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Protease repertoires of periodontal regenerative cells

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Protease Repertoires of Periodontal Regenerative Cells

by

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THESIS

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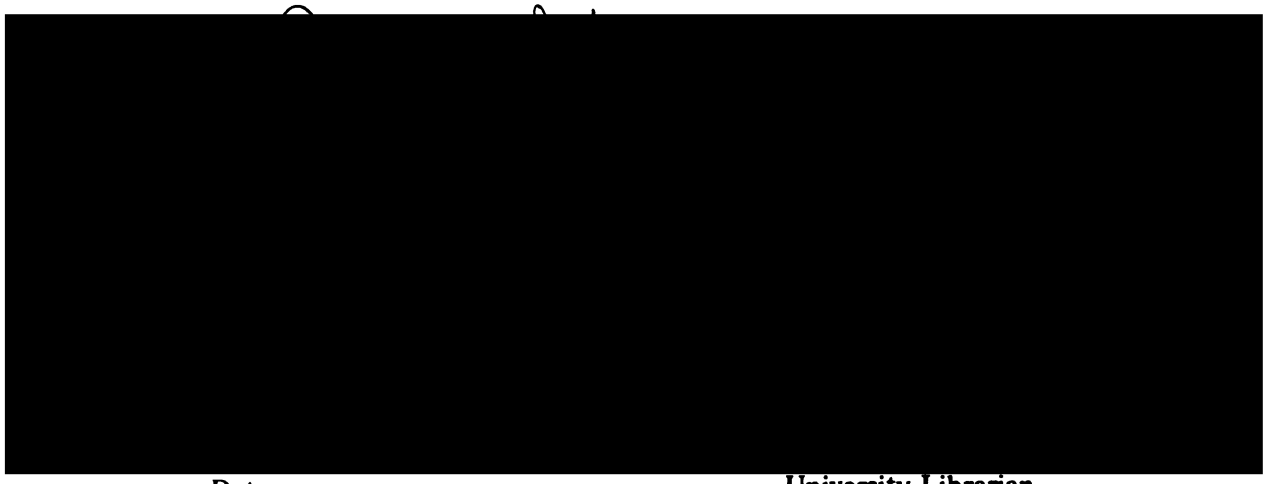

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DEDICATION

For you Dad,

I would like to dedicate this thesis to you dad, for your unwavering faith in me and all the love you expressed to me over the years. You always wished that you would live long enough to see me "hang my shingle" and that didn't happen so.....Dad, I dedicate my shingle to you and I hang it with great pride in your honor.

Love Lisa, your #3 daughter.

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INTRODUCTION

Guided Tissue Regeneration and Guided Bone Regeneration

Periodontitis is defined as inflammation involving and destroying the supporting alveolar bone and periodontal ligament (American Academy of Periodontology 1986). The lesion of periodontitis is characterized by inflammation, subgingival plaque and calculus, loss of alveolar bone and periodontal ligament, and apical positioning of the pocket and junctional epithelium. Clinically, the lesion is recognized by gingival redness, bleeding, and enlargement or recession. Periodontal pockets that extend apical to the cementoenamel junction are present, and loss of alveolar bone can be seen in intraoral radiographs (Caton 1989).

Therapy for treatment of periodontitis involves the elimination of bacterial plaque. When periodontitis is resolved, an anatomic defect remains in the periodontium. This anatomic defect is characterized by reformation of gingival fibers, substantial reduction of inflammation, persistent loss of bone and ligament, and formation of a long junctional epithelium (Caton and Zander, 1976; Caton et al, 1980, 1989; Listgarten, 1967; Listgarten and Rosenberge 1979; Tagge et al 1975). Clinically, on elimination of subgingival bacterial plaque, substantial changes can be observed. Clinical signs of gingival inflammation, ie, redness and bleeding, disappear. Periodontal pockets are reduced in depth as a result of gingival recession and gain of clinical attachment (Caton et al 1982; 1989; Cercek et al 1983; Proye et al 1982; Tagge et al 1975). However, increased probing depths, loss of clinical attachment, and radiographically observed

bone loss remain (Caton 1989). Substantial efforts have been made to alter this anatomic defect as part of periodontal therapy.

Thus, periodontal therapy involves two primary components: elimination of bacterial plaque and elimination of the anatomic defects produced by periodontitis. There are two primary approaches to eliminating these anatomic defects: resective and regenerative, both surgical. Resective surgery seeks to eliminate periodontal defects by removal of gingival and bony walls; this is accomplished by gingivectomy, osseous resection, and apically positioned flaps (Ochsenbein 1960; Ramfjord et al 1987). Regenerative surgery seeks to eliminate periodontal defects by creating new bone and periodontal ligament and coronally displacing the gingival attachment and margin.

The distinction between regeneration and repair must be clarified. Periodontal regeneration means healing after periodontal surgery that results in the formation of a new attachment apparatus, consisting of cementum, peridontal ligament, and alveolar bone. Periodontal repair implies healing after periodontal surgery without restoration of the normal attachment apparatus. Repair of a periodontal defect can be mediated by formation of a long junctional epithelium and bone fill, as well as root resorption or ankylosis. A combination of these healing responses often occurs. Thus, increased bone volume and density (bone fill) is commonly observed in angular bony defects in dental radiographs (Polson and Heijl 1978; Rosling et al 1976). Histologic studies have demonstrated, however, that this new bone is usually not connected to the root surface because a long junctional epithelium intervenes (Caton et al 1980; Caton and Zander

1975; Caton and Kowalski 1976). A partial regeneration, that is a cementum-mediated new fibrous attachment to a pathologically exposed root surface, can also take place without new bone formation.

Several types of periodontal treatment have been used to achieve periodontal regeneration. These have traditionally included scaling, root planing, gingival curettage, and various types of flap procedures. Changes reportedly produced by these therapies include pocket depth reduction mediated by gingival recession and gain of clinical attachment. When angular bony defects are not corrected, they often are remodeled by a process of bone fill and crestal resorption (leveling) (Polson and Heijl 1978).

Guided tissue regeneration (GTR) is a clinical procedure intended to restore the attachment apparatus of periodontally diseased teeth. This procedure includes the use of ePTFE membranes. Guided bone regeneration (GBR) is a similar procedure used to augment edentulous ridges often in conjunction with implant placement. This procedure includes the use of ePTFE augmentation membranes. Both of the therapies provide occlusive membranes to exclude gingival fibroblast and keratinocytes.

Histological evaluation is the only reliable method to determine the true efficacy of periodontal therapies aimed at creation of a new attachment apparatus consisting of new cementum, bone, and periodontal ligament. Animal models are used for these evaluations because of ethical reasons, the need to limit variation, and the need to provide controls. Many animal models have been utilized with success, but a nonhuman primate model is generally accepted as the

most useful for direct extrapolation of data to humans. Such a model was created by preparing contralateral defects with the same amount of bone and periodontal ligament loss (Caton and Zander 1975; Caton and Kowalski1976). Typically, the therapy to be tested is done on one side of the arch and results are compared histologically to those on the contralateral side to determine the effect of the experimental treatment on levels of bone, cementum, and periodontal ligament. In this way, it can be ascertained whether the experimental treatment produced repair or regeneration. These determinations are especially important for testing the safety and efficacy of new techniques, drugs, and devices (Caton et al 1992).

Results from these controlled animal studies and from evaluation of human block sections for periodontal regeneration have suggested that conventional periodontal surgery results in repair, rather than regeneration (Caton et al 1980; Listgarten and Rosenberg 1979). This knowledge, along with the increasing sophistication of investigators and technologies has led many research laboratories to a systematic and scientific approach to the study of periodontal wound healing. This involves the definition of variables involved in periodontal wound healing and the experimental manipulation of these variables to solve the puzzle of how to achieve periodontal regeneration (Caffesse et al 1985; Gantes et al 1988; Garrett et al 1978; Martin et al 1988; Melcher 1976; Melcher et al 1987; Nyman et al 1982; Polson and Caton 1982; Polson and Proye 1982; Polson 1986; Terranova et al 1986; Terranova and Wikesjo1987; Wikesjo and Nilveus 1990).

For regeneration to occur, cells with the capability to synthesize cementum, bone and periodontal ligament must occupy the periodontal defect and produce these specialized tissues. It is currently thought that these periodontal progenitor cells reside in the periodontal ligament and/or the alveolar bone remaing around the tooth (Melcher, 1976: Melcher et al 1987; Nyman et al 1982).

Devices, bioactive substances, and technical manipulations have been developed to favor repopulation of the periodontal defect by periodontal progenitor cells and discourage repopulation by cells that do not have the capability to produce periodontal supporting tissues. Coronally positioned or anchored flaps delay apical proliferation of gingival epithelium and connective tissue and allow time for coronal migration of periodontal ligament and bone cells (Gantes et al 1988; Garrett et al 1978; Martin et al 1988). Physical barriers made of polytetrafluoroethylene and bioabsorbable materials promote guided tissue regeneration by physically inhibiting gingival epithelium and connective tissue and favoring repopulation of the periodontal defect by cells from the periodontal ligament and alveolar bone (Caton et al 1992). The abilities of growth factors and attachment proteins applied to the periodontal defect to stimulate periodontal progenitor cells and inhibit undesirable cells are being investigated (Terranova et al 1986; Terranova and Wikesjo 1987) Finally, bone augmentation may act as devices allow coronal migration of periodontal progenitor cells.

When the gingival flap is replaced against the tooth during a periodontal flap pocedure, the most aggressive tissue during the initial phases of wound healing is gingival epithelium. The

epithelium proliferates apically on the tooth aspect of the flap and becomes attached to the tooth forming a long junctional epithelium. This effectively prevents connective tissue from gaining access to the root surface and precludes periodontal regeneration. Many techniques, including coronal flap manipulation, fibrin linkage, placement of physical barriers, root submergence and use of bioactive inhibitory substances, are available to inhibit gingival epithelium(Caton and Zander 1976; Caton et al 1980; Listgarten and Rosenberg 1979).

Cellular and Molecular Biology of Periodontal Wound Healing

Previous studies had indicated that the presence and severity of adult periodontitis is directly related to the presence of long-standing plaque and calculus (Loesche 1982; Tanner et al 1979). The initiation of the disease and the subsequent conversion of an established lesion to an advanced lesion (periodontitis), characterized by destruction of the connective tissue attachment to the root surface, has been studied, but the biologic mechanisms involved are not understood (Loesche 1982; Tanner et al 1979). The current research examines the biologic, biochemical and molecular biological events necessary to repair the destruction, once the destructive disease has been initiated.

Recent clinical investigations with focus on regeneration of the periodontium have attempted to define factors involved in the formation of a new connective tissue attachment to periodontally diseased or denuded root surfaces (Selvig 1983; Terranova et al

1986, 1987). One essential biologic event involved in tissue regeneration is specific cell-directed migration (chemotaxis). Chemotaxis is an essential feature of many biologic processes in health and disease. Examples are ectodermal migration in development, movement of endothelial cells in the process of neovascularization, movement of fibroblasts in wound healing, neurite outgrowth, movement of polymorphonulcear leukocytes to areas of infection, and movement of tumor cells to form metastases (Akers et al 1981; Azizkhan et al 1980; Gaus-Muller et al 1980; Grotendorst et al 1981; McCarthy and Furcht 1984; McCarthy et al 1983; Schiffmann and Gallin 1979; Seppa et al 1982; Terranova et al 1985).

Fibronectin, laminin and certain proteoglycans have been implicated in the directed movement of different cell types. Other extracellular matrix proteins or fragments of larger molecules have also been shown to possess chemotactic properties (Terranova and Lyall 1986). In addition, growth factors (polypeptide mitogens) have recently been reported to be chemotactic for various cell types. The extended form of b-FGF (basic fibroblast growth factor) has shown to be a potent chemoattractant for human endothelial cells (Terranova et al 1985). Other growth factors that have been shown to possess chemoattractant activity include PDGF (platelet-derived growth factor) NGF (nerve growth factor), and TGF-α and TGF-β (transforming growth factors alpha and beta) (Terranova and Lyall 1986;Terranova and Wikesjo 1988).

The repair of injury begins as soon as tissue damage occurs. The release of polypeptide growth factors have been shown to be

involved in tissue repair (Sporn and Roberts 1986). For example, PDGF, TGF-α and TGF–β are three polypeptides that have been shown to play an important role. PDGF is initially released from the alpha granules of platelets and is a potent mitogen for fibroblasts in the presence of either TGF-α or EGF. Furthermore, PDGF stimulates the production of both types I and IV collagenase (shown to be chemotactic for endothelial cells) by fibroblasts and thus contributes to the remodeling of matrix components, an essential feature of tissue repair (Deuel et al 1985; Ross et al 1986). Peptides resembling TGF-α have been found in platelets, activated macrophages, and in the ascites fluid of cirrhotic patients. TGF-α with it high degree of homology to EGF and to the β2 chain of laminin, has been shown for the growth potential and integrity of most epidermal cells (Assoian and Sporn 1986). TGF-β appears to have a particularly important role in the repair process. This peptide is found in relatively high concentrations in platelets, in activated T-lymphocytes, as well as in macrophages. TGF-β stimulates the formation of collagen in humans or rodent fibroblasts and when injected subcutaneously in newborn mice causes rapid fibrotic and angiogenic response at the site of injection. Another recently discovered source of TGF-β is bone. Transforming growth factor beta is present in bone in amounts almost 100-fold greater than those found in soft tissues and has some degree of homology with the bone morphogenic protein B2A (BMP-2A), BMP-2B and BMP-3. In vitro studies indicate that TGF-β can control the effects of several other polypeptide growth factors including PDGF, TGF-α, EGF, α-FGF, β-FGF, and IL-2 (interleukin-2)

(Assoian et al 1984; Bowen-Pope and Seifert 1985; Kehrl et al 1986; Mehlman and Maciag 1985; Seyedin et al 1986).

Although there may be an autocrine action of these peptides in injured cells, it would appear that their paracrine action, driven by their production and release by various inflammatory cells, accounts for the key role of these peptides in the repair process (Bowen-Pope and Seifert 1985; Kehrl et al 1986; Mehlman and Maciage 1985). However, autocrine function may play an important role. This role has been demonstrated in traumatized cultures and arterial smooth muscle cells that synthesize and release peptides that resemble PDGF. The function of some of these known peptides, that are similar to PDGF, are being investigated in relationship to cells of the periodontium (Terranova and Wikesjo 1988b). In addition, other factors isolated from cementum may have the potential to be mitogenic or chemotactic for cells of osseous or ligament origin (Terranova et al 1994) .

Among the least understood aspects of generalized tissue repair are the combinations and temporal sequence of growth factors that initiate and control cell motility and proliferation. Possibly a unique autocrine factor is responsible for initiation of this event. An autocrine motility factor (AMF) was identified for melanoma cells. This factor was found to be unique, based on amino acid analysis, and to have a molecular weight of 55,000 (Liotta et al 1986). Its action may be associated with membrane changes in phospholipid methylation. Similar membrane changes have been implicated in the motility of leukocytes. In other systems, marked increases in

phospholipid methylation have been observed in hormonal effects on hepatocytes, adipocytes, and bladder epithelial cells.

By using a newly developed assay system, the potential to test the activity of various biologic responses, using a dentin substrate, is now a reality. The assay has two parts, the first allows the measurement of the chemotactic activity of the test substanc bound to dentin. The cells must actively move through a filter (eg, Nucleopore [Pleasanton, CA]) in response to biologic response modifiers bound to dentin. The second part measures the ability of the dentin-bound factors to stimulate directed movement and proliferation of periodontal tissue cells on dentin surfaces. The use of these assays has demonstrated that human periodontal ligament cells migrate to fibronectin, PDGF, a-FGF, b-FGF, and TGF-β induce a proliferative response in periodontal ligament cells grown on surface-demineralized dentin or in tissue culture (Terranova and Wikesjo 1988a). Human gingival epithelial cells were shown to migrate to laminin, laminin fragments, and EGF. Laminin was also shown to timulate gingival epithelial cell proliferation on native dentin surfaces (Terranova et al 1986 1987). These in vitro finding suggest that biologic conditioning of the root surface (dentin) may enhance mesenchymal cel attachment, migration, and proliferation. These events may subsequently lead to improved healing after surgical therapy.

Most if not all mesenchymal cells have receptors for the growth factors and have been examined. The lack of migration of periodontal ligament cells to TGF-α and EGF is surprising because cells of mesenchymal origin have been shown to have a limited number of

receptors of high afinity for TGF-α (EGF) type molecules (Terranova 1986, 1987).

The above mentioned studies examined factors that could confer a selective advantage on periodontal ligament cells for stimulated adhesion, migration, and proliferation. No one factor alone or in combination with other factors was shown to have any unique ability to stimulate periodontal ligament cells (Terranova 1986 1987).

Periodontal Tissue Destruction

Much of the connective tissue degradation that takes place in periodontal diseases is mediated by proteolytic enzymes, previous studies have focused on the action of proteinases released by invading PMNs and macrophages, and bacterial enzymes (Meikle et al 1986a). Periodontal pathogens might mediate connective tissue degradation in periodontal disease by the ability of antigens from their cell walls to stimulate cytokine production by circulating mononuclear phagocytes. These cytokines could, in turn, induce MMP synthesis by resident gingival cells and resident macrophages, thereby initiating degradation (Meikle et al, 1986a, 1989; Heath et al, 1987). A critical step may be the interaction of bacterial antigens with inflammatory cells, resulting in the production of IL-1, which in turn could induce MMPs. Thus, cytokines may induce the connective tissues of the periodontium to 'self destruct' through stimulation of MMP production (Reynolds, Hembry and Meikle, 1994).

Antigens from the walls of bacteria commonly present in dental plaque (Gram-negative and positive organisms) can induce

connective tissue breakdown by a mechanism which involves a primary interaction with circulating mononuclear cells (Heath et al, 1987). Human gingival fibroblasts in vitro respond directly to lipopolysaccharide (LPS) preparations in terms of prostaglandin E_2 production; they were only induced to synthesize collagenase in response to supernatants from mononuclear cells stimulated with either LPS or lipoteichoic acid (Heath et al 1987). Conclusions were that if a cytokine-mediated mechanism plays an important role in connective tissue destruction during periodontitis, then the disease can probably be triggered by a large variety of bacteria or by combinations of different bacteria. It is thought that disease is more likely to be initiated by a critical number of organisms, which varies according to the susceptibility of the patient, and that the population of bacteria will always consist of the mixture of strains which is best able to exploit the environmental conditions pertaining at any one time (Reynolds, Hembry and Meikle, 1994). The particular cytokines responsible remain incompletely characterized, but much circumstantial evidence and some direct evidence suggest that IL-1 alpha and IL-1 beta are likely to be important.

Collagenase has been identified in gingival explant culture supernatants (Fullmer and Gibson, 1966; Bennick and Hunt, 1967; Birkedal-Hansen et al, 1974; Woolley et al, 1978; Heath et al, 1982) and in gingival crevicular fluids at levels that correlate with disease activity (Golub et al, 1976; Uitto and Raeste, 1978; Dryshatalskyj, 1986; Villela et al, 1987). A relationship has also been established between latent and active forms of collagenase extracted from gingival tissues and the degree of inflammation (Overall et al, 1987).

Collagenase activity can be found in the crevicular fluid of patients with periodontitis in much larger amounts than in control subjects (Larivee et al, 1986; Bilela et al, 1987; Reynolds et al, 1994). This collagenase seems to be only partly tissue-derived and mostly from PMNs (Villela et al 1987; Suomalainen et al 1989; Gangbar et al, 1990; Sorsa et al, 1990; Lee et al 1991). TIMP levels could be measured only in healthy individuals or in clinically healthy sites (Larivee et al, 1986). Increased collagenase activity was found in tissue extracts from specimens from inflamed gingiva, and MMP activities from the three major groups were found when inflamed gingiva was cultured (Overall et al, 1987).

Currently MMPs collagenase, gelatinase A, Stromelysin-1 and their specific inhibitor TIMP-1 can all be immunolocalized in gingival tissues, both from patients with periodontitis and from patients under going crown-lengthening procedures (presumably healthy tissue). Cells secreting MMPs and TIMP-1 were observed at sites that histologically showed signs of connective tissue remodeling (Reynolds, Hembry and Meikle, 1994).

TIMP-1 and collagenase transcripts are present at significantly higher levels in diseased gingival tissue (Trabandt et al, 1992; Nomura et al, 1993). Also, tetracycline, which inhibits the activity of several MMPs, including collagenase, reduces the severity of periodontal breakdown (Rifkin et al, 1993; Vernillo et al, 1994).

In previous reports of collagenase immunolocalization in human gingival biopsy specimens from patients with periodontitis, the enzyme was frequently associated with inflammatory cells, but its precise cellular origin was uncertain (Woolley and Davies, 1981).

However, recently, human gingival fibroblasts were shown to produce IL-1β, IL-6, IL-8 and TNF-α in response to LPS from gram negative bacteria (Agarwal et al, 1995). These results support the idea that gingival cells of mesenchymal origin can function as accessory immune cells by producing proinflammatory cytokines that act in an autocrine and paracrine manner during periodontal disease destruction.

The Role of Growth Factors in Periodontal Repair and Regeneration

For efficient tissue repair to be achieved, cells from around a wound must proliferate, migrate into the wounded area, and once within the wound, lay down extracellular matrix. As a result of this process, the tissue void created by the wounding insult fills up with new cells and their surrounding matrix. For tissue regeneration to be achieved, the cells that occupy the wound must be the same type and oriented in the same pattern as the cells that originally occupied that space. In addition, the extracellular matrix produced by those cells must be of the same type and orientation and must form the same structures as those that were present before the insult occurred. To achieve repair, structural, but not necessarily functional, integrity must be restored (Kiristy and Lynch, 1993).

The pathogenesis, and therefore regeneration/repair, of periodontal lesions is perhaps one of the most complex to occur in the body. At least six tissue types are involved in the repair of a periodontal lesion: The gingival epithelium, gingival connective tissue, periodontal ligament, tooth root surface cementum, alveolar

bone, and all the corresponding vasculature. Nowhere else in the body are epithelium and mineralized and nonmineralized connective tissues juxtaposed in such proximity. For regeneration of the periodontium to occur, all of these components must be restored to their original position and architecture. Thus the cells that form these tissues must repopulate a wound site and produce the appropriate matrix (Lynch and Giannobile, 1994; Lynch, Buser, and Hernandez, 1991; Terranova, Franzeti, Lyall, Wikesjo, 1987).

Barrier membranes facilitate periodontal regeneration by allowing only cells from the tissues originally present to repopulate the root surface following surgical therapy. However, such barriers do not promte the cellular processes necessary for repair or regeneration (ie, proliferation, migration and matrix synthesis) (Aukhil and Iglhaut 1988). How does the body promote these processes so essential to all tissue repair and regeneration? The current understanding leads us to believe that polypeptide growth factors are key regulators of these processes (Lynch , Colvin, Antoniades, 1989b; Lynch 1990; Lynch and Williams 1990; Lynch and Giannobile, 1994).

Growth factor is a general term used to denote a class of naturally occurring proteins that function in the body to promote the mitogenesis (proliferation), directed migration and metabolic activity of cells. Thus, the three key cellular events in tissue repair are mitogenesis, migration and matrix synthesis and remodelling. Numerous growth factors have been isolated from tissues and characterized. We now know that growth factors are cell specific, meaning that a particular growth factor acts only on defined cell

types. For example, keratinocyte growth factor (KGF) will stimulate epithelial cells but not fibroblasts or endothelial cells, while fibroblast growth factor (FGF) will stimulate several cell types, including fibroblasts and endothelial cells (Canalis, McCarthy and Centrella, 1989a; Gospodarowicz, Schweigerer and Neufeld, 1987; Greenhalgh, Sprugel, Murray and Ross, 1990; Joyce, Jingushe, Scully and Bolander, 1990b; Lynch, Colvin and Antoniades, 1989b; Lynch, 1990).

Growth Factors Which Promote Periodontal Regeneration

Platelet-derived growth factor is chemotactic for periodontal ligament osteoblasts and fibroblasts, thus, will likely promote coronal migration of periodontal ligament cells. The in vitro data show that PDGF and IGF-1 promote fibroblast and osteoblast proliferation, collagen (especially EGF-1) and noncollagen (especially PDGF) protein synthesis, and bone matrix formation. Nearly all these activities are increased even further when PDGF and IGF-1 are combined. The combination of PDGF and IGF-1 would enhance regneration of all the componenets of the periodontium. (1) PDGF and IGF-1 are chemotactic and mitogenic for periodontal ligament fibroblast; (2) PDGF is chemotactic for osteoblasts; (3) IGF-1 promotes collagenous protein synthesis and PDGF promotes primarily noncollagenous protein synthesis in bone cultures.; (4) the combination of PDGF and IGF-1 promotes greater bone matrix formation than any individual growth factor; and (5) PDGF and IGF-1 interact synergistically to promote significant collagen formation and healing in soft tissue

wounds (Pierce 1991; Lynch and Giannobile 1994; Gilardetti et al 1992; Pfeilshifter et al 1990).

Connective tissue degradation in health and periodontal disease. The roles of matrix metalloproteinases and thier natural inhibitors.

The remodeling of connective tissues is an integral feature of normal growth and development, and diseases such as arthritis, have long been associated with the breakdown of the collagenous matrix of cartilage and bone. The resorption is brought about by both resident connective tissue and infiltrating cells. It is tightly regulated by a complex interplay of cell-cell and cell-matrix interactions involving the production of enzymes, activators, inhibitors and regulatory molecules such as cytokines and growth factors. The accelerated breakdown of connective tissues occurring in pathological situations, such as arthritis and tumor invasion and metstasis, may be due to a failure in the normal regulation mechanisms (Reynolds, Hembry and Meikle, 1994).

The endopeptidases (proteinases) are key enzymes in degradative processes, since the protein components of most matrix macromolecules are the predominant determinants of tissue structure and function. In vitro experiments, matrix macromolecules can be degraded by endopeptidases from the four major classes (metallo, serine, cysteine, and aspartic active sites residues). Their relative role in vivo, vary with the situation, depending upon the tissue environment and types of cells involved, particularly whether inflammatory cells are present. The extent of matrix turnover

occurring extracellularly compared with intracellularly is also variable. Except in special circumstances, such as bone resorption or other situations where the cell is in close apposition with its matrix, extracellular degradation is usually thought to occur at neutral pH values. Consequently, proteinases of metallo and serine families will be optimally funtional and could be responsible for the initial phase of degradative process. Connective tissue cells synthesize and secrete a family of MMPs (matrix metalloproteinases), which can synergistically digest the macromolecules of connective tissue matrices (Murphy and Reynolds, 1993).

Matrix Metalloproteinases (MMPs)

The connective tissue MMP family, which consists of enzymes derived from many types of mesenchymal cells, hemopoietic cells, including monocytes and macrophages, and keratinocytes, are metal-binding proteinases secreted as proenzyme forms requiring extracelluar activation (Reynolds, Hembry and Meikle, 1994). Their activity is further regulated by secreted inhibitors (TIMPs) (Murphy et al 1991a). Alignments of the amino acid sequences of MMPs show that there is a high degree of similarity between the enzymes in each group (about 80%) and between groups (about 50%) (Murphy, Murphy and Reynolds, 1991c). The properties of each MMP group vary little with either the specific cell type, and differences in the substrate specificity involve only fine differences between their primary structures. MMPs have an extended and conserved Zn^{2+} binding site, and this site has a degree of homology with bacterial MMPs. There are also regions of homology that may have importance

in the activation of latent proforms to active enzymes. MMPs all require Ca^{2+} for stability and exhibit a preferred cleavage specificity for the N-terminal side of hydrophobic residues (Reynolds, Hembry and Meikle, 1994).

Matrix metalloproteinases are believed to play a key role in the tissue destruction connected to inflammatory diseases such as periodontitis (Werb, 1989). At present, 10 different MMP family members have been found. Based on their substrate specificities, they can be divided into three subgroups: interstitial collagenases (MMP-1, and MMP-8), stromelysin (MMP-3 and MMP-10), and type IV collagenases (MMP-2 and MMP-9). The cellular origin and role of interstital collagenases in oral fluids has been studied (Overall et al, 1989; Sorsa et al, 1988; Uitto et al 1990). The two type IV collagenases (MMP-2, 72-kDa and MMP-9, 92-kDa), which are capable of degrading denatured interstitial collagens (gelatins; Seltzer et al 1981, 1990), laminin (Okada et al, 1990), elastin (Senior et al, 1991), fibronectin (Okada et al, 1990), and basement membrane zone associated collagens type IV (Fessler et al, 1984) and type VII (Selzer et al 1989).

Even MMP-2 and MMP-9 are very similar in their structure and substrate specificity, their cellular origins are different. MMP-2 is synthesized by various fibroblasts (Salo et al, 1985, 1991), endothelial cells (Kalebic et al, 1983), and osteoblasts (Rifas et al, 1989). MMP-9 is expressed by polymorphonuclear leukocytes (PMNs), (Hibbs et al 1985), macrophages (Mainardi et al, 1984) and epithelial cells (Woodley et al 1987; Salo et al, 1991). The PMN-Cl gene is only expressed by PMN leukocytes whereas the highly

homologous FIB-CL is expressed by fibroblasts (Golberg et al, 1986; Stricklin et al,1977), keratinocytes (Lin, Wells, Taylor, Birkendal-Hansen, 1987), endothelial cells, (Moscatelli, Jaffeand Rifkin, 1980; Herron, Banda, Gavrilovic, and Werb, 1986), monocytes/macrophages (Welgus et al, 1985; Campbell et al, 1987), osteoblasts (Otsuka, Sodek and Lime back, 1984; Quinn et al, 1990), and chondrocytes (Lefebvre et al 1990). The highly glycosylated PMN enzyme has a considerably larger molecular mass than FIB-CL, but the two protein cores are virtually identical size. The two collagenases differ markedly in terms of transcriptional regulation. PMN-type collagenase is rapidly released from specific granule storage sites of triggered PMNs whereas FIB-CL is synthesized on demand by initiating transcription of the corresponding gene. This process causes a lag period of 6 to 12 hours before enzyme can be detected in the extracellular environment (Birdedal-Hansen, 1993).

The Mr 72K GL is perhaps the most widely distributed of all MMP and has been indentified in fibroblasts (Seltzer, Adams, Gr

and Eisen, 1981), keratinocytes (Salo, Lyons, Rahemtulla, Birkedal-Hansen, Larjava, 1991), endothelial cells (Kalebic et al, 1983), monocytes/macrophages (Garbisa et al, 1986), osteoblasts, (Overall and Sodek, 1987), and chondrocytes (Lefebvre et al, 1991). Mr 72K-GL does not appear to be expressed by PMN leukocytes but is present in circulating form in plasma (Hibbs et al 1985). The Mr 92K-GL is produced by PMN leukocytes (Hibbs, et al, 1985; Murphy, Ward, Hembry, Reynolds, Kuhn, Tryggvason, 1989), Ketatinocytes (Salo et al, 1991; Wilhelm et al, 1989) and monocytes/macrophages

(Mainardi et al 1984), and occasionally by fibroblasts. The Mr 72K GL is expressed constitutively by most cells in culture and responds only moderately to growth factors which induce the Mr 92K GL. The Mr 72K GL and 92K GL cleave a number of peptide bonds in denatured collagen chains to yield small peptides (Seltzer et al 1981; Salo et al 1991). Besides gelatin, gelatinases also cleave type IV, V, VII, and X collagens (Seltzer et al, 1991; Seltzer et al 1989), and elastin (Overall, Wrana, and Sodek, 1991).

The stromelysin group of MMP includes SL-1 and SL-2 which have virtually identical substrate specificities and cleave a wide range of extracellular matrix proteins (proteoglycan core protein, type IV and V collagen, fibronectin, and laminin) (Nicholson, Murphy and Breathnach, 1989). SL-1 is expressed by stromal cells either constitutively or after induction by growth factors/cytokines IL-1 (MacNaul et al, 1990), EGF (Kerr, Holt and Matrisian, 1988),TNF-α (MacNaul et al, 1990), PDGF (Kerr, Holt and Matrisian, 1988)] or phorbol esters (Chin, Murphy, and Werb, 1985; Wilhelm, Collier and Kronberger, 1987). The enzyme does not appear to be expressed by PMN leukocytes and keratinocytes in the human, but a SL-1 (or SL-2) homologue is induced in murine skin epidermis by treatment with tumor promoter, TPA (Willhelm, Collier, and Kronberger et al, 1987; Matrisian, Bowden and Krieg et al, 1986). Sl-2 is expressed less abundantly than SL-1 and does not appear to respond to growth factors (EGF and IL-1) or phorbol esters (Birkedal-Hansen, 1993).

In the oral cavity, the main source of type IV collagenases has been suggested to be PMN cells (Gangbar et al, 1990; Overall et al, 1991), which enter the oral cavity through the gingival sulcus.

However, detectable amounts of type IV collagenases can also be seen in healthy subjects who have low numbers of oral leukocytes (Shiott and Loe, 1970). Therefore other cells in addition to PMNs could also secrete these enzymes into the oral cavity. In chronic inflammatory periodontal disease, proteolytic enzymes responsible for the tissue destruction may originate from several cellular sources, including PMNs, macrophages, activated connective tissue fibroblasts and epithelial cells, and pathogenic oral bacteria. MMPs have the primary role in the degradation process, since they can degrade most of the extracellular matrix components (Makela, Salo, Uitto, and Larjava, 1994).

Bacterial components stimulate macrophages and lymphocytes to produce inflammatory mediators which are thought to further affect fibroblasts to produce MMPs (Meikle et theral, 1989; Overall et al, 1991; Birkendal-Hansen, 1993) and FIB-CL (fibroblast collagenase) and are the major MMPs of fibroblast origin (Chin et al, 1985). It has been observed that stromelysins can activate fibroblast type collagenase (Unemori et al, 1991). Most of the cells capable of secreting SL (stromelysin) and FIB-CL also produce TIMP (Tissue Inhibitor of Metalloproteinases) (MacNaul et al, 1990). Cawston and co-workers (1987) suggested that TIMP is locally produced and its main role is defending connective tissues in the very local area around the cell from which metalloproteinases are secreted.

Inhibition of Matrix Metalloproteinases

Active MMPs are captured by alpha-macroglobulins in a unique venus-fly-trap mechanism activated by cleavage of a bond in the "bait region" (Sottrup-Jensen, 1989; Sottrup-Jensen, Sand,

Kristensen and Fey, 1989). The rapid capture rates, particularly with CL, suggest that alpha-macroglobulins play an important role in the regulation of MMP activity.

Tissue inhibitors of metalloproteinases (TIMPs) form a classical non-covalent bimolecular complex with the active forms of MMPs, and in some instances with latent MMP precursors. TIMPs appear to regulate matrix degradation both by proteinase elimination and by blockage of autolytic MMP activation (DeClerck et al, 1991; Howard, Bullen and Banda, 1991). Three members of the TIMP family, TIMP-1, TIMP-2 (Herron, Banda, Clark, Gavrilovic and Werb, 1986; Carmichael et al, 1986; Dochery, Lyons and Smith,1985; Stetler-Stevenson et al 1989; Boone et al, 1990), and TIMP-3 which has been found in the extracellular matrix of many cells but not in the conditioned media (Kishnani et al, 1994), and a fourth, previously uncharacterized proteinase inhibitor has also been observed (Kapila, Kapila and Johnson, 1996). TIMPs are widely distributed in tissues and fluids (Murphy, Docherty, Hembry and Reynolds, 1991; Welgus, Bauer and Stricklin, 1986), and are expressed by many cell types including fibroblasts (Stricklin andWelgus, 1983), keratinocytes (Lin, 1987), monocytes/macrophages (Campbell, Cury, Lazarus, and Welgus,1987) and endothelial cells (Herron, Banda, Clark Gavrilovic and Werb, 1986). TIMP-1 is a Mr 28K glycosylated protein with a Mr 20K protein core (Carmichael, Sommer and Thompson, 1986; Stricklin and Welgus, 1983). TIMP-1 forms complexes with active collagenase but not procollagenase in a 1:1 stoichiometry (Cawston, et al, 1983; Welgus et al, 1985). Complex formation with active MMP does not appear to depend on cleavage of the inhibitor and fully functional

inhibitor can be recovered from the comple (Murphy, Koklitis and Carne, 1989). TIMP-1 also forms a complex with the zymogen form of Mr 92K GL(Goldberg et al 1989; Howard, Bullen and Banda, 1991; Howard, Bullen and Banda, 1991). TIMP-2 a Mr 22,000 unglycosylated protein, is express by fibroblasts and endothelial cells and perhaps by other cells as well (Herron, Banda, Clark, Gavrilovic and Werb, 1986; Declerck et al, 1989; Stetler-Stevenson et al 1989). The inhibitor forms a complex with the zymogen form of Mr 72K GL (Goldberg et al 1989; Welgus, Bauer and Stricklin, 1986). TIMP-2 is two to ten fold more effective than TIMP-1 against the 2 GL whereas TIMP-1 appears to more effectively inhibit CL (Howard, Bullen and Banda, 1991). The two TIMP genes are regulated differently, TIMP-1 expression is stimulated by growth factors (EGF, TNF-α, IL-1, TGF-β), phorbol esters, retinoids, and glucocorticoids, whereas TIMP-2 expression is down-regulated by TGF-β and fails to respond to phorbol esters (Overall, Wrana and Sodek, 1991; Stetler-Stevenson et al 1990; Clark et al, 1987; Coulombe et al, 1988; Nomura et al 1989; Mawatari et el 1989).

Cytokines and Periodontal Disease

IL-1 activity has been identified in gingival crevicular fluid samples from clinically inflamed sites in human subjects (Mergenhagen, 1984) and in peripheral blood monocyte culture supernatants from periodontitis patient (Meikle et al, 1986b). In more recent work IL-1beta and tumor necrosis factor alpha (TNF-α) have been cultured from peripheral blood mononuclear cells from patients with chronic periodontal disease and compared with controls

(McFarlane et al, 1990; Meikle et al, 1992). Interleukin-2 and interleukin-4 levels (presumably from lymphocytes) are also increased in disease (McFarlane and Meikle, 1991). These and many other studies amply demonstrate that in periodontal disease, there is a stimulated release of cytokines, especially by circulating mononuclear cells, and that these modify the host response.

The role of cytokines, matrix metalloproteinases and tissue inhibitors of metalloproteinases in connective tissue homeostasis.

Connective tissue homeostasis involves a multitude of perfectly coordinated anabolic and catabolic processes at the cellular and extracellular level (Hammacher et al, 1988). Cellular homeostasis in tissues is likley to be the result of a balance among complex interactions of antagonistic and synergistic molecules, with cytokines, matrix metalloproteinases and natural inhibitors of matrix metalloproteinase playing major roles. The roles of the individual cytokines is still vague. It is not possible to single out one most important pathway of tissue homeostasis. Some cells seem to be influenced by many cytokines, but others demand specific influence by one cytokine only. Some cytokines have a broad spectrum of target cells, whereas others act on one cell type only. The individual responses to cytokine stimulation vary greatly. Most studies are in vitro, however, cytokines do not occur as single entities in vivo. Results have beome available from studies that combined serval cytokines to affect cellular targets (Hefti, Hassel and Thomson, 1993; Lynch and Giannobile, 1993).

Overall et al, 1991, suggests that there is simultaneous existence of 4 different functional stages and phenotypes (Overall, Wrana and Sodek, 1991) of fibroblast cells: 1) a cell in proliferation; 2) a cell that participates in matrix synthesis and secretion; 3) a cell that participates in matrix resorption; and 4) a cell in apoptosis. Each phenotype is characterized by the expression of specific genes induced by a cytokine or combinations of several cytokines. Platelet-derived growth factor, fibroblast growth factor and interleukin-1 have been characterized as being inducers of anabolic cell activities, whereas interferon-γ and prostaglandin E_2 are inhibitors of such activities. Transforming growth factor beta has been shown to increase the synthesis of functional tissue inhibitors of metalloproteinases in gingival fibroblasts, while reducing collagenase expression in the same cells (Overall, Wrana and Sodek, 1991). Such reciprocity assigns TGF-β a complex role in extracellular matrix homeostasis.

Proteinase cascade model of tissue breakdown and imbalance of MMPs and TIMPs.

No one proteinase can be considered of overriding importance in matrix turnover, and a complex cascade of proteolytic events best explains how matrix destruction takes place. In situations without inflammatory cells, resorption is initiated by specific attack on matrix macromolecules by members of the family of MMPs, synthesized by stimulated connective tissue cells. This initial attack on the integrity of extracellular macromolecules facilitates further extracellular degradation and also intracellular degradation by

phagocytosis of fragments into phago-lysosomes. These processes may be augmented by the infiltration of inflammatory cells, mechanical damage, and bacterial infection (Reynolds, Hembry and Meikle, 1994).

Degradation of collagen in the bone-resorbing compartment underlying the osteoclast has been studied (Everts et al, 1992). This degradation involves both MMPs and cysteine-proteinases and extends earlier studies showing that both classes of proteinase are important. Evidence continues to build that there are extracellular and intracellular pathways for collagen degradation (Delaisse and Vaes, 1992), and bone resorption that illustrate the complexity of the degradative process.

Evidence that tissue destruction in disease processes might result from an imbalance of MMPs over TIMPs first came from the findings that a reduction in TIMP synthesis compared with MMPs in synovial explants from a rabbit arthritis model (Cambray et al, 1981), and later from studies of human rheumatoid synovium in culture. In recent work, others found that extracts from osteoarthritic cartilage have considerably elevated levels of MMPs and only moderately increased levels of TIMP, leading to an imbalance (Dean et al, 1989).

SPECIFIC AIMS

Previous studies have directly related the severity of adult periodontal disease to the presence of long standing plaque (Loesche 1982; Tanner et al 1979). Proteases such as collagenase have been shown to play an important role in the loss of bone associated with

periodontal disease (Overall 1990). Recently human cells have been shown to produce collagenase in response to lipopolysaccharide from gram negative bacteria (Saarialho-Kere 1993). Kawata et al, have also demonstrated that fimbriae from *P. gingivalis* stimulates the expression of IL-1 in macrophages. IL-1 is a cytokine known to stimulate cells to produce collagenase (Argarwall 1995). Studies such as these have established a relationship between bacterial cytokines and proteases in periodontal disease.

One of the major problems in bone regenerative therapy has been the maintenance of regenerated tissue. Often patients may regenerate tissue clinically only to subsequently lose the newly formed tissue. The basis for the subsequent loss of tissue is an important clinical issue since it directly relates to patient care and the success of regenerative procedures. We hypothesize that based on the association between periodontal disease and proteases,, success or failure of these procedures may be associated with alterations in protease activity. The alterations in protease activity should be associated with the resident cell populations in the newly regenerated tissue.

To address this hypothesis we have conducted experiments of cells randomly harvested from membranes used in regenerative procedures to determine the protease expression. We also compared the protease expression with clinical success and failure of these regenerative procedures.

MATERIALS AND METHODS

Membrane Retrieval and Isolation of Cells

Gore-tex® membranes and GTAM™ were retrieved from patients following treatment by guided tissue regeneration procedures in the postgraduate periodontology clinic at the University of California, San Francisco. The Gore-tex® membranes had been used to regenerate bone around periodontally involved teeth, whereas GTAM™ were used in edentulous ridge augmentation procedures. After removal, the membranes were placed into Alpha Modified Eagle's Medium (aMEM) containing 10% fetal bovine serum (FBS) supplemented with antibiotics (1/100 dilution of an antibiotic mixture containing 10^4 units/ml penicillin, 10 mg/ml streptomycin sulfate, and 25 mg/ml fungizone). The membranes were then sectioned, immobilized in tissue culture dishes and cultured until cells adherent to the membrane were observed migrating onto the culture dish (usually at 10 to 14 days). Cells were then passaged in the same medium without fungizone and were frozen at passage three or four.

Human periodontal ligament cells were scraped from the midroot portion of periodontally healthy bicuspids extracted for orthodontic therapy (Richards and Rutherford, 1988). Human gingival fibroblasts were obtained from distal wedge incisions over maxillary tuberosities removed during periodontal surgery. Both types of cells were established and maintained in the aMEM as described previously.

von Kossa staining for mineralized nodule formation in vitro

Confluent cell isolates were cultured in nodule growth medium containing aMEM supplemented with 15% FBS, antibiotics, 50 mg/ml ascorbic acid and 10 mM b-glycerophosphate. Visualization of nodules was enhanced by staining using the von Kossa technique. Cells were fixed and treated with 5% aqueous silver nitrate followed by exposure to ultraviolet light to turn calcium salts visibly brown. Non-specific staining was then removed by a 2 min wash with 5% sodium thiosulfate followed by three rinses with distilled water (Bellows et al, 1990).

Substrate zymograms and reverse zymograms

Gelatin and casein substrate gel electrophoresis (zymography) was performed as previously described by Fisher, Cui and Zhang et al, 1989. Polyacrylamide gels (10%), containing 2 mg/ml gelatin (EIA grade, Biorad, Hercules, CA) or a-casein (Sigma, St. Louis, MO) were loaded with 20 ml serum-free medium conditioned for 48 hours by each cell isolate . Gels were run under nonreducing conditions. Sodium dodecyl sulfate was removed from the gels with 2.5% Triton X-100 washes, and gels were incubated for 24 and 72 hours respectively at 37°C in Hanks balanced salt solution. Gels were then stained with Coomassie Brilliant Blue dye and destained with water/acetic acid(10%)/methanol(40%). Upon destaining, cleared bands of active protease activity could be detected on a blue background.

Reverse zymograms were used to detect proteinase inhibitors from the same conditioned medium, using 10% polyacrylamide gels

containing 1 mg/ml of gelatin as described previously (Fisher and Werb, 1991). Reverse zymography was similar to substrate zymography except that after being washed with Triton X-100, the gels were incubated for 15-30 min in 4-aminophenylmercuric acetate-activated skin fibroblast-conditioned medium, which contains a mixture of matrix-degrading proteinases. This activated medium degrades most of the gelatin in the gel except where inhibitors are present. The gels were then incubated for 18 hours, stained with Coomassie blue and destained as described for the substrate zymograms. This produces dark blue inhibitor bands on a light blue background.

RESULTS

The cells rescued from regenerative membranes were initially tested for their ability to form mineralized nodules *in vitro*. This assay was used to screen for isolates exhibiting osteogenic potential. Time for formation of these nodules varied from 24.7 days for the GTR cell isolates to 21.0 days for the GBR cells. Although these cells were morphologically similar to fibroblasts, they tended to grow more slowly. Cells derived from GTR membranes also exhibited some polarity in confluent cultures and formed interdigitating stripes (Wakabayashi et al, 1996).

Two isolates of human gingival fibroblasts and five periodontal ligament cells were tested to determine the baseline repertoire of proteinases and inhibitors in cells associated with regenerative procedures. As shown in Fig. 1A, gelatin zymograms demonstrated that both of these cell types produced two major proteinases

migrating at approximately 72 kDa and 52 kDa. These bands have been shown to correspond to the 72 kDa gelatinase and interstitial collagenase respectively (Wakabayashi, Wong, Richards and Johnson, 1996). The reverse zymograms (Fig.1B) identified a protein migrating at approximately 30 kDa which has been identified by western blot as TIMP-1 (Kapila YL, Kapila S and Johnson PW, accepted for publication). Casein zymography (Fig. 1C) identified a proteinase migrating at approximately 55 kDa which has similarly been shown to be stromelysin (Kapila YL, Kapila S and Johnson PW, accepted for publication).

One of the periodontal ligament cell isolates, (Fig. 1A, lane 5) demonstrated multiple proteinases which was inconsistent with the other isolates from gingival fibroblasts and periodontal ligament cells. Figure 1B, lane 5 demonstrates comparatively less protein migrating at 30 kDa, consistent with less TIMP-1 in this sample compared to the other cell isolates. This particular isolate also demonstrated multiple proteinases associated with the casein zymography, unlike the other isolates represented in the sample. The periodontal cell isolate represented in the various zymograms (Fig. 1A,B & C lane 5) was inconsitent with the other islolates and therefore considered as an abberancy; it was not used in analyzing the data.

Fig. 2 depicts a series of gelatin zymograms comparing gelatinolytic proteinases produced by cells rescued from GBR and GTR procedures. The samples are grouped so that conditioned medium from the twelve cell isolates derived from GBR (labelled A to L) can be compared with medium from the twelve GTR cell isolates

(labelled a to l). Proteinase profiles from all the GBR cell isolates can be observed in each vertical series of gels (for example gels 1, 2 and 3). Profiles for all GTR cell isolates are shown in each horizontal series (for example gels 1 and 4). The six zymograms are arranged to compare each sample from GBR cell isolates with each sample from GTR cell isolates on the same gel. Since zymography detects an activity rather than an absolute concentration of proteinases, the series compensates for differences in banding intensity associated with small differences in processing of each gel.

The lanes representing the GBR isolates (Fig. 2, labeled A-L) reveal fewer proteinases as compared to the GTR isolates (Figure 2, labeled a-l) on the same gelatin zymograms. For example, GTR cell isolates represented in figure 2, lane d and g have multiple proteinases expressed on the gelatin zymograms as compared to the GBR isolates, lane C and G. The clinical outcomes table relating to the GTR samples (d and g), indicate the membranes were in place for 4 weeks. Clinically, isolate d was exposed to the oral environment and had minimal gain in clinical attachment. GTR cell isolate represented by lane g of figure 2 had a clinical history of purulent exudate while the membrane was in place and also had minimal clinical attachment gain.

Relating the clinical outcomes of the cell isolates from the GBR samples in figure 2, lane C and G, indicates the membranes had been in place for 15 months and 2 months, respectively. The membrane was not exposed to the oral environment in isolate C throughout the duration of its' placement. Isolate G had exposure to the oral cavity after its' placement. Implants were able to be placed in both cases.

Fig. 3 illustrates the same samples assayed by reverse zymography for TIMP-1. As in Fig. 2 the gels are arranged to compare conditioned medium from each individual cell isolate with each other on the same gel. Again using isolates from GTR isolates represented by d and g of figure 3, the banding pattern representing the presence of TIMP-1 activity is weak for isolate g and is strong for isolate d. There is also a strong profile of multiple proteinases shown in these gels for isolate d. The GTR isolates d, f, and k have strong bands demonstrating the presence of TIMP-1, but also have strong multiple proteinase profiles on the gels as well. The GBR isolates C and G, have a strong banding pattern for the presence of TIMP-1 activity and a much less active and numerous proteinase profile.

In general, the isolates from GBR procedures demonstrated in the reverse zymograms (Fig. 3), show strong bands (indicating presence of TIMP-1), for lanes B, C, D, G, L and a less dense banding pattern for isolate F. Compared to the GBR isolates, the GTR isolates demonstrate a weaker banding appearance indicating less TIMP-1 in the samples. The exception to this is represented by isolate d which reveals both strong proteinase activity as well as TIMP-1 presence.

Fig. 4 depicts a similar series of casein zymograms which detect stromelysin. The GBR isolates depicted in figure 4 (isolates A-L), reveal a consistent banding pattern, especially noted in figure 4D, E, F. The bands are not particularly strong in appearance, but are consistently present. For example, isolates C and G have, a similar banding appearance when comparing them on the various casein zymograms. The GTR cell isolates are variable in their presence and in the strength of their appearance. This observation is well depicted

in comparing the isolates d and g. Isolate d has a very strong banding pattern as shown in fig. 4A, B, and C. In contrast is the banding pattern depicted in isolate g figure 4D, E, and F, which is very weak.

Selected samples of conditioned medium of cells isolated from GBR procedures were compared with samples of medium conditioned by either PDL cells or gingival fibroblasts. As shown in Fig. 5A, profiles of gelatinolytic proteinases of cells isolated from GBR procedures were similar to those of the PDL cells and gingival fibroblasts in that all cell isolates produced predominently 72 kDa gelatinase and interstitial collagenase. Concentrations of TIMP-1 (Fig. 5B) appeared greater in most of the medium from GBR procedures than in medium from either fibroblasts or PDL cells. Some of the selected GBR cell isolates also produced significant amounts of stromelysin (Fig. 5C). Since the same volume of medium for a given cell isolate was loaded on each of the three gels, the three gels give an approximate indication of relative amounts of each proteinase and inhibitor.

Similar analyses were performed with cells isolated from GTR procedures. As shown in the gelatin zymograms of Fig. 6A, many samples of conditioned medium from these cells exhibited additional, lower molecular weight proteinases not produced by either the gingival fibroblasts or PDL cells. The reverse zymograms of the same samples in Fig. 6B, indicate that relative amounts of TIMP-1 in the samples from the GTR procedures are less than amounts observed in the gingival fibroblasts and PDL cells. The observed amount of TIMP-1 exhibited in figures 5B and 6B appear to be similar when comparing the samples of PDL and GF cell isolates.

As was observed with conditioned medium of cells from GBR, some cell isolates from the GTR procedures also exhibited caseinolytic activity associated with stromelysin (Fig.4). Periodontal ligament cell isolates revealed weak caseinolytic activity on figure 1C. The gingival fibroblast isolate demonstrated a stronger activity level for stromelysin compared to the PDL isolates, however this observation was inconsistent between gels.

In summary, cells were isolated from twelve membranes used in each type of regenerative procedure (GTR and GBR) and screened for the ability to form mineralized nodules *in vitro.*, a property of cells with osteogenic potential. Five independently derived human PDL cell isolates and two gingival fibroblast isolates were examined to determine repertoires of proteinases and inhibitors. Using zymography and reverse zymography, regenerative cells which formed nodules were examined for the expression of gelatinolytic and caseinolytic proteases as well as for proteinase inhibitors. These proteinase and inhibitor profiles were then compared with the clinical outcome for each procedure.

Both PDL and gingival fibroblast cell isolates demonstrated activities of two major proteinases which migrate at approximately 75 kDa and 52 kDa on the gelatin zymograms (Fig. 1A). These bands correspond to 72 kDa gelatinase and the 52 kDa intersitital collagenase. Very little caseinolytic activity, which was shown to be stromelysin, (Kapila YL, Kapila S, Johnson PW, accepted for publication) was detected in the PDL and gingival fibroblast isolates. Reverse zymography demonstrated a consistent and strong banding pattern which indicated the presence of TIMP-1 (Wakabayashi RC,

Wong F, Richards DW and Johnson PW, 1996) for each PDL and GF cell isolate.

Table I documents portions of the clinical history associated with these procedures. Outcomes for the GTR procedures were determined by whether or not these procedures generated new clinical attachment at subsequent recall evaluation. Outcomes for GBR were determined by whether or not sufficient bone was regenerated to allow subsequent implant placement. The results of these experiments demonstrate that in cases where there is a gain of clinical attachment and an ability to place implants (GBR), there is an association with the presence of the 72 kDa gelatinase and interstitial collagenase only. The clinical cases which did not have gain in attachment (GTR) are associated with multiple protienases in their profiles. By these criteria, GBR procedures were more successful than GTR surgeries.

DISCUSSION

Zymography detects activities of multiple proteases which are capable of digesting a specific substrate. The technique is useful in screening for the presence or absence of matrix-degrading proteins but determines enzyme activity rather than actual concentrations. Since activity is usually proportional to concentration, the assays also provide a rough comparison of relative amounts of specific proteases within samples. Because the processing and staining of each gel is subject to individual variation, the best comparisons are made between proteases within a given gel.

The regenerated bone arising from GTR and GBR procedures is presumeably derived from different osteogenic cell types. The periodontal ligament cell is primarily associated with GTR while immature osteoblasts form the bone associated with GBR. In addition, these two regenerative techniques differ in the type of wound healing associated with the process. GBR procedures heal by primary intention and are isolated from the oral cavity. GTR procedures occur in association with teeth and are therefore in communication with the oral cavity. Since bacteria have been shown to be associated with GTR membranes, (Selvig et al, 1990), presumeably they play a major role in the healing and regeneration process. Bacterial products may also modulate the protease profiles of cells associated with GTR. Wakabayashi (submitted) found that cell isolates recovered from exposed GTAM and all Gore-tex membranes produced IFN-γ. Where as cell isolates recovered from unexposed GTAM did not produce detectable amounts of IFN-γ. IL-1β was produced by all cell isolates from GTR regenerative procedures but was not detected in any of six isolates rescued from from GBR regenerative procedures. Their findings indicate that cells from regenerative tissue modulate expression of their cytokines and that exposure of the tissue to the oral cavity during healing may cause variable expression of these factors.

Simion (1995) found a more mature and complex bacterial flora on exposed membranes not treated with topical chlorhexidine gel as opposed to exposed membranes treated with topical chlorhexidine. The membranes treated with the chlorhexidine topical gel for 4 weeks after exposure maintained a relatively simple flora

consisting of cocci and short rods. Membranes not treated with chlorhexidine gel after exposure exhibited a more mature flora consisting mainly of filamentous bacteria. Nowzari (1995), found sites free of pathogens on the membrane surface toward the tooth gained the most clinical attachment, even in the presence of various pathogens on the gingiva facing the membrane surface. Sites with little or no attachment gain demonstrated several periodontal pathogens on both sides of the membrane. Pathogens on the tooth facing surface of the membrane in particular, seemed to be a critical determinant on the outcome of GTR and appear essential to control in order to obtain results. These findings underscore the importance of controlling microbial pathogens in guided tissue regeneration procedures.

To further emphasize the importance of controlling microbial pathogens in GTR procedures, Bragger et al (1995), concluded that a low incidence of gingival inflammation was a prerequisite for the maintenance of attachment level gain by GTR. Cortellini (1994), completed a risk assessment analysis of his patients that recieved GTR procedures and correlated the maintenance care each patient recieved, over a 4 year period after a GTR procedure was performed. GTR sites in patients receiving only sporadic care had a 50-fold increase in risk of probing attachment level loss between 1 and 4 years, with respect to patients undergoing regular recall visits. It was concluded that stability of gained clinical attachment is dependent upon stringent oral hygeine.

GTR procedures are always associated with teeth where as GBR procedures are not usually associated with teeth. This is significant

for two reasons. First, there is a communication between the oral cavity and the membrane which is in association with teeth through the gingival sulcus. This becomes a direct channel for bacteria to contaminate the membrane surface. The second point to consider is the issue of primary closure during the surgical procedure. It is always the intention of the surgeon to gain primary closure during GTR and GBR procedures. However, many times it is not possible to gain primary closure around a tooth, thus the wound heals by secondary intention rather than primary intention. Healing by secondary intention occurs because the wound edges are unable to be approximated when suturing the wound closed. Therefore there is a space of open wound in which the membrane is exposed to the oral environment, again providing access for the bacteria to contaminate the membrane.

The zymograms depicted in Figs. 2, 3 and 4 were designed to initially screen for multiple proteases and to allow comparisons to be made between samples from all GTR and GBR procedures within the same gel. Additional gels were then used to compare selected samples from each procedure with each other and with a sample from PDL cells and gingival fibroblasts (Fig. 5 and 6). Each sample was therefore examined multiple times.

The substrate gels in this study indicate that the cell isolates derived from GTR procedures often expressed multiple gelatinolytic proteases. They also exhibited reduced levels of TIMP-1 when compared with cells from GBR procedures. Hayakawa (1994), looked at whole saliva from healthy and diseased sites. The whole saliva from healthy subjects consisted mainly of procollagenase, while the

whole saliva from patients with periodontal disease consisted mainly of active collagenase. An increase in TIMP-1 concentration and decrease in collagenase activity were observed after initial therapy in the periodontal patients.

Hou et al, (1995) found the amount of IL-1β in the GCF to be closely associated with periodontal status. Strong correlations were found between total amount of IL-1β and gingival index or pocket depth. Remarkable reductions in IL-1β were exhibited in pateints after initial therapy.

The results obtained by Sorsa (1988) indicate that collagenase in extracts of inflamed gingival tissue and the sulcular fluid, is most likely derived from PMN cells. On the other hand, collagenase produced by non-inflammed gingival explants in culture is probably derived from gingival fibroblasts. Agarwal (1995), indicated that in addition to providing structure and support, gingival fibroblasts may also function as accessory immune cells and play an important role in the initial inflammatory reaction as well as amplification of the immune response. These results point to the possibility that long term success and stability of regenerative procedures may also be modulated by the resident cells as well as the initial inflammatory cell reaction. The initial inflammatory cell reaction is also important in the initial remodeling phase. Since the stability of regenerated tissues may be modulated by expression of these proteins, further studies are indicated to explore the mechanisms related to the role of proteases in the regenerative process.

The criteria for evaluating the outcome of regenerative procedures in this study is based on clinical determinations. The GTR

procedures were judged by changes in clinical attachment levels, usually one year postoperatively. GBR procedures were considered successful if sufficient bone was regenerated to allow implant placement. By these criteria, the GTR procedures described in this study were less successful than the GBR procedures at regenerating new mineralized tissue. These data indicate that expression of proteases and protease inhibitors may correlate with the clinical success or failure of periodontal regenerative procedures.

Future prospects for improved healing and regeneration of periodontal tissues may be elucidated from identification and isolation of informational molecules that are stored in the connective tissue matrices. The identification of these molecules as well as a better understanding of their functions may open new perspectives in our understanding of the biology of periodontal wound healing and may provide novel approaches to periodontal regeneration procedures (Pitaru, 1994). Kapila et al (1996), found that the 120 kDa cell-binding region of the fibronectin fragment which contains the RGD central cell-binding region, can induce expression of multiple proteinases. This 120kDa fragment also alters expression of one of the three TIMPs elaborated by PDL cells. Factors such as the 120 kDa fragment, that increase protease activity are likely to play a significant role in exacerbating destruction of the periodontal tissues. This increase in the secretion of multiple proteases in PDL cells may be an important aspect of the periodontal disease process (Kapila et al, 1996). Recombinant human TIMP-1 and TIMP-2 was found to inhibit bone resorption induced by either parathyroid hormone or 1,25-dihydroxy vitamin D_3 in cultured neonatal mouse calvariae.

These results suggest that endogenous TIMPs play a central role in regulating both physiological and pathological bone resorption (Hill, Reynolds and Meikle, 1993).

Cytokines also play an important role in the pathogenesis of periodontitis. Cytokines found in periodontal tissue exhibit biological activities that are known to be involved in the progression of disease, including activation of osteoclasts and fibroblasts. Increasing knowledge of the cytokine network may open new pathways for periodontal treatment by controlling processes involved in tissue breakdown. For example, IL-1β is an important mediator in the pathogenesis of periodontitis (Kjelden et al, 1993). IL-1β has been found in much higher numbers in chronic adult periodontits patients than IL-1α and TNF-α (Stashenko et al,1991). Also, significantly higher IL-1β inhibiter in GCF of inflamed sites has been found when compared to clinically healthy sites (Kabashima et al, 1991). These studies further clarify the complex processes involved in periodontal tissue breakdown. Identification of these processes may one day lead to ways of identifying markers of tissue breakdown; the aim being to establish ways to prevent breakdown.

Future therapy may also include the use growth factors at the time of surgery to stimulate the regeneration of the periodontal attachment apparatus during the early phases of periodontal wound healing, including cementum with inserting collagen fibers and alveolar bone. Lynch et al (1991), completed a study which looked at the short term application of a combination of growth factors (PDGF/IGF-1). These growth factors have been shown to regulate DNA and protein synthesis in bone cells in vitro, and to interact

synergistically to enhance soft tissue wound healing in vivo. Computer aided histologic analysis of the tissue biopsies found a 5 to10 fold increase in bone and cementum formation in PDGF/IGF treated sites compared to control sites recieving placebo. A physiologic periodontal ligament was also formed between new bone and new cementum. A single bolus administered to a bony site can stimulate rapid osteogenesis and a single application at the time of surgery can stimulate the regeneration of the periodontal attachment apparatus, including cementum with inserting collagen fibers and alveolar bone during early phases of periodontal wound healing. Becker et al (1992), histologically demonstrated that sites treated with ePTFE membrane plus PDGF/IGF-1 had the highest bone density compard with sites treated with ePTFE membranes alone or with DFDBA. Unraveling the complex interactions between periodontal tissue cells, proteases, inhibitors to those proteases, growth factors, cytokines, and extracellular matrix molecules points to a new era of periodotal treatment. This is an era that is aimed at the molecular level of interactions to establish ways to prevent periodontal tissue breakdown and to enhance regenerative procedures.

Matrix Metalloproteinase Family (Birkedal-Hansen,1993)				
Enzyme	**Abbrev.**	**MMP#**	**Mr**	**Substrates**
Interstitial Collagenases	FIB-CL	MMP-1	57,000-52,000	Collagen I, II, III, VII, VIII, X, gelatin
Fibroblast-type collagenase	PMN-CL	MMP-8	75,000	Same as FIB-CL
PMN-type collagenase				
Stromelysins: Stromelysin-1	SL-1	MMP-3	60,000-55,000	PG core Protein, Fibronectin, Laminin, Collagen IV, V, IX, X, Elastin
Stromelysin-2	SL-2	MMP-10	60,000-55,000	Same as SL-1
Gelatinases Mr 72K Gelatinase Type IV Collagenase	Mr 72K GL	MMP-2	72,000	Gelatin, collagen IV, V, VII, X, Elastin, Fibronectin
Mr 92K Gelatinse Type IV Collagenase	Mr 92K GL	MMP-9	92,000	Gelatin Collagen IV, V, Elastin
Other:				
Stromelysin-3	SL-3	MMP-11	n.d.	n.d.
PUMP-1	PUMP-1	MMP-7	28,000	Fibronectin, Laminin, Collagen IV, Gelatin, proCL PG Core Protein
Macrophage Metallo-elastase	MME	?	55,000	Elastin

MMP numbering according to Nagase et al. 1992.

Table 1: Clinical outcomes of GTR and GBR procedures

GBR Procedures

Cell Isolates	Membrane Retention (Weeks)	Membrane Exposure	Clinical Bone Augmentation
A	10	no	yes
B	24	no	yes
C	60	no	yes
D	8	no	yes
E	6	yes	yes
F	8	yes	yes
G	8	yes	yes
H	8	yes	yes
I	8	no	yes
J	8	yes	yes
K	12	yes	yes
L	4	yes	yes

GTR Procedures

CELL ISOLATES	Membrane Retention (Weeks)	Membrane Exposure	Attachment Gain
a	4	yes	no
b	4	yes	no
c	5	yes	no
d	4	yes	no
e	6	yes	no
f	6	yes	no
g	3	yes	no
h	5	yes	yes
i	9	no	yes
j	12	yes	no
k	6	no	yes
l	12	no	yes

Table 2. Mineralized nodule formation of cells retrieved from membranes used for GTR(A) and GBR(B). Data represent time required for mineralized nodule formation.

A.

CELLS	NODULE FORMATION(DAYS)
C 22	13
GORE 30	43
G	33
K 8/22	52
CL 30	26
CB 31	26
K 9/15	34
K 8/29	35
L	32
Average ± Standard error	32.66 ± 3.65

B.

CELLS	NODULE FORMATION(DAYS)
GTAM 10	13
REG	24
GTAM 11	21
GTAM 25C	20
GTAM 3	42
GTAM 8i	25
GTAM 12	42
GTAM 19i	28
GTAM 20i	21
Average ± Standard error	26.22 ± 3.28

Figure 1

Proteinase and inhibitor expression of human gingival fibroblasts and PDL cells. Cell isolates from different patients were analyzed using gelatin zymography (A), reverse zymography (B), and casein zymograms (C). Molecular weights are indicated to the left. Lanes I to V contain conditioned medium from PDL cells while lanes VI and VII contain medium contitioned by gingival fibroblasts.

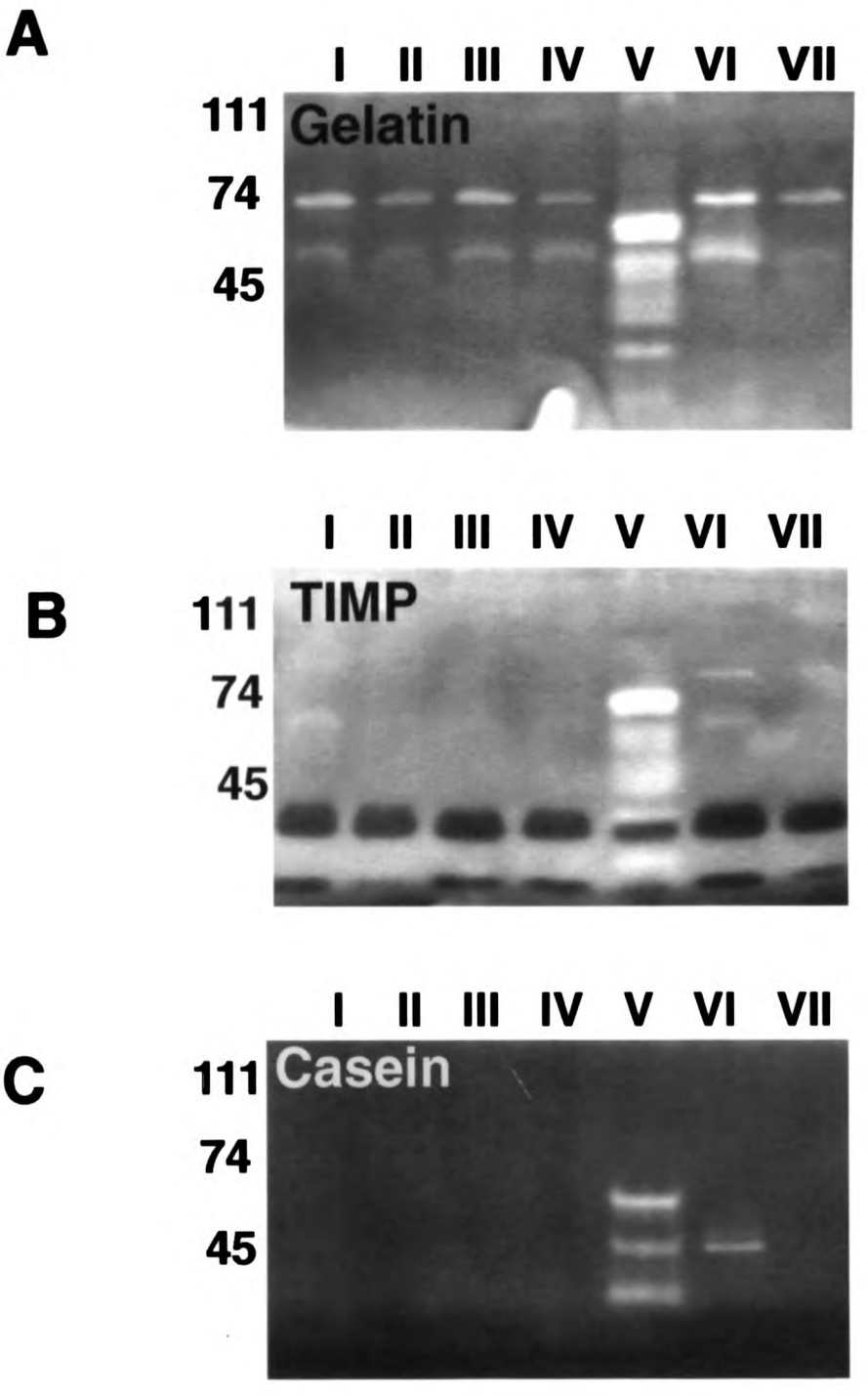

A
I II III IV V VI VII
111
74
45
Gelatin
B
I II III IV V VI VII
111
74
45
TIMP
C
I II III IV V VI VII
111
74
45
Casein

Figure 2

Screening for gelatinolytic activity of cells isolated from GBR and GTR. Replicate samples of medium conditioned by cells isolated from regenerative membranes were analyzed on multiple gelatin zymograms to allow comparison of gelatinolytic activity from individual cell isolates.

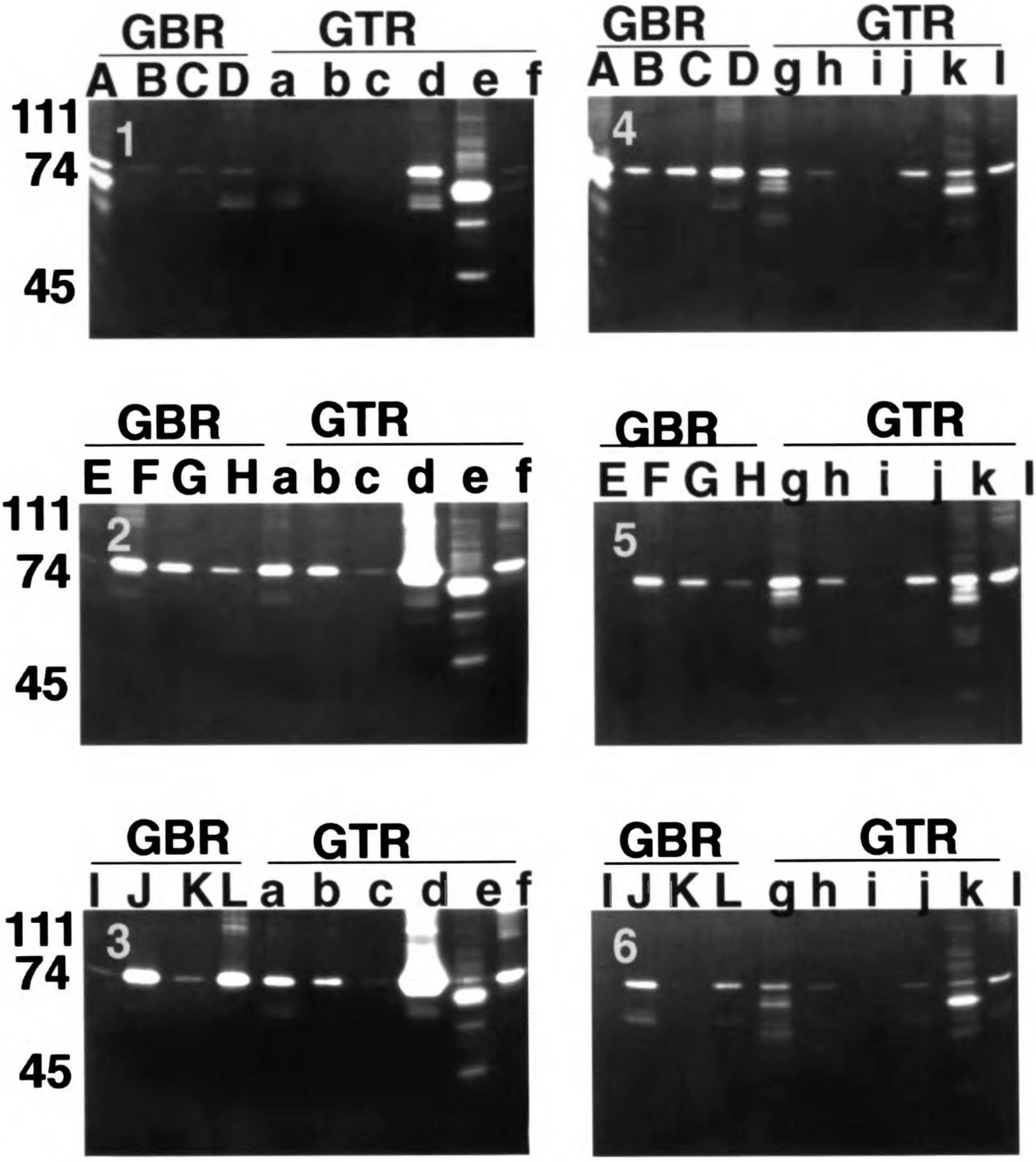

GBR
GTR
A B C D a b c d e f
111
74
45
1
GBR
GTR
A B C D g h i j k l
4
GBR
GTR
E F G H a b c d e f
111
74
45
2
GBR
GTR
E F G H g h i j k l
5
GBR
GTR
I J K L a b c d e f
111
74
45
3
GBR
GTR
I J K L g h i j k l
6

Figure 3

TIMP-1 expression of cells isolated from GTR and GBR. Reverse zymograms were used to compare expression of the proteinase inhibitor TIMP-1 in cell isolates.

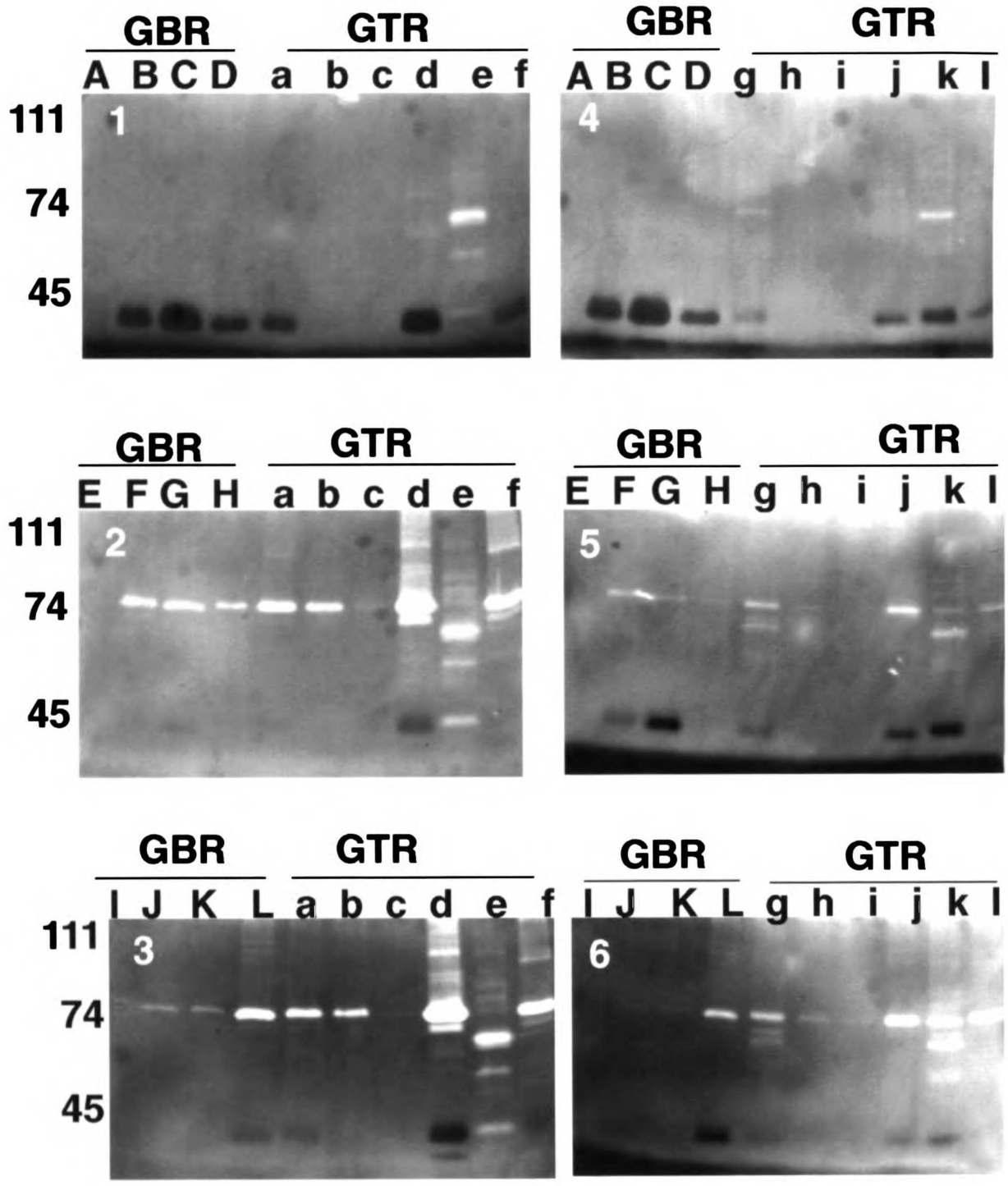

GBR
GTR
A B C D a b c d e f
111
74
45
1
GBR
GTR
A B C D g h i j k l
4
GBR
GTR
E F G H a b c d e f
111
74
45
2
GBR
GTR
E F G H g h i j k l
5
GBR
GTR
I J K L a b c d e f
111
74
45
3
GBR
GTR
I J K L g h i j k l
6

Figure 4

Expression of stromelysin in cells recovered following GTR and GBR. Multiple casein zymograms were used to compare stromelysin expression betweeen cell isolates.

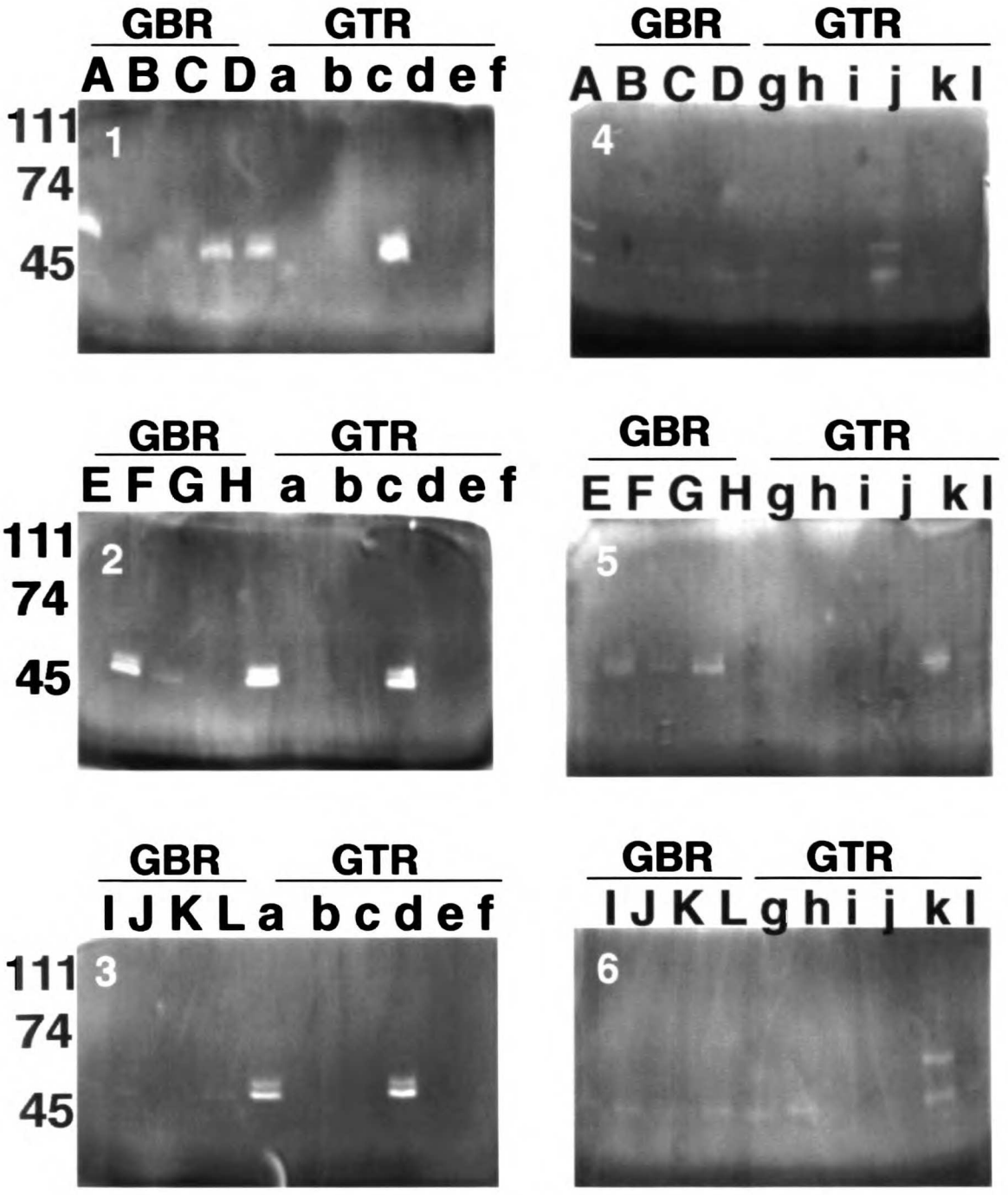
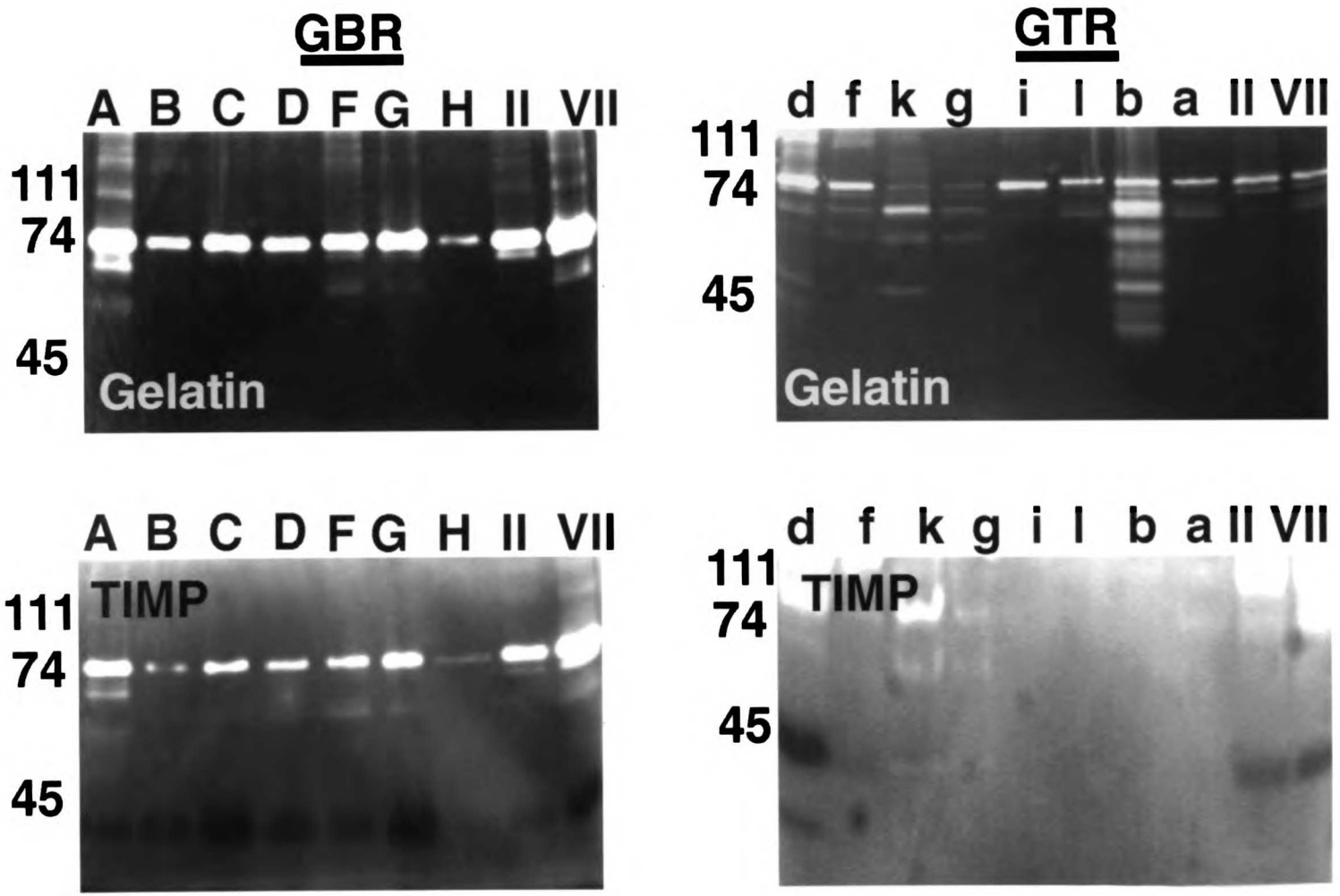

GBR
GTR
A B C D a b c d e f
111
74
45
1
GBR
GTR
A B C D g h i j k l
4
GBR
GTR
E F G H a b c d e f
111
74
45
2
GBR
GTR
E F G H g h i j k l
5
GBR
GTR
I J K L a b c d e f
111
74
45
3
GBR
GTR
I J K L g h i j k l
6

Figure 5

Comparison of expression of gelatinases and TIMPs in cells derived from GBR and GTR procedures. Medium conditioned by cells derived from GBR procedures was analyzed by gelatin zymography and reverse zymography (Gels to the left). Similar assays for medium conditioned by cells derived from GTR procedures are shown to the right.


GBR
A B C D F G H II VII
111
74
45
Gelatin
GTR
d f k g i l b a II VII
111
74
45
Gelatin
A B C D F G H II VII
111
74
45
TIMP
d f k g i l b a II VII
111
74
45
TIMP

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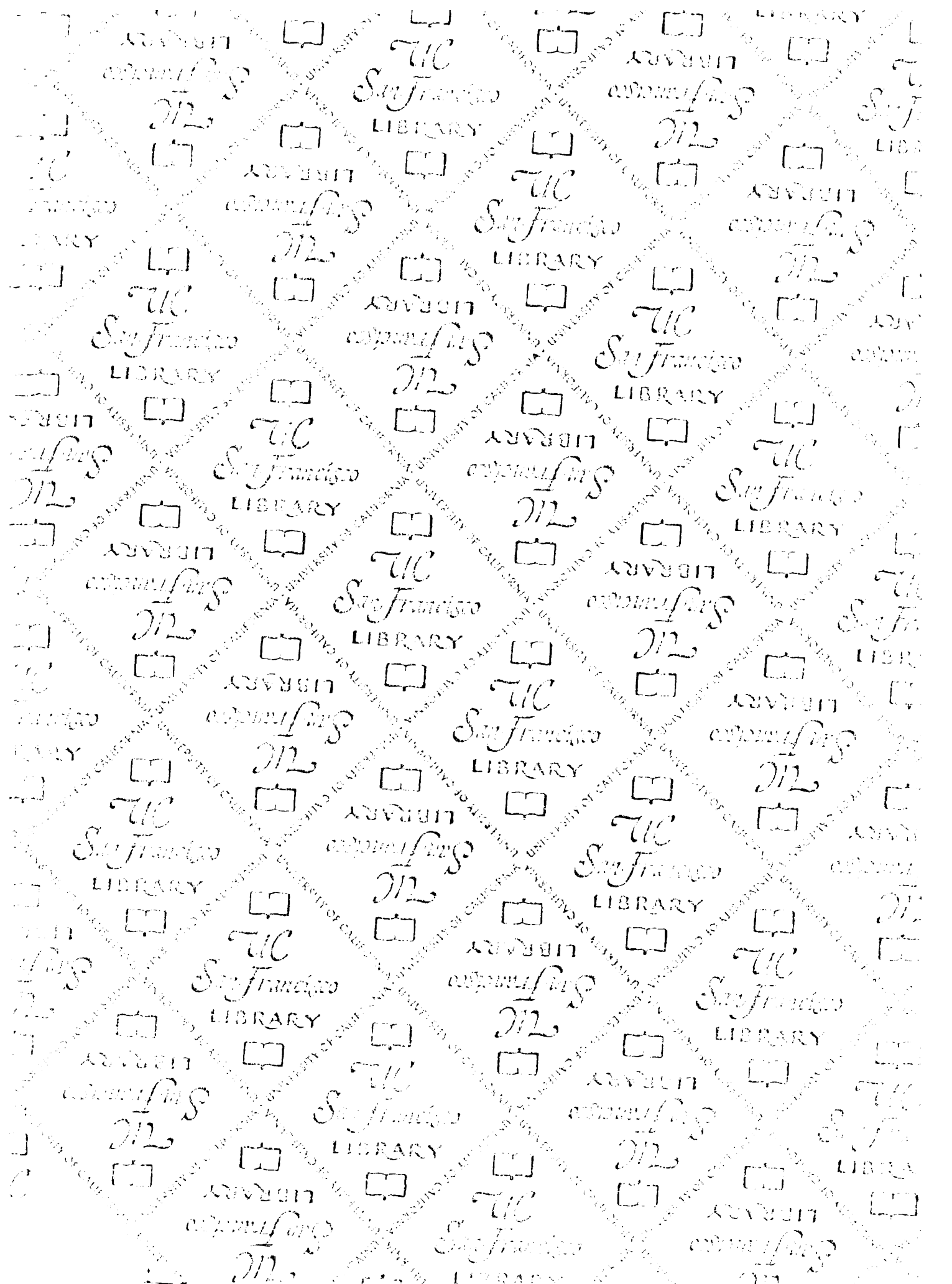
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