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Mechanical induction of TGF-[beta]1 and TGF-[beta]2 in cultured human periodontal ligment cells

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MECHANICAL INDUCTION OF TGF-β1 AND TGF-β2 IN CULTURED HUMAN PERIODONTAL LIGAMENT CELLS

by

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PREFACE

Acknowledgements

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MECHANICAL INDUCTION OF TGF-β1 AND TGF-β2 IN CULTURED HUMAN PERIODONTAL LIGAMENT CELLS

Michael S. Lyons, DDS

ABSTRACT

It is well documented that several growth factors modulate the activities of osteoblasts, osteoclasts and fibroblasts, and further that these cells synthesize specific growth factors in response to various biochemical signals. However the effects of mechanical forces, such as those applied during orthodontic tooth movement, in the induction of these growth factors by bone-related cells remain largely unknown. For this reason, and because of the known effects of TGF-β in inducing osteogenesis and suppressing osteoclast activity, we examined the potential role of this growth factor in mechanically induced bone modeling. Early passage periodontal ligament fibroblasts were plated in serum-free medium on collagen-coated flexible-bottom plates, and subjected to stretching by a microprocessor-regulated system at a frequency of either 6 cycles per hour at 20% strain or 6 cycles per minute at 5% strain. Non-stretched control plates were maintained under similar conditions. The cell-conditioned medium was retrieved at 6, 12, 24, 36, 48 and 72 hours and assayed for total protein, as well as TGF-β1 and β2, while the cells were fixed in 2% PBS-buffered paraformaldehyde for immunocytochemistry. Protein assays revealed that the total protein in the conditioned media from the stretched cells was not significantly different from the non-stretched controls, while ELISAs on the cell-conditioned medium revealed a significantly greater synthesis of TGF-β1 and TGF-β2 by mechanically stimulated cells than by control cells as early as 12 hours in some cases and by 72 hours in all cases ($p<.05$). Immunocytochemistry demonstrated a relatively uniform staining for TGFβ1 in cells coated on flexible-bottom plates, and more cells stained positively in the experimental than in the control plates. Our preliminary findings

using cytochalasin B also indicate that disturbances in the cytoskeleton may at least be partly responsible for induction of TGF-βs under mechanical loading. Further preliminary studies suggest that changes in catabolic activity, as evaluated by the expression of matrix metalloproteinases, do not accompany the increased synthesis of TGF-βs by mechanically strained PDL cells. Our findings indicate that a specific regimen of mechanical stimulation of periodontal ligament cells leads to increased expression of TGF-β1 and TGF-β2 and suggest a potential role of this growth factor in the osteogenic response associated with tooth movement.

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A. BACKGROUND AND SIGNIFICANCE

INTRODUCTION

In 1778, Hunter suggested that "to extract an irregular tooth would answer but little purpose, if no alterations could be made in the situation of the rest; but we find that the very principle upon which teeth are made to grow irregularly is capable, if properly directed, of bringing them even again. This principle is the power which many parts (especially bones) have of moving out of the way of mechanical pressure" (Hunter, 1778). Wolff, in the late 19th century, proposed that the architecture of bone is greatly influenced by the functional loads under which it is placed (Wolff, 1892). Since that time, the effects of strain on bone *in-vivo* have been examined extensively, and Wolff's initial observations remain largely intact.

Currey (1984) suggested that the remodeling process is necessary for the bone to better withstand the applied forces. Several studies provide evidence supporting this view. *In-vivo* experiments have shown that denervation and/or resection of the muscles of mastication (Moore, 1976; Gardener et al., 1981; Byrd, 1984), changes in texture of the diet (Bouvier and Hylander, 1981), or altered mandibular position (Petrovic et al., 1975; Curtis et al., 1991; Kapila et al., 1990) induce changes in the bone density and morphology of the craniofacial skeleton. Similar alterations in bone morphology have been demonstrated in long bones subjected to increased or decreased functional loads (Shaw et al., 1987; Pead et al., 1988; Vailas et al., 1988). Additionally, long-term immobilization and weightlessness have been shown to reduce the rate of bone formation and have a profound influence on bone density (Globus et al., 1986).

Many *in-vitro* studies have yielded similar results. Several investigators (Meikle et al., 1979, 1980; Yen et al., 1984; Miyawaki and Forbes, 1987) have shown that coronal suture explants, when exposed to tensile mechanical forces, will increase collagen synthesis as well as cellular proliferation. Advances in organ and cell culture techniques

have allowed a more precise examination of these phenomena at the cellular and sub-cellular levels. The development of a commercially available flexible-bottomed culture dish (Petriperm, Tekmar Corp., Cincinnati, OH) marked the beginning of a series of studies investigating the effects of mechanical strain on all aspects of cell physiology and morphology. Osteoblasts, for example, will dramatically increase PGE_2 production within 20 minutes of the application of tensile forces (Harrell et al., 1977). Hasegawa and co-workers (1985) subjected calvarial osteoblasts to both continuous and intermittent compressive forces for 2 hours and found that there was a 64% increase in the number of cells synthesizing DNA as well as a 33% increase in the synthesis of collagenous proteins.

A reliable and reproducible model of mechanically induced bone modeling is that of orthodontic tooth movement. Such a model has previously been used to better comprehend bone responses *in-vivo* and *in-vitro*(Shanfield et al., 1986; Ngan et al., 1990). It has been shown that bone modeling in this model is mediated partially by a number of specific soluble factors including prostaglandin-E2 (PGE_2), interleukin-1 (IL-1) as well as some of growth factors (Shanfield et al., 1986; Ngan et al., 1990). The specific roles of these factors and the conditions under which they are expressed, however, have yet to be determined. In these studies we utilize an *in-vitro* analog of orthodontic tooth movement to study the role of members of a family of specific growth factors, the TGF-βs, in mechanically induced bone modeling.

MECHANICAL LOADING REGIMENS AND THEIR SIGNIFICANCE

In any *in-vitro* study it is generally desirable that the test conditions mimic the *in-vivo* environment. Since loads placed on tissues vary greatly, and because the magnitude and type of force perceived by the cells are largely unknown, the model systems used in mechanical loading studies vary greatly in terms of magnitude, duration, frequency and direction of load application. In order to develop an *in-vitro* tooth movement analog it is

useful to characterize the forces delivered by orthodontic appliances to the bone and the periodontal ligament.

Various analytical techniques have been applied to studies for the biomechanical responses of the periodontium to mechanical forces. Strain gauges (Tanne et al., 1985), photoelastics (Caputo et al., 1974), holographics, and finite element modeling (FEM, Tanne et al., 1987) have all been employed. It had long been believed that for the efficient movement of teeth a continuous force is desirable. However, a recent paper has proposed a concept that due to the physical limitations of the appliance that require the application of force away from the center of resistance of the tooth, the loads delivered by orthodontic appliance systems may indeed be intermittent in nature (Isaacson et al., 1993). It has also been determined by histologic and biophysical studies that the applied load is generally quite small, in the range of 1-5% strain (Tanne et al., 1992; Norton et al., 1992). For these reasons most orthodontic tooth movement model systems have been designed to deliver light, discontinuous mechanical loads.

TISSUE AND CELL RESPONSES TO MECHANICAL LOADING

Nearly all tissues are subjected at one time or another to mechanical forces. These forces may be derived internally, such as in muscle-bone interactions, or externally as is seen in situations of high gravity (van Kampen et al., 1985) and weightlessness (Globus et al., 1984; Mack et al., 1967; Cann et al., 1983; Rambaut et al., 1985; Bikle et al., 1987; Yamaguchi et al., 1989). The response of the tissues varies greatly depending on the character of the applied load and the responding cells.

Increased hemodynamic loading has been shown to result in hypertrophy of the vessels of hypertensive patients. Numerous studies in which aortocaval shunts mimicked hypertension have demonstrated this phenomenon repeatedly (Lavandero et al., 1993; Kiriazis et al., 1992; Ding et al., 1991). Sumpio and his colleagues (Sumpio et al., 1987, 1988) sought to examine the effects of intermittently applied strain on aortic endothelial

cells. They found that these cells altered their morphology and increased rates of both cell division and DNA synthesis. Cell division in smooth muscle cells was decreased, however collagen synthesis in this cell line was increased. Ives (1991) showed that vascular endothelial cells will align themselves perpendicularly to the applied strain. Furthermore, neonatal rat vascular smooth muscle cells subjected to cyclic strain *in-vitro* increased the basal rate of thymidine incorporation threefold, and increased cell numbers by 40% (Wilson et al., 1993).

The response of skeletal tissue to mechanical forces has been known for some time. The primary response of these tissues manifests as bone remodeling (Ramfjord and Enlon, 1971; Curtis et al., 1991). While this process of remodeling is not well understood at the biochemical and cellular levels, it is recognized that the changes produced in the macroscopic bony architecture provides the tissue the ability to better withstand the applied loads (Wolff, 1892; Currey et al., 1984).

The application of compressive forces to long bones in culture has resulted in a 50% increase in glucose consumption, increased thymidine incorporation and decreased extracellular fluid space by as much as 8% (Rodan et al., 1975). Similar studies by Klein-Nulend and co-workers demonstrated that compressive forces applied to growth plate cartilage significantly increased cartilage calcification. Furthermore, these studies revealed that intermittent compressive force was twice as effective as continuous force in promoting growth plate calcification (Klein-Nulend et al., 1980).

These studies confirm that skeletal tissues will respond to mechanical loading both *in-vivo* and *in-vitro*. However, the intracellular events that transform physical stimulation to biochemical events are not well known. Cytoskeletal alterations, strain sensitive ion channels, second messenger systems and cytokine release, among others, have all been suggested as possible mediators of these events, but substantial evidence for most of these is still lacking.

MECHANISMS OF RESPONSE OF BONE CELLS TO MECHANICAL LOADING

The events immediately subsequent to the mechanical perturbation of a cell are poorly understood. The intermediary mechanisms that could potentially play a role in mechanotransduction are numerous. Local membrane events, such as strain-sensitive ion channels, have been suggested (Guharay et al., 1984; Lansman et al., 1987). Others have linked the transduction events directly to perturbation of the cytoskeleton, which in turn leads to the activation of second-messengers systems such as cAMP and inositol phosphate (Sachs, 1989; Komuro et al., 1991; De Groot et al., 1990; Olesen et al., 1988; Lansman et al., 1987; Watson, 1990; Ryan, 1989). It is possibe that these ultrastructural events lead to a signaling cascade that ultimately results in changes in DNA synthesis and the production of proteins involved in tissue remodeling.

An understanding of the cellular and molecular mechanisms that enable cells and tissues to adapt to mechanical alterations of their environment is central to the practice of orthodontics. Compressive and tensile forces applied to the crowns of teeth are transmitted to the surrounding alveolar bone, and the bone responds to these forces by remodeling. The remodeling process allows for a permanent relocation of the teeth into a more functional and esthetic relationship. Traditionally orthodontists have relied on the pressure/tension hypothesis, in which bone is resorbed in sites of pressure and deposited in areas of tension, to explain this phenomenon.

More recently, an attempt has been made to understand the underlying biochemical mechanisms of bone remodeling in response to orthodontic forces. Arachidonic acid and its major metabolites, the prostaglandins and leukotrienes, have been shown to influence mechanically induced bone remodeling in a number of models. Based on Harell's earlier work, Yamasaki and co-workers (1950) injected prostaglandins into the periodontal ligament space of rats. The rate of orthodontic tooth movement increased dramatically, as did osteoclast formation. These effects were diminished by the injection of indomethacin, a potent inhibitor of prostaglandin synthesis. Blocking prostaglandin synthesis through the

use of cyclooxygenase inhibitors has resulted in reduced bone remodeling in long bone fractures as well (Sudman et al., 1979).

The recent focus on cytokine research has shown that any number of locally produced soluble factors may influence bone and connective tissue remodeling, including but not limited to the interleukins, tumor necrosis factors, interferons, polypeptide growth factors, and colony stimulating factors. The immunolocalization of IL-1α and IL-1β in the periodontal ligament space of cat teeth undergoing orthodontic force application has provided strong evidence of the role of cytokines in these events (Davidovitch et al., 1988).

TRANSFORMING GROWTH FACTOR-β (TGF-β) AND BONE REMODELING

TGF-β belongs to a family of polypeptides that includes the activins, inhibins and bone morphogenetic proteins. It was discovered more than 15 years ago as a product of murine sarcoma virus-transformed cells. Since that time it has been found that nearly all cells produce TGF-β in one isoform or another, and that almost all normal cells possess receptors for this family of peptides (Massague et al., 1987; Delarco et al., 1978).

There are several well-defined isoforms of TGF-β and are designated numerically as TGF-β1 through TGF-β5. TGF-β1 and TGF-β2 are produced in many cell types, while TGF-β3 is expressed mainly within cells of mesenchymal origin suggesting a different role for this protein than for TGF-β1 or β2. To date, TGF-β4 has only been detected in chick embryo chondrocytes, while TGF-β5 has been found in *Xenopus* embryos and in some adult tissues. A heterodimer, TGF-β1.2, has been identified in porcine platelets. The most common isoforms, TGF-β1 and TGF-β2, are found in the highest concentrations in human or porcine platelets and in mammalian bone (Roberts and Sporn, 1992; Lyons and Moses, 1990; Derynck et al., 1988).

Sequencing of the cDNAs of TGF-β indicates that it is initially synthesized and secreted as part of a larger precursor molecule that is biologically inactive. Proteolytic cleavage of the precursor is mediated by a subtilisin-like protease, resulting in a

noncovalently-bound complex consisting of a dimer of both the precursor remainder and the mature TGF-β. The mature TGF-β can be released from this complex through a variety of treatments *in-vitro*, however the mechanisms of *in-vivo* activation is not yet known. The mature TGF-β is a multifunctional, highly conserved disulfide-linked dimer consisting of two identical subunits. The active polypeptide has an apparent molecular weight of 25 kDa (Roberts and Sporn, 1992; ten Dijke et al., 1988; Jakowlew et al., 1988; Derynck et al., 1988; Kondaiah et al., 1990; Derynck et al., 1985).

The actions of TGF-β are mediated by binding to cell surface receptors. Five TGF-β receptors have been identified and are labeled numerically as Types I-V (Cheifetz et al., 1986; Massague et al., 1990). All receptor types are co-expressed on most cell types, with the exception of Type IV which is found on pituitary cells only (Segarini, 1990 & 1991). The production of TGF-β resistant mutants and the restoration of TGF-β sensitivity by complementation shows that Type I and Type II recptors are the primary mediators of the biological response to TGF-β (Boyd and Massague, 1989; Laiho et al.,1991).

The broad range of activities of TGF-β include growth and differentiation of many tissues, morphogenesis, embryogenesis and chemoattraction. It may act as a promoter or inhibitor of cell growth depending upon the cell type, assay conditions, the state of cell differentiation and the presence of other growth factors (Bonewald et al., 1990). It is considered an important mediator of extracellular matrix formation, generally stimulating matrix formation and inhibiting degradation (Sporn et al., 1987; Sporn and Roberts, 1990). It directly affects the differentiation and proliferation of osteoblasts and inhibits osteoclast activity, making it a major contributor to the cellular control of bone remodeling (Sporn et al., 1987; Sporn and Roberts, 1990; Mundy, 1990). In general, TGF-β1 and TGF-β2 are considered functionally equivalent in terms of biologic activity *in-vitro* (Sporn et al., 1987; Sporn and Roberts, 1990; Mundy, 1990; Rizzino, 1988; Graycar et al., 1990; Ridley et al., 1989; ten Dijke et al., 1990; Cheifetz et al., 1990)

Recent studies have shown that TGF-β1 and TGF-β2 may be important mediators of wound healing. It is released in wound sites by platelet degranulation, resulting in the recruitment of other effector cells, matrix synthesis and the secretion of other factors which, in combination with TGF-β, mediate angiogenesis and fibrosis. Its effects in bone and cartilage repair, suppression of immune response in autoimmune diseases and transplant rejection and in the moderation of ischemic damage following heart attacks (Roberts and Sporn, 1992; Sporn and Roberts, 1990; Sporn et al., 1983; Rosa et al., 1988; Mustoe et al., 1987; Lefer et al., 1990; Figari et al., 1990) support this hypothesis.

RATIONALE, HYPOTHESIS AND SPECIFIC AIMS

In a landmark experiment published in 1905, Sandstedt showed that force-induced tissue changes surrounding orthodontically treated teeth are limited to the PDL and its alveolar bone margin . Following three weeks of treatment it was observed that there was new bone growth in the stretched PDL, and bone resorption in the area of PDL compression (Sandstedt, 1904). This remodeling process has since been shown to be associated with various cytokines and soluble factors (Shanfeld et al., 1986; Ngan et al., 1990).

TGF-βs role in bone appositional events has been postulated (Sporn et al., 1987; Sporn and Roberts, 1990; Mundy, 1990; Rizzino, 1988; Graycar et al., 1990; Ridley et al., 1989; ten Dijke et al., 1990; Cheifetz et al., 1990). Because of the activation of osteoblast proliferation, differentiation and metabolic activity, and the inhibition of osteoclastic activity by TGF-β, it is likely that this growth factor contributes to the coupling of bone resorption and bone formation. It is also possible that one of the direct responses of bone related cells to mechanical forces is increased synthesis of TGF-β, which may in turn contribute to bone anabolic response and its increased ability to adapt to altered loads as proposed by Wolff. This concept, however, has not been examined to date. To begin addressing this concept, we hypothesized that the expression of TGF-β is specifically increased by PDL cells

subjected to mechanical loading. The purpose of these studies was to evaluate the changes in expression of TGF-β1 and TGF-β2 in cultured human periodontal ligament cells subjected to specific regimens of loading. The specific aims of this study were to:

1. Determine the effects of cyclic tensile mechanical forces on TGF-β1 and β2 expression in cultured human periodontal ligament (PDL) cells.

2. Conduct preliminary studies on possible cellular mechanisms for changes in levels of TGF-βs expressed by mechanically loaded PDL cells.

3. Evaluate whether alterations in catabolic activity accompany changes in levels of TGF-βs by assaying for specific matrix degrading enzymes, the matrix metalloproteinases.

B. MATERIALS AND METHODS

MATERIALS

All media and media supplements, including αMEM, fetal calf serum, penicillin/streptomycin, lactalbumin hydrolysate (LAH) and fungisone were purchased from Fisher Scientific (Pittsburgh, PA). Recombinant human TGF-β1 (rhTGF-β1) used as assay standards and mouse monoclonal anti-TGF-β1 courtesy of Rik Derynck (UCSF). Turkey anti-TGF-β1 was acquired from Collaborative Biomedical Products (Bedford, MA). Rabbit anti-turkey IgG obtained from Zymed Corp. (South San Francisco, CA). All immunocytochemistry reagents were from Vector Labs (Burlingame, CA). TGF-β2 ELISA kit from R&D Systems (Minneapolis, MN). Protein assay kit from Bio-Rad (Hercules, CA). Flexercell plates from Flexercell Inc. (McKeesport, PA).

RETRIEVAL, ISOLATION AND CULTURING OF PDL CELLS

Primary cell cultures of periodontal ligament (PDL) cells were established from healthy first premolars extracted for orthodontic reasons from four young adult patients. Teeth were extracted under sterile conditions from consenting patients and immediately placed in culture media (αMEM with 20% fetal calf serum [FCS] and 1% fungisone). Within half an hour of extraction tissues comprising the periodontal membranes were scraped from the apical two-thirds of the root surface with a sterile scalpel and placed in a 100mm polystyrene cell culture plate. A cover glass, held in place by sterile vacuum grease, was placed over these tissue explants in order to maintain contact between the tissue and the cell culture plate. Fresh media (αMEM with 20% FCS and 1% fungisone) was added and the explants incubated at 37^{o}C in humidified atmosphere with 95% air, 5% CO_2. This media was replaced every 2-3 days for approximately 2-4 weeks until; adequate numbers of cells were observed to be growing out from the explants. The plates were washed twice with PBS and the cells were trypsinized and re-seeded onto new plates. The

media supplement was also reduced at this time to 10% FCS and the fungisone replaced by 1% penicillin and 1% streptomycin. The cells were split at 90% confluence and repassaged through 3-4 passages.

Fourth or fifth passage cells were split into six well, collagen coated plates with a rubber base. The rubber bases of these plates are either supported on a hard plastic base (Flex II plates) which do not permit distortion of the base, or are unsupported (Flex I plates, Flexcell International. Corporation, McKeesport, PA) which are stretched when exposed to a vacuum from beneath. Eight plates each were seeded at 25,000 cells/well for both the experimental and control groups. The media was changed every 2-3 days until the cells were 90% confluent, when the cells were washed (x3) with PBS and the media was changed to a serum free media (αMEM supplemented with 0.2% LAH, penicillin and streptomycin). After a further 24 hours the plates were again washed with PBS, fresh serum-free media added, and the plates placed in the mechanical loading apparatus for the application of the prescribed magnitude and duration of tensile mechanical forces.

METHODS FOR APPLICATION OF MECHANICAL FORCES

Instrumentation:

The Flexercell Strain Unit FX-2000 (Flexcell International Corp., McKeesport, PA) was used to apply tensile mechanical forces to the cultured PDL cells. This system consists of a microprocessor regulated series of valves and baffles (Figure 1a) connected via tubing to a rubber gasket designed to house specially designed plates (Figure 1b). The culture plates are comprised of six wells each with a deformable rubber base. Control plates have the same cell culture surface as experimental plates attached above a non-deformable plastic base. The microprocessor can be programmed to apply a wide spectrum of frequency and magnitude of vacuum pressure to the base of the plates, stretching the deformable bases in the process, and in turn, the cells attached to the substrate. The cell cultures are maintained in the incubator at 37^{o}C with 5% CO_2 and 95% air.

Loading regimens

For this study, two regimens were utilized: a high magnitude (20% elongation), low frequency (6 cycles/hour) deformation; and a low magnitude (5% elongation), high frequency (6 cycles/minute) deformation. All trials were carried out over a period of 72 hours, interrupted briefly at regular intervals throughout the experimental period for collection of the conditioned media and matrix-cell extract samples at 6, 12, 24, 36, 48 and 72 hours. The vacuum applied to the base of the plates results in a deformation of the elastic culture surface (Figure 2).

Preliminary studies

Since culture plates are available precoated with four substrates, namely Type I collagen, elastin, amino groups and carboxyl groups, initial studies were done to determine the best available substrate on which to plate the PDL cells. Periodontal ligament cells were cultured on each substrate and maintained with αMEM/10% FCS in an humidified incubator. After 24 hours the cells were thoroughly washed with PBS and the media was changed to a non-serum supplemented media (αMEM/0.2% LAH). The cells were evaluated subjectively at 12, 24 and 48 hours for uniformity of distribution and adherence to the culture surface as well as phenotypic appearance. Each plate was videoimaged at the beginning and end of the trial (Figure 3).

CHEMICALLY-INDUCED ALTERATION IN CELL SHAPE

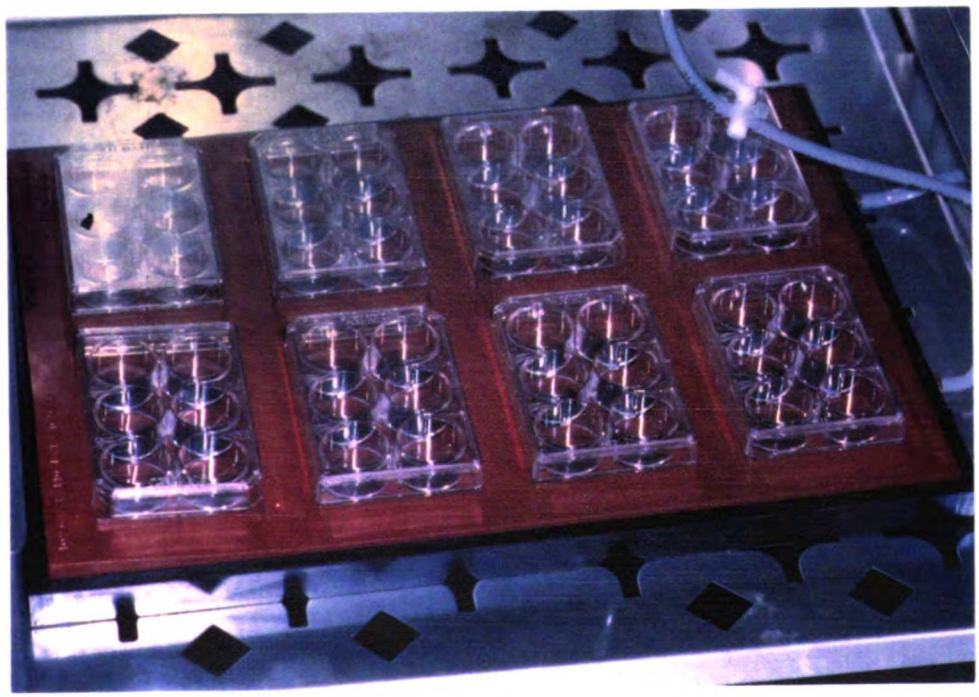
It has been proposed that the transduction of mechanical perturbation into cellular response is mediated by cytoskeletal alterations (Aggeler et al., 1984; Allan et al., 1980; Bissell et al., 1982; Folkman et al., 1978). Cytochalasin B, an agent that alters the shape of the cell through binding and disruption of the cytoskeleton, was utilized to examine the effects of cytoskeletal alteration on TGF-β expression.

Third passage PDL cells were cultured in 100mm cell culture plates as previously stated. The media was changed every 2-3 days until the cells were 90% confluent, when

a

Figure 1: Microprocessor (a) and plate assembly (b) of Flexercell apparatus.

b



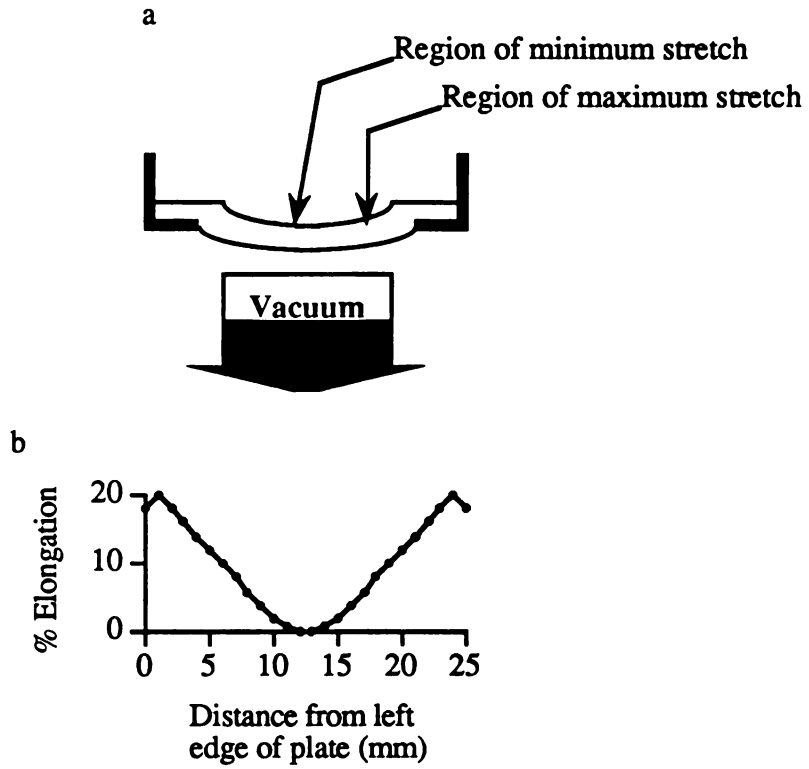


Figure 2: Vacuum application of tensile mechanical forces in Flex I cell culture plate (a) and graphic representation of non-uniform distribution of force across the well (b).

the cells were washed (x3) with PBS and the media was changed to a serum free media (αMEM supplemented with 0.2% lactalbumin hydrolysate [LAH], penicillin, and streptomycin). After a further 24 hours the plates were again washed with PBS, fresh serum-free media added, and 5 μg/ml of Cytochalasin B (Fisher Scientific, Pittsburgh, PA) added to two plates. Two plates had no additional supplements and were used as controls. Conditioned media samples were collected as described below and stored at -70°C until assay.

SAMPLE COLLECTION

PDL cell-conditioned media and matrix-cell extract were retrieved at 6, 12, 24, 36, 48, and 72 hours from 3 to 4 experimental and control wells each and from the cytochalasin B experiments. Prior to collection, gelatin (final concentration 100 μg/ml), pepstatin A

(final concentration 2μg/ml), and PMSF (final concentration 120 μg/ml) were added to the conditioned media in order to prevent protein degradation and binding of TGF-β to the wall of the storage vessel. Cell and extracellular matrix extract was retrieved with the use of a cell scraper following solubilization by 200 μl/well of 50mM Tris/1% SDS. All samples were stored at -70^{O} C until assay.

TOTAL PROTEIN ASSESSMENT

Total protein content was determined by use of a commercially available assay compatible with phenol present in the conditioned media (Bio-Rad, Hercules, CA) and by using BSA in SFM as a standard. 160 μl of each sample and standard were mixed with 40 μl of dye solution and incubated in a 96 well plate for at least 5 minutes. The optical density was read in an automated plate reader (ICM, Costa Mesa, CA) at a wavelength of 595 nm. Total protein concentration for the unknown samples was determined from the standard curve.

Since gelatin was added to the conditioned media prior to sample collection, it was necessary to determine the effects of this addition the total protein assessment. This was done by comparing the OD of blank serum-free media with that of serum-free media containing 100 mg/ml of gelatin. Addition of gelatin did not affect the color development with this particular assay and demonstrated similar OD as the non-gelatin containing blank serum-free media.

QUANTIFICATION OF TGF–β

Acid activation

Since the biologically inactive form of TGF-β is not recognized in most immunodetection techniques, and because TGF-β is secreted in its inactive form, it was necessary to acid activate all samples prior to assay. For each 100μl of conditioned media, 10 μl of 1.2N HCl was added, the samples vortexed and incubated in a 37^{O}C water bath

for 20 to 30 minutes, following which the samples were neutralized with 20 µl of neutralizing buffer (1.44N NaOH/1.0 M HEPES).

ELISA

TGF-β1: 100 µl/well of 1.0 µg/ml mouse anti-hTGF-β1 in coating buffer (0.05M Na_2CO_3, pH=9.6) were incubated in a 96 well ELISA plate (Costar, Cambridge, MA) with gentle agitation at 4°C for 20 to 24 hours. The wells were washed with a wash buffer (PBS/0.05% Tween 20) three times and blocked for 2 hours with 0.5% BSA in PBS (with 0.05% Tween 20, 0.01% Thimerosol). The assay diluent was removed and 100 µl/well of activated rhTGF-β1 standards (62.5 pg/ml to 4000 pg/ml) or activated samples were placed in the wells and incubated for 2 hours. Following the removal of the samples, 100µl/well of the binding antibody, 4.5 µg/ml turkey anti-TGF-β1 in binding buffer (100mM Tris-HCl, 10mg/ml BSA, 0.05% Tween 20, 150mM NaCl, 0.02% NaN_3), were added and incubated for 1 hour. The binding antibody solution was removed and 100 µg/ml of the signaling antibody (1:2000 Horseradish peroxidase [HRP] conjugated rabbit anti-turkey IgG in 100mM Tris-HCl, 20 mg/ml BSA, 2.5% rabbit serum, 0.05% Tween 20, 150mM NaCl, and 0.02% NaN_3) were added and incubated for 1 hour. The signaling antibody solution was removed and 100 µl/well of the substrate solution (OPD, 3% v/v H_2O_2, 0.1M Citric acid, 0.2M Na_2HPO_4) were added and incubated for 30 minutes. The color reaction was halted with 100µl/well of 4.5N sulfuric acid and the plate was read in an automated plate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 490 nm. All samples were incubated at room temperature with gentle agitation unless otherwise indicated. Each well was thoroughly washed 3-5 times with wash buffer between each step.

TGF-β2: All samples were acid activated as stated above and assayed with a commercially available TGF-β2 ELISA according to the manufacturers instructions (R&D Systems, Minneapolis, MN). Activated rhTGF-β2 standards (31.3 pg/ml to 2000 pg/ml) or activated samples were placed in wells which had been pre-coated with the coating

antibody and incubated for 2 hours, following which the contents of the wells were removed and each well washed thoroughly. Two hundred µl of a solution containing an enzyme conjugated binding antibody was then added to each well and incubated for 2 hours, followed by a thorough wash of each well. One hundred µl of substrate solution was added to each well and incubated for 20 minutes and the color reaction halted with 50 µl of a stop solution and the absorption read on a plate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 405 nm.

Following subtraction of background levels, a standard curve was generated and compared to the unknown samples to calculate TGF-β1 and TGF-β2 content in pg/ml. All unknown values were multiplied by 1.3 to account for the dilution effects of acid activation.

IMMUNOCYTOCHEMISTRY

Immunolocalization of TGF-β1 was done to determine if expression of these growth factors may be related to magnitude of loading, since the magnitude of strain varies along the cross-section of the well (Figure 2). Flexercell plates were subjected to the same loading regimens as described previously. The cell-conditioned media was collected and cell monolayers washed twice with TBS, and fixed permeabilized with 0.5% paraformaldehyde/0.1% Triton X100 for 15 minutes at 4^{o}C. The cells were then blocked with diluted horse serum in TBS for 30 minutes. Following another wash with TBS, the cells were incubated with a 1:100 dilution of mouse anti-TGF-β1 in TBS or 1:1000 non-immune mouse IgG as a control for 1 hour. Biotin conjugated horse anti-mouse secondary antibody was added and incubated for 1 hour. The cells were then incubated with a solution containing streptavidin conjugated alkaline phosphatase for 45 minutes. Following repeated washings in AP buffer, the cells were incubated in a substrate solution of p-Nitro blue tetrazolium chloride (NBT) / 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphatase buffer with 5mM levamisole until the appropriate color

reaction was observed under the microscope. The reaction was halted with dilute EDTA after approximately 15 minutes. All incubations were done at room temperature with gentle agitation unless otherwise indicated. Each well was washed three times with TBS before proceeding to the next step. The elastic Flexcell bases were released from the plastic supports with a sharp scalpel and mounted with an aqueous mounting media on glass microscope slides and photomicrographed.

SUBSTRATE ZYMOGRAPHY

In order t o evaluate whether changes in levels of TGF-β did indeed reflect a potential matrix anabolic response rather than an increased turnover of matrix macromolecules, we did preliminary studies to evaluate any alterations in secreted proteinases by gelatin substrate zymography.

Cell conditioned media from 6 wells each of control and 20% strain / 6cph were electrophoresed at 15ºC on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) containing 2 mg/ml of gelatin as described previously (Heussen and Dowdle, 1980; Chin et al.., 1985). After electrophoresis, the SDS was removed by washing the gels in 2.5 % Triton X-100 for 30 minutes with one change of washing media. The gelatin gels were incubated at 37ºC for 20 hours in incubation buffer (50 mM Tris-HCl buffer, pH 8, 5 mM $CaCl_2$, 0.02% NaN_3). The optimum time of incubation was determined from enzyme-substrate kinetic studies as described below. The gels were stained with 0.5% Commassie blue and destained in 10% acetic acid and 40% methanol until proteinase bands were clearly visible. Proteinase bands were further characterized by incubating zymograms in incubation buffer containing 0.3 mM 1,10-phenanthroline, a metalloproteinase inhibitor.

Enzyme-kinetic profiles were obtained by running gelatin gels with serial dilutions of a sample demonstrating high proteinase activity. This was done to determine the optimum time-of-incubation for the levels of gelatinolytic activities observed in our samples so that quantitative comparisons of gelatinolytic activities could be made between

conditioned media from control and mechanically loaded cells. Serial dilutions of a sample of synovium conditioned media (courtesy, S. Kapila) demonstrating high proteinase activities were electrophoresed in 10% gelatin-impregnated SDS-PAGE gel as described above, and incubated for 6, 20 or 44 hours, and stained and destained in a standardized manner. Images of the gels were video-digitized by a CCD camera (NEC T1-24A, Japan) and image software (Image Version 1.42, NIH, Bethesda, MD). The imaging of the gels were standardized by capturing them at the same focal length and exposure, and the intensity and area of gelatinolytic activity was quantified. Video-densitometric analysis revealed that, for the level of proteinase activities in our samples, 20 hours of incubation provided a log-linear gelatin degradative activity profile (Fig. 1b), indicating this as the optimum incubation time for quantitation of gelatin gels. The undiluted conditioned media was run in one lane of every gel done subsequently on experimental and control conditioned media to ensure that none of the samples exceeded this proteinase activity, and to help standardize for inherent variabilities between gels. The gelatinolytic activity of these samples was determined as described above by digitization followed by densitometry of proteinase activities.

STATISTICAL ANALYSIS

All experimental total protein and TGF-β concentrations were compared to their corresponding control values with a statistical software program (StatView 4.0,.Abacus Concepts, Berkeley, CA) using multiple comparisons analysis of variance (ANOVA). Where ANOVA demonstrated significant differences, a multiple comparisons test, Fishers PLSD, was used to evaluate the time points at which the control and experimental levels of TGF-β1 and β2 were significantly different. Data from gelatin zymograms and Cytochalasin B studies were analyzed using a Student's t-test. The statistical significance levels for all analyses were set at $p<0.05$.

C. RESULTS

CELL CULTURE SUBSTRATE SELECTION

PDL cells cultured on various substrates were evaluated on the basis of uniformity of distribution, phenotypic appearance and substrate adherence. These characteristics were determined to be optimal on both collagen Type I and elastin coated plates both in serum containing and serum-free media (Figure 3). We opted to use the type I collagen coated plates rather than the elastin for all subsequent experiments since the natural matrix environment of PDL cells is richer in type I collagen than in elastin (Hou et al., 1993; Uitto et al., 1992).

TOTAL PROTEIN ASSESSMENT

For both regimens of loading no statistically significant difference in total protein concentration was found between conditioned media from the non-strained (control) and strained (experimental) PDL cells (Table I). In general, the total protein content in the conditioned media from both the experimental and control cells increased approximately 1.5 to 2 fold over the 72 hour experimental period (Figures 4). The variability in protein concentration, as reflected by the standard deviations, was slightly greater in the experimental versus the control conditioned media. The total protein content in the high magnitude / low frequency loading regimen (20% strain and 6 cph) was approximately twice that of the low magnitude / high frequency regimen (5% strain and 6 cpm) at all time points in both the experimental and control conditioned media samples.

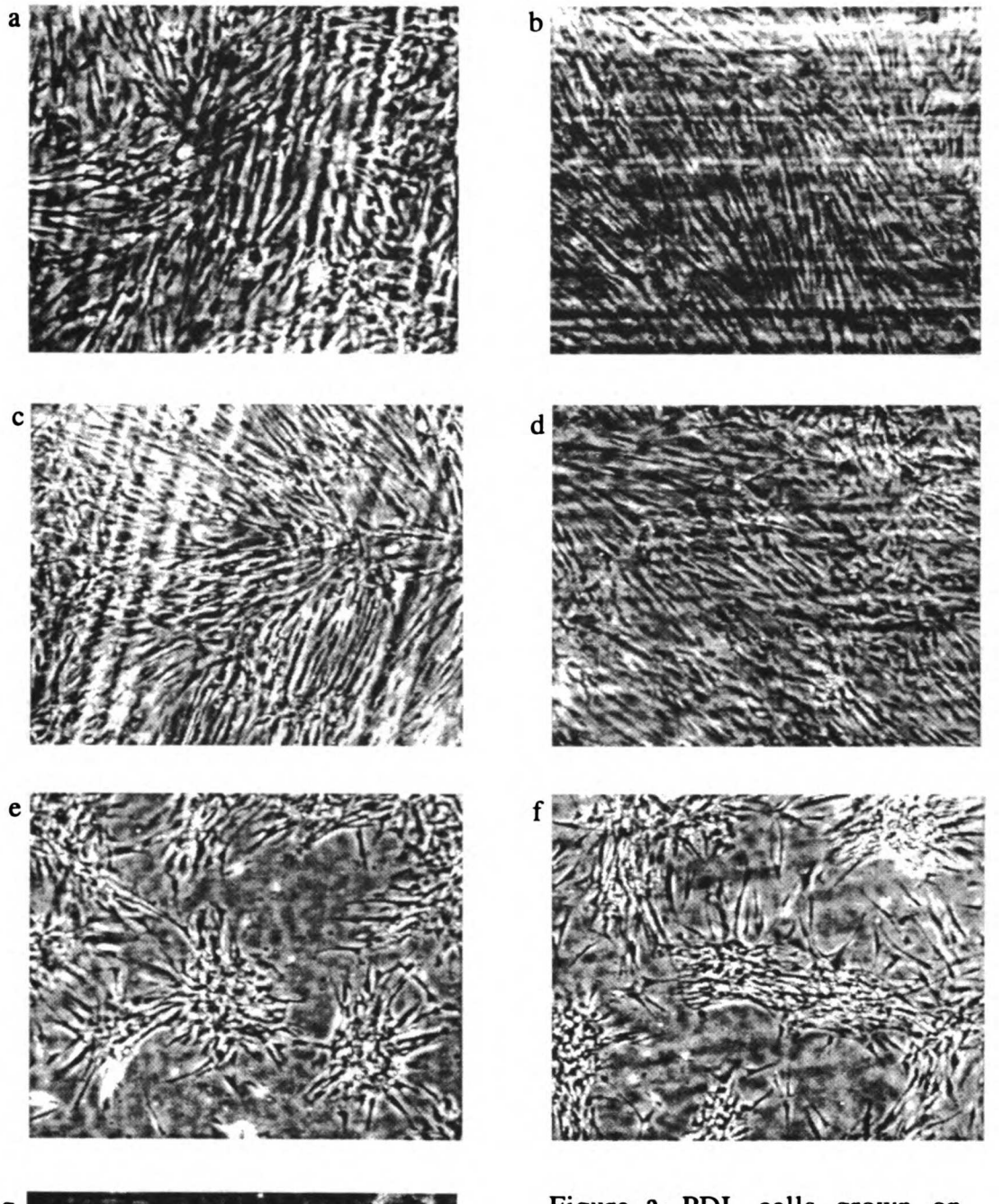


Figure 3: PDL cells grown on various Flexcell substrates with serum supplemented media (a, c, e and g) and after 48 hours in serum-free media (b, d and f). Substrates are collagen I (a and b), elastin (c and d), amino terminal (e and f), and carboxyl terminal (g). The cells grown on the carboxyl substrate were contaminated after 48 hours and are therefore not represented here.

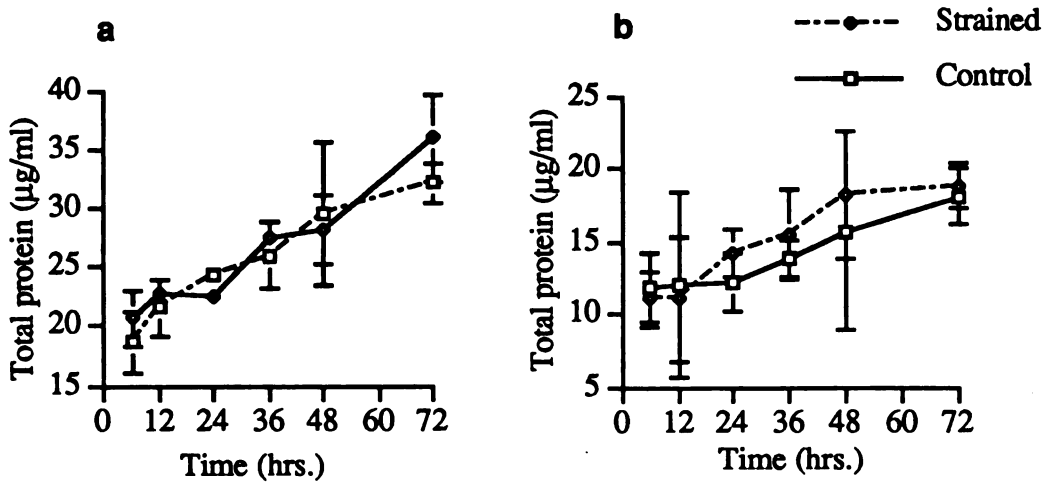


Figure 4: Total protein content in the conditioned media of cultured PDL cells when subjected to 20% strain at 6 cph (a), and 5% strain at 6 cpm (b).

Table I: Means ± SD of total protein content from the conditioned media of PDL cells subjected to 20% strain at 6cph and 5% strain at 6cpm.

	20% Strain, 6cph Total protein (µg/ml)			5% Strain, 6cpm Total protein (µg/ml)		
Time (hrs)	Control	Exper.	P-Value	Control	Exper.	P-Value
6	20.7± 2.4	18.7± 2.6	0.37	11.8± 2.4	11.1± 1.9	0.82
12	22.7± 0.3	21.6± 2.3	0.65	11.8± 6.4	11.7± 4.2	0.72
24	22.5± 0.7	24.4± 0.3	0.39	12.2± 2.0	14.2± 1.7	0.51
36	27.6± 0.6	26.0± 2.8	0.41	13.8± 1.4	15.5± 3.1	0.58
48	28.2± 3.0	29.5±6.1	0.57	15.7± 6.9	18.2± 4.4	0.06
72	36.1± 3.7	32.2± 1.7	0.11	18.1± 1.9	18.8± 1.6	0.81

MODIFICATION AND OPTIMIZATION OF TGF-β1 ELISA

In order to determine the TGF-β1 content in the conditioned media we modified and optimized an ELISA for TGF-β1 from that developed previously by Danielpour (1993). This involved the selection of the solid-phase, determination of optimal concentrations for coating antibody, optimization of buffer, pH and other assay conditions, and reduction of background (Kemeny, 1991).

Choice of solid phase: The choice of microtitre plate is essential to the success of the assay, as failure to bind at this initial step guarantees failure of all subsequent steps. The three basic requirements for a suitable solid-phase are:

1. The coating material should actually bind to the plate.
2. Having bound it should not fall off.
3. Having bound firmly it should retain as much of its immunological activity as possible.

For these reasons we opted to use the microtitre plates recommended for ELISAs (Costar, Cambridge, MA), which provide most of these features. Furthermore, because the capacity of most microtitre plates to bind protein is limited, the most variable aspect of the assay is the ability to optimally coat the plate. This is largely dependent on the concentration of coating antibody used to coat the wells. At lower concentrations of coating antibody inadequate amounts or uneven binding of the antibody may result. On the other hand, at higher concentrations there is a greater tendency for the antibody molecules to bind with one another rather than to the plate. The range of coating antibody concentrations at which there is no interference with binding to the plastic surface is called the *zone of independent binding*. The optimal concentration to use is the one that gives the steepest dilution curve. The approximate recommended concentrations of coating antibody in most ELISAs is 1 μg/ml (Danielpour et al., 1993; Kemeny, 1991). In order to determine the optimal coating antibody concentration for the TGF-β1 ELISA, a microtitre plate was coated with serial dilutions of the primary antibody ranging from 0 μg/ml to 8 μg/ml. The coated wells were then exposed to an HRP conjugated anti-mouse antibody (1:2000) and it's substrate (OPD, 3% v/v H_2O_2, 0.1M Citric acid, 0.2M Na_2HPO_4). The color reactions were measured on the plate reader and plotted as represented in Figure 5a. These findings indicate that the optimal binding for TGF-β1 coating antibody is greater than 0 μg/ml but at or less than of 1 μg/ml.

In order to further titer the optimal concentration of coating antibody, dilution curves were derived from an ELISA using rhTGF-β1 and two concentrations of anti-TGF-β1 coating antibody. Microtitre wells were coated either with 0.5 μg/ml or 1 μg/ml of TGF-β1 coating antibody, washed, serial dilutions of rhTGF-β1 added and immunodetected as described previously (page 15). The results suggest that the TGF-β1

coating antibody concentration of 1 μg/ml provides a slightly steeper curve than the coating antibody concentration of 0.5 μg/ml (Figure 5b). As a result of these two experiments, 1 μg/ml was selected for use in the coating of the microtitre plates in all subsequent experiments.

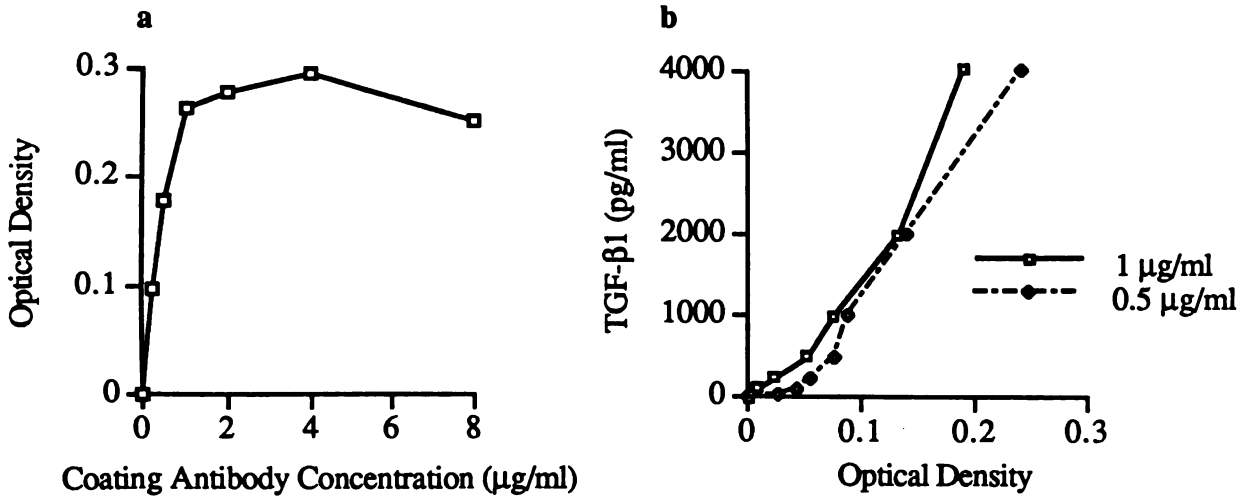


Figure 5: Binding of serially diluted anti-TGF-β1 coating Ab to microtitre wells (a), and dilution curves of 0.5 μg/ml and 1μg/ml anti-TGF-β1 coating Ab (b).

Choice of buffer and pH: The charge and hydrophobicity of the antibodies play an important role in proper binding. These variables are controlled by the pH of the buffer solution. For the purposes of this assay the coating buffer reagents (0.05M Na_2CO_3) and pH of 9.6 were selected on the advice of the provider of the coating antibody as well as data from Danielpour et al. (1993).

Assay conditions: Time and temperature for incubation are inversely related for adequate binding. The coating conditions of overnight incubation at 4°C were selected based on the recommendation of the antibody supplier. Subsequent room temperature incubations were based on the recommendation of Danielpour et al. (1993). There is relatively little range of variation for volume selection. However, since uneven coating is often a problem at the lower volumes, we utilized a volume of 100 μl for coating the wells.

Background levels: In the ELISA technique, as in most immunoassays, the greatest source of difficulty is with non-specific binding or short circuiting. Short circuiting arises when the reagents bind to each other as well as, or instead of, the sample. It is distinguished from non-specific binding in being a specific immune reaction. These undesirable reactions manifest as high background levels. Very high background levels were seen in the first generation of these assays, indicating non-specific binding or short-circuiting (Figure 6). To determine the source of the problem it was essential to isolate the undesirable reaction(s). Given the large number of variables within an ELISA, this difficulty was diagnosed by a systematic process of elimination. In this study a test matrix was designed in which each major reagent was independently eliminated and replaced with a sham reagent, namely PBS. Background levels were assessed and the problem was systematically isolated.

Table II indicates that the high background levels were probably due to non-specific binding of the signaling antibody. This test, however, did not determine to what the antibody was binding. There are several possible binding sites for the signaling antibody, namely the plastic surface of the microtitre plate, the coating antibody, the bound serum free media, or the binding antibody. We, therefore, further evaluated the potential reason for binding of the signaling antibody. As in the previous study, selected reagents were individually and systematically replaced by PBS. Table III indicates that when the signaling antibody and the coating antibody are present together, the background levels increase dramatically, suggesting a short-circuit or non-specific binding of these two elements.

In order to determine the assay conditions that would minimize these effects, a similar test matrix was carried out with variations in the composition of BSA and normal rabbit serum (NRS) in the buffer solution of the signaling antibody. The proportion of blocking sera was varied in these buffers while maintaining the composition of the other components (100mM Tris-HCl, 0.05% Tween 20, 150mM NaCl, and 0.02% NaN_3). The

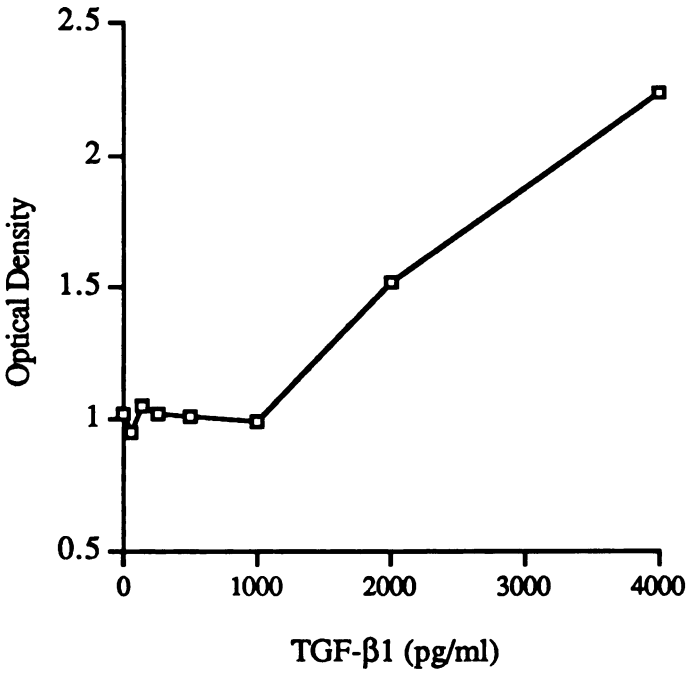


Figure 6: High background levels at lower sample concentrations of TGF-β1.

Table II: Selective sham substitution of major reagents with PBS demonstrate that elimination of the signaling antibody greatly reduces background levels. "Reagents" column lists the reactants used in that portion of the microtitre plate. C=coating Ab, M=serum free media, B=binding Ab, S=signaling Ab, Sb=substrate, 0=sham PBS substitution, OD=optical density.

Reagents	Mean (±SD) OD
C-M-B-S-Sb	.510 ± .05
C-O-B-S-Sb	.474 ± .06
C-O-O-S-Sb	.402 ± .02
C-O-O-O-Sb	.035 ± 0.0

Table III: Sham PBS substitution of major reagents, focusing on the signaling Ab reaction. C=coating Ab, M=serum free media, B=binding Ab, S=signaling Ab, Sb=substrate, 0=sham PBS substitution, OD=optical density.

Reagents	Mean (±SD) OD
C-M-B-S-Sb	.489 ± .05
C-O-O-S-Sb	.375 ± .04
O-O-O-S-Sb	.041 ± 0.0
C-O-O-O-Sb	.044 ± 0.0

buffers contained the following ceoncentration of sera: Buffer A-10 mg/ml BSA, 5% normal rabit serum; Buffer B-20 mg/ml BSA, 5% rabbit serum; Buffer C-10 mg/ml BSA, 2.5% rabbit serum; and Buffer D-20 mg/ml BSA, 2.5% rabbit serum. Table IV shows the results of this experiment. The findings suggest that the least background is obtained when the buffer contains twice the recommended concentration of BSA and half the recommended concentration of normal rabbit serum (buffer D). Figure 7 represents a standard dilution curve using activated rhTGF-β1 and the modified reagent concentrations, indicating that the background effects have been significantly reduced as a consequence of these modifications to the assay (compare to Figure 6).

Table IV: Effect on background levels of varying signaling Ab buffer reagents. A,B,C,D represent the various buffer conditions used. Buffer solution "A" is the recommended buffer. Buffer solution "D" had double the concentration of BSA and half the concentration of normal rabbit serum. C=coating Ab, M=serum free media, B=binding Ab, S=signaling Ab, Sb=substrate, 0=sham PBS substitution, OD=optical density.

	Mean (±SD) OD			
Reagents	**A**	**B**	**C**	**D**
C-O-O-S-Sb	375 ± .04	.111 ± .05	.061 ± 0.0	.048 ± .01
O-O-O-S-Sb	.041 ± 0.0	.042 ± 0.0	.041 ± 0.0	.040 ± 0.0
C-O-O-O-Sb	.044 ± 0.0	.043 ± 0.0	.043 ± 0.0	.044 ± 0.0

TGF-β1 AND β2 EXPRESSION BY STRAINED AND NON-STRAINED PDL CELLS

Findings on the levels of TGF-β1 and β2 are expressed both as total concentration in conditioned media (absolute concentration in pg/ml), as well as levels of these growth factors relative to total protein (relative concentration in pg/μg). A time-related increase in levels of both TGF-β1 and β2 was observed in conditioned media from both strained and non-strained PDL cells from 0 to 72 hours. Both the absolute and relative levels of TGF-β1 and β2 were substantially lower in both the non-strained and strained cells in the 20% strain / 6cph experiment than those in the 5% strain / 6cpm experiment. Significantly higher absolute and relative levels of TGF-β1 and β2 were found in conditioned media from strained versus non-strained cells at several time points under both loading regimens

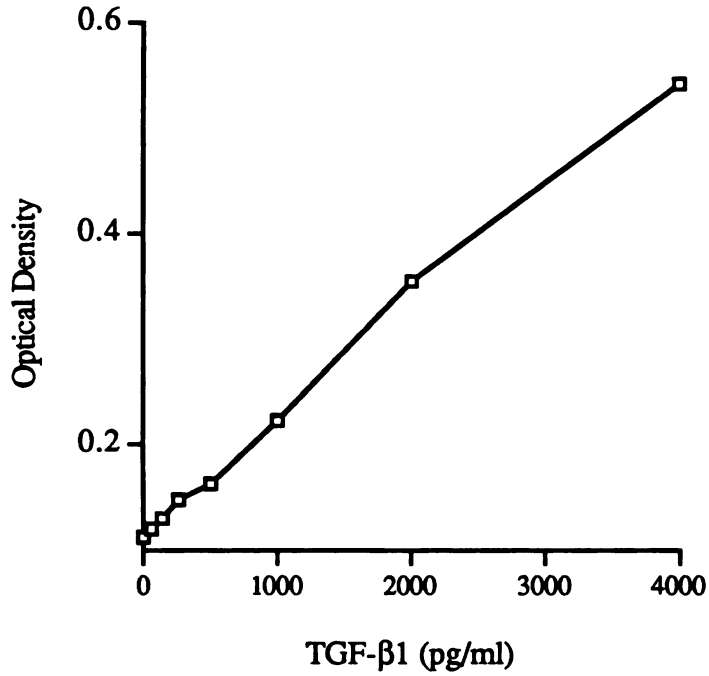


Figure 7: ELISA of standard serial dilution of TGF-β1 using the modified reagents as listed in the materials and methods section.

(Figures 8-11). In general the differences in secreted levels of TGF-βs were more marked between non-strained cells and cells exposed to the low magnitude / high frequency strain, than that between control cells and cells exposed to the high magnitude / low frequency regimen of loading.

TGF-β1

In the high magnitude / low frequency experiments, the increase in absolute levels of TGF-β1 in controls was approximately three-fold from 6-72 hours, while the levels expressed by experimental cells increased approximately four-fold. When standardized against the total protein expression the control levels increased only mildly after the 6-hour time point while the experimental levels more than doubled (Figure 8 a & b). While in general the experimental conditioned media samples had a higher absolute and standardized concentrations of TGF-β1 than control conditioned media, these differences were not significant until the 72 hour time point (Table V).

Upon exposure to the 5% strain / 6cpm regimen of loading the absolute levels of TGF-β1 increased by as much as eight-fold as compared to a five-fold increase for the non-strained controls from 6 to 72 hours of culture. When these figures are standardized against total protein the control levels approximately doubled, while the experimental levels increased approximately five-fold over this period of time (Figure 9 a & b). Statistically significant differences in TGF-β1 levels between control and experimental conditioned media in absolute concentrations are seen from 24 to 72 hours of culture, while the standardized data reveal significant differences at 12 and 72 hours (Table VI).

TGF-β2

The increase in absolute levels of TGF-β2 in the cells exposed to 20% strain was greater than 40-fold, while that in their non-strained controls was 12-fold from 6 to 72 hours of culture. When standardized against the total protein content the experimental levels increased approximately 30-fold, compared to the four-fold increase seen in the non-strained controls over the same period (Figure 10 a & b). While in general the experimental conditioned media samples had a higher absolute and standardized concentrations of TGF-β2 throughout the trial, these differences were not statistically significant until the 72 hour time point (Table VII). This finding is similar to that observed for TGF-β1 levels for cells subjected to the same loading regimen.

When the cells are subjected to 5% strain the TGF-β2 levels in the conditioned media increased more than 350-fold after the 12 hour time point, compared to approximately 40-fold for the non-strained controls. When standardized against the total protein content the levels of the controls increased approximately ten-fold while the strained levels increased more than 140-fold over the same period (Figure 11 a & b). Statistically significant differences between control and experimental conditioned media in the absolute concentration of TGF-β2 were noted after 24 hours of culture, and at the 24, 48 and 72 hour time points in the standardized samples (Table VIII).

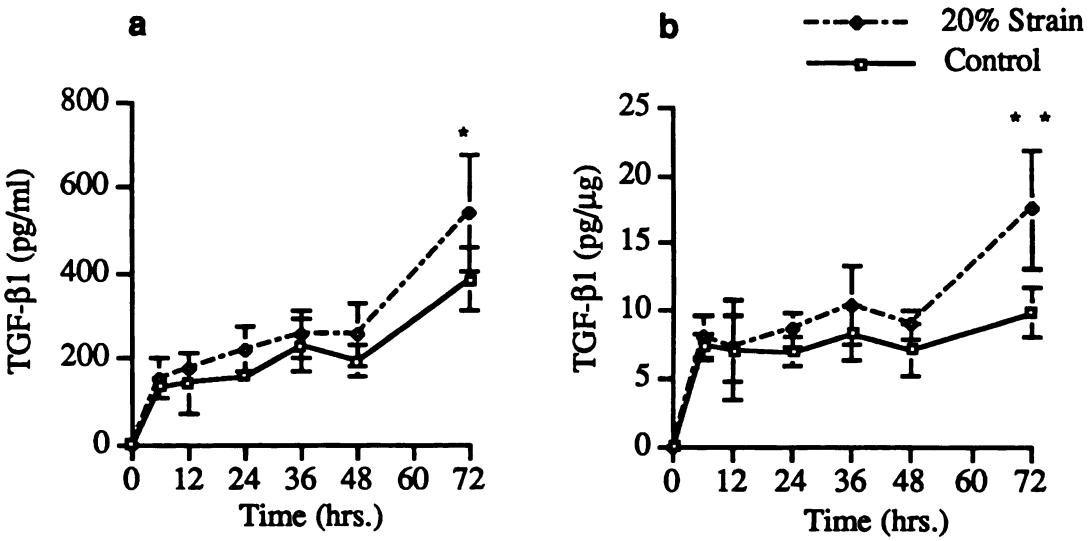


Figure 8: Absolute TGF-β1 concentration in the conditioned media of PDL cell cultures subjected to 20% strain at 6cph (a) and standardized against total protein content (b). *p<.01, **p<.0001.

Table V: TGF-β1 concentration (pg/ml; means ± SD) in the conditioned media of cell cultures subjected to 20% strain at 6cph and standardized against total protein content (pg/μg).

	TGF-β1 (pg/ml)			TGF-β1 (pg/μg)		
Time (hrs)	Control	Exper.	P-Value	Control	Exper.	P-Value
6	134.8 ± 6.8	154.7 ± 45.1	.71	7.5 ± 0.88	8.0 ± 1.6	0.76
12	142.8 ± 72.8	178.5 ± 24.8	.50	7.1 ± 3.7	7.3 ± 2.4	0.43
24	158.6 ± 18.2	222.2 ± 51.9	.23	7.0 ± 1.0	8.6 ± 1.2	0.41
36	229.9 ± 60.8	257.9 ± 54.6	.60	8.4 ± 2.0	10.5 ± 2.9	0.28
48	194.4 ± 38.3	257.9 ± 72.5	.23	7.2 ± 1.9	9.0 ± 1.3	0.34
72	383.9 ± 74.8	539.8±137.0	.006	9.9 ± 1.9	17.5 ± 4.4	0.0005

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Immunostaining of TGF-β1 in the cell monolayers showed an increased staining intensity within the cytoplasms of the stretched cells when compared to the non-stretched control cells . Low-power visualization indicated the majority of staining was within the cytoplasms of the mechanically perturbed cells, with very little staining in the extracellular matrix (Figure 12).

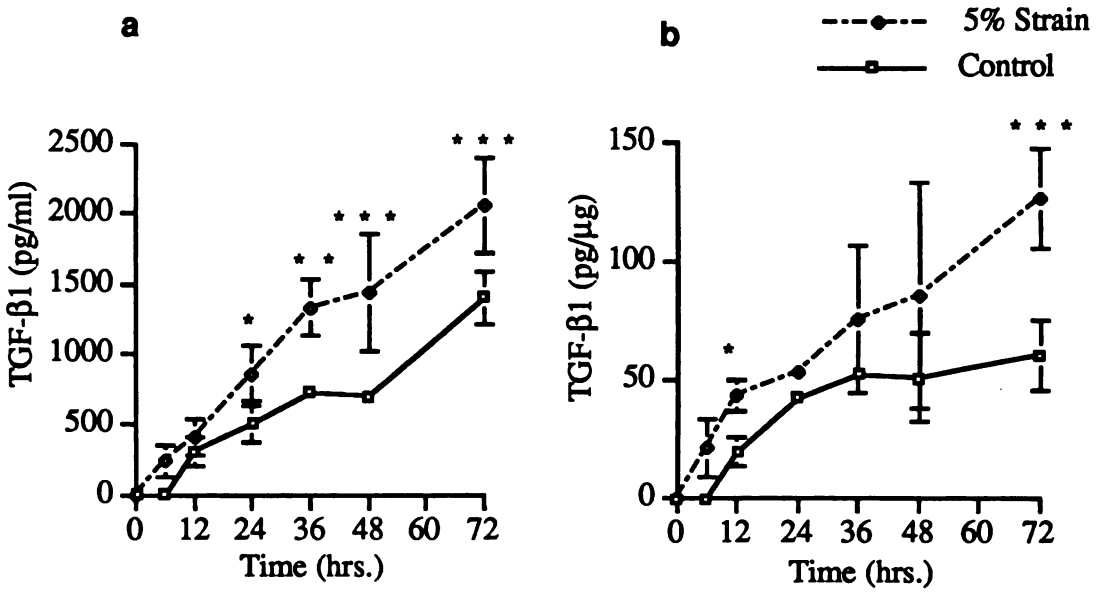


Figure 9: Absolute TGF-β1 concentration in the conditioned media of PDL cell cultures subjected to 5% strain at 6cph (a) and standardized against total protein content (b). *p<.05, **p<.001, ***p<.0001.

Table VI: TGF-β1 concentration (pg/ml; means ± SD) in the conditioned media of cell cultures subjected to 5% strain at 6cpm and standardized against total protein content (pg/μg).

	TGF-β1 (pg/ml)			TGF-β1 (pg/μg)		
Time (hrs)	Control	Exper.	P-Value	Control	Exper.	P-Value
6	0 ± 0	238.3 ± 118.9	0.16	0 ± 0	21.1 ± 12.2	0.09
12	308.9 ± 102.2	400.8 ± 129.8	0.58	19.8 ± 5.9	44.2 ± 6.6	0.04
24	503.8 ± 128.8	858.8 ± 199.4	0.02	42.8 ± 4.7	54.3 ± 5.6	0.35
36	729.2 ± 52.1	1333.5 ± 198.4	0.001	53.1 ± 4.2	76.3 ± 31.3	0.79
48	701.3 ± 49.3	1438.6 ± 410.3	0.0001	51.0 ± 18.9	85.7 ± 48.0	0.23
72	1409.0 ± 187.6	2058.0 ± 345.5	<0.0001	61.1 ± 14.9	127.0 ± 21.0	<0.0001

SUBSTRATE ZYMOGRAPHY

Conditioned media from both the control and mechanically stimulated cells demonstrated only one gelatinolytic band at approximately 72 kDa. Since this band was inhibited when similar gels were incubated in 1,10 phenanthroline, a metalloproteinase inhibitor, and because of its electrophoretic mobility, this gelatinolytic activity is likely that due to 72-kDa gelatinase. In addition to the little discernible levels of expressed 72-kDa

gelatinase in mechanically stimulated cells, no additional bands attributable to other proteinases commonly observed on gelatin zymograms, such as 92-kDa gelatinase or collagenase, were observed in conditioned media from experimental cells.

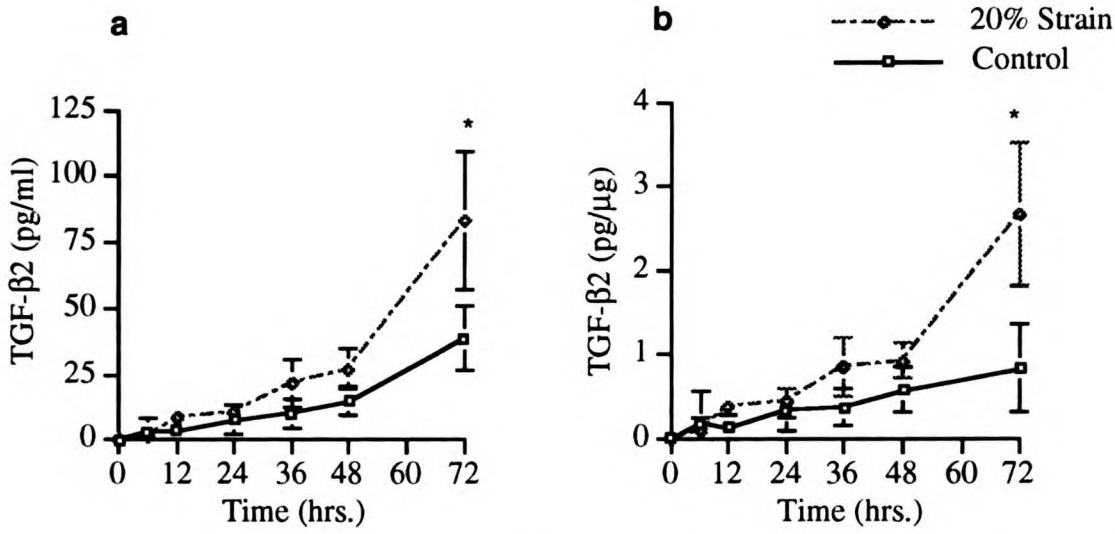


Figure 12: Absolute TGF-β2 concentration in the conditioned media of PDL cell cultures subjected to 20% strain at 6cph (a) and standardized against total protein content (b). *p<.0001.

Table VII: TGF-β2 concentration (pg/ml; means ± SD) in the conditioned media of cell cultures subjected to 20% strain at 6cph and standardized against total protein content (pg/μg).

	TGF-β2 (pg/ml)			TGF-β2 (pg/μg)		
Time (hrs)	Control	Exper.	P-Value	Control	Exper.	P-Value
6	2.9 ± 5.7	1.8 ± 3.5	.88	0.19 ± 0.37	0.08 ± 0.16	0.69
12	3.5 ± 4.3	8.4 ± 3.2	.49	0.14 ± 0.16	0.37 ± 0.15	0.38
24	7.5 ± 5.6	10.8 ± 4.4	.64	0.34 ± 0.26	0.43 ± 0.18	0.72
36	10.1 ± 5.8	21.9 ± 9.0	.10	0.37 ± 0.22	0.86 ± 0.35	0.07
48	14.7 ± 5.5	27.0 ± 7.2	.09	0.57 ± 0.27	0.93 ± 0.21	0.17
72	38.5 ± 12.3	82.9 ± 26.5	<.0001	0.83 ± 0.52	2.66 ± 0.86	<0.0001

TGF-β EXPRESSION IN RESPONSE TO CHEMICALLY-INDUCED ALTERATION IN CELL SHAPE

Following the exposure to Cytochalasin B (CB), the cells were observed to undergo dramatic changes in cell shape. The normally spindle shaped cells became stellate in appearance with extensive and numerous cytoplasmic projections. ELISAs performed

on the conditioned media samples indicated that both TGF-β1 and β2 expression is increased after 48 hours of incubation with CB (Figure 15). TGF-β1 levels in the experimental conditioned media (4446.1 pg/ml) more than doubled relative to control levels (1900.2 pg/ml), while TGF-β2 levels in the experimental conditioned media samples (414.1 ± 117.1 pg/ml) showed an approximate six-fold increase relative to the control levels (59.8 ± 29.3).

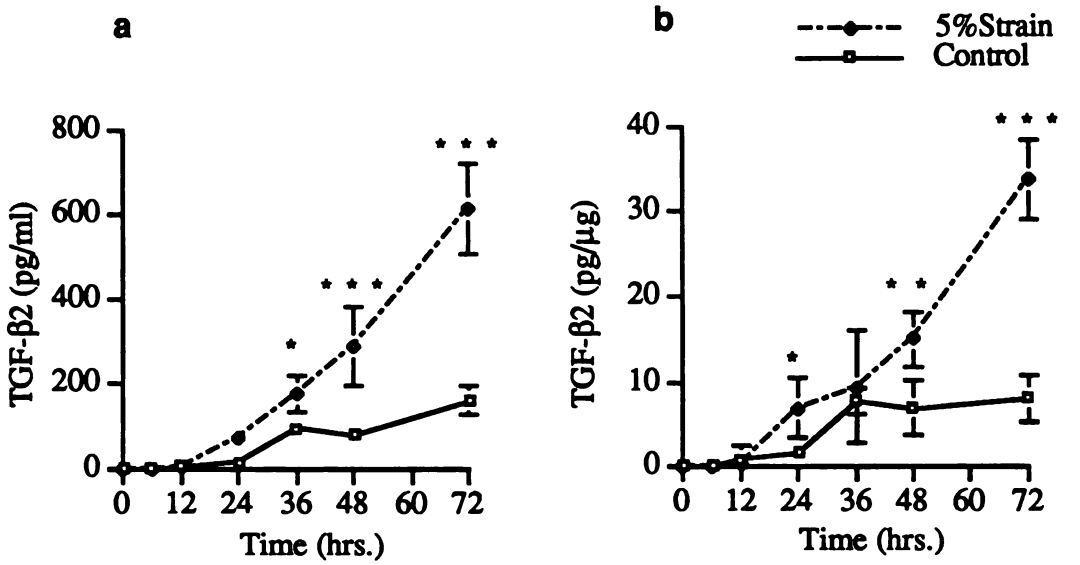
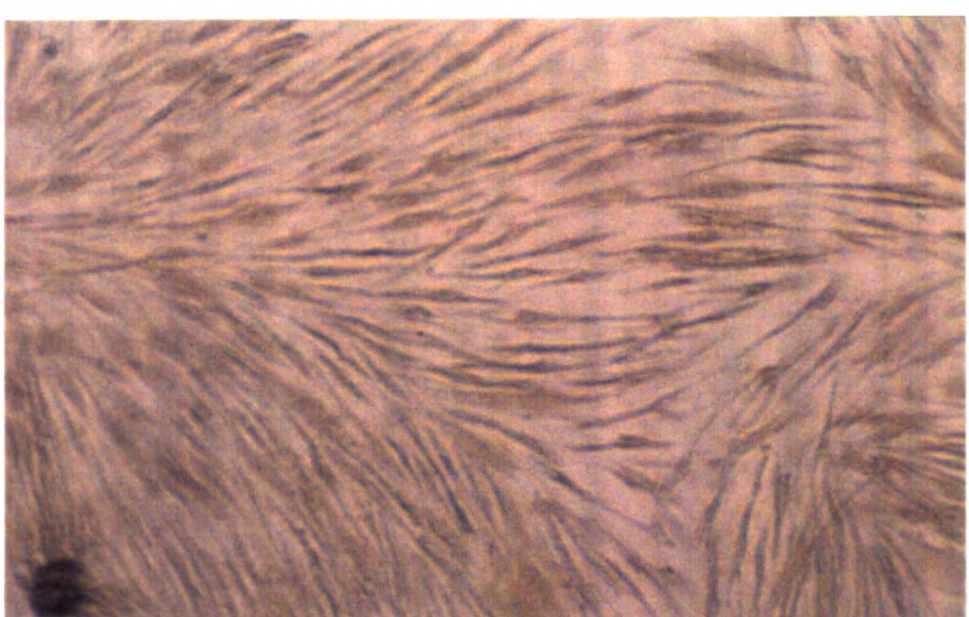


Figure 11: Absolute TGF-β2 concentration in the conditioned media of PDL cell cultures subjected to 5% strain at 6cpm (a) and standardized against total protein content (b). *p<.05, **p<.001, ***p<.0001

Table VIII: TGF-β2 concentration (pg/ml; means ± SD) in the conditioned media of cell cultures subjected to 5% strain at 6cpm and standardized against total protein content (pg/μg).

	TGF-β2 (pg/ml)			TGF-β2 (pg/μg)		
Time (hrs)	Control	Exper.	P-Value	Control	Exper.	P-Value
6	0	0	-	0	0	-
12	3.7 ± 4.9	1.7 ± 1.9	0.95	0.83 ± 1.5	0.23 ± 0.28	0.78
24	15.0 ± 8.6	71.6 ± 29.2	0.09	1.56 ± 0.4	6.9 ± 3.5	0.019
36	93.4 ± 14.6	176.8 ± 45.7	0.014	7.7 ± 1.6	9.4 ± 6.6	0.44
48	79.4 ± 11.6	291.1 ± 93.1	<0.0001	6.9 ± 3.2	14.9 ± 3.3	0.0006
72	160.8 ± 35.9	610.5±106.6	<0.0001	8.1 ± 2.8	33.7 ± 4.6	<0.0001

a



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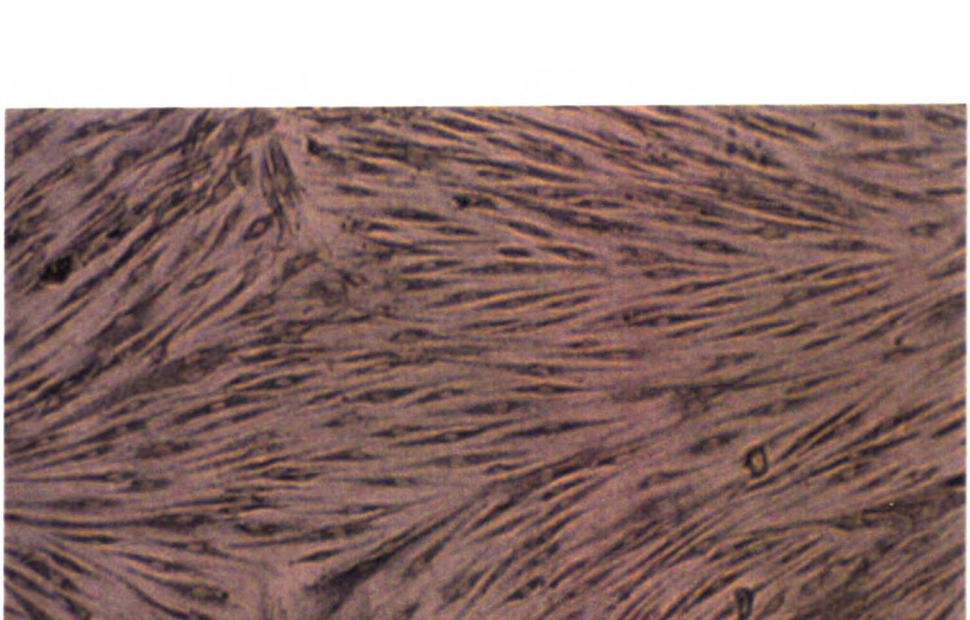

Figure 12: Non-strained control (a) and strained experimental (b) PDL cells immunochemically stained for TGF-β1.

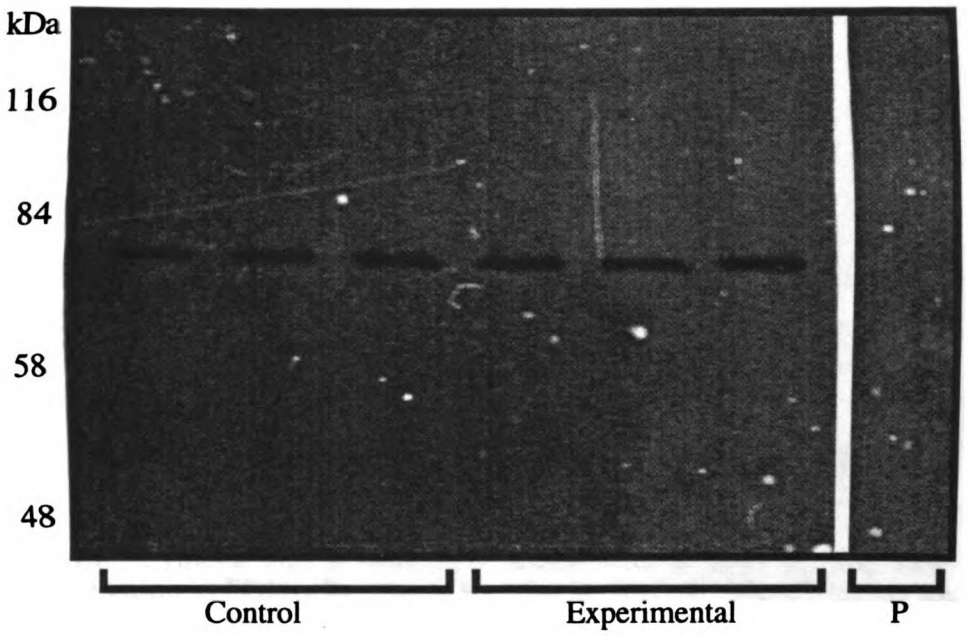

Figure 13: Negative image of gelatin substrate zymogram with conditioned media from control PDL cells and experimental cells subjected to 20% elongation at 6 cph. P represents lane from gel incubated with 1, 10 phenanthroline.

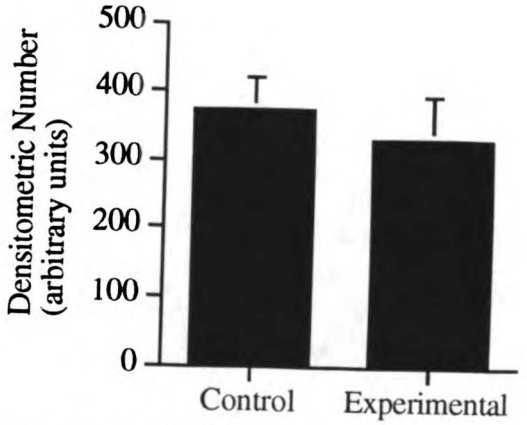

Figure 14: Densitometric analysis of zymogram from conditioned media of PDL cells subjected to 20% strain at 6cph. Control: 371.3 ± 47.2, Experimental: 331.6 ± 62.8; n = 6, p = .06.

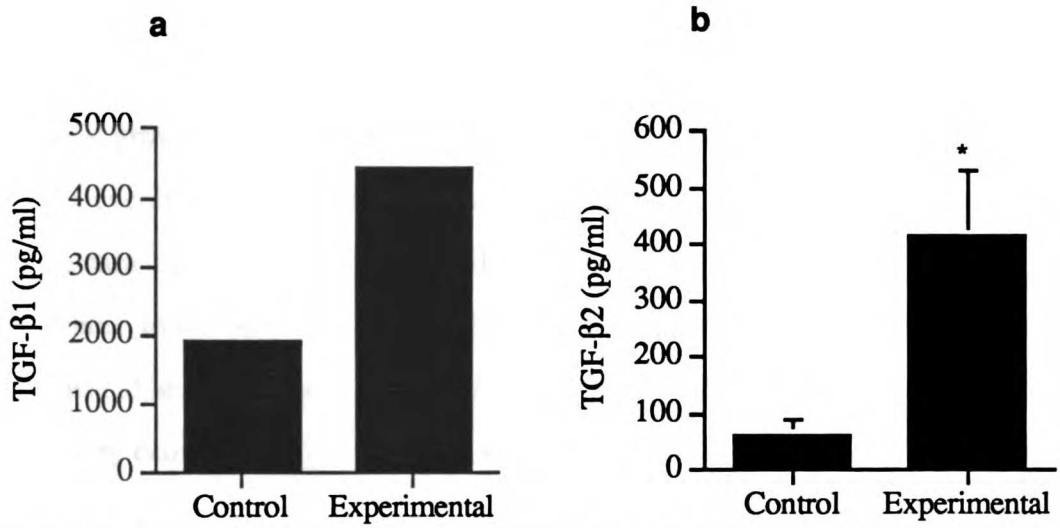

Figure 15: Cytochalasin B induced expression of TGF-β1 (a) and TGF-β2 {(mean ± SD),b} in cultured PDL cells (*p=.005).Data for TGF-β1 is preliminary and represents a sample size of one, while the sample size for TGF-β2 is 6.

D. DISCUSSION

The biology of bone remodeling involves a series of complex interactions between cytokines, growth factors, bone cells and bone matrix. Although an understanding of these complex events is far from complete, we have attempted to characterize these processes as they pertain to mechanically induced bone modeling in general, and specifically to orthodontic tooth movement. Mechanically induced tooth movement is successfully implemented largely due to the capacity of stressed living skeletal tissues to model. One of the primary cellular events subsequent to the application of orthodontic force is stretching of the periodontal ligament cells (Tanne et al.., 1992; Cobo et al.., 1993; Brudvik et al.., 1993; Fukui et al.., 1993). The response of these cells to tensile loading forces, while difficult to characterize *in-vivo*, may be studied *in-vitro* using currently available cell culture and assay techniques. Although these *in vitro* systems have shown that alterations in levels of specific mediators of bone modeling, such as IL-1 and PGE, in response to the application of mechanical forces (Harrell et al.., 1977; Binderman et al.., 1984 and 1988), none have demonstrated the induction of TGF-β, a potent mediator of bone formation, in cells subjected to tensile mechanical forces *in-vitro*.

The findings of this study indicate that the expression of TGF-β1 and β2 in PDL cells increases upon exposure to intermittent tensile mechanical forces. TGF-βs have been shown to influence the extracellular matrices produced by many different cell types. For example, in cells of mesenchymal origin, such as fibroblasts, TGF-βs mediate increases in extracellular matrix macromolecules by increasing their synthesis and by decreasing their degradation (Rizzino, 1988). Since the PDL is primarily composed of cells of mesenchymal origin, namely fibroblasts, osteoblasts and cementoblasts, it is likely that any increases in expression of these growth factors in response to mechanical forces would result in a net matrix anabolism in these tissues.

Further support for the potential increase in net matrix anabolism of PDL cells in response to mechanical stimulation is provided by our preliminary findings on substrate

zymograms. These studies show that cyclic loading of PDL cells does not increase their expression of 72-kDa gelatinase, the only matrix metalloproteinase that is constitutively expressed by PDL fibroblasts. The findings also reveal no induction of other gelatinolytic proteinases by these cells when subjected to mechanical loading, indicating no net increase in matrix degradative activity attributable to these enzymes. However, further detailed studies are recommended to evaluate for these and other matrix metalloproteinases by more sensitive assays, as well as to assess for any alterations in the synthesis of specific inhibitors of these enzymes, the tissue inhibitors of metalloproteinases (TIMPs), by mechanically stimulated PDL cells.

In order to properly address our hypothesis, it was essential to develop a reproducible and reliable assay for the quantification of TGF-βs within the conditioned media of the PDL cells. The ELISA used in these studies served this purpose well. Additional sources of complexity in these experiments included the ability to manipulate the time course, frequency, magnitude and duration of the mechanical strain regimen providing literally hundreds of potential loading regimens for testing. Given an unlimited amount of time and resources, appropriate test matrices could be designed to alter these variables independently of the others in order to determine the effects of each on the expression of any protein. We, however, chose to conduct our experiments using high magnitude / low frequency and low magnitude / high frequency loading regimen on the basis of previous studies, and because these regimens likely lie within the range of physiologic and therapeutic loads experienced by cells (Tanne et al., 1992; Norton et al., 1992).

While two separate regimens were utilized in this investigation, it would be erroneous to draw conclusions based on comparisons between the two, as differences in the control levels of TGF-βs between the groups suggest that a variable other than the parameters of the loading regimen influenced the findings of the two experiments. This unknown variable may be differences in cell numbers, and variations between the cell lines used for the two experiments, as well as the lengthy period of time over which the media

for the 20% strain / 6 cph experiment was stored. For these reasons this discussion is limited to within-group comparisons for TGF-β content in conditioned media.

The quantification of TGF-β within the conditioned media is an important aspect of the characterization of PDL cell response to mechanical forces. However, the extreme hydrophobicity of TGF-βs is likely to result in these growth factors being drawn out of the conditioned media and into the matrix secreted by the cells into which they may be readily incorporated. This scenario is supported by findings that the absolute and standardized concentrations of TGF-β1 and β2 in conditioned media from control cells, and several of the strained cells plateaued during the experiment (Figures 8 to 11). Several of the control samples even demonstrated an apparent decrease in the concentration of TGF-βs in the conditioned media between 36 and 48 hours of culture (Figures 8, 9 and 11). These findings may have resulted because the rate of absorption of TGF-βs into the extracellular matrix approximated or exceeded the rate of production and release by the cells into the media. Therefore, for a better comprehension of the dynamics of TGF-β induction and distribution into various compartments within a culture plate, assessment of TGF-β levels in the extracellular matrix is necessary. As described in Materials and Methods, the cell and extracellular matrices were collected at time points corresponding to the collection of the conditioned media and these samples were assayed for TGF-βs. However, ELISAs on these samples were not successful probably due to an incompatability between reagents used to solubilze the cells and matrix and the ELISA. It is likely that this incompatability was due to the high concentration of detergent (SDS) within the extraction buffer. We hope to overcome this obstacle by solubilizing the cells and matrix with reagents compatible with the ELISAs.

The issue of TGF-βs within the cellular and extracellular matrix compartments may be partially addressed through further development of the immunolocalization technique presented here. The approach for these experiments may involve an appropriate sampling technique to quantitate the number and intensity of positively stained cells within the control

and experimental wells at various time-points throughout the experimental period. In addition, such experiments may provide insights into whether TGF-βs are induced in direct response to mechanical loading since the physical properties of the Flexcell plate allow for the non-uniform distribution of tensile forces within an individual well (Figure 2). An increased proportion and intensity of positively stained cells within the region of maximum stretch relative to the regions of minimum stretch would lend validity to the hypothesis that induction of TGF-βs results in direct response to the strain placed on the cells. Although a subjective evaluation of the immunostaining of TGF-β1 demonstrated approximately even staining along the cross-section of the well, a more objective quantitative evaluation as suggested above, may help to further confirm our observations. Such an analyses was not done in these series of experiments due to problems of visualization of the cells on a thick elastic base with poor optical quality. Further work is being done to optimize methods for visualization and counting cells cultured on this elastic media.

In order to further understand the early events in the mechanical transduction of TGF-βs in PDL cells, evaluations on early response genes as well as mRNA levels for TGF-βs are necessary. However, the latter experiments are difficult because of the small quantities of mRNA retrievable from each well. This limitation necessitates either the pooling of mRNA from 6 wells, thereby reducing the sample size, or using sensitive mRNA assays, such as reverse transcriptase-polymerase chain reaction (RT-PCR) or RNA protection assay. These methods are currently being developed for use with these experiments.

The process of recognizing and responding to mechanical stimuli is still poorly understood. Possibly the simplest explanation for the increased expression of TGF-βs seen in these studies is an increase in cellular metabolism in response to the mechanical stimuli. Others have suggested that altered cellular proliferation contributes to the perceived increase in cytokine expression in mechanically perturbed cells (Pead et al.l, 1988; Sumpio, 1987). In either of these situations, an increase in total protein content would accompany

these phenomena. However, as indicated in Figure 4 and Table I, no significant differences were observed in levels of total protein expressed by control and experimental cells in both loading experiments. Furthermore, standardization against these total protein levels, in fact, support the notion that TGF-β1 and β2 expression was specifically upregulated in response to the tensile mechanical strain used in these experiments. It is, therefore, likely that cellular or subcellular mechanotransduction events may mediate these phenomena, as suggested by Wang et al.. (1993).

Many sensory functions including touch, hearing, baroreception, proprioception, and gravity sensation involve specialized mechanotransduction mechanisms (Wang et al., 1993). Several of these mechanisms have been suggested as possible mediators of mechanically induced bone formation. While strain-sensitive ion channels, adenylate cyclase and protein kinase C all change their activity in response to applied stress (Sachs, 1989; Komuro et al., 1987; Watson, 1990), these signaling pathways are likely to lie downstream from the initial mechanoreception event at the cell surface. It has recently been proposed that these events are mediated by the cytoskeleton and its natural interaction with the extracellular matrix (Sachs, 1989; Watson, 1990; Murti et al., 1992). Cell shape, which is affected by various components of the extracellular matrix, such as fibronectin, collagen and laminin, can effect the synthesis and stability of mRNA (Benecke et al., 1978). Other data suggest a direct link between changes in the cytoskeleton and the production of certain gene products (Wicha et al., 1982; Werb et al.., 1986; Medina et al., 1987). Transmembrane extracellular matrix receptors, such as those from the integrin family, are excellent candidates for mechanoreceptors because they bind actin-associated proteins within focal adhesions and thereby physically link the extracellular matrix with the cytoskeleton (Wang et al., 1993; Albelda and Buck, 1990; Burridge et al., 1988). We, therefore, utilized cytochalasin B, an agent that alters the shape of the cell through binding and disruption of the cytoskeleton, in a preliminary study to assess the effects of cytoskeletal alterations on induction of TGF-βs. These experiments demonstrated that

TGF-β levels are increased in response to cytochalasin B, suggesting that cytoskeletal elements may serve, at least in part, as mediators of mechanotransduction (Figure 15). These experiments, however, are preliminary and deserve more thorough investigation.

The dynamics of TGF-β expression, particularly TGF-β2 (Figures 10 and 11), suggest that expression of this growth factor following the initital induction in response to mechanical stimulation may be autoregulated. Since TGF-β1 induces shape changes, at least in osteoblasts (Rosen et al.., 1988), it is possible that the increased secretion of TGF-βs, brought about initially by mechanical perturbations of the cytoskeleton, may further enhance the expression of TGF-βs by the effects of this growth factor on the cytoskeleton. Furthermore, TGF-β1 has also been shown to positively regulate it's own expression in a number of different cell lines (van Obberghen-Schilling et al., 1988), and TGF-β2 has been shown to increase the expression of TGF-β1 in at least one cell line (Bascom et al., 1989). The finding that TGF-β1 increases the production of fibronectin in fibroblasts (Bassols et al., 1988; Blatti et al., 1988; Penttinen et al., 1988), and that the addition of fibronectin may alter phenotypic expression in a manner similar to TGF-β induced shape changes (Rosen et al., 1988), again suggests that the mediator of many of these events may be the cytoskeleton. The biphasic expression of TGF-β1 in the conditioned media of the mechanically strained PDL cells may be due to such autoregulatory events. While the early rise in TGF-β1 expression in the conditioned media samples from the stretched cells does not differ significantly from that of the control samples, the late onset increase suggests an indirect response mediated by an intermediary factor or TGF-β itself. Future studies hoping to investigate these complex regulatory mechanisms might selectively block TGF-β1 and β2 receptors in order to observe the effects of these manipulations on the dynamics of expression of these growth factors.

The major findings of the present study are that TGF-β1 and β2 expression in cultured PDL cells is significantly increased in response to intermittent tensile mechanical forces. These findings, together with preliminary experiments that the levels of

gelatinolytic proteinases are not elevated in PDL conditioned media, would indicate that this response of PDL cells to mechanical stimulation is primarily anabolic since TGF-βs are known to induce matrix synthesis and inhibit matrix degradation by connective tissue cells (Rizzino, 1988). While the regulatory mechanisms for these events are still unknown, the increased expression of TGF-βs upon disruption of the cytoskeleton with cytochalasin B indicates that the cytoskeletal-extracellular matrix interactions may play an important regulatory role.

From the preceding discussion, several areas for future research are recommended. These include further examination of the effects of perturbations of the cytoskeleton and cell-matrix interactions in inducing TGF-βs, optimization of methods for visualization of cells, development of quantitative immunocytochemical techniques, development of methods to assay mRNA and to further detailed examination for the mechanisms for TGF-β regulation in mechanically stimulated cells.

Findings from this study may provide considerable insights into the biologic mechanisms underlying bone formation in mechanically induced bone modeling and new bone formation. The observations made here could have potential implications in orthopedic treatments such as those for the decreased bone density seen in disuse atrophy, denervation and weightlessness (Mack et al., 1967; Cann et al., 1983; Rambaut et al., 1985; Bikle et al., 1987; Yamaguchi et al., 1989). Furthermore, these findings may suggest potential therapeutic benefits of TGF-βs in orthodontics and orthopedics. For example, rapid formation of bone following tooth movement or sutural expansion achieved by utilization of optimal loading regimens which enhance the expression of TGF-βs will have definite advantages in decreasing treatment time and increasing post-treatment stability.

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