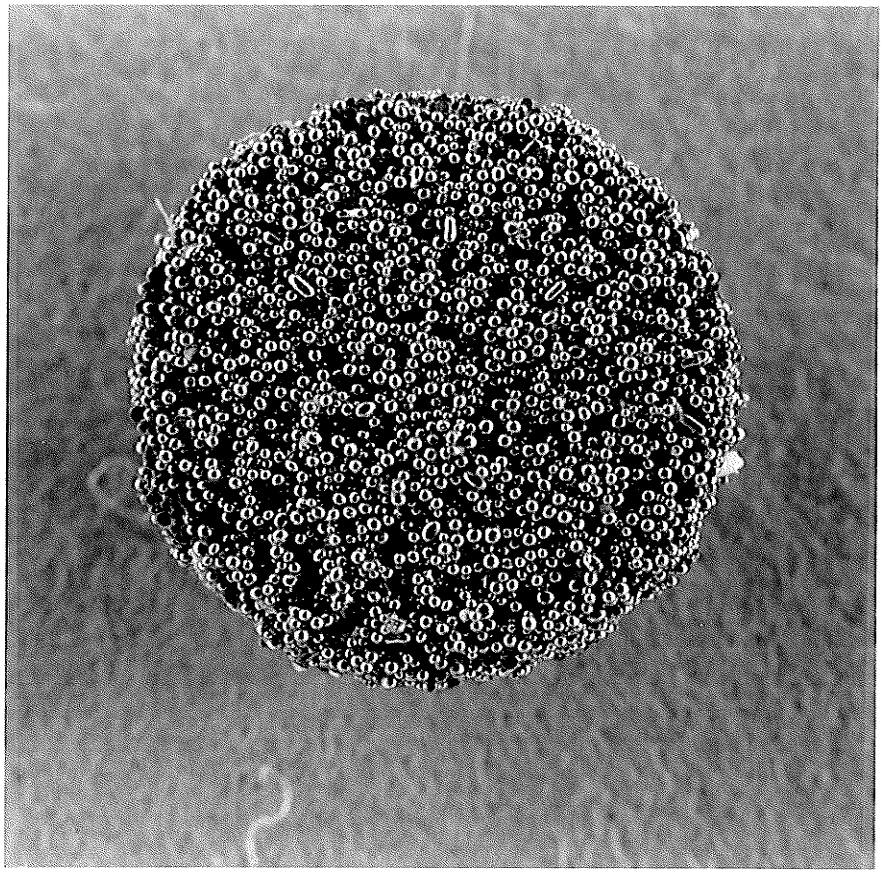

Fig. App.1-1 Sintered surface of disk

Composition.....Ti-6Al-4V

Mean weight of disks.....0.104 gm

Standard deviation.....0.010 gm

Dimensions; diameter.....5.0 mm

thickness.....1.0 mm

Appendix-2

PROPORTION OF TYPE III ALPHA-CHAINS TO THE TOTAL OF TYPE I & III ALPHA-CHAINS FROM CELLS

TREATMENT	III / I & III %
CONTROL	22.351
	25.728
	26.820
	25.183
CONTROL and DISK	27.611
	25.471
EXPERIMENTAL	30.290
	31.100
	31.100
	33.820

PROPORTION OF TYPE III ALPHA-CHAINS TO THE TOTAL OF TYPE I & III ALPHA-CHAINS FROM MEDIUM

TREATMENT	III / I & III %
CONTROL	23.28
	15.26
	15.26
CONTROL and DISK	20.81
	22.24
EXPERIMENTAL	48.68
	33.77

Appendix-3

PROPORTION OF TYPE III ALPHA-CHAINS TO THE TOTAL OF TYPE I & III ALPHA-CHAINS FROM CELLS

TIME	CONTROL	EXPERIMENTAL
6 Hours	25.2	18.0
	20.7	25.7
	18.5	24.1
	24.3	
12 Hours	27.9	30.2
	24.0	31.0
	32.1	26.4
	22.8	25.5
24 Hours	31.4	28.8
	29.9	18.2
	24.3	25.3
	25.2	
36 Hours	23.9	29.1
	31.3	46.3
	29.8	32.3
		31.4
3 Days	26.0	25.2
	25.5	28.8
	30.0	24.3
	28.3	
5 Days	25.0	15.7
	13.4	19.0
		17.8
		21.9

Appendix-4

PROPORTION OF TYPE III ALPHA-CHAINS TO THE TOTAL OF TYPE I & III ALPHA-CHAINS FROM MEDIUM

TIME	CONTROL	EXPERIMENTAL
6 Hours	0.0	0.0
	10.7	24.3
	0.0	23.8
	0.0	13.0
12 Hours	0.0	8.7
	0.0	16.0
	0.0	0.0
	0.0	0.0
24 Hours	0.0	0.0
	0.0	0.0
	0.0	0.0
	7.9	0.0
36 Hours	0.0	0.0
	7.3	0.0
	3.4	34.1
	0.0	0.0
3 Days	5.1	0.0
	4.7	0.0
	12.7	11.9
	0.0	5.7
5 Days	0.0	5.0
	0.0	0.0
	0.0	0.0
	0.0	3.9

Appendix-5

TIME-COURSE STUDY OF COLLAGEN PHENOTYPE SYNTHESIS IN RAT INTERPARIETAL SUTURE FIBROBLASTS UNDER SHEAR-LIKE STRESS IN VITRO

A study similar to that carried out in Chapter IV was carried out using 5 week old white male Sprague-Dawley rats instead of Swiss mice. Immediately after the cells were subcultured onto the glass slides, it was found that the cells appeared to spread throughout the petri dish. In addition, the glass slide remained thinly covered with cells until after 8 days, once the entire dish had become monolayered.

Of greater significance is the observation that the disks never became attached to the glass slide via a cell bridge. This was the finding after waiting for a period of 25 days. Observation of the glass slide after the disk had fallen off revealed virtually no areas of irregular, torn tissue that had been disrupted with the removal of the disk.

From this experiment it is apparent that not all suture cells share the same basic properties. It is possible that mouse cells have a stronger chemotactic

attraction to collagen and hence are stimulated to migrate and form an attachment.

Appendix-6

PILOT STUDY TO ESTABLISH IF GRAVITY CAN BE USED TO GENERATE A CONTINUOUS FORCE SYSTEM ON CELLS GROWN IN VITRO

One of the initial studies that I conducted aimed at demonstrating that a fibroblast cell sheet could attach a root slice to a glass coverslip and suspend it against the force of gravity. The value of this system was that a continual force would be applied to the cells.

The experiment utilized mouse interparietal suture fibroblasts which were obtained in the same manor as has been outlined in chapters III and IV. The cells were subcultured onto 25 mm X 25 mm coverslips upon which were two rows of root slices. The root slices had been cut from teeth that had been removed for orthodontic reasons. The root slices varied in mass and surface area but had a uniform thickness of 0.5 mm. The root slices were surface demineralized to enhance cell attachment and then sterilized in ethylene oxide. The system was grown for 3 weeks to allow for the formation of a collar of cells around the root slices. At this time we were

able to hold the coverslip inverted without the loss of the root slices. Additional studies were done to better understand the nature of the cell attachment. Scanning evidence suggested that a collar of cells formed around the root slices and attached them to the coverslip. Inversion of the sample caused the cells around the root slice to become stressed and appear "stretched" in the scanning images.

This approach was considered limited in potential for use in experiments as the force system was highly variable but the principle of utilizing gravity to create the force system was retained in subsequent model systems.