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Characterization of cells adherent to membranes recovered after periodontal guided tissue regenerative therapy



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# CHARACTERIZATION OF CELLS ADHERENT TO MEMBRANES RECOVERED AFTER PERIODONTAL GUIDED TISSUE REGENERATIVE THERAPY

by

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

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in

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San Francisco

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## DEDICATION

I wish to dedicate this thesis to my always
supportive and loving parents, brother, and his family
who always give me the strength to let my dreams come true.
Thanks to all my Aunties and Uncles who always give me the reassuring
feeling of having multiple moms and dads. All your love has always
been an inspiration.
This is for my grandfather. May I be the Dentist that
he would have wanted me to be.

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Thanks to those that helped push me to my limits. . . to help me know how far connective tissue and Gore-tex® can go, how much positive architecture is possible, why there are paralleling pins in implant kits, that young kids heal well from iatrogenic root exposure, that occlusion really is important, and that scaling and root planing and oral hygiene instruction really work. But most of all, giving back to me the sane notion that private practice should be the way of life for me.

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# ABSTRACT

A novel way is described to culture cells adherent to membranes used in periodontal guided tissue regeneration. Cell lines had similar fibroblast-like characteristics when first established *in vitro*, but when confluent, Gore-tex lines exhibited palisading orientations, a feature associated with periodontal ligament cells. All Gore-tex and GTAM lines but not gingival fibroblasts formed mineralized nodules *in vitro* as determined by von Kossa staining. Time of nodule formation between the two cell type lines revealed no significant difference ($p = 0.063$) although 4 of 6 GTAM lines formed nodules faster than any Gore-tex line.

Metalloproteinase production of all cell lines was examined using gelatin zymography and Western blots. All but one cell line produced 72K gelatinase (MMP-2). Gelatin zymography demonstrated that all lines produced a 55K doublet associated with collagenase suggesting that all these cells can be induced by cytokines and hormones to produce collagenase (MMP-1). Cells cultured from a membrane left *in situ* as long as 15 months, produced 55K collagenase, suggesting long-term induction of regenerated cells.

Some cell lines from both GTAM and Gore-tex lines produced a wide variety of proteases. For example, Gore-tex 27 exhibited a prominent band at 30K that could be associated with of PUMP-1 (MMP-7). No significant differences were seen in TIMP-1 production between the cells from Gore-tex and GTAM membranes ($p = .265$). Gore-tex and GTAM lines that formed higher molecular weight bands on TIMP-1 Western blots also exhibited a band migrating at 92K on substrate gels, suggesting a complex with 92K gelatinase and TIMP-1.

Results suggest that cells migrating to areas under the membranes have the capability to form mineralized matrices but also retain the ability to produce collagenases that could degrade immature regenerative tissue if exposed to inflammatory mediators. Since little collagenase was produced by cells recovered from membranes left for extended time periods, membrane removal should be prolonged as long as possible. Careful maintenance of the regenerative area is also important before and after membrane removal to minimize the inflammatory response around the regenerative tissue.

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| | |
|---|---|
| $1,25\text{-}(OH)_2D_3$ | 1,25-dihydroxyvitamin $D_3$ |
| CM | Conditioned medium |
| G-tex | Gore-tex® membrane |
| GF-11 | Gingival fibroblast cell line |
| GTAM | Guided Tissue Augmentation Material™ membrane |
| IL-1ß | Interleukin-1 Beta |
| K | Kilodalton (molecular weight measurement) |
| KcP4LAHCM | Keratinocyte cell line |
| MG-63 | Human osteosarcoma cell line |
| MMP | Matrix Metalloproteinase |
| MMP-1 | Fibroblast-type collagenase or collagenase |
| MMP-2 | 72K Gelatinase |
| MMP-7 | PUMP-1 or Putative Metalloproteinase - 1 |
| MMP-9 | 92K Gelatinase |
| $M_r$ | Molecular weight |
| PDL 814 | Periodontal ligament cell line |
| PTH | Parathyroid hormone |
| TIMP-1 | Tissue inhibitor of metalloproteinase - 1 |
| TIMP-2 | Tissue inhibitor of metalloproteinase - 2 |
| TNF-α | Tissue necrosis factor-alpha |

## INTRODUCTION

The traditional aim of periodontal therapy has been to arrest the progression of periodontal infection and to maintain the remaining periodontal structures (Stahl 1977). It was once thought that destruction of periodontal tissues caused by periodontitis was irreversible and predictable regeneration was not possible (Stahl 1977). With the advent of techniques of guided tissue regeneration, partial regeneration of lost periodontal tissues can be achieved (Karring et al. 1985). A basic understanding of the events and characterization of the cells and extracellular components comprising this regenerative tissue will be the key to maximizing this therapy for periodontitis patients.

Periodontal disease is a general term used to describe specific diseases that affect the gingiva and the supporting connective tissue and alveolar bone, which anchor the teeth in the jaws (Williams 1990). The periodontal diseases are among the most common chronic disorders of man. With the declining incidence of dental caries in the general population, periodontal disease has become the most common cause of tooth loss in adults (Shaw 1987). For many years, it has been recognized that periodontal diseases are caused by the accumulation of bacteria on the surface of the tooth. Although the exact causative bacteria have not yet been identified, a number of anaerobic microaerophilic gram-negative bacteria have been implicated (Socransky 1970).

Periodontitis affects the periodontal attachment apparatus surrounding the teeth. The periodontal attachment apparatus comprises alveolar bone, the periodontal ligament with its cells and fibers anchoring into cementum on the root surface, gingival connective tissue, and junctional epithelium (Williams and Zager 1978). Overlying these supporting structures are the gingiva and the alveolar mucosa. In the process of periodontitis, some or all of these components are destroyed and epithelium migrates down the root surface, forming periodontal pockets. Thus, periodontitis causes a loss of connective tissue, resorption of the alveolar bone, and formation of periodontal pockets (Williams 1990). In cases of periodontitis, after periodontal surgery repair usually occurs by formation of a long junctional epithelium (Listgarten 1967). During periodontal surgery the diseased tisssue is removed and the fastest migrating cells will repopulate that space. Epithelium will migrate the fastest and form along the root surface excluding

the other cell types (Gottlow et al. 1984). The techniques of guided tissue regeneration were developed to preferentially allow migration and proliferation of the cell types responsible for restoration of the original periodontal attachment apparatus.

**Development of the Periodontal Attachment Apparatus**

A basic understanding of the development of the periodontal attachment apparatus is important in order to understand the principles of guided tissue regeneration. The tissues of the attachment apparatus are derived from the dental follicle (Ten Cate et al. 1971, Ten Cate and Mills 1972). The dental follicle is first recognized as a condensation of ectomesenchymal cells and tissue surrounding the developing tooth (Ten Cate et al. 1971). The development of the roots begins after enamel and dentin formation has reached the future cementoenamel junction. The enamel organ plays an important part in root development by forming Hertwig's epithelial root sheath, which molds the shape of the roots and initiates dentin formation on the root surface. Cementum formation in the developing tooth is preceded by the deposition of dentin along the inner aspect of Hertwig's epithelial root sheath. Once dentin formation is under way, breaks occur in the epithelial root sheath allowing the newly formed dentin to come in direct contact with connective tissue of the dental follicle. Cells derived from this connective tissue are responsible for cementum formation (Ten Cate et al. 1971).

As the crown and the root develop, the surrounding periodontal ligament (PDL) becomes organized as well (Furstman and Bernick 1965). The main function of this connective tissue complex is to support and anchor each tooth within the alveolar housing (Levy and Bernick 1968). The ligament is maintained in the periodontal ligament space (0.15 to 0.4 mm) intervening between the cementum covering the root and the bone of the alveolar process (Hassell 1993). The mesenchymal cells and fibers of the PDL space are first disorganized. The collagen fibers (principal fibers) measure 300 to 500 nm in diameter. These principal fibers are attached to bone by Sharpey's fibers, thus anchoring the tooth into the surrounding alveolar bone. As development proceeds, a fairly dense layer of connective tissue substance is laid down near the surface of the cementum, with the orientation of the matrix and fibers generally paralleling the long axis of the tooth. Before eruption, PDL fibroblasts near the cemental surface, especially

in the coronal one third of the root, become oriented in an oblique direction, and a fibrillar matrix is deposited (Levy and Bernick 1968). It is not until the tooth erupts fully in the dental arch and is subjected to functional occlusal forces that the periodontal tissues become fully differentiated. This involves innervation from the mesencephalic nucleus and from the trigeminal ganglion (Byers and Matthews 1981, Byers 1985, Byers 1986); maturation of the vascular supply from nutrient canals from the cribiform plate of the alveolar bone, from branches of the arteries supplying the teeth, and from the vessels of the free gingival margin (Hayashi 1932); formation of blind-end lymphatic vessels (Levy and Bernick 1968), and the functional orientation of the fibers. This orientation of the fibers changes from parallel to perpendicular to the root surface (Levy and Bernick 1968, Furstman and Bernick 1965).

**Definition of Terms Used in Regenerative Therapy**

Prior to the World Workshop in Periodontics in 1989 (sponsored by the American Academy of Periodontology), nomenclature associated with regeneration therapy was often used interchangeably. Many terms were used in overlapping fashion when describing the outcome of periodontal surgery. At that meeting, the following terms were proposed.

**Repair:** healing of a wound by tissue that does not fully restore the architecture or the function of the part (i.e., ankylosis, healing by long junctional epithelium, connective tissue adhesion)

**Reattachment:** to attach again. The reunion of connective tissue with a root surface on which viable periodontal ligament tissue is present.

**New attachment:** the reunion of connective tissue with a root surface that has been deprived of its periodontal ligament. This reunion occurs by the formation of new cementum with inserting collagen fibers. (Note: independent of alveolar bone growth)

**Regeneration:** reproduction or reconstitution of a lost or injured part to its prediseased state. This involves coronal movement of the margin of the alveolar bone, functionally oriented, perpendicular fibers inserting into reformed cementum, and a periodontal ligament space.

**Early Wound Healing Events**

An understanding of early wound healing events around tooth surfaces is important to the understanding of guided tissue regeneration. One must be aware that healing events in periodontal defects are complicated by the fact that one healing surface is opposed to an avascular, calcified, rigid root surface. The sequence of healing in periodontal defects is commonly divided into three overlapping phases: 1) inflammation, 2) granulation tissue formation, and 3) matrix formation and remodeling (Wikesjö et al. 1992). The first event includes formation of a fibrin clot and aggregation of erythrocytes and PMNs (polymorphonuclear leukocytes). After the first day, PMNs predominate and strands of fibrin gradually become more distinct throughout the clot, binding to the connective tissue as well as the dentin surface. Because the clot must closely adhere to the rigid, avascular radicular surface, some advocate that the initial stabilization of this clot is most critical to wound healing around teeth (Linghorne and O'Connell 1950, Polson and Proye 1983, Wikesjö and Nilvéus 1990). This clot is mostly fibrin and fibronectin (Clark et al. 1982). The degradation of erythrocytes begins at this time.

At day 3, granulation tissue forms at the dento-gingival interface. Macrophages can now be seen along with fibroblasts. At day 7, the wound is dominated by cell-rich granulation tissue with fibroblasts and fibronectin (Diegelmann et al. 1975 and Kurkinen et al. 1980). In this early granulation tissue, the fibronectin is associated with the type III collagen which eventually will predominate. As the matrix remodels, type III collagen is degraded and replaced by type I collagen in the mature matrix. (Kurkinen et al. 1980). At day 14, the collagen fibers in the periodontal ligament space now becomes associated with the dentinal surface. After 21 days, cementum formation is detected (Selvig et al. 1988, Ririe 1980). Up to 4 months after surgery, collagen adhesion (collagen fibers predominantly oriented parallel to the hard tissue surface) may still be present (Cole et al. 1980). Thus, as outlined above, there is a very complex spatiotemporal sequence of events. This requires strict regulation of the cells and remodeling of the matrix components to intermediary components prior to complete tissue regeneration.

**Development of the Theory of Guided Tissue Regeneration**

Guided tissue regeneration is based on the principles first proposed by Melcher in 1976. He hypothesized that there were four separate compartments of connective tissue in the periodontium: the lamina propria of the gingiva, the periodontal ligament, cementum, and bone. The connective tissue cells in each of these compartments represented different phenotypes, and it was the phenotype of the cells repopulating the area after wound healing that determined the regenerative response. After a review of the wound healing studies done up to that time, Melcher hypothesized that periodontal ligament regeneration can only come from the periodontal ligament itself.

Interesting enough, a year later in 1977, Stahl presented his views on the regeneration of the periodontium. In his essay, Stahl questioned the ability to form new cementum in which new fibers can reattach once the cementum has been exposed to the microbial environment. He sited several studies that showed that this was seldom seen. The only promising possibilities, that were unpredictable at best, were those seen with autogenous bone grafts (Dragoo and Sullivan 1973, Froum et al. 1975). He hypothesized that regeneration of new cementum that can support functionally oriented periodontal ligament fibers is the most important factor in achieving regeneration.

Stahl and others (Stahl 1977, Levine and Stahl 1972, Aleo et al 1975) questioned whether or not this regeneration could be achieved on diseased root surfaces or root surfaces contaminated by long-term exposure to the oral cavity. Karring et al. (1985) addressed this question. Orthodontic elastic ligatures were placed around teeth in green monkeys in order to induce breakdown of the supporting tissues until it progressed to midroot level. Three months after ligature removal, the crowns and the exposed cementum of the teeth were removed by instrumentation. The remaining root structure of these teeth were submerged under the gingival tissues by repositioning the gingival flaps over the roots. After 3 months, all roots showed newly formed cementum with inserting collagen fibers ranging from 0.1 mm to 2.6 mm. Of the 16 roots, 6 became exposed through the gingival tissue within the first week. Epithelium was shown to migrate apically down the root surfaces of all these erupted root surfaces. All the other 10 roots that remained submerged showed some areas of bone regeneration; whereas, no regrowth of alveolar

bone was observed adjacent to any of the exposed roots. The new attachment occurred up to the coronal extent of new cementum. Coronal to this area where the roots were in direct contact to gingival connective tissue, the root surfaces showed some areas of resorption. Therefore, it was shown that new attachment can occur on previously diseased root surfaces provided that the root surfaces are isolated from the oral cavity and migrating epithelium.

Isidor et al. (1986) looked at the significance of coronal growth of periodontal ligament tissue for new attachment formation. Angular bony defects were surgically created around teeth in four adult green monkeys. The periodontal ligament tissue and the root cementum were removed to the bottom of the bony defects. In half the roots, an elastic ligature was placed at the bottom of the defects. All the roots were submerged under the gingival tissues by repositioning the gingival flaps coronal over the roots. After 3 months of healing, the animals were sacrificed and block sections of the roots were prepared. New attachment had formed above the level of the defect in 10 of the 14 control teeth but only 1 of the 18 test roots had new attachment. This supports the suggestion that the periodontal ligament is a prerequisite for new attachment formation.

**The Role of Periodontal Ligament Cells**

From the above studies, it was suggested that new attachment can be achieved on previously diseased root surfaces as long as precursor cells are allowed access to migrate to these areas. Also, it has been implicated that precursor cells that give rise to cementoblasts and the fibroblasts to produce new periodontal fibers originate from the periodontal ligament space. The following studies also support this.

In one study, PDL cells were reimplanted around teeth to determine if this can induce new attachment. This novel experiment was reported by Boyko et al. (1981). They extracted premolar teeth from beagle dogs, harvested the periodontal ligament cells and gingival fibroblasts, and cultured them *in vitro* for one or two passages. Maxillary and mandibular lateral incisors were extracted, the crowns removed, and root canals instrumented and obliterated using zinc-oxide-eugenol cement. The root surfaces were scraped and demineralized for 48 hours in 0.5M EDTA ([Ethylenedinitrilo]tetraacetic acid tetra sodium salt), pH 7.4. After demineralization, the roots were incubated either with harvested PDL cells or gingival fibroblasts, or no cells (control) for

10 days. The roots were then implanted into prepared edentulous areas. The dogs were sacrificed at 8 weeks and *en bloc* sections were prepared for histologic examination. On control roots or roots bearing gingival connective tissue cells, ankylosis of bone and tooth and resorption of cementum were observed. Roots bearing periodontal ligament connective tissue cells showed similar changes but not as widespread. Only roots transplanted with PDL cells showed areas covered by fibrous connective tissue organized to resemble periodontal ligament, with attachment to both tooth and bone by collagen fibers. Therefore, it was concluded that only PDL cells can contribute to new attachment.

Studies have also shown that cells harvested from the periodontal ligament space have phenotypic characteristics of mineralizing cells. Arceo et al. (1991) reported that PDL cells, but not gingival fibroblasts, were capable of forming mineral-like nodules *in vitro.* Nojima et al. (1990) showed that PDL cells can respond to osteoblastic inducers. In this study, bone *gla* protein (BGP) (also known as osteocalcin) was detected in PDL cells but not in gingival fibroblasts and BGP was increased if 1,25-dihydroxyvitamin $D_3$ ($1,25-(OH)_2D_3$) was added to the PDL cultures. BGP is a known osteoblastic marker (Skjødt et al. 1985). Furthermore, the production of 3',5'-cyclic adenosine monophosphate (cAMP) in PDL cells was greatly increased in response to human parathyroid hormone. Mukai et al. (1993) observed that rat periodontal ligament cells formed a mineralized matrix resembling osteoid *in vitro.* This tissue included osteoblast-like cells, osteocyte-like cells, a collagenous matrix, a mineral composed of hydroxyapatite, and intense alkaline phosphatase activity.

Other evidence that supports that the periodontal ligament space contains progenitor cells needed to regenerate a new attachment was reported by Buser et al. (1990). They sectioned the coronal half of teeth in monkeys and placed ITI implants in close proximity to the retained roots. They found new cementum and perpendicularly oriented, embedding periodontal ligament fibers forming around the implant. This lends support to the concept that periodontal ligament cells can regenerate the attachment apparatus because without the proximity of the implant to an intact PDL, no new cementum can around an implant and only osseointegration with bone structure is seen.

**Use of Barrier Membranes to Assist Guided Tissue Regeneration**

From the studies in the 1980's by Karring et al. (1985), Isidor et al. (1986) and others (Aukhil et al. 1987, Bowers 1988, Houston et al. 1985), it was demonstrated that regeneration of a functional periodontal attachment apparatus could be achieved around teeth previously deprived of cementum. It was apparent that the epithelium and connective tissue from the lamina propria must be excluded to guide tissue growth from the apical portion of the defects.

Currently, techniques to achieve guided tissue regeneration of the periodontium involve the use of barrier membranes and adjuncts such as root conditioning agents (i.e. citric acid and tetracycline), graft materials including autografts (intraoral and extraoral), alloplastics (hydroxyapatite) and allografts (freeze-dried demineralized bone). The barrier membranes fall into two types: resorbable and non resorbable. The resorbable membranes include collagen membranes (Chung et al. 1990), polylactic acid barriers (Magnusson et al. 1990), and Vicryl (polygalactin 910, a copolymer of glycolide and lactide) (Fleisher et al. 1988). The standard material in use today in the nonresorbable category is polytetrafluorethylene (ePTFE) or Gore-tex® (W.L. Gore & Associates). The main purpose of the grafts and the membranes is to maintain the space once occupied by the diseased tissue long enough for tissue to regenerate into a functional periodontal attachment apparatus.

The use of a barrier membrane in humans was first published by Nyman et al. in 1982. In this case report, they reported the use of a Millipore® filter (Millipore S. A.) as the barrier on a lower left lateral incisor. They removed the tooth *en bloc* and prepared the biopsy for histologic evaluation. New cementum with inserting collagen fibers was observed and within the pre-existing angular bony defect, new supporting bone had been formed but no coronal regeneration of alveolar bone was observed. Thus, they were the first to report that regeneration could be obtained with the use of a barrier membrane.

Further reports were soon published supporting the use of membranes to achieve regeneration. Gottlow et al. (1984) reported the first cases comparing Millipore® and Gore-tex® membranes in 3 monkeys (*Macaca cynomolgus*). The results showed that with either barrier, a new functional attachment apparatus around teeth was obtainable. Two years later Gottlow et al. (1986) repeated an experiment using Gore-tex® membranes on human

subjects . All 12 teeth treated with the Gore-tex® membranes showed some regeneration.

Magnusson et al. (1985) presented variable results of regeneration after the use of Millipore® filters in 6 *Macaca nemestrina* monkeys. Surgical defects were created where the buccal bone and the cementum were removed. The defects were then either covered with the filters (7 teeth) or left alone (8 teeth) and then flaps coronally sutured over the defects. The monkeys were monitored for 6 months after which 7 test teeth and 8 control teeth were compared by histological examination. Regrowth of alveolar bone and perpendicular, functionally oriented fibers had occurred to a varying degree adjacent to the test roots. However, no relationship was found between the amount of new cementum formation and attachment fibers and the degree of regrowth of the alveolar bone. In the majority of the control roots, no new cementum was formed but a long junctional epithelium was lining the root surface. Therefore, they concluded that reformation of cementum and a functionally oriented periodontal ligament (new attachment) is independent of reformation of the surrounding alveolar bone.

Schupbach et al. (1993) examined this new attachment at higher magnifications using scanning and transmission electron microscopy. Resorbable polyurethane membranes were used over surgically created defects in dogs. They reported that full regeneration occurred in parts of the roots only if some of the original cementum remained on the root surface. Repair was observed if peripheral dentin was removed by root planing as this layer was not reestablished. New attachment, regardless of bone regeneration, always occurred by intermingling linkage between new cementum fibrils and pre-existing tissue fibrils. Thus, accentuating the point that there must be a complex spatial and temporal coordination of events to gain full regeneration of the attachment apparatus and that repair and regeneration can occur simultaneously.

Since these pioneering studies, it has been well-established that membranes can be used to assist in tissue regeneration around teeth (Figure 1 (A)). Studies have been published supporting the use of Gore-tex® and other membranes in routine periodontal therapy with and without adjunctive procedures such as graft placement and citric acid conditioning (Warrer et al. 1988, Becker et al. 1988, Caffesse et al. 1990, McClain and Schallhorn 1993). Although all these studies report favorable gain in clinical parameters

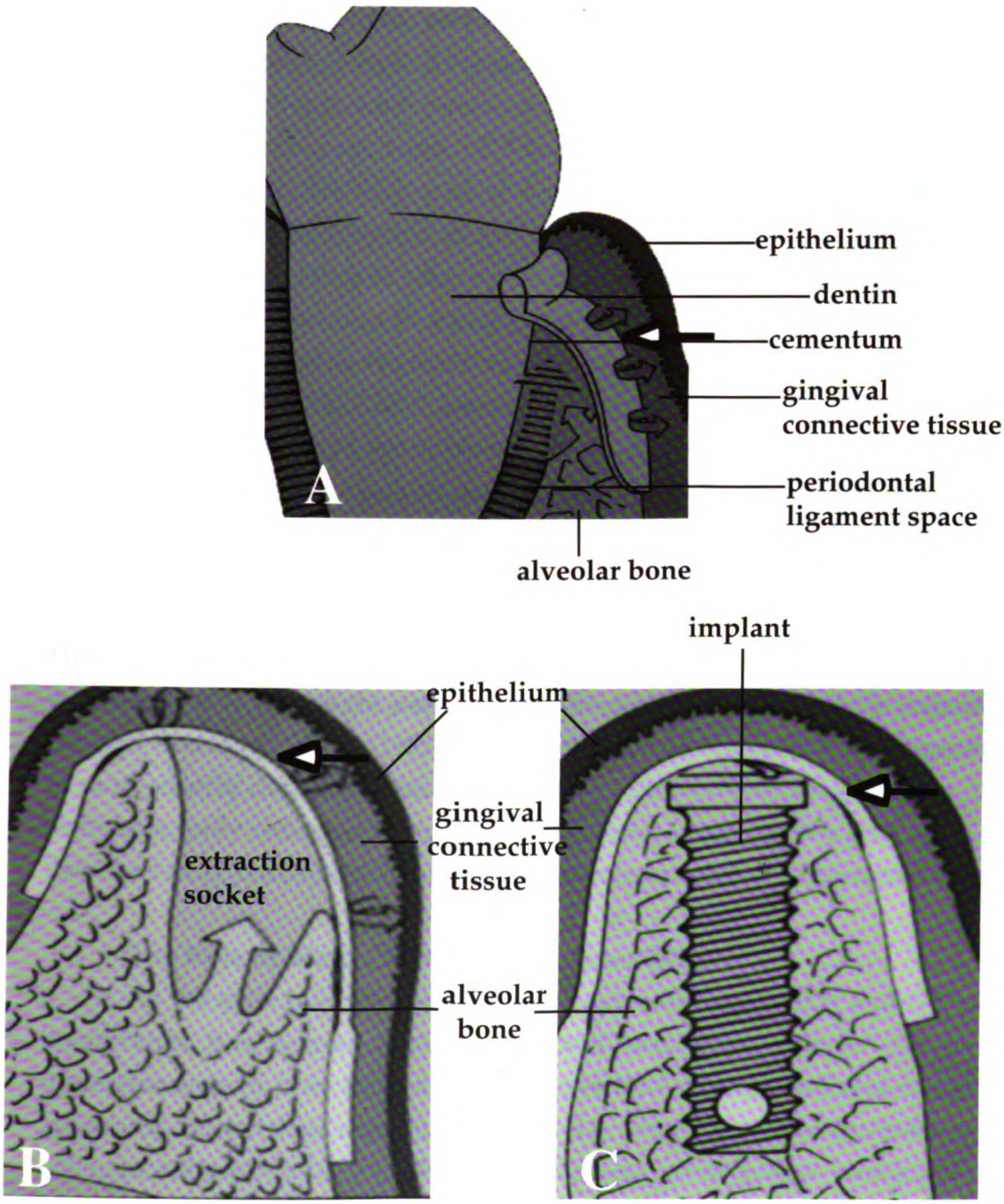


Figure 1. Schematic diagrams of the function of Gore-tex® membrane and GTAM™. (A) Gore-tex® membrane placed around a periodontally diseased tooth. (B) GTAM™ membrane placed over an extraction socket. (C) GTAM™ membrane placed in conjunction with implant placement. (⬅) pointing to the membrane in each diagram.

(decrease in clinical probing depth, increase in clinical attachment level, decrease bleeding on probing) and histological parameters (changes in bone height, width, and volume), no study has reported perfect success of regeneration of periodontal defects.

The use of Gore-tex® materials has been adapted to regenerate osseous structures in edentulous areas. GTAM™ (Gore-tex® Augmentation Material) materials are now widely used to augment an alveolar ridge in conjunction with an implant to achieve maximum osseointegration (Figure 1 (C)) (Nyman et al. 1990) or after extraction of a tooth (Figure 1 (B)) (O'Brien et al. 1994). After extraction of a tooth, the socket region is filled in with bone but the alveolar crest is resorbed to an unpredictable level (Pietrokovski and Massler 1967). The GTAM™ membrane is placed over the socket to exclude gingival fibroblasts and epithelium from migrating into the space once occupied by the tooth. Seibert and Nyman (1990) demonstrated in two adult beagle dogs that surgically created defects in extraction sockets could be successfully augmented using a membrane or a membrane plus porous hydroxyapatite graft 3 months following the creation of the defect. Histological analyses showed that this regenerative tissue filling the defect was bone. It is this slight variation on the original guided tissue regeneration concept that Buser et al. (1993) suggest the use of "Guided Bone Regeneration" to more clearly define the desired outcome. Strictly speaking, in this technique, only osteogenic precursors will have access to this area and therefore only osseous structures will be formed under these membranes.

**The Role of Matrix Metalloproteinases (MMPs)**

As the cells migrate into the area under the Gore-tex® and GTAM™ membranes, they secrete elements that will form a new matrix. An understanding of how this matrix matures into a functional attachment apparatus rather than reparative tissue (i.e. long junctional epithelium, collagen adhesion with parallel oriented fibers, or only new attachment with no osseous regeneration) would be essential to guaranteeing successful regeneration.

Remodeling is essential to the maturation of tissue. Tissue is made up of cells and extracellular matrix (ECM). The major structural proteins, collagens, support the basic structure of the ECM, while the major adhesive glycoproteins, fibronectin and laminin, mediate the binding of cells to ECM

(McDonald 1988, Ruoslahti 1988, Martin & Timple 1987). As the matrix matures, it will be remodeled by surrounding cells secreting degradative enzymes. Matrix metalloproteinases (MMP) are some of these enzymes. To date, nine MMPs have been identified. They include interstitial collagenases (Fibroblast-Collagenase [FIB-CL] and PMN [Polymorphonuclear]-Collagenase), stromelysins (SL-1, SL-2, SL-3), gelatinases (72K gelatinase/type III collagenase [$M_r$ 72K GL], 92K gelatinase/type IV collagenase [$M_r$ 92K GL]), putative metalloproteinase-1 (PUMP-1), and macrophage metalloelastase (MME) (Table 1). The MMPs are secreted as proenzymes, requiring further modification by proteinases to become activated (Birkedal-Hansen 1993). These enzymes share a number of common structural and functional features but differ somewhat in terms of substrate specificity (Table 1). However, there is an apparent redundancy with overlapping of the substrate specificity. For instance, virtually all of the enzymes cleave gelatin and fibronectin, and most cleave type IV and V collagens.

The high frequency with which MMP or inhibitor transcripts or proteins can be detected in cells, tissue, and interstitial fluids during development and reparative processes suggests that these enzymes play a major role. A number of studies have provided evidence for involvement of MMPs and their inhibitors in these processes. These include embryonic growth and differentiation (Nomura et al. 1989), trophoblast invasion (Fisher et al. 1989), skeletal growth and remodeling (Sellers et al. 1978, Dean et al. 1985, Delaisse et al. 1988, Nomura et al. 1989, Flenniken and Williams, 1990), and development of tooth germs (Nomura et al. 1989).

The degradation of the matrices must be tightly regulated. Because of the potential danger of uncontrolled degradation, there are many pathways that MMPs are regulated. Growth hormones and cytokines mediate some transcriptional regulation (MacNaul et al. 1990, Kerr et al. 1988). $\alpha$-Macroglobulins bind and inactivate proteinases such as MMPs by cleaving part of the molecules and causing a conformational change (Sottrup-Jensen et al. 1989). Another form of MMP regulation is through noncovalent bond formation with tissue inhibitor of metalloproteinases (TIMPs). TIMPs inhibit MMP activity as well as retard precursor activation (DeClerck et al. 1991).

Of the growing number of known TIMPs, TIMP-1 has been the most widely studied. It is a 29K mannose-rich sialoglycoprotein (Stricklin & Welgus, 1983). TIMP-1 forms complexes with FIB-CL (Welgus et al. 1985) and

| Enzyme | Abbreviation | MMP #$^{\Delta}$ | $M_r$ | Extracellular matrix substrates |
|---|---|---|---|---|
| Fibroblast-type collagenase | FIB-CL | MMP-1 | 57,000/52,000 | Collagen I,II,III,(III>>I)VII,VIII,X; gelatin; Proteoglycan core protein |
| $M_r$ 72K gelatinase/type IV collagenase | $M_r$ 72K GL | MMP-2 | 72,000 | Gelatin; collagen IV,V,VII,X,XI; elastin; fibronectin; proteoglycan core protein |
| Stromelysin-1 | SL-1 | MMP-3 | 60,000/55,000 | Proteoglycan core protein; fibronectin; laminin; collagen IV,V,IX,X; elastin; procollagenase |
| Putative metalloproteinase-1 | PUMP-1 | MMP-7 | 28,000 | Fibronectin; laminin; collagen IV, gelatin; procollagenase; proteoglycan core protein |
| PMN-type collagenase | PMN-CL | MMP-8 | 75,000 | Same as FIB-CL (I>>III) |
| $M_r$ 92K gelatinase/type IV collagenase | $M_r$ 92K GL | MMP-9 | 92,000 | Gelatin; collagen IV,V; elastin; Proteoglycan core protein Fibronectin* |
| Stomelysin -2 | SL-2 | MMP-10 | 60,000/55,000 | Same as SL-1 |
| Stromelysin-3 | SL-3 | MMP-11 | not determined | Not determined |
| Macrophage metalloelastase | MME | *** | 53,000 | Elastin |

Table 1. List of Matrix Metalloproteinases

$\Delta$ - MMP numbering according to Nagase H, Barrett AJ, Woessner JF Jr. Matrix Spec. Supp. 1: 421-424, 1992.

* - MMP-9 also found to cleave fibronectin (Partridge et al 1993), not originally reported by Nagase et al.

*** - discovered after Nagase et al. article

to the Mr 92K GL (Wilhelm et al. 1989). TIMP-1 expression is stimulated by epidermal growth factor (EGF), tissue necrosis factor-α (TNF-α), Interleukin-1 (IL-1), transforming growth factor-ß (TGF-ß), phorbol esters, retinoids, and glucocorticoids (Clark et al. 1987, Edwards et al. 1987, Mawatari et al. 1989).

The association of MMPs in the destruction of periodontal disease has been studied. MMPs have been isolated from the gingival crevicular fluid from periodontally-diseased sites (Sorsa et al. 1988). Teng et al. (1992) reported that during periods of attachment loss, there was a 2-fold increase of mean active gelatinase activity in saliva collected from recurrent periodontitis patients. Because of these reports, therapy has been developed to decrease the degradation of periodontal tissues due to collagenases. The use of tetracycline and their chemically-modified analogs for their collagenase inhibition properties has been examined in periodontitis patients (Rifkin et al. 1993). Some success had been reported with patients on long-term low doses of these drugs. Schroeder et al. (1992) reported decreased loss of attachment in periodontitis patients when these patients were on low dose (20 mg twice daily) doxycycline for 3 months. The drug dosage was below the dose effective for its antibacterial effects but high enough for collagenase inhibition. This was confirmed by no development of doxycycline-resistant microorganisms in their subgingival plaque.

The reports in the periodontal literature have only looked at MMPs and their role in the pathogenesis of periodontitis. However, no reports have been published exploring the role of MMPs and their TIMPs in remodeling the matrix formed after guided tissue regeneration procedures. As stated earlier, MMPs are important in the development and remodeling of tissues; therefore, understanding the role that they play after guided tissue regenerative therapy could have a great impact on gaining and preserving a more predictable result.

Although studies pertaining to the molecular characteristics of the extracellular matrix produced after regenerative procedures are nonexistent, there are studies looking at the cell types attached to barrier membranes. Studies have characterized the microflora (Tempro & Nalbandian 1993, Demolon et al. 1993, Wang et al. 1994) or the epithelium adherent to the membrane (Salonen & Persson 1990, Pritlove-Carson et al. 1992, Grevstad and Leknes 1992). Selvig et al. (1990, 1992) reported that some connective tissue elements were adherent to the membranes. These elements included

firbrous structures suggestive of collagen fibers and cells with a fibroblast-like appearance. Machtei et al. (1994) reported enlarged fibroblasts on the inner surface of the membranes correlated with decrease in furcation dimension after 12 months. These fibroblasts were not further characterized.

It is likely that the fibroblast-like cells adherent to the membrane contribute to the development of the final structure of the regenerative tissue. No reports have been published characterizing these fibroblast-like cells associated with the membranes. Examination phenotypes of these cells will assist in a better understanding of the regenerative process. This manuscripts reports initial characterization of the fibroblast-like cell types adherent to Gore-tex® and GTAM™. Part of the MMP repertoire as visualized by gelatin zymography, TIMP-1 production, and the ability to form mineralized nodules as visualized by von Kossa staining are examined for cell lines isolated from these membranes.

## MATERIALS AND METHODS

### Patient Population and Membrane Retrieval

Gore-tex® membranes and GTAM™ (W.L. Gore and Associates) were retrieved from patients following guided tissue regeneration procedures in the postgraduate periodontology clinic at the University of California, San Francisco. Informed consent was signed by the patients to undergo the procedures. All patients were in good general health and were taking no medications. The membranes were retrieved from 11 patients (7 males and 4 females) ranging from 21 to 69 years (mean 42.38 ± 15.83). Patients were given a 2 week regimen of doxycycline (200 mg stat, then 100 mg qd) immediately after placement of the membranes. Also, patients were asked to rinse with Peridex® (0.12% Chlorohexidine) twice a day during the healing phase.

The Gore-tex® membranes were used for regenerative treatment of periodontally diseased teeth (Figure 1 (A)). The periodontally involved teeth required no restorative treatment and were without endodontic or periapical involvement. Five Gore-tex® membranes were placed over interproximal defects (G-tex 18,19,27,30,31) and one membrane was placed in a Class II furcation defect (G-tex 3). [Note: The number designation of the cell lines represents the location that the membranes were placed based on the Universal numbering system of teeth.] All Gore-tex membranes were left in place between 6 to 8 weeks.

GTAM™ were recovered from edentulous ridge augmentation procedures either after extraction of a tooth (GTAM 11,12,25) (Figure 1 (B)) or in conjunction with implant placement (Figure 1 (C)) (GTAM 5i, 8i, 10i) [Note: The lower case "i" designates membrane in conjunction with an implant.]. The GTAM membranes for ridge augmentation (GTAM 11, 12, 25) were left in place 6 to 8 weeks. The GTAM™ membranes placed in conjunction with an implant (5i, 8i, 10i) were in place 2, 6, and 15 months, respectively, before retrieval. The three GTAM lines isolated from membranes around implants were not exposed to the oral cavity during the healing phase but all other membranes were at least partially exposed. The membranes for GTAM 12 and 25 were retrieved from the same patient.

### Isolation of Cell Lines

After removal, the membranes were placed into Alpha Modified Eagle's Medium (αMEM) (obtained from the UCSF Cell Culture Facility)

containing 10% heat-inactivated fetal bovine serum (FBS) (UCSF Cell Culture Facility) and supplemented with antibiotics (100 units/ml penicillin, 0.10 mg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B) (UCSF Cell Culture Facility). The membranes were then cut into 3x3 $mm^2$ pieces with tissue scissors and immobilized under sterile cover slips held in place on tissue culture dishes by sterile vacuum grease. The membrane "explant" was then cultured in the same medium until cells adherent to the membrane were observed migrating onto the culture dish or the lower surface of the cover slip (usually at 10 to 14 days). The cultures were incubated at 37°C and equilibrated in 5% $CO_2$ and air. These cells were then detached with STV (0.05% Trypsin, 0.02% Versene [EDTA], in Saline A [0.1% glucose, 0.058% $NaHCO_3$]) (UCSF Cell Culture Facility) and passaged in the same medium. Cells were frozen at passage three or four. This was carried out by detaching the cells with STV collecting the cells and storing the cells in 1 ml of FBS with 5% Dimethyl sulfoxide (DMSO) (Fisher Scientific) at -70°C.

Human periodontal ligament cells (PDL 814) were scraped from the midroot portion of periodontally healthy bicuspids extracted for orthodontic therapy and were established as cell lines in the same medium as previously described (Richards & Rutherford, 1988). After 3 washes in phosphate-buffered solution, the PDL tissue attached to the middle third of the root was removed by scraping with a scalpel. The coronal and apical portions of the ligament were not used to avoid contamination by gingival and pulpal cells. Human gingival fibroblasts (GF-11) were obtained from distal wedge incisions over maxillary tuberosities removed during routine periodontal surgery (Richards & Rutherford, 1988). Keratinocytes ($KcP_4LAHCM$) were cultured from the same tissue. Gingival and PDL tissue were minced into small pieces (less than 0.5 mm) and treated with 2.4 units/ml dispase (Boehringer Mannheim Biochemicals) for 10 minutes(1 unit of dispase will release Folin-positive amino acids and peptides equivalent to 1 µmol tyrosine in 1 min.). Epithelium was then separated from the underlying dermis by microscopic dissection and treated with STV for 15 minutes to produce a single-cell suspension. Keratinocytes were established from the epithelial sections and grown in keratinocyte growth medium (KGM-Clonetics) (Oda & Watso, 1990). MG-63, the osteogenic sarcoma line used as an example of a human bone cell line, was purchased from the American Type Culture Collection. CA 928 is a

squamous cell carcinoma line included as a standard on the gelatin substrate gels because of its ability to produce various metalloproteinases.

**von Kossa Staining for Mineralized Nodule Formation**

After cells were grown to confluency in 6 well plates with αMEM, 10% FBS and supplemented with antibiotics (100 units/ml penicillin, 0.10 mg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B), the medium was changed to one that is known to induce mineralized nodule formation (Bellows et al. 1990). This NGM (Nodule Growth Medium) contained αMEM, 15% FBS and was supplemented with antibiotics (100 units/ml penicillin, 0.10 mg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B), 50μg/ml ascorbic acid (Sigma), and 10mM ß-glycerophosphate (Sigma). Following nodule formation, cultures were fixed for 30 minutes *in situ* with 10% neutral buffered formalin (NBF) (Sigma), washed, and stained with the von Kossa technique for visualization of mineralized nodules (Bellows et al. 1986). This technique involved exposure to 5% aqueous silver nitrate (Sigma) for 15 minutes in the dark, then exposure in UV light for 60 minutes until the calcium salts in the wells were visibly brown. Nonspecific staining was removed from the specimens by a 2 minute washing with 5% sodium thiosulfate (Sigma) and then rinsing with distilled water 3 times for 5 minutes. Nodules were visible under light microscopy 4X (Nikon Diaphot)

**Gelatin Substrate Gels**

Gelatin substrate gel electrophoresis (zymography) was performed as previously described (Fisher et al., 1989). Polyacrylamide gels (10%), containing 2mg/ml pig skin, type I gelatin (Sigma), were loaded with 20 μl serum-free αMEM that had been conditioned by each cell line for 72 hours (CM) and run under non-reducing conditions. CM was store at -20°C until electrophoresis was performed. The samples were not boiled or reduced, and the stacking gel did not contain substrate. CM were diluted with an equal volume of sample buffer [0.5 M Tris-HCl, pH 6.8 (Tris[hydroxymethyl]aminomethane)[Fisher Scientific], 10% (v/v) glycerol[Fisher Scientific], 0.4% (v/v) SDS (sodium dodecyl sulfate)(Sigma), 0.05% (v/v) bromophenol blue(Sigma)]. The gels (0.75mm thick) were prepared in a small gel apparatus (Hoefer Scientific Instruments). The electrophoresis was done at 100V until the leading front was seen migrating

to one inch from the bottom of the gel. After electrophoresis, sodium dodecyl sulfate was removed from the gels with a 2.5% Triton X-100 (Fisher Scientific) wash for one hour, and then gels were incubated for 24 hours at 37°C in Hanks balanced salt solution (UCSF Cell Culture Facility). After incubation, gels were then stained with 0.1% Coomassie Brilliant Blue R-250 dye (Bio-Rad) in water:methanol (Fisher Scientific):acetic acid (Fisher Scientific) (5:5:1, v/v) for 30 minutes and then destained with 45% methanol (v/v), 3% acetic acid. Upon destaining, cleared bands of active gelatinase activity could be detected on a blue background. Molecular weight standards were ß-Galactosidase (MW=119K), Fructose 6-Phosphate Kinase (MW=98K), Pyruvate Kinase (MW=80K), Fumarase (MW=64K), Lactic Dehydrogenase (44K), and Triosephosphate Isomerase (39K) (BRL Inc).

**Western Blots**

20 µl aliquots of conditioned serum-free medium from each of the cell lines were analyzed by Western blots. Before analysis, total protein concentrations in each of the samples were determined by an amido black colorimetric assay (Wilson 1992). This assay consisted of prepared samples of CM compared to standard concentrations of diluted stock solutions of 2 mg/ml Bovine Serum Albumin (BSA) (Pierce) The samples were prepared by adding 500 µl of sample to 50 µl of 1M Tris and 2% SDS and 150 µl 90% Trichloroacetic acid (TCA) (Fisher Scientific). This mixture was vortexed and incubated for 2 minutes at room temperature (RT). 200 µl of 6% TCA was added to the samples and the samples were then placed in isolated spots on a Millipore® HA filters (0.45µM) that was washed with distilled water once placed on a holder attached to an Erlenmeyer flask hooked up to vacuum. Once all the samples were placed on the filter, the filter was washed with 10 ml 6%TCA. The filters was removed and left to stand for 12 hours. The filters were then placed in amido black stain (.1% amido black (Sigma) in 45% methanol (v/v) and 10% acetic acid (v/v) for 20 minutes. The filters were washed in distilled water 3 times for 30 minutes. The filters were then destained in 90% methanol and 2% acetic acid 2 times for 20 minutes. Final rinsing in distilled water was done 3 times for 1 minute. The filters were then blotted dry and the stained spots on the filter were cut out. These filter spots were placed in 12x75 ml tubes (Fisher Scientific) and 1 ml of elution buffer (25mM NaOH, 0.5mM EDTA, 50% EtOH) was added to each tube. The

tube was then vortexed intermittently for 10 minutes until no more dye was apparent on the filter paper. The prepared samples and BSA standards were transferred to a cuvette and read at 630 nm on a Milton Roy Spectronic 1201 spectrophotometer.

The protein concentrations of the CM were standardized to 1 mg/ml secreted cellular protein. In some instances, this required concentration of CM of the appropriate amount of protein sample. CM was placed in an Ependorf ultracentrifuge 5413 for 10 minutes, then concentrated in a Savant Speed Vac Concentrator (Model #RH 40-11) for 15 minutes. This was reconstituted in 20 µl of nonreducing sample buffer (40% glycerol, 0.25M TRIS-HCl pH 6.8, 0.8% SDS, 0.05% Bromophenol blue) before loading on 10% acrylamide mini gels (Bio-Rad). The CM was run on the mini gels at 100V until the leading front dye was seen migrating to 1 inch from the bottom of the gel. The protein was transferred to nitrocellulose paper (Whatman) by Western blotting at 300V for 45 minutes in a mini-gel transfer apparatus (Bio-Rad). To minimize nonspecific background antibody binding, blots were soaked in 5% T-Blotto (5% non-fat dried milk (Carnation), .1% (v/v) Tween-20 (Bio-Rad) in PBS (Phosphate buffered saline [UCSF Cell Culture Facility]). Blots were probed with 1/200 concentrations of primary antiserum in PBS for 1 hour, then washed twice with T-PBS buffer (.1% [v/v] Tween-20 in PBS) and incubated with 1/1000 concentrations of secondary antibody conjugated either to alkaline phosphatase as in the case of the blots to the 72K and 92K gelatinases and the TIMP-1 blot of the conditioned media of representative cell lines (Figure 7) or conjugated to Horseradish Peroxidase as in the case of the TIMP-1 blots of the Gore-tex and GTAM cell lines (Figure 9). The blots were then washed again 2 times in TBST (50mM Tris pH7.5, 150mM NaCl (Sodium Chloride)(Fisher Scientific), 0.6% Tween 20). In the blots to 72K, 92K and TIMP-1 of CM of representative lines (Figure 7 and 8), the bands were developed due to a color reaction with 66 µl of 50 mg/ml stock solution of nitro blue tetrazolium (Promega) and 33 µl of 50 mg.ml stock solution of 5-Bromo-4-chloro-3-indoyl phosphate (Promega) in 10 ml of T-PBS buffer solution. The reaction was stopped once bands were visible by diluting in distilled water. The bands on the TIMP-1 blots of the Gore-tex and GTAM lines were developed by the enhanced chemiluminescence (ECL) technique. This was carried out by adding 5 ml of ECL Detection Reagent 1 to 5 ml of ECL Detection reagent 2, incubating for 1 minute, and exposing both blots

simultaneously to Hyperfilm-ECL for 1 minute (Amersham Corp). Densitometric scan analysis (Scan Analysis™ 2.11, Biosoft) was done on the bands on the TIMP-1 blots to quantitate relative amounts of TIMP-1 secreted into the conditioned medium of the G-tex and GTAM cell lines as compared to conditioned medium of GF-11.

Antisera to 72K and 92K gelatinases were a gift from Dr. M.S. Hibbs, University of Connecticut; antiserum to TIMP-1 was a gift from Dr. Z. Werb, University of California, San Francisco, a purified TIMP-1 protein was a gift from Dr. H. Welgus, St. Louis University.

**Data Analysis**

The difference in TIMP-1 production between the GTAM cell lines and the Gore-tex cell lines as measured by scan densitometry was analyzed by using a two-tailed paired-difference Student *t*-test (Excel 4.0, Microsoft Corp). The difference in the time for mineralized nodule formation between the two lines was also measured by a two-tailed paired difference Student *t*-test (Excel 4.0, Microsoft Corp).

## RESULTS

### Morpholgy of Cell Lines

Cells adherent to Gore-tex® membranes and GTAM™ were compared with human periodontal ligament cells, human gingival fibroblasts, and human keratinocytes. Except for keratinocytes, these cells were morphologically similar in that all had the spindle-shaped outline associated with fibroblasts (Figure 2). The main morphological difference was noted at confluence, when cells cultured from Gore-tex membranes tended to form palisades of interdigitating cells in repeating units. Figure 3 shows a membrane "explant" that had been left in culture until adherent cells grew to confluence and illustrates the periodicity of these cells.

### Mineralized Nodule formation

The von Kossa staining to visualize mineralized nodule formation showed that all cell lines of Gore-tex and GTAM were capable of forming nodules. After cells were grown to confluency, these lines were grown in NGM until the nodules became visible by von Kossa staining. The time and pattern of staining varied among the cell lines. The gingival fibroblasts showed no staining even after growing them in NGM for 70 days (Figure 4 (A)). The PDL cells formed discrete nodules (Figure 4 (B)) after 35 days. All the Gore-tex lines also tended to form discrete nodules resembling the PDL nodules (Figure 4 (C)) after a mean average of 30.75 days ± 8.94 (Table 2). All the GTAM lines tended to show a more diffuse staining pattern (Figure 4 (D)) with a shorter time to nodule formation at a mean average of 23.67 ± 4.38 days (Table 2). The difference in time for nodule formation between the two cell types was not found to be statistically significant ($p = 0.063$) although 4 of 6 GTAM lines formed nodules more quickly than any of the Gore-tex lines.

Dexamethasone at 10 nM is known to induce osteoprogenitor cells to differentiate as measured by the increase in the number of nodule formation *in vitro* (Bellows 1990). Growth of PDL 814, GF-11, Gore-tex, and GTAM was attempted in NGM supplemented with 10 and 1 nM dexamethasone. But because all lines except the GF-11 line did not survive more than 2 days in this medium, no further culturing was done with the addition of dexamethasone. Warters (1992) also reported apoptosis in T-cell Hybridomas when exposed increasing concentrations of dexamethasone. At the similar concentration of 10 nM, he reported about a 25% cell death rate.

All cell lines were split several times in order to perform all the analyses. Separate cultures of each cell line were necessary in order to harvest the cell lines' conditioned medium (CM), freeze the cell lines for future use, and for examining nodule formation. Sufficient CM from all cell lines were collected for zymography and western blot analyses. Unfortunately, two Goretex lines were not analyzed for nodule formation. Culture dishes for Gore-tex 3 lines were contaminated prior to completion of von Kossa staining. Nodule formation for Gore-tex 27 was undetermined since this cell line proliferates too slowly and over half the cells did not survive splitting once exposed to STV for detachment off the culture dishes.

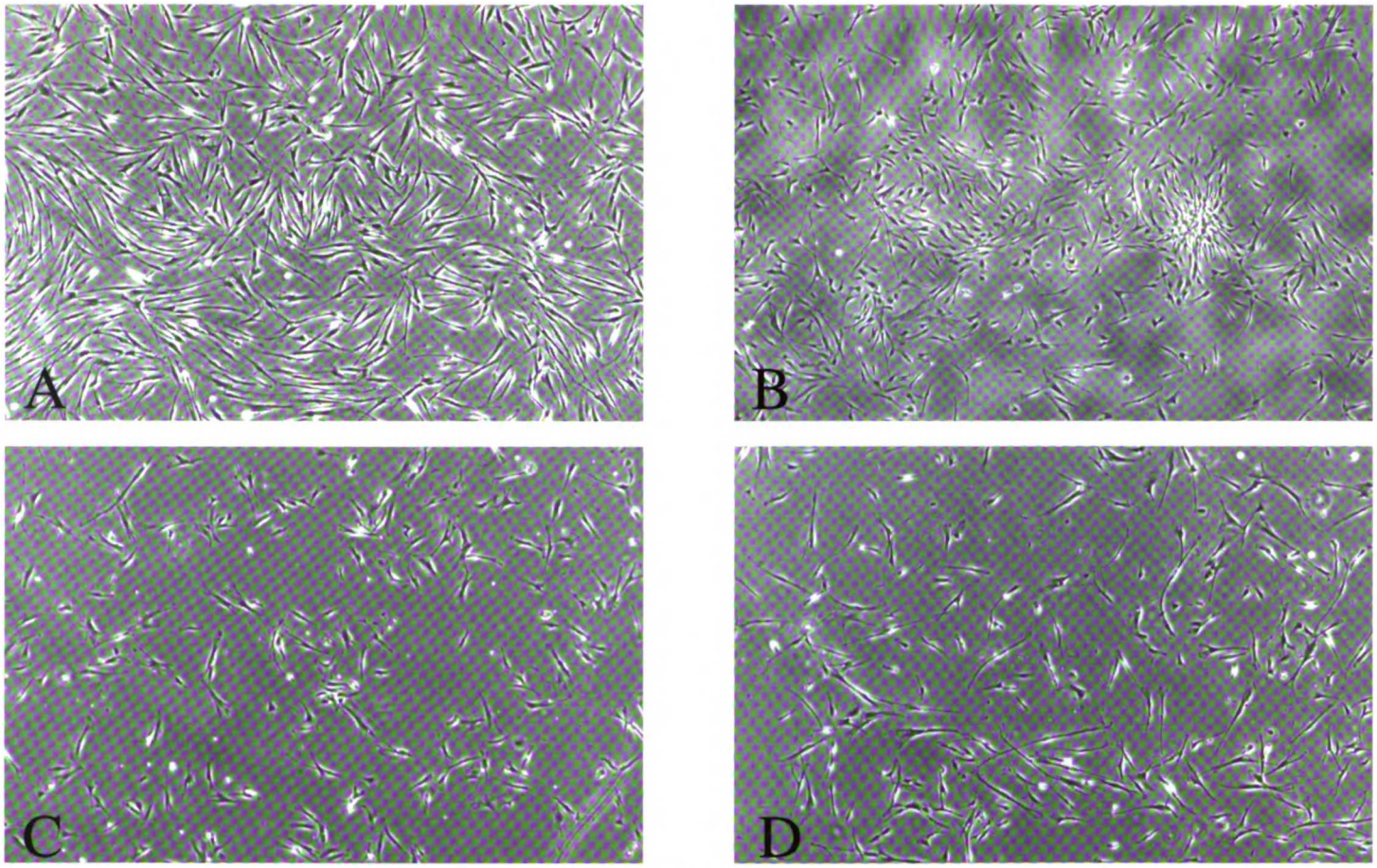


Figure 2. Morphology of isolated cell lines. (A) GF-11 = gingival fibroblasts. (B) PDL 814 = periodontal ligament cells. (C) G-tex 3 = cells cultured from a Gore-tex® membrane placed around the upper right first molar (tooth number 3). (D) GTAM 11 = cells cultured from a GTAM™ membrane placed for ridge augmentation of the upper right cuspid area (tooth number 11). The number designation of the cell lines represents the location that the membranes were placed based on the Universal numbering system. Note the similarities in cell morphology among the cell lines.

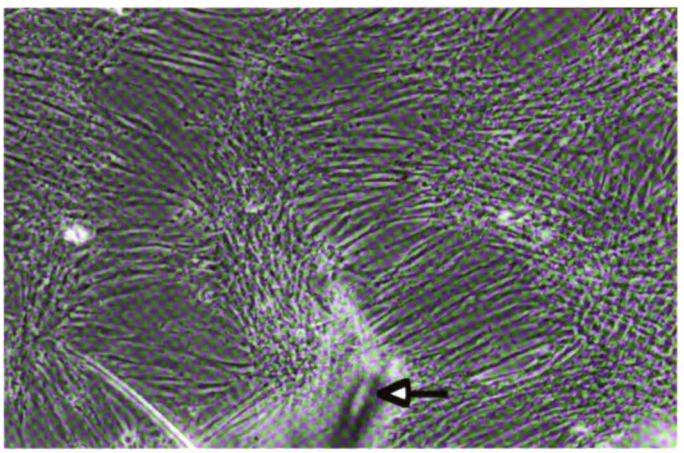

Figure 3. Cells allowed to grow to confluency ( about 4 weeks) cultured from an "explanted" Gore-tex® membrane. Arrow is pointing to the membrane. The figure illustrates pallisading of of interdigitating cells.

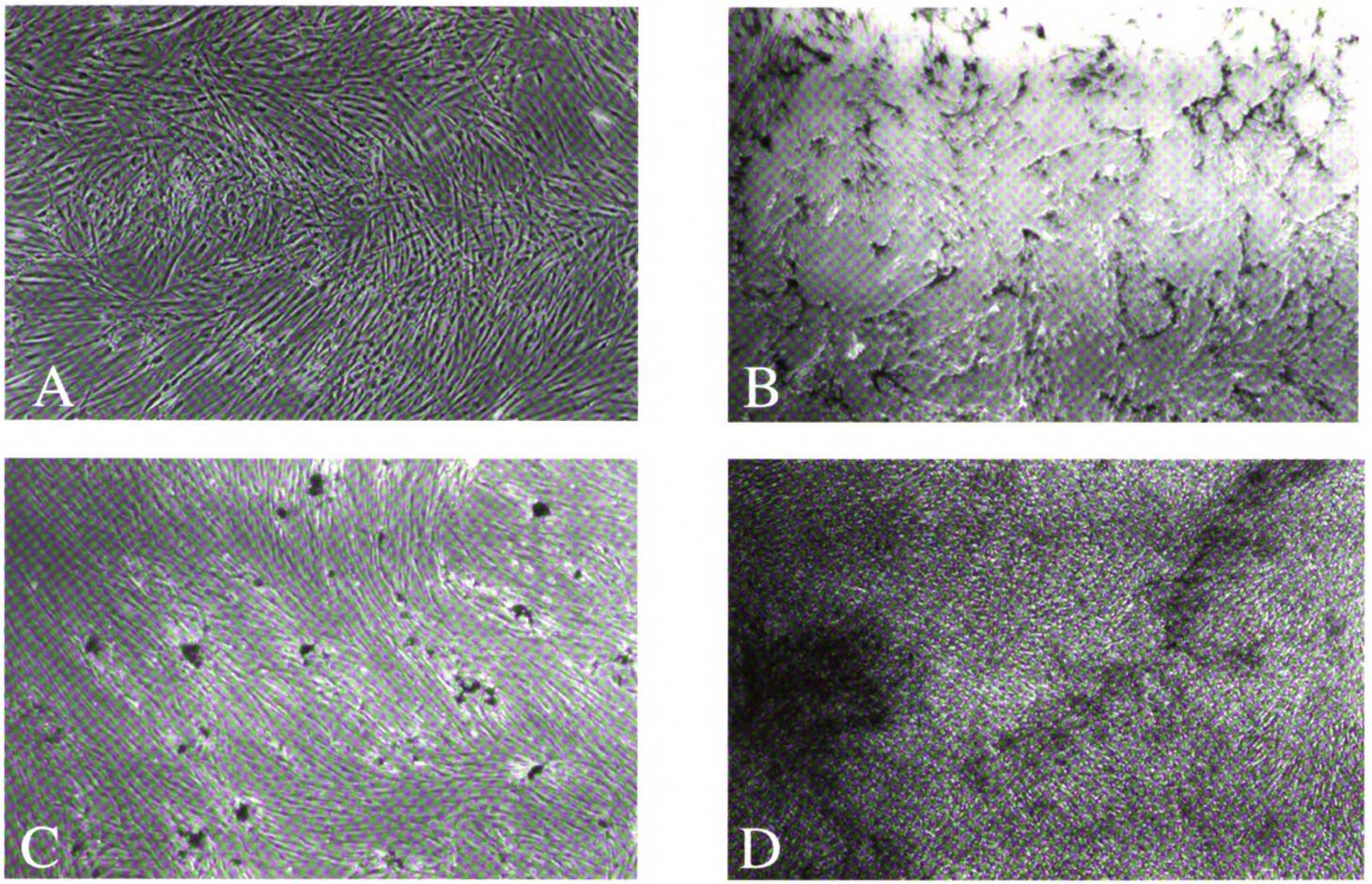


Figure 4. von Kossa staining of cell lines for the formation of mineralized nodules. (A) = GF-11 cells after 8 weeks. No staining was seen. (B) = PDL 814 cells after 5 weeks. Staining was seen in discrete nodules. (C) = G-tex 19 cells after 5 weeks. Staining was seen in discrete nodules. (D) = GTAM 11 cells after 3 weeks. Staining was seen in a diffuse pattern.

A

| CELL LINE | NODULE FORMATION (DAYS) |
|---|---|
| Gore-tex 3 | undetermined° |
| Gore-tex 18 | 25 |
| Gore-tex 19 | 35 |
| Gore-tex 27 | undetermined• |
| Gore-tex 30 | 35 |
| Gore-tex 31 | 28 |
| Average for Gore-tex lines | 30.75 ±4.38¤ |
| PDL 814 | 35 |
| GF-11 | no mineralization * |

B

| CELL LINE | NODULE FORMATION (DAYS) |
|---|---|
| GTAM 5i | 21 |
| GTAM 8i | 25 |
| GTAM 10i | 13 |
| GTAM 11 | 21 |
| GTAM 12^ | 42 |
| GTAM 25^ | 20 |
| Average for GTAM lines | 23.67 ±8.94¤ |
| PDL 814 | 35 |
| GF - 11 | no mineralization * |

Table 2. Nodule formation for (A) Gore-tex (B) GTAM cell lines.

° - dishes contaminated prior to completion of von Kossa staining
• - cell line proliferated too slowly for von Kossa stain determination
* - cells grown for 70 days
^ - cell lines cultured from same patient
¤ - p = .063 - not statistically significant

**Gelatin Substrate Gels**

Zymography separates different proteinases and inhibitor activities by electrophoresis which allows for the determination of their molecular weight ($M_r$) as well as other specific properties (such as molecular size changes occurring upon activation of the zymogens). Also, the size of the bands give insight to the relative abundance of the proteinase (Heussen and Dowdle 1980). Gelatin zymography detects proteinases that can degrade gelatin. The known metalloproteinases that can degrade gelatin include the collagenases (MMP-1, fibroblast type, MMP-8, PMN type) and the gelatinases (MMP-2, 72K and MMP-9, 92) and putative metalloproteinase-1 (MMP-7, PUMP-1) (Nagase et al 1992). The presence of SDS activates proenzymes without changing their apparent molecular weight ($M_r$) (Heussen and Dowdle 1980). Furthermore, although SDS may dissociate some MMP-TIMP complexes or prometalloproteinase-TIMP complexes, some higher molecular weight complexes is still seen. The unactivated proenzymes of the known MMPs are seen as one clear band except MMP-1, fibroblast-type collagenase. MMP-1 is a doublet at around 55K (57K-52K) since there are two forms of the proenzyme (Eisen et al. 1970, Stricklin et al. 1977).

Gelatin substrate gels (zymography) demonstrate that the gelatinase profiles of conditioned media from all the cell types were different (Figure 5 and 6). The Keratinocyte line exhibit a prominent band at 92K and a faint doublet at 55K (Figure 5 and 6). The CA 928 line was used as a standard to show the various bands of gelatinases and collagenases (Figure 6). It forms a prominent band at 92K, 72K, a doublet at 55K and a doublet at about 40K. The MG-63 line exhibits a very prominent band at 72K, a faint doublet at 55K and some higher molecular weight bands (Figure 5). The GF-11 line also exhibits multiple banding (Figure 5 and 6) with a prominent band at 72K, a doublet at 55K. The PDL 814 line exhibits bands at 72K, a faint doublet at 55K, and banding at higher molecular weights (Figure 5).

The GTAM lines exhibit fairly consistent banding patterns on the gels (Figure 6). Gels of GTAM lines all contain a doublet at 55K. All but the GTAM 12 show a band at 72K. The GTAM 11 and 25 have multiple banding patterns and a band at 92K. Also, GTAM 11 exhibits a prominent doublet at about 85K. GTAM 5i, 8i, and 10i only show the 72K and the doublet at 55K. The GTAM 12 line shows only the 55K doublet.

Banding pattern of the Gore-tex lines overlaps those of the other cell types. Gore-tex 3 and 18 have a banding pattern most resembling the PDL 814 (Figure 5 and 6), with bands at 72K, 55K doublet, and higher molecular weight bands. Gore-tex 19 and 30 show a similar banding pattern to those seen in the GTAM lines with bands at 72K and a doublet at 55K. Gore-tex 27 and 31 have multiple banding patterns with all the bands mentioned above plus additional higher molecular weight bands. Furthermore, Gore-tex 27 also has one prominent band at about 30K, not seen in any other cell line.

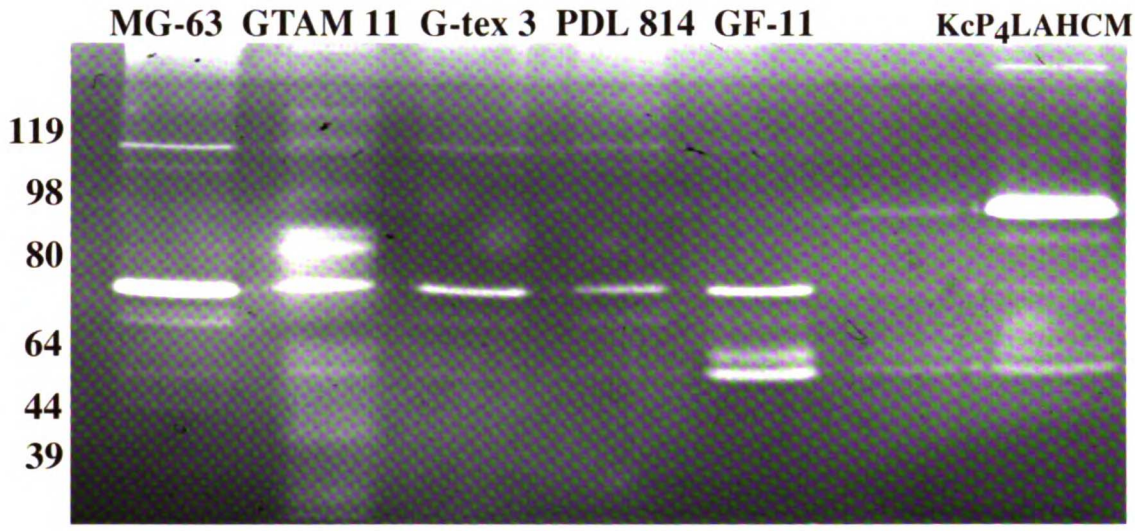


Figure 5. Gelatin zymogram of representative cell lines. Abbreviations are as listed in the table of abbreviations. Molecular masses in kilodaltons are indicated to the left of the figure. Note the similar banding pattern between G-tex 3 and 814 PDL. Both have a faint doublet at 55K, a prominent band at 72K, and higher molecular weight bands. GTAM 11 shows a prominent band at 72K, a doublet at 55K, a faint band at 92K, a prominent doublet at 85K, and higher molecular weight bands. The MG-63 shows a prominent band at 72K, a faint doublet at 55K, and higher molecular weight bands.

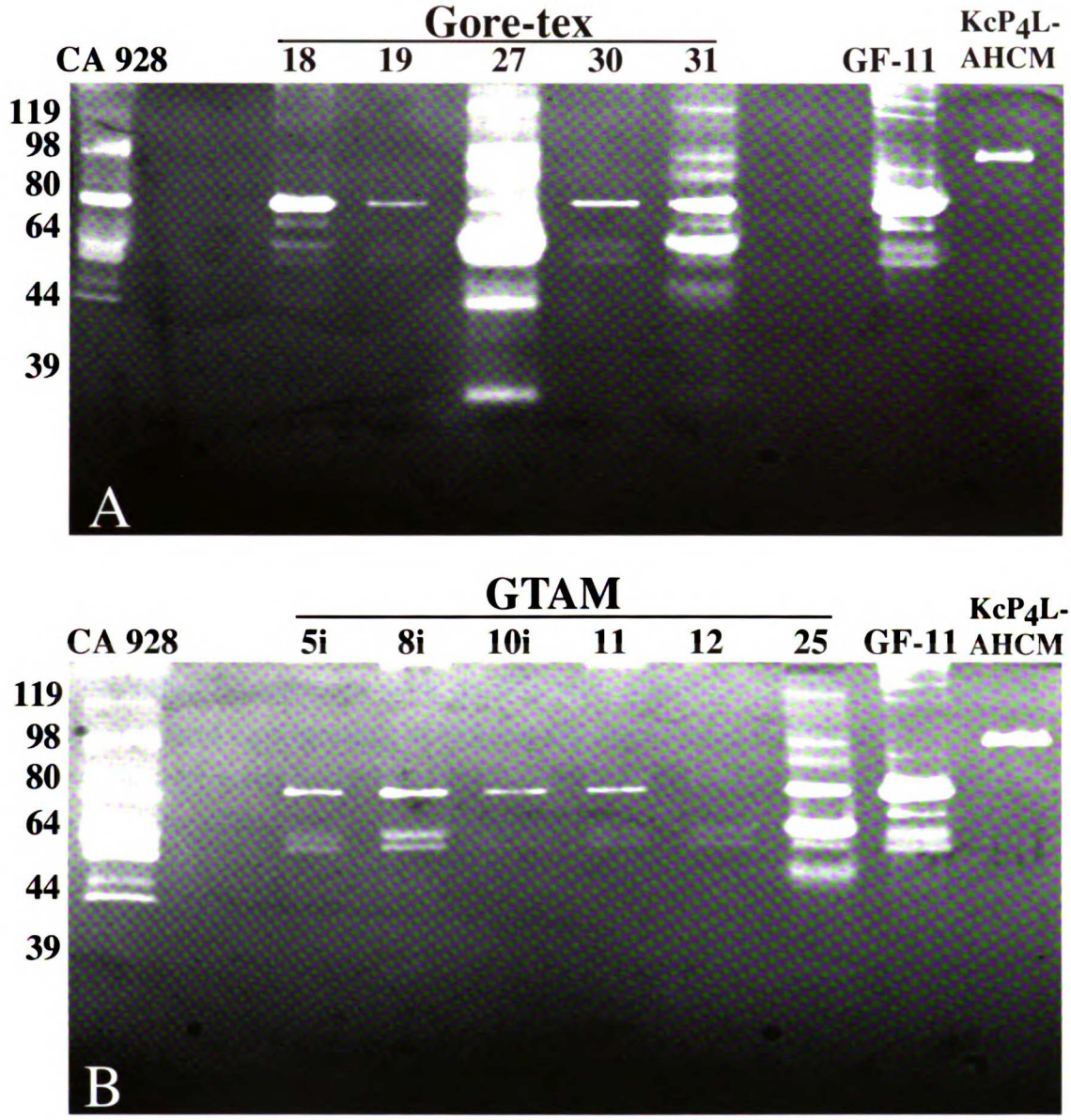


Figure 6. Gelatin zymogram of cell lines cultured from (A) Gore-tex and (B)GTAM membranes. The number designation of the cell lines represents the location that the membrane was placed based on the Universal numbering system. Small case "i" designates a GTAM placed in conjunction with an implant. Other abbreviations are as listed in the table of abbreviations. Molecular masses in kilodaltons are indicated to the left of the figures. Note the similar banding pattern of GTAM 5i, 8i, 10i,and 11. Multiple banding seen with GTAM 25. GTAM 25 shows a band at 92K. No 72K gelatinase apparent in GTAM 12. Note the similar banding patterns of Gore-tex 19 and 30 to the GTAM lines. Gore-tex 18 is similar but with an additional band at 92K and some faint higher molecular weight bands. Multiple banding seen with Gore-tex 27 and 31. Also, Gore-tex 27 shows a prominent band at 30K. All Gore-tex and GTAM lines show a doublet banding at 55K.

**Western Blots**

Bands at 72K were identified as being the 72K gelatinase by probing Western blots of conditioned medium from the cell lines with a polyclonal antiserum raised against a synthetic peptide corresponding to a portion of the sequence for human 72K gelatinase. The antiserum reacted strongly with this band in the cells harvested from G-tex 3, from PDL 814, and GF-11. Weaker banding was seen with the MG-63 and the GTAM 11. No binding was seen in keratinocytes, even though this lane was loaded with five times the amount of sample used for the other lanes.

The band on zymograms at 92K from keratinocytes was identified as the 92K gelatinase by probing a second Western blot with a polyclonal antiserum raised against the whole purified human enzyme (Unemori et al. 1991). The antiserum detected a band at 92K in keratinocytes as well as a band at 72K in MG-63 cells. This antiserum has previously been shown to cross-react with the 72K gelatinase in human synovial fibroblasts (Unemori et al. 1991). No other bands were seen on this blot, although GTAM 11 appears to have a faint band comigrating at 92K on the substrate gel (Figure 5).

Although analysis of the Western blot of the 92K gelatinase revealed no bands in the GTAM 11 lane (Figure 7(A)), there appeared to be a band at 92K comigrating with the prominent 92K band seen in the keratinocyte line on the substrate gel (Figure 5).

This apparent discrepancy between immunoblotting and zymography techniques has been reported by other investigators. Apodaca et al. (1990) reported a similar finding when looking at immunoprecipitation of collagenase. They reported definite bands on the substrate gel pertaining to the doublet produced by collagenase that was not detected by immunoprecipitation. They suggested that the substrate gel is more sensitive than immunoprecipitation. Adler et al. (1990) has suggested comigrating bands seen on zymograms may be due to uncharacterized metalloproteinases.

Western blots were also probed with poylclonal antiserum to TIMP-1 (Figure 8 and 9). All cell lines produced a band at about 30K. These were seen in MG-63, GTAM 11 and 25, Gore-tex 27 and 31, and GF-11. By densitometric scanning, the bands were analyzed for relative amounts of TIMP-1 production compared with GF-11 TIMP-1 production on the same blot. Although not statistically significant ($p = .265$), the GTAM lines tended to produce more TIMP-1 (Table 3) (average was $1.00 \pm .53$ [Note: The 1.00 average designates

that TIMP-1 production from GTAM was equal to TIMP-1 production from GF-11]). The Gore-tex lines produced lower amounts (.63 ± .47). The PDL and the keratinocytes produced the lowest amounts of TIMP-1, .17 and .05, respectively. Higher molecular weight bands were also seen in the GTAM 11 and 25, Gore-tex 18, 27, 31, MG-63, and GF-11. The amount of TIMP-1 did not appear to correspond to the time for nodule formation (Table 3). However, only GTAM and Gore-tex lines that had a band at 92K on substrate gels (Figure 5 and 6), showed higher molecular weight complexes on the TIMP Western blots (Figure 8 and 9).

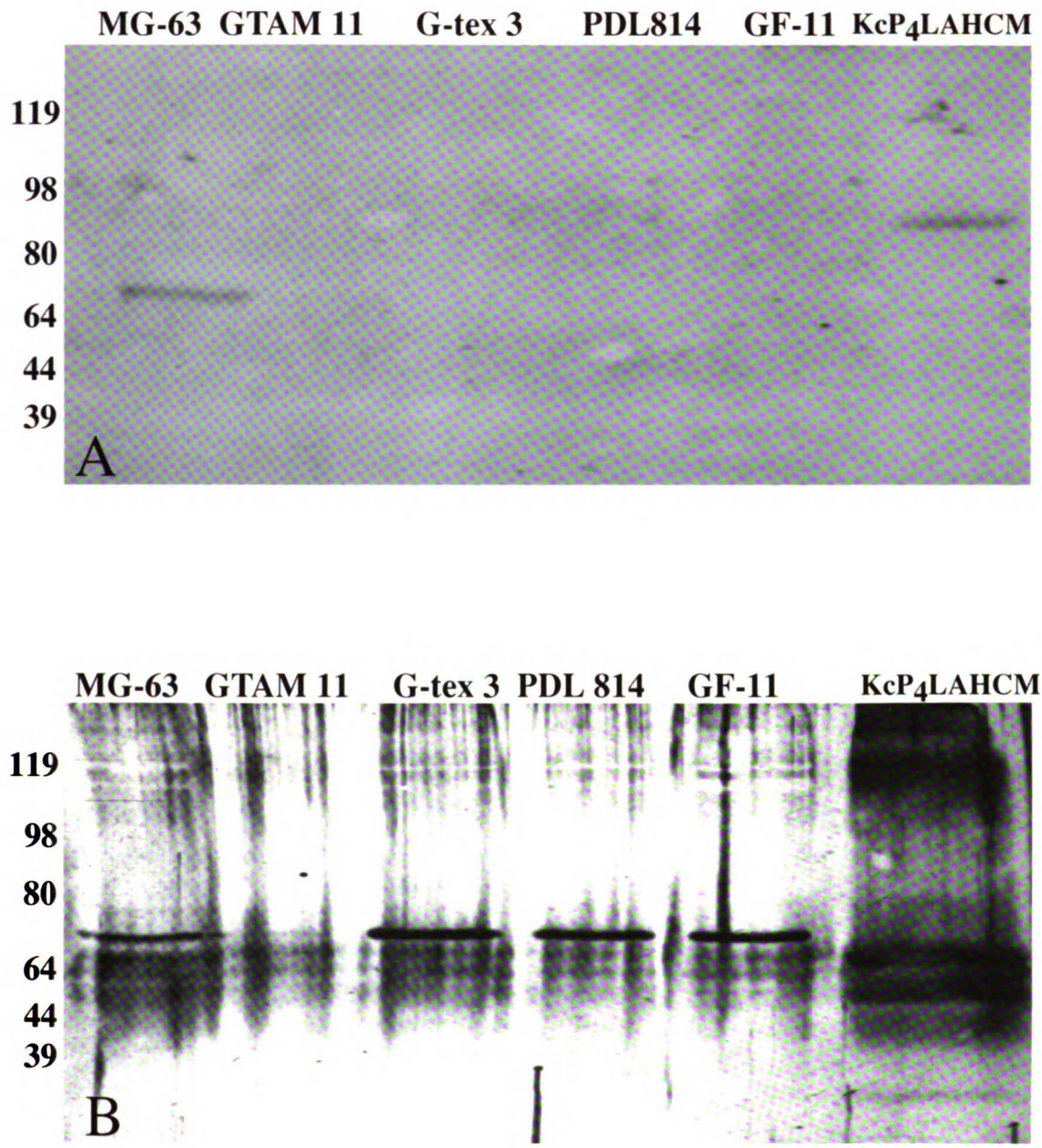


Figure 7. Western blot of conditioned media from representative cell lines to (A) the 92K gelatinase and (B) the 72K gelatinase. Molecular masses in kilodaltons are indicated to the left. Note on (A) the $KcP_4LAHCM$ line shows a band at the 92K and there is a cross-reactivity band near 70K for the MG-63. Note on (B) the $KcP_4LAHCM$ does not show a band at 72K. All other lines show a band at 72K. The GTAM 11 band at 72K is faint.

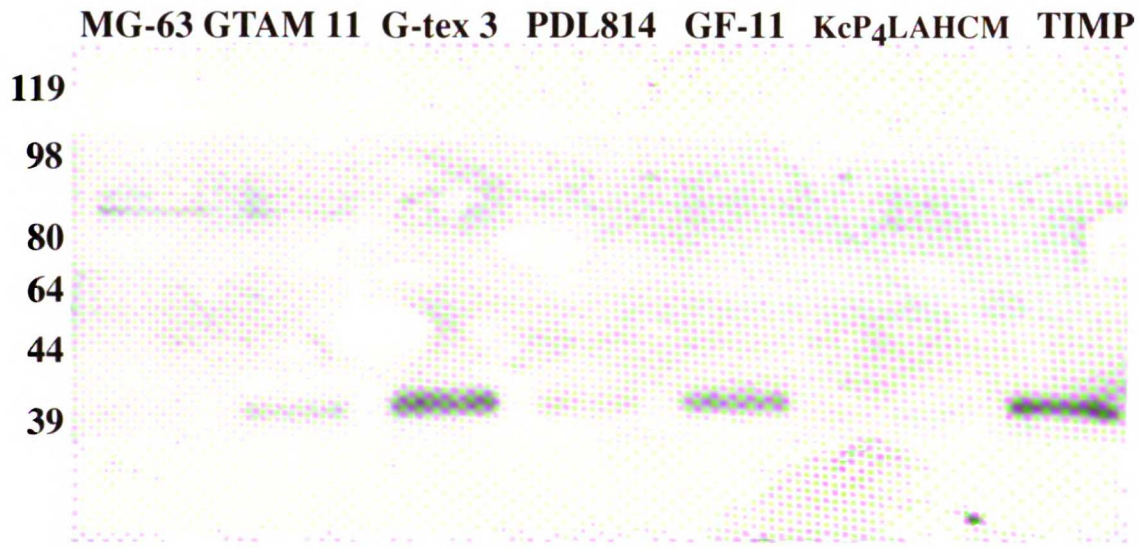


Figure 8. Western blot to TIMP-1 of conditioned media from representative cell lines. Molecular masses in kilodaltons are indicated. The last lane is a purified TIMP-1 standard. Note G-tex 3 produced more TIMP-1 than GF-11. Also note higher molecular weight bands in the MG-63 and GTAM 11 lanes.

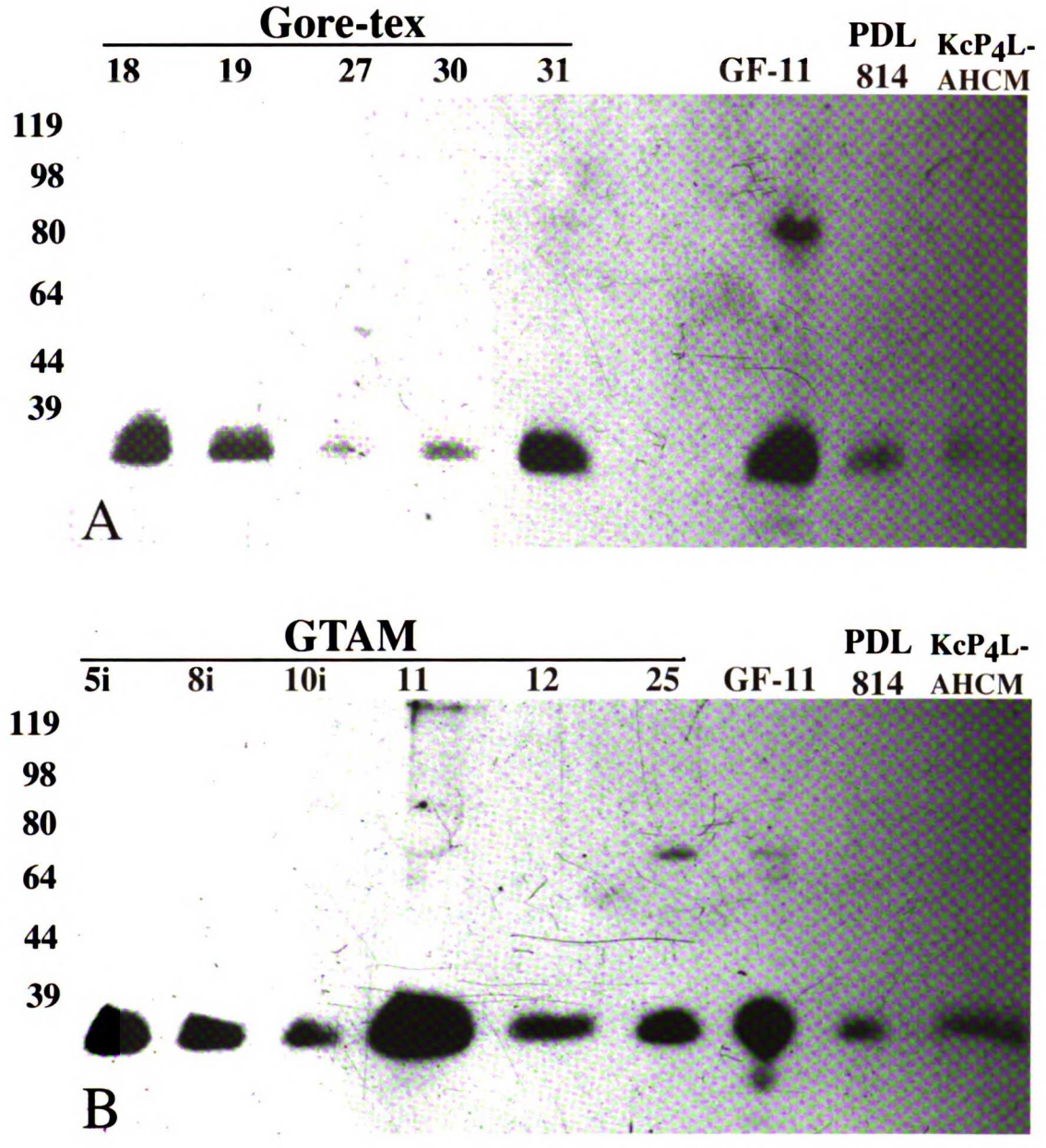


Figure 9. Western blot to TIMP-1 of conditioned media from (A) Gore-tex and (B) GTAM lines. The number designation of the cell lines represents the location that the membrane was placed based on the Universal numbering system. Small case "i" designates a GTAM placed in conjunction with an implant. Other abbreviations are as listed in the table of abbreviations. Molecular masses in kilodaltons are indicated to the left of the figures. Note that all cell lines show some TIMP-1 production. Production of TIMP-1 is less in the $KcP_4LAHCM$ and PDL 814 lines. Overall, the GTAM cell lines produce more TIMP-1 than the Gore-tex lines. Also note higher molecular weight bands in the Gore-tex 27 and 31, GTAM 11 and 25, and the GF-11.

| Cell Line | TIMP-1 production†ø¤/ higher molecular weight bandsπ | Gelatinase production | Collagenase production | Other gelatinase and collagenase production‡ | Nodule formation (Days) ◊ |
|---|---|---|---|---|---|
| GTAM 5i | 1.22 | 72K | 55K - doublet | -- | 21 |
| GTAM 8i | 1.06 | 72K | 55K - doublet | -- | 25 |
| GTAM 10i | .43 | 72K | 55K - doublet | -- | 13 |
| GTAM 11 | 2.00 / + | 92K,72K | 55K - doublet | ++ | 21 |
| GTAM 12^ | .51 | -- | 55K - doublet | -- | 42 |
| GTAM 25^ | .80 / + | 92K,72K | 55K - doublet | ++++ | 20 |
| Gore-tex 3 | 1.52 | 72K | 55K - doublet | + | undetermined° |
| Gore-tex 18 | .73 / + | 92K,72K | 55K - doublet | + | 25 |
| Gore-tex 19 | .45 | 72K | 55K - doublet | -- | 35 |
| Gore-tex 27 | .07 / + | 92K,72K | 55K - doublet | +++++ | undetermined• |
| Gore-tex 30 | .22 | 72K | 55K - doublet | -- | 35 |
| Gore-tex 31 | .79 / + | 92K,72K | 55K - doublet | ++++ | 28 |
| PDL 814 | .17 | 72K | 55K - doublet | + | 35 |
| GF-11 | 1.00 | 72K | 55K - doublet | +++ | no mineralization* |
| KcP4LAHCM | .05 | 92K | 55K - doublet | ++ | no mineralization |

Table 3. Summary table of results

Table 3. Summary table of results.

† - expressed as a ratio of scanned densitometric units of cell line to the corresponding gingival fibroblasts on same blot. GTAM lines/GF (48809) (Figure 9 (B)), Gore-tex/GF (52318) (Figure 9 (A), Gore-tex 3/GF (47178) (Figure 8)

ø - GTAM average = 1.00 ± .53 Gore-tex average = .63 ±.47

¤ - p = .265 - not statistically significant

π - as shown on the Western blots of TIMP (Figure 8 and 9)

‡ - as shown on the corresponding gelatin zymography (+ = relative amounts of other bands present on gel) (Figure 5 and 6 (A) & (B))

◊ - p = .063 - not statisitically significant

-- - no bands present

^ - cell lines cultured from same patient

° - dishes contaminated prior to completion of von Kossa staining

• - cell line proliferated too slowly for von Kossa stain determination

* - cells grown 70 days

## DISCUSSION

### Cell Cultures and Mineralized Nodule Formation

Guided tissue regeneration relies on manipulating the sequence of healing in surgical wounds to favor repopulation of the wound site with cells that can regenerate either bone, cementum, and periodontal ligament in the case of periodontal defects or bone as in ridge augmentation. Selvig et al. (1990 and 1992) and Machtei et al. (1994) have discussed fibroblast-like cells adherent to Gore-tex® membranes after regenerative procedures around teeth. Culturing these cells adherent to the membranes was therefore a logical consequence of these observations and proved to be a readily available means of obtaining regenerative cells for further investigation.

This manuscript reports a novel way to culture cells adherent to barrier membranes. All cell lines cultured from both the Gore-tex® and the GTAM™ membranes had the fibroblast-like shape reported by Selvig et al. (1990 and 1992) and Machtei et al. (1994) (Figure 2). and had similar morphologies especially when subconfluent. It was not until the cells were grown to confluency that some differences became visible. No orientation was evident with the GF-11 or GTAM lines. However, the lines from the Gore-tex® membranes tended to form palisading units in rows (Figure 3). This was also seen with a PDL cell line isolated from unerupted third molars (data not shown) and to some extent in the PDL 814 line from bicuspids. Garant and Cho (1979) have shown that rat and mouse PDL cells maintain a cytoplasmic polarization *in vivo*. They tend to have their nuclei at one end with the organelles on the other. They are also elongated with their long axes parallel to the principal fiber bundles. Cho et al. (1992) reported this same orientation was retained in PDL cells cultured *in vitro*. This cell growth pattern was similar in the Gore-tex® lines.

All cell lines from the Gore-tex® and GTAM™ membranes had the capability to form mineralized nodules when grown in the presence of ascorbic acid and ß-glycerophosphate. Although not statistically significant, the Gore-tex cell lines tended to form nodules more slowly and in a discrete pattern (Figure 4 (C)) and (Table 2 and 3)]. GTAM lines tended to show a more diffuse pattern of staining [(Figure 4 (D)). The Gore-tex cell lines tended to form nodules about the same time and had a similar discrete pattern as the 814 PDL line [(Figure 4 (B)) and (Table 2 and 3)]. This was maybe due in part to the similar palisading growth pattern. Also, this observation may suggest

that the Gore-tex and the PDL 814 lines have a more heterogeneous population of cells, consisting of cells that can form mineralized nodules (i.e. osteoblasts and cementoblasts) and those which can't (i.e. undifferentiated precursor cells and fibroblasts). GTAM lines may be more homogenous consisting of only osteoblasts and their precursors.

Various studies have reported different additional requirements in order to induce mineralized nodule formation. Cho et al. (1992) reported that PDL cells formed nodules only when cultured in medium containing 50 µg/ml ascorbic acid and 5 µM dexamethasone, not in ß-glycerophosphate. Bellows et al. (1990) reported that 10 nM dexamethasone, a synthetic steroid, can induce osteoprogenitor cells to differentiate as measured by increased nodule formation. However, Warters (1992) reported a 25% cell death rate of T-cell hybridomas exposed to 10nM dexamethasone. Dexamethasone was also added to the growth medium of the Gore-tex, GTAM, and PDL 814 cell lines. None of the cell lines survived exposure to the dexamethasone at 10 nM or 1 nM. Melcher et al. (1986) has reported that PDL cells were not capable of mineralized nodule formation but cells cultured from fetal rat calvariae could form cementum-like tissue when cultured with 10mM ß-glycerophosphate and 50 µg/ml ascorbic acid. Recently Mukai et al (1993) demonstrated bone-like nodules formed by rat PDL cells cultured with 10mM ß-glycerophosphate and 50 µg/ml ascorbic acid. Differences in the reports could be due to variations in culturing and technique. Melcher et al (1986) used cells at the 11th passage whereas Mukai used cells at the 4th passage. Only cells at the 4th passage were used in the experiments of this thesis. Further studies exploring the mechanism of additives in the culture media of osteoprogenitors and regnerative cells should be initiated.

Qualitative differences between the nodules formed by cell lines have been reported. Mukai et al. (1993) reported nodules from PDL cells looked bone-like with elements of cells incorporated into the matrix, a collagenous matrix, a mineral composed of hydroxyapatite, and intense alkaline phosphatase activity. Cho et al (1992) reported nodules from PDL cells lacked elements seen in a mineralized matrix of bone such as cells within the matrix which would be suggestive of osteoblasts or osteocytes. Also, they reported a polarity of cells which is indicative of PDL cells *in vivo*. However, even Mukai et al. (1993) admitted that since there are no available markers to distinguish cementum from bone or cementoblasts from osteoblasts *in vitro*,

they could not rule out that the matrix was cellular cementum. Although no scanning electron microscopy was done in this study, the qualitative differences of the overall staining patterns and the difference in time to nodule formation could be suggestive of two types of nodule formation.

**MMPs of a PDL Cell Line (814 PDL)**

No data describing substrate gels of periodontal ligament cell lines has been published. Gels of CM of PDL 814 revealed bands at 72K, a faint doublet at 55K and complex banding at higher molecular weights (Figure 5). Therefore, this suggests this line can produce the 72K gelatinase and collagenase. From *in vitro* observations, others (Gould 1983, McCulloch 1985, Roberts et al. 1987, Piche et al. 1989, Somerman et al. 1990 & 1992 Hou & Yeager 1993) have found the cell population harvested from the periodontal ligament is heterogenous. PDL 814 would be a representation of one PDL subpopulation. It will be interesting to compare these findings to future investigations of PDL profiles on gelatin substrate gels and correlate these results with clinical significance.

**MMPs of GTAM and Gore-tex Lines**

Several significant finidings have been discovered from analysis of the gelatin zymography of the Gore-tex and GTAM lines. The most prominent was the fact that all the Gore-tex and GTAM lines were able to synthesize MMP-1, the fibroblast type collagenase (55K doublet) and all but one line (GTAM 12) showed a band at 72K. Johansen et al. (1992) showed that in three separate human osteoblastic cell cultures (cultured from trabecular bone of femoral heads), the major proteins secreted were the 72K gelatinase, osteonectin, the C-terminal propeptides of the $\alpha_1$– and $\alpha_2$ chains of type I collagen, TIMP-1, and $ß_2$ macroglobulin. On the other hand, normal fetal osteoblastic cells from one 20-week old human fetus secreted osteonectin and the C-terminal propeptides of the $\alpha_1$– and $\alpha_2$ chains of type I collagen. Gelatinase and TIMP could not be detected. Therefore, they concluded that fetal osteoblasts primarily express proteins that are matrix constituents, whereas the adult human osteoblasts secrete additional proteins that function in matrix turnover.

Recently, Rifas et al. (1994) also reported that fully differentiated human osteoblasts released copious amounts of 72K gelatinase and TIMP-1

and TIMP-2. Only a very faint band was seen at 55K regardless of whether the cells were grown on plastic or a type I collagen substratum. Even after incubation with bone resorptive factors (parathyroid hormone, Phorbol 12 myristate 13-acetate, 1,25-$(OH)_2D_3$, human recombinant Interleukin-1ß [hr IL-1ß], human recombinant tumor necrosis factor-α [hrTNF-α], very little collagenase was induced in the osteoblast lines. On the other hand osteogenic tumor lines produced collagenase and this production could be increased if bone resorptive factors were added. Collagenase production tended to be greater in the lines that produced less alkaline phosphatase (Alk P'tase). Alk P'tase was used as a marker for differentiation, with greater enzymatic reactivity implying greater differentiation in the osteogenic tumor lines. Therefore, they concluded less collagenase is produced in more differentiated osteoblast-like cells and less differentiated osteoblast-like cells could be induced to produce collagenase in the presence of bone resorptive factors.

Meikle et al. (1992) showed that human osteoblasts cultured from adult trabecular bone of the femoral head can also be induced by PTH or monocyte conditioned medium to secrete collagenase, 92K gelatinase and stromelysin. They found that stimulation was greater if osteoblasts were cultured on type I collagen films. If the osteoblasts were cultured on glass slides and unstimulated, they did not produce collagenase, 92K, or stromelysin. These two papers indicate that less differentiated osteoblasts can be induced to produce collagenase and bone nodules. These considerations suggest that the GTAM cell lines represent less differentiated osteoblastic lines that can produce mineralized nodules and have responded to cytokine signals *in vivo* prior to harvesting (because they produce the induced 55K collagenase). Furthermore, these characteristics are retained when cultured on plastic culture dishes *in vitro*. Future studies to confirm this finding could be pursued such as investigating whether collagenase mRNA transcription is increased in these regenerative cells is increased after exposure to TNF-α or IL-1ß.

The three GTAM lines retrieved around implants (GTAMi's) had very similar banding patterns on the substrate gels. They all produced only the 72K gelatinase and the 55K doublet. The GTAM lines retrieved after ridge augmentations showed more variability. The GTAM 11 and 25 showed multiple bands, whil the GTAM 12 line showed only the doublet at 55K. Part of the explanation could be due to the fact that the three GTAMi's were left in

place longer (at least 3 months) and were not exposed. The ridge augmentation GTAMs were left in at a shorter interval regardless of exposure. This could possibly mean that the GTAMi lines represented more mature cells. Lekholm et al. (1993) examined the effect of early versus late removal of GTAM membranes around implants in dogs over a 16 week interval. They reported that sockets with implants and membranes left in place for the duration of the 16 weeks had 100% bone fill. Sockets with implants where the membrane and the regenerated tissue were removed after 4 weeks then reentered at 16 weeks had 42% bone fill. Interestingly enough, the sockets where the membranes were removed (after 4 weeks) but the regenerative tissue was left intact, showed the least amount of fill (21%). The removed regenerative tissue after 4 weeks showed inflamed connective tissue, containing spicules of newly formed bone. Possibly, exposure of the immature regenerative tissue after membrane removal triggered the inflammatory cells to release cytokines that initiated the cascade of events that lead to further resorption of this new regenerative tissue.

Resorption of bone is believed to be caused by the coordination of osteoblastic and osteoclastic activity. Mineralized bone is resorbed by osteoclasts (Chambers 1980). The coordination is hypothesized because in mouse calvaria, when osteoclasts and osteoblasts are exposed to bone resorbing agents, only osteoblasts can produce collagenase (MMP-1) (Heath et al., 1984 and Sakamoto & Sakamoto 1984). Delaisse et al. (1988) also have reported that bone-resorbing agents (IL-1ß, TNF-$\alpha$ , 1,25-$(OH)_2D_3$, PTH) cause an accumulation of procollagenase in the nonmineralized matrix of mouse calvaria and that degradation of this nonmineralized matrix can be inhibited by a purified preparation of human TIMP. Chambers et al. (1985) hypothesized that it is this degradation of the unmineralized matrix that initiates the degradation of mineralized matrix by osteoclasts. It is possible that the tissue seen under GTAM membranes represents an extension of the nonmineralized matrix of bone and that this matrix is highly susceptible to collagenases.

Although Rifas et al. (1994) stated that mature human osteoblasts are not capable of collagenase production even in the presence of bone resorbing agents or monocyte conditioned medium, it is apparent that cells under GTAM membranes in this study are capable of not only producing mineralized nodules but also producing collagenase that can contribute to the

degradation of this tissue. Two lines, GTAM 11 and 25, were also shown to produce multiple molecular weight banding with more collagenases and gelatinases, and this may lead to a greater potential to contribute to the degradation of this immature tissue.

The observation of multiple MMP production may be significant in clinical treatment. Lekholm et al. (1993) have reported results suggesting that the least bone fill was seen in test sites where the membrane but not the regenerative tissue was removed after 4 weeks. Removal of the membrane requires a second surgical procedure that will again initiate the cascade of events of wound repair. One of the consequences includes the recruitment of leukocytes. These leukocytes will release cytokines such as TNF-$\alpha$ and IL-1ß which are known to stimulate collagenase production. Ellies and Aubin (1990) have reported that cytokine release at sites of inflammation and infection may alter normal bone remodeling process resulting in pathologic bone destruction or bone formation. The osteoblasts, although capable of bone formation might be triggered to release copious amounts of collagenase that may offset any bone regeneration. This could also help explain why most studies on the clinical results of guided tissue regeneration with Gore-tex® report recession and shrinkage at the regenerated site (Handelsman 1991, McClain & Schallhorn 1993, Blumenthal 1993, Cortellini et al. 1993).

In this study, the GTAM membranes that were exposed and all the Gore-tex® membranes showed variable banding patterns. The coronal portion of Gore-tex® membranes are usually exposed because they are abutted next to the tooth surface and some recession of the gingival tissue is usually expected. There is therefore communication between the regenerating site and the oral cavity. In their study of the use of ePTFE membranes in dogs, Haney et al. (1993) reported that complete gingival coverage of the barrier membrane appeared critical for optimal healing. They reported that exposure of the membrane exhibited an inflammatory infiltrate underneath the membrane and none or minimal bone regeneration. The lack of bone regeneration in this instance may be mediated by cytokines released by the inflammatory infiltrate causing increase MMP production in these regenerative cells, thereby degrading the newly formed osteoid matrix.

Overall, the Gore-tex lines had variable enzymatic profiles. Two cell lines (Gore-tex 19 and 30) were similar to the profiles of the GTAMi's, two cell lines (Gore-tex 3 and 18) were similar to PDL 814, and two cell lines (Gore-tex

27 and 31) had very unique multiple banding patterns. This variability accentuated the heterogeneity of these cell lines. This overlap is not surprising if one considers all the complex events that must take place to fully regenerate hard and soft tissues under these membranes. One would expect to find a variety of cells adherent to these membranes with osteoblast-like qualities, PDL cell-like qualities, and fibroblast-like qualities.

High molecular weight banding patterns seen in some GTAM and Gore-tex lines may be associated with increased migration of these cells. Yamada et al. (1990) have shown that cells can be seen migrating on curretted root surfaces up to 7mm from the base of the denuded root surfaces after 7 weeks of healing under membranes. Fisher et al. (1989) have shown that there is a temporal and spatial regulation of MMP production in cytotrophoblasts cells *in vitro*. They showed a greater production of higher molecular weight complexes in the extracts of cells during the first trimester when the cytotrophoblasts are most actively invading and migrating. Apodaca et al. (1990) noted normal astrocytes also produced these multiple banding patterns when induced by inflammatory mediators that may be important in the migration of these cells. Others (Adler et al. 1990, Bernhard et al. 1990) have shown that production of MMP is associated with invasion and metastasis of tumor lines.

In the case of guided tissue regeneration, cells travel a relatively large distance (periodontal defects can be up to 1-1.5 cm). This may be facilitated by degradation of granulation tissue by cellular secretion of MMPs and other matrix degrading enzymes. Salo et al. (1994) reported that the granulation tissue in wound healing has a high quantity of MMP-9 (92K gelatinase) which is not associated with macrophages. Their results suggest that other cell types, such as endothelial cells and fibroblasts, may participate in MMP-9 expression in the granulation tissue. MMP-9 is known to degrade fibronectin (Partridge et al. 1993) which may be important for cells to migrate through a fibrin-fibronectin matrix. Further studies looking at the ability of these regenerate cells to migrate should be addressed.

The Gore-tex 27 line is unique. Not only did it produce multiple high molecular weight proteases, but it produced a prominent band at about 30K that was not seen in any other line. This band may represent PUMP-1 (MMP-7). PUMP-1 was first isolated in the postpartum rat uterus (Woessner and Talpin 1988) and has been shown to have a wide spectrum of substrates that

includes casein, gelatins, fibronectin, and proteoglycan. Murphy et al. (1989) provided evidence that PUMP-1 also degrades type IV collagen and elastin. Murphy et al. (1989) found mRNA transcripts to PUMP-1 in the involuting uterus and Miyazaki et al. (1990) reported that after a survey of conditioned media from 30 nonmalignant and malignant human cell lines, only one rectal carcinoma line secreted PUMP-1. They reported that PUMP-1 shows a band at 28K on gelatin substrate gels. Marti et al. (1992) isolated PUMP-1 from conditioned medium of mesangial cells of the kidney glomerulus. This was mediated by synergistic induction with IL-1ß and TNF-α . They also showed that PUMP-1 is expressed *in vivo* during an acute glomerular inflammatory process. Overall and Sodek (1990) reported that normal human fibroblasts secrete PUMP-1 when induced by Concanavalin A (Con A). Con A, a lectin from the jack bean plant (*Conavalia ensiforms*), induces a resorptive cellular phenotype in human fibroblasts by binding to saccharides containing α-D glucose and D-fructose (Rosenblith et al 1973). They speculate that because this enzyme exhibits such a broad substrate activity, secretion of PUMP-1 may contribute to further injury. Previously, this enzyme has not been reported in regnerative tissue. Secretion of this broad spectrum degradative enzyme may potentiate degradation of regenerative tissue and compromise the outcome. Further efforts to purify this 30K protein from the Gore-tex 27 line should be pursued.

Furthermore, only after induction, certain normal human cells have been shown to produce PUMP-1 *in vitro*.. The only other cell line reported to produce PUMP-1 is a tumor line. The Gore-tex 27 cell line retains the ability to produce PUMP-1 on plastic culture dishes without induction, transformation, or the addition of any outside agent. Therefore, culturing of regenerative cells from periodontal membranes could prove to be an invaluable source from which the study of induced normal human cell lines can be investigated.

**TIMP-1 Production**

TIMP-1 production has been associated with the maintenance and formation of bone. As stated above, Johansen et al. (1992) found that human osteoblasts secrete a copious amount of TIMP. Everts et al. (1993) examined TIMP in rabbit calvarial bone explants with immunolabeling. They found that TIMP was in higher concentrations in osteoblast-like cells lining both the

outer bone surface as well as the endosteal spaces and in osteocyte lacunae. The periosteum was almost negative. Therefore, it is suggested that TIMP is important in the basal remodeling and turnover of bone. Furthermore, Meikle et al. (1991) reported that in mouse calvaria, free TIMP in the culture medium was associated with less bone resorption. Nomura et al. (1989) reported a high expression of TIMP in mouse osteogenic structures such as the calvaria using antisense RNA probes to TIMP. Although not statistically significant ($p = 0.063$), in the GTAM lines, there was a tendency for higher TIMP production than was seen in GF-11 or Gore-tex lines. Lack of statistical significance may be related to the relative small number of cell lines and the overlapping characteristics that the various Gore-tex lines share with the GF-ll and the GTAM lines. The role of TIMP as an inhibitor of matrix degradation and bone resorption has been proven; however, the extent of its actions in periodontal regeneration has not been established.

TIMPs have also been shown to form high molecular weight complexes with MMPs. TIMP-1 has been shown to form complexes with 92K gelatinase (Wilhelm et al. 1989) and the active form of fibroblast collagenase (Welgus et al. 1985) whereas, TIMP-2 aggregates with 72K gelatinase(Goldberg et al. 1989). In the absence of TIMP-1, 92K gelatinase has been shown to form a dimer and a complex with fibroblast collagenase which is active against both gelatin and fibrillar type I collagen (Goldberg et al. 1992). This complexing of 92K may explain the presence of some of the higher molecular weight banding patterns seen on the substrate gels and the higher molecular weight bandings observed on the TIMP Western blots.

## CLINICAL IMPLICATIONS

Although this report does not correlate the clinical outcome of the regenerative procedures with the characteristics of the cell cultures established *in vitro*, some findings from this study may have an impact on the procedural aspects of periodontal regeneration. First, all cells migrating and adhering to the barrier membranes used around periodontal defects as well as alveolar ridge and implant augmentation defects have been shown to be capable of forming mineralized nodules. Therefore, full bone regeneration should be possible.

Precise spatiotemporal constraints and regulations are necessary in order to gain new attachment instead of repair. This probably requires breakdown of a series of matrices by a variety of the MMPs and other degradative enzymes. The sequence of MMP expression may be critical to induce regeneration rather than repair. Lynch et al. (1989) showed that a single topical application of 1 μg/ml of Platelet-derived Growth factor (PDGF) and Insulin-like growth factor (IGF) in an aqueous gel on root surfaces after root debridement enhanced the regeneration of new cementum and bone formation in dogs. Since these agents may affect production of MMPs, further studies investigating the role of cytokines and MMPs in regeneration should be addressed.

The finding that collagenase was produced in all cell lines cultured may have important consequences. Collagenase was produced even from the cell line cultured from the GTAM™ membrane retrieved after 15 months. This implies that some resorption of the mineralized tissue can be expected after any membrane removal. In some lines, multiple collagenases and gelatinases were produced. The events leading to the production of degradative enzymes are unclear, but could be due to a combination of factors such as exposure or early maturation of the tissue (prior to 8 weeks). The Gore-tex® Guided Tissue Regeneration Workshop Manual (1992) advocates the removal of material 4 to 6 weeks postoperatively. This might seem premature since prolonging removal of the nonresorbable membrane may increase regenerated bone (Lekholm et al 1993) and inhibit release of factors that can induce osteoblasts to release more collagenase. Such a protocol may preserve the regenerative tissue.

Membrane exposure may impair regeneration. As seen in the Haney et al. (1993) study, exposure of the membrane resulted in a greater inflammatory

infiltrate with less favorable clinical results. The cells adherent to the unexposed GTAM™ membranes tended to produce the least amount of collagenase which may be due to less exposure to inflammatory mediators. Therefore, exposure of membranes should be avoided at all cost. This could be facilitated by vertical releasing incisions to coronally reposition the flap or to allow for laterally sliding pedicles over the membranes. Flaps sutured passively over the membrane to gain primary closure should therefore be a goal of the clinician to produce more favorable results.

Because inflammatory mediators such as TNF-α and IL-1ß can cause increased collagenase production in osteoblasts (Meikle et al. 1993), steps should be taken to minimize inflammation in the regenerative site. This can be facilitated by good oral hygiene and plaque debridement before, during, and after guided tissue regenerative procedures. Maintenance of these areas must be a priority since these areas may still be susceptible to breakdown many weeks after retrieval of the membrane.

Guided tissue regeneration offers the periodontist another invaluable modality for the treatment of periodontal defects and alveolar ridge defects. Although full regeneration of these defects is seldom reported with current methods available, partial regeneration of these defects improves the prognosis for these teeth. A comprehensive understanding of the spatiotemporal mechanisms involved in the processes of regeneration and repair will aid in achieving more predictable clinical results.

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