THE EFFECT OF MECHANICAL FORCE ON COLLAGEN PHENOTYPE SYNTHESIS OF PERIODONTAL TISSUES IN VITRO

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

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ABSTRACT

Collagen is the main extracellular component of all connective tissues. Its metabolism is therefore an essential part of the biochemical aspects of growth and development (Baily and Robbins, 1976,; Kivirikko and Risteli, 1976; Bornstein and Traub, 1979). In addition, regulated synthesis of the phenotypes may be crucial in the dynamic processes of tissue repair and stability (Bornstein and Sage, 1980) both of which are integral components of orthodontics and dentofacial orthopedics.

Two groups of soft tissue periodontal fibers have been implicated in the degree of resulting stability following orthodontic therapy: the supraalveolar group of fibers and the principal fibers of the periodontal ligament (Edwards, 1988). Both of these fibers have been shown to be made up of a large number of collagen phenotypes dominated by types I and III (Narayanan et al., 1983a). Although the turnover of collagen in the gingival tissues is more rapid than in most other tissues (Page and Ammons, 1974), the collagens of the periodontal ligament are altered at an even more accelerated rate (Sodek, 1976).

The application of physiologic levels of force has been shown to stimulate the production of both collagen and noncollagen proteins in vitro (Sumpio et al., 1988a, 1988c). However, different regimens of force application may be transduced into varying cell responses (Banes et al., 1988). The proportion of type III/type I + III collagen synthesis has been

shown to be an indication of the cellular response to mechanical stress (Yen et al., 1989b).

In the first investigation, the effect of force magnitude, duration and frequency on synthesis patterns of collagen phenotypes in cell populations isolated from rat gingiva in vitro was studied. Gingival fibroblast-like cells were grown from palatal gingival explants from 3 week old male rats and subcultured in collagen coated Flex I^R culture dishes. Confluent cell cultures were subjected to cyclic force regimens of loading and relaxation of 180 cycles per hour (cph), 30 cph and 0.5 cph as well as a continuously applied force regimen. Cultures were harvested after 2, 4, 6, 8 and 24 hours of loading at a maximum strain of either 24 or 10 percent. Control cells received no stress.

5 μCi/ml of [^{14}C]-Glycine were added for the last 2 hours of culture. Type I and type III collagen alpha chains were separated by SDS-PAGE with a delayed reduction step.

Densitometric scans of fluorograms from dried gels demonstrated a significant increase in the proportion of newly-synthesized type III collagen in the cultures stressed intermittently but not in the constantly stressed or control cultures. Peak responses in type III collagen synthesis appeared after 4 to 8 hours of stress and decreased at longer periods. No significant difference was demonstrated between the strain levels over the experimental period. These findings suggests a delayed response to intermittent force to which the cells eventually adapt as part of their microenvironment.

In the second investigation, the effects of mechanical force application and duration as well as cell passage on collagen phenotypes in cell populations of rat gingiva *in vitro* were investigated. As in the previous investigation, gingival fibroblast-like cells were grown from palatal gingival explants from 3 week old male rats. Confluent cultures grown from explant under a cyclical force application and first passage cell cultures, both confluent and nonconfluent, were subjected to cyclical force regimens of loading and relaxation of 30 cph for 2, 4, 6, 8 and 24 hours at a maximum strain of 24 percent. Control cells were confluent first passage cultures which received no force application. The cells were labeled and processed for fluorographic scanning as in the previous investigation.

A significant increase in the proportion of newly-synthesized Type III collagen synthesis appeared after 2 hours of stress in the explant cultures and remained throughout the experimental period. This early increase was also shown in the first passage nonconfluent cultures, however this significance dissipated after 4 hours only to return at the 24 hour time period. First passage confluent cells showed a latent response at the early time period reaching significant increases at 4, 6 and 8 hours before returning to nonsignificant levels after 24 hours of force application. These findings suggest that primary cell cultures grown in a dynamic environment may adapt or select for cell populations adaptable to levels of heightened response to intermittent force. This hypothesis may also hold true to

nonconfluent first passage cells as visual inspection of the wells revealed increased cell numbers and cell alignment throughout the investigation.

DEDICATION

TO MY LOVING WIFE, BRIDGET

FOR HER UNWAVERING SUPPORT AND ENCOURAGEMENT

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CHAPTER I

LITERATURE REVIEW

INTRODUCTION

The hard and soft connective tissue changes that occur within the periodontal tissues as a result of the application of orthodontic forces has been studied extensively (see Storey, 1975; Reitan, 1985; Rygh, 1986; and Roberts et al., 1981 for reviews). Prolonged application of forces that exceed the tissues' bioelastic limits result in tooth movement through the changes in the connective tissues and the blood vascular system leading to the proliferation and migration of cells and remodeling of the tissues.

In the stages of tooth movement, a number of microenvironments within the tooth supporting structures are established. Areas of the tooth pulling away from the alveolar bone cause tension through stretching of the periodontal ligament leading to bone apposition. In contrast, areas in which the tooth root is compressing the periodontal ligament against the alveolar bone surface result in pressure leading to bone resorption.

The initial biodisruptive stages of tooth movement are characterized by an interruption in nutrition, ischemia, cell death, connective tissue disruption and inflammation. These classic signs of tissue hyalinization are most distinct in regions of compression. After this initial stage of necrosis, a period of tissue repair begins with bone resorption. This resorption may be termed frontal, whereby the bone surface next

to the compressed periodontal ligament is removed or undermining, in which the endosteal bone is removed behind the hyalinized zones.

Areas of tension result in orientation of collagen fibers in the direction of the applied force if the force application is low; however, higher force applications lead to disruption of the collagen fibers and inflammation, but once tooth movement ceases tissue repair begins through removal of cell debris, bone apposition and mitotic activity in the periodontal ligament realigning the principle fibers.

In certain types of orthodontic movements, the supra-alveolar structures are involved. Unlike the principle fibers of the periodontal ligament, these gingival fibers appear to be very resistant to realignment resulting in the instability of orthodontic corrections (Edwards 1968, 1970 and 1988). It has been proposed that this resistance is the result of a slower collagen turnover in the gingival tissues than in the periodontal ligament (Skougaard et al., 1969; Sodek, 1976), but the true nature of this phenomenon is still unknown.

The mechanisms involved in the transduction of the external stimulus into cellular activity are not thoroughly understood. It is thought that the force systems are transduced both extracellularly and intracellularly into biological signals through a multiplicity of responses within the tissues such as cell-surface receptor proteins, activation of membrane-bound enzymes and changes of ion concentrations within the cells.

These changes lead to a series of events resulting the signaling of progenitor cells to differentiate into the specialized cells necessary for remodeling of the periodontium.

A better understanding of the specialized processes of transduction, differentiation and migration will provide a greater understanding of the biology of the supporting tissues of the teeth and the dynamic environment in which they exist, promoting a more efficient and stable application of our clinical techniques.

The following review of the literature will discuss the concepts relating to the biological consequences resulting from the application of orthodontic forces within the periodontal tissues and will identify areas in which questions still remain unanswered.

HISTOLOGICAL RESPONSE OF THE PERIODONTIUM TO ORTHODONTIC THERAPY

Periodontal Ligament Response to Orthodontic Treatment

(A) Collagen Degradation in Physiologic Tooth Movement

The view that untreated teeth will continue to drift, especially in the developing dentition is generally accepted Ten Cate et al., 1976). This phenomenon of physiologic migration is characterized by active removal of bone on the side toward tooth movement with reconstruction of the periodontal ligament.

This active resorption is indicated by the presence of osteoclasts with ruffled cell membranes within the Howship's lacunae on the alveolar bone surface (Rygh, 1984). Once the resorption is completed, cells deposit new layers of bone to which periodontal ligament fibers reestablish their attachment. This reattachment is present on both the alveolar bone and the cemental surfaces (Rygh, 1973a; Kurihara and Enlow, 1980). Simultaneous with this repair are the beginnings of other areas of bone resorption.

Only selective areas of collagen resorption take place during the movement insuring that the majority of the supporting apparatus remains intact (Rygh, 1986).

The exact nature of the remodeling and relinking of the collagen fibers is poorly understood. Although previously thought that collagen remodeling was a completely extracellular process, it has been shown that fibroblasts have the ability to

both produce and breakdown collagen simultaneously (Ten Cate et al., 1976). It is hypothesized that these cells participate in a vital role in collagen remodeling and relinking (Ten Cate, 1972; Listgarten, 1973; Ten Cate and Deporter, 1974; Eley and Harrison, 1975; Beertsen and Everts, 1977; Melcher et al., 1978; Shore and Berkovitz, 1979).

In support of the theory that fibroblasts play a vital role in collagen synthesis and reattachment, Ten Cate (1972) observed no collagen-containing profiles in the osteoblasts lining the alveolar walls nor in the cementoblasts on the root surface. He did observe fibroblasts extensions containing collagen profiles which projected between the cementoblasts suggesting that the fibroblasts remodel the collagen bundles right up to the surface of the cementum.

The degradative process that occurs in physiologic migration occurs so slowly and in such limited locations that confirmation of this theory is very difficult to demonstrate. However, three intracellular profiles have been suggested (Ten Cate et al., 1976). In the initial stage a well developed collagen fibril is phagocytosed by a fibroblast forming a phagosome made up of the fibril in a clear matrix surrounded by a membrane. In the second stage, a phagolysosome is formed when a primary lysosome synthesised by the Golgi apparatus fuses to the phagosome resulting in an increased cell density. During the final stage, enzymatic degradation of the fibril leads to a progressive loss of the characteristic striation of the collagen.

Ten Cate et al. (1976) has further demonstrated these stages through electron microscopy: (1) a banded fibril during degradation surrounded by an electron-lucent zone, (2) a banded fibril surrounded by an electron-dense zone with or without swellings at its lengths, and (3) an unbanded fibril.

It is interesting to note that the stages of collagen degradation previously described are classical for macrophage phagocytosis. Indeed, macrophages have been shown to degrade collagen intracellularly. These changes were reported in uterine involution after pregnancy (Luse and Hutton, 1964). This has yet to be proven in physiologic tooth movement as the presence of macrophages within the cell population of the periodontal ligament has not been demonstrated.

(B) Periodontal Ligament Degradation in Therapeutic Tooth Movement

Remodeling in the periodontal ligament is much more dramatic during orthodontic tooth movement than during physiologic tooth migration. Even with the application of light force systems, areas of hyalinization within the periodontal ligament are evident (Rygh, 1972, 1973b; Buck and Church, 1972). This has led to the description of orthodontic therapy as a pathological process from which the tissue recovers.

This process of damage and repair, typically found in areas of pressure, is the rate limiting step in orthodontic tooth movement. Rygh has shown that removal of most of the hyalinized

tissue occurs prior to repair (Rygh, 1974). As a result these areas are found to delay tooth movement (Skillen and Reitan, 1940). The fact that physiologic tooth migration proceeds in a rather orderly fashion suggests that a better understanding of the differences between therapeutic and physiologic tooth movement could enhance our clinical abilities.

Collagen fiber alterations in zones of hyalinization are not readily assessed by light microscopy due to masking by the surrounding ground substance. Collagen fibrils can be observed through the use of the electron microscope (Reitan, 1959; Kvam, 1972). In these studies fibrils appear densely packed and disoriented, but demonstrate the characteristic cross-banding. However other investigations claim that the fibrillar nature of collagen fibers is lost (Buck and Church, 1972).

Rygh (1973b and 1974) confirmed that the majority of the collagen fibrils in the areas of hyalinization retained their integrity. Thus, it is thought that the appearance of these areas is possibly due to compression and disintegration of the fiber bundles, not from degradation of the collagen fibrils or the collagen molecules (Kvam, 1973). That portion of collagen fibrils that does degenerate, split longitudinally into nonstriated filaments, a pattern similar to periodontal disease (Selvig, 1966).

The removal of hyalinized tissues from within the periodontal ligament space by osteoclasts differentiating from marrow spaces and peripheral areas of the ligament along with

progenitor cells transported into the area through increased vascularity (Gainelly, 1969; Khouw and Goldhaber, 1970) is not thought to be the same mechanism for removal of collagen (Rygh, 1984). It is though that invading macrophages push cellular extensions into the area. The penetration of these extensions begins the degradation process through enzymatic activity resulting in phagocytosis of the collagen fibrils. This process is similar to wound repair in other connective tissues (Ross and Odland, 1968).

All of the tissue damaged due to the compression is removed. Included in these tissues are the collagen fibers of the periodontal membrane in the hyalinized areas even though the degradation of collagen due to pressure rarely results in loss of cross-striation (Rygh, 1984). The knowledge that fibroblasts have the ability to phagocytize collagen in physiologic tooth movement (Ten Cate, 1972), raises the possibility that these cells may also play a role in the removal of the degraded collagen.

Once cleared, the periodontal area is invaded by an increased number of fibroblasts (Reitan, 1985). Continuity of the periodontal ligament is restored through intense cellular activity giving rise to functionally orientated collagen fibers (Rygh, 1973a).

Along with the elimination of the hyalinized tissues, new layers of cementum and bone are deposited. These new matrices may provide attachment of the newly synthesised collagen

fibrils although some fibers still remain embedded in the old cemental surface (Rygh, 1973a).

On the tension side of therapeutic tooth movement, fiber lengthening must occur after the initial stretching phase is completed in order to allow for tooth movement (Rygh, 1984). Several hypothesises have been put forth to explain this mechanism. Among these hypothesises is existence of an intermediate plexus (Sicher, 1954, 1959). Although a controversial feature of the anatomy of the periodontium, the concept of an intermediate plexus is that, in the middle region of the ligament, the collagen of the principle fiber bundles is constantly being broken and reformed to permit tooth movement. It has also been suggested that groups of fibers separate from the principle fiber to combine with neighboring fiber bundles (Orban et al.,1958).

The more recent investigations leading to the discovery that fibroblasts are capable of both synthesizing and degrading collagen explains the remodeling mechanism. The findings show that new fibers are secreted in conjunction with new bone deposition. As the fibroblasts migrate away from the bone, they may secrete either new fibers or new fibrils which are incorporated into the existing fibers (Garant and Cho, 1979).

Increased degradation and synthesis of collagen on the tension side of orthodontic tooth movement (Freeman and Ten Cate, 1978) is supported by studies which show a high amount of intracellular collagen-containing profiles within fibroblasts

(Rygh, 1976). Additional breakdown of fibers in this area is mediated by macrophage-like cells provided through a high degree of vascularity between the collagen fibers (Rygh, 1984). Other sources of cells may include pericytes and endothelial cells of the invading blood vessels.

Gingival Response to Orthodontic Treatment

The gingival tissues that surround the teeth are subjected to frictional and compressive forces in the normal process of mastication. The character of this tissue shows that it is adaptable to this environment. (See Stern, 1976 for a detailed description of the oral mucosa.)

The healthy gingiva is a highly collagenous connective tissue. Within this tissue, specifically orientated bundles of collagen fibers have been observed orientating themselves from the cementum on the root surface into the gingival tissues from the alveolar bone margin into the gingival tissues, and from the cementum of one tooth transversing the interdental bone and inserting into the cementum of the adjacent tooth. This last group of fibers, the transseptal fibers, form a ligamentous structure which joins all of the teeth within an arch (Narayanan and Page, 1983).

As in the periodontal ligament, the principal cell within the gingiva is the fibroblast which accounts for about 5.6 percent of the total connective tissue volume (Schroeder et al.,

1973) and two-thirds of all cells present in a healthy gingiva (Brecx et al., 1988). In addition to these cells are large numbers of mast cells and blood leukocytes. The primary defense towards any aggression against the healthy gingiva is mediated by a constant influx of neutrophils and lymphocytes from the gingival blood vasculature (Page and Schroeder, 1976; Brecx et al., 1988).

In physiologic tooth movement, Ten Cate et al. (1976) noted the presence of a significant number of fibroblasts containing phagocytosed collagen fibrils in the lower part of the transseptal fiber system of mouse molars. This is in agreement with the histologic picture within the principal fiber bundles of the periodontal ligament; however, few studies are present in the literature to date providing quantitative data with respect to collagen metabolism in the supra-alveolar region.

With the application of orthodontic force, the gingival fibers are stretched displacing the gingival tissues in the direction of tooth movement. The distortion of these fibers remains for an extended period even though the principal fibers of the periodontal ligament readjust rapidly. The transseptal fibers have even been shown to maintain their integrity after tooth extraction (Erickson et al., 1945). The exact cause for these differences still remains to be explained, but a number of investigators have associated these fibers with orthodontic relapse (Reitan, 1959; Thompson, 1959; Edwards, 1968, 1970, 1988; Brain, 1969; Parker, 1972). This resistance has even been noted

to result in resorption of the adjacent bone (Reitan, 1959), the formation of bone spicules (Edwards, 1968) and movement of adjacent teeth (Erickson et al., 1945).

One possible explanation for this phenomenon is that although collagen turnover is more rapid in gingiva than in many other tissues (Claycomb et al., 1967; Page and Ammons, 1974), the collagens of the periodontal ligament are remodeled at an even more accelerated rate (Kameyama, 1975; Skougaard et al., 1969; Sodek, 1976). Another possibility may be related to the fact that many of the gingival fibers are not attached directly into the alveolar bone. Thus the reduction in length of these fibers by changes in the osseous attachment never occurs.

The discovery of the presence of oxytalan and elastin fiber formation within the gingival connective tissues (Fullmer and Lillie, 1958; Fullmer, 1966; Edwards, 1968; Parker, 1972; Lopez et al., 1976) led to the hypothesis that the elastic nature of these fibers may be responsible to the instability after orthodontic treatment. Edward's investigations (1968, 1970 and 1988) into the existence of these fibers led to the discovery of an increased number of oxytalan fibers with orthodontically rotated teeth, which was not eliminated with a retention period. However these fibers may actually represent altered collagen fibers (Fullmer et al., 1974).

Early investigators were apparently so perplexed by the persistent nature of the gingival collagen bundles that they believed that no process could alter them (Erickson et al.,

1945).

Recent evidence shows that tooth movement on rat molars results in detachment of the transseptal fibers from the alveolar bone associated with an increase in vascular activity (Rygh, 1984). As well, the presence of oxytalan fibers seemed to follow the blood vessels with no increase in number.

Although there is no substantial evidence which can explain the mechanism by which the gingival connective tissues apply a force capable of moving teeth, many orthodontists advocate the use of a circumferential supracrestal fiberotomy which transects the transseptal fibers (Edwards, 1970 and 1988). Histological examination of teeth rotated following this procedure shows less extensive alterations to the cementum and bone as compared to teeth rotated without the procedure. The gingival fibers of the treated teeth presented with a fibrous pattern and density similar to nontreated teeth (Brain, 1969, Edwards, 1968; Parker, 1972). It is also clinically important to note that these same investigators state a significant increased stability in the surgically treated teeth following orthodontics.

COLLAGEN REMODELING IN PERIODONTAL TISSUES

Biochemistry of Collagen

Collagen is the main extracellular component of healthy connective tissues (Baily and Robins, 1976; Kivirikko and Risteli, 1976; Bornstein and Traub, 1979). The most abundant structural protein of vertebrates, it comprises approximately 25 percent of the body's total protein (Parry and Craig, 1988).

Collagen is responsible for the structural stability and compartmentalization of all major organ systems (Bornstein and Traub, 1979) and is thought to play a vital role in the control of cellular differentiation and embryogenesis (Hay 1981).

It is synthesized by a variety of cell types predominately mesenchymal in origin, including fibroblasts, chondroblasts, osteoblasts and odontoblasts, as well as by epithelial cells which are known to secrete basement membrane protein (Barbanell et al., 1978).

A variety of genetically distinct types of collagen have been isolated and each connective tissue contains a unique spectrum of these types depending on its functional requirements. However, few of the collagen types have been shown to have a specific tissue distribution (Bornstein and Sage, 1980).

(A) Structure of the Collagen Molecule

The collagen molecule is characteristically described as a right hand triple-stranded helical structure. Comprising the helical structure of the molecule are three collagen polypeptide chains referred to as alpha chains made of about 1050 amino acids. These alpha chains (also referred to as polypeptides because of the peptide bond linking the amino acids) are wound around each other in the form of a regular superhelix resulting in a collagen molecule about 300nm long and 1.5nm in diameter. (Miller, 1988; Alberts et al., 1989)

Each alpha chain is a repeating triplet (gly-X-Y) consisting of a glycine and two other amino acids (Bansal et al., 1975). Glycine, the smallest amino acid, makes up every third residue throughout the central region of the alpha chain, allowing the three helical alpha chains to pack tightly together to form the final collagen molecule. The Y position is usually occupied by the amino acids proline or hydroxyproline. Due to its ring structure, proline stabilizes the left handed helical conformation of the individual alpha chains with three amino acid residues per turn. Hydroxyproline is almost exclusively found in collagen. Changes in the hydroxyproline content of the molecule may represent changes in collagen metabolism. The X position is often occupied by alanine, lysine or hydroxylysine, but may be occupied by any amino acid (Alberts et al., 1989; Bansal et al., 1975).

(B) Biosynthesis and Degradation of Collagen

Although collagen performs a role in the structural stability of connective tissues, it is continuously turned over in the extracellular matrix (Berg, 1986). This complex process is composed of numerous specific complex steps (Prockop et al., 1976). This sequence of events is similar to that of other secretory proteins (Palade, 1975; Farquhar and Palade, 1981) in that collagen is synthesized on membrane-bound ribosomes and has a signal sequence at the amino terminus of the polypeptide.

Once the individual collagen polypeptide chains are synthesized, they are injected into the rough endoplasmic reticulum as precursors, referred to as pro-alpha chains (Palmiter et al., 1979). While in the lumen of the rough endoplasmic reticulum, specific proline and lysine residues are hydroxylated into hydroxyproline and hydroxylysine, respectively (Kivirikko and Myllyla, 1981). In addition, glycosylation of specific hydroxylysine residues begins (Prockop et al., 1976; Kivirikko and Myllyla, 1981). Three individual pro-alpha chains are then hydrogen bonded to form a triple-stranded helical molecule referred to as procollagen (Alberts et al., 1989).

The transfer of procollagen from the lumen of the rough endoplasmic reticulum into the extracellular space involves transport through the Golgi apparatus (Fessler and Fessler, 1978; Bornstein and Traub, 1979) and into condensing granules and secretory vesicles (Weinstock and Leblond, 1974; Karim et al., 1979).

Once secreted, the procollagen precursors of type I, II and III collagen is acted upon by specific proteolytic enzymes which cleave small polypeptides from the N-telopeptide portion of the molecule (Berger et al., 1985; Tanzawa et al., 1985). This conversion results in the collagen molecule, known as tropocollagen. Individual tropocollagen molecules are estimated at 1.5nm in diameter, but once formed they associate in the extracellular space to form collagen fibrils with diameters of 10-300nm (Tanzer, 1973; Alberts et al., 1989). These collagen fibrils aggregate further to form a collagen fiber (Barbanell et al., 1978; Prockop et al., 1979a, 1979b).

Connective tissue cells are known to orchestrate the size and organization of the collagen fibrils through selection of gene expression, controlling the order of polypeptide cleavage, secretion of different types and amounts of noncollagen matrix macromolecules, and by altering the amount of cross-linking of the molecules (Alberts et al., 1989). In addition, fibroblasts have been shown in vitro to alter the collagen they have secreted by physically moving over it and pulling on it, compacting it into sheets and drawing it out into cables (Stopak and Harris, 1982).

The rapid intracellular degradation of newly synthesized collagen has been demonstrated in type I (Bienkowski et al., 1978a, 1978b), type II (Duchene et al., 1981), type III, and IV collagens (Paloti, 1983) and is important as a post translational control mechanism (Berg, 1986). This intracellular degradation

has also been shown in a wide range of tissues and cells including tendon fibroblasts (Duchene et al., 1981), lung (Bienkowski et al., 1984), bone (Sakamoto et al., 1979), skin fibroblasts and muscle cells (Imberman et al., 1982), and gingiva (Schneir et al., 1984). These discoveries have led to the hypothesis that this degradation is universal for all collagen producing cells.

Intracellular degradation has been divided into two major modes - basal and enhanced (Bienkowski, 1983; Berg et al., 1980). The exact mechanism and location of basal degradation remains to be established, but is thought to occur between the endoplasmic reticulum and the Golgi apparatus (Berg, 1986). Enhanced degradation of collagen is known to occur in the lysosomal system (Berg, 1986). It is still unknown whether defective collagen is routed to the lysosomes in the Golgi apparatus or at other locations in the secretory path.

Collagen in the extracellular matrix is also degraded although once cross-linked it is largely resistant to proteolytic enzymes. The cross-linking of the fibril along with the triple-helical structure of the collagen molecule protects the peptide bonds from enzymatic attack and provides increased stability to denaturation (Kuhn, 1987).

Extracellular collagen degradation is done by a family of collagenases that are responsible for both collagen turnover in tissue homeostasis and remodeling (Gross, 1976; Gross and Nagar, 1965; Woolley and Evanson, 1980). The individual enzymes attack

collagen at specific sites on the molecule (Harris, 1978; Jeffery, 1986) such that the breakdown of collagen may require several enzymes such as proteases to split the cross-linked nonhelical ends of the molecule, vertebrate collagenase to cleave the molecule into two pieces, and a final proteolytic enzyme to digest the polypeptide chains after denaturation (Weiss, 1976).

(C) Distribution and Physiology of Collagen Phenotypes

It can be seen from this information that collagen is not homogeneous. The concept of heterogeneous molecules has led to the discovery of eleven structurally and genetically distinct collagen types identifiable by the arrangement of the twenty different alpha chains which have been discovered to date. (Bornstein and Sage, 1980)

The main types of collagens constituting the interstitial connective tissues are types I, II, and III (Miller, 1976). The amino acid content of these collagens is characterized by approximately 33% glycine and 20% of the amino acids proline and hydroxyproline (Sage and Bornstein, 1979).

It is doubtful that any of the collagen types have a specific tissue distribution, however quantitative differences in the proportions of varying types to specific anatomical locations clearly exists and is required for normal function.(Burnstein and Sage, 1980)

The conventional collagen known as type I is the most

abundant and accounts for about 90% of body collagen. It is found in skin, tendon, bone (ossified and endochondral), internal organs, cornea, intervertebral disc, dentin, gingiva and ligaments including the periodontal ligament. It has low amounts of hydroxylysine and carbohydrates. It is composed of two identical and an additional alpha chains and is given the molecular formula of [alpha 1 (I)]$_2$ alpha 2 (I) (Alberts et al., 1989).

Type II collagen has been localized in cartilage, intervertebral disc, sclera of the eye, lung, bone, and notochord (Bornstein and Sage, 1980). It is distinctively high in hydroxylysine and carbohydrates. It is composed of three identical alpha chains and given the molecular formula [alpha 1(II)]$_3$ (Alberts et al., 1989).

Type III collagen, [alpha 1(III)]$_3$, contains the highest levels of glycine (Sage and Bornstein, 1979). It is characteristically high in hydroxyproline and low in hydroxylisine and carbohydrates (Alberts et al., 1989). It is found to co-exist with type I collagen in a variety of tissues including skin, lung, heart, uterus, nerve, liver, placenta, umbilical cord, spleen, intestine, kidney, lymph nodes, the dental papilla and pulp, and the periodontal ligament (Bornstein and Sage, 1980). The proportions of type I and III have been shown to vary with tissue (Butler et al., 1975; Sodek and Limeback, 1979), age (Epstein, 1974), and metabolic state of the tissue (Narayanan et al., 1980, 1983; Narayanan and Page, 1983a;

Miller, 1976; Gay et al., 1978).

The basement membrane collagens, types IV and V, generally contain more residues with bulky hydrophobic side chains and significantly fewer alanyl and arginyl residues than other collagen types. (Sage and Bornstein, 1979)

Type IV collagen has the molecular composition of [alpha 1(IV)]$_2$ alpha 2(IV) and is the major collagen in the basement membrane, but has also been located in liver, skin, placenta, aorta and the lens capsule. It is very high in hydroxylysine in addition to being high in carbohydrates (Alberts et al., 1989).

Type V collagen was first discovered in fetal membranes (Burgeson et al., 1976), however studies have shown that it exists in a variety of connective tissues along with types I and III or II (Rhodes and Miller, 1978). It is distinctive in that it localizes in pericellular spaces and near basement membranes (Madri and Furthmayer, 1979; Roll et al., 1980). Its molecular formula is [alpha 1(V)]$_2$ alpha 2(V) (Rhodes and Miller, 1978).

Collagen Phenotype Synthesis as a Biological Signal

Collagen is the main extracellular component of all healthy connective tissues (Baily and Robbins, 1976; Kivirikko and Risteli, 1976; Bornstein and Traub, 1979). It is not a homogeneous protein and a quantitative difference in the

distribution of the various collagen phenotypes within a tissue has been shown to be necessary for normal function. In addition, regulated synthesis of the phenotypes may be crucial in the dynamic processes of development and tissue repair (Bornstein and Sage, 1980).

Physiological demands on a connective tissue have been shown to result in metabolic activity within connective tissues resulting in changes of the amount and relative proportions of the different phenotypes, especially types I and III which are often localized within the same tissue (Meikle et al., 1982; Yen et al., 1989a, 1989b; Duncan et al., 1984). Type III collagen is important in the events of rapid remodeling such as fetal skin growth (Chung and Miller, 1974; Epstein, 1974), wound healing (Gay et al., 1978) and inflammation (Weiss et al., 1975). The high presence of type III collagen in tissues such as the aorta, uterine wall and the periodontal ligament may indicate a stress bearing role (Chung and Miller, 1974; Butler et al., 1975).

As a result, changes in collagen phenotype proportions, especially that of type I and type III, may serve as important parameters to indicate the metabolic state of the connective tissue involved, and may act as signals for changing cellular activities.

Orthodontic tooth movement has been described as an inflammatory wound healing response (Storey, 1973; Ten Cate et al., 1976) comparable to other situations with rapid collagen synthesis and remodelling and where the proportion of type

III/type I + III is significantly influenced. Thus, collagen phenotype ratio can serve as an important parameter, indicating the metabolic state of tissues undergoing inflammatory and physical stress bearing roles in response to orthopedic and orthodontic stress.

Role of Collagen in the Periodontium

(A) Collagen Distribution within the Periodontium

The periodontium is described as being made up of the gingiva comprising epithelium and connective tissue, the alveolar bone, the root cementum and the periodontal ligament. The anatomic arrangement is responsible for the tissues' composition, arrangement and turnover. In addition, these tissues are capable of preserving the integrity of the functional unit when under the delivery of physiologic levels of force resulting from mastication, deglution, phonation and enamel wear (Claycomb et al., 1967). These facts are critical in understanding the collagen distribution and turnover within the periodontium.

As stated previously in this review, these connective tissues are highly collagenous containing collagen fibers with specific orientations and distribution. In addition, the connective tissues consist of noncollagenous glycoproteins, glycosaminoglycans, and small amounts of oxytalan and elastin

(Narayanan and Page, 1983a).

Gingiva has been shown to have the greatest distribution of collagen phenotypes. Present in this tissue are types I, III, IV and V. Type I is the most abundant making up about 91% while type III makes up approximately 9%. Only small amounts of the basement membrane collagens are present (Sodek and Limeback, 1979; Hammouda et al., 1980; Narayanan, 1980, 1983a). Once the teeth are lost, the gingiva of the edentulous ridge is known to change composition where only 85% of the collagen is type I while 14% is type III and less than 1% of type V is detected (Narayanan et al., 1981b).

In the periodontal ligament, type III collagen is thought to have the greatest distribution within the periodontium estimated at 15-20%. Type I comprises the majority of the remaining collagen with trace amounts of type V present (Butler et al., 1975a; Sodek and Limeback, 1979).

In the cementum, the collagen make up is 95% type I and less than 5% type III. It is thought that a small amount of the type III measured in the cementum may actually be periodontal fibers embedded in the tissue (Butler et al., 1975b; Birkedal-Hanson, 1977; Christner et al., 1977). The alveolar bone has been shown to have a 100% Type I collagen content (Narayanan and Page, 1983a).

(B) Ultrastructure and Metabolism of Collagen in the Periodontium

As previously reported in this review, histological investigations have verified the presence of three reasonably well-defined systems of collagenous fiber bundles in the connective tissue of the marginal gingiva. These seem to be functionally responsible for (a) the maintenance of the tone of the marginal gingiva, (b) the maintenance of a close adherence of the gingiva to the tooth, (c) the prevention of the epithelial migration in an apical direction along the root surface of the tooth and (d) the incorporation of the gingival margin with the underlying tissues (Arnim and Hagerman, 1953).

Ultrastructural investigation into healthy human gingiva has confirmed that this connective tissue is made of two distinct patterns of type I and III collagen. The first pattern is composed of type I collagen preferentially organized in large dense bundles while the second pattern is composed of a fibrous and fibrillar type III collagen network (Chavrier et al., 1984). Thus, type I collagen predominates in the distinct gingival fibers, where stability is required, although the exact distribution of collagen phenotype among the individual fiber groups has yet to be determined. Since the second pattern is mostly type III collagen, it is thought that this area has a greater need and ability to remodel. However, despite the differences in procollagen processing and phenotype distribution, type I and type III collagen are metabolized at similar rates

during rapid turnover in the periodontal tissues (Sodek and Limeback, 1979).

Collagen synthesis in rat palatal mucosa decreases markedly from 2.5 to 13 weeks of age when determined by an *in vitro* labelling technique. Beyond 13 weeks, the rate of collagen synthesis remained unchanged and at a relatively low level. The findings suggests that collagen in the soft oral connective tissues undergoes fibrillogenesis more characteristic to tissues during rapid growth or during healing, rather than tissues during a normal adult stage of metabolism (Schneir *et al.*, 1976).

The collagenous fibers in the alveolar crest in the rat molar begin to mature and organize at 15 days postnatally, while the transseptal fibers do not appear until 21 days (Miura *et al.*, 1970). The initial principal fibers are evident in the occlusal third of the periodontal ligament at 25 days (Bernick, 1960). The onset of occlusion at 28 days leads to thickening of the fiber bundles and continuity from the alveolar bone to the cementum (Bernick, 1960).

Chromatographic analysis of the cross-linking patterns of the collagen in bovine periodontium showed that the ratios of two main cross-links, dihydroxylysinonorleucine to hydroxylysinonorlucine, were similar to that of skin collagen (Kuboki *et al.*, 1981) Although they are quite similar, oral collagen synthesis occurs at a higher level than skin during normal growth (Claycomb *et al.*, 1967; Page and Ammons, 1974), possibly at a higher metabolic level than any mature collagen in

the adult connective tissues (Sodek, 1978). The reason for this high turnover in the periodontal ligament may be due to the propensity for eruption (Rippen, 1978).

Crumley (1964) showed that different rates of collagen formation existed in different locations of the healthy rat periodontium. Bone collagen appeared to form more rapidly than collagen of the periodontal ligament which formed faster than the cementum collagen.

Beertsen et al. (1978) reported no differences in collagen turnover in the cemental, middle or alveolar areas of the periodontal ligament. Additional investigations have shown the collagens of the periodontal ligament to remodel at a faster rate than collagens of the gingiva (Kameyama, 1975; Skougaard et al., 1969; Sodek, 1976). Studies using ^{3}H-proline have shown the half-lives of the periodontal ligament in the mouse to be 5.7 days, the transseptal tissue to be 8.4 days and the gingiva to be 25 days (Minkoff and Engstrom, 1979; Minkoff et al., 1981).

KNOWN RELATIONSHIPS BETWEEN FORCE AND CELLULAR RESPONSE

Hypothesized Control Mechanisms of Orthodontic Tooth Movement

The science of tooth movement has been practiced for centuries. Through this time, many advances have been made in the understanding of the sequelae of orthodontic tooth movement. Although the histologic responses are well documented, the exact method by which orthodontic forces are transduced into biological events is not thoroughly understood. With the understanding that an accurate understanding of the factors responsible for the initiation of cell-mediated responses will optimize orthodontic treatment, several hypotheses have been put forth for investigation into this phenomenon.

(A) Pressure-Tension Theory

The pressure-tension theory originally proposed by Sandstedt (1904, 1905a, 1905b) and later supported by Schwartz (1932) and Gainelly (1969) is the classical hypothesis for tissue response to orthodontic force. This hypothesis puts forth the concept that vascular occlusion resulting from pressure within the periodontal ligament results in the histological events of hyalinization, seen as cell free zones, and undermining bone resorption, seen as osteoclasts on the endosteal surface. Thus, the cellular activity was thought to be a direct response to pressure/tension within the local tissue.

Alternative forms of this theory were presented by Baumrind (1969) and Kardos and Simpson (1980). Baumrind's interpretation of the this theory was that the differences existing between the pressure and tension sides during tooth movement would result in differential amounts of cell replication rates. He predicted the reduction of cell replication in the areas of pressure due to its vascular occlusion along with an increased cell replication in the areas of tension due to stretching of the periodontal ligament fibers.

This hypothesis was found to be incorrect. Although experiments using radioactive proline do indicate that there is a high rate of collagen turnover in the periodontal ligament, probably higher than anywhere else in the body, even when not undergoing orthodontic tooth movement (Carneiro and Fava de Moraes, 1965; Skougaard et al., 1970a, 1970b; Kameyama, 1973, 1975.), studies have also shown increased levels of proline incorporation in the areas of both tension and pressure as a result of orthodontic tooth movement (Crumley, 1964; Koumas and Matthews, 1969; Baumrind and Buck, 1970).

A more recent form of this theory was described by Kardos and Simpson (1980) who predicted that the histological picture was a result of thixotrophy or alterations in viscosity within the system. It was thought that compression within the periodontal ligament space caused a reduction in the viscosity of the collagenous matrix allowing for rapid cell movement away from the area. This cellular activity would result in the loss of

tissue architecture explaining the appearance of hyalinized zones.

(B) Oxygen-Tension Theory

Bien (1966) hypothesized that constriction of capillaries by compressed periodontal ligament fibers formed a series of cirsoid aneurysms. A decreased blood pressure resulted in the formation of minute oxygen bubbles which could diffuse through the vessel wall below each site of stenosis. Such oxygen bubbles lodged against the bone surface was felt to lead to osseous resorption.

Investigations have shown that it is possible to regulate resorption in the mouse calvaria *in vitro* through manipulation of the oxygen concentration in the culture medium (Goldhaber 1958, 1961, 1966; Stern *et al.*, 1966). Increased vascularity of the periodontal ligament in areas of frontal resorption have been observed (Gianelly, 1969; Khow and Goldhaber, 1970) further implicating increased oxygen tension as the stimulus to bone resorption. Although it has yet to be proven whether increased vascularity results primary or secondary to bone resorption, Rygh (1984) hypothesises that "the more rapid the tooth movement response has to be, the more extensive is the activation of vascularization and the greater is the part of the remodeling process of the periodontal ligament played by the cells of the blood vessels which invade the regions where rapid changes are urgent."

(C) Piezo-Electric Theory

The mechanical stress initiated through orthodontic force leading to the deflection of alveolar bone (Baumrind, 1969; Grimm, 1972) has been shown to generate electric charge polarizations within the tissues referred to as a piezo-electric response (Fukada and Yusada, 1957; Bassett and Becker, 1962). Electronegative regions were demonstrated in the areas of bone apposition and electropositive regions in the areas of bone resorption (Bassett et al., 1964; Bassett, 1968; Pawluk and Bassett, 1970; Zengo et al., 1973, 1974, 1976). Additional investigations into the effect of applied currents during orthodontic treatment have provided evidence of increased cell numbers within the ligament along with increased cellular activity resulting in accelerated orthodontic tooth movement Davidovitch et al., 1978a, 1978b, 1979, 1980a, 1980b).

Thus, the association between piezo-electrical stimulation and bone remodelling is well established. However, the transduction of this stimulation into biological responses is still unresolved, although hypotheses of electron migration through biologic membranes (Jahn, 1962), proton charge transport through the cytoplasm (Eigen and DeMaeyer, 1958) and amphoteric ion exchanges (Jahn, 1968) have been promoted.

(D) Cyclic AMP Theory

Low levels of intracellular cyclic adenosine monophosphate (cAMP) are associated with initiation of proliferation among bone

progenitor cells (Norton et al., 1977). Immunocytochemical investigations into this relationship give confirmation to this relationship during the proliferation stages of orthodontically stimulated osteogenesis (Davidovitch, 1976a).

Cyclic AMP and cyclic guanosine monophosphate (cGMP) are characterized as intracellular second messengers because they are known to convert membrane effects into cellular responses (Sutherland and Rall, 1960; Sutherland et al., 1962, 1965; Hardman and Sutherland, 1969).

The primary stimulus, or first messenger, can take a variety of forms including systemically acting on hormones such as calcitonin (Murad et al., 1970; Rodan and Rodan, 1974; Nagata et al., 1975), parathyroid hormone (Chase and Auerbach, 1970; Vaes, 1970; Murad et al., 1970; Peck et al., 1973; Rodan and Rodan, 1974; Nagata et al., 1975), prostaglandin (Goldring et al., 1979) calcium ion concentrations (Whitfield et al., 1979), electrical stimulation (Davidovitch et al., 1978a, 1979, 1980a, 1980b; Rodan et al., 1978), and the mechanical stretch of cells (Binderman and Cox, 1977; Harell et al., 1977; Somjen et al., 1980).

Once activated by the first messenger, cell membrane-bound enzymes, adenylate cyclase and guanylate cyclase are converted into the second messengers adenosine triphosphate (ATP) and guanosine triphosphate (GTP), respectively, to produce cAMP and cGPM (Sutherland et al., 1962; Hardman and Sutherland, 1969; White et al., 1969). These metabolites have been demonstrated to serve as co-factors in enzymatic phosphorylation reactions

influencing the metabolic functions of the cell (Greengaard, 1978). Thus a cAMP-mediated mechanism, involving direct perturbation and/or a bioelectrical signal, appears to initiate proliferation in osseous progenitor cells, an feature vital in the activation of bone remodeling.

(E) Optimal Force Theory

The optimal force theory suggests that the optimal force is the minimal force required to produce the desired tooth movement (Smith and Storey, 1952; Storey and Smith, 1952). It is thought that the periodontal ligament has a threshold to pressure. Pressure within the ligament above such a threshold will result in tooth movement. Thus, the areas of pressure at the root surface-alveolar bone interface are thought to be the rate limiting locations since bone resorption is required.

The level of pressure can be determined by the amount of force applied to the tooth divided by the area of the periodontal ligament over which that force is distributed.

Although forces greater than the threshold may produce tooth movement, they may also produce iatrogenic sequelae. Exceeding heavy force levels have been shown to result in an increased movement of the anchorage unit. Extreme forces may even decrease tooth movement due to increased levels of undermining resorption and gross inflammatory responses on the tension side.

Furthermore, the range which has proven to result in the most rapid tooth movement has also shown to result in poor

quality of bone remodeling. Although this remodeling will continue quite rapidly, the potential for relapse is high. In comparison, the low range of forces deliver slower but steadier tooth movements and are preceded by lesser degrees of undermining resorption, and result in the formation of more mature bone, and have a decreased potential for relapse. Thus, the optimal threshold is thought to be quiet low (Storey, 1973).

Cellular Response to Hypofunction within the Periodontium

Answering the question of how physical forces are transduced into remodeling means understanding that within the oral cavity physiologic force applications serve as extracellular stimuli which may be vital in normal cellular function. Collagen fibers within the periodontium of mice have been shown to atrophy with disuse resulting in the loss of epithelial attachment, detachment of the periodontal ligament and resorption of the alveolus (Cohn, 1965). This loss of function and reduction of mechanical strength of the periodontal ligament are also reported in rats (Kinoshita et al., 1982).

In an autoradiographic study of the overall collagen turnover in normal and hypofunctional molars of young and adult rats, Rippen (1976 and 1978) noted a higher turnover rate in the total length of the periodontal ligament of the hypofunctional teeth in both age groups. The fastest turnover was in the

younger animals, especially in the crestal areas. The findings along with the associated eruption of these teeth lead to the suggestion that the turnover rate was dependent on the amount of remodeling required for this movement.

Since phagocytosis of collagen is thought to be an expression of collagen turnover, these findings are supported by the data obtained by Kanoza et al. (1980) who found an increase in both type I and type III collagen synthesis in hypofunctional rat molars after three days (Ten Cate, 1972; Beertsen and Everts, 1977).

Ultrastructural findings within hypofunctional mouse molars (Beertsen, 1987) adds further confirmation to these findings. Electron microscopy showed up to a 50% decrease in the volume density of extracellular collagen in the periodontal ligament accompanied by a two-fold increase in the fibrillar collagen ingested by the cells. This could explain the net loss of collagen fibrils within the extracellular space with decreased function.

Experimental Responses to Mechanical Stress

The concept of mechanical stress playing an intregal part in cell dynamics was investigated as early as 1926 when Bunting and Eades noted that the application of mechanical tension to fresh wounds resulted not only in fibroblasts aligning in the direction

of the tension, but also in the mitotic spindles orienting parallel to the applied force with the newly formed cells separating along the lines of tension (Bunting and Eades, 1926). They concluded from their investigation that not only may mechanical tension determine the polarity of cell division along with the line of growth of developing fibroblasts, but that fibroblasts already formed may shift position as a result of the change in tension.

The difficulty in developing a suitable model delayed rigorous investigation into the effect of physical forces on cells. In an attempt to rectify this problem, Arem and Madden (1976) implanted Proplast bars connected by scar collagen into rats. The model was then subjected to cyclic repulsion forces through magnetic field activation. They showed that in contrast to the randomly oriented collagen fibers of the nonstressed controls the stressed fibers were oriented in the direction of tension. Not only was this reorientation evident, but the newer scars could be altered morphologically without any apparent affect on the older scars.

Buck (1980) performed an <u>in vitro</u> investigation into the effect of directional stretching on the orientation of fibroblasts. In the experiment, fibroblasts were cultured on an apparatus constructed of silicon rubber undergoing intermittent elongations and recoils at 15 second intervals. He found that, in contrast to the unstretched substratum, the cells of the stretched cultures tended to be bipolar lying with their long

axes across the direction of stretching and that mitosis ocurred across the direction of stretching after only 18 to 24 hours. This characteristic alignment of the rhythmical stretched cells was hypothesized to protect them from the longitudinal stretching.

The application of physiological levels of force have also been shown to produce an osteogenic stimulus capable of increasing bone mass (Rubin and Lanyon, 1984) while removal of this force resulted in a decline in bone mass through remodeling (Rubin and Lanyon, 1984). Additional experiments have shown that the rate of change in strain affects bone remodeling in that faster strain induces far more new bone formation (O'Connor et al., 1982).

Bassett and Becker (1962) further investigated the effect of stress on bone by placing electrodes in the midshaft of feline fibulae. When the applied stress caused the bone to bow, the area under compression developed a negative potential with respect to other areas. Furthermore, the amplitude of electrical currents generated was dependent upon the rate and magnitude of bony deformation. Removal of the inorganic fraction from bone abolished its ability to generate these potentials. This suggests that the stress-generating electrical phenomena may be responsible for directing the activity of bone cells. Additional work demonstrated the orientation of collagen fibrils by weak, direct, electric currents. The implantation of current generating electrodes into the femora of dogs demonstrated that

low levels of direct electrical current results in new bone growth preferentially in regions of relative electro-negativity (Bassett et al., 1964).

Acting on the hypothesis that electromechanical relationships play a role in the functional demands of the oral cavity, Zengo et al. (1973 and 1974) demonstrated the existence of bioelectric potentials in the tooth-alveolar bone complex by applying tipping forces to beagle teeth. Application of the findings to the pressure-tension theory of tooth movement demonstrated that areas of osteoblastic activity were electronegative and areas of osteoclastic activity were electropositive. These experiments also showed that the potentials are affected by the presence of a viable periodontal membrane and gingival attachments. The implantation of potential generating electrodes into beagle jaws supported previous findings that osteogenesis occurs at the cathode (Zengo et al., 1975).

Immunohistochemical investigations into this phenomenon by Davidovitch et al. (1980a, 1980b) showed that D.C. electrical currents in cats undergoing orthodontic tooth movement resulted in increased osteogenesis near the cathode and resorption near the anode resulting in accelerated tooth movement. Accompanying these findings were an increase number of osteoblasts and periodontal ligament cells which stained intensely for cAMP and cGMP adjacent to the anode and cathode. These findings support previous findings of increased DNA synthesis (Rodan et al., 1978)

and cAMP (Norton et al., 1977) in cartilage cells stimulated by oscillating electric fields.

To study the effect of force on bone, Hinrichsen and Storey (1968) placed helical springs in growing bones of guinea pigs. Tensile and compressive force applications to cranial bones resulted in little movement of the spring arms after 40 days. Macroscopic examination showed little change in the bone. In contrast to the cranial bones, endochondral cartilage showed inhibited growth along with alteration in growth direction with the application of tensile and compressive forces. Tensile forces applied across the midsagittal suture induced a rapid lateral movement of the bones for the first fourteen days which eventually remained stationary with a net increase in expansion when compared to the untreated controls. Dramatic histological changes of bone thickening, loss of normal sutural architecture, and loss of definition in the bony margin of the suture were evident. The application of compressive stress to these sutures resulted in obliteration of the suture line as the parietal bones became abutted.

Harrell et al. (1977) and Somjen et al. (1980) investigated the effects of force on osteoblasts cultured on polystyrene dishes in vitro which were in turn subjected to constant strain through the activation of an orthodontic screw fitted to the dish's bottom. They observed the stimulation of cell division through [^{3}H]thymidine incorporation into DNA (Rodan et al., 1978; Somjen et al., 1980) and found that prostaglandin E_2 synthesis

increased rapidly reaching its peak within 20 minutes of stimulation, then declining (Harrell et al., 1977 and Somjen et al., 1980). Coinciding with this increase in prostaglandin E_2 was cellular production of cyclic adenosine monophosphate which was at a maximum around 15 minutes poststimulation (Harrell et al., 1977 and Somjen et al., 1980).

The results of these studies suggests that physical forces are transduced into biochemical events in bone cells through a stimulus receptor mechanism which is mediated through prostaglandin E_2 and stimulates cyclic AMP and DNA synthesis.

In order to study the variation in effects between intermittent compressive force (ICF) and continuous compressive force (CCF) on growth plate cartilage, cultures from sixteen day old mouse metatarsal rudiments were subjected to the force of compressed air (Klein-Nullend et al., 1986 and Van Kampen et al., 1985). High density cultures were shown to react to ICF in 24 hours by decreasing cell proliferation and increasing proteoglycan synthesis. The aggregating capacity of this new proteoglycans and their coherence with other matrix components were enhanced with the ICF. In five day experiments both ICF and CCF increased cartilage calcification. However, the ICF evoked a greater cellular response resulting in about twice the deposition of calcium-phosphate mineral in the matrix. Thus, it appears that mechanical loading may be an important regulator of biomineralization and in this situation that discontinuous stimulation evokes a higher response than continuous stimulation.

An additional study by De Witt et al. (1984) on chick chondrocytes receiving a cyclical 5.5% strain for a 24 hour period showed a 2.4-fold increase in [^{3}H]thymidine incorporation into DNA indicating that intermittent mechanical loading results in an increase in DNA synthesis. In addition to this finding, there was an increase in the rate of synthesis and size of the proteoglycans in the mechanically loaded cultures. In contrast, there was not a significant difference in the amount of protein, including collagen, in the cultures subjected to strain.

The loss of joint function whether experimental or pathologic has been shown in association to decrease thickness of the articular cartilage and breakdown of the extracellular matrix, eventually resulting in chondrocyte degeneration (Malemud and Sokoloff, 1974; Caterson and Lowther, 1978). However, abnormally high loading of joints results in the loss of proteoglycans from the articular cartilage, fibrillation of the tissue and in time necrosis (Rodan et al., 1978; Binderman et al., 1974). Thus mechanical loading within the physiologic range may be important for maintaining the integrity of the tissue.

Investigating the effect of force levels on collagen synthesis in fibrous joints, Yen et al. (1989b) inserted helical springs to expand the interparietal suture of adolescent mice. An increase in the proportion of Type III to Types I and III collagen directly related to the level and rate of sutural expansion was noted. It appeared that the light forces initiated a response in the proportion of collagen phenotypes which was

much closer to the physiologically developing sutures than did the heavier forces (Yen et al., 1989a).

Other investigations into the response of mechanical stress on the interparietal sutures have indicated a two-fold increase in collagen synthesis (Meikle et al., 1979) in newborn rabbits. Later experiments using the same model indicated increases in enzyme activity for collagenase, gelatinase, and NMP III with no alteration in the degradation of structural proteins (Meikle et al., 1980).

Using radioautography to study expansion of interparietal sutures, Chiang (1981) found significant changes in protein synthesis within four hours with peak values occurring after one week of application. In addition, notable expansion of the sutures accompanied by stretched and disoriented fibers at this time. After four weeks of application, the original suture width was reestablished with immature bone.

Duncan et al. (1984) used a periodontal organ culture system capable of receiving orthodontic type forces to evaluate changes in collagen phenotypes and prostaglandin synthesis in vitro. They found significant increases in the proportion of Type III collagen synthesis during periods of active stress, but no alteration in the relative level of prostaglandin synthesis were discernible.

Leung et al. (1976 and 1977) investigated rabbit aortic smooth muscle cells grown on elastin membranes in vitro. By cyclically stretching the membranes by 10% beyond their resting

length 52 times per minute for 8 hours and 56 hours, they demonstrated that the synthesis of types I and III collagen increased to the same degree, as did the synthesis of other matrix proteins, while cell proliferation and DNA synthetic rates were not increased. Moreover this stimulation of protein and collagen synthesis did not deteriorate over the different time periods studied. In contrast to these findings, neither chondroitin 4-sulfate nor dermatan sulfate synthesis were found to increase.

They noted intracellularly a more abundant amount of ribosomes, both free and associated with rough endoplasmic reticulum in the cyclically stressed cultures while the stationary cultures presented more myelin figures and lysosomes.

These results reported by Leung et al. (1976 and 1977) were further substantiated by Sottiurai et al. (1983) who, by using the same system and cyclic regimen for 8, 48 and 56 hour periods, showed similar two- to fivefold increases in protein and collagen synthesis. Cyclic stretching also resulted in the formation of five times more abundant rough endoplasmic reticulum and the dilated cisterna along with the preservation of myofilaments depending on the duration of the stress. In contrast, immobility resulted in an apparent decrease in myofilament content and cytoplasmic degradation in the individual cells. They concluded that the absence of stimulation apparently reduced cell function to basal maintenance levels favoring involution and degenerative changes, while stimulation increased cellular

biosynthesis.

Although little is known about the cell rests of Malassez, they have been found to be stimulated to a state of proliferation as a result of orthodontic tooth movement (Gilhouse-Moe, 1972). This stimulation is characteristically found only in the areas under tension while that area under compression tends to be devoid of cells.

To investigate this phenomenon, Brunette (1984) attached an appliance previously described by Harrell et al. (1977) to a Petriperm culture dish which has a flexible plastic membrane. Through this model, he applied 4.2% elongation. A significant increase in the number of epithelial cells synthesizing DNA was observed after just 30 minutes of force application. This rise in DNA synthesis more than doubled by two hours and at all times the stretched cultures presented a higher percentage of labelled cells when compared to the unstretched controls, although after six hours this result was no longer significant.

Electron micrographs of the stretched and unstretched cells indicated a greater amount of filamentous structures and increased number of desmosomes per unit length of the cell membrane in the stretched cells (Brunette, 1984). It may be hypothesized that the mechanical stretching resulted in alterations in the attachment of cells to each other

Another cell line in which role of physical forces has been investigated are the endothelial cells lining the walls of blood vessels. These cells are consistently exposed to the

oscillating forces produced by blood flow which may play a vital role in the cell's pathobiology through its affect on cell shape, alignment and attachment (Chien et al., 1981).

To investigate the role of shear stress on cell shape and alignment, Ives et al. (1983) cultured human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC). These cultures were exposed to shear stress of up to 50 dynes cm^{-2} through the utilization of hydrostatic pressure provided through an electromagnetic flow loop. The behavior of the two cell lines was found to be very different. The BAEC, described as a polygonal monolayer prior to testing, elongated and aligned within 24 hours at all shear stresses. In contrast, the HUVEC resisted reorientation until stressed for a period of 137 hours. These results suggest a difference between human and bovine endothelial cell behavior in vitro with the HUVEC being more resistant to change in cell shape and alignment inferring the possibility of less adaptability in vivo.

Responses to Deformation Via the Flexercell Strain Unit

The large variety of models used and areas investigating the effects of physical forces in vitro prompted Banes et al. (1985) to develope a stress-producing instrument (Flexercell Strain Unit, Flexcell Corp., McKeesport, Pa. - Refer to Chapter II - The Flexercell Strain Unit for a detailed description of

this system.) which applies compression or tension of variable strengths and durations to cells in vitro.

Initial experiments using the Flexercell Unit were preformed on plates of tendinocytes cultured from Flexor hallucis longus tendons of 6-week-old White Leghorn chickens (Banes et al., 1985 and 1987). A regimen of 25 seconds stress and 5 minutes relaxation for 3500 cycles at 0.13% distortion of the well produced no significant synthesis changes of a 45 x 10^3 M_r protein that co-migrates with actin, a key cellular component associated with myosin in cell movement and contraction (Pollard and Weihing 1974) However, a 52 x 10^3 protein that comigrates with tubulin, a major cytoskeletal element involved in the maintenance of cell form was found to decrease (Olmstead and Borisy, 1973).

To further investigate the effects of cyclic strain on tendons, Banes et al. (1988) isolated internal fibroblasts from flexor tendons 2-4 weeks of age and subjected the cultures to a maximum elongation of 24% for 3 or 10 cycles per minute for hours to 8 days. By day three the cells subjected to 3 cycles per minute began to align in the region of maximum strain (24%), whereas the cells receiving 10 cycles per minute had aligned in the 5-24% strain regions of the well. Similar alignment findings have been noted for human dermal and scar fibroblasts (Henderson et al., 1988). Cytochalasin b was found to prevent this alignment, but indomethacin nor colchicine did not, indicating that this alignment is dependent on actin

polymerization. Although this experiment showed that the internal fibroblasts aligned to minimize strain, additional experimentation showed that these same low level cyclic deformations did not increase generation times (Banes et al., 1988).

In contrast to other cell lines in which mechanical stimulation has induced DNA synthesis and cell division, cyclic stressing of the tendinocytes with the Flexercell Unit at 10% elongation of the well with a regimen of 10 seconds stress followed by 10 seconds relaxation for 1 to 7 days resulted in decreasing cell numbers (Banes et al., 1987) in similarity to the internal fibroblasts. Thus stress may actually inhibit tendon cell division to prevent hypercellularity and weakness in vivo.

Application of the same force system to bovine aortic endothelial cells and human scar fibroblasts provided significant increases in DNA synthesis and cell number over the seven day periods Banes et al., 1987). These findings suggest that there may be an in vitro response in a cell line of a positive or negative nature which is characteristic of that cells function in vivo.

Investigation into other fibroblast cell lines resulted in similar findings (Levin et al., 1988). Human pulpal fibroblasts cyclically stress to 10 seconds stress followed by 10 seconds relaxation for seven days showed that cell division and DNA synthesis were depressed by day 3. This same experiment revealed significant differences in protein synthetic patterns for 45

proteins and a 7-fold increase in actin polymerization.

As previously discussed, earlier works with osteoblast-like cells under mechanical strain have shown an increase in growth rate, increase in DNA synthesis and alignment perpendicular to the strain vector (Harrell et al., 1977 and Somjen et al., 1980). In support of these findings, Buckley et al. (1988, 1988, and 1990) have applied a maximum of 24% elongation at a cyclic regimen of 10 seconds stress and 10 seconds relaxation for periods of up to three days on the Flexercell Unit on osteoblast-like cells from the calvaria of 4-week-old chicks. These studies have shown that in response to the mechanical stress these cells are stimulated to increase their growth rate, thymidine incorporation and to alter their alignment much as in the earlier studies. In addition, they have shown that in response to stress these osteoblasts will alter their morphology, surface topography, and mineralize within 24 hours. In three day tests collagen and noncollagen proteins where found to have decreased by the first day.

The majority of scientific investigation utilizing the Flexercell Strain Unit has been focused towards the effects of mechanical stress on vascular endothelial and smooth muscle cells.

Sumpio and Banes (1988a, 1988b, 1988c) examined porcine aortic smooth muscle cells by exposing them to cyclic deformation of 10 seconds of 24% maximal elongation and 10 seconds relaxation for periods up to 7 days. Investigation into the growth rate of

these cells when stressed and nonstressed revealed a significant decrease in generation time by the first day (Sumpio and Banes, 1988b). This deceleration was maximal from days 3 to 5 after which the generation time returned to a rate comparable to the nonstressed cultures. [^{3}H]thymidine incorporation showed DNA synthesis paralleled the rate of cell division found in the growth curve experiment. This inhibition of proliferation is in contrast to the investigation of Leung et al. (1977). However this difference may be the result of numerous factors including the rate of mechanical stretching, the amount of strain provided through each stress, surface interactions, or the level of confluency of the cultures. In addition, the smooth muscle cells in this study tended to align perpendicular to the direction of the strain vector, especially in the areas of maximal elongation (Sumpio and Banes, 1988b).

Despite the low frequency of cycles used in Sumpio's experiment (1988a) compared to Leung et al. (1977), the results after three days indicated a qualitatively similar result in that cyclic stressing stimulated smooth muscle cell production of both collagen and noncollagen protein.

Sumpio et al. (1988b, 1988d, 1990) further investigated these effects of mechanical perturbation provided through the Flexercell Strain Unit on bovine aortic endothelial cells. Repeating the cyclic regimen of 10 seconds elongation to a maximum of 24% elongation followed by 10 seconds relaxation for a 5 days they found striking changes in cell morphology and

expression of the cytoskeletal protein, F-actin.

The more organized presence of actin along with the appearance of stress fibers in the cyclically stressed cells is in agreement with the hypothesis that these fibers represent a reaction to unfavorable or stimulatory conditions in vivo (White et al., 1983, Savion et al., 1982, and Harris et al., 1980). It has been shown that the stress fibers may be related to an increased need for adhesion to the substrate (Harris et al., 1980).

In addition to the presence of stress fibers, the cells appeared larger, more rounded and contained peripheral vacuolization. The significance of these cell populations is unproven, but it has been shown that more rounded cells have a lower rate of DNA synthesis as compared to flat cells (Asuprunk and Folkman, 1977). However, additional experiments under similar conditions showed that the mechanical stretching stimulated deoxyribonucleic acid synthesis and cell proliferation (Sumpio et al., 1987, 1988b). In this experiment cells subjected to cyclic tension had a significantly decreased generation time over a seven day period with the greatest rate of growth occurring on the first day. Furthermore, DNA synthesis paralleled the growth rate in both the static and stressed cultures.

In contrast to osteoblasts (Buckley, 1988 and 1990) and smooth muscle cells subjected to shear stress (Sumpio and Banes 1988b) the endothelial cells in this system did not align

themselves in the direction of maximum shear, but remained randomly oriented (Sumpio et al., 1988b; Sumpio and Banes, 1988b). The reason for this relative resistance to alignment is unexplained. It could be that a higher amount of stimulation is necessary to cause breakdown of the cell attachments exposed to the maximal strain vector or that the cellular response toward alignment or reorientation in these cells has a preference for lower amounts of stimulation. It has been suggested that there may be a "stretch receptor" coupled to adenylate cyclase that could modulate endothelial cell function with hemodynamic changes (Letsu et al., In Press).

Using the same force system, Sumpio reported differential synthesis of proteins, with both stimulation and inhibition of certain proteins in the "flexed" cultures. Of nearly 1000 proteins analyzed on autoradiographs, 14 were significantly increased, 20 were absent or repressed including collagen, and proteins such as actin, alpha-tubulin, and beta-tubulin were not significantly altered. The mechanisms for the stimulation and inhibition of protein synthesis in stressed cells is still not known.

Due to its potent antiaggregation of platelets and vasodilator potential, prostacyclin synthesis by endothelial cells under mechanical stress in vitro has been a recent area of investigation (Sumpio and Banes, 1988a; Upchurch et al., 1989). Upchurch et al. (1989) cultured bovine venous and arterial endothelial cells and subjected them in the Flexercell Unit to a

maximal of 17% elongation at a cyclic regimen of 60 cycles per minute. The results indicated that venous endothelial cells secreted significantly more prostacyclin than cells from arterial sources. Of special interest is the finding that both venous and arterial endothelial cells secreted significantly less prostacyclin when mechanically stimulated. This would support the belief that cells from different environments may react differently to the same regimen of deformation.

Another study by Upchurch et al. (1989) using the same system at a maximal elongation of 24% at 3 cycles per minute on bovine aortic endothelial cells indicated that the basal production of prostacyclin was unaltered with the force application. However, the addition of arachidonic acid significantly enhanced prostacyclin production of the stressed cells. Thus the cyclic deformation, while not increasing the amount of prostacyclin secreted may enhance the cells ability to produce prostacyclin while suppressing some of the enzymes involved in the arachidonic acid pathways.

The results of these in vitro investigations are that the classic culture environment may be an inaccurate representation of the dynamic environment of cells in vivo. It may be that removing cells from their dynamically active environment and placing them into a static condition actually causes them to react inappropriately or at least suboptimally.

CHAPTER II

THE FLEXERCELL STRAIN UNIT

THE FLEXERCELL STRAIN UNIT

The flexercell strain unit is a computerized vacuum-operated strain- providing instrument which enables the investigator to apply a defined, controlled, static or variable duration cyclic tension or compression to growing cells *in vitro*.

Culture Plates

The Flex I^{R} culture plates are made of conventional polystyrene with six cell culture wells with silicone rubber (silastic) bottom surfaces capable of up to 200% stretch. (This amount of elongation is sufficient to tear most tissue culture cell sheets. For this reason, a more physiological range of 1 to 20% elongation of the membrane is suggested.)

A 34.54 mm diameter well is formed in the bottom of each well, leaving a rim of 3.89 mm. Silicone rubber cured to a membrane thickness of 2.24mm is then poured into the well to form the well's bottom. (The modiolus of elasticity of the silicone rubber may vary from 0.107 to 0.284 kg/m^2 depending on the curing process.) The upper surface of this silicone rubber can then be treated chemically with an amino-rich hydrophilic substrate (positively charged surface), a carboxyl substrate (negatively charged surface), genetic type I collagen and elastin matrix surfaces to allow for cell attachment and growth. Each

well has approximately 5 cm^2 of surface area (30 cm^2 per plate).

The genetic Type I collagen surface presents collagen peptides to cells. Experiments have shown that approximately 2.1×10^{14} collagen molecules form covalent bonds per cm^2. Thus, theoretically, there should be approximately 10^{14} derivatizable sites per cm^2 resulting in a greater than 1 to 1 relationship between available sites and actual peptides, indicating complete surface coverage of the rubber. Plating efficiency of cells on the collagen surface is reported to be in the range of 50 to 95%. This variation is dependent on the cell type and the confluency level at the time of passage (Flexnet, 1990).

The elastin membranes have been shown to be a suitable substrate to which cells will adhere (Leung et al., 1977). Additionally, these membranes are well preserved and cohesive with minimal fatigue of their ability to recoil to their original length after trial stretching or agitation periods of 7 days at frequencies up to 100 cycles per minute with amplitudes up to 25% of the resting length (Leung et al., 1977).

Light microscopic studies of cells on this surface has revealed that the cells are deformed and regain their original shape as the substrate is elongated and relaxed (Leung et al., 1977).

Transmission and scanning electron micrographs have shown that cell cultures growing on elastin membranes form numerous attachments to the elastin plates by terminating directly into the elastin membrane as well as to each other. Furthermore,

these attachments persist after prolonged stretching or agitation (Leung et al., 1977 and 1977).

Real time video analyses of Flex I^R plates indicated that when a flexible membrane was stretched, the adherent cells also stretched and remained adherent, demonstrating that the deformation to the flexible membrane was translated to the cells (Gilbert et al., 1989). In addition, cell cultures have been shown to proliferate to these well bottoms while undergoing cyclical stretching (Sumpio et al., 1987, 1988a)

Flex II^R culture plates are of identical size with rigid-bottomed well surfaces made of the same substrate material present in the Flex I^R plates. The Flex II^R plates are designed to be used for the culture of control cells which are to be compared to the stressed cells grown on the flexible Flex I^R plates.

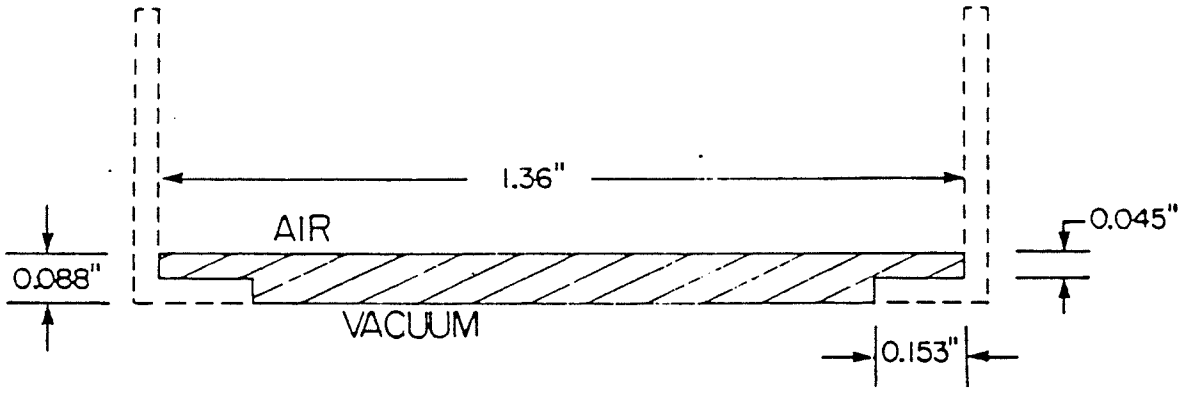

FIGURE II-1: Diagrammatic representation of a single well of a Flex I^R culture dish. As shown, the membrane is attached to the inner wall of the well, the upper surface of the rim, and the inner edge of the rim.

System Description

The Flexercell Strain Unit consists of a computer system controller and monitor, a control module, a vacuum baseplate and gasket specially designed to hold eight Flex culture plates, and a vacuum pump and air line.

The microcomputer includes one disk drive, 256k memory, and a color monitor. Varying cyclic programs to control frequency and duration of membrane elongations can be designed and implemented by the investigator through utilization of the Flexware^{R}, Flexercell^{R} Operations System Disk (Flexercell^{R} Copywrite Protected Software, Flexcell^{R} Corporation, 1396 Washington Blvd., Box 890, McKeesport, PA 15132). This allows for reproducibility of unique investigation programs.

A vacuum pump capable of producing and maintaining a negative pressure of 25mm of mercury in 5 minutes is required as well as an air line which can be regulated to .004 kg/m^2 of pressure. This air line is necessary to apply positive pressure to return the membrane to its original rest position but is only required in cyclic programs of 50 cycles per minute or faster.

The control module is used to regulate the negative or positive pressures applied in the system. Vacuum and air inlet and outlet lines are attached to this module as well as the microcomputer. Proper usage of the fine and course control knobs on the front of the module enable proper evacuation of atmosphere and volume beneath the culture plates. The unit contains an in

line pressure transducer which monitors the baseplate's pressure. Gauges located on the front of the control module indicate the system pressure of the vacuum reservoir as well as the baseplate pressure. A readout of pressure transducer is reported on the monitor as the program is being run.

The vacuum outlet line is threaded through a hole on the top or the back of a CO_2 incubator to a coupling attachment on the baseplate. It is important that the vacuum line be as short as possible to gain rapid response to the programed elongation and relaxation.

The Flex culture plates with their six deformable bottom wells are fitted into an elevated gasket on the baseplate so that negative pressure provided through a vacuum system can distort the bottoms downward to a hemispherical shape.

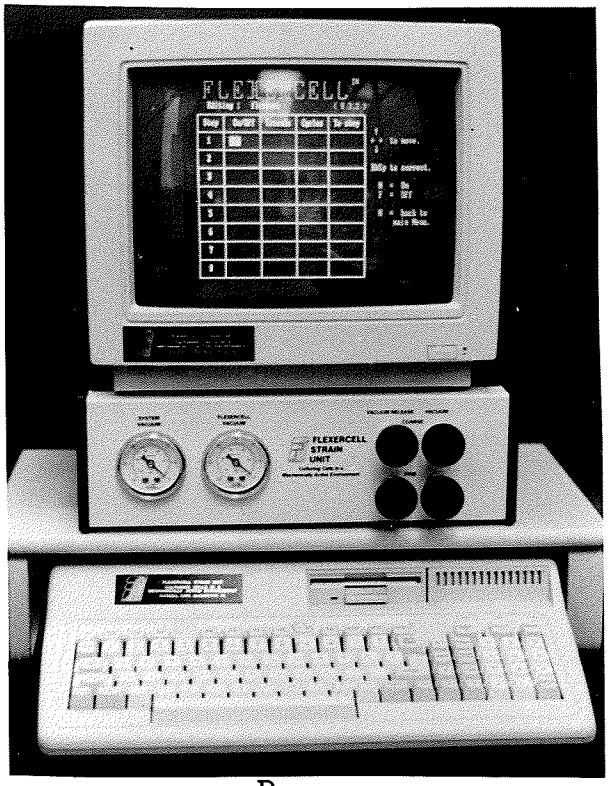

FIGURE II-2: Flexercell Strain Unit^R. The vacuum gauge on the far left of the control module measures the amount and stability of the system vacuum. The Flexercell vacuum gauge (second to the left) measures the vacuum applied to the silicone membranes of the culture dishes.

Viewing of the Cells

The cells in a Flex I^R culture dish may be observed with a phase contrast microscope by applying a round coverslip with immersion oil between the coverslip and the well's bottom. The coverslip corrects for the refractive index change caused by the rubber surface permitting visualization of the cells.

Strain Profile of the Membrane

The nonlinear relationship between the percent elongation of the silicone rubber membrane and the amount of applied vacuum applied has been demonstrated through finite element analysis, empirically and mathematically.

(A) Finite Element Model

Using ANSYS, an axisymmetric model of the circular membrane was developed consisting of a total of 112 isoparametric (STIF42) elements (Gilbert et al., 1989) Each element having four nodes. The silicone rubber was treated as a homogeneous, isotropic material with a Poisson's ratio of 0.49 and an elastic modulus of .213 kg/m^2.

Fifteen of the nodes connected to the plate. These nodes were constrained to zero displacements, UX-UY-UZ-0. The symmetry boundary condition (SYMBC was used to specify the symmetry axis

corresponding to the center of the membrane. Due to the large deflections of the membrane relative to thickness being modeled, it was necessary to apply large displacement analysis (KAY(6)-1), specify stress data through level 4 in the POSTR command, and apply incremental amounts (10 loading steps of -0.00035 kg/m^2 each) of negative pressure to distort the membrane. The analysis was done on a DEC MicroVax II.

The solution yielded a deformation of the membrane as seen in figure 3 for a pressure of -0.0035 kg/m^2. The maximum deflection was 6.502 mm at the center of the membrane. This calculated deflection was matched to the actual deflection measured on a cast from a deformed membrane.

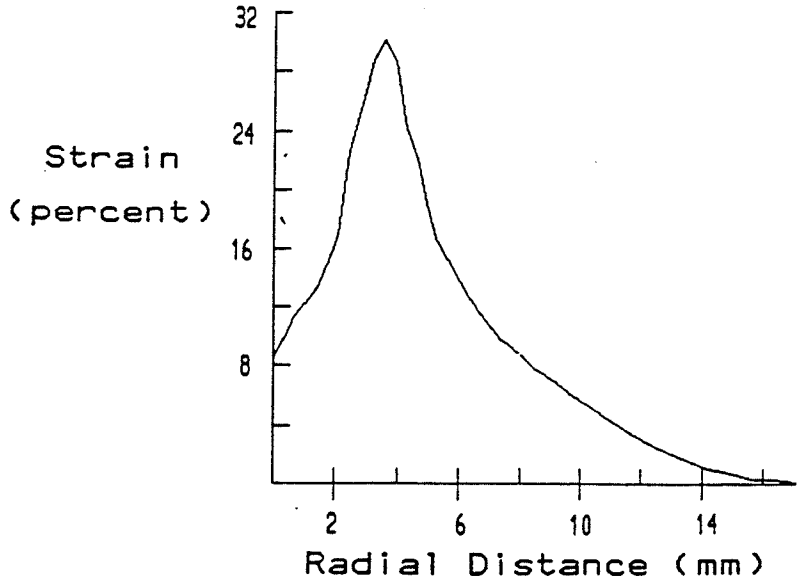

FIGURE II-3: Radial strain profile along the superior surface of the membrane from the periphery to the center at -0.0035 kg/m^2. Maximum strain of approximately 30 percent is measured above the edge of the well rim, 3.18 mm from the wall of the plate well.

(B) Empirical Formula

Thirteen concentric circles and axes and two pairs of diametric axes, 5^{o} offset from those of the first were embossed on the superior surface of the membrane with a die and marked with xerographic ink (Figure II-4). Using a compound light microscope with an attached video camera (magnification of 133x), images of the radial width of the of each individual circle were stored on an Amiga 1000 computer.

A vacuum 0.0035 kg/m^2 was applied to distort the silicone membrane downward. Incremental images of the radial widths of each ring was once again stored on computer. Once the images were stored, rapid-curing acrylic polymer was poured into the distorted well and allowed to cure forming a cast of the well to which the xerographic in was transferred.

Deformed and undeformed images of the rings were analyzed to determine the radial elongation of strain by recording the coordinates of the inner and outer edges of each ring and viewing the images from the side. The angle of the membranes could then be determined. The radial widths of each ring was calculated by dividing the width of the surface as viewed from the superior aspect by the cosine of the membrane angle at that point. The average percent strain was then calculated.

The change in distance of the rings was found to be greatest near the periphery of the well decreasing to almost zero increase distance at the well's center. The largest increase was determined to 3-4mm from the well wall where the membrane passed

over the rim of the plate.

The measurements were repeated for varying vacuum levels. The percent elongation versus the vacuum level were plotted showing a nonlinear function with the degree of elongation in a specific location on the membrane is proportional to the degree of vacuum applied.

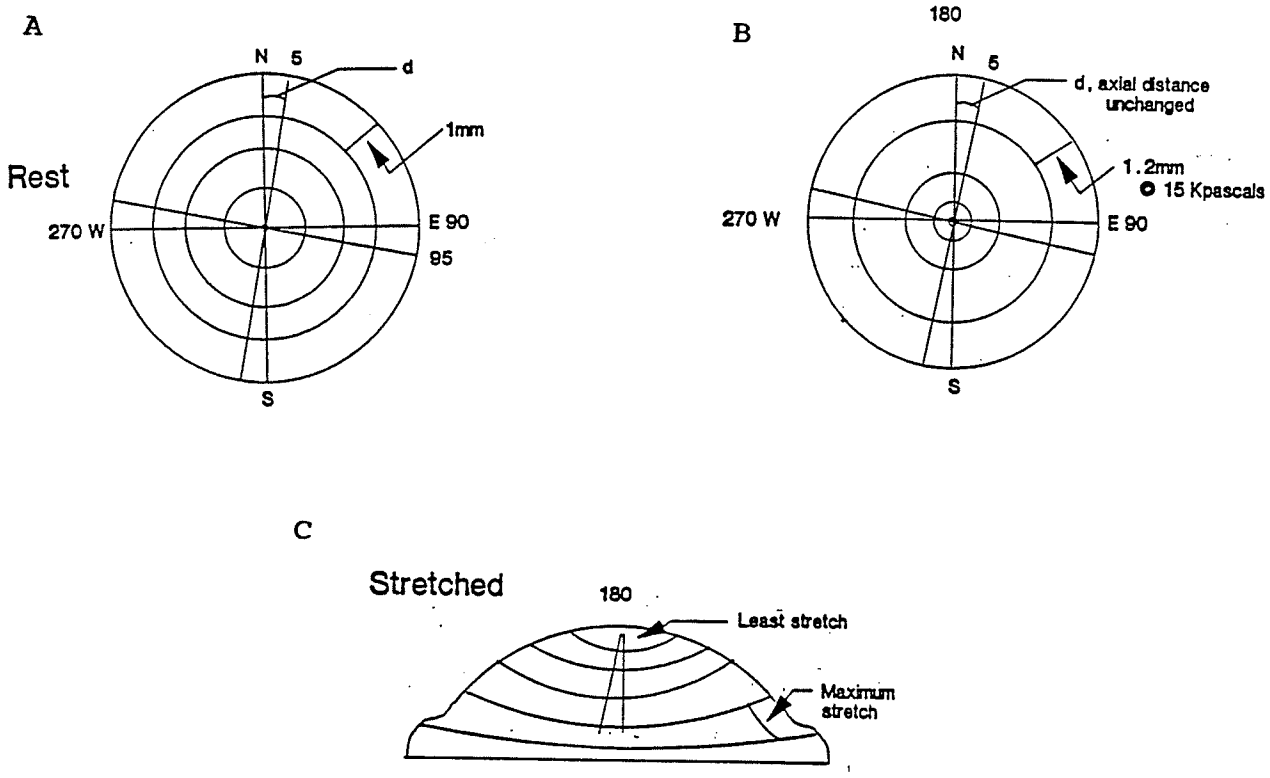

FIGURE II-4: Method of empirical measurement. (A) Representation of the concentric rings and axes tattooed to the silicone membrane at rest. (B) Representation of a stretched membrane illustrating that the distortion (increased distance) of the rings decreases to zero as the center of the membrane is reached. In addition, the axial distances (north, south, east and west) remain unchanged. (C) Representation of an acrylic casting showing the transformation of the xerographic ink from which the direct measurements were obtained.

(C) Mathematical Model

A mathematical solution of the relationship between the applied vacuum and the percent elongation was derived using a diaphragm, clamped at the periphery, and pulled downward (Banes et al., 1990) The solution showed that the strain is greatest at the periphery and least at the center of the well. This solution describes the relationship to the given response of a cell, R, and the characteristics of the amplitude of an applied force, A; the duration of the applied force, t_1; the time between deformation cycles, t_2; the duration between supercycles, t_3; the number of cycles of applied deformation, c; the strain rate as deformation reaches maximum A, e*1; the strain rate as deformation declines from maximum A, e*2; the shear rate, T, if the fluid flow is a factor; and the characteristics of the chemical substrate, s.

The expression is:

$$R = f[A(t_1,t_2,t_3)c(e*1,e*2,T,s]n$$

*The n indicates that the expression is repetitive.

CHAPTER III

STATEMENT OF THE PROBLEM

STATEMENT OF THE PROBLEM

The most perplexing phase of orthodontic treatment in many circumstances is the retention of the teeth in their newly defined positions. Although the significant influence of the structural components of the head and neck to the success of orthodontics and dentofacial orthopaedics is obvious, the immediate area of force application is primarily confined to the supporting structures of the teeth. Two soft tissue periodontal fibers have been implicated in the degree of resulting stability following treatment: the supraalveolar group of fibers and the principal fibers of the periodontal ligament. The main component of both of these tissues is collagen.

The histological process of remodelling in response to orthodontic and orthopaedic stresses in the periodontal tissues is well documented, however, the specialized processes of transduction, differentiation and migration are still poorly understood. The majority of information in these areas gathered through the use of tissue culture has been deduced from in the "classic" experimental techniques. However, this static environment may be inappropriate for investigating cells which normally reside in a dynamic environment.

The resultant impact of changes in force magnitude, duration and frequency on cultured cells has yet to be fully investigated. The application of stress to cells in vitro may act as a first messenger influencing a wide range of metabolic

functions of the cell. The effects of a dynamic environment on the cells of the periodontium can be investigated in vitro through the application of the Flexercell Strain Unit^{R}.

Investigations have demonstrated that tissues within the periodontium remodel collagen at different rates with bone remodelling at a higher rate when compared to the periodontal ligament which in turn remodels faster than the gingival tissues. Because collagen is a nonliving material, any remodeling which occurs ultimately depends on the ability of the living cells to transduce the mechanical forces into biological signals. As a result the proportion of Type III collagen relative to the total collagen synthesis can be used as an indication of the cellular response to mechanical stress.

The initial area of investigation into the problem of instability would be the gingival tissues since these tissues have been implicated in relapse and are known to remodel slower than the other tissues of the tooth supporting apparatus. Collagen synthesis in the rat palatal mucosa has been shown in vitro to be most rapid at 2.5 weeks of age. For this reason, 3 week old rats were used in this investigation.

In the first investigation, the effects of magnitude, duration and frequency of mechanical force application on collagen synthesis in gingival tissues were determined in vitro. These findings were compared to cell cultures in a static environment. This provided insight into which type of force system is appropriate for rapid and efficient remodeling of the

gingival and other fibrous tissues.

The second experiment investigated the effects of cell passage and confluency on collagen synthesis in gingival tissues *in vitro* as they related to an intermittent force system over time. These findings related the effects of cell selection or adaptation to their physical surroundings in culture.

Investigations into the resulting effects of force magnitude, duration and frequency, and their total impact on cell cultures remains to be studied. However, the results of these investigations into the production of collagen in a variety of culture environments may reflect similar processes *in vivo* providing valuable insight into the functional role of mechanical force on transduction ultimately leading to the establishment of an "optimal force system" such that the therapeutic objectives of treatment can be achieved efficiently, without iatrogenic effects and with stable results.

CHAPTER IV

THE EFFECT OF MECHANICAL FORCE ON COLLAGEN PHENOTYPE SYNTHESIS IN GINGIVAL TISSUES IN VITRO

THE EFFECT OF MECHANICAL FORCE ON COLLAGEN PHENOTYPE SYNTHESIS IN GINGIVAL TISSUES IN VITRO

SUMMARY

Gingival fibroblast-like cells were grown from palatal gingival explants from 3 week old male rats and subcultured in collagen coated flexible bottom dishes (Flex I^R). The purpose of this study was to compare the effect of force magnitude, duration and frequency on synthesis patterns of collagen phenotypes in mixed cell populations isolated from rat gingiva *in vitro*. Confluent cell cultures were subjected to cyclic force regimens of loading and relaxation of 180 cycles per hour (cph), 30 cph and 0.5 cph as well as a continuously applied force regimen. Cultures were harvested after 2, 4, 6, 8 and 24 hours of loading at a maximum strain of 24 or 10 percent. Control cells received no stress. 5 μCi/ml of [^{14}C]-Glycine were added for the last 2 hours of culture. Type I and type III collagen α-chains were separated by SDS-PAGE with a delayed reduction step. Densitometric scans of fluorograms from dried gels demonstrated a significant increase in the proportion of newly-synthesized type III collagen in the cultures stressed intermittently but not in the continuously stressed or control cultures. Peak responses in type III collagen synthesis appeared after 4 to 8 hours of stress and decreased at longer periods at both force magnitudes. This suggests a delayed response to intermittent force, independent of the force magnitude, to which the cells eventually adapt as part of their microenvironment.

INTRODUCTION

Possibly the most frustrating, and still unexplained, phenomenon in clinical orthodontics and dentofacial orthopedics is the tendency of teeth to relapse from their newly established positions. Although the significance of the structures of the craniofacial complex to successful treatment cannot be understated, the immediate location of force application is the teeth and their supporting structures, the gingiva, alveolar bone, and fibrous attachments. A number of hypotheses have been put forward regarding this problem and many practitioners may suspect that its cause is multifactoral. Implicated in these hypotheses are two soft tissue fibers: the supraalveolar fibers and the principal fibers of the periodontal ligament (Reitan, 1959; Thompson, 1959; Edwards, 1968, 1970, 1988; Brain, 1969; Parker, 1972).

With the application of orthodontic force, the gingival fibers are stretched displacing these tissues in the direction of the tooth movement. The distortion of these fibers remains for an extended period even though the principal fibers of the periodontal ligament are known to remodel rapidly (Skougaard et al., 1969; Page and Ammons, 1974; Kameyama, 1975; Sodek, 1976).

Collagen is the main component of the gingival fiber bundles, and its biosynthesis and degradation by fibroblasts is a closely regulated process constantly influenced by extracellular conditions (Hance and Crystal, 1977; Muller et al., 1981;

Saltzman et al., 1982) including the immediate mechanical environment (Leung et al., 1976, 1977; Yen et al., 1980, 1989a, 1989b; Meikle et al., 1982). A variety of genetically distinct collagen phenotypes have been isolated and each connective tissue contains a unique spectrum of these types depending on its functional requirements (Bornstein and Sage, 1980). As a result, changes in the collagen phenotype ratio, especially that of type I and type III, may serve as important parameters indicating the metabolic state of the connective tissue involved and may act as a signal for changes in cellular activity (Epstein, 1974; Weiss et al., 1975, Reddi et al., 1977; Gay et al., 1978). Changes in collagen phenotype synthesis due to the application of continuous mechanical stress has been demonstrated to result in an increase in the proportion of newly-synthesized type III collagen in the sutures of a variety of animal models (Yen et al., 1980, 1989a, 1989b; Meikle et al., 1982). However, different regimens of force application may be transduced into varying cell responses (Banes et al., 1988).

The purposes of this investigation were to determine and compare the changes in the proportion of newly-synthesized type III collagen in fibroblast-like cell populations in vitro isolated from rat gingiva through the application of varying mechanical force magnitudes, durations and frequencies. Three-week-old Sprague Dawley male white rats were selected because time studies have indicated this as the age of greatest collagen remodeling of the rat palatal mucosa (Schneir et al., 1976).

MATERIALS AND METHODS

(A) Primary Cell Culture

Sprague Dawley male white rats, 3 weeks of age, inbred in our facility, were sacrificed by cervical dislocation following ether anesthesia (Mallinckrodt Inc., Paris, Kentucky). The palatal tissue was divided sagitally into halves and immediately removed by surgical excision and placed into growth medium (Refer to Figure IV-1). The growth medium consisted of Dulbecco's α-minimum essential medium (α-MEM) with L-Glutamine (Gibco Laboratories, Grand Island, N.Y.), 400 units per ml penicillin G (Sigma Chemical Co., St. Louis, MO), 0.56 mg per ml streptomycin sulphate (GIBCO Lab, Grand Island, N.Y.), 0.2 mg per ml ascorbic acid (Fisher Scientific Co., Fair Lawn, N.J.), 1 ml per litre gentamycin reagent (Gibco Laboratories, Life Tech. Inc., Grand Island, N.Y.) and 2.2 mg per ml sodium bicarbonate (Fisher Scientific Co., Fair Lawn, N.J.), pH 7.2. The medium was sterilized using a Sterivex-GV 0.22μm filter (Millipore Corp. Bedford, Mass.).

After washing in the medium for thirty minutes, each half was then cut into small pieces (1.5mm x 1.5mm) and placed into 60mm polystyrene tissue culture dishes (Corning Glass Works, Corning, N.Y.). Sterile cover slips with sterile silicone grease (Dow Corning Corp., Midland, Mich.) where lightly placed over the tissue to facilitate adherence to the dish. 5ml of medium, as described previously with the addition of 10% fetal bovine serum

(Bocknek Lab. Inc., Canada) and 0.1ml/litre fungizone (Gibco Laboratories, Life Tech. Inc., Grand Island, N.Y.) was added to the cultures.

The cultures were immediately incubated (Fisher Scientific CO^2 Incubator, Fisher Scientific Co., Fair Lawn, N.J.) in a humidified atmosphere of 5% CO^2 at 37° C. The growth medium was changed every two days. The cells were monitored daily with a phase contrast microscope (Nikon, Japan).

(B) Subculturing Technique

Within a period of 9-12 days the primary tissue cultures had reached confluency and were deemed ready to be subcultured. The dishes were washed 3 times in cold Hanks Buffered Saline Solution (HBSS) immediately followed by 2ml of cold trypsin ([1:250] 0.5 g/L; EDTA - 4Na 0.2 g/L in Ca^{++}, Mg^{++} free HBSS [Sigma Chemical Co., St. Louis, Mo.]) for 60 seconds. The excess trypsin was decanted and the dishes were incubated for a period of 3 minutes in a humidified atmosphere of 5% CO^2 at 37° C. Cell detachment from the dish was determined by the rounded appearance of the cells when viewed under the phase contrast microscope. The cells were suspended in 3ml of growth medium with a sterile pasteur pipette. A 100μl sample volume was removed to determine the number of cells present in the suspension by using Coulter counter values (Coulter Electronics Inc., Hialeah, Florida).

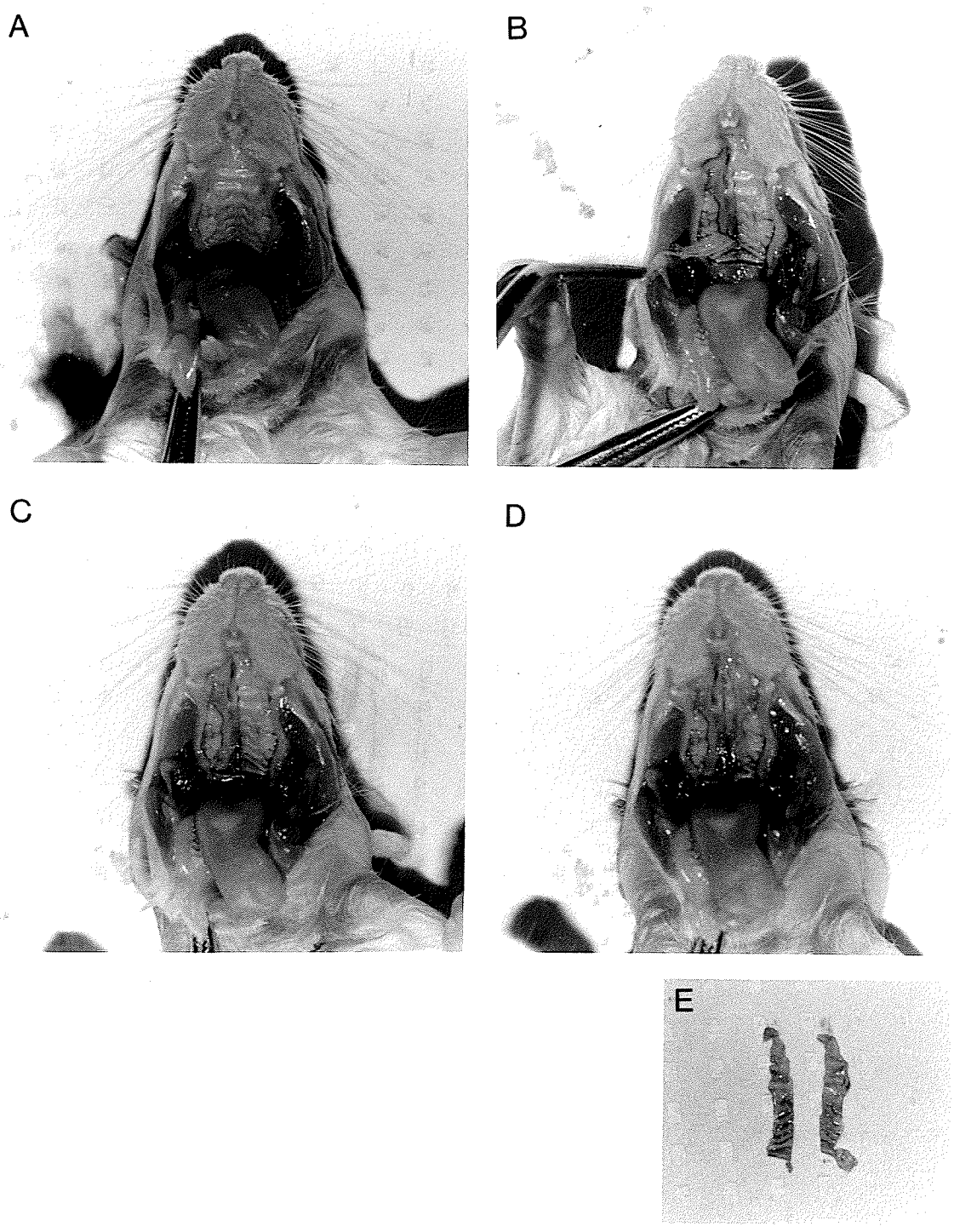

FIGURE IV-1: Surgical removal of the rat's palatal gingival tissues. (A) TMJ dislocated and oral cavity exposed in preparation for (B) the sagittal incision and (C & D) subsequent removal of tissues in preparation for (E) explant.

(C) Secondary Cell Cultures

The appropriate aliquot of the primary culture suspension required to seed at a concentration of 40,000 cells/ml medium was calculated and added to the six wells (35mm per well) of the Flex I^R dishes. The flexcell dishes were placed into a humidified atmosphere of 5% CO^2 at 37^o C for a 24 hour period. After this period, cellular attachment was confirmed with the phase contrast microscope and the subculture medium was decanted. The dish were then rinsed in sterile growth medium to ensure the removal of residual matters and 1.5ml a sterile growth medium with 10% fetal bovine serum, 0.2mg/ml ascorbic acid and 0.01ml/L fungizone were added to each of the six wells of the flexcell dish. The wells were monitored under the phase contrast microscope and the medium was changed every two days until the cells reached confluency, usually by day 3.

(D) Radioautography

When confluency was reached, the cultures were prepared for labelling of newly-synthesized collagen. Experimental procedures required a minimal of six wells per experimental time period, duration, and frequency.

Fresh growth medium with 10% FBS and 0.2mg/ml ascorbic acid were added. The dishes were placed into incubation on the flexcell baseplate and subjected to cyclic force regimens of loading and relaxation of 180 cycles per hour (cph), 30 cph and 0.5 cph as well as to continuous force. Control cells were

treated the same with no stress application. Cultures were harvested after 2, 4, 6, 8 and 24 hours of loading at maximum strains of 24 and 10 percent maximum elongation (-20 and -10 kPascals of pressure application respectively) of the dish's membrane. 5μCi/ml [^{14}C(u)]-glycine (Specific Activity 110.70 mCi/mmol) (ICN Biomedicals, St. Laurent, Que.) were added for the last two hours of each experiment.

To terminate the culture, the medium was first removed with a pipette and placed into a glass test tube in preparation for pepsin extraction. The cell layer was harvested with two washes of 1 and 0.5ml of 0.5M acetic acid. The cellular suspensions were pipetted and placed into a glass test tube. The cell samples were sonicated for about 30 seconds to disperse the cells. Both the acetic acid cell extracts and the medium samples were frozen in -70^{o} C in preparation for biochemical analysis.

(E) <u>Pepsin Extraction of Radio-labelled Collagen</u>

The frozen samples were lyophilized and subjected to limited pepsin digestion for collagen solubilization. Each sample was digested in a pepsin solution (0.1mg/ml in 0.5N acetic acid; Sigma Chemical Co., St. Louis, MO) at 16^{o} C for a total of 4 hours. The cell and medium samples were then dialyzed for 24 hours and 5 days, respectively, against 1% acetic acid at 4^{o} C. 100μl of each sample were placed in mini-scintillation vials with 5ml of scintillation cocktail (Aquasol, New England Nuclear, Boston, Mass.) and counted for [^{14}C]-glycine in a liquid

scintillation counter (Nuclear Chicago Isocap 300, Searle Instrumentation, Toronto, Ont.). The remaining sample was frozen to -70° C and lyophilized in preparation for gel electrophoresis.

(F) Collagen Separation and Quantification

Collagen α-chains and procollagens were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 20cm slab gel. The procedure used was a modified method previously described by Laemmli (1970) and utilized a 7.5% cross-linked separating gel, a 2.5% stacking gel, and tris/glycine buffers (Bio-Rad Laboratories, Richmond, California). Type I and type III collagen α-chains were separated using the interrupted electrophoresis method of Sykes *et al.* (1976).

Freeze-dried samples were dissolved in 70μl of reservoir buffer containing 2M urea, 2% sodium dodecyl sulphate, and 0.1% bromophenol blue, and were heated at 60° C for 30 minutes to denature the collagen. Samples were introduced to the sample wells and electrophoresis was performed for 1 hour at 130 volts under non-reducing conditions. Electrophoresis was stopped and samples were then reduced by the addition of 20% β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) to the sample wells to allow type III α-chains to penetrate the gel. Reduction was carried out for 60 minutes, then electrophoresis was resumed 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 dehydrated with two washes of dimethyl sulfoxide (Fisher Scientific, Fair Lawn, NJ) and impregnated with 2,5-diphenyloxazole / dimethy sulfoxide (Dupont-New England Nuclear, Boston, Mass.) as described by Bonner and Laskey (1974). The gels were placed on filter paper (Bio-Rad Laboratories, Richmond, CA), dried on a slab drier (Bio-Rad, Richmond, CA) and exposed to Kodak XRP-1 radiographic films (Kodak Canada, Inc., Toronto, Ont.) at -70^{o} C for a period of three to four weeks as indicated by the scintillation counts of the samples.

Individual sample tracks from the developed fluorographs were scanned at 550 nm with a spectrophotometer (Bio-Rad Model 620 Video Densitometer, Matsushita Electric Industrial Co. Ltd., Japan). The proportions of type III α-chains relative to the total of type I and type III α-chains were calculated from the integrator (Bio-Rad Model 3392A integrator, Richmond, CA) readings. The values obtained for these collagen ratios were subjected to statistical analysis using a three-way analysis of variance.

RESULTS

(A) Microscopic Observations

Observations made through the phase contrast microscope of the explantation of gingival tissues under the described experimental conditions resulted in an initial slow period of cell growth rate in the first five days of culture followed by a rapid increase resulting in a confluent multilayer network within ten days. These cells, resembling fibroblasts in there stellate appearance resulting from their multipodial extensions (Refer to Figures IV-2 and IV-3), radiated out from the tissue explant. It is interesting to note that as confluency was reached the cells within the culture, although having no specific orientation, appeared to take on a bipodial shape.

The subculture technique resulted in a confluent multilayer within a three day period (Refer to Figures IV-4 and IV-5). These first passage cells visually appeared to closely resembling the primary culture in their stellate appearance along with a tendency toward no axial alignment and bipodial shape at confluency. These factors did not appear to be affected by any of the force regimens employed in the investigation.

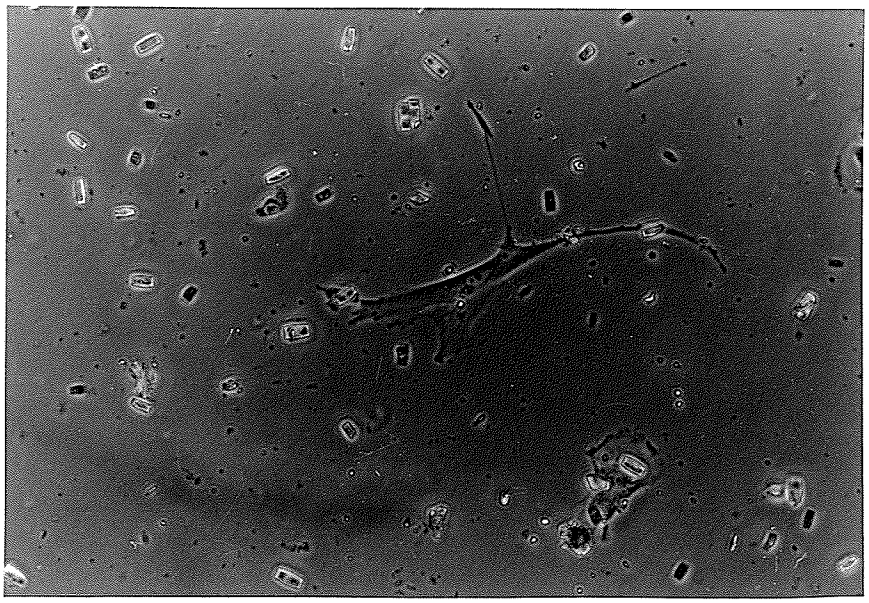

FIGURE IV-2: Phase contrast micrograph of an individual fibroblast-like cell cultured from the initial explant. Note the stellate appearance resulting from the multipodial extensions.

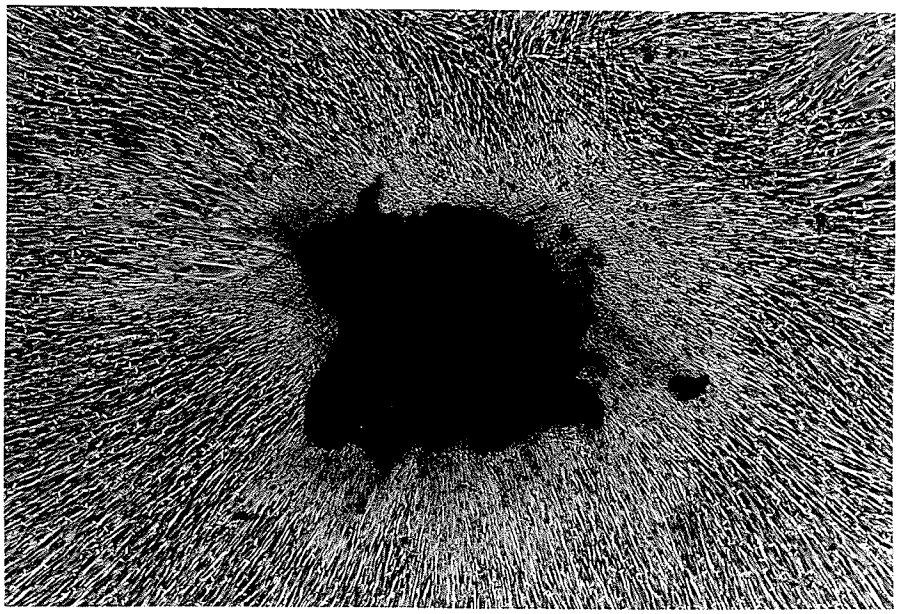

FIGURE IV-3: Phase contrast micrograph of fibroblast-like cells at confluency radiating from the explant tissue. Note the bipodial appearance of the cells as well as the lack of cell orientation in the lower left of the micrograph.

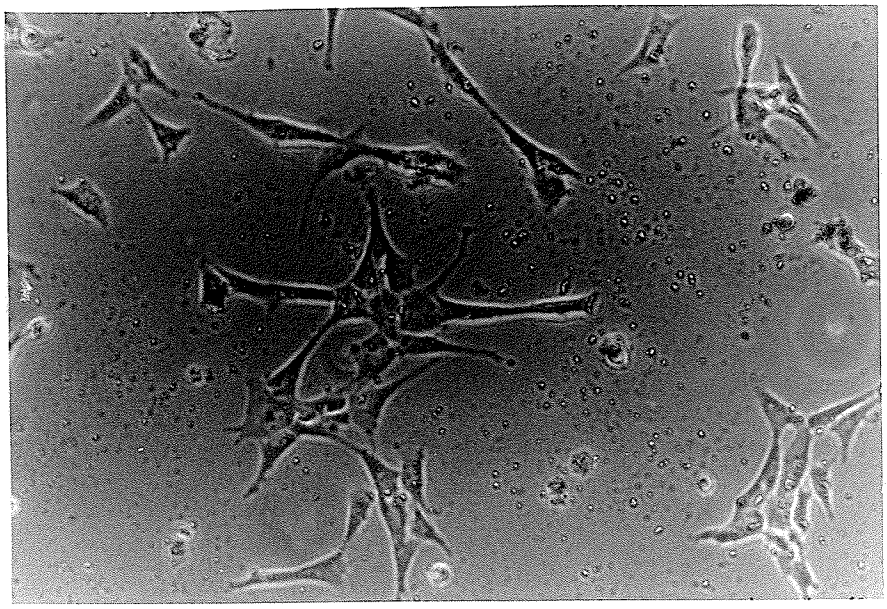

FIGURE IV-4: Phase contrast micrograph of an individual fibroblast-like cell attached to the collagen coated silastic membrane of the Flex IR culture dish after subculture. Note the resemblance of this stellate cell to that of the primary culture shown in Figure IV-2.

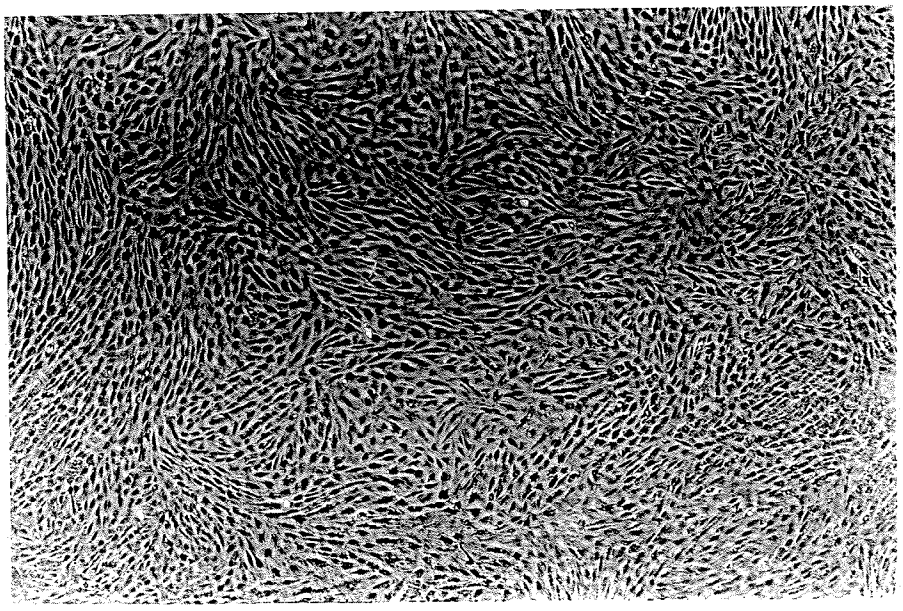

FIGURE IV-5: Phase contrast micrograph of fibroblast-like cells at confluency three days after subculturing into the Flex I^R dish. Note the random orientation of the cells and their bipodial appearance in the multilayer.

(B) Radioautographic Quantification

The mean values of type III α-chains relative to the total of type I and type III α-chains under the experimental force regimens are summarized in Tables IV-1 and IV-2 and graphically represented in Figures IV-6, IV-7, IV-8 and IV-9. As previously noted, these collagen phenotype ratios have been calculated from the raw data obtained through densitometric scanning of fluorograms processed from both the cell (Appendices B, C and D) and medium layers (Appendices E, F and G) of the tissue culture.

A delayed response to all frequencies and magnitudes of force application was noted at the 2 hour time period in both the cell and medium layers. This was soon replaced by a phase of increased proportions of newly-synthesized type III collagen in the intermittent force regimens (180 cph, 30 cph and 0.5 cph) at the 4, 6, and 8 hour periods followed by a return to the initial responses after 24 hours of force application.

It should be noted that the difficulty of pepsin digestion in the medium layer made quantification of these fluorograms a very demanding procedure. As a result the corresponding ratios, their standard deviations, and the statistical significance of the collagen phenotypes secreted into the medium layer vary somewhat from the cell layer data. Photographic comparisons of fluorograms from both the cell and medium layers illustrating collagen bands separated by SDS-PAGE are represented in Appendix R.

No statistically significant interaction was found between

the variables of force and time (Appendix H), $p=0.2627$, in the cell layer data at either the -20 kPa or -10 kPa force level and minimal significance (Appendix M), $p=0.0444$, was found in the medium layer data. As a result, these ratios were combined for analysis of the variables of time vs frequency. (Statistical results of the individual force levels at time, frequency, and time vs frequency are shown in Appendices J through L and N through Q.)

Statistical analysis of the mean ratios of newly-synthesized type III collagen confirmed that a significant interaction existed between the variables of time and frequency (Tables IV-3 and IV-4) in both the cell and medium layers. These data confirm the existence of a highly significant ($p<0.01$) rise in the type III collagen percentages in the intermittent force when compared to the continuous and control values at the 4, 6, and 8 hour experimental periods. No highly significant differences in the force levels were found to exist between the intermittent force regimens throughout the experimental period in the cell layer data (Tables IV-5 and IV-6).

FIGURE IV-6: Graphical representation of the mean percentages of type III to the combined type I and III collagen phenotypes from the cell layer of subcultured gingival fibroblast-like cells at the force magnitude of -20 kPascals or 24% elongation of the membrane over time. The legends represent the intermittent force regimens of elongation followed by relaxation of 180 cycles per hour (cph), 30 cph, and 0.5 cph as well as the continuously (constant) elongated and nonstressed (control) regimens.

FIGURE IV-7: Graphical representation of the mean percentages of type III to the combined type I and III collagen phenotypes from the cell layer of subcultured gingival fibroblast-like cells at the force magnitude of -10 kPascals or 10% elongation of the membrane over time. The legends represent the intermittent force regimens of elongation followed by relaxation of 180 cycles per hour (cph), 30 cph, and 0.5 cph as well as the continuously (constant) elongated and nonstressed (control) regimens.

FIGURE IV-8: Graphical representation of the mean percentages of type III to the combined type I and III collagen phenotypes from the medium layer of subcultured gingival fibroblast-like cells at the force magnitude of -20 kPascals or 24% elongation of the membrane over time. The legends represent the intermittent force regimens of elongation followed by relaxation of 180 cycles per hour (cph), 30 cph, and 0.5 cph as well as the continuously (constant) elongated and nonstressed (control) regimens.

FIGURE IV-9: Graphical representation of the mean percentages of type III to the combined type I and III collagen phenotypes from the medium layer of subcultured gingival fibroblast-like cells at the force magnitude of -10 kPascals or 10% elongation of the membrane over time. The legends represent the intermittent force regimens of elongation followed by relaxation of 180 cycles per hour (cph), 30 cph, and 0.5 cph as well as the continuously (constant) elongated and nonstressed (control) regimens.

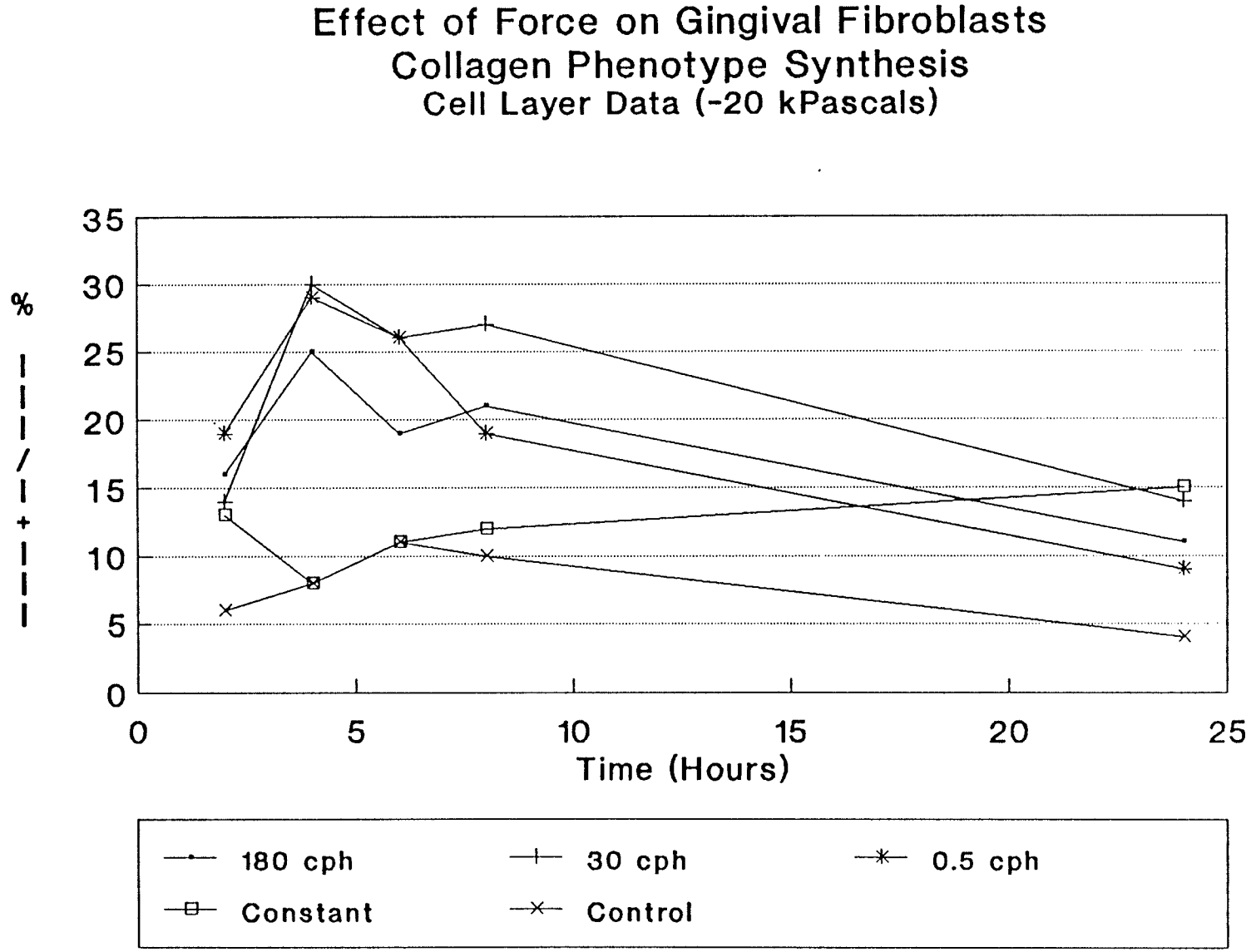
Effect of Force on Gingival Fibroblasts
Collagen Phenotype Synthesis
Cell Layer Data (-20 kPascals)
%
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Time (Hours)
180 cph
30 cph
0.5 cph
Constant
Control

FIGURE IV-6

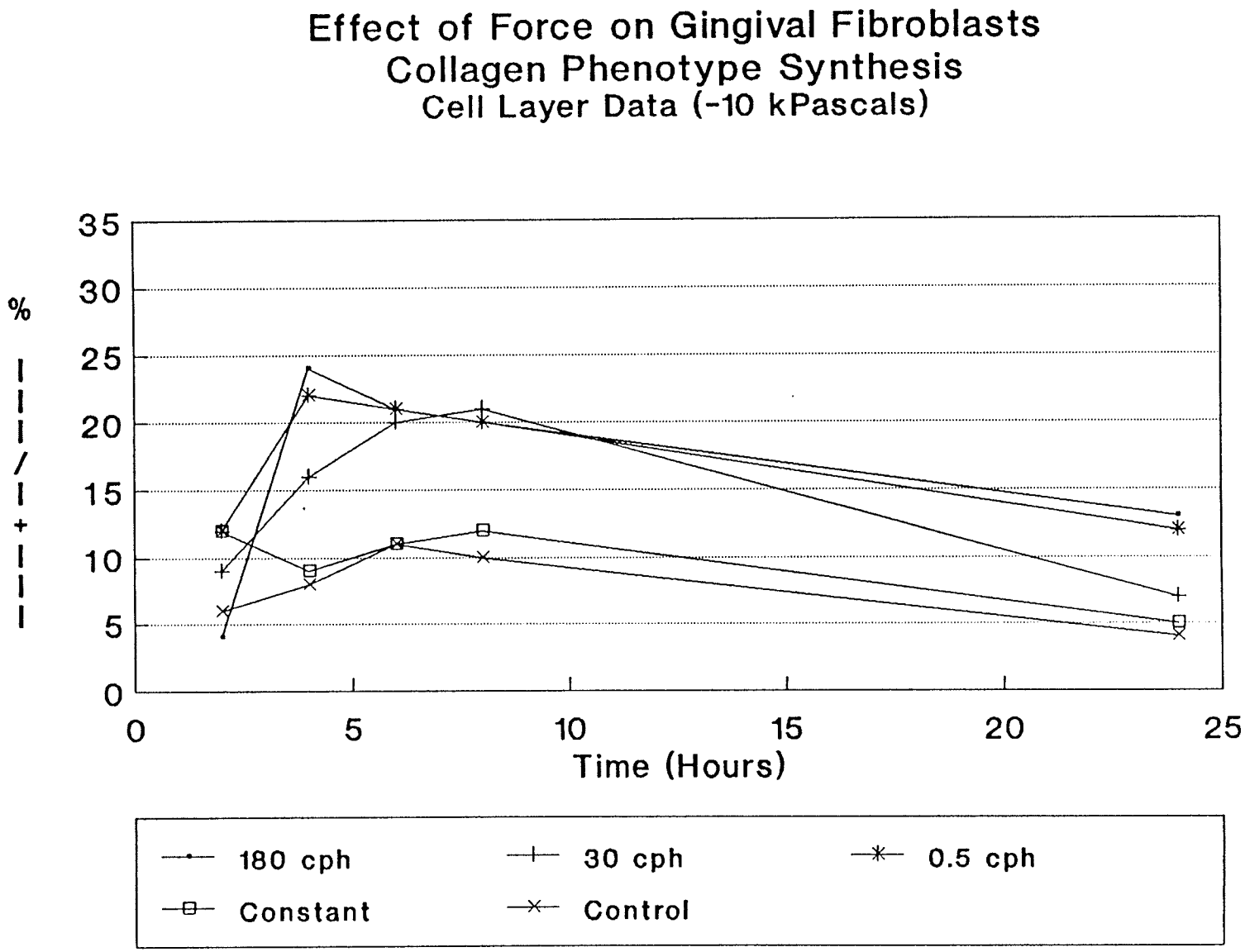
Effect of Force on Gingival Fibroblasts
Collagen Phenotype Synthesis
Cell Layer Data (-10 kPascals)
%
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Time (Hours)
180 cph
30 cph
0.5 cph
Constant
Control

FIGURE IV-7

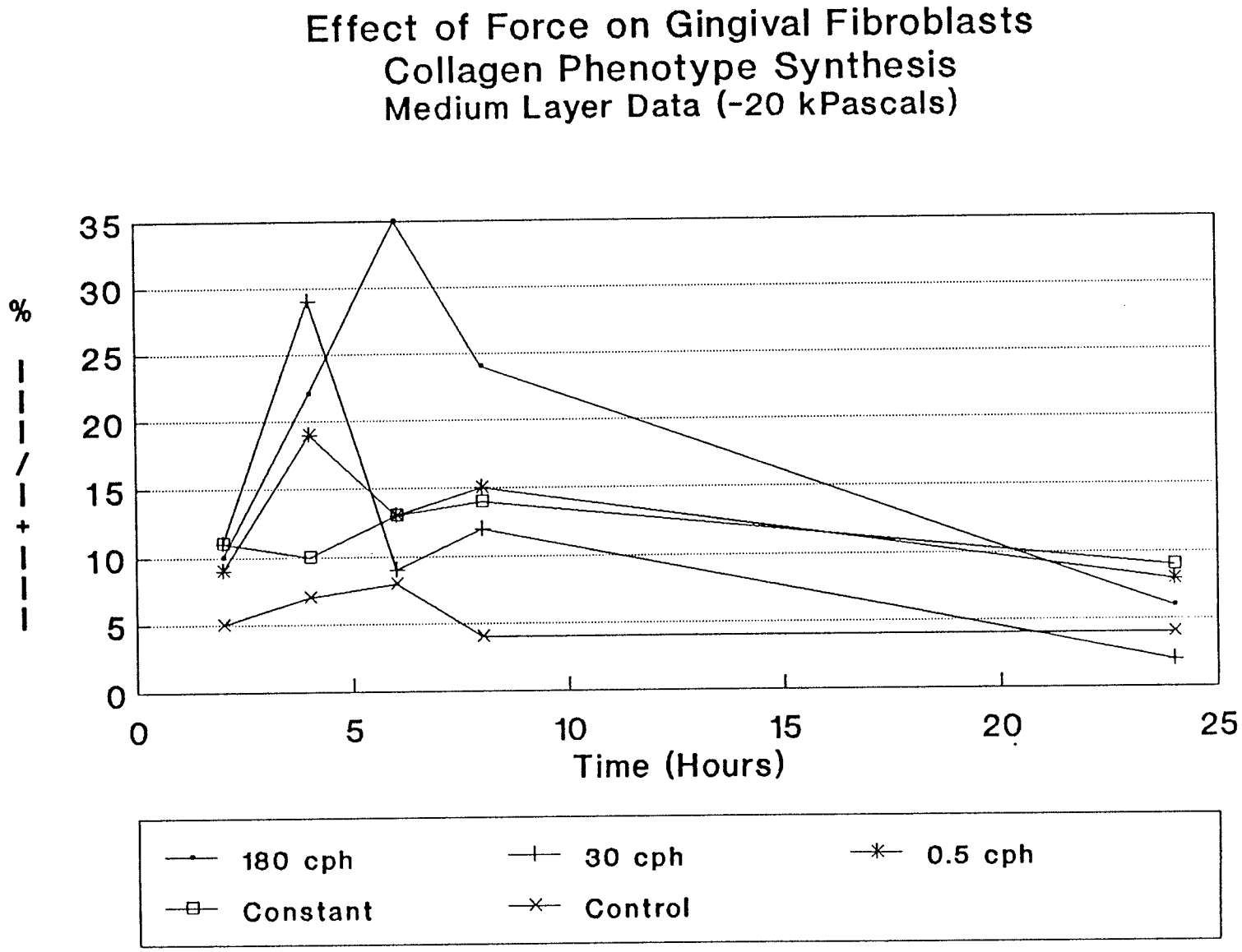
Effect of Force on Gingival Fibroblasts
Collagen Phenotype Synthesis
Medium Layer Data (-20 kPascals)
%
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Time (Hours)
180 cph
30 cph
0.5 cph
Constant
Control

FIGURE IV-8

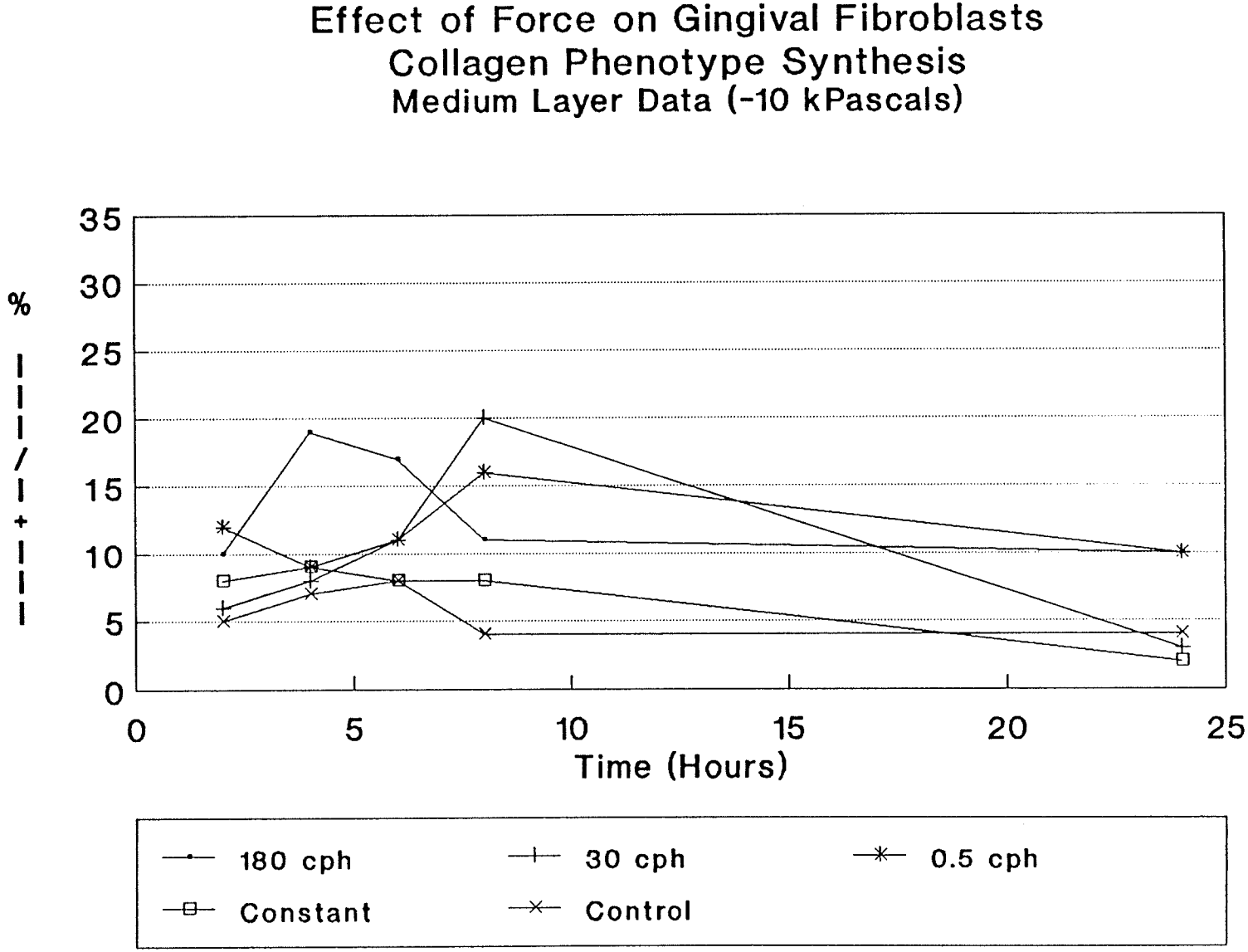
Effect of Force on Gingival Fibroblasts
Collagen Phenotype Synthesis
Medium Layer Data (-10 kPascals)
%
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Time (Hours)
180 cph
30 cph
0.5 cph
Constant
Control

FIGURE IV-9

TABLE IV-1

EFFECT OF FORCE ON GINGIVAL COLLAGEN PHENOTYPES IN VITRO
TYPE III / TYPE I + TYPE III
CELL LAYER DATA

FORCE LEVEL -10 kPascals

	2 HOURS		4 HOURS		6 HOURS		8 HOURS		24 Hours	
FREQ.	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD
180 cph	4.00	1.00	23.67	5.77	21.00	4.58	19.67	6.35	17.00	10.58
30 cph	8.33	2.31	16.67	7.57	20.33	2.08	21.33	6.66	6.67	6.51
0.5 cph	12.00	3.00	21.33	4.16	20.67	4.62	19.67	2.89	12.00	3.46
Const.	12.33	5.86	9.00	2.65	11.33	3.79	11.67	6.11	5.33	1.15
Control	6.00	5.20	8.33	1.15	11.33	3.51	9.67	3.79	4.33	4.51

FORCE LEVEL -20 kPascals

	2 HOURS		4 HOURS		6 HOURS		8 HOURS		24 Hours	
FREQ.	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD
180 cph	15.67	3.06	25.67	0.58	19.33	2.52	20.67	3.06	10.67	0.58
30 cph	13.67	3.06	30.00	3.00	25.67	9.07	27.33	2.08	14.67	1.15
0.5 cph	18.67	6.66	29.38	5.77	26.33	5.69	19.00	2.65	8.67	2.08
Const.	13.67	4.04	8.00	1.00	10.33	2.08	11.67	1.53	14.67	0.58
Control	6.00	5.20	8.33	1.15	11.33	3.51	9.67	3.79	4.33	4.51

TABLE IV-2

EFFECT OF FORCE ON GINGIVAL COLLAGEN PHENOTYPES IN VITRO
TYPE III / TYPE I + TYPE III
MEDIUM LAYER DATA

FORCE LEVEL -10 kPascals

	2 HOURS		4 HOURS		6 HOURS		8 HOURS		24 Hours	
FREQ.	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD
180 cph	9.33	2.08	19.00	5.00	17.67	0.58	11.00	3.61	10.33	3.21
30 cph	5.67	5.69	8.33	1.15	11.00	6.93	20.00	6.24	3.00	3.61
0.5 cph	12.33	4.62	9.00	1.73	10.67	5.03	16.33	4.73	10.00	4.36
Const.	8.33	0.58	9.00	2.65	8.33	4.16	7.67	5.86	1.67	2.08
Control	5.00	7.00	7.00	3.00	8.00	2.65	4.33	2.52	4.00	2.65

FORCE LEVEL -20 kPascals

	2 HOURS		4 HOURS		6 HOURS		8 HOURS		24 Hours	
FREQ.	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD
180 cph	10.00	5.00	21.67	6.35	34.67	7.23	24.00	2.65	5.67	2.31
30 cph	11.33	2.31	29.67	3.06	9.00	8.89	12.67	2.52	2.33	1.53
0.5 cph	9.67	3.06	19.00	9.17	13.33	1.53	15.33	5.03	8.00	3.61
Const.	10.67	1.53	10.33	1.53	13.00	7.00	14.00	5.29	8.67	2.08
Control	5.00	7.00	7.00	3.00	8.00	2.65	4.33	2.52	4.00	2.65

TABLE IV-3

Analysis of Variance of Mean Values for Cell Layer Data
Collagen Type III / Type I + Type III Percentage
Force Levels Combined

TIME vs FREQUENCY

2 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.6715	0.0478*	0.2512
30 CPH	0.6715		0.1175	0.4677
0.5 CPH	0.0478*	0.1175		0.3970
CONSTANT	0.2512	0.4677	0.3970	

4 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.6280	0.8085	0.0001**
30 CPH	0.6280		0.4677	0.0001**
0.5 CPH	0.8085	0.4677		0.0001**
CONSTANT	0.0001**	0.0001**	0.0001**	

6 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.3042	0.2273	0.0010**
30 CPH	0.3042		0.8557	0.0001**
0.5 CPH	0.2273	0.8557		0.0001**
CONSTANT	0.0010**	0.0001**	0.0001**	

8 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.1321	0.7619	0.0026**
30 CPH	0.1321		0.0715	0.0001**
0.5 CPH	0.7619	0.0715		0.0063**
CONSTANT	0.0026**	0.0001**	0.0063**	

24 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.2512	0.2051	0.1655
30 CPH	0.2512		0.9035	0.8085
0.5 CPH	0.2051	0.9035		0.9035
CONSTANT	0.1655	0.8085	0.9035	

p = 0.0001
Standard Error = 1.94
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

TABLE IV-4

Analysis of Variance of Mean Values for Medium Layer Data
Collagen Type III / Type I + Type III Percentage
Force Levels Combined

TIME vs FREQUENCY

2 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.7102	0.6711	0.9576
30 CPH	0.7102		0.4265	0.7501
0.5 CPH	0.6711	0.4265		0.6329
CONSTANT	0.9576	0.7501	0.6329	

4 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.6711	0.0459*	0.0010**
30 CPH	0.6711		0.1136	0.0037**
0.5 CPH	0.0459*	0.1136		0.1695
CONSTANT	0.0010**	0.0037**	0.1695	

6 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.0001**	0.0001**	0.0001**
30 CPH	0.0001**		0.5244	0.8318
0.5 CPH	0.0001**	0.5244		0.6711
CONSTANT	0.0001**	0.8318	0.6711	

8 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.7102	0.5956	0.0358*
30 CPH	0.7102		0.8734	0.0822
0.5 CPH	0.5956	0.8734		0.1136
CONSTANT	0.0358*	0.0822	0.1136	

24 Hours

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.0917	0.7501	0.3677
30 CPH	0.0917		0.0459*	0.4265
0.5 CPH	0.7501	0.0459*		0.2238
CONSTANT	0.3677	0.4265	0.2238	

p = 0.0023
Standard Error = 2.21
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

TABLE IV-5

Analysis of Variance of Mean Values for Cell Layer Data
Collagen Type III / Type I + Type III Percentage

FORCE vs FREQUENCY

FORCE LEVEL -10 kPascals

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.1698	0.9694	0.0001**
30 CPH	0.1698		0.1583	0.0076**
0.5 CPH	0.9694	0.1583		0.0001**
CONSTANT	0.0001**	0.0001**	0.0001**	

FORCE LEVEL -20 kPascals

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.0282*	0.2518	0.0002**
30 CPH	0.0282*		0.2846	0.0001**
0.5 CPH	0.2518	0.2846		0.0001**
CONSTANT	0.0002**	0.0001**	0.0001**	

P = 0.0481
Standard Error = 1.23
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

TABLE IV-6

Analysis of Variance of Mean Values for Medium Layer Data
Collagen Type III / Type I + Type III Percentage

FORCE vs FREQUENCY

FORCE LEVEL -10 kPascals

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.0538	0.3655	0.0015*
30 CPH	0.0538		0.2992	0.1923
0.5 CPH	0.3655	0.2992		0.0205*
CONSTANT	0.0015*	0.1923	0.0205*	

FORCE LEVEL -20 kPascals

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT
180 CPH		0.0023**	0.0023**	0.0001**
30 CPH	0.0023**		0.9732	0.4020
0.5 CPH	0.0026**	0.9732		0.3835
CONSTANT	0.0001**	0.4020	0.3835	

P = 0.4735
Standard Error = 1.40
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

DISCUSSION

The application of intermittent mechanical force regimens to the tissue cultures resulted initially in a retarded response to the perturbation followed with a rapid increase in the proportion of type III collagen. This rise was superceded by an almost equivalent decline in type III percentage by the final experimental period. This general pattern resembles findings in situations where rapid extracellular matrix production is necessary, as in embryonic tissues (Epstein, 1974), wound healing (Barnes et al., 1976, Gay et al., 1978), matrix-induced bone formation (Reddi et al., 1977), and sutural growth, development, and repair (Yen et al., 1989a, 1989b).

Because collagen is a nonliving material, any alterations in collagen phenotype synthesis ultimately depends on the ability of the gingival cells to transduce the mechanical forces into biological signals. As a result, these findings suggest that the mechanical strain of the silastic membrane is indeed perceived as stress, possibly on the plasma membranes, by the attached gingival cells of the tissue culture.

The initial delay in biosynthesis of type III collagen percentage could be hypothesized as resulting from alterations in specific biochemical changes within the cell, possibly causing modifications in transcription or translation retarding the formation of the procollagen molecule or a post-translational alteration which effects the molecules secretion into the

extracellular matrix since the treatment with SDS-PAGE, as used in this investigation, results in the separation of both procollagen and collagen into their appropriate α-chains.

The recovery of the cell population to produce significantly higher percentages of type III collagen can be the result of an increase in cell function and/or a shift in the cell population. It is suspected that these findings are the result of cellular changes in transcription and translation although mechanical force application in vitro has been shown to cause increases in cell numbers in a matter of minutes (Harrell et al., 1977; Somjen et al., 1980). Should the latter be the case, it could be suggested that the application of intermittent force regimens selects for functionally distinct subpopulations of fibroblasts which produce enhanced levels of type III collagen. The decline of the intermittent forces to nonsignificant levels of type III percentages indicates the adaptation of the microenvironment within the tissue culture to the force regimen much as a return to physiologic levels of type III collagen percentage represents the completion of remodeling in vivo.

Of importance is the finding that no significant difference was established between the ability of any of the intermittent force regimens to produce higher percentages of type III collagen even at different force magnitudes. In addition, the continuous force regimens resulted in no significant increase in type III percentage at either magnitude investigated when compared to the unstressed control values. If high proportions

of type III collagen implies histodifferentiation and rapid remodeling, then this outcome suggests that a variety of force applications may be optimal for the stimulation of remodeling in the gingival tissues as long as they are of an intermittent nature and that light force magnitudes may be as efficient as heavy force magnitudes in initiating this process.

CONCLUSIONS

1. A dynamic environment for the study of cell biology *in vitro* has been applied in the investigation of the effect of mechanical force magnitude, frequency and duration on cellular activity. Although the regulatory mechanisms responsible for the changes in cellular activities are still largely unknown, collagen phenotypes may still serve as important parameters of these changes.

2. Mechanical deformation applied to cells in culture may alter some functional and/or structural characteristics of the cells.

3. Initial injury from mechanical stresses of an intermittent frequency leads to significant adaptive cellular responses to which cells in an *in vitro* environment will adapt.

4. Forces of a light magnitude initiate a response equivalent to those resulting from greater magnitudes in both intermittent and continuous force regimens.

5. The application of continuous mechanical force regimens *in vitro* do not significantly alter the amount of Type III collagen percentage when compared to unstressed cell cultures.

CHAPTER V

THE EFFECT OF MECHANICAL FORCE, CELL PASSAGE, AND CONFLUENCY ON COLLAGEN PHENOTYPE SYNTHESIS IN GINGIVAL TISSUES IN VITRO

THE EFFECT OF MECHANICAL FORCE, CELL PASSAGE, AND CONFLUENCY ON COLLAGEN PHENOTYPE SYNTHESIS IN GINGIVAL TISSUES IN VITRO

SUMMARY

Explants of palatal gingival tissue from 3 week old male rats cultured to confluency in collagen coated flexible bottom dishes (Flex I^R) under a cyclic mechanical force regimen of loading and relaxation of 30 cycles per hour (cph) at a maximum strain of 24 percent were compared to cultures grown from the same tissues under "classical" static conditions and subcultured to confluent and nonconfluent stages in Flex I^R culture dishes. The tissue cultures were subjected to a force regimen of 30 cph and harvested after 2, 4, 6, 8 and 24 hours of loading at 24 percent elongation. First passage cells grown under static conditions served as control samples. 5 μCi/ml of [^{14}C]-Glycine were added for the last 2 hours of culture. Type I and type III collagen α-chains were separated by SDS-PAGE with a delayed reduction step. Densitometric scans of fluorograms from dried gels demonstrated a significant increase in the proportion of newly-synthesized type III collagen in the primary cultures grown in a dynamic environment compared to the control values throughout all of the experimental period. Following an initial period of no change, the confluent cultures demonstrated a rise in these percentages which leveled off at extended periods. In

contrast to this, early increases were found in nonconfluent cultures. However, the significance of these findings dissipated after 4 hours only to return returned to a high level again after 24 hours. These findings suggest the possibility that gingival explant and subcultured cells grown in a dynamic environment may have a greater ability to remodel collagen than confluent cells from a static environment. These results may also reflect the effects of contact guidance and contact inhibition of movement and/or cell-to-cell communication in high density cell cultures.

INTRODUCTION

Orthodontic therapy can be described as a controlled pathological event resulting from the application of mechanical forces from which the tissues normally recover. In contrast, Cohn (1965) demonstrated that tissues of the periodontium will undergo degenerative changes as a result of hypofunction leading to detachment of the free gingiva, and the alveolar and transseptal fibers. This has led to the general belief that although the application of extreme mechanical forces may result in iatrogenic effects some physiological level of force may be required for normal function of the gingival tissues.

Collagen is the main component of the healthy gingival tissues, and its biosynthesis and degradation by fibroblasts is a closely regulated process constantly under the influence of extracellular conditions (Hance and Crystal, 1977; Muller et al., 1981; Saltzman et al., 1982) including the immediate mechanical environment (Leung et al., 1976, 1977; Yen et al., 1980, 1989a, 1989b; Meikle et al., 1982). A variety of genetically distinct collagen phenotypes have been isolated and each connective tissue contains a unique spectrum of these types depending on its functional requirements (Bornstein and Sage, 1980). As a result, changes in the collagen phenotype ratio, especially that of type I and type III, may serve as important parameters indicating the metabolic state of the connective tissue involved and may act as a signal for changes in cellular activity (Epstein, 1974; Weiss

et al., 1975, Reddi et al., 1977; Gay et al., 1978). Changes in collagen phenotype synthesis due to the application of continuous mechanical stress has been demonstrated to result in an increase in the proportion of newly-synthesized type III collagen in the sutures of a variety of animal models (Yen et al., 1980, 1989a, 1989b; Meikle et al., 1982). However, different regimens of force application may be transduced into varying cell responses (Banes et al., 1988).

It has been proposed that pathogenesis of the gingival connective tissues results from cell injury to a selection of specific fibroblast subpopulations rather than a general cellular injury (Hassell et al., 1976; Hassell, 1981). Furthermore, it has been theorized that the gingiva harbors cells with the capacity to regenerate the periodontal tissues (Bowers et al., 1982; Fernyhough and Page, 1983). These proposals have been further supported in studies demonstrating the manifestation of functionally distinct subpopulations of fibroblasts within human gingiva, such that some fibroblasts produce both type I and type III collagen, while others produce only type I (Engel et al., 1980; Hassel and Stanek, 1983). These findings suggest that although gingival fibroblasts appear to be morphologically homogeneous, there may exist functionally different subpopulations within cultures as a result of maturation or differentiation of the cell as well as cell selection through tissue culture techniques.

Underlying the study of fibroblast-like cells derived in

vitro is the assumption that these cells have properties or potentials that are specific to the tissue in vivo. While it is well-established that cells undergo alterations in biochemical expression with subculture in vitro (Limeback and Sodek, 1979; Aubin et al., 1982) the consistent production of collagen and other cellular activities within human gingiva have been observed up to the 11th cell passage (Sommerman et al., 1988).

The purposes of this investigation were to determine and compare the effects of cell passage, level of confluency and mechanical environment through changes in the proportion of newly synthesized type III collagen in mixed cell populations in vitro resulting from intermittent force applications.

MATERIALS AND METHODS

(A) Cell Cultures

Sprague Dawley male white rats, 3 weeks of age, inbred in our facility, were sacrificed by cervical dislocation following ether anesthesia. The palatal tissue harvested was immediately placed into sterile Dulbecco's α-MEM containing 400 units/ml penicillin G, 0.56 mg/ml streptomycin sulfate, 0.2 mg/ml ascorbic acid, 1 ml/L gentamycin reagent, and 2.2 mg/ml sodium bicarbonate, pH 7.2. After washing in the medium for thirty minutes, the palatal tissues were divided sagitally into halves.

Each half was then cut into small pieces $1.5mm^2$. All procedures were conducted in the laminar flowhood excepting the dissection.

Explants were randomly separated and treated under the following conditions: Group A (primary culture) tissues were placed into 60 mm polystyrene culture dishes. Sterile cover slips with sterile silicone grease where lightly placed over the tissue to facilitate adherence to the dish. 5ml of medium, as previously described with the addition of 10% FBS and 0.1 ml/L fungizone was added to the culture. Group B (Explant) tissues were placed into collagen coated Flex I^R culture plates pre-warmed with 100% FBS (excess decanted off) and incubated at 37^o C for 1 hour with 5% CO^2 in a humidified atmosphere to allow the explants to adhere to the dish. 1.5ml per well of growth medium (as previously described) plus 10% FBS and 0.1 ml/L fungizone was then added and incubation continued for 24 hours. At 24 hours, the growth medium was changed in both groups.

Group A received incubation with medium changes every 2 days until confluency was reached within 9-12 days. When confluent, the medium was removed, the cells washed three times with cold Hanks BSS and trypsinized as described in Chapter IV. 4×10^4 cells/ml were seeded into each of the 6 wells of collagen coated Flex I^R dishes. The flexcell dishes were placed into the incubator at 5% CO^2 at 37^o C for a 24 hour period. At this time, the tissue cultures were lightly rinsed in sterile growth medium to ensure the removal of residual matters. 1.5ml/well of fresh sterile growth medium was added. The group was then subdivided

at random. The first subdivision were grown to confluency as in Chapter IV. The second subdivision was immediately placed on the baseplate gasket of the Flexercell Strain Unit^{R} in preparation for radioautography not allowing for confluency of these cultures to be reached.

Group B was placed into the baseplate gasket of the Flexercell Strain Unit^{R} and a force regimen of 30 cph at -20 kPascals was placed on the culture dishes. The medium was changed every two days for a period of 6 days. At this time, confluency was confirmed under the phase contrast microscope.

(B) Radioautography, Pepsin Extraction, and Electrophoresis

Following visualization under the phase contrast microscope, fresh growth medium with 10% FBS and 0.2 mg/ml ascorbic acid was added to each well of the Flex I^{R} plates in both groups. The dishes were placed into incubation on the flexcell baseplate and subjected to a 30 cph force regimen of -20 kPascals for periods of 2, 4, 6, 8 and 24 hours. 5 μCi/ml [^{14}C(u)]-glycine (Specific Activity 110.70 mCi/mmol) were added for the last two hours of each experiment. Mediums were pipetted off and the cells harvested in 2 mls of 0.5 HNAc followed by another 2 ml wash. The pooled samples were sonicated 30 seconds and frozen in -70^{o} C. The frozen samples were lyophilized and subjected to limited pepsin digestion, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and fluorographic quantification as described in Chapter IV. The values were subjected to statistical analysis

using a two-way analysis of variance.

Control cells received no loading and were processed and quantified in the same manner as the experimental groups. (These values were taken from the control values of Chapter IV. Please refer to this chapter for a more detailed description.)

RESULTS

(A) Microscopic Observation

Phase contrast microscopic viewing of cell cultures from tissue explants growing in the dynamic environment at day 3 showed morphologic changes of in cell architecture resulting from the loss of cellular extensions in comparison to cells grown in a static environment (Refer to Figure V-1). The tissues seemed to recovery from the initial insult reaching confluency by the fifth day regaining the appearance of unstressed cells at confluency (Refer to Figure V-2). Although no investigation was done to confirm increases in cell number and the wells involved were of a smaller diameter, it appeared that the cells in this culture replicated exceptionally fast reaching confluency in half the time of the unstressed cultures.

Morphology of gingival fibroblasts from nonconfluent cultures viewed under the phase contrast microscope immediately after removal of mechanical stress showed distortion of the cell shape to a bipodial appearance and alignment perpendicular to the

direction of force application in the outer third of the well at the sixth hour of experimentation (Refer to Figure V-4). These findings are in contrast to what was seen in nonconfluent cultures prior to the application of mechanical force (Refer to Figure V-3). As in the dynamically grown explant cultures, cell replication appeared to heighten resulting in confluency by the 24 hour experimental period as opposed to the usual 72 hours in unstressed subcultures. The cell alignment was still present in the outer third of the well (Refer to Figure V-5).

Confluent subcultures showed no morphologic changes compared to the unstressed controls (Refer to Figures IV-4 and IV-5).

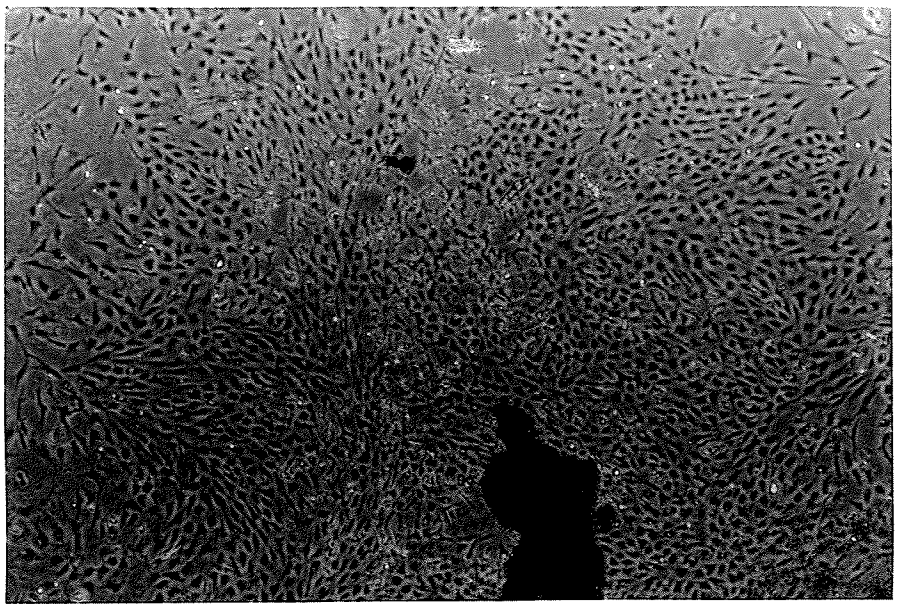

FIGURE V-1: Phase contrast micrograph of a mixed cell population growing from the palatal tissue explant in a dynamic environment on day 3. Note the apparent loss of cellular extensions and rounded appearance of many of the cells in this population in contrast to the confluent culture seen in figure V-2.

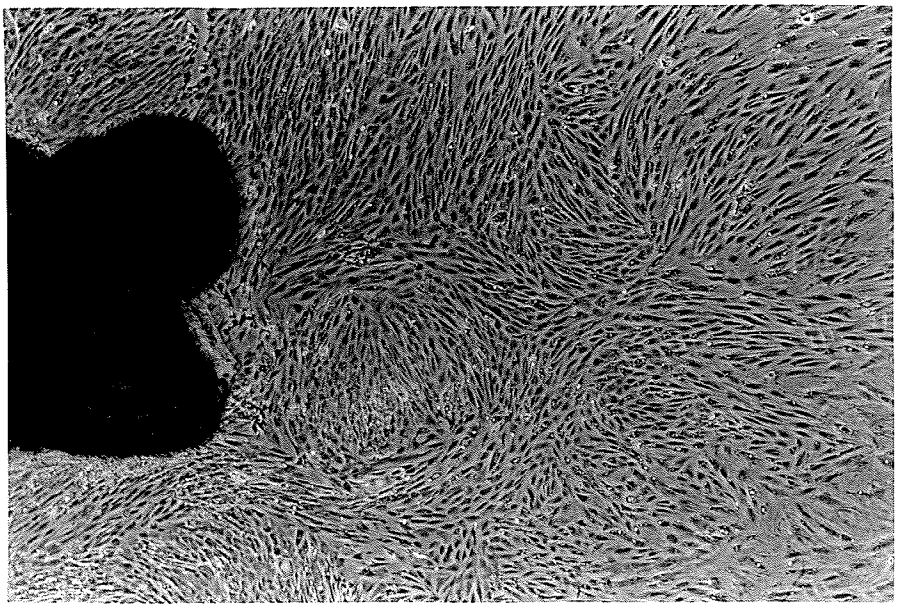

FIGURE V-2: Phase contrast micrograph of a mixed cell population growing from the palatal tissue explant in a dynamic environment on day 5. Note the morphologic similarities of size, shape, and orientation to the multilayer of unstressed first passage cells in figure IV-5.

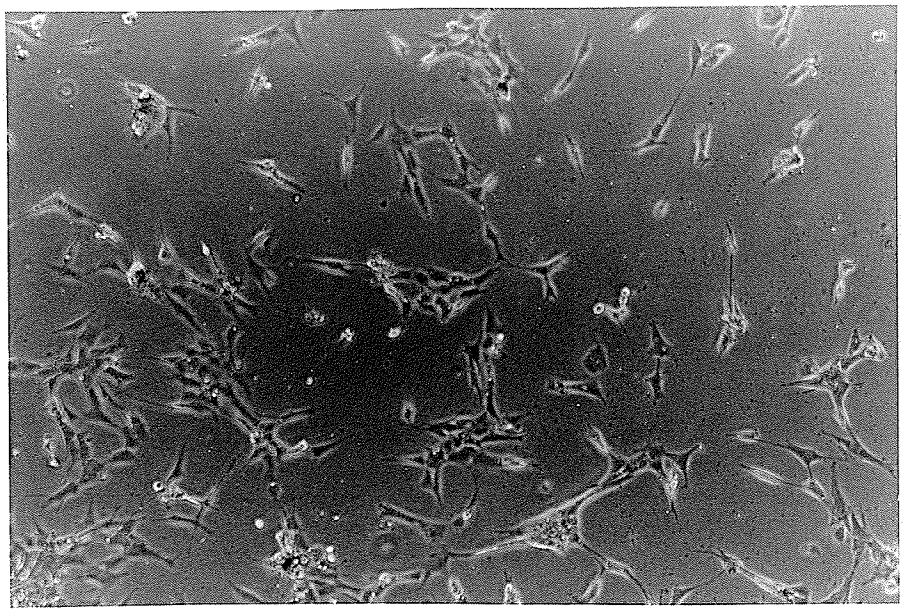

FIGURE V-3: Phase contrast micrograph of the outer third of a Flex I^R well containing attached fibroblast-like cells in nonconfluent subcultures just prior to mechanical force application. Note the multipodial appearance of these cells.

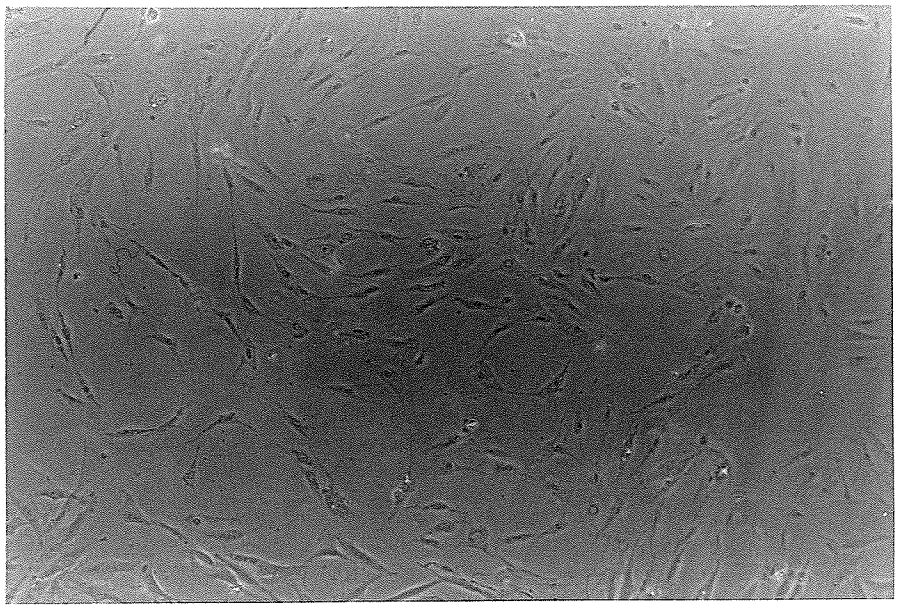

FIGURE V-4: Phase contrast micrograph of the outer third of a Flex I^R well containing attached fibroblast-like cells in nonconfluent subcultures 6 hours after the application of mechanical force. Note the change in size and the bipodial appearance of the majority of cells as well as the alignment of cells perpendicular to the strain application in contrast to the cells in figure V-3.

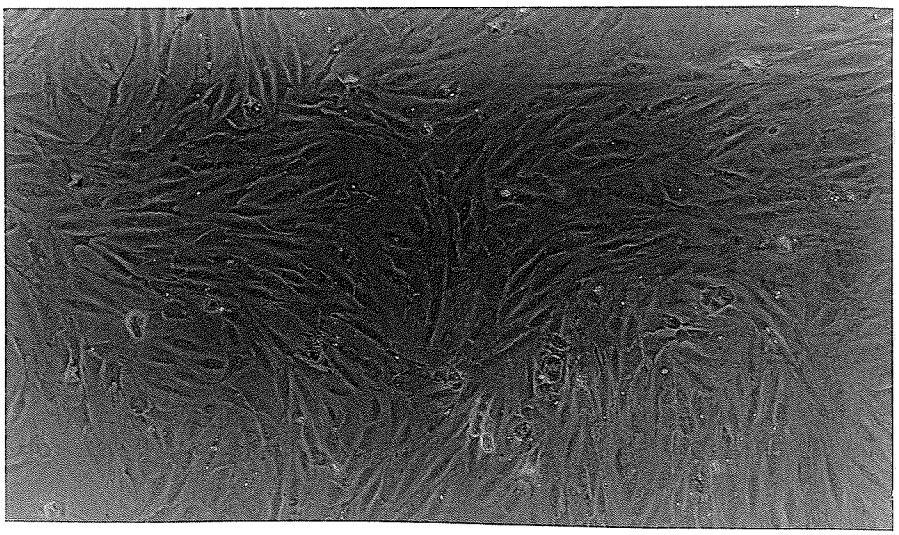

FIGURE V-5: Phase contrast micrograph of the outer third of a Flex I^R well containing attached fibroblast-like cells in nonconfluent subcultures 24 hours after the application of mechanical force. Note the large increase in cell number, the pattern of cellular alignment perpendicular to the force application, and the recovery of the cells' size while maintaining the bipodial appearance.

(B) Radioautographic Quantification

The mean values and standard deviations of the type III α-chains relative to the total of type I and type III α-chains under the experimental conditions are summarized in Table V-1. These results are graphically represented in Figure V-6. As previously noted, these collagen phenotype ratios have been calculated from the raw data obtained through densitometric scanning of fluorograms processed from the cell layer of the tissue cultures (Appendices B, D and T).

Statistical analysis of the mean ratios confirmed a significant interaction existed between the experiment and time variables (Table V-2). (Individual statistical results for the variables of experiment and time are shown in Appendix S.) These findings confirm a significant increase in the proportion of newly-synthesized type III collagen in the primary cultures grown in a dynamic environment compared to the control values throughout all of the experimental period. Following an initial period of no change, the confluent cultures demonstrated a significant rise in these percentages which leveled off at extended periods. In contrast to this, early increases were found in the nonconfluent cultures, however the significance of these findings dissipated after four hours returning again to a significantly high level at the 24 hour experimental period.

TABLE V-1: Table of the mean values and standard deviations of type III percentage of the combined type I and III collagen phenotypes from the cell layer of statically grown confluent and nonconfluent subcultures and dynamically grown primary cell cultures to which an intermittent force regimen of 30 cph at a -20 kPascals force magnitude has been applied, as well as nonstressed controls, for 2, 4, 6, 8 and 24 hour experimental periods.

TABLE V-2: Analysis of variance of experiment vs time for the dynamically grown primary cultures, nonconfluent and confluent statically grown subcultures, and the control cultures.

FIGURE V-6: Graphical representation of the mean percentages of type III to the combined type I and III collagen phenotypes from the cell layer of the tissue cultures exposed to a cyclical force regimen of loading and relaxation of 30 cycles per hour at a maximal elongation of 24% (-20 kPascals) over a 24 hour experimental period. The legends represent the experiments of dynamically grown explants, nonconfluent and confluent statically grown subcultures, and nonstressed subcultured controls.

TABLE V-1

EFFECT OF MECHANICAL FORCE, CELL PASSAGE, AND CONFLUENCY ON COLLAGEN PHENOTYPE SYNTHESIS IN GINGIVAL TISSUES IN VITRO

TYPE III / TYPE I + TYPE III

Regimen: 30 CPH at -20 kpascals

	2 HOURS		4 HOURS		6 HOURS		8 HOURS		24 HOURS	
EXPERIM	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD	MEAN	STD
EXPLANT	36.00	16.00	24.00	9.00	22.00	1.00	38.00	6.00	25.00	10.00
CON SUB	8.33	2.31	16.67	7.57	20.33	2.08	21.33	6.66	6.67	6.51
NON SUB	19.00	5.00	19.00	9.00	20.00	1.00	15.00	3.00	24.00	3.00
Control	6.00	5.20	8.33	1.15	11.33	3.51	9.67	3.79	4.33	4.51

TABLE V-2

Analysis of Variance of Mean Values
Effect of Force and Passage on Gingival Collagen Synthesis
Type III / Type I + Type III Percentage
Regimen: 30 CPH at -20 kPascals

EXPERIMENT vs TIME

EXPERIMENT	EXPLANT	PASS 1 NON	PASS 1 CON	CONTROL
2 HOURS				
EXPLANT		0.0346*	0.0023**	0.0001**
PASS 1 NON	0.0346*		0.2945	0.0166*
PASS 1 CON	0.0023**	0.2945		0.1584
CONTROL	0.0001**	0.0166*	0.1584	
4 HOURS				
EXPLANT		0.3869	0.2945	0.0045**
PASS 1 NON	0.3869		0.0598	0.0399*
PASS 1 CON	0.2945	0.0598		0.0002**
CONTROL	0.0045**	0.0399*	0.0002**	
6 HOURS				
EXPLANT		0.7097	0.2421	0.0524*
PASS 1 NON	0.7097		0.1261	0.1121
PASS 1 CON	0.2421	0.1261		0.0028**
CONTROL	0.0524*	0.1121	0.0028**	
8 HOURS				
EXPLANT		0.0001**	0.0524*	0.0001**
PASS 1 NON	0.0001**		0.0225*	0.3542
PASS 1 CON	0.0524*	0.0225*		0.0020**
CONTROL	0.0001**	0.3542	0.0020**	
24 HOURS				
EXPLANT		0.9012	0.0775	0.0122**
PASS 1 NON	0.9012		0.0994	0.0166**
PASS 1 CON	0.0775	0.0994		0.4214
CONTROL	0.0122**	0.0166**	0.4214	

p = 0.0164
Standard Error = 3.77
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

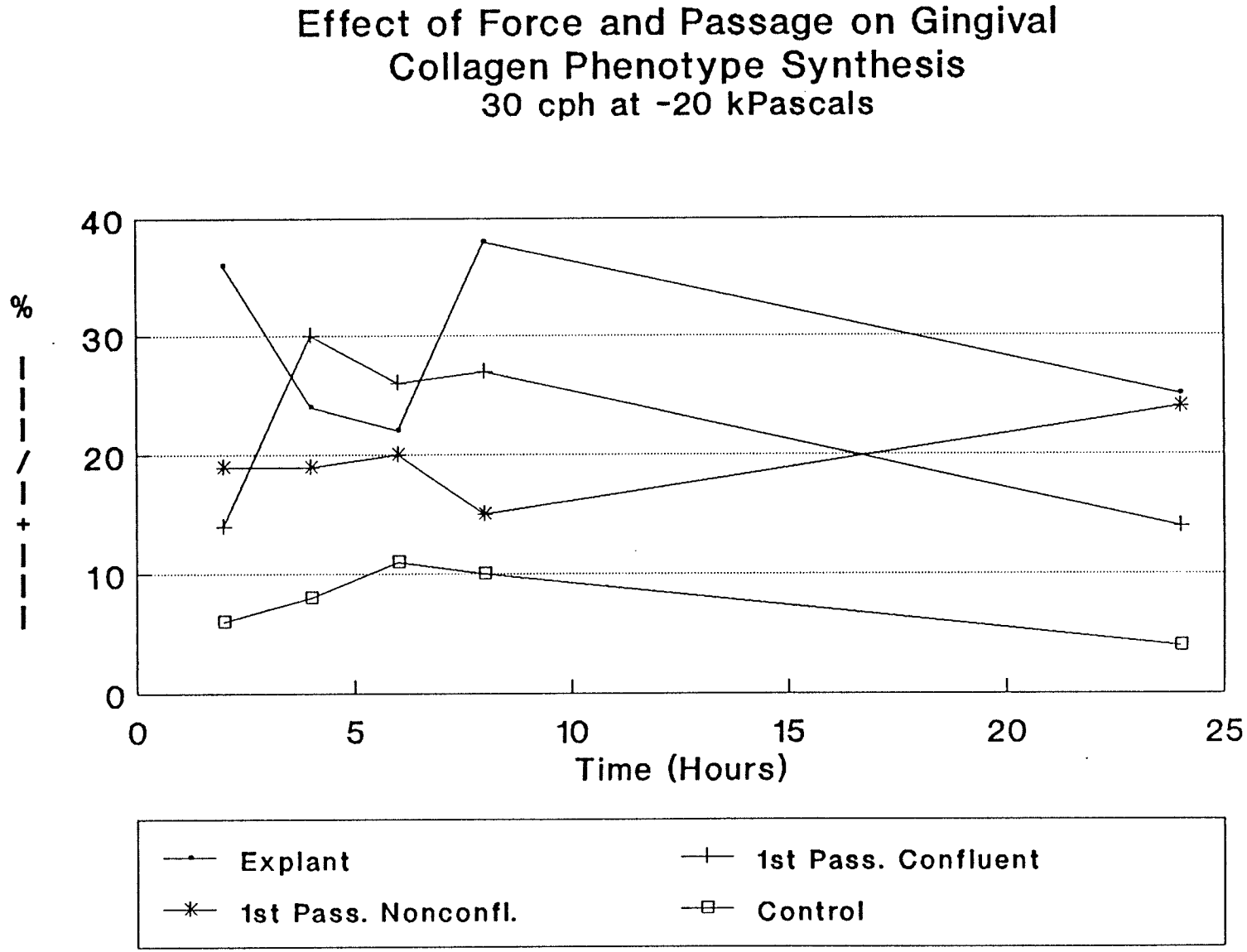
Effect of Force and Passage on Gingival
Collagen Phenotype Synthesis
30 cph at -20 kPascals
%
40
30
20
10
0
0
5
10
15
20
25
Time (Hours)
Explant
1st Pass. Confluent
1st Pass. Nonconfl.
Control

FIGURE V-6

DISCUSSION

The changes observed through microscopic observations suggest an initial period of morphologic adjustment within both the explant and nonconfluent cultures exposed to mechanical stress. These cell cultures were observed to regain the morphologic appearance of gingival tissues grown in a static environment coincident to enhanced replication rates leading to confluency. These findings suggest physical and functional alterations within the tissue culture. However, these observations do not show any alteration in cellular morphology with subculture until confluency is reached. This apparent difference may be the result of contact guidance, contact inhibition, and/or cell to cell metabolic interactions. Differences of this nature have been shown to exist between gingival cells *in vitro* and *in vivo* (Ten Cate, 1972; Engel *et al.*, 1980), however quantitative studies into DNA synthesis and cell numbers as well as electron microscopic observations into the cell microstructure are necessary to support these observations.

The knowledge that a subpopulation of heterogeneous fibroblasts are present within the gingival tissues leads to the conclusion that the possibility of a shift in cell populations may occur as a result of the stresses. However, it could be that the different subpopulations of cells residing within the gingival tissues executing specialized functions perceive this

mechanical stimulus within the context of their differentiated roles.

The significance of heightened type III collagen as a result of mechanical force in all of the tissue cultures quantitatively supports theories that mechanical strain of the silastic membrane is indeed perceived as stress by the attached gingival cells. The findings also suggest that the homogeneous morphologic appearance of these cultures at confluency is misleading. There may a be functionally different heterogeneity within the culture population. These shifts could also be a function of maturational or differentiative states of the cells populations as a result of the mechanical stresses.

The lack of a lag period prior to the maintenance of significantly high levels of type III collagen percentages within explanted cultures grown dynamically may be misleading as a period of morphologic alterations was noted early in the cell culture prior to confluency and the autoradiographic investigation. Hence, the consistently high proportions actually reflect the capacity of these cultures for long term function at significantly enhanced levels of this phenotype. Furthermore, the period of retarded type III proportion prior to a significant return to increased levels in the nonconfluent cultures was coincident with the morphologic alterations seen microscopically. This insinuates that a significantly higher level may also be maintained long term in this environment, even after confluency is reached.

The differential in responses to the intermittent force expressed as contrasting phenotype percentages between the explant and nonconfluent subcultures in comparison to the confluent subcultures may be due to the selection of specific cell populations or alterations in the metabolic functions of cells within the cultures prior to confluency. . This ability to shift subpopulations of cells or permanently alter the existing cell function may be lost on confluency due to intercellular interaction. The conclusions drawn from this investigation give no support for changes resulting from a single cell passage as long as the tissue is grown to confluency under an environment of intermittent stress.

Although differences in cell population and function resulting from an in vitro environment are known to exist and have not been accounted for here, this investigation does shows evidence that observations made from in vitro investigations using tissue cultures grown in the "classic" static techniques may be misleading for tissues such as gingiva which normally grow under dynamic conditions in vivo. Thus, a better understanding into the remodeling process within the periodontal support system may be gained through the application of a dynamic environment to in vitro investigations into the synthesis of collagen and other biological functions of cell populations.

CONCLUSIONS

1. Evidence has been presented supporting hypotheses that tissue cultures from rat palatal gingiva, both primary and first passage, demonstrate adaptive responses to the application of intermittent mechanical stress by increasing their rate of cell proliferation, altering their morphology, and aligning themselves perpendicular to the force application.

2. Gingival cells grown in a dynamic environment of intermittent stress *in vitro* will adapt to by producing significantly heightened percentages of type III collagen.

3. Significant differences in the collagen phenotype expression have been shown to exist between confluent cultures grown dynamically and confluent subcultures grown statically after the application of intermittent stress in that the confluent subcultures eventually adapt to baseline levels of type III percentages with time.

4. Statically grown nonconfluent subcultures respond differently to intermittent stress application than confluent subcultures by gaining a significantly higher level of type III collagen proportion with the initial

stimulation and maintaining this high proportion once achieving confluency at the extended investigatory period.

CHAPTER VI

PILOT STUDIES

PILOT STUDY #1

THE EFFECT OF FORCE AND PASSAGE ON SUTURAL COLLAGEN SYNTHESIS

INTRODUCTION

An integral part of clinical orthodontics and dentofacial orthopedics is the mechanical manipulation of fibrous joints, both sutural and periodontal, of the craniofacial complex through remodeling of hard and soft tissues. The main constituent of these fibrous joints is collagen. Its synthesis and secretion by fibroblasts is a highly controlled process under constant influence of extracellular conditions including mechanical manipulation (Meikle et al., 1982; Yen et al., 1989a, 1989b; Duncan et al., 1984). Collagen, however, is not a homogeneous protein and the distribution and proportions of the phenotypes within tissues are necessary for normal function (Bornstein and Sage, 1980). Thus, changes in collagen phenotype proportion, especially between type I and type III, may serve as an important parameter indicating the metabolic state of the connective tissue as well as a signal of changes in its cellular activity (Chung and Miller, 1974; Epstein, 1974; Butler et al., 1975).

Changes in collagen phenotypes synthesis indicating an increase in type III percentage as a result of mechanical stress in the interparietal suture have been demonstrated (Yen et al., 1980, 1989a, 1989b; Meikle et al., 1982). However, no investigations into the effect of intermittent force application

or cell passage *in vitro* on sutural tissues have been performed. The purpose of this investigation is to determine and compare the changes in the type III ratio of newly synthesized collagens in rat sutural mixed cell populations cultured *in vitro* in a dynamic environment to first passage cells cultured in a static environment to which a force has been applied at 2, 4, 6, 8, and 24 hour time periods. The comparison of these findings to each other and to control first passage cells never exposed to mechanical manipulation may aid in further understanding the effects of mechanical forces and cell passage on tissue culture.

MATERIALS AND METHODS

(A) Cell Cultures

Interparietal sutures from Sprague Dawley male white rats 3 weeks of age were removed by surgical excision immediately following sacrifice by cervical dislocation. The tissue harvested was immediately placed into α-MEM containing 400 units/ml penicillin G, 0.56 mg/ml streptomycin sulfate, 0.2 mg/ml ascorbic acid, 1 ml/L gentamycin reagent, and 2.2 mg/ml sodium bicarbonate. The tissues were scrapped clean of blood and

periostium and rinsed twice in fresh medium. All procedures were conducted in the laminar flowhood excepting the dissection.

The sutures were cut into 4 to 6 pieces approximately $2mm^2$. Explants were randomly separated and treated under the following conditions: Group A (1st Passage) tissues were placed into 60 mm culture dishes pre-warmed with 100% FBS (excess decanted off). Group B (Explant) tissues were placed into collagen coated Flex I^R culture plates pre-warmed with 100% FBS (excess decanted off). Both groups were incubated at 37^o C for 1 hour with 5% CO^2 in a humidified atmosphere to allow the explants to adhere to the dish. 5 ml per dish and 1.5 ml per well, for Group A and B respectively, of growth medium (as previously described) plus 10% FBS and 0.1 ml/L fungizone was then added and incubation continued for 24 hours. At 24 hours, the growth medium was changed in both groups.

Group A received incubation with medium changes every 2 days until confluency was reached at day 6. When confluent, the medium was removed, the cells washed three times with cold Hanks BSS and trypsinized as described in Chapter IV. 4×10^4 cells/ml were seeded into each of the 6 wells of collagen coated Flex I^R dishes. The flexcell dishes were placed into the incubator at 5% CO^2 at 37^o C for a 24 hour period. At this time confluency was confirmed under a phase contrast microscope.

Group B was placed into the baseplate gasket of the Flexercell Strain $Unit^R$ and a force regimen of 30 cph at -20 kPascals was placed on the culture dishes. The medium was

changed every two days for a period of 6 days. At this time, confluency was confirmed under the phase contrast microscope.

(B) Radioautography, Pepsin Extraction, and Electrophoresis

Following visualization under the phase contrast microscope, fresh growth medium with 10% FBS and 0.2 mg/ml ascorbic acid was added to each well of the Flex I^R plates in both groups. The dishes were placed into incubation on the flexcell baseplate and subjected to a 30 cph force regimen of -20 kPascals for periods of 2, 4, 6, 8 and 24 hours. 5 μCi/ml [^{14}C(u)]-glycine (Specific Activity 110.70 mCi/mmol) were added for the last two hours of each experiment. Mediums were pipetted off and the cells harvested in 2 mls of 0.5 HNAc followed by another 2 ml wash. The pooled samples were sonicated and frozen in -70^{o} C. The frozen samples were lyophilized and subjected to limited pepsin digestion, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and fluorographic quantification as described in Chapter IV. The values were subjected to statistical analysis using a one analysis of variance.

Control values were obtained from previously reported data for comparative purposes only. These cultures were from sutural explants of 3 week old male white Sprague-Dawley rats at first passage cultured on Petriperm culture dishes (Heraeus Inc., S. Plainfield, N.J.). Control cells received no loading and were processed and quantified in the same manner as the experimental groups.

RESULTS

No morphologic differences could be noted between the two tissue culture groups as both cultures appeared to consist of fibroblast-like cells due to their stellate shape. Much like the gingival cell lines investigated in Chapters IV and V, the cells changed from a multipodial to a bipodial appearance by the time confluency was reached. In contrast to the gingival cells, no phase of cell adjustment through changes in shape, size, or realignment could be seen microscopically although the dynamically growing cell line did appear to show an increased growth rate.

The mean values of the type III α-chains relative to the total of type I and type III α-chains under the experimental conditions are summarized in Table VI-1 and are graphically represented in Figure VI-1. These ratios have been calculated from the raw data obtained through densitometric scanning of fluorograms. (Refer to Appendix U for the raw data from the dynamically grown primary cultures and the statically grown subcultures. Raw data from controls is not listed as it was used for comparative purposes only.)

Statistical analysis of the mean ratios confirmed a significant interaction between the two experimental conditions at the 24 hour investigative period ($p = 0.0002$). No other significance could be demonstrated. However, comparison of these values to the rather consistently low control values lead

toward the conclusion that a difference may exist between the controls and the dynamically cultured explants throughout the entire experiment. As well, following an initial period of delay, differences may exist at the 6 hour period which return to control levels after 24 hours.

TABLE VI-1

THE EFFECT OF FORCE AND PASSAGE ON SUTURAL COLLAGEN SYNTHESIS
TYPE III / TYPE I + TYPE III
Regimen: 30 CPH at -20 kPascals

	2 HOURS		4 HOURS		6 HOURS	
EXPERMT	MEAN	STD	MEAN	STD	MEAN	STD
EXPLANT	35	10	30	5	29	4
SUBCULT	17	3	20	4	25	4
CONTROL	10	1	10	1	8	2

8 HOURS		24 HOURS	
MEAN	STD	MEAN	STD
27	3	42	10
21	5	21	2
9	2	10	1

TABLE VI-1: Table of the mean values and standard deviations of type III percentage of the combined type I and III collagen phenotypes from the cell layer of statically grown confluent subcultures and dynamically grown primary cell cultures controls to which an intermittent force regimen of 30 cph at -20 kPascals force magnitude has been applied, as well as nonstressed controls, for 2, 4, 6, 8, and 24 hour experimental periods.

FIGURE VI-1: Graphical representation of the mean percentages of type III to the combined I and III collagen phenotypes from the cell layer of dynamically grown primary cultures and confluent statically grown subcultures exposed to intermittent force over a 24 hour period. Also represented are ratios from cultures statically grown on petriperm culture dishes. (See the next page.)

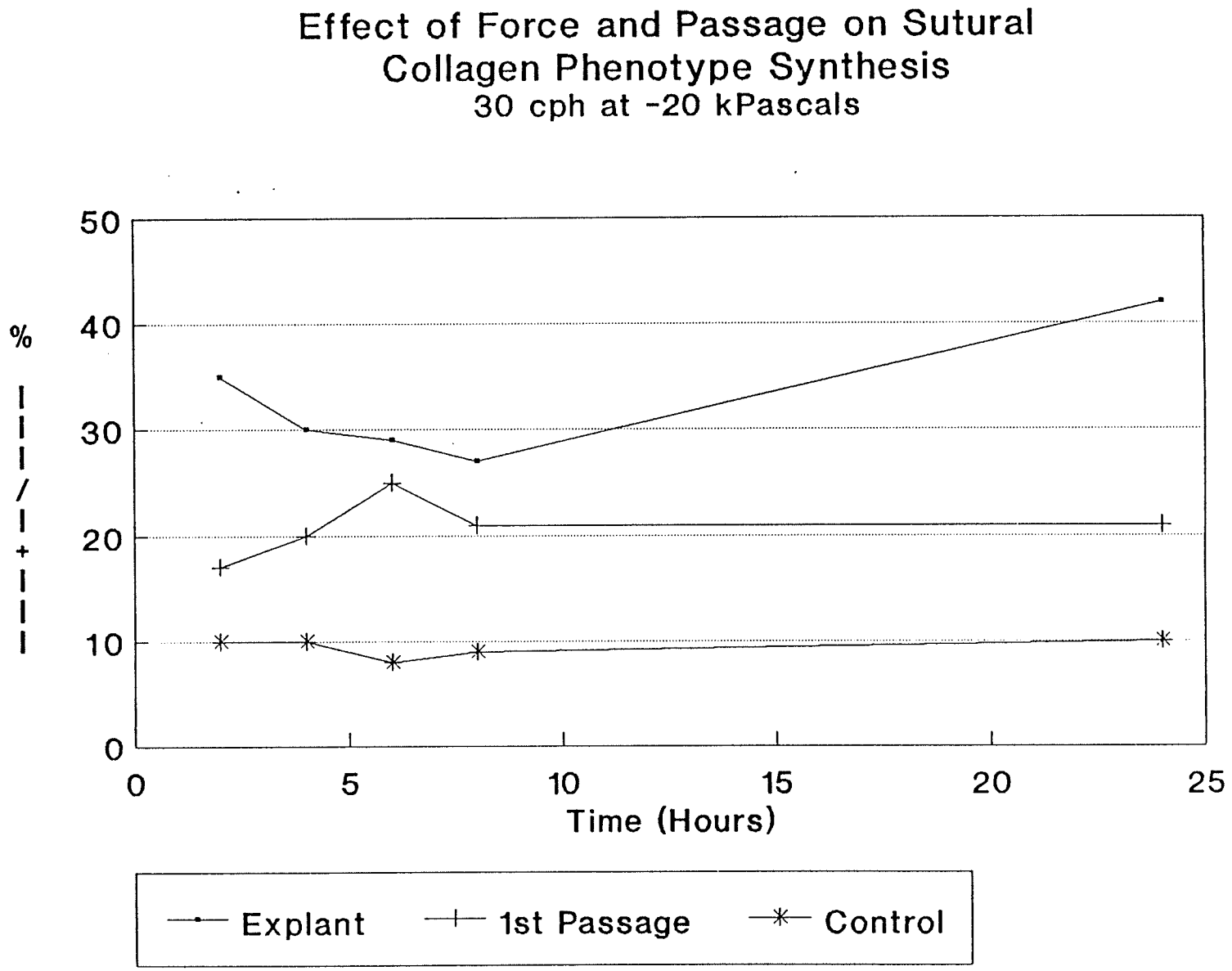
Effect of Force and Passage on Sutural
Collagen Phenotype Synthesis
30 cph at -20 kPascals
%
50
40
30
20
10
0
0
5
10
15
20
25
Time (Hours)
Explant
1st Passage
Control

FIGURE VI-1

The selection of this age of animal for the model was based of convenience of tissue extrapolation since these animals were being sacrificed primarily for the experiments in Chapters IV and V. It is interesting to note that at three weeks of age the rat is known to begin a period of considerable increase in body weight (Yen et al., 1989a). No significance has been shown between this increase in body weight and newly-synthesized type III collagen in the interparietal suture, but the period between the second and fourth week of life do account for about a third of the total post-natal bony growth of the rat interparietal suture (Massler and Schouri, 1951). This time has also been shown to be a period characterized by changes in growth rate, bony reorganization, and sutural structure (Moss, 1954; Young, 1962; De Angelis, 1968). All of these factors correspond with a transition from high to low percentages of type III collagen.

The possibilities of a period of delay followed by a high level of type III collagen proportion from which the tissue eventually recovery in the first passage cells along with the continually heightened proportion in the dynamically grown cell lines are very similar to the results in Chapters IV and V. These tissues are similar in that collagen is their predominant structural protein and they both have a great ability to remodel. Even the general proportions at differing time periods and experimental condition appear found in the studies presented in

this reference appear very comparable. As a result, these findings come as no surprise. Hencemore, many of the same conclusions can be drawn from this investigation.

Sutural tissues do appear to demonstrate adaptive responses to mechanical stresses in vitro by increase their rate of cell division and changing their collagen phenotype distributions. It may also be possible to select for functionally different subpopulations within the tissue culture or cause the functioning cells to alter their metabolic activities with this stress. However, the same findings may hold true that cells allowed to reach confluency prior to the application of an intermittent force will return to baseline levels after extended applications. For these reasons, future in vitro investigations into fibrous joint remodeling and collagen synthesis may obtain results more closely resembling the in vivo situation if the cultures are grown under the application of mechanical force.

PILOT STUDY #2

THE EFFECT OF FORCE ON PERIODONTAL LIGAMENT CELL COLLAGEN PHENOTYPE SYNTHESIS

INTRODUCTION

The connective tissues of the periodontium are composed of collagens, noncollagenous glycoproteins, glycosaminoglycans and oxytalan. The collagens make up 60% of the periodontal ligament and gingival volume. In the periodontal ligament 99% of this collagen is phenotypically type I and type III. (Narayanan and Page, 1983). (See Chapter II - Literature Review for a more detailed description.)

Investigations have revealed that the turnover of collagen within the periodontal ligament occurs at a very high rate compared to other tissues, even gingiva (Carneiro and Fava de Moraes, 1965; Skougaard et al., 1970a, 1970b; Sodek, 1976, 1978; Rippen, 1978). Studies have also revealed a constant turnover in the undisturbed dentition (Beertsen et al., 1978). The activity of this collagen synthesis has been shown to occur at varying times (Miura et al., 1970) and at varying rates (Caneiro, 1965) within different locations of the periodontal ligament in the rat molar.

Changes in extracellular volume density of the collagens within the periodontal ligament have been demonstrated to decrease with the removal of physiologic function (Beertsen,

1987) accompanied with degenerative changes (Cohn, 1965) and supraeruption (Konoza et al., 1980). Furthermore, increases in collagen synthesis and cell replication can be stimulated through remodeling within the periodontal ligament as a result of orthodontic force applications (Stallard, 1963; Crumely, 1964; Baumrind and Buck, 1970; Diaz, 1978; Reitan, 1985).

Cranial sutures and the periodontal ligament are both fibrous joints which are known to remodel with force application. Changes in the phenotypic proportions toward a higher type III ratio to the combined type I and type III collagens have been associated with periods of rapid remodeling in sutures of a variety of animal models (Yen et al., 1980, 1989a, 1989b; Meikle et al., 1982).

The practice of orthodontics and dentofacial orthopedics depends on the ability to mechanically manipulate the remodeling processes within these fibrous joints. both sutural and periodontal, through the controlled application of force. It can be concluded from this knowledge that alterations in these collagen proportion can be used as signal of metabolic adjustments resulting from force applications in vitro. The aim or this investigation is to provide some insight into the effect of intermittent and continuous force applications on cells from different locations within the periodontal ligament in culture.

MATERIALS AND METHODS

(A) Cell Cultures

Bicuspids extracted in the course of orthodontic treatment were obtained immediately post-extraction. The teeth were immediately placed into α-MEM containing 400 μ/ml penicillin G, 0.56 mg/ml streptomycin sulfate, 0.2 mg/ml ascorbic acid, 1 ml/L gentamycin reagent, and 2.2 mg/ml sodium bicarbonate. The tissues were rinsed clean of blood with fresh medium. The gingival attachment was removed using a sharp scalpel and the teeth were rinsed twice more in fresh medium. All procedures were conducted in the laminar flowhood following the extraction.

The root surface was then scrapped with a sharp scalpel by vertical thirds (gingival, middle, and apical) into 35 mm polystyrene culture dishes pre-warmed with 100% FBS (excess decanted off). The dishes were incubated at 37^{o} C for 1 hour with 5% CO^{2} in a humidified atmosphere to allow for adherence of the explants. 2 ml per dish of growth medium (as previously described) plus 10% FBS and 0.1 ml/L fungizone was then added and incubation continued for 24 hours. At 24 hours, the growth medium was changed.

The tissue cultures received incubation with medium changes every 2 days until confluency was reached at day fourteen. When confluent, the medium was removed, the cells washed three times with cold Hanks BSS and trypsinized as described in Chapter IV. 4×10^{4} cells/ml were seeded into each of the 6 wells of collagen

coated Flex I^R dishes. The flexcell dishes were placed into the incubator at 5% CO^2 at 37° C for a 24 hour period. The medium was changed at this time and every two days there after until confluency was confirmed under a phase contrast microscope.

(B) Radioautography, Pepsin Extraction, and Electrophoresis

Following visualization under the phase contrast microscope, fresh growth medium with 10% FBS and 0.2 mg/ml ascorbic acid was added to each well of the Flex I^R plates. The dishes were placed into incubation on the flexcell baseplate at -20 kPascals of 30 cph, 180 cph or continuous force regimens for 2, 4 and 8 hour time periods. 5 μCi/ml [^{14}C(u)]-glycine (Specific Activity 110.70 mCi/mmol) were added for the last two hours of each experiment. Mediums were pipetted off and the cells harvested in 2 mls of 0.5 HNAc followed by another 2 ml wash. The pooled samples were sonicated and frozen in -70° C. The frozen samples were lyophilized and subjected to limited pepsin digestion, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and fluorographic quantification as described in Chapter IV.

OBSERVATIONS AND CONCLUSIONS

Observations made through the phase contrast microscope showed fibroblast-like cells with the expected stellate appearance in both the explant and first passage cultures (Refer to Figures VI-2 and VI-3). Both cultures appeared morphologically the same. Much like the gingival and sutural cells, the cells in these cultures did seem to change from multipodial to bipodial extensions as cell to cell contact increased. However the periodontal ligament cells appeared thinner and with longer extensions, more morphologically comparable to the sutural cells. This may be expected if the natural environment affects the morphology of the cells in culture since both tissue lines are derived from fibrous joints. No observations of these cells was preformed during or after force application. This is with regret as this investigator found these cells to be very temperamental to <u>in vitro</u> techniques resulting in only a partial study due to time restraints.

The raw values, averages, and standard deviations of the type III collagen ratios obtained in these investigations are presented in Appendix V. Bar graphs reflecting the averages are shown in Figures VI-4, VI-5, and VI-6. It should be noted that in many of these cases a less than significant number of samples is represented. Thus, any conclusion made from these findings should be guarded.

The proportions at 2 hours of intermittent force show the

an increase in the level of type III collagen to exist in the middle third of the periodontal ligament while the gingival and cervical thirds are at control levels. This comes as a surprise since normal physiology resulting in a greater amount of pressure and tension to the proximal and distal ends of the periodontal ligament would tend toward selection of cells in these areas which are more adaptive to a dynamic environment. Even high rates of synthesis and turnover of collagen are known to exist in the cervical and apical thirds (Carneiro, 1965). The lack of a period of adaptation in the middle third also is in contrast to findings in the gingival and sutural tissues.

The 4 hour values at intermittent forces show a drop in all three root thirds with the middle third still maintaining the greatest ratio although all three results are now below the 2 hour control value.

The experiments at 8 hours are of both continuous and intermittent force applications. They reveal slightly higher differences in type III proportion with intermittent forces. Furthermore a much lower percentage of the collagen is again found in the apical third in comparison the middle third even when exposed to a continuous force application. If it is true that a greater response to force application is present in the middle third of the periodontal ligament, then the values represented between the intermittent and continuous force application may be even greater.

The periodontal ligament appears to contain cells that can

differentiate into a number of functioning forms including fibroblasts, osteoblasts, cementoblasts (Melcher, 1976). Should this be a reflection of a heterogeneous subpopulation within the periodontal ligament, the general responses found in the middle third may reflect a selected population of cells in vivo which has been explanted into the experimental environment.

The main conclusion of these investigations is that a significant difference may exist in the periodontal ligament's remodeling response with intermittent force, but not with continuous force application. As well, differences may exist in this ability between vertical thirds of the periodontal ligament. Future investigations of significant numbers, longer time periods, and force application during cell growth and passage are still required to show comparisons between the periodontal ligament and other tissues. If these results are as similar as they appear, then general comparisons can be made leading to a greater understanding of the periodontal tissues' remodelling abilities.

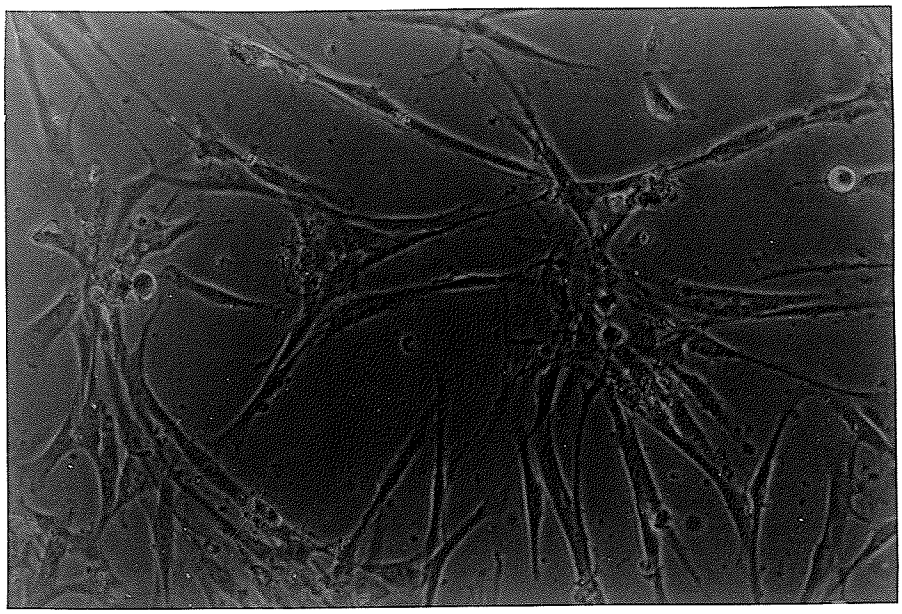

FIGURE VI-2: Phase contrast micrograph of an individual fibroblast-like cell cultured from the initial explant. Note the stellate appearance and similarities to the gingival cells in Figure IV-2.

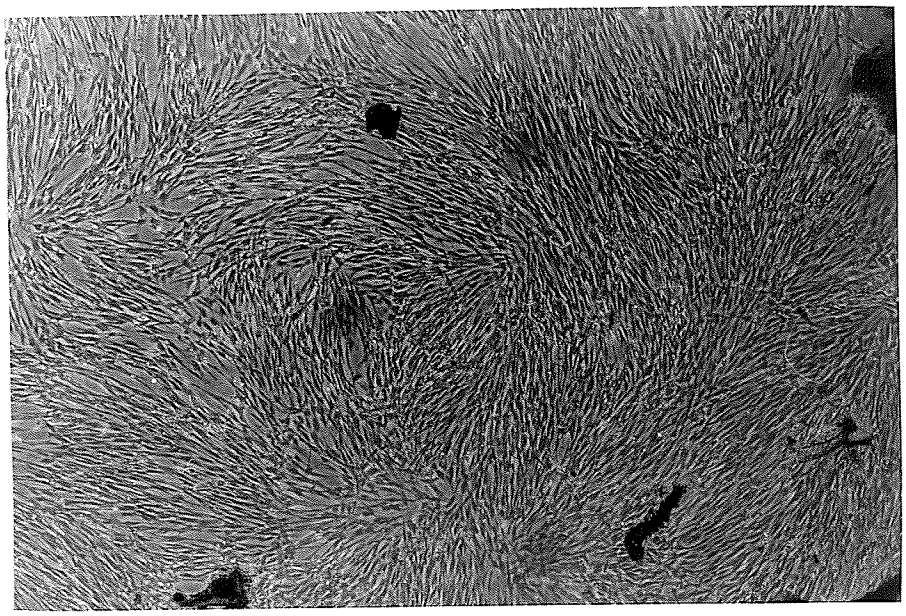

FIGURE VI-3: Phase contrast micrograph of fibroblast-like cells at confluency prior to subculture. Note the random orientation and bipodial appearance as well as the thinness of the cells compared to the culture in Figure IV-3.

FIGURE VI-4: Bar graph representation of the percentages of type III to the combined type I and III collagen phenotypes at two hours for nonstressed controls and cultures exposed to an intermittent force regimen of 180 cph at -20 kPascals.

FIGURE VI-5: Bar graph representation of the percentages of type III to the combined type I and III collagen phenotypes at four hours for cultures exposed to an intermittent force regimen of 180 cph at -20 kPascals.

FIGURE VI-6: Bar graph representation of the percentages of type III to the combined type I and III collagen phenotypes at eight hours for cultures exposed to intermittent 180 cph and continuous force regimens at -20 kPascals.

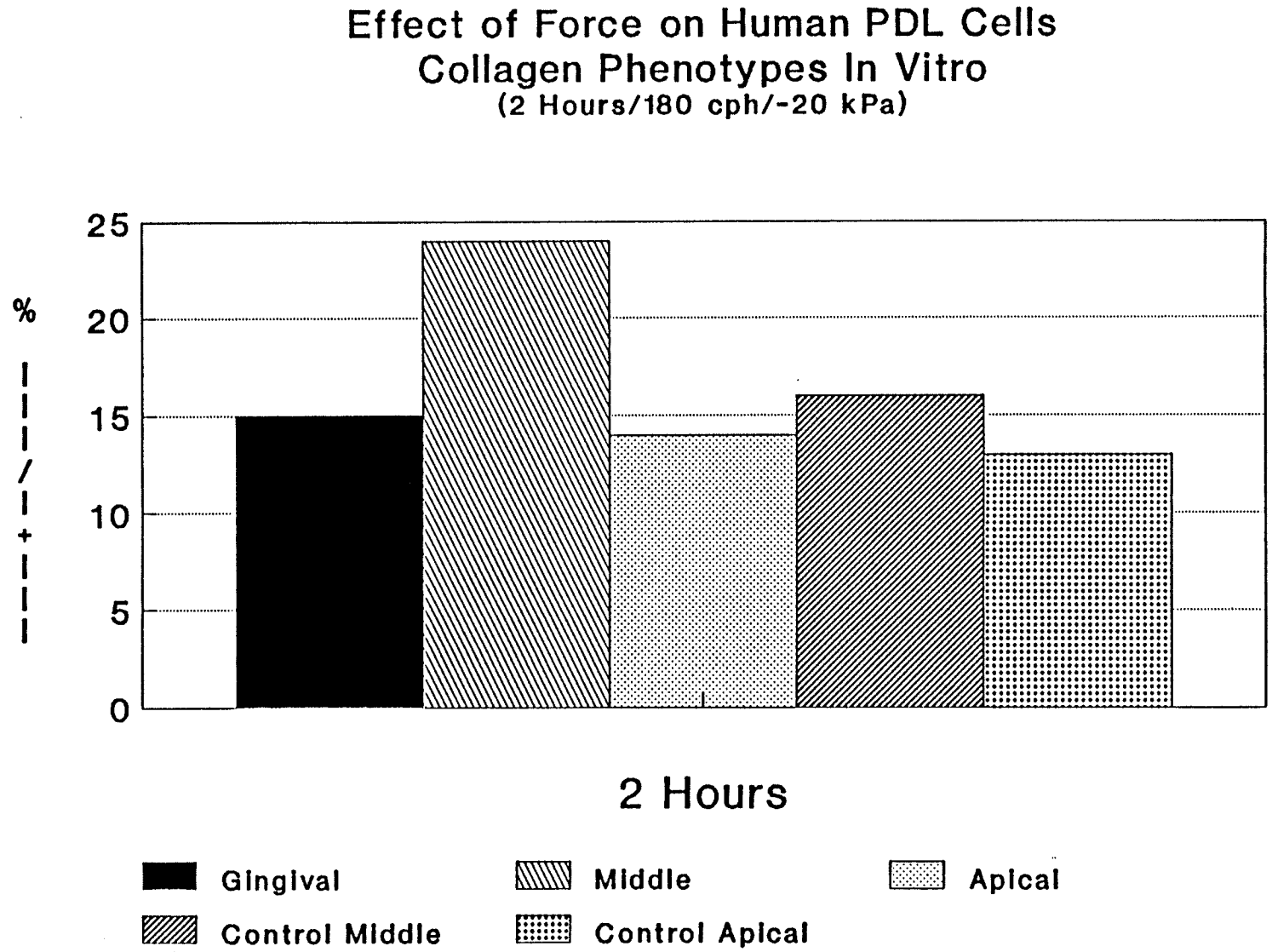
Effect of Force on Human PDL Cells
Collagen Phenotypes In Vitro
(2 Hours/180 cph/-20 kPa)
%
25
20
15
10
5
0
2 Hours
Gingival
Middle
Apical
Control Middle
Control Apical

FIGURE VI-4

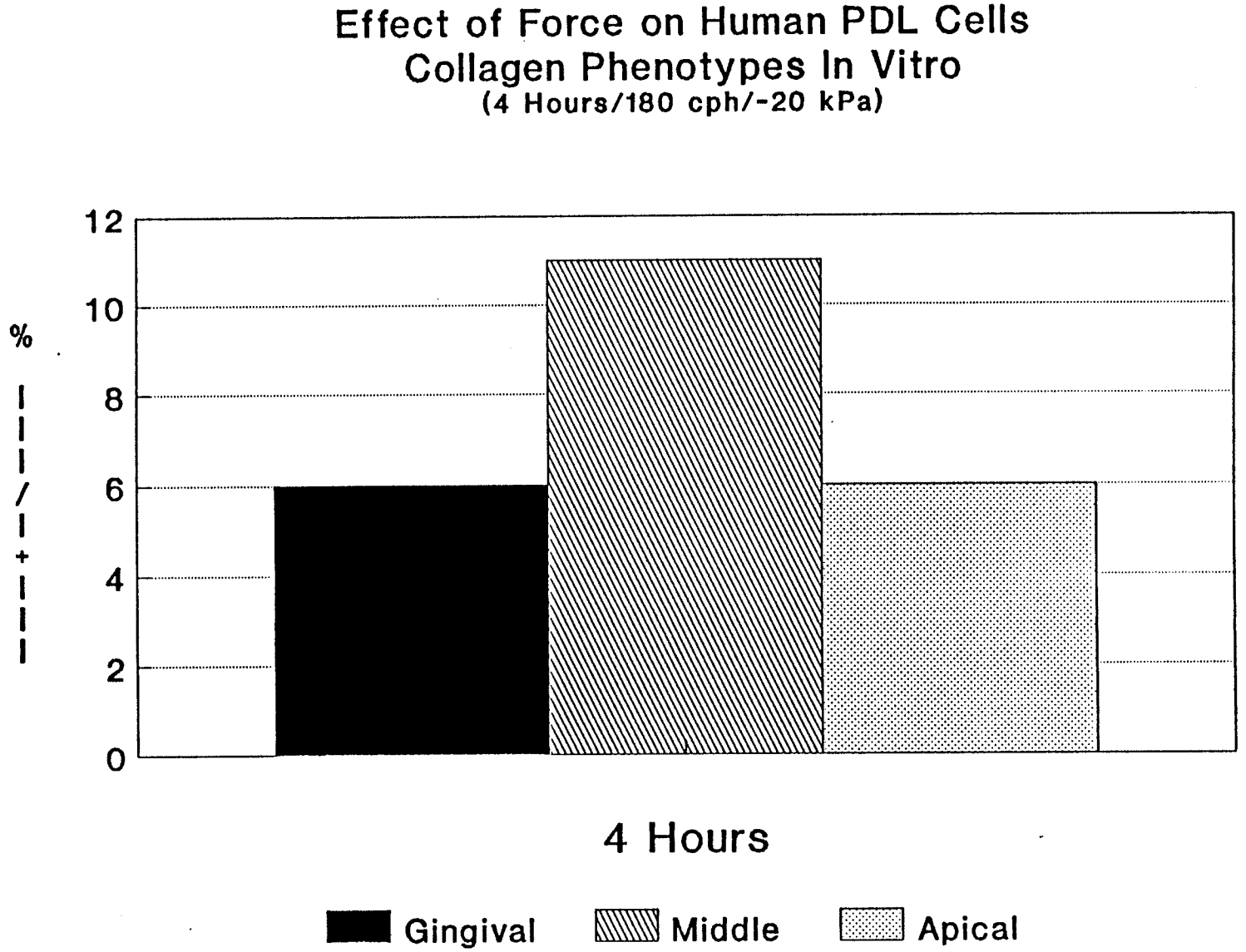
Effect of Force on Human PDL Cells
Collagen Phenotypes In Vitro
(4 Hours/180 cph/-20 kPa)
12
10
8
6
4
2
0
%
4 Hours
Gingival
Middle
Apical

FIGURE VI-5

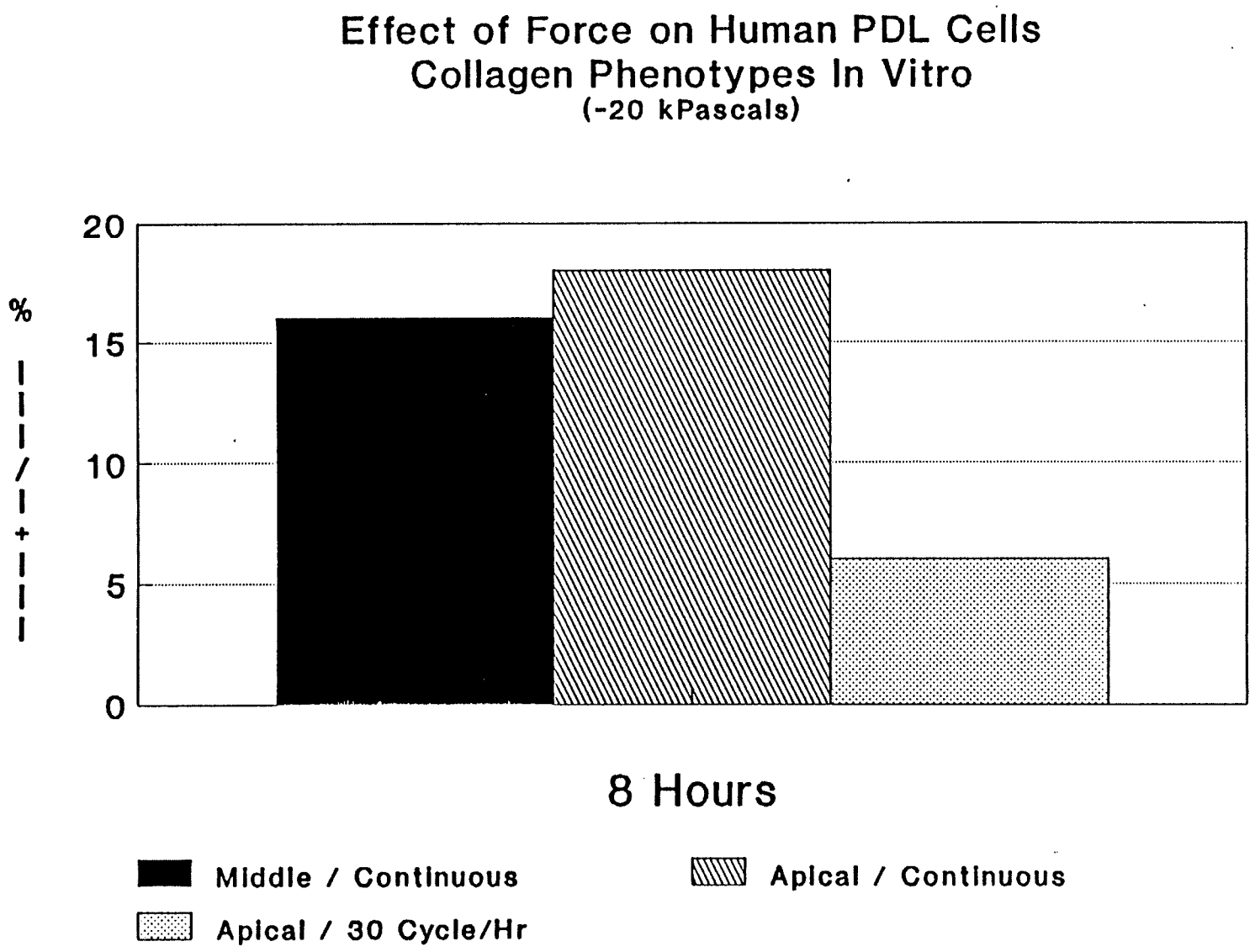
Effect of Force on Human PDL Cells
Collagen Phenotypes In Vitro
(-20 kPascals)
%
20
15
10
5
0
8 Hours
Middle / Continuous
Apical / Continuous
Apical / 30 Cycle/Hr

FIGURE VI-6

CHAPTER VII

SUMMARY AND FUTURE CONSIDERATIONS

SUMMARY AND FUTURE CONSIDERATIONS

These series of investigations have shown that tissue cultures from rat palatal gingival grown and subcultured in the "classical" static *in vitro* environment respond to the application of mechanical force of various magnitudes by altering the ratio of type III collagen to the total newly-synthesized type I and type III collagen.

Significant differences have been demonstrated between the application of continuous and intermittent forces in these cultures. From these studies, it appears that minimal changes in collagen phenotype biosynthesis occur as a result of continuous force applications while a variety of intermittent force applications have a similar capacity to stimulate metabolic changes in this regard. The application of intermittent force regimens results in an initial period of delay followed by significant increases in type III collagen proportions which eventually returned to baseline levels after 24 hours.

Primary gingival cell cultures grown in the presence of this intermittent mechanical environment responded by maintaining significantly higher levels of type III proportions demonstrating the capacity for a dynamic environment to select for cells or alter the function cells. Similar findings were found in nonconfluent subcultures which were exposed to the intermittent force applications.

Based on morphologic observations made through the phase

contrast microscope it appears that cells, both of primary cultures and subcultures, growing in this environment react with an early period of change in shape, size, and alignment which have been shown to relate well with the delayed response found in type III collagen ratio. A period of increased growth leading to confluency and the return of "normal" morphologic appearance was also observed following this delay which was related to the increases found in type III proportions. Future studies into cAMP, DNA, and cell counts remain to be done to confirm this increase in cell number and replicative abilities.

These experiments have also shown that once confluency was reached the expression of collagen phenotypes within the cultures is determined. This leads to the assumption that cell to cell interactions must play an important role in the gingival tissues culture's ability to alter the cell population's dynamics.

Whether these changes are the result of cell selection among the subpopulations of cells or adaptive alterations in cellular function still remains to be shown. It may be possible to not only select from among the existing cells, but to determine the maturational state of progenitor cells through their mechanical environment. This too remains to be confirmed.

It was shown that cells grown in the presence of an intermittent force at a magnitude of 24 percent elongation will alter there alignment perpendicular to the force application. However, this was found only in the outer third of the well. It is known that this is the area of the well which receives the

greatest amount of distortion as a result of force application. Thus, future investigations at even greater force magnitudes should be performed to confirm that the similarities found between the force systems tested is truly an equivalent response and not the result of only a limited number of cells being significantly altered.

Similar responses to intermittent force applications have been suggested to occur in vitro in fibrous joints, both sutural and periodontal, although differences are thought to occur within vertical thirds of the ligament. Because the different tissues within the periodontal support system are known to remodel at differing rates, more detailed studies into these tissues' abilities to alter collagen phenotypes and other metabolic products as a result of varying force frequencies, durations, and magnitudes will provide further information needed to compare and contrast these tissues.

It is the objective of in vitro investigations to simulate a natural environment as closely as possible in order for the findings to be applicable to the in vivo condition. It appears from these findings that the tissues of the periodontal support system, which are known to exist in vivo in a physiologically dynamic environment react differently when grown statically and dynamically in vitro. Thus, it is conceivable that a static environment is suboptimal for culturing these cells and may even result in misleading conclusions.

The application of these in vitro findings requires further

support through long term studies and *in vivo* applications. However, a greater understanding of how the gingiva and other periodontal tissues react to mechanical force will lead to the establishment of an optimal force system which will stimulate predictable and efficient tissue remodelling resulting in controlled noniatrogenic stable tooth movements as well as control of the craniofacial complex.

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APPENDICES

APPENDIX A

PERCENT ELONGATION CONFIRMATION OF FLEX I^{R} SILASTIC MEMBRANES

PERCENT ELONGATION CONFIRMATION OF FLEX I^{R} SILASTIC MEMBRANES

INTRODUCTION

Previous studies using the Flexercell Strain Unit^{R} have demonstrated a linear response between the amount of negative pressure transmitted through the vacuum and the percentage of elongation of the Flex I^{R} well bottoms (Banes, et al., 1990). This distortion of the silicone rubber (silastic) well bottoms has been calculated through finite element analysis, an empirical formula and a mathematical model. (Refer to Chapter II - The Flexercell Strain Unit^{R} in this publication for a more detailed description.)

The purpose of this investigation is to confirm the percent elongation of the silastic membranes at -10 and -20 kPascals of pressure at both the initiation and completion of a 24 hour time period experiment.

MATERIALS AND METHODS

Flex I^R culture plates for each level of vacuum pressure and time period were removed from packaging as shipped by the Flexcell Corporation. The diameter of each well bottom was embossed with a pair of perpendicular axes by an indelible marker (Staedtler Lumocolor 313, Nurnberg, West Germany).

The plates were then placed onto the baseplate gasket and inserted into the incubator under the normal cell culture environment. The appropriate force application of 0, -10, or -20 kPascals was then applied to the plates through the Flexercell Strain Unit^R for 2 and 24 hour experimental periods. During the last thirty minutes of the experimental time, the baseplates were removed so that a clear fast-setting acrylic could be poured into the wells. Once filled, the baseplate was returned to the experimental conditions for the remainder of the time period. One plate was treated in the above described manner, but never introduced into the incubator. An additional plate was embossed only. The siliastic surfaces were removed and the measurements obtained were used as control values.

After experimentation, the acrylic plugs and silastic bottoms were separated from the Flex I^R culture plates with light pressure. Measurement of the axes of the silastic bottoms were then divided by their appropriate axes on the acrylic plug to obtain a percent elongation per vacuum level and time period.

RESULTS

Experimental values obtained at a normal room environment and at an incubated environment were identical. The control values were identical to those obtained at 0 kPascals (unloaded) under the experimental conditions.

Both the -10 kPa and the -20 kPa pressures demonstrated no measurable permanent increases in the distortion of the well bottoms throughout the time periods.

The percentage elongation across the diameter of the well's bottoms was consistent for all wells in and between both axes at a given force level.

The calculated values for the percent elongation of the silastic membranes were 0 percent at 0 kPascals, 10 percent at -10 kPascals, and 20 percent at -20 kPascals. The average value per well surface and the calculated percent elongation are represented in table A1.1.

Table. A-1: Average Percent Elongation Values

Experiment	Ave. Diameter Unloaded	Ave. Diameter Loaded	Percent Elongation
Control	23.0mm	23.0mm	0
0 kPascals (Room Temp.)	23.0mm	23.0mm	0
0 kPascals (Incubated)	23.0mm	23.0mm	0
-10 kPascals (Incubated)	23.0mm	25.25mm	9.7
-20 kPascals (Incubated)	23.0mm	28.5mm	23.8

Discussion and Conclusions

The results of this experiment confirm the hypothesis that the -10 kPascals and the -20 kPascals pressure applications result in different degrees of membrane elongation. Those percent elongations being 10 and 20 percent respectively. It is assumed that the alterations of the surface area are translated to the cells attached.

In addition it is apparent that the amount of heated generated by the acrylic setting or by incubation has no measurable effect on the silastic substrate. Likewise, there is no permanent deformation of the membrane over the 24 hour time period.

These findings support the idea that the forces delivered to the cell layers are consistent over the 2, 4, 6, 8, and 24 hour time periods. This consistency of the Flex I^{R} culture plates and the ability to control the degree, amount, and frequency of force application through the Flexercell Strain Unit^{R} gives the system broad applications for monitoring biochemical changes in response to stress in a dynamic environment.

Effect of Force on Gingival Collagen Phenotypes

Cell Layer Raw Data (-20 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
180 cph	2 Hr.	07.06	37.86	07.77	13	16	2
		17.65	62.68	11.75	19		
		13.50	58.77	16.57	15		
180 cph	4 Hr.	24.56	44.82	30.40	25	25	0
		25.38	43.28	30.75	26		
		21.09	47.16	13.97	26		
180 cph	6 Hr.	16.70	50.39	31.98	17	19	2
		17.27	70.13	05.76	19		
		19.98	51.90	18.92	22		
180 cph	8 Hr.	18.00	45.55	36.03	18	21	2
		18.27	46.43	25.18	20		
		21.76	54.54	15.73	24		
180 cph	24 Hr.	10.62	53.19	35.73	11	11	1
		07.04	39.95	24.63	10		
		11.41	52.17	36.23	11		
30 cph	2 Hr.	12.87	63.64	20.86	13	14	2
		10.51	59.36	21.85	11		
		15.30	60.66	14.05	17		
30cph	4 Hr.	18.49	28.17	09.29	33	30	2
		30.43	43.60	25.95	30		
		20.94	31.00	25.11	27		
30 cph	6 Hr.	30.09	44.14	17.22	36	26	7
		18.77	53.65	11.98	22		
		15.19	56.91	08.31	19		
30 cph	8 Hr.	28.67	45.27	25.14	29	27	2
		27.88	39.78	32.32	28		
		22.00	55.54	09.93	25		
30 cph	24 Hr.	11.06	49.40	19.00	14	14	1
		13.89	60.25	14.64	16		
		10.99	49.40	18.59	14		

Effect of Force on Gingival Collagen Phenotypes

Cell Layer Raw Data (-20 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
0.5 cph	2 Hr.	09.57	51.00	14.33	13	19	5
		16.25	66.38	11.21	17		
		23.24	57.30	09.81	26		
0.5 cph	4 Hr.	35.81	37.70	26.00	36	29	5
		23.69	60.18	08.75	26		
		25.39	60.38	12.48	26		
0.5 cph	6 Hr.	14.00	39.26	16.14	20	26	4
		15.98	22.42	13.85	31		
		11.48	15.59	14.35	28		
0.5 cph	8 Hr.	18.02	45.55	36.07	18	19	2
		16.89	49.34	32.45	17		
		18.72	51.64	14.03	22		
0.5 cph	24 Hr.	06.68	58.15	27.06	07	09	2
		06.47	43.10	29.85	08		
		08.95	51.21	21.66	11		
Constant	2 Hr.	07.94	49.95	25.57	10	13	3
		08.57	42.21	17.30	13		
		15.64	49.91	22.37	18		
Constant	4 Hr.	06.97	42.12	32.11	09	08	1
		06.70	45.34	35.33	08		
		06.52	55.37	30.93	07		
Constant	6 Hr.	11.58	51.23	31.23	12	11	2
		10.79	43.68	42.71	11		
		08.59	58.59	34.96	08		
Constant	8 Hr.	12.81	48.89	34.27	13	12	1
		10.13	33.57	55.73	10		
		12.09	52.79	33.06	12		
Constant	24 Hr.	11.04	45.37	17.84	15	15	0
		13.51	58.63	16.54	15		
		12.33	56.76	17.48	14		

Effect of Force on Gingival Collagen Phenotypes

Cell Layer Raw Data (-10 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
180 cph	2 Hr.	0.483	74.04	31.07	.04	04	1
		04.16	61.43	12.63	05		
		02.65	61.15	15.39	03		
180 cph	4 Hr.	27.43	49.75	22.61	27	24	5
		16.27	52.16	29.17	17		
		15.23	28.25	12.64	27		
180 cph	6 Hr.	16.56	64.70	18.02	17	21	4
		25.24	40.30	33.01	26		
		18.40	57.08	16.59	20		
180 cph	8 Hr.	10.38	50.97	03.22	16	20	5
		22.49	48.58	12.10	27		
		13.05	53.93	15.02	16		
180 cph	24 Hr.	13.85	58.06	17.78	15	13	3
		12.83	67.69	16.21	13		
		08.68	74.02	10.01	09		
30 cph	2 Hr.	07.24	60.13	31.63	07	09	2
		08.03	45.66	17.92	11		
		07.46	67.75	24.77	07		
30cph	4 Hr.	06.82	65.96	17.56	08	16	6
		19.04	64.20	11.69	20		
		10.67	24.88	13.28	22		
30 cph	6 Hr.	18.64	55.75	11.52	22	20	1
		16.83	52.49	12.00	21		
		16.86	67.38	07.83	18		
30 cph	8 Hr.	13.28	58.19	21.08	14	21	5
		24.03	50.71	15.80	27		
		22.49	45.75	28.58	23		
30 cph	24 Hr.	00.00	37.69	22.71	00	07	5
		05.53	68.85	5.08	07		
		12.10	69.10	14.54	13		

Effect of Force on Gingival Collagen Phenotypes

Cell Layer Raw Data (-10 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
0.5 cph	2 Hr.	14.27	50.62	32.87	15	12	2
		10.72	56.29	20.17	12		
		09.26	67.47	21.41	09		
0.5 cph	4 Hr.	18.01	45.64	36.03	18	22	4
		19.86	74.67	03.69	20		
		23.49	48.55	17.06	26		
0.5 cph	6 Hr.	18.02	45.52	36.04	18	21	4
		18.02	45.58	36.06	18		
		25.66	68.19	04.59	26		
0.5 cph	8 Hr.	18.64	40.28	23.38	23	20	2
		18.02	45.58	36.03	18		
		16.40	61.44	11.03	18		
0.5 cph	24 Hr.	09.68	33.29	25.54	14	12	3
		13.35	41.12	40.35	14		
		07.55	75.23	06.19	08		
Constant	2 Hr.	09.71	53.46	34.22	10	12	5
		06.24	42.99	30.29	08		
		18.28	59.63	17.64	19		
Constant	4 Hr.	06.46	56.17	32.18	07	09	2
		05.67	42.86	20.88	08		
		11.13	64.21	18.45	12		
Constant	6 Hr.	11.92	45.46	32.52	13	11	3
		07.36	47.81	44.75	07		
		13.46	72.02	07.83	14		
Constant	8 Hr.	05.39	71.43	23.17	05	12	5
		12.01	50.65	30.51	13		
Constant	24 Hr.	13.58	49.66	16.62	17	05	1
		05.66	59.09	35.17	06		
		06.47	60.68	32.68	06		
		03.86	60.75	35.02	04		

Effect of Force on Gingival Collagen Phenotypes

Cell Layer Raw Data (0 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
Control	2 Hr.	03.04	58.08	37.26	03	06	4
		03.34	64.07	32.22	03		
		09.88	55.77	15.37	12		
Control	4 Hr.	08.65	53.89	37.42	09	08	1
		08.73	55.36	34.85	09		
		06.02	61.45	14.03	07		
Control	6 Hr.	10.75	56.65	27.23	11	11	3
		06.81	62.13	17.36	08		
		13.63	55.49	21.05	15		
Control	8 Hr.	04.60	29.24	21.09	08	10	3
		07.24	65.67	24.53	07		
		13.19	62.67	17.87	14		
Control	24 Hr.	03.94	65.67	24.53	04	04	4
		00.00	45.66	36.03	00		
		06.15	46.89	14.46	09		

Effect of Force on Gingival Collagen Phenotypes

Medium Layer Raw Data (-20 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III .%	AVG %	STD
180 cph	2 Hr.	13.25	64.92	12.91	15	10	4
		09.98	72.40	15.35	10		
		05.04	78.83	14.59	05		
180 cph	4 Hr.	22.02	40.62	14.40	29	22	5
		16.39	59.20	13.65	18		
		14.01	43.84	19.43	18		
180 cph	6 Hr.	27.79	43.24	20.80	30	35	6
		28.68	39.50	23.79	31		
		34.77	31.45	14.96	43		
180 cph	8 Hr.	25.35	56.58	10.30	27	24	3
		20.88	50.65	18.91	23		
		20.66	60.31	14.90	22		
180 cph	24 Hr.	03.90	26.35	25.18	07	06	2
		02.84	76.85	14.75	03		
		06.86	74.60	10.83	07		
30 cph	2 Hr.	09.65	84.02	02.41	10	11	2
		13.61	75.01	07.30	14		
		09.51	79.39	04.86	10		
30cph	4 Hr.	23.85	35.76	22.89	29	29	3
		27.67	50.18	06.85	33		
		21.97	45.00	15.65	27		
30 cph	6 Hr.	17.64	63.36	11.24	19	09	7
		05.72	76.59	09.96	06		
		01.62	78.13	04.89	02		
30 cph	8 Hr.	13.71	74.04	05.34	15	12	2
		12.65	71.26	14.79	13		
30 cph	24 Hr.	09.33	45.91	41.47	10	02	1
		02.63	53.43	18.47	04		
		01.04	86.92	11.49	01		
		01.57	81.94	09.93	02		

Effect of Force on Gingival Collagen Phenotypes

Medium Layer Raw Data (-20 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
0.5 cph	2 Hr.	0 6.19	83.72	02.01	07	09	2
		08.54	68.99	17.30	09		
		11.44	56.70	23.06	13		
0.5 cph	4 Hr.	15.95	74.22	03.62	17	19	8
		09.35	59.05	19.78	11		
		28.59	38.86	30.58	29		
0.5 cph	6 Hr.	13.98	58.28	18.35	15	13	1
		12.13	60.51	20.21	13		
		11.22	71.66	11.17	12		
0.5 cph	8 Hr.	14.04	50.43	22.16	16	15	4
		18.91	55.75	20.53	20		
		09.65	83.96	04.77	10		
0.5 cph	24 Hr.	06.16	65.05	20.27	07	08	3
		04.45	75.45	07.96	05		
		10.64	68.17	12.04	12		
Constant	2 Hr.	10.33	63.62	20.35	11	11	2
		12.10	76.19	08.69	12		
		07.52	51.96	25.87	09		
Constant	4 Hr.	07.95	70.67	14.61	09	10	1
		10.43	78.47	11.01	10		
		10.84	62.74	15.76	12		
Constant	6 Hr.	08.06	76.30	12.45	08	13	5
		09.11	67.87	14.67	10		
		17.16	42.00	24.28	21		
Constant	8 Hr.	09.82	71.96	15.16	10	14	4
		11.45	77.74	05.80	12		
Constant	24 Hr.	17.07	50.52	18.20	20	09	2
		07.66	64.12	23.03	08		
		06.47	77.67	13.64	07		
		10.50	63.62	22.98	11		

Effect of Force on Gingival Collagen Phenotypes

Medium Layer Raw Data (-10 kPascals)

Experiment	Time	Densitometer Readings					
		Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
180 cph	2 Hr.	10.02	76.98	09.53	10	10	2
		06.49	74.19	06.48	07		
		10.18	69.76	12.34	11		
180 cph	4 Hr.	11.24	61.36	06.79	14	19	4
		17.27	30.18	24.78	24		
		17.78	67.59	05.90	19		
180 cph	6 Hr.	16.06	71.67	07.47	17	17	0
		15.85	50.31	22.54	18		
		16.66	63.86	14.24	18		
180 cph	8 Hr.	09.11	50.21	18.08	12	11	3
		06.21	62.89	14.12	07		
		12.03	48.16	27.99	14		
180 cph	24 Hr.	13.15	71.13	09.43	14	10	3
		07.86	68.67	21.18	08		
		08.26	60.46	20.37	09		
30 cph	2 Hr.	01.17	75.74	17.16	01	06	5
		03.20	74.02	13.20	04		
		11.21	58.43	21.45	12		
30cph	4 Hr.	08.57	80.42	03.13	09	08	1
		06.59	82.85	07.08	07		
		08.53	71.22	12.44	09		
30 cph	6 Hr.	06.51	81.95	06.38	07	11	5
		17.69	63.84	13.32	19		
		06.87	72.35	15.36	07		
30 cph	8 Hr.	13.35	68.99	05.28	15	20	5
		16.82	40.29	35.69	18		
30 cph	24 Hr.	22.25	56.90	02.39	27	03	3
		00.00	21.90	14.65	00		
		01.60	86.28	10.09	02		
		07.04	86.38	04.80	07		

Effect of Force on Gingival Collagen Phenotypes

Medium Layer Raw Data (-10 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
0.5 cph	2 Hr.	14.12	77.86	03.71	15	12	4
		06.55	61.29	26.79	07		
		13.71	58.09	21.21	15		
0.5 cph	4 Hr.	09.03	24.96	59.61	10	09	1
		06.37	82.04	06.29	07		
		07.98	58.29	17.55	10		
0.5 cph	6 Hr.	09.37	77.18	04.82	10	11	4
		14.60	63.95	13.62	16		
		05.77	80.92	03.55	06		
0.5 cph	8 Hr.	16.01	52.64	20.92	18	16	4
		18.38	55.41	19.64	20		
		10.23	64.12	21.76	11		
0.5 cph	24 Hr.	05.16	79.60	13.79	05	10	3
		10.85	55.41	14.61	12		
		12.74	64.12	18.29	13		
Constant	2 Hr.	07.29	79.60	11.75	08	08	1
		06.98	66.52	10.22	08		
		07.93	66.23	17.05	09		
Constant	4 Hr.	11.53	72.21	19.85	12	09	2
		07.72	74.50	10.57	08		
		06.76	63.64	02.40	07		
Constant	6 Hr.	11.60	78.05	02.68	13	08	3
		04.86	87.19	03.94	05		
		06.11	66.67	20.13	07		
Constant	8 Hr.	08.98	79.51	02.55	10	08	5
		11.36	70.06	15.29	12		
Constant	24 Hr.	01.31	79.33	13.42	01	02	2
		00.00	92.65	06.93	00		
		04.16	85.88	09.20	04		
		01.20	76.67	17.06	01		

Effect of Force on Gingival Collagen Phenotypes

Medium Layer Raw Data (0 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
Control	2 Hr.	00.00	66.30	10.20	00	05	6
		11.66	60.23	16.13	13		
		01.78	85.64	10.27	02		
Control	4 Hr.	09.42	62.64	19.46	10	07	3
		03.45	81.63	10.91	04		
		06.30	80.11	02.93	07		
Control	6 Hr.	06.63	86.07	05.76	07	08	2
		05.38	80.06	11.11	06		
		10.53	65.78	21.87	11		
Control	8 Hr.	06.81	65.45	20.63	07	04	2
		03.74	79.90	12.84	04		
		01.81	62.60	15.38	02		
Control	24 Hr.	03.25	79.30	13.71	03	04	2
		06.69	78.20	08.46	07		
		01.96	76.32	04.69	02		

APPENDIX H

Analysis of Variance of Mean Values for Cell Layer Data
Collagen Type III / Type I + Type III Percentage

FORCE vs TIME

FORCE LEVEL -10 kPascals

TIME	2 Hours	4 Hours	6 Hours	8 Hours	24 Hours
2 Hours		0.0001**	0.0001**	0.0001**	0.5777
4 Hours	0.0001**		0.7318**	0.8303	0.0002**
6 Hours	0.0001**	0.7318		0.8977	0.0001**
8 Hours	0.0001**	0.8303	0.8977		0.0001**
24 Hours	0.5777	0.0002**	0.0001**	0.0001**	

FORCE LEVEL -20 kPascals

TIME	2 Hours	4 Hours	6 Hours	8 Hours	24 Hours
2 Hours		0.0001**	0.0115*	0.0309*	0.0971
4 Hours	0.0001**		0.1474	0.0678	0.0001**
6 Hours	0.0115*	0.1474		0.6998	0.0001**
8 Hours	0.0309*	0.0678	0.0678		0.0002**
24 Hours	0.0971	0.0001**	0.0001**	0.0002**	

P = 0.2627
Standard Error = 1.37
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Analysis of Variance of Mean Values for Cell Layer Data
Collagen Type III / Type I + Type III Percentage
-20 kPascals Force Level

TIME

TIME	2 HOURS	4 HOURS	6 HOURS	8 HOURS	24 HOURS
2 HOURS		0.0001**	0.0005**	0.0041**	0.0374*
4 HOURS	0.0001**		0.2302	0.0639	0.0001**
6 HOURS	0.0005**	0.2302		0.4995	0.0001**
8 HOURS	0.0041**	0.0639	0.4995		0.0001**
24 HOURS	0.0374*	0.0001**	0.0001**	0.0001**	

p = 0.0001
Standard Error = 0.97
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

FREQUENCY

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT	CONTROL
180 CPH		0.0069**	0.1512	0.0001**	0.0001**
30 CPH	0.0069**		0.1798	0.0001**	0.0001**
0.5 CPH	0.1512	0.1798		0.0001**	0.0001**
CONSTANT	0.0001**	0.0001**	0.0001**		0.0089**
CONTROL	0.0001**	0.0001**	0.0001**	0.0089**	

p = 0.0001
Standard Error = 0.97
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Analysis of Variance of Mean Values for Cell Layer Data
Collagen Type III / Type I + Type III Percentage
-20 kPascals

TIME vs FREQUENCY

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT	CONTROL
2 HOURS					
180 CPH		0.5175	0.3329	0.5175	0.0028**
30 CPH	0.5175		0.1095	1.0000	0.0158*
0.5 CPH	0.3329	0.1095		0.1095	0.0001**
CONSTANT	0.5175	1.0000	0.1095		0.0158*
CONTROL	0.0028**	0.0158*	0.0001**	0.0158*	
4 HOURS					
180 CPH		0.1640	0.2377	0.0001**	0.0001**
30 CPH	0.1640		0.8289	0.0001**	0.0001**
0.5 CPH	0.2377	0.8289		0.0001**	0.0001**
CONSTANT	0.0001**	0.0001**	0.0001**		0.9139
CONTROL	0.0001**	0.0001**	0.0001**	0.9139	
6 HOURS					
180 CPH		0.0442*	0.0268*	0.0050**	0.0120*
30 CPH	0.0442*		0.8289	0.0001**	0.0001**
0.5 CPH	0.0268*	0.8289		0.0001**	0.0001**
CONSTANT	0.0050**	0.0001**	0.0001**		0.7458
CONTROL	0.0120*	0.0001**	0.0001**	0.7458	
8 HOURS					
180 CPH		0.0346*	0.5894	0.0050**	0.0008**
30 CPH	0.0346*		0.0090**	0.0001**	0.0001**
0.5 CPH	0.5894	0.0090**		0.0206*	0.0037**
CONSTANT	0.0050**	0.0001**	0.0206*		0.5175
CONTROL	0.0008**	0.0001**	0.0037**	0.5175	
24 HOURS					
180 CPH		0.1983	0.5175	0.1983	0.0442*
30 CPH	0.1983		0.0561*	1.0000	0.0015**
0.5CPH	0.5175	0.0561		0.0561	0.1640
CONSTANT	0.1983	1.0000	0.0561		0.0015**
CONTROL	0.0442*	0.0015**	0.1640	0.0015**	

p = 0.0001
Standard Error = 2.17
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Analysis of Variance of Mean Values for Cell Layer Data
Collagen Type III / Type I + Type III Percentage
-10 kPascals Force Level

TIME

TIME	2 HOURS	4 HOURS	6 HOURS	8 HOURS	24 HOURS
2 HOURS		0.0002**	0.0001**	0.0001**	0.7666
4 HOURS	0.0002**		0.5289	0.7385	0.0004**
6 HOURS	0.0001**	0.5289		0.7666	0.0001**
8 HOURS	0.0001**	0.7385	0.7666		0.0002**
24 HOURS	0.7666	0.0004**	0.0001**	0.0002**	

p = 0.0001
Standard Error = 1.26
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

FREQUENCY

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT	CONTROL
180 CPH		0.1854	0.9704	0.0002**	0.0001**
30 CPH	0.1854		0.1737	0.0108**	0.0004**
0.5 CPH	0.9704	0.1737		0.0002**	0.0001**
CONSTANT	0.0002**	0.0108**	0.0002**		0.2685
CONTROL	0.0001**	0.0004**	0.0001**	0.2685	

p = 0.0001
Standard Error = 1.26
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Analysis of Variance of Mean Values for Cell Layer Data
Collagen Type III / Type I + Type III Percentage
-10 kPascals

TIME vs FREQUENCY

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT	CONTROL
2 HOURS					
180 CPH		0.2835	0.0508	0.0422*	0.6190
30 CPH	0.2835		0.3633	0.3217	0.5620
0.5 CPH	0.0508	0.3633		0.9339	0.1396
CONSTANT	0.0422*	0.3217	0.9339		0.1194
CONTROL	0.6190	0.5620	0.1396	0.1194	
4 HOURS					
180 CPH		0.0860	0.5620	0.0006**	0.0004**
30 CPH	0.0860		0.2485	0.0608	0.0422*
0.5 CPH	0.5620	0.2485		0.0033**	0.0021**
CONSTANT	0.0006**	0.0608	0.0033**		0.8682
CONTROL	0.0004**	0.0422*	0.0021**	0.8682	
6 HOURS					
180 CPH		0.8682	0.9339	0.0193*	0.0193*
30 CPH	0.8682		0.9339	0.0288*	0.0288*
0.5 CPH	0.9339	0.9339		0.0236*	0.0236*
CONSTANT	0.0193*	0.0288*	0.0236*		1.0000
CONTROL	0.0193*	0.0288*	0.0236*	1.0000	
8 HOURS					
180 CPH		0.6785	1.0000	0.0508	0.0157*
30 CPH	0.6785		0.6785	0.0193*	0.0053**
0.5 CPH	1.0000	0.6785		0.0508	0.0157*
CONSTANT	0.0508	0.0193*	0.0508		0.6190
CONTROL	0.0157*	0.0053**	0.0157*	0.6190	
24 HOURS					
180 CPH		0.0127*	0.2167	0.0053**	0.0026**
30 CPH	0.0127*		0.1881	0.7401	0.5620
0.5CPH	0.2167	0.1881		0.1016	0.0608
CONSTANT	0.0053**	0.7401	0.1016		0.8035
CONTROL	0.0026**	0.5620	0.0608	0.8035	

p = 0.0634
Standard Error = 2.83
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Analysis of Variance of Mean Values for Medium Layer Data
Collagen Type III / Type I + Type III Percentage

FORCE vs TIME

FORCE LEVEL -10 kPascals

TIME	2 Hours	4 Hours	6 Hours	8 Hours	24 Hours
2 Hours		0.2777	0.1785	0.0315*	0.2313
4 Hours	0.2777		0.7927	0.2777	0.0239*
6 Hours	0.1785	0.7927		0.4096	0.0121*
8 Hours	0.0315*	0.2777	0.4096		0.0010**
24 Hours	0.2313	0.0239*	0.0121*	0.0010**	

FORCE LEVEL -20 kPascals

TIME	2 Hours	4 Hours	6 Hours	8 Hours	24 Hours
2 Hours		0.0001**	0.0019**	0.0072**	0.0579
4 Hours	0.0001**		0.2313	0.1010	0.0001**
6 Hours	0.0019**	0.2313		0.6524	0.0001**
8 Hours	0.0072**	0.1010	0.6524		0.0001**
24 Hours	0.0579	0.0001**	0.0001**	0.0001**	

P = 0.0444
Standard Error = 1.56
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Analysis of Variance of Mean Values for Medium Layer Data
Collagen Type III / Type I + Type III Percentage
-20 kPascals Force Level

TIME

TIME	2 HOURS	4 HOURS	6 HOURS	8 HOURS	24 HOURS
2 HOURS		0.0001**	0.0005**	0.0070**	0.0373
4 HOURS	0.0001**		0.2559	0.0446*	0.0001**
6 HOURS	0.0005**	0.2559		0.3664	0.0001**
8 HOURS	0.0070**	0.0446*	0.3664		0.0001**
24 HOURS	0.0373*	0.0001**	0.0001**	0.0001**	

p = 0.0001
Standard Error = 1.19
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

FREQUENCY

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT	CONTROL
180 CPH		0.0006*	0.0006**	0.0001**	0.0001**
30 CPH	0.0006**		0.9685	0.3266	0.0001**
0.5 CPH	0.0006**	0.9685		0.3078	0.0001**
CONSTANT	0.0001**	0.3266	0.3078		0.0015**
CONTROL	0.0001**	0.0001**	0.0001**	0.0015**	

p = 0.0001
Standard Error = 1.19
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Analysis of Variance of Mean Values for Medium Layer Data
Collagen Type III / Type I + Type III Percentage
-20 kPascals

TIME vs FREQUENCY

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT	CONTROL
2 HOURS					
180 CPH		0.7245	0.9297	0.8601	0.1898
30 CPH	0.7245		0.6596	0.8601	0.0985
0.5 CPH	0.9297	0.6796		0.7915	0.2206
CONSTANT	0.8601	0.8601	0.7915		0.1383
CONTROL	0.1898	0.0985	0.2206	0.1383	
4 HOURS					
180 CPH		0.0384	0.4817	0.0041**	0.0003**
30 CPH	0.0384*		0.0066**	0.0001**	0.0001**
0.5 CPH	0.4817	0.0066**		0.0254*	0.0025**
CONSTANT	0.0041**	0.0001**	0.0254*		0.3798
CONTROL	0.0003**	0.0001**	0.0025**	0.3798	
6 HOURS					
180 CPH		0.0001**	0.0001**	0.0001**	0.0001**
30 CPH	0.0001**		0.2548	0.2927	0.7915
0.5 CPH	0.0001**	0.2548		0.9297	0.1625
CONSTANT	0.0001**	0.2927	0.9297		0.1898
CONTROL	0.0001**	0.7915	0.1625	0.1898	
8 HOURS					
180 CPH		0.0041**	0.0254*	0.0105*	0.0001**
30 CPH	0.0041**		0.4817	0.7245	0.0313*
0.5 CPH	0.0254*	0.4817		0.7245	0.0052**
CONSTANT	0.0105*	0.7245	0.7245		0.0132*
CONTROL	0.0001**	0.0313*	0.0052**	0.0132*	
24 HOURS					
180 CPH		0.3798	0.5379	0.4289	0.6596
30 CPH	0.3798		0.1383	0.0985	0.6596
0.5CPH	0.5379	0.1383		0.8601	0.2927
CONSTANT	0.4289	0.0985	0.8601		0.2206
CONTROL	0.6596	0.6596	0.2927	0.2206	

p = 0.0001
Standard Error = 2.66
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Analysis of Variance of Mean Values for Medium Layer Data
Collagen Type III / Type I + Type III Percentage
-10 kPascals Force Level

TIME

TIME	2 HOURS	4 HOURS	6 HOURS	8 HOURS	24 HOURS
2 HOURS		0.1250	0.0503*	0.0159*	0.1250
4 HOURS	0.1250		0.6577	0.3537	0.0030**
6 HOURS	0.0503*	0.6577		0.6260	0.0008**
8 HOURS	0.0159*	0.3537	0.6260		0.0002**
24 HOURS	0.1250	0.0030**	0.0008**	0.0002**	

p = 0.0009
Standard Error = 1.06
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

FREQUENCY

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT	CONTROL
180 CPH		0.0127*	0.2344	0.0001**	0.0001**
30 CPH	0.0127*		0.1731	0.0883	0.0113*
0.5 CPH	0.2344	0.1731		0.0030**	0.0020**
CONSTANT	0.0001**	0.0883	0.0030**		0.3769
CONTROL	0.0001**	0.0113*	0.0020**	0.3769	

p = 0.0001
Standard Error = 1.06
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Analysis of Variance of Mean Values for Medium Layer Data
Collagen Type III / Type I + Type III Percentage
-10 kPascals

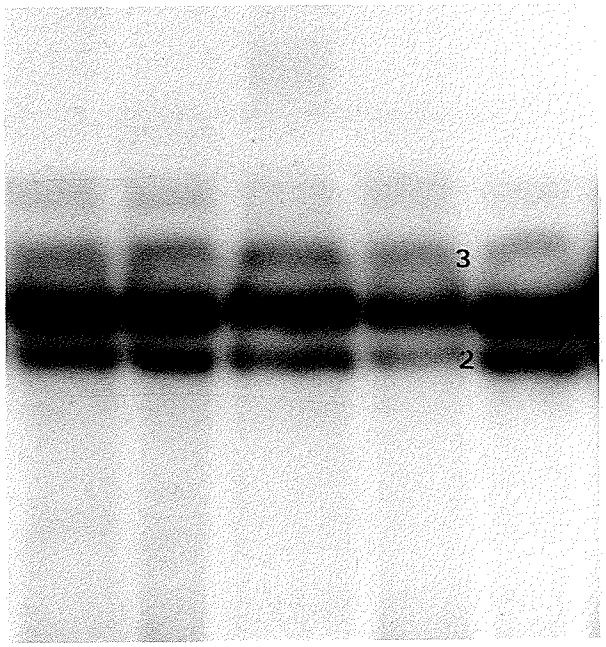
TIME vs FREQUENCY

FREQUENCY	180 CPH	30 CPH	0.5 CPH	CONSTANT	CONTROL
2 HOURS					
180 CPH		0.2781	0.3739	0.7661	0.2010
30 CPH	0.2781		0.0517	0.4290	0.8428
0.5 CPH	0.3739	0.0517		0.2373	0.0330*
CONSTANT	0.7661	0.4290	0.2373		0.3237
CONTROL	0.2010	0.8428	0.0330*	0.3237	
4 HOURS					
180 CPH		0.0025**	0.0043**	0.0043**	0.0008**
30 CPH	0.0025**		0.8428	0.8428	0.6918
0.5 CPH	0.0043**	0.8428		1.0000	0.5525
CONSTANT	0.0043**	0.8428	1.0000		0.5525
CONTROL	0.0008**	0.6918	0.5525	0.5525	
6 HOURS					
180 CPH		0.0517	0.0414*	0.0074**	0.0057**
30 CPH	0.0517		0.9210	0.4290	0.3739
0.5 CPH	0.0414*	0.9210		0.4886	0.4290
CONSTANT	0.0074**	0.4290	0.4886		0.9210
CONTROL	0.0057**	0.3739	0.4290	0.9210	
8 HOURS					
180 CPH		0.0097**	0.1170	0.3237	0.0517
30 CPH	0.0097**		0.2781	0.0006**	0.0001**
0.5 CPH	0.1170	0.2781		0.0125*	0.0008**
CONSTANT	0.3237	0.0006**	0.0125*		0.3237
CONTROL	0.0517	0.0001**	0.0008**	0.3237	
24 HOURS					
180 CPH		0.0330*	0.9210	0.0125*	0.0640
30 CPH	0.0330*		0.0414*	0.6918	0.7661
0.5CPH	0.9210	0.0414*		0.0161*	0.0788
CONSTANT	0.0125*	0.6918	0.0161*		0.4886
CONTROL	0.0640	0.7661	0.0788	0.4886	

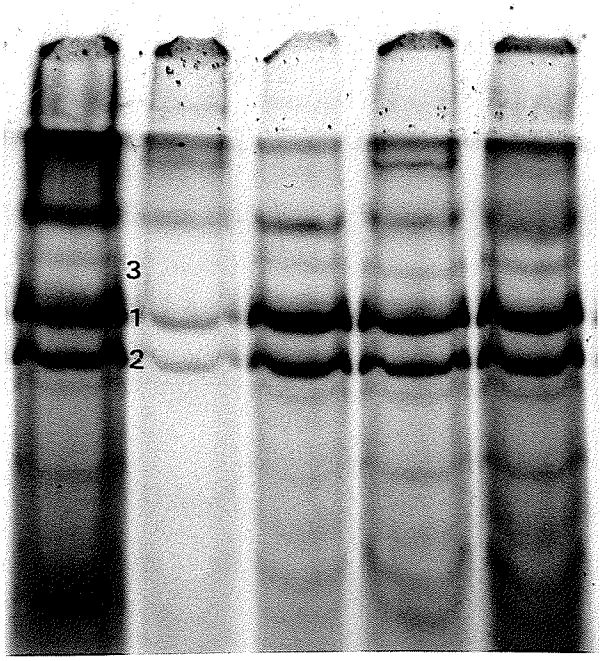
p = 0.0111
Standard Error = 2.36
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

APPENDIX R

PHOTOGRAPHS OF FLUOROGRAMS ILLUSTRATING COLLAGEN BANDS SEPARATED BY SDS-PAGE



Medium Layer



Cell Layer

1 = $alpha_1$ (I)
2 = $alpha_2$ (I)
3 = $alpha_1$ (III)

APPENDIX S

Analysis of Variance of Mean Values
Effect of Force and Passage on Gingival Collagen Synthesis
Type III / Type I + Type III Percentage
Regimen: 30 CPH at -20 kPascals

TIME

TIME	2 HOURS	4 HOURS	6 HOURS	8 HOURS	24 HOURS
2 HOURS		0.2545	0.2807	0.0727	0.7800
4 HOURS	0.2545		0.9505	0.4958	0.3869
6 HOURS	0.2807	0.9505		0.4577	0.4214
8 HOURS	0.0727	0.4958	0.4577		0.1261
24 HOURS	0.7800	0.3869	0.4214	0.1261	

p = 0.3739
Standard Error = 1.89
No significant difference at P = 0.05 or 0.01 confidence level.

EXPERIMENT

EXPER.	CONTROL	EXPLANT	PASS 1 C	PASS 1 N
CONTROL		0.0001**	0.0001**	0.0001**
EXPLANT	0.0001**		0.0010**	0.0375*
PASS 1 C	0.0001**	0.0010**		0.1700
PASS 1 N	0.0001**	0.0375*	0.1700	

p = 0.0001
Standard Error = 1.69
* Denotes significant difference at P = 0.05 confidence level.
** Denotes significant difference at P = 0.01 confidence level.

Explant: Initial harvest grown to confluency.
PASS 1 C: First passage confluent dishes.
PASS 1 N: First passage nonconfluent dishes.

Effect of Force and Passage on Gingival Collagen

Raw Data (30 cph at -20 kPascals)

		Densitometer Readings					
Experiment	Time	Type III	Type I Alpha 1	Type I Alpha 2	III/I+III %	AVG %	STD
Explant	2 Hr.	38.49	36.66	17.83	41	36	16
		48.73	31.20	13.68	52		
		13.22	77.99	03.64	14		
Explant	4 Hr.	13.25	45.90	31.53	15	24	09
		21.38	68.18	08.24	22		
		29.51	27.29	25.52	36		
Explant	6 Hr.	20.07	43.34	23.86	23	22	01
		18.52	51.14	22.36	20		
		22.07	69.05	04.06	23		
Explant	8 Hr.	27.92	42.13	17.74	32	38	06
		39.56	27.37	18.47	46		
		30.52	36.03	17.51	36		
Explant	24 Hr.	28.29	42.58	28.82	28	25	10
		30.47	46.97	12.37	34		
		10.10	74.66	04.65	11		
1st Passage Nonconfluent	2 Hr.	18.61	63.96	12.80	20	19	05
		23.17	49.54	18.78	25		
		12.50	67.31	15.30	13		
1st Passage Nonconfluent	4 Hr.	06.91	66.45	16.15	08	19	09
		27.77	48.91	16.91	30		
		19.49	61.38	14.19	21		
1st Passage Nonconfluent	6 Hr.	17.32	64.44	11.74	19	20	01
		20.24	63.63	08.87	22		
		16.50	56.55	13.31	19		
1st Passage Nonconfluent	8 Hr.	16.38	61.92	10.66	18	15	03
		11.28	62.01	19.35	12		
1st Passage Nonconfluent	24 Hr.	12.39	58.08	20.26	14	24	03
		20.56	55.19	15.31	23		
		18.19	61.20	11.53	20		
		25.88	52.67	13.62	28		

Effect of Force and Passage on Suture Collagens

Raw Data (30 CPH at -20 kPascals)

Experiment	Time	Densitometer Readings			III/I+III %	AVG %	STD
		Type III	Type I Alpha 1	Type I Alpha 2			
Explant	2 Hr.	41.72	46.60	07.55	44	35	10
		18.53	64.33	07.72	20		
		37.18	39.06	17.68	40		
Explant	4 Hr.	27.46	36.23	13.90	35	30	05
		24.49	40.01	19.57	29		
		19.11	54.57	04.64	24		
Explant	6 Hr.	30.93	31.95	30.00	33	29	04
		20.88	46.93	17.25	25		
		25.51	52.45	09.18	29		
Explant	8 Hr.	26.61	50.55	16.05	29	27	03
		18.91	48.79	17.74	22		
		27.47	45.63	18.32	30		
Explant	24 Hr.	36.95	38.39	14.46	41	42	10
		47.78	29.85	10.63	54		
		25.99	54.90	04.86	30		
1st Passage	2 Hr.	19.27	48.93	29.94	20	17	03
		10.95	43.74	24.80	14		
1st Passage	4 Hr.	22.10	65.87	07.90	23	20	04
		12.91	65.82	12.11	14		
		19.34	62.28	05.89	22		
1st Passage	6 Hr.	20.89	47.14	24.17	23	25	04
		22.45	68.33	06.29	23		
		29.67	37.22	29.86	31		
1st Passage	8 Hr.	13.70	54.29	30.83	14	21	05
		23.44	52.60	12.17	27		
		21.68	58.40	14.69	23		
1st Passage	24 Hr.	18.82	50.53	23.67	20	21	02
		20.61	57.28	11.03	23		
		17.74	52.13	22.58	19		

Effect of Force on Human Periodontal Ligament Cell

Collagen Phenotype Synthesis In Vitro

(-20 kPascals)

Experiment	Time	Densitometer Readings			III/I+III %	AVG %
		Type III	Type I Alpha 1	Type I Alpha 2		
180 CPH (Middle 1/3rd)	2 Hr.	06.83	84.90	07.03	06	15
		14.13	60.00	20.18	15	
		20.14	35.49	32.41	22	
180 CPH (Gingiv. 1/3rd)	2 Hr.	35.33	39.93	19.12	37	24
		09.92	57.80	27.98	10	
180 CPH (Apical 1/3rd)	2 Hr.	15.44	57.72	24.34	16	14
		13.27	72.60	11.80	14	
		11.09	57.29	21.38	12	
Control (Middle 1/3rd)	2 Hr.	14.81	46.95	33.23	16	16
		16.45	56.39	20.01	18	
		12.89	40.79	32.27	15	
Control (Apical 1/3rd)	2 Hr.	13.92	47.73	31.63	15	13
		09.45	47.53	22.85	12	
180 CPH (Gingiv. 1/3rd)	4 Hr.	02.03	22.46	05.36	07	07
180 CPH (Middle 1/3rd)	4 Hr.	11.29	77.94	09.53	11	11
180 CPH (Apical 1/3rd)	4 Hr.	05.71	56.99	30.15	06	06
180 CPH (Apical 1/3rd)	8 Hr.	05.43	65.03	14.10	06	06
Constant (Middle 1/3rd)	8 Hr.	11.47	60.66	18.93	13	16
		09.01	57.08	15.95	11	
		13.91	28.28	14.01	25	
Constant (Apical 1/3rd)	8 Hr.	14.71	38.94	13.65	22	18
		10.89	46.81	19.05	14	
		11.29	38/01	15.01	18	