EMDOGAIN® INTERACTION WITH EXTRACELLULAR MATRIX PROTEINS AND THEIR EFFECTS ON CELL ADHESION, SPREADING AND MIGRATION

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ABSTRACT

Studies over the past twenty years have demonstrated that the cells of Hertwig's epithelial root sheath secrete enamel matrix proteins and that these proteins are involved in the formation of acellular extrinsic fiber cementum during development and regeneration of periodontal tissues (Slavkin and Boyde, 1975; Hammarstrom et al., 1997b). However, the cellular and molecular mechanisms involved in the process of periodontal regeneration induced by enamel matrix proteins are poorly understood. This in vitro study was designed to investigate Emdogain's interaction with extracellular matrix (ECM) proteins and regulation of adhesion, spreading and migration of epithelial cells and periodontal ligament fibroblasts. We examined the binding interaction between Emdogain and the ECM proteins, fibronectin; collagen types I and IV; and laminin I using ELISA. Binding of Emdogain to fibronectin and collagen type I (10 μg/ml) was inversely related to Emdogain coating concentration and resulting precipitation. That is, fibronectin and collagen type I bound to non-precipitated Emdogain and precipiation of Emdogain at higher concentrations did not bind fibronectin or collagen type I. Type IV collagen and laminin I did not bind to any concentration of Emdogain-coated surfaces. Epithelial cells plated on Emdogain-coated surfaces (1-30,000 μg/ml) in the presence of cylcoheximide, showed minimal adhesion (5%) compared to type I collagen-coated surfaces (positive control) and no spreading was found at 2 hours. When fibronectin or collagen type I (10 μg/ml) was used along with Emdogain, epithelial cell adhesion was significantly enhanced (3-5 fold higher compared to Emdogain alone). PDL fibroblast adhesion and spreading on Emdogain (1-30,000 μg/ml) was concentration-dependent with the

maximum adhesion and spreading on 10,000 μg/ml (40% and 10% of positive control at 2 hours, respectively). Fibronectin or collagen type I (10 μg/ml) used along with Emdogain for surface coating enhanced adhesion to a level that was comparable with positive control. Cell spreading was also improved and reached 30% compared to 70% on positive control. Collectively these data indicate that Emdogain supports adhesion and spreading of PDL fibroblasts but not epithelial cells. Addition of ECM proteins enhances cell binding to Emdogain that is more significant for PDL fibroblasts compared to epithelial cells.

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CHAPTER ONE – REVIEW OF THE LITERATURE

1. Wound Healing

Wound healing occurs as a consequence of well-organized, interrelated events and involves a variety of cell types acting in concert to re-establish the integrity of injured tissues. The healing of an incisional or excisional wound has been studied extensively in nonoral sites; however, the basic biological events apply to the tooth-mucogingival flap interface as well. Therefore, I will briefly provide a historical review of general wound healing before describing in details the focus of my research that is periodontal wound healing and regeneration.

The overall healing response can be divided into three overlapping phases including:

1. inflammation; 2. granulation tissue formation and contraction; 3. matrix maturation and remodelling.

1.1. Inflammation

1.1.1. Hemostasis Tissue injury results in damage to blood vessels which leads to exposure of subendothelial structures. Connective tissue components of the subendothelium (e.g. collagens type IV and V, laminin and proteoglycans) are known to promote platelet aggregation and activation (Barnes et al. 1980, Chiang et al. 1980, Weksler et al. 1988). Activated platelets release a variety of products such as, von Willebrand factor, platelet derived growth factor (PDGF) and thromboxane A_2 (Weksler

et al. 1988) and are also involved in activation of the coagulation cascade leading to formation of the blood clot that seals off the vessels and stops bleeding. The growth factors and cytokines present in the fibrin clot initiates the wound repair process (Aukhil, 2000).

1.1.2. Polymorphonuclear Leukocyte Chemotaxis The infiltration of polymorphonuclear leukocytes is an early response after tissue injury that occurs in response the local release of a variety of leukocytic chemoattractants during hemostasis (e.g. kallikrein, plasminogen activator, PDGF) (Malech, 1988). The neutrophils begin to decontaminate the wound of foreign particles, debris and bacteria through the release of enzymes and toxic oxygen products (Clark, 1996).

1.1.3. Monocyte Chemotaxis Following an initial trauma and after neutrophil accumulation, monocytes/macrophages accumulate in the inflammatory site, presumably in response to chemoattractants (Ohura et al., 1987). These cells participate in debridement, microbicidal events and orchestration of several later events involved in tissue repair (Wahl et al., 1992) such as, release of growth factors and cytokines (Grotendorst et al., 1989). Macrophages and monocytes are also important in matrix remodelling. Macrophages and monocytes are a source of collagenase and along with neutrophils and fibroblasts, they contribute to the degradation and removal of collagen from damaged tissue (Wahl et al., 1992).

1.2. Re-epithelialization and granulation Tissue Formation

1.2.1. Re-epithelialization The blood clot that forms shortly after injury acts as a temporary barrier which is later covered by migrating and proliferating epithelial cells

from the free edges of the tissue surrounding the defect. The stimuli for cell migration and proliferation are unknown, however several mechanisms have been suggested including the elaboration of chemotactic factors, contact guidance or the loss of neighbouring cells (Stenn et al., 1988). In the normal gingival tissues, the basal layer of epithelium is attached to the basal lamina. The keratinicytes use receptors on their surface, known as integrins, to bind to laminin in the basal lamina. In the normal tissues, keratinocytes use the integrins $\alpha 6\beta 4$ to bind to laminin in the basal lamina, and these integrins have intracellular links with the keratin cytoskeletal network (Martin, 1997). In preparation for migration, the keratinocytes at the edge of the surgical wound have to dissolve the hemidesmosome attachment and begin to express other integrins that are more suitable for the wound environment. The migrating keratinocytes will start expressing the integrins $\alpha 5\beta 1$ and $\alpha V\beta 6$ (for binding to fibronectin and tenascin respectively), the integrins $\alpha V\beta 5$ for binding to vitronectin and, finally, reorganize the distribution of the integrins $\alpha 2\beta 1$ (collagen receptor) (Clark, 1996; Aukhil, 2000). Several growth factors seem to be key players in regulating the proliferation of keratinocytes in healing wounds. Among the key factors are the EGF, TGF-β, heparin-binding epidermal growth factor and keratinocyte growth factor (Nanney et al., 1984).

1.2.2. Granulation tissue formation The formation of granulation tissue and the provisional matrix, especially the cross-linking and reorganization of nascent collagen is a critical step in wound healing. The formation of granulation tissue involves several correlated events including fibroblast migration and proliferation, connective tissue deposition and angiogenesis. One of the key cells in collagen synthesis and remodeling is the fibroblast. Growth factors are released during hemostasis by activated platelets, endothelial cells, infiltrating monocytes and lymphocytes. The major growth factors that

have been extensively studied includes, platelet derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β). Collectively they encourage fibroblast migration into the wound area and promote fibroblast proliferation (Wong and Wahl, 1989). Migrating fibroblasts play a central role in depositing the fibronectin-rich, provisional matrix (Grinnell et al., 1981; Kurkiren et al., 1980). While we currently do not have a detailed understanding of the regulatory mechanisms involved in the production of granulation tissue by fibroblasts, in vitro studies have suggested a role for thrombin (Mosher and Vaheri, 1978), epidermal growth factor (EGF) (Chen, 1977) and TGF-β (Ignotz and Massaugue, 1986; Roberts et al., 1986) in regulating the formation of collagen, fibronectin and other provisional matrix components by fibroblasts.

The deposition of a provisional matrix is followed by wound contraction that is defined as the mechanism by which the edges of the wound are drawn toward the center. Around 7-10 days after wounding, some of the fibroblasts in the wound transform into myofibroblasts and express α-smooth muscle actin. Such transformation allows these myofibroblasts to generate strong contractile forces that are required for wound contraction (Martin, 1997). This process of wound contraction reduces the wound size so that smaller amounts of connective tissue deposition and epithelialization are required to reconstitute the lost tissue (Clark, 1996; Mast, 1992).

1.3. Maturation of Provisional Matrix and Remodelling

The last and longest step of healing involves the replacement of the provisional matrix (rich in fibronectin, proteoglycans and types I, III and IV collagen) by a more mature fibrous matrix. This process is characterized by the continued synthesis, cross-

linking and remodelling of collagen, which gives rise to large bundles of collagen type I, thereby providing the tissue with increasing tensile strength. Notably, growth factors such as TGF-β can modulate fibroblast remodeling of connective tissue (Ignotz and Massague, 1986; Roberts et al., 1986). As described briefly above, the integrated process of wound closure consists of several steps. Each step plays an important role in the final outcome of the process which is restoration of tissue architecture. Since the emphasis of this literature review is on periodontal wound healing and the regulation of these processes, in the next section I will focus on the biology of periodontium in health and during wound healing.

2. BIOLOGY OF PERIODONTIUM IN HEALTH AND DURING WOUND HEALING

2.1. Biology of periodontium

The periodontium, the tissues investing and supporting the teeth, includes alveolar bone, root cementum, periodontal ligament and gingival (epithelium and connective tissue):

2.1.1. Gingiva compromised of gingival epithelium and connective tissue, is a portion of the oral mucosa that covers the tooth-bearing part of the alveolar bone and the cervical neck of the tooth. The epithelial component shows regional variations including oral gingival epithelium, sulcular epithelium and junctional epithelium. These 3 epithelia differ ultrastructurally (Schroeder and Listgarten, 1997), and there are distinct phenotypic differences in their expression of various cytokeratins and cell surface markers (Mackenzie and Gao, 1993). Oral epithelium extends from the mucogingival junction to

the tip of the gingival crest and is a stratified squamous keratinising epithelium. The sulcular epithelium is the epithelial lining of the gingival sulcus, which in health is a small crevice of approximately 0.5 mm in depth. It is a stratified squamous epithelium and is often parakeratinized. In health, the epithelial connective tissue interface is demarcated by rete pegs, and with developing inflammation they become elongated (Bartold et al., 2000). The junctional epithelium forms the tissue attachment of the gingiva to tooth surface. The epithelial cells of junctional epithelium produce an internal basal lamina and the attachment of the gingiva to tooth is by numerous hemidesmosomes (Listgarten, 1975). In contrast the basal cells are separated from the connective tissue by the external basal lamina. The interface between the junctional epithelium and the underlying connective tissue is relatively smooth, unlike the condition found in the oral gingival epithelium. Throughout the junctional epithelium, numerous migrating polymorphonuclear leukocytes are present in health but dramatically increase in number with the accumulation of dental plaque and are closely associated with the development of gingival inflammation (Tonetti et al., 1995). The junctional epithelium tapers from its coronal end, which may be 10 to 20 cells wide, to 1 or 2 cells at its apical termination, located at the cementoenamel junction in healthy tissue (Cho and Garant, 2000). The gingival connective tissue is a largely fibrous connective tissue. Type I collagen is the main collagen species in gingival connective tissue (Narayanan et al., 1980). Densely packed collagen bundles are anchored to the acellular extrinsic fiber cementum just below the terminal point of the junctional epithelium forming the connective tissue attachment. The stability of this connective tissue attachment is a key factor in limiting the migration of the junctional epithelium. Type III collagen appears to be preferentially

localized as thinner fibers in a reticular pattern near the basement membrane at the epithelial junction (Narayanan et al., 1985). Proteoglycans (mainly dermatan sulfate) are also ubiquitous constituents of the gingival connective tissue and appears to be localized in close association with collagen fibers (Bartold et al., 1981). Fibroblasts are the most prominent cells in gingival connective tissue and their principle function is to synthesize and maintain the components of the extracellular matrix. Gingival fibroblasts show considerable variation in morphology depending on site to site microenvironmental variations in cytokines and other biological mediators (Bartold et al., 2000). In general, the main function of the gingival tissue is to protect the root surface and alveolar bone from the external oral environment and to provide support for the teeth in their alveolar housing.

2.1.2. Root Cementum is an avascular mineralized tissue covering the entire root surface. It forms the interface between root dentin and the periodontal ligament. Cementum is approximately 50% hydroxyapatite and 50% collagen and noncollagenous proteins (Carranza, 1996). Traditionally, cementum has been classified as cellular and acellular cementum depending on the presence and absence of cementocytes in cementum, further grouped into intrinsic and extrinsic fibers formed by cementoblasts or by fibroblasts, respectively (Schroeder, 1986). Intermediate cementum is an ill-defined zone near the cemento-dentinal junction of certain teeth that appears to contain cellular remnants of Hertwig's sheath embedded in calcified ground substance (Carranza, 1996). Acellular afibrillar cementum is located over cervical enamel at the CEJ (Schroeder, 1988). Its major structural components are glycosaminoglycans (Schroeder, 1986) and its functional significance is unknown. Cellular intrinsic fiber cementum contains

cementocytes embedded in a collagenous matrix of intrinsic collagen fibers. These collagen fibers are oriented mostly parallel to the root surface and course in a circular fashion around the root (Schroeder, 1986). Cellular intrinsic fiber cementum is found in old resorption lacunae and in root fracture sites. Cellular mixed stratified cementum is located primarily on the apical one third of the root and in the furcation area of multirooted teeth. It is composed of alternating layers of acellular extrinsic fiber cementum/acellular intrinsic fiber cementum, and is covered by a thin layer of acellular extrinsic fiber cemetum for attachment to the periodontal ligament (Schroeder, 1986). Cellular mixed stratified cementum serves to reshape the root surface in order to compensate for physiological and nonphysiological shifting of the teeth in their socket (Schroeder, 1986). Acellular extrinsic fiber cementum covers 40% to 70% of the root surface and is comprised of collagen fibers and glycosaminoglycans. It serves the exclusive function of anchoring the root to the periodontal ligament. Cementoblasts and cementocytes are involved in the secretion of intrinsic fibers (in contrast to the extrinsic fibers that are a product of periodontal ligament fibroblasts). Mature cementoblasts are relatively large cells with a highly basophilic cytoplasm and share similar morphological features with osteoblasts, suggesting that these two cell types might originate from a common progenitor pool located in the periodontal ligament and the marrow spaces of the adjacent alveolar bone (Cho and Garant, 2000). In general, cementum provides a medium for the attachment of collagen fibers that bind the tooth to surrounding structures. Moreover, cementum formation is critical for appropriate maturation of the periodontium, both during development as well as that associated with regeneration of periodontal tissues. (See Table 1. for summary)

Table 1. Types of Cementum

	Components		Location	Function
	Cells	**Fibers**		
Acellular Afibrillar Cementum (AAF)	—	—	Over cervical enamel at CEJ	Unknown
Cellular Intrinsic Fiber Cementum (CIFC)	Cementocytes	Intrinsic collagen fibers	In old resorption lacunae and root fracture sites	Reparative
Cellular Mixed Stratified Cementum (CMSC)	Cementocytes	Intrinsic and extrinsic collagen fibers	Apical 1/3 of the roots and furcations areas	Adaptive response to external forces
Acellular Extrinsic Fiber Cementum (AEFC)	—	Extrinsic collagen fibers	Coronal 1/3 of the roots	Anchorage

2.1.3. Periodontal ligament is a complex, vascular, and highly cellular soft connective tissue that attaches the root of the tooth to the inner wall of the alveolar bone. The mechanical strength of periodontal ligament derives largely from the molecular structure of the type I collagen molecule and its ordered arrangement into fibers (Liu et al., 1995). These fibers are anchored by their insertion into bone or cementum as Sharpey's fibers. Once embedded in either the wall of the alveolus or the tooth, Sharpey's fibers calcify to a significant degree (Johnson, 1983) and are associated with an abundance of noncollagenous proteins, notably osteopontin and bone sialoprotein (Bosshardt et al., 1998). The principle fibers of the periodontal ligament are arranged in 6 groups: transseptal, alveolar crest, horizontal, oblique, apical and interradicular (Carranza and Ubios, 1996). Maintenance and remodelling of periodontal ligament collagen fibers (Ten Cate, 1976), together with the embedding and calcification of their terminal portion to form Sharpey's fibers (Johnson, 1987), require the concerted action of numerous cell types (Garant and Cho, 1979) and multiple, synchronized signalling mechanisms to coordinate these activities. Central to these integrated activities is the periodontal ligament fibroblasts, whose responsibilities include the formation and remodelling of the periodontal ligament fibers, and presumably a signalling system to maintain periodontal ligament width (McCulloch and Melcher, 1983). Periodontal ligament fibroblasts are rather regularly dispersed throughout the ligament and are generally oriented with their long axes parallel to the direction of the collagen fibrils. A subpopulation of osteoblast-like fibroblasts, rich in alkaline phosphatase, has been identified in the periodontal ligament (Liu et al., 1997). These cells have the capacity to give rise to bone cells and cementoblasts. They are also responsible for the production of

acellular extrinsic fiber cementum in the mature periodontal ligament (Groeneveld et al., 1995). Periodontal ligament fibroblasts are also needed to maintain the normal width of the periodontal ligament by preventing the encroachment of bone and cementum into the periodontal ligament space (Melcher, 1970). While their roles in the periodontal ligament are not yet clear, a number of reports have identified additional extracellular matrix components including collagen types V and VI, chondroitin sulfate, proteoglycans, fibronectin, tenascin and undulin (Kagayama et al., 1996; Lukinmaa et al., 1992; Zhang et al., 1993). In general the functions of the periodontal ligament are physical mainly attachment of teeth to the bone, maintenance of the gingival tissues in their proper relationship to the teeth and shock absorption; formative and remodelling by participating in formation and resorption of cemetum and bone in response to different stimuli; and nutritional and sensory.

2.1.4. Alveolar process is the portion of the maxilla and mandible that forms and supports the tooth socket. The alveolar process consists of the thin alveolar bone proper that forms the alveolar wall of the tooth socket, the inner and outer cortical plates, and spongy bone between the alveolar bone proper and the cortical plates (Carranza, 1996). The bone matrix is formed from a scaffold of interwoven collagen fibers within and between which small, uniform, plate-like crystals of carbonated hydroxyapatite are deposited. The inorganic matter constitutes 65-70% of the bone structure (Sodek and McKee, 2000). Collagen comprises 80-90% of organic components in mineralized bone tissues. Type I collagen is the principle collagen and together with type V collagen, forms heterotypic fiber bundles that provide basic structural integrity of connective tissue (Rao et al., 1979; Bronckers et al., 1986). Other proteins, including proteoglycans, acidic

glycosylated and non-glycosylated proteins associate with and regulate the formation of collagen fibrils and mineral crystals, or provide continuity between matrix components and between the matrix and cellular components (Sodek and McKee, 2000). The most active secretory cells in bone, the osteoblasts, line a large percentage of the bone surfaces and are primarily responsible for the production of the organic matrix of bone (Holtrop, 1990). The organic matrix produced by osteoblasts consists predominantly of type I collagen (Heinegard et al., 1989). Following maturation, osteoblasts may undergo apoptosis, become encased in matrix as osteocytes or remain in the bone surface as bone-lining cells. Osteoblasts that become osteocytes occupy spaces (lacunae) in bone and are defined as cells surrounded by bone matrix (Bonucci, 1990). Of central importance in the ability of bone to respond to biological regulatory factors and functional forces is the capacity of the large, multinucleated osteoclasts to resorb bone. The coupling of bone resorption with bone formation constitutes one of the fundamental principles by which bone is remodelled throughout life (Turner and Pavalko, 1998). In general, alveolar bone has a specialized function in the support of teeth. While there are architectural specifications for alveolar bone that relate to its functional role, the basic cellular and matrix components, as well as the cellular and molecular activities involved in its formation and remodelling are consistent with other bone tissues.

In the periodontium, epithelium, soft and mineralized connective tissue come together to form a junction, referred to as the dentogingival junction. Chronic inflammation associated with periodontal diseases, invades the integrity of this junction causing loss of underlying bone and periodontal ligament. Periodontitis is defined as inflammation involving and destroying the supporting alveolar bone and periodontal

ligament (American Academy of Periodontology 1986). Previous studies have shown that the presence and severity of the disease is directly related to the presence of long-standing plaque and calculus (Loesche, 1982; Tanner et al., 1979). The lesion of periodontitis is characterized by marked inflammation, subgingival plaque and calculus, loss of alveolar bone and periodontal ligament and apical positioning of the junctional epithelium. Periodontal 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 periodontal ligament, and formation of a long junctional epithelium (Caton and Zander, 1976; Caton et al., 1980, 1989; Listgarten and Rosenberg, 1979). Clinically, on elimination of subgingival bacterial plaque, substantial changes can be observed. Clinical signs of gingival inflammation, i.e., 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). 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. These therapies result in wounding of the already inflamed periodontal tissues and the consequence depends largely on the cellular and molecular events associated with wound healing.

2.2. Biology of periodontal wound healing (Repair vs. Regeneration)

Periodontal repair implies healing after periodontal surgery without restoration of the normal attachment apparatus. Repair of the periodontal defect can be mediated by

formation of a long junctional epithelium and bone fill, as well as root resorption, ankylosis, and fibrous adhesion. A combination of these various healing responses often occurs. Thus, increased bone volume and density is commonly observed in angular bony defects in dental radiographs (Polson and Heijl, 1978; Rosling et al., 1976). Histologic studies have demonstrated, because of an intervening long junctional epithelium this new bone is usually not connected to the root surface (Caton et al., 1980; Caton and Zander, 1975). In another form of repair, collagen fibers form bundles parallel to the root surface. This type of healing has often been referred to as "collagen adhesion" (Selvig et al., 1995). Root resorption mainly in the cervical area can also take place during the healing process. This has been shown in 2 studies using experimental circumferential defects in beagle dogs (Bogle et al., 1985; Wikesjo and Nilveus, 1991). Finally ankylosis may develop, possibly because progenitor cells that migrate coronally and form new periodontal attachment are overrun by bone-forming cells (Kling et al., 1985; Wikesjo et al., 1988).

Periodontal regeneration is defined as the reproduction or reconstitution of a lost or injured part so that form and function of lost structures are restored (Americal Academy of Periodontology, 1992). In consequence, periodontal regeneration includes regeneration of alveolar bone, cementum, periodontal ligament and gingiva. This is the ultimate goal of periodontal therapy. Therefore, the regenerative events of periodontal wound healing require recruitment of progenitor cells, which have the potential to differentiate into specialized regenerative cells followed by proliferation of these cells, and synthesis of the specialized components of the connective tissues they are attempting to restore.

The histological observations suggest that many of the cellular and molecular events in the healing of periodontal wounds are similar to those seen in wounds elsewhere in the body except that, in the periodontal wounds, there is a mineralized tissue interface at the junction of epithelium and connective tissue. In addition, the healing site, because of the tooth, communicates with the oral environment during all phases of wound healing. Immediately following periodontal surgical procedures, the tissues represent surgically wounded sites and a cascade of cellular and molecular events set in to initiate wound healing. When a mucogingival flap is repositioned against the root surface, the epithelium due to its remarkable capacity to regenerate following injury, will continue to migrate along the root surface as long as there are no attached collagen fibers on the root surface (Bartold, 2000). This process is a fundamental principle of periodontal wound healing and explains the formation of long junctional epithelium. Since epithelium migrates at a much faster rate than the formation of new connective tissue attachment to a debrided root surface, the epithelial attachment will form at the expense of new connective tissue attachment. However, some new connective tissue attachment and new cementum formation can also occur in the apical region of the wound indicating that these tissues if given appropriate time and environment can regenerate. Therefore, excluding or delaying rapid re-epithelialization of the flap wound is an essential requirement to achieve periodontal regeneration (Wikesjo and Selvig, 2000).

Early wound healing events at the dentogingival interface have been studied in details in monkeys, using an extraction and reimplantation model (Polson and Proye, 1983). Teeth were extracted; the coronal third of the root planed and half of the root-planed teeth were demineralised with citric acid and then reimplanted in their extraction

sites. Observations of healing were made at 1, 3, 7, and 21 days. At 1 day all specimens had a fibrin clot adhering to the root surface. However, by 3 days in the nonmineralized specimens the fibrin clot maintained its adhesion to the root surface and there was no apical migration of the junctional epithelium. An important finding of this study was that initial healing in this wound healing model is very similar to that seen in epidermal sites; a fibrin clot fills the space between the surgical flap and the root surface and it does seem to adhere to the root surface, at least initially. Moreover, these observations support the concept that if this fibrin adhesion can be maintained to the root surface, there will be no apical migration of the junctional epithelium. The same as in epidermal wounds, the fibrin clot seems to block epithelial invagination into the healing wound. More recently, Wikesjo et al., 1991a, studied the early healing events at the interface of the healing wound and dentinal surfaces. Full-thickness mucoperiosteal flaps were surgically implanted into created bony concavities in edentulous alveolar ridges in two beagle dogs. The defects were created in a manner to allow a time-lapse study of initial and early healing events. Within minutes, plasma proteins, primarily fibrinogen precipitated onto the wound surfaces and provide an initial basis for adherence of a fibrin clot. Within hours, the wound site was stabilized by the formation of a fibrin clot that adheres to the root surface, and there is a heavy infiltrate of neutrophils. In 6 hours, the root surface became lined by neutrophils, which decontaminated the wound by phagocytosing injured and necrotic tissue. Within 3 days, granulation tissue became evident at the wound site and, although fibroblasts could be identified within the wound, the site was still heavily infiltrated by inflammatory cells. During this phase the fibrin clot was slowly degraded and the inflammatory reaction moved into its late phase as the neutrophil infiltrate

gradually decreased while the influx of macrophages increased. The macrophage contributed to wound debridement by removing effete red blood cells, neutrophils, and the residual tissue debris. The macrophage also had another important role including release of growth factors which supported fibroblast proliferation, matrix production and angiogenesis and therefore, played a key role in the transition from inflammation to granulation tissue formation (Riches, 1996). By day 7, the site was rich in newly formed granulation tissue and the collagen fibers appeared to align in a parallel array along the root surface. By day 14 the collagen fibers showed some signs of attachment to the root surface, with subsequent cementum formation not appearing until the third week (Wikesjo et al., 1991a). These observations confirm the earlier findings of Polson and Proye, 1983, that a fibrin clot adherent to the root surface is a part of early periodontal wound healing. The results of these two studies suggest that the early period of wound healing in periodontal sites may be critical. The developing clot must form and adhere to the root surface for enough time to allow for proper wound maturation, including connective formation and development, before a new connective tissue attachment to the root surface can occur. If these first series of events is disrupted or if the initial attachment of fibrin and/or immature connective tissue is ruptured, then a pattern of healing including a long junctional epithelium is likely to occur (Polson and Proye, 1983). In another study by Wikesjo et al., (1991b), the fibrin clot at the tooth-mucogingival flap interface was experimentally compromised, using a heparin solution. The histologic analysis showed that the nonheparinized defects healed with a mean connective tissue reattachment of 95% of the surgically created defects. Heparinized defects healed with only a mean of 50% connective tissue reattachment. The coronal 50%

of the heparinized root surfaces demonstrated a marginal tissue recession of approximately 25% and a long junctional epithelium of approximately 25%. There were no statistically significant differences in the amount of bone and cementum formation between the two groups. Taken together, the studies by Wikesjo et al., 1991a; 1991b, and the earlier work by Polson and Proye, 1983, suggest that apical migration of the gingival epithelium in periodontal wounds may not be spontaneous but may instead result from breakdown of the root surface-fibrin clot interface, thus allowing epithelial migration along the inner surface of the wound margin.

There appears to be at least two important components to the dynamics of this root surface-fibrin clot interface during healing that affect the maintenance of a stable interface: *tensile strength of the healing wound* and *biologic acceptance of the root surface. The tensile strength of the healing wound* can be defined as the strength of the root surface-fibrin-clot interface to resist tearing or rupture from mechanical forces (Wikesjo et al., 1991b), such as forces that mobilize the wound margin. Kling et al., 1981; 1985, in an animal model, studied the effect of flap positioning to a location where it had little impact on the healing site or stabilizing the flap by suturing it in a manner that prevented its recession and limited its movement. In the study using coronal flap displacement (Kling et al., 1981), surgically created furcation defects in premolars of beagle dogs were treated with regenerative technique including full-thickness flap elevation, root planning and citric acid demineralisation. Then the flap margins were coronally positioned at a mean of 4.5 mm coronal to the cemento-enamel junction (CEJ). Histologic evaluation of the areas showed that in 9 of 15 defects treated with the coronally positioned flaps a new connective tissue attachment was present in the

furcation area. Whereas, of 16 defects in which the flap margin was sutured at the CEJ, one healed with a new connective tissue attachment and the other defects demonstrated an epithelized furcation. In another study, Kling et al., 1985, tested the effect of crown-attached sutured in the same model as previously discussed. In this study the flaps were positioned just coronal to the CEJ and were closed in the control group with interrupted interproximal sutures. In the experimental group the flap margins were positioned similarly and were closed with interproximal sutures that were bonded to the crown so that the margins were secured and stabilized in a position approximately 1 mm coronal to the CEJ. These crown-attached sutures prevented the flap margin from receding apically and helped to stabilize it. Histologic analysis showed complete new connective tissue attachment in 13 of 14 defects treated with crown-attached sutures, while all the 10 control defects showed a junctional epithelium healing pattern. Taken together, the results of these studies suggest that stability of the flap or wound margin may be important to the achievement of new connective tissue attachment and regeneration. Mobility of this margin will likely lead to tears or ruptures in the fibrin linkage to the root surface, allowing apical migration of the junctional epithelium along the inner surface of the wound, which would preclude the possibility of regeneration (Egleberg, 1987). The second and equally important component of fibrin clot adhesion to the root surface is *the biologic acceptance of the root surface*. Root surfaces being prepared in regenerative procedures have undergone significant changes from the norm. Theoretically, they resemble a biomaterial that needs to be altered before substantial regeneration can occur. There are several factors that may produce a surface that is biologically less than ideal for achieving and/or maintaining a stable wound healing interface. These factors include

contamination by bacteria and bacterial products like endotoxins (Aleo et al., 1974); a phenomenon of "smear layer" postinstrumrentation (Polson et al., 1984); and finally surface contamination by saliva (Heaney, 1990). These factors may affect the tensile strength of the fibrin clot adhesion or may interfere with or alter its formation. Both circumstances could lead to healing via a long junctional epithelium. In some studies, root surfaces in periodontal defects have been partially demineralised by acid conditioning (Hancock, 1989; Polson, 1986). This process removes the smear layer of instrumentation, debris, blood elements and saliva (Polson et al., 1984). Partial demineralisation of the root surface following root planning exposes the outer layer of the collagenous matrix of dentin (Garrett et al., 1978). It also removes the endotoxins from the root surface (Fine et al., 1980). This surface has been shown to enhance new connective tissue attachment to root surfaces in various animal models (Bogle et al., 1983; Polson and Proye, 1983). However, results in human trials have been unconvincing (Hancock, 1989). In vitro studies have shown that surface demineralisation of dentin surfaces will enhance the ability of these surfaces to serve as a reservoir for biologically active extracellular matrix proteins or growth factors that theoretically could alter the wound healing environment in a positive manner (Terranova and Wikesjo, 1987). Furthermore, extracellular matrix proteins (Alger et al., 1990; Caffesse et al., 1985), polypeptide growth factors (Lynch et al., 1989) and blood elements including fibronectin (Ripamonti et al., 1987) have been applied to the root surface in attempts to biologically modify the surface and improve its acceptability to the cellular elements necessary to form a new connective tissue attachment.

While preclinical models have provided enhanced understanding of parameters of wound healing critical for periodontal regeneration and clinically relevant regeneration has been demonstrated, attempts at reconstruction of clinical defects usually have resulted in modest regeneration success. The studies presented here suggest that this limited success may in part be related to the clinical restrictions with regard to flap management and maintenance of wound integrity during the early healing phase. Another determining factor is the native regenerative potential in a periodontal defect under optimal conditions for wound healing. The generation of highly specialized cell populations that can remodel and heal damaged tissues in a temporally and spatially appropriate manner is thought to be essential for the regeneration purposes in healing periodontium. There is some suggestion that a small population of periodontal ligament cells in the mature periodontium have the capacity to undergo differentiation and produce cells that can synthesize bone, cementum and the extracellular matrix of the periodontal ligament (Lin et al,. 1994; Roberts et al., 1987). Also it is highly likely that other sources of cementoblast or osteoblast progenitor cells include marrow stroma and paravascular and endosteal fibroblasts (McCulloch and Melcher, 1983). The specific factors associated with regenerating tissue that may prove to have potential as trigger factors in regenerative therapies will be discusses later in this review.

3. LIMITATIONS OF CONVENTIONAL THERAPEUTIC MODALITIES

Periodontal therapy involves two primary components, elimination of the bacterial plaque and elimination of the anatomic defects produced by periodontitis. Traditionally, the accepted techniques used for connective tissue reattachment have been

scaling and root planning, subgingival curettage, modified Widman flap procedure and open flap curettage (Goldman, 1949; Carranza, 1996; Takei and Carranza, 1996; Ramfjord and Nissle, 1974). Changes reportedly produced by these therapies include pocket depth reduction mediated by gingival recession and gain of clinical attachment (Knowles et al., 1979; Pihlstrom et al., 1981 and 1983; Ramfjord et al., 1987; Lindhe et al., 1982; Lindhe and Nyman, 1984; Becker et al., 1988 and 1990; Kaldahl et al., 1988 and 1990; Kalkwarf et al., 1992). When angular bony defects are not corrected, they are often remodelled by a process of bone fill and crestal resorption (leveling) (Polson and Heijl, 1978). Until the mid-1970s, the gain of clinical attachment and bone fill produced by conventional periodontal therapy was interpreted to indicate that a true regeneration of the periodontium had occurred. Several longitudinal research centers around the world have demonstrated that conventional periodontal therapy, followed by good periodontal supportive therapy, is effective in stabilizing periodontal status and maintaining periodontal health (Becker et al., 1984; Knowles et al., 1979; Lindhe and Nyman, 1984; Lindhe et al., 1982; Pihlstrom et al., 1983; Ramfjord et al., 1987). Osseous surgery effectively reduces probing depth, but is accomplished by clinical attachment loss. Root planning and modified Widman flap procedures result in the greatest gains in clinical attachment, but do not reduce proding depths as effectively as osseous surgery (Kaldahl et al., 1988).

The clinical methods of evaluating periodontal therapy are periodontal probing, examination of radiographs and reentry procedures (Caton, 1989). Pre-treatment and post-treatment measurements are compared to determine the effect of the therapy. These clinical methods of evaluation, however, cannot distinguish between periodontal repair

and periodontal regeneration. Clinical attachment level measurements are important in evaluation of therapies outcome. However, large gains in clinical attachment can occur after therapy without regeneration of new periodontal attachment. These false gains are the result of resolution of the gingival inflammation, bone fill, reformation of the gingival collagen fibers and formation of a long junctional epithelial attachment (Caton et al., 1980). Similarly, while radiographic and reentry examinations, can evaluate the gross presence of bone, they cannot reveal if the bone is connected to the tooth by new periodontal ligament and cementum (Caton, 1989).

Histologic evaluation is the only reliable method to determine the true efficacy of periodontal therapies. Caton et al., (1980), presented a histologic analysis of healing following 4 treatment modalities in a monkey model. The treatments tested were root planning and gingival curettage; modified Widman flap procedure; modified Widman flap procedure combined with transplantation of previously frozen autogenous red marrow and cancellous bone; and modified Widman flap procedure in combination with implantation of beta tricalcium phosphate. The histologic sections revealed that healing following all four different procedures resulted in the formation of long junctional epithelium along the treated root surfaces, with no new connective tissue attachment. Listgarten and Rosenberg, 1979, conducted a histologic study to evaluate healing following periodontal surgery in human infrabony defects. Defects were treated by the use of osseous autografts, allografts or open flap curettage alone. Histological analysis 12 months after the treatment showed the junctional epithelium proliferated below the alveolar crest in all treatment groups, with the epithelium occupying from 52-85% of the distance from the alveolar crest to the bottom of the original osseous defect. With respect

to bone fill of the original defect, the grafted sites showed a more favourable results than the areas treated with open flap curettage alone. Steiner et al., 1981, studied the possibility of obtaining new connective tissue attachment to periodontally diseased root surfaces following replaced flap procedure in patients with advanced chronic periodontitis. Block biopsies 4 months after the procedure demonstrated that none of the specimens showed soft connective tissue adhering to the tooth or evidence of new cementum. Similar to the results shown previously the treated root surfaces were covered by a thin junctional epithelium (Caton et al., 1980; Steiner et al., Listgarten and Rosenberg, 1979).

In conclusion, although the clinical studies have shown success in closure of infrabony pockets, gain in probing attachment level, and minimal bone resorption with conventional periodontal treatment, the histologic studies have revealed that minimal or no new attachment is achieved, and the rest of the marginal seal is established by a long junctional epithelium. This knowledge, along with the increasing sophistication of technologies, has led many researchers to a scientific approach to the study of periodontal wound healing. This involves the definition of the variables involved in periodontal wound healing and the experimental manipulation of these variables in order to solve the problem of how to achieve periodontal regeneration (Melcher, 1976; Melcher et al., 1987; Caffesse et al., 1985; Gantes et al., 1988; Polson, 1986; Wikesjo and Nilveus, 1990).

4. REGENERATIVE THERAPIES

The ultimate goal of periodontal treatment includes the arrest of progressive periodontal disease and the predictable regeneration of those parts of the periodontium that were destroyed by the disease (Kalkwarf, 1974; Stahl, 1977). The surgical techniques used for periodontal regeneration are osseous grafting; guided tissue regeneration (GTR); biological approaches; and various combinations of these procedures. This review will focus on the 2 concepts of GTR and biological mediators for periodontal regeneration.

4.1. Guided tissue regeneration

Melcher, 1976, first presented the basic concepts which had led to the development of the clinical techniques collectively known as GTR. He suggested that selected cell populations residing in the periodontium can produce new cementum, alveolar bone and periodontal ligament, provided that these populations are given the opportunity to occupy a periodontal wound. Such opportunity arises when other cell populations, such as epithelial cells or gingival fibroblasts, which also would invade the wound space are effectively excluded. This provision to exclude specific tissues during the healing phase of a periodontal defect wound has generated an impetus for the development of periodontal devices, commonly called barriers or membranes, for GTR.

Nyman et al., 1982a and 1982b were the first to use a membrane in periodontal healing studies in monkeys and humans. A Millipore filter was placed between the gingival tissue on one side and the exposed root surface and the surrounding alveolar bone on the other, so that the epithelium and gingival connective tissue would be diverted

from the root surface. The results showed that new cementum with inseting connective tissue fibers formed on 50% of the test sites, suggesting that new attachment, following the principle of GTR is a strong possibility. The same principle was applied in a study by Gottlow et al., 1984, in a monkey model using a non-resorbable membrane. The coronal half of the buccal roots was surgically exposed and plaque was allowed to accumulate for 6 months after which a buccal flap was raised, the roots were planed and a membrane was placed over the denuded root surfaces. The results after 3 months showed new cementum with inserting collagen fibers on the previously exposed surfaces of both tests and controls, however, the test sites showed considerably more new attachment, indicating that the placement of a membrane favored repopulation of the wound area by cells originating from the periodontal ligament. Also bone regeneration occurred to a varying extent. Magnusson et al., 1985, used Millipore filter in monkeys in a manner similar to that used by Nyman et al., 1982a, and showed that approximately 50% of the root surfaces in the test sites were covered by new attachment. Whereas control sites healed by a long junctional epithelium. Aukhil et al., 1986, experimented on beagle dogs using filters to attempt coronal circumferential migration of cells. The results showed that while new connective tissue was mostly seen at the apical part of the specimens, some specimens showed the formation of long junctional epithelium. Root resorption, which was sometimes seen, preceded the formation of new cementum. The authors suggested that contact between periodontal ligament cells and root dentin is necessary for the progenitor cells to differentiate into formative cells such as cementoblasts. Caffesse et al., 1990, evaluated the effects of GTR in the treatment of class II furcation defects in dogs using Gore-Tex membrane. Results showed different degree of fill attained by

epithelium, new connective tissue and bone. Statistically, GTR gave significantly better results in the amount of connective tissue and bone fill achieve. Gottlow et al., 1986, published another report on new attachment formation, this time on humans with advanced periodontitis, using Gore-Tex membrane. They assessed the predictability of such formation when the principles of GTR were applied. After treatment, 5 of the teeth were removed for histologic analysis. Histologically, new bone growth seemed to be restricted to areas that had infrabony lesions prior to treatment. Bone regrowth and new attachment appeared to be unrelated phenomena. The authors attributed this variation to variables such as the amount of recession, the type of defect, and the availability of the periodontal ligament cells. Taken together, the histologic studies indicate significantly better results with the use of GTR in treatment of class II furcation and vertical osseous defects. However, much work remains regarding predictability.

In respect with clinical outcomes of GTR therapy, Laurell et al., 1998, published an article reviewing studies in the past 20 years on the surgical treatment of infrabony defects. Treatments included open flap curettage (OFC); OFC plus osseous grafts; and GTR. In order to assess the changes in pocket reduction, clinical attachment level gain, and bone fill with the various treatment modalities, data were pooled for meta-analysis in each treatment category. The results indicated that GTR offers the best results in terms of clinical attachment level gain or bone fill. In addition, the correlation between clinical attachment level gain or bone fill and defect depth was significantly larger for GTR than for the other treatment. The benefit of bone grafts as an adjunct to OFC or GTR to increase clinical attachment level gain was not demonstrated in the clinical studies surveyed (Guillemin et al., 1993; Chen et al., 1995; Gouldin et al., 1996). Also, the

studies surveyed, were not able to demonstrate any advantage of root conditioning as an adjunct to GTR (Handelsmann et al., 1991; Kersen et al., 1992), which was also the case for OFC (Renvert and Egelberg, 1981). It was also shown that the amount of bone fill, that evidently can be achieved by OFC alone, together with the crestal resorption and the residual defect total 3.5 mm. Thus to benefit from GTR procedures, the intrabony defect has to be at least 4 mm deep. Review of the clinical studies on GTR treatment of furcation defects, shows improved clinical attachment levels and bone fill in mandibular class II furcation defects, while treatment with debridement and flap repositioning alone provides little or no improvement (Lekovic et al., 1991; Lekovic et al., 1998). However, treatment of maxillary, proximal class II furcation defects, as well as mandibular or maxillary class III furcation defects does not seem to offer any degree of predictability of furcation closure (Mellonig et al., 1994; Pontoriero and Lindhe, 1995a; Pontoriero and Lindhe, 1995b; Pontoriero et al., 1989). It should be noticed that although there are several clinical studies showing significant improvement of class II furcation and intrabony osseous defects with GTR treatment, but most of them are not accompanied with histologic analysis. Therefore, it is not known if improved probing attachment levels and bone fill are associated with new connective tissue attachment to the treated root surface.

4.2. Biological mediators

Various biological approaches to the promotion of periodontal regeneration have been used. These can be divided into the use of growth and differentiation factors; and application of extracellular matrix protein and attachment factors.

4.2.1. Growth and differentiation factors The first demonstrations of periodontal regeneration induced by growth factors was presented by Lynch et al., 1989; 1991. A combination of platelet-derived growth factor (PDGF) and insulin growth factor-I (IGF-I) was used in dogs with natural periodontitis and histologic evaluation 14 days after treatment showed new cementum and bone formation in the test site, while the control sites exhibited a long junctional attachment without evidence of regeneration. Later, the effects of PDGF alone or in combination with IGF-I were evaluated in nonhuman primate models and they all confirmed the earlier results suggesting that PDGF and IGF-I may stimulate periodontal regeneration (Rutherford et al., 1992; 1993; Giannobile et al., 1996). In 1997a, Howell et al., performed a clinical trial to evaluate the safety and efficacy of PDGF/IGF-I combination for periodontal regeneration in infrabony and furcation defects. Using re-entry approach 6-9 months after treatment, significant increases in bone formation were observed with high dose of growth factors (150 μg/ml) but not with the low dose (50 μg/ml) and no safety issues observed. The authors concluded that surgical implantation of PDGF/IGF-I combination support significant periodontal regeneration in humans in a dose-dependent fashion. Other combinations of growth factors have also been tested. Combination of PDGF, basic fibroblast growth factor (bFGF), and TGF-β1 was used in a fenestration model in dogs and the results showed significantly more bone regeneration in the control sites compared to test sites, indicating that this growth factor combination did not stimulate periodontal regeneration and in fact, inhibited it. (Selvig et al., 1994). In another study, the effect of TGF-β1 combined with GTR was tested in dogs and the results were compared with GTR alone. It was shown that bone and cementum regeneration was only limited to the most apical area

of all defects and was no different in growth factor compared with control defects indicating that in this approach for regeneration in this preclinical model may have limited clinical potential (Wikesjo et al., 1998).

Several bone morphogenic protein (BMP) molecules and preparations have been tested in preclinical models of periodontal regeneration. Ripamonti et al., 1994, reported on periodontal regeneration following surgical implantation of BMP-2 and –3 in surgically induced furcation defects in monkeys. Histological evaluation showed significant amounts of new bone, periodontal ligament and cementum in test sites compared with controls. Ishikawa et al., 1994, used recombinant BMP-2 mixed with autologous blood to stimulate periodontal regeneration in surgically induced 3-wall intrabony defects in dogs. Following a one-month healing period, both tests and controls demonstrated new bone and cementum, however, the coronal extent of the newly formed tissues was significantly greater in defects receiving BMP-2 compared to control. Clinical trials have been initiated in the evaluation of recombinant BMP-2. BMP-2 has been used for maxillary sinus augmentation (Boyne et al., 1997); and alveolar ridge augmentation or preservation (Howell et al., 1997b). The results for sinus augmentation study demonstrated bone formation associated with BMP-2 in all 11 patients (Boyne et al., 1997). However, significant bone formation could not be established following the ridge augmentation procedures. No serious adverse effect was reported in any of the cases (Howell et al., 1997b).

Taken together, the studies on the role of growth factors in periodontal regeneration indicate a potential for some of these molecules to induce regeneration of periodontal tissues. However, this approach is not commonly used in the clinic. One

reason is the high costs associated with it. Another possibility is that although theoretically these growth factors make sense at the cellular level and can be shown to be effective in animal models, when used in humans with all inherent variables, the effects become less obvious due to other growth factors in the environment. Therefore the magnitude of the response is not overwhelming compared to existing therapies.

4.2.2. Extracellular matrix proteins A variety of proteins and extracellular matrix components control where cells migrate, how they adhere and how they function. Fibronectin is a glycoprotein present in serum and produced by a wide variety of cell types (Caffesse and Quinones, 1993). Its major function is to aid in the attachment of cells to the extracellular matrix, and it thus plays a pivotal role in tissue regeneration and wound healing. Fibronectin, applied in conjunction with citric acid demineralisation of the root surface, has been shown to increase the level of connective tissue attachment by approximately 2 mm compared with flap surgery alone in a dog model (Caffesse et al., 1985). These results were confirmed by another study using dog model (Smith et al., 1987). However, contrasting results were observed following root surface demineralisation and fibronectin application in supra-alveolar periodontal defects in a dog study (Wikesjo et al., 1988). These results were confirmed in a human histological case study by Alger et al., 1990. In another human study, the clinical effects of placement of fibronectin in periodontal defects following root demineralisation was compared with flap surgery alone. The results after one year showed that while there was a difference between the 2 groups, the differences in pocket depth and clinical attachment levels were slight (Caffesse et al., 1988). Thus, this treatment modality remains of uncertain clinical benefit.

Enamel matrix proteins are a family of extracellular matrix proteins that regulate the initiation and growth of hydroxyapatite crystals during mineralization of the enamel. The expectation is that this material will direct the formation of cementum, based on the circumstantial evidence that, during embryonic development, these enamel proteins are involved in the formation of cementum (Slavkin, 1976). The formation of cementum is dependent on a matrix-cell interaction, and in vitro studies have demonstrated that mesenchymal cells of the dental follicle develop a hard tissue matrix believed to be cementum when exposed to enamel matrix proteins (Hammarstrom, 1997). The expected primary effect of enamel matrix derivative is generation of cementum, however, it has also been shown that exposure of periodontal ligament cells to enamel matrix derivative results in increased cell proliferation, total protein synthesis, and numbers of mineralized nodules formed (Gestrelius et al., 1997b). Enamel matrix proteins and their role in cementum development and regeneration will be thoroughly discussed in the next section of this review.

5. CEMENTUM DEVELOPMENT AND REGENERATION

Cementum serves as the biological and structural link between the root surface and the collagenous network of the periodontal ligament. As discussed earlier base on the presence or absence of enclosed cells and on the contents, origin and direction of collagenous fibers different types of cementum have been distinguished including: *Acellular extrinsic fiber cementum* (AEFC), *Acellular afibrillar cementum* (AAC), *Cellular mixed stratified cementum* (CMSC), and *Cellular intrinsic fiber cementum* (CIFC) (Schroeder, 1986). Since AEFC is found mainly on the cervical and middle root it is the cementum type that is most affected by the destructive processes of periodontitis.

However, as described by Ten Cate 1997, the type of new cementum that forms on the root surfaces following regenerative therapies is almost always cellular cementum, rather than acellular cementum. As it has been suggested by Ten Cate 1997, acellular and cellular cementums might be inherently different tissues with distinctly different developmental origins. Acellular cementum is most likely an odontogenic tissue, originating either from dental follicle cells or odontogenic epithelium. On the other hand, evidence is also emerging to support the theory that cellular cementum may be a bone-like tissue with origins in alveolar bone (Ten Cate, 1997). If this is the case, the formation of AEFC may be restricted to early development only and it may not be possible to regenerate AEFC on mature root surfaces. Another morphological and structural difference between regenerative and developmental cementum is in the manner by which AEFC attaches to the underlying dentin that may be unique. The fusion between AEFC and dentin may be related to specific cell, substrate and extracellular matrix conditions existing during root development that may facilitate a functional dentin-cementum union. On the other hand, the histological sections obtained from regenerated tissues show the presence of separations between dentin and newly formed cementum suggesting that cellular cementum formed in these situations may not fuse to dentin in a manner similar to that observed during development (MacNeil and Somerman, 1999). These observations provoke several questions. First is the difference in cementum quality in development and regeneration clinically significant and is periodontal regeneration truly regenerative or more like a form of repair.

The focus of the next sections will be on cementogenesis and putative factors controlling development and regeneration of periodontal tissues.

5.1. Root Development

Root development is initiated through the contribution of the cells of the (1) enamel organ, (2) dental papilla, and (3) dental follicle. The cells of the outer enamel epithelium come into contact with the inner enamel epithelium at the base of the enamel organ, i.e., the cervical loop. With completion of the crown, the cells of the cervical loop continue to grow away from the crown and become root sheath (Hertwig's) cells. On its interior, the root sheath encloses the cells of the dental papilla, and on its exterior, it is surrounded by the cells of the dental follicle. The inner root sheath cells will induce the adjacent cells of the dental papilla to become odontoblasts which, in turn, will form root dentin. The third component in the root formation, the dental follicle, is the tissue that surrounds the enamel organ and the dental papilla. It will give rise to cells that form the supporting structures of the tooth, i.e., the cementum which covers the surface of the root, the periodontal ligament, and the inner layer of the alveolar bone (Avery, 1987).

Initial cementogenesis and root formation are intimately related. In the classical theory of cementogenesis Hertwig's epithelial root sheath (HERS) induces the mesenchymal cells of the dental papilla to form the mantle predentin before it disintegrates and leaves the root surface. This allows the predentin to come in direct contact with connective tissue of the dental follicle. The mesenchymal cells of the dental follicle exposed to the newly formed dentin are then believed to differentiate, and become cementoblasts (Selvig, 1963; Armitage, 1986; Cho and Garrant, 1988; Bosshardt and Schroeder, 1991). The sheath root cells degenerate after they have completed their formative and odontoblast-stimulating functions. The newly differentiated cementoblasts first elaborate the organic matrix or cementoid. This matrix consists of collagen fibers

and a ground substance composed of protein polysaccharides (proteoglycans). Then, the organic matrix mineralizes, and cementum is laid down in successive layers or increments until its full thickness is reached. Thereafter, the cementoblasts enter a quiescent state near the cementum front, ready to function for further growth or repair. Adjacent fibroblasts elaborate collagen fibers, which become embedded in the cementum matrix, to provide attachment of the tooth to the surrounding bone. The embedded portions of the periodontal ligament fibers in the cementum are known as Sharpey's fibers that will become the means of attachment of the principle fibers of the periodontal ligament. The periodontal ligament originates from the dental follicle and is the specialized, soft, connective tissue ligament that provides the attachment for the teeth to the adjacent bone at the other end. Some delicate fiber bundles of the forming periodontal ligament appear first as root formation begins. The innermost follicular cells near the forming root differentiate into cementoblasts and lay down cementum. The outermost follicular cells differentiate into osteoblasts and furnish the lining of the bony socket. The most centrally located cells in the ligament differentiate into fibroblasts which produce collagen fibers that will become embedded in both forming cementum and bone (Avery, 1987). (Fig. 1)

Although it has been generally accepted that cells of dental follicle give rise to cells forming the periodontium, at the cellular and molecular level, the specific cell types and the required stimuli to activate these cells to function as cementoblasts, osteoblasts or periodontal ligament cells has not yet been established. In the next section I will focus on the controversies in the literature regarding factors capable of triggering cells to function in formation of a periodontium.

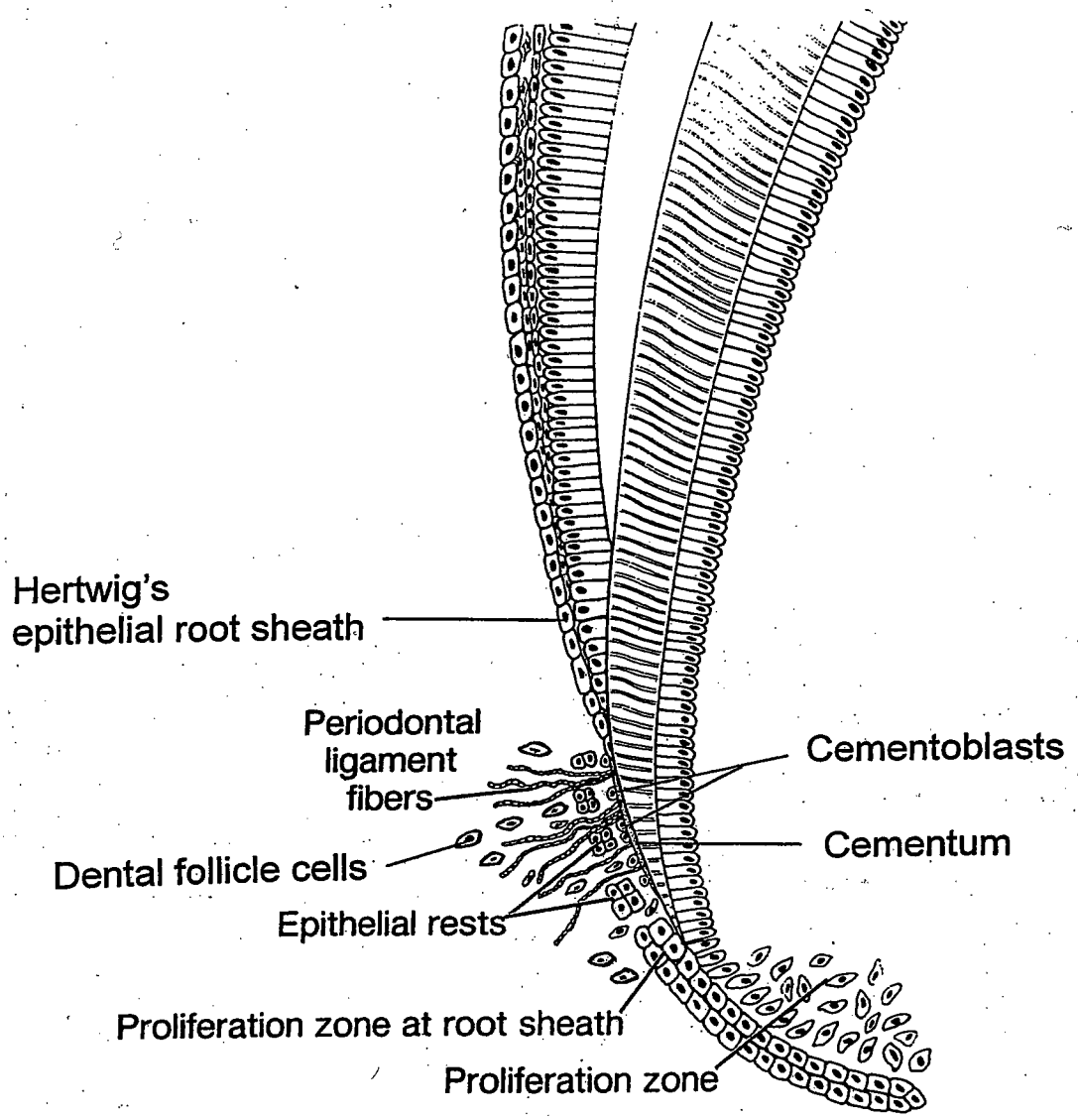

Figure 1. Schematic representation of root and cementum formation. The cells of the outer and inner enamel epithelium form the bilayer of cells known as the Hertwig's epithelial root sheath which lays down the precursor matrix necessary for differentiation of dental follicle cells into cementoblasts. (Modified from: Avery, J.K. 1987, Development of root and supporting structures [Figure 8.5.]. In: Oral development and histology. pp 97).

5.2. Controversies

Histological and tissue recombinant studies examining cells and tissues during periodontal development indicate that cells derived from the neural crest, that is, dental ectomesenchymal cells (dental papillae and dental follicle cells), when appropriately triggered, are capable of acting as cementoblasts, periodontal ligament fibroblasts and osteoblasts, thus controlling formation of cementum, periodontal ligament and alveolar bone, respectively (Ten Cate et al., 1971; Yoshikawa and Kollar, 1981; Palmer and Lumsden, 1987; Osborn and Price,1988). In a similar fashion, investigations directed at determining the cells responsible for eliciting regeneration of the periodontium have suggested that periodontal ligament fibroblasts, as well as paravascular and endosteal fibroblasts when appropriately induced, have the capacity to synthesize periodontal ligament, cementum and alveolar bone (Gould et al., 1980; Gould, 1983; Lin et al., 1994; McCulloch and Melcher, 1983; McCulloch et al., 1987; McCulloch, 1993; Somerman et al., 1988). As with cells responsible for development of the periodontium, the exact cells responsible for the regeneration of the periodontium have not been established. While, data suggest that populations of cells exist within the periodontal ligament having the capacity to function as cementoblast/osteoblast-like cells, there is also evidence that a population of cells within the periodontal ligament, both during development and during regeneration, secrete factors that can regulate mineralization by inhibition, thus prevent the fusion of the tooth root with surrounding bone (Melcher, 1970; Ogiso et al., 1991).

As mentioned earlier, there is evidence suggesting that exposure of follicular cells to dentin is a sufficient stimulus for cementoblast differentiation (Armitage, 1986; Bosshardt and Schroeder, 1991). On the other hand, recombinations between slices of

root dentin and follicular cells have shown that an exposed dentin surface alone is not enough for differentiation of cementoblasts (Thomas and Kollar, 1988).

Accumulating data suggest that cells derived from oral epithelium may also participate in formation of cementum. It has been proposed that epithelial root sheath cells derived from the enamel organ can influence cementum formation by several mechanisms including a) release of specific factors resulting in induction of follicle cells to differentiate into cementoblasts (Slavkin et al., 1989; Luo et al., 1991; Fong et al., 1996; Hammarstrom et al., 1996; Lindhe, 1997) and/or b) actual transition of epithelial root sheath cells into cementoblasts as reported both *in vivo* and *in vitro* (Boyer et al., 1996). c) Another possibility is that epithelial root sheath cells undergo apoptosis, allowing follicle cells to contact the root surface and that subsequently, the interaction of follicle cells with the tooth surface promotes follicle cell differentiation into cementoblasts. In this situation the triggering factors may be dentin-related and/or products previously secreted by epithelial root sheath cells (Slavkin et al., 1989; MacNeil and Thomas, 1993; Bosshardt and Nanci, 1997). Signaling molecules important in early stages of tooth development such as bone morphogenetic proteins and parathyroid hormone-related proteins may or may not be involved in root formation and development of periodontal ligament. For example, *in situ* hybridization and immunohistochemical analysis for temporal and spatial expression of bone morphogenetic proteins within epithelial root sheath, follicle cells and periodontal ligament region during root development have not shown any evidence of epithelial-mesenchymal signaling (Nieminen et al., 1998). It is possible that epithelial products are involved in signaling of mesenchymal cells within the root area, but are different from those linked with crown

development. Developmental studies have provided markers, such as bone sialoproteins, osteopontin and octeocalcin, that can now be used to determine which cells express these factors during regeneration and to determine whether these factors are altered by specific therapies (Lekic et al., 1996). The molecule bone sialoprotein has been highlighted in order to provide an example of a molecule considered to have a role in controlling periodontal cells, both during development and regeneration of tissues. Bone sialoprotein is expressed during cementum formation and with continued root formation, bone sialoprotein remains localized to the root surface and is expressed only by root surface cells and cementoblasts (Lekic et al., 1996; MacNeil et al., 1996; D'Errico et al., 1997). Moreover some properties of bone sialoprotein such as containing an RGD adhesion domain and several stretches of polyglutamic acid that promote cell attachment and binding to hydroxyapatite respectively, makes this molecule a candidate for promoting cementum formation (Sommerman et al., 1991; Hunter and Goldberg, 1993). Other data suggest that some molecules may act to control the extent of mineralization during root development. In this regard type XII collagen has been suggested to have some role in regulating the degree of mineralization during root formation, since its expression within the periodontal ligament region coincides with formation and alignment of periodontal ligament fibers perpendicular to bone and tooth (MacNeil et al., 1998). Moreover, it was demonstrated by Lekic et al., 1996, that at early stages of periodontal healing in a rat model, expression of osteopontin was localized to the periodontal ligament region and appeared to define a border between alveolar bone and periodontal ligament.

While several candidate molecules are considered to be required for formation of the periodontium, specific factors responsible for periodontal formation have not been

confirmed. Recent evidence suggests that the newly discovered enamel-like products including amelin, amelogenin and sheathlin may have some role in controlling cementogenesis. This has resulted in the use of enamel proteins in an attempt to restore periodontal tissues lost as a consequence of disease (Lindhe, 1997). The focus of the next section will be on introduction of enamel matrix proteins and their possible role in cementum development and regeneration.

5.3. Enamel matrix proteins

An association between enamel and cementum formation is compatible with the fact that coronal cementum is a normal structure on the enamel surface in a variety of animals such as sheep, cows, rabbits and guinea-pigs (Listgarten, 1968; Ainamo, 1970; Listgarten and Shapiro, 1974, Schroeder, 1986). Moreover, in human teeth, in about 60% to 65% of cases cementum overlaps the enamel (Carranza, 1996). In general, the coronal cementogenesis seems to be initiated by exposure of the developing enamel to the cells of dental follicle shortly after the breaking up of the reduced enamel epithelium (Listgarten, 1968; Ainamo; 1970). In sheep and rabbits this process results in a complete coverage of the enamel by a continuos layer of cementum (Ainamo, 1970; Listgarten and Kamin, 1969), while in guinea-pig molars numerous separate cementum pearls are spread over the enamel surface separated by the reduced enamel epithelium (Listgarten and Shapiro, 1974).

5.3.1. Background The idea that enamel-related proteins from the epithelial root sheath are involved in the formation of acellular cementum was proposed by Slavkin and Boyde in 1975. Schonfeld and Slavkin 1977, showed enamel-matrix proteins on the root

surfaces of rabbit incisors. Later, Lindskog, 1982a,b, and Lindskog and Hammarstrom, 1982, using scanning electron microscopic and autoradiographic studies on monkey incisors, showed that the inner layer of the Hertwig's epithelial root sheath had a secretory stage and that enamel matrix like material was formed on the root surface prior to cementum formation. These findings were also supported by ultrastructural and immunohistochemical studies by Bosshardt and Nanci, 1997, showing that enamel matrix proteins were deposited on the dentin surface at the apical end of the roots of developing human and porcine teeth. Slavkin and coworkers, 1988, demonstrated that acellular cementum contains proteins that are immunologically related to proteins present in the enamel matrix. The enamel matrix is a temporary structure in the development of the crown and most of it is degraded and eliminated during the final mineralization of the enamel (Brookes et al., 1995). Hammarstrom, 1997a, has shown that in the cervical region of the roots of human teeth, the enamel matrix sometimes becomes mineralized, while in the other areas, it may be degraded.

The major proteins of the enamel matrix are known as amelogenins, comprising about 90% of the matrix. It has also been shown that amelogenin exists in several different sizes, forming supramolecular aggregates that are highly hydrophobic. Amelogenins are supposed to play a role in the developing enamel crystals and later influence their growth. The remaining 10% includes proline-rich non-amelogenins, tuftelin, tuft protein, serum proteins and at least one salivary protein (Brookes et al., 1995). More recently, Krebsbach et al., 1996 and Cerny et al., 1996, using cloning and DNA sequencing showed additional proteins such as ameloblastin and amelin associated with enamel matrix. A study by Fong et al., 1996, has shown that amelin is expressed by

the cells of HERS in rat molars during root formation. However, *in situ* hybridization studies on developing mouse molars indicated that the enamel proteins expressed during root formation are not identical to amelogenin (Luo et al., 1991). In 1997, Hammarstrom presented a study investigating the distribution of amelogenin in the forming apical end of the root of developing human premolars and rat molars. The result of the study supported the idea originally proposed by Slavkin and Boyde, 1975 that enamel matrix proteins are involved in the formation of cementum. In developing human teeth amelogenin is present in the area where cementogenesis was initiated and a cementum-like tissue was formed when cells of the dental follicle were exposed to enamel matrix. Moreover, analysis of ground sections of human teeth showed a thin layer of highly mineralized enamel extending from the coronal enamel between the dentin and cementum. These observations suggest that deposition of enamel matrix on the dentin surface precedes formation of acellular cementum in human teeth (Hammarstrom, 1997a). This is also in agreement with findings of Owens, 1978, that showed a brief secretory phase in the Herwig's epithelial root sheath in developing teeth of dogs in the same apical region as amelogenin was found in the study by Hammarstrom. The second part of Hammarstrom's study explored the question if exposure of endogenous and exogenous enamel matrix to dental follicular cells could induce cementum formation. Experimental exposure of enamel matrix to the mesenchymal follicular cells in developing rat molars showed that an acellular collagenous layer was formed on the surface of the exposed enamel matrix. Exposure of the experimental cavities in the root surfaces in adult monkeys to crude porcine enamel matrix showed healing with a thick layer that was histologically identical to acellular cementum. This layer was well attached

to the underlying dentin and collagenous fibers extended out from the cementum layer. Therefore, the exogenous enamel matrix had induced the same kind of tissue reaction as endogenous enamel matrix did in the experiment on the rat molars. The healing of the cavities where no enamel matrix was placed was characterized by deposition of an unevenly thick layer of cellular, hard tissue that was poorly attached to the denuded dentin. This cellular hard tissue that was formed in the control cavities seemed to be similar to the hard tissue formed at the root surfaces after application of membranes to selectively promote regeneration of the periodontal tissues (Gottlow et al., 1984). Taken together, these findings support that idea that enamel matrix proteins are involved in the development of cementum and that these proteins may be used as a means to regenerate acellular extrinsic fiber cementum.

5.3.2. Constituents As mentioned earlier the major proteins of the enamel matrix are known as amelogenins forming about 90% of the matrix. Based in current data, enamel matrix is a complex mixture of several matrix proteins including: (1) full-length and truncated isoforms of amelogenin having the same N-terminal, and usually C-terminal, sequences but with certain internal sequences missing as a result of alternative splicing of mRNA (Uchida et al., 1997); (2) probably more than one non-amelogenin protein, including those currently classified by cloning or conventional biochemical techniques as (a) tuftelin (Deutsch et al., 1991), (b) sheathlin (Uchida et al., 1991), ameloblastin (Krebsbach et al., 1996), amelin (Fong et al., 1996) (analogous proteins), (c) sulfated enamel protein (Smith et al., 1995) and (d) porcine enamelin (Fukae et al., 1996).

Amelogenins have a hydrophilic C-terminal end, but they are otherwise highly hydrophobic and show unpredictable solubility behavior in solutions of various ionic

strengths, temperatures, and pH (Moradian-Oldak et al., 1994a). They tend to aggregate at 37°C and neutral pH and may form quanternary supramolecular structures, called nanospheres (Fincham et al., 1994). Compared to amelogenins, the knowledge about the non-amelogenin group of enamel matrix proteins is very limited at the present, in part because some of these proteins are known only as a result of cloning, and their exact biochemical properties as they are secreted *in vivo*, and their fate, have not been established (Krebsbach et al., 1996; Hu et al., 1997b,c). It is suspected from comparisons of cDNA sequences that ameloblastin and amelin are the same molecule, and that ameloblastin/amelin are the rat equivalent of porcine sheathlin (Snead 1996; Hu et al., 1997b). Based on molecular weights deduced for the cloned proteins and the likelihood of post-translational sugar modifications, there is a chance that ameloblastin/amelin/sheathlin may correspond to sulfated enamel protein (Krebsbach et al., 1996; Hu et al., 1997b; Uchida et al., 1997). The status of porcine enamelin is also unclear at this time. The possible secretory forms or fragmented forms of this protein have not been seen clearly in developing rat incisor enamel by direct in vivo radiolabeling, by which the sulfated enamel proteins were originally identified (Smith et al., 1995). Another component of enamel matrix, tuft proteins/tuftelin, may serve some purposes different from those associated with ameloblastin/amelin/sheathlin, sulfated enamel protein, and/or porcine enamelin (Zeichner-David et al., 1997). Tuftelin contains the amino acid cycteine (Deutsch et al., 1991) which is not present in amelogenin (Simmer and Snead, 1995) or in any non-amelogenin known today (Krebsbach et al., 1996; Smith and Nanci, 1996; Hu et al., 1997b,c), allowing tuftelin to form potential intra- and intermolecular bonds impossible for the other matrix proteins. Moreover,

tuftelin is present in mature enamel (Deutsch et al., 1991) not a fate observed for all amelogenins and at least one non-amelogenin protein (sulfated enamel protein), which are both lost as enamel matures (Moradian-Oldak, 1995; Smith and Nanci, 1996).

In the next section of this review, I will discuss the studies conducted to evaluate the role of enamel matrix derivatives in periodontal regeneration.

5.4. Enamel matrix proteins and periodontal regeneration

5.4.1. ***In vitro studies*** As mentioned earlier, amelogenin family is the hydrophobic constituent of the enamel matrix proteins (Fischer and Termine, 1985; Aoba et al., 1987). The characteristic of such proteins is that they aggregate and become practically insoluble at physiological pH and body temperature. Amelogenin has been reported to form insoluble supramolecular aggregates representing molecular weights of 2-3 million Daltons under physiological conditions (Fincham, 1994). The solubility increases at acid or alkaline pH and low temperature. In order to facilitate use of enamel matrix proteins as a matrix for regeneration of periodontal defects, a suitable formulation for its application onto affected dental root surfaces is required. Based on the molecular characteristics of enamel matrix proteins, a suitable liquid formulation should have a non-neutral pH and allow gradual reprecipitation of the matrix when physiological conditions are reestablished. Hammarstrom et al., (1997), explored the usefulness of a number of different vehicles, including alginates, dextrans and celluloses in a dehiscence model in monkeys. When enamel matrix derivatives (EMD) were dissolved in different vehicles and applied on the denuded dentin in the dehiscence model, only the propylene glycol alginate (PGA) vehicle resulted in significant periodontal regeneration. PGA is a

propylene glycol ester of alginate acid. The esterification of carboxyl groups in alginic acids results in macromolecules giving high viscosity, even at a low pH and in the presence of ions such as calcium. Gestrelius et al., 1997a, conducted a study to explore the behaviour and kinetics of enamel matrix derivatives in PGA vehicle solution. The results showed that EMD can be dissolved in PGA at an acidic pH, resulting in a highly viscous solution. At neutral pH and body temperature the viscosity decreases and EMD precipitates. Ellipsometry, biospecific interaction analysis and total internal reflection fluorescence were used for analysis of multilayers of EMD on mineral or protein surfaces. The results showed that EMD adsorbs both to hydroxyapatite and collagen and to denuded dental root surfaces. It forms insoluble spherical complexes, and detectable amounts remain at the site of application on the root surface for two weeks, as shown with radiolabelled protein in rats and pigs. Moreover, scanning electron microscopic and morphometric examination on monkeys revealed that EMD in PGA significantly promotes repopulation of periodontal ligament cells during the first weeks after application compared to controls. Therefore, it was concluded that PGA solution fulfills the essential requirements of a vehicle to facilitate application of EMD during periodontal surgery. Gestrelius et al., 1997b, conducted a study to determine the ability of EMD to influence specific properties of periodontal ligament cells in vitro. Cells properties that were examined included migration, attachment, proliferation, biosynthetic activity and mineral nodule formation. Immunoassays were used to determine whether or not EMD retained known polypeptide factors such as PDGF, TNF-α and TGF-β. The results showed that none of the polypeptide factors tested in the study were present in EMD. Under the in vitro conditions of this study it was shown that EMD enhanced proliferation

of the PDL cells, but not of epithelial cells; increased total protein production by PDL cells; and promoted mineral nodule formation of PDL cells. However, EMD had no effect on migration or attachment and spreading of PDL cells. The authors concluded that the current data support the hypothesis that EMD can act as a matrix for cells at a periodontal regenerative site. Monique et al., 2000, investigated the effects of EMD on the behavior of human periodontal ligament and gingival fibroblasts in vitro, with specific focus on their attachment properties, the expression of alkaline phosphatase activity, the release of TGF-β and their proliferation rate. The results indicated that gingival fibroblasts barely attached and spread on EMD, whereas periodontal ligament fibroblasts attached and spread within 24 hours. Furthermore, the expression of alkaline phosphatase and release of TGF-β were significantly enhanced under the influence of EMD. However, EMD did not influence the proliferative rate in either of the cell lines. It was suggested that a more rapid attachment of periodontal ligament fibroblasts to EMD might contribute during the initial stages of periodontal healing to selective outgrowth and colonization of exposed root surfaces in vivo. Using an in vitro system to evaluate wound-fill rates, EMD has been shown to be a significant stimulator of human periodontal ligament cells (Hoang et al., 2000). This effect by EMD was greater than the effect of PDGF-BB, a proliferation agent of human PDL cells. Consistent with the effect of PDGF, EMD also stimulated wound-fill rates of gingival fibroblasts. The authors suggested that since this effect was observed at early time points after wounding, one explanation for EMD stimulation of clinical periodontal regeneration is by its induction of the PDL cell proliferation.

5.4.2. Animal studies Periodontal regeneration after application of enamel matrix proteins was investigated in a buccal dehiscence model in monkeys (Hammarstrom et al., 1997). Porcine enamel matrix was locally applied on buccal root surfaces of maxillary canine to 1st molar after removal of the alveolar plate, exposed periodontal ligament and cementum. Evaluation of the healing under light microscope 8 weeks later, showed an almost complete regeneration of acellular cementum, firmly attached to the dentin and with collagenous fibers extending over to newly formed alveolar bone in areas where enamel matrix proteins were applied. Therefore, it was concluded that it is possible to induce regeneration of all the periodontal tissues in a way that mimics the normal development of the periodontal tissues. In another study the ability of enamel matrix proteins to regenerate periodontal tissues was tested in a monkey model (Gestretius et al., 1997a). Scanning electron microscopic examination 7-14 days after application of enamel matrix proteins on surgically exposed root surfaces, revealed enhanced repopulation of fibroblast cells, whereas the control surfaces were covered by a layer of bacterial plaque. The effect of enamel matrix proteins on periodontal wound healing was evaluated in degree III furcation defects in a dog model (Araujo et al., 1998). A combination of resorbable barrier membrane and enamel matrix proteins was used in the test areas compared to controls where barrier membrane was used alone. 4 months later, the furcation defects were clinically closed in both the test and control groups and the defects were found to harbor bone and periodontal ligament tissue which appeared to be in structural continuity with a newly formed root cementum. In the Test group, however, in the apical portion of the furcation defect a thin layer of acellular cementum had been laid down, whereas the corresponding tissue in the coronal portion in the test group, and also

in the Control group was a thick layer of cellular cementum. The current observation seems to support that enamel matrix proteins may have the ability to create an environment conducive for the formation of acellular cementum. More recently, treatment of infrabony defects with guided tissue regeneration and enamel matrix proteins was investigated in an experimental study in monkeys (Sculean et al., 2000). Surgically produces infrabony defects were treated with one of the 4 therapies including GTR, EMD, combination of GTR and EMD or coronally positioned flap (control). 5 months later the histological sections showed formation of long junctional epithelium in the control group, whereas periodontal regeneration (new periodontal ligament, new cementum with inserting connective tissue fibers and new bone) to a varying extent was present in all other 3 treatment modalities.

5.4.3. Clinical studies Heijl, 1997a, presented a case report where periodontal regeneration with EMD was investigated in one human experimental defect. EMD was applied on a surgically created buccal dehiscence defect in a mandibular incisor. 4 months later the microscopic examination revealed formation of a new acellular extrinsic fibre cementum, and a new periodontal ligament with inserting and functionally-oriented collagen fibres and an associated alveolar bone was also present. This was the first human study to show that EMD could provide a regenerative technology with a potential for true periodontal regeneration. More recently a series of case reports were presented to evaluate the clinical outcome following the application of EMD in treatment of intrabony periodontal defects. In a report of 32 cases, EMD was used in 2- and 3-walled intrabony defects. 8 months later, the clinical measurements showed a mean clinical attachment level gain of 3 mm and a mean reduction of 4.3 mm in probing pocket depth (Sculean et

al., 1999a). In another series of case reports, EMD was used in treatment of angular defects in 108 patients. Clinical evaluation after 12 months showed a mean probing attachment level gain of 4.6 mm and a probing pocket depth reduction of 5.2 mm. Radiographic assessments revealed a reduction in defect size corresponded to an average bone fill of 69% of the original defect. A histologic evaluation of periodontal healing following regenerative therapy with EMD was presented in a 10-case series by Yukna and Mellonig, 2000. Six months after application of EMD in 10 intrabony defects in 8 patients with advanced adult periodontitis, the histologic evaluation of the sites showed evidence of regeneration (new cementum, new bone and new periodontal ligament) in 3 specimens; connective tissue attachment/adhesion only in 3 specimens; and a long junctional epithelium in 4 specimens. The results indicated that use of EMD can results in periodontal regeneration on previously diseased root surfaces in humans, but on an inconsistent basis. In 1999b, Sculean et al., presented a study with the aim of histological analysis in humans the healing of advanced intrabony defects following treatment with EMD or GTR. The clinical results revealed in 6 months a mean clinical attachment level gain of 3 mm and 3.2 mm in the EMD and GTR groups respectively. The histological analysis showed in the EMD group a mean 2.6 mm of new cementum with inserting collagen fibers but this was not always followed by bone regeneration. In the GTR group, there was a mean 2.4 mm of new attachment that was accompanied by a varying amount of new bone. The newly formed cementum in both groups showed predominantly cellular character. The results following EMD therapy in this study, was similar to the corresponding outcome variables following GTR, confirming the previous studies that treatment of infrabony defects with EMD enhances the formation of a new attachment.

This was further supported by a prospective controlled clinical trial that compared the effect of various regenerative procedures at sites with angular bone defects. Clinical examinations 12 months after therapy showed that either the use of barrier membranes or application of EMD in angular defects enhanced outcome variables such as probing pocket depth and probing attachment gain (Pontoriero et al., 1999). A comparison between EMD used alone or in combination with bovine porous bone mineral (BPBM) in the treatment of intrabony defects was explored by Lekovic et al., 2000. 21 paired intrabony defects were treated either with EMD or with EMD combined with BPBM in a split-mouth design. Clinical evaluation and re-entry surgeries 6 months later revealed a significantly greater reduction in probing depth and more attachment level gain in the EMP/BPBM group compared to EMP group. Moreover, the there was significantly greater defect fill in favor of the EMP/BPBM group indicating that BPBM has the ability to augment the effects of EMD in treatment of intrabony periodontal defects. The long-term effect of EMD treatment as an adjunct to modified widman flap (MWF) was investigated in a placebo-controlled, multicenter trial. The clinical attachment gain was 2.1 mm in the test group at 36 months, compared to 1.5 mm in controls (MWF alone). The radiographic bone level continued to increase over the 36 months at the test sites, while it remained close to the baseline level at the control sites indicating that using EMD as an adjunct to MWF in treatment of infrabony defects will promote an increased gain of radiographic bone and clinical attachment compared to control (Heijl et al., 1997b). The clinical use of enamel matrix derivative has been shown to be safe, also after multiple applications (Zetterstrom et al., 1997). A total of 107 patients requiring periodontal surgery received the EMD at two separate intrabony defects adjacent to single-rooted

teeth. Within 2-6 weeks following treatment of the first defect, the second defect received surgery including application of EMD. 33 patients with similar periodontal defects served as surgical controls. Serum samples were analyzed for total and specific antibody levels. The results suggested that none of the antibody levels differed from baseline, demonstrating that the immune potential is low to the enamel matrix proteins. Clinical and radiographic analysis at 8 months and after 3 years indicated a significant difference between protein-treated and non-treated teeth. The EMD treatment under these conditions resulted in a 2.5 to 3.0 mm increase in clinical attachment and bone level.

In summary, the use of enamel matrix proteins to enhance periodontal regeneration is based on the scientific discovery that these proteins are active during embryogenesis of cementum, periodontal ligament and supporting bone. Based on in vitro results and safety studies, efficacy was tested in animal models. Results of primate studies suggested that enamel matrix proteins in conjunction with a mucoperiosteal flap resulted in the formation of periodontal supporting tissues which were similar to those formed during tooth development. The newly-formed tissues were characterized by acellular cementum firmly attached to the underlying dentin, a functionally oriented periodontal ligament and new alveolar bone. The results of human clinical trials demonstrated substabtial gain of supporting alveolar bone and clinical attachment, results which were consistent with the animal efficacy results. While a strong scientific rationale exists for the use of a enamel matrix proteins in regeneration of periodontal tissues, the exact cellular and molecular events that lead to progression of tissue formation is poorly understood and the conditions for stimulating predictable periodontal tissues with enamel matrix proteins are not known. Further studies are required to determine the therapeutic

potential for these molecules such that they may be used to optimally stimulate and direct specific points along tissue formation cascade.

CHAPTER TWO - AIM OF THE STUDY

Periodontal diseases are characterized by inflammation involving and destroying the tooth-supporting tissues including cementum, periodontal ligament (PDL) and alveolar bone. The ultimate goal of periodontal therapy is periodontal regeneration i.e. restoration of original structure and function of the periodontal tissues that have been previously damages by periodontal disease. Therefore, the regenerative events of periodontal wound healing require repopulation of progenitor cells, which have the potential to differentiate into specialized regenerative cells. It has been shown that distinctive progenitor cells in PDL are able to proliferate, migrate and ultimately produce more differentiated cells that can synthesize bone, cementum and PDL as has also been shown in the developing periodontal ligament (Gould et al., 1977; McCulloch et al., 1987; Palmer and Lumsden, 1987). However, the healing of periodontal wounds following conventional periodontal treatments has been confounded by the tendency for rapid epithelial proliferation and resultant coverage of the dentogingival wound area (Caton et al., 1980; Listgarten and Rosenberg, 1979).

Special treatment procedures have been introduced to re-establish new tooth-supporting tissues including osseous grafting, guided tissue regeneration and biological approaches. A recent approach to achieve periodontal regeneration is to mimic the events that take place during root development. There is increasing evidence that the cells of Hertwig's epithelial root sheath secrete enamel matrix proteins during root formation. These proteins are involved in the formation of acellular extrinsic fiber cementum during development as well as regeneration of periodontal tissues (Slavkin and Boyde, 1975;

Slavkin, 1976; Lindskog, 1982a and b; Hammarstrom 1997a; Hammarstrom et al., 1997b).

To further explore the role of enamel matrix proteins (Emdogain®) in periodontal regeneration, specific aspects of epithelial cells and PDL fibroblasts behavior in cell culture were examined in this study. Specific cellular activities tested were cell adhesion, spreading and migration, all known to be important in periodontal wound healing. This thesis examined the hypothesis: Emdogain® promotes periodontal regeneration by enhancing the adhesion, spreading and migration of PDL fibroblasts, but not epithelial cells.

To test this hypothesis the objectives were to:

1. Examine the binding interaction between Emdogain and the extracellular matrix proteins, fibronectin; collagen types I and IV; and laminin.

2. Examine Emdogain effects on epithelial and PDL fibroblasts cell adhesion, spreading and migration.

3. Examine if integrin proteins regulate the adhesion of PDL fibroblasts to Emdogain coated wells.

CHAPTER THREE - MATERIALS AND METHODS

1. EMDOGAIN® SURFACE COATING (STUDY OF PRECIPITATION)

Precipitation of Emdogain protein at neutral pH was examined using scanning electron microscopy (SEM). Freeze dried commercially available Emdogain (BIORA, Malmo, Sweden) was dissolved in 10 mM acetic acid (Gestrelius et al., 1997). Eight-chamber slides (Labtek; Naperville, IL) were coated with different concentrations of Emdogain solution ranging (1 to 30,000 μg/ml) at 4°C over night. The highest concentration used is the therapeutic dose that is applied in a periodontal surgical site. The slides were subsequently rinsed 4× with PBS to remove the excess Emdogain and processed for scanning electron microscopy by 1 hour fixation in 2.5% (w/v) glutaraldehyde and 1 hour post-fixation in 2% OsO_4 (w/v) in PBS, pH 7.2, at 4°C. Samples were DC-sputtered with 15-20 nm gold in an Edwards coating unit (Gibco, Grand Island, NY), and examined with a Cambridge Stereoscan microscope (Cambridge, UK).

2. EXTRACELLULAR MATRIX PROTEIN BINDING TO EMDOGAIN®

Interactions between Emdogain (0-30,000 μg/ml) and 4 different extracellular matrix (ECM) proteins (10 μg/ml) including: fibronectin (Bovine; Chemicon; Temecula, CA); collagen type I (Bovine tendon; Upstate Biotech; Lake Placid, NY); collagen IV (Human placenta; Sigma; Saint Louis, Missouri); and laminin I (Human placenta; Sigma; Saint Louis, Missouri) was evaluated with ELISA. Primary polyclonal antibodies

recognizing Fibronectin (F3648; Sigma); Collagen type I (T40113R; Biodesign); Collagen type IV (PS057; CedarLane); and Laminin (AB19012; Chemicon) were used. ELISA was used to also study, the interaction between increasing concentrations of fibronectin (5-100 μg/ml) and 2 concentrations of Emdogain (10 and 10,000 μg/ml).

96-well plates (Nunc; Brand Products, Denmark) were coated with Emdogain (0-30,000 μg/ml) at 4°C over night followed by rinsing 4× with PBS to remove the unbound Emdogain and induce the expected precipitation of bound proteins at neutral pH. Plates were blocked with 1% heat-denatured bovine serum albumin (BSA) in PBS for 30 minutes. Subsequently the wells were incubated with 10 μg/ml of ECM proteins (as listed above) at room temperature for 3 hours followed by rinsing 4× with PBS to remove unbound ECM protein. The wells were incubated with the relevant primary antibody (as listed above) for 1 hour at room temperature. Wells were then washed followed by 1 hour incubation with the secondary antibody (Anti-Rabbit IgG-Peroxidase conjugated; Boehringer Mannheim; Germany). Color was developed with ABTS substrate consisting of 20 ml buffer (0.1 M Na-acetate, 0.05 M Na-H2PO4); 1 ml ABTS (22 mg/ml in distilled water); and 0.2 ml diluted H_2O_2 (0.2 ml of 30% stock solution in 7 ml distilled water). Four replicates were assayed for each condition. The optical density of wells was measured with a microtiter plate reader set to 570 nm absorbance. To correct for possible non-specific binding of the secondary antibody to coated surfaces in each condition, samples incubated only with the secondary antibody were used. To determine possible binding of the primary antibodies to Emdogain and BSA, wells coated with Emdogain and BSA alone were prepared. The mean readings from these 2 controls were added

together and subtracted from the mean reading obtained from each set of quadruplicate sample containing Emdogain and one ECM protein.

To determine the amount of ECM proteins binding to varying Emdogain concentrations, additional wells were coated with 0-50 μg/ml of the ECM proteins. A standard curve (0-50 μg/ml) was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was determined by regression analysis. The relative concentration of ECM protein bounded to Emdogain in each condition, was calculated using this regression equation.

3. EMDOGAIN®-CELL INTERACTIONS

3.1. Cell lines and cell cultures

3.1.1. Epithelial cell line

Epidermal keratinocyte cell line HaCaT (Dr. Hubert Fusenig, Germany Cancer Center, Heidelberg, Germany) are spontaneously transformed keratinocytes and represent normal epidermal keratinocytes in most of its properties. They are not invasive and are able to differentiate under proper conditions (Boukamp et al., 1988). HaCaT cells were routinely maintained in 25 cm^2 tissue culture flasks (Falcon; Becton Dickinson Labware, NJ) in the presence of Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Irvine, UK) supplemented with 23 mM sodium bicarbonate, 20 mM Hepes (Gibco Biocult, Paisley, UK), antibiotics (50 μg/ml streptomycin sulfate, 100 U/ml penicillin) and 10% heat-inactivate fetal bovine serum (FBS). Cells were incubated at

37°C under 5% CO_2. The medium was changed every 2-3 days. Cells were allowed to grow until confluence, where they were detached with a brief incubation with 0.25% trypsin (in EDTA, in Hanks Solution) at 37°C. Trypsin activity was inhibited with 10% FBS in DMEM and the cells were recovered by centrifugation (1000 RMP for 3 minutes) and seeded in 75 cm^2 tissue culture flasks (Falcon; Becton Dickinson Labware, NJ) at a cell population density of 10^6 cells/flask.

3.1.2. Fibroblast cell line:

Primary cultures of human periodontal ligament fibroblasts (PDLF) were obtained from healthy periodontal tissues in individuals (aged 20-30 years), undergoing extractions for non-periodontic reasons. Tissues were collected from the middle 1/3 of the roots using a scalpel. The explants were cultured in 24-well multi-chamber slide (Labtek, Naperville, IL) containing growth media consisting of 85% (v/v) DMEM, 5% antibiotics (v/v) and 10% FBS. Cultures were left undisturbed for 10 days at 37°C in a humidified atmosphere of 5% CO_2- 95% air. The cultures were expanded as described for HaCaT cells. Cells from passages 4-12 were used for all the experiments.

3.2. Cell adhesion and spreading assays

Adhesion and spreading of epithelial and fibroblast cells to the provisional matrix are essential for wound healing. To explore the role of Emdogain in periodontal wound healing and regeneration, adhesion and spreading of PDLFs and HaCaT cells to Emdogain or Emdogain + ECM proteins were evaluated by plating the cells on 96-well plates, precoated with 3 different substrates: (a) Emdogain (1-30,000 μg/ml); (b)

Emdogain (0.1-10 μg/ml) + Collagen type I (10 μg/ml); or (c) Emdogain (10-1000 μg/ml) + Fibronectin (10 μg/ml). Coating of the surfaces were performed similar to that described for ELISA experiments. Briefly, the surfaces were coated with Emdogain (0-30,000 μg/ml) at 4°C over night, blocked with 1% heat-denatured bovine serum albumin (BSA) in PBS for 30 minutes and then incubated with an ECM protein (10 μg/ml) at room temperature for 3 hours. Surfaces coated with BSA1% were used to measure the non-specific binding. In group (a) that the surfaces were coated with Emdogain, Collagen type I (50 μg/ml) was used as positive control. In group (b) Emdogain + Collagen type I, surfaces coated with equivalent concentrations of collagen type I served as positive controls; and finally in group (c) Emdogain + Fibronectin, surfaces coated with equivalent concentrations of fibronectin were used as positive controls. The equivalent concentrations were determined with performing an ELISA for each protein as described earlier.

Cells in T-75 flasks were trysinized (0.25% trypsin) and trypsin activity was blocked with 0.25% trypsin inhibitor. The cells were recovered by centrifugation and re-suspended in DMEM ± 10% FBS. In serum free condition, 50 μM cycloheximide was added to the media to prevent *de novo* protein synthesis and cells were then seeded onto 96-well plates precoated with ECM proteins (as described earlier) at a cell density of 1×10^4 and 3×10^4 cells/well for PDLF and HaCaT cells, respectively. Four replicate cultures were performed for each condition. The experiment was stopped at 2 hours in serum-free media, and at 2 or 4 hours in serum-supplemented media. For the adhesion assay, at the designated time points, the unattached cells were removed by washing the wells 3 times with PBS followed by permeabilization with 0.1% Triton and thorough

rinsing with PBS. The cells were then fixed in PBS (+ calcium and magnesium) containing formaldehyde (4%) and sucrose (5%) and stained with 0.1% crystal violet. A phase-contrast microscope was used to count the cells. Three representative fields from four replicate wells were studied.

For cell spreading assays, at specific time points the cells were fixed with addition of 2.5% glutaraldehyde directly to the culture medium. The cells were stained with 0.1% crystal violet and observed with a phase contract microscope. Cells that were surrounded by a lamellar cytoplasm were considered to be spread. Three representative fields from four replicate wells were studied. The morphology of the cells grown on Emdogain, were photographed with a Zeiss camera attached to a Zeiss Microscope, using a 25× objective.

3.3. Integrin-blocking assay

A dynamic reciprocity between cells and their surrounding extracellular matrix requires cell adhesion and spreading that are mainly mediated through cell surface receptors known as integrins. Therefore, an integrin-blocking assay was performed to investigate if adhesion and spreading of PDLFs on Emdogain-coated surfaces are integrin-dependant. αv and β1 integrin subunits were chosen to be tested due to their fundamental role in adhesion of fibroblast to the provisional matrix. Blocking monoclonal antibodies specific to block αv (L230; Houghton et al., 1982) and/or β1 (mAb13; Akiyama et al., 1989) integrin subunits were used. A 96-well plate was coated with Emdogain (10,000 μg/ml). Fibronectin (10 μg/ml)-coated surfaces served as the positive controls. PDLFs (10,000 per well) were seeded onto the wells in DMEM

containing 50 μM cycloheximide. Prior to seeding, the cells were incubated on ice with the anti-integrin antibodies (20 μg/ml) for 15 minutes. Cells were allowed to attach to the Emdogain or fibronectin pre-coated surfaces at 37°C until about 50% of control cells with no antibody appeared to be spread. The cells were then fixed by adding formaldehyde 4% or glutaraldehyde 2.5% and stained with 0.1% crystal violet. Cell adhesion and spreading was evaluated with a phase-contrast microscope as described earlier.

3.4. Cell migration assay

Cell migration was examined using 24-well plates (Costar; Corning Inc, NY) coated with 1-30,000 μg/ml Emdogain. Wells coated with BSA 1% and collagen type I 50 μg/ml were used as negative and positive controls, respectively. HaCaT cells (30,000 per well) or PDLFs (10,000 per well) were seeded in DMEM + 10% FBS into custom-made stainless steel cylinders with an opening of 2.8 mm in diameter in the center. The cells were let to attach for 8 hours before cylinder removal and wells were rinsed with PBS to remove the unattached cells. Cells were allowed to migrate outwards from the cell colony in the presence of DMEM + 10% FBS for 72 hours. After fixation in PBS containing formaldehyde (4%) and sucrose (5%), cell colonies were stained with 0.1% crystal violet. Twenty-fold magnified image was used to measure the average diameter of the cell colony. The increase in the diameter of a cell colony was calculated by subtracting the diameter of BSA1% cell colony at 3 days.

In another set of experiments, cell migration was investigated in the presence of porcine TGF-β1 (R and D systems; Minneapolis, Minn). 24-well plates were coated with Emdogain 10,000 μg/ml; BSA 1% (negative control); or Collagen type I 50 μg/ml (positive control). PDLFs and HaCaT cells were seeded into the cylinders as above. After removing the cylinders, cells were allowed to migrate in DMEM + 1% in the presence or absence of 10 ng/ml TGF-β1 for 72 hours. The colonies were then fixed and evaluated for cell migration. Twenty-fold magnified image was used to randomly measure the diameter of the cell colony 5×, then averaging the value for each colony. The increase in the diameter of a cell colony was calculated by subtracting the diameter of the original cell colony. The concentration of TGF-β1 used for HaCaT cells was based on the series of experiments conducted in our lab showing the highest level of HaCaT cell migration with 10 ng/ml TGF-β1 (unpublished data). For PDLF migration, a preliminary experiment was conducted to determine the concentration of TGF-β1 that induces optimal level of PDLF migration. PDLFs seeded on collagen type I were exposed to different concentrations of TGF-β1 ranging from 10 to 60,000 pg/ml. Analysis of the results did not show any significant difference in cell migration rates between various concentrations of TGF-β1. Therefore it was decided to use 10 ng/ml TGF-β1 in order to be consistent with epithelial cell experiment. After the migration analysis was completed the role of TGF-β1 on proliferation activity of HaCaT cells seeded on Emdogain (10,000 μg/ml) was evaluated by dissolving the crystal violet stained cells with 10% acetic acid and measuring the color density with a microtiter plate reader set to 570 nm absorbance.

4. STATISTICAL ANALYSIS

For quantitative data, means and standard errors of means were calculated. Comparisons between paired samples were performed using Student's t-test (paired two sample for means). Comparisons between multiple samples were performed using analysis of variance (ANOVA), followed by Post Hoc test (Bonferroni).

CHAPTER FOUR - RESULTS

1. EMDOGAIN® SURFACE COATING (SEM ANALYSIS)

The major proteins of Emdogain, known as amelogenins, are highly hydrophobic and form large molecular aggregates and that become insoluble at physiological pH and temperature (Fischer and Termine, 1985; Aoba et al., 1987). The solubility increases at acid or alkaline pH and low temperature. In this study, Emdogain (1-30,000 μg/ml) was dissolved under acidic conditions (Gestrelius et al., 1997) and the surfaces were coated with this acidic solution at 4°C. Later, the surfaces were neutralized with PBS in room temperature, resulting in precipitation of Emdogain. SEM analysis of the coated surfaces showed that precipitation started to form at the concentration of 1000 μg/ml Emdogain and increased with higher concentrations (Fig. 2). There was no Emdogain precipitation at concentrations of <1000 μg/ml (data not shown). On surfaces coated with 30,000 μg/ml Emdogain, the precipitated aggregates were observable with a phase-contrast microscope.

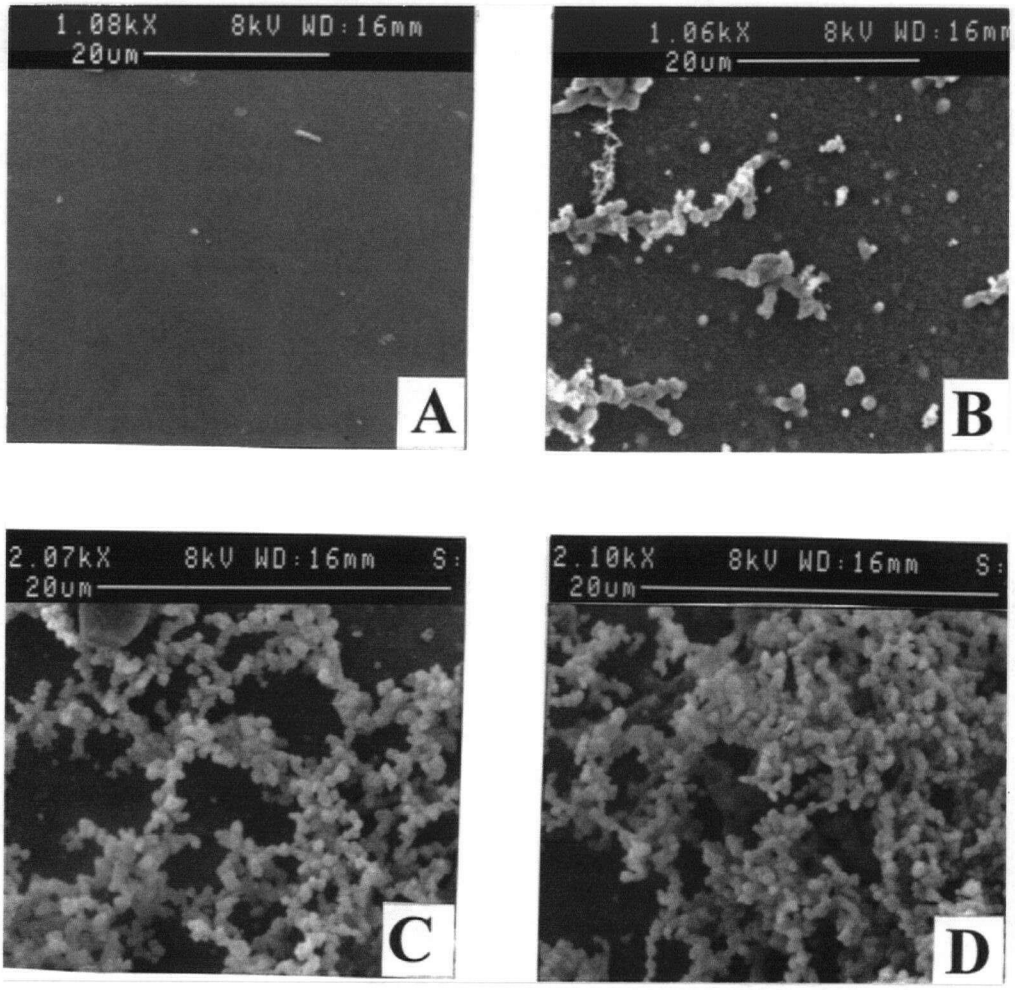

Figure 2. Precipitation of Emdogain on tissue culture plates. Tissue culture surfaces were coated with an acidic solution of Emdogain (1-30,000 μg/ml), neutralized with PBS and processed for scanning electron microscopy. (A) Control (0 EMD); (B) 1000 μg/ml EMD; (C) 10,000 μg/ml EMD; and (D) 30,000 μg/ml EMD.

2. EXTRACELLULAR MATRIX PROTEIN BINDING TO EMDOGAIN®

2.1. Emdogain-Fibronectin interactions

Binding of fibronectin (10 μg/ml) to Emdogain-coated surfaces was dependant on the Emdogain coating concentration. Fibronectin binding was significantly increased ($p<0.005$) with Emdogain concentrations of 0.1 to 1000 μg/ml. Fibronectin binding to Emdogain increased from the baseline (0.21 μg/ml) to a maximum (1.7 μg/ml) at the Emdogain concentration of 100 μg/ml. The binding then decreased to 0.4 μg/ml at the concentration of 10,000 μg/ml Emdogain (Fig. 3). When the concentration of fibronectin was increased from 5 to 100 μg/ml, the amount of fibronectin that bound to two Emdogain concentrations increased as well. More fibronectin bound to the lower concentration of Emdogain (10 μg/ml) compared to the higher concentration (10,000 μg/ml). At the highest fibronectin concentration that was tested (100 μg/ml), 22 μg/ml of fibronectin bound to 10 μg/ml Emdogain and 11 μg/ml bound to 10,000 μg/ml Emdogain (Fig. 4).

2.2. Emdogain-Collagen type I interactions

Binding of type I collagen (10 μg/ml) to Emdogain (0.001-10,000) exhibited a similar binding pattern as was found for fibronectin. Binding of type I collagen was significantly increased ($p<0.05$) with Emdogain concentration of 0.1-1 μg/ml. However,

in contrast to fibronectin the maximum binding (0.7 μg/ml) was reached at 1 μg/ml Emdogain coating (Fig. 5).

2.3. Emdogain-Collagen IV or Laminin I interactions

Type IV collagen and laminin I did not bind to Emdogain-coated surfaces. All values were below the baseline level for all the concentrations of Emdogain, indicating no interactions between these proteins (Fig. 6; Fig. 7).

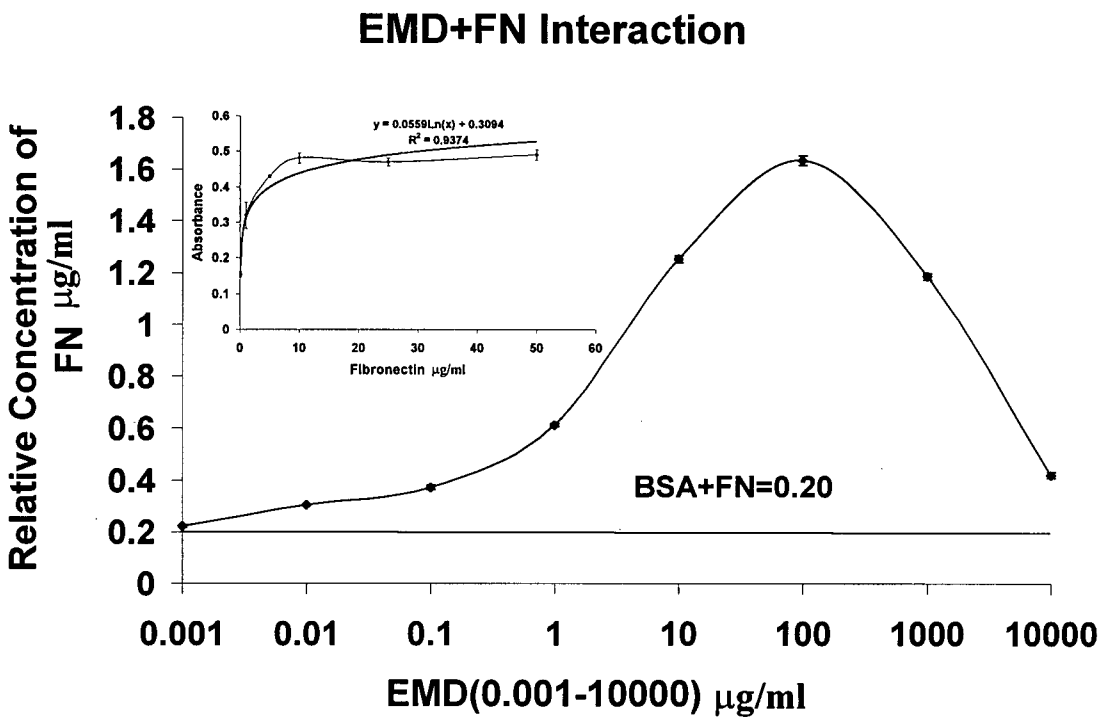

Figure 3. Emdogain-Fibronectin interaction. Emdogain-coated surface (1-10,000 µg/ml) were blocked with BSA 1% and then incubated with fibronectin (10 µg/ml) for 3 hours at room temperature. To determine the amount of fibronectin bounded to each concentration of Emdogain, ELISA was used. Fibronectin-coated surfaces (0-50 µg/ml) were used to construct the standard curve (Top left graph). The relative concentration of fibronection (µg/ml) in each condition was calculated using the regression equation. (n=4 for each condition; BSA 1% + Fibronectin 10 µg/ml= baseline). EMD 0.1 to 1000 µg/ml are significantly different from baseline, $p<0.05$

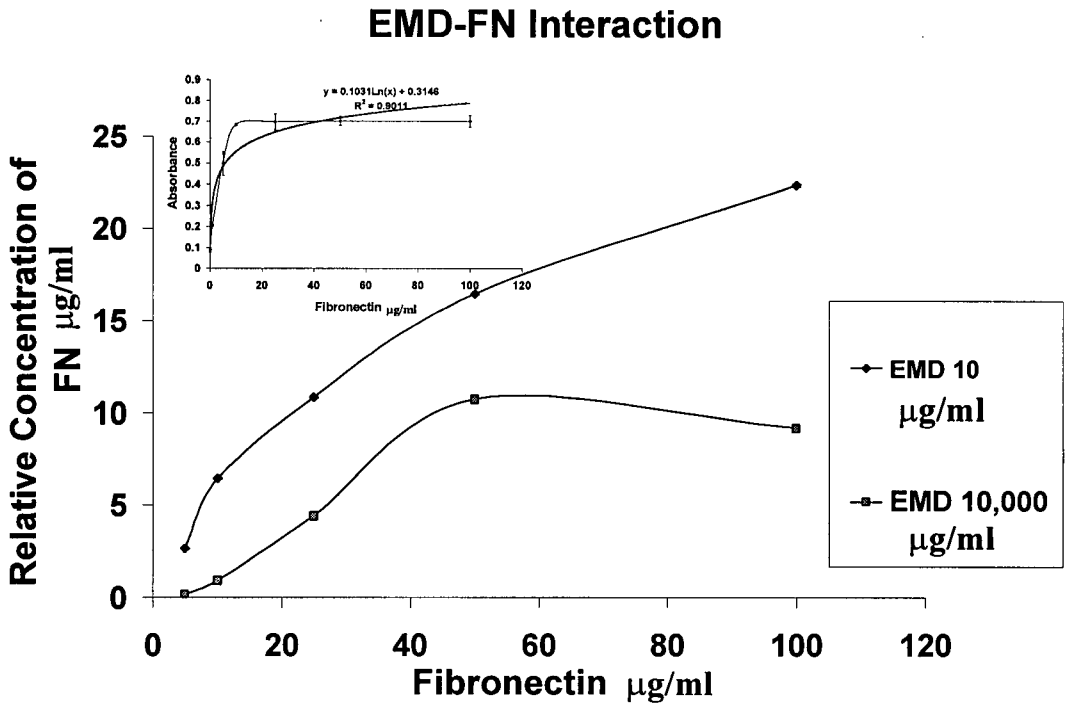

Figure 4. The effect of increasing concentrations of fibronection on Emdogain-Fibronectin interaction. Emdogain-coated surface (10 and 10,000 µg/ml) were blocked with BSA 1% and incubated with fibronectin (5-100 µg/ml) for 3 hours at room temperature. ELISA was preformed to determine the amount of fibronectin bound to each Emdogain concentration. The top left graph represents the standard curve for fibronection (0-100 µg/ml). Relative concentrations of fibronection bound to Emdogain expressed in µg/ml. (n=4 for each condition; BSA 1% + Fibronectin 10 µg/ml= baseline). All points for EMD 10µg/ml and EMD 10000µg/ml are significantly different, $p<0.05$

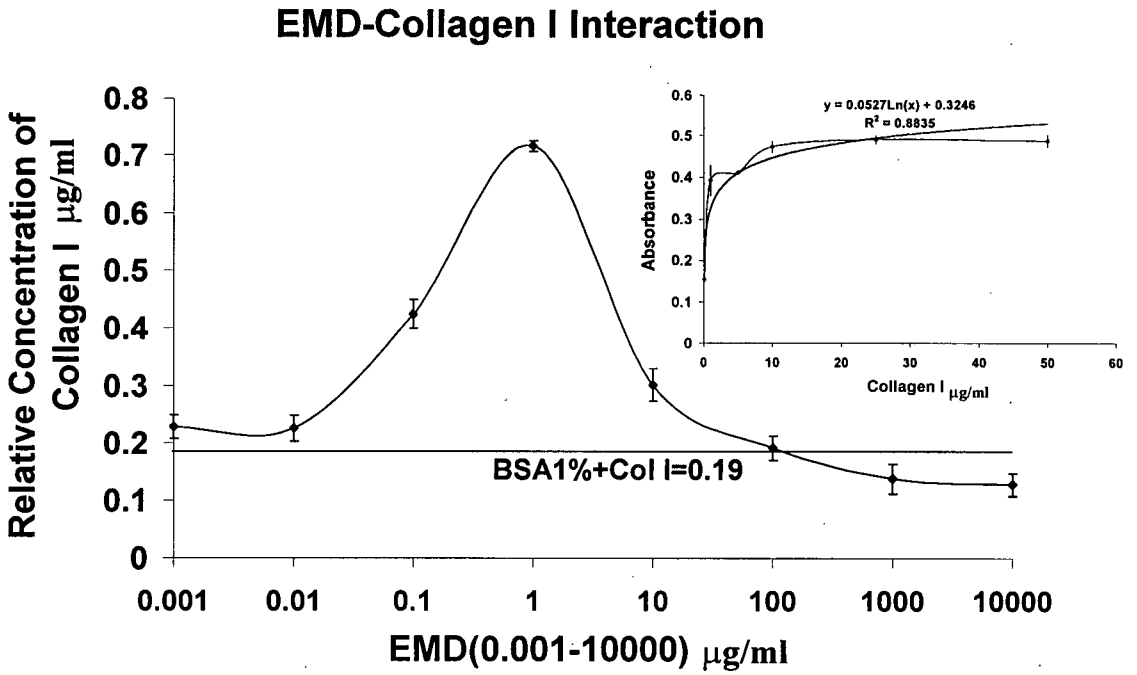

Figure 5. Emdogain-Collagen type I interaction. Emdogain-coated surface (10 and 10,000 µg/ml) were blocked with BSA 1% and incubated with collagen type I (10 µg/ml). ELISA was preformed to determine the amount of collagen type I bound to each Emdogain concentration. The top left graph represents the standard curve for collagen type I (0-50 µg/ml). Relative concentrations of collagen type I bounded to Emdogain expressed in µg/ml. (n=4 for each condition; BSA 1% + Collagen type I 10 µg/ml= baseline). EMD 0.1 to 1µg/ml are significantly different from baseline, $p<0.05$

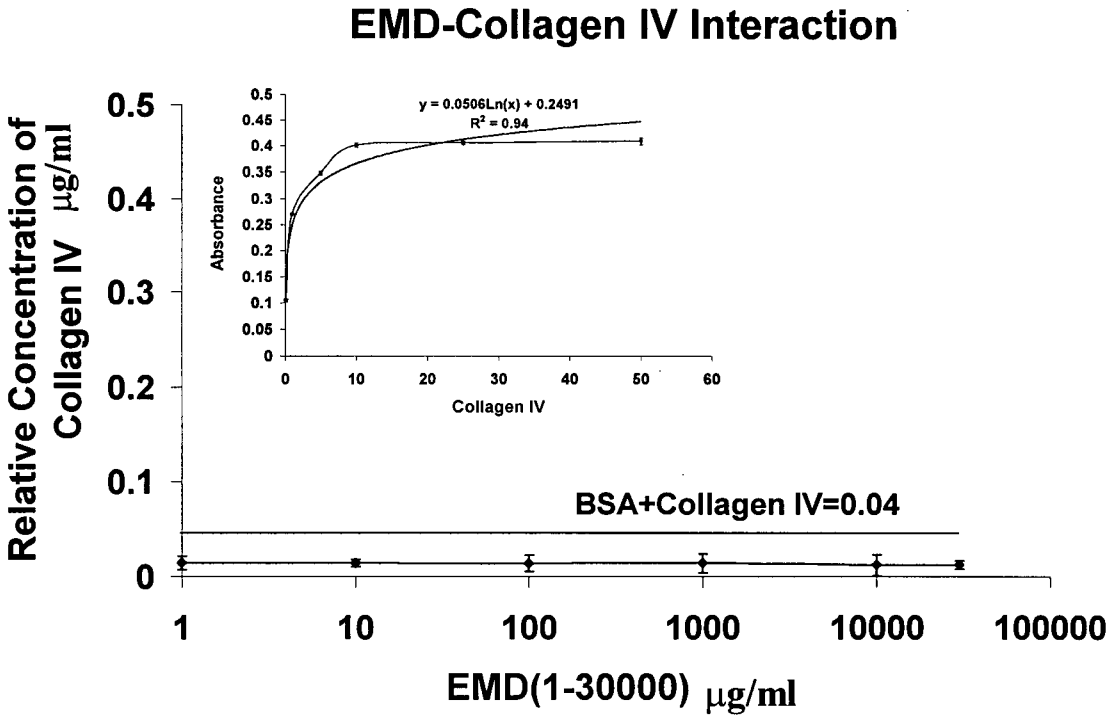

Figure 6. Emdogain-Collagen type IV interaction. Emdogain-coated surface (10 and 30,000 µg/ml) were blocked with BSA 1% and incubated with collagen type IV (10 µg/ml). ELISA was preformed to determine the amount of collagen type IV bound to each Emdogain concentration. The top graph represents the standard curve for collagen type IV (0-50 µg/ml). Relative concentrations of collagen type IV bounded to Emdogain expressed in µg/ml. (n=4 for each condition; BSA 1% + Collagen type IV 10 µg/ml= baseline)

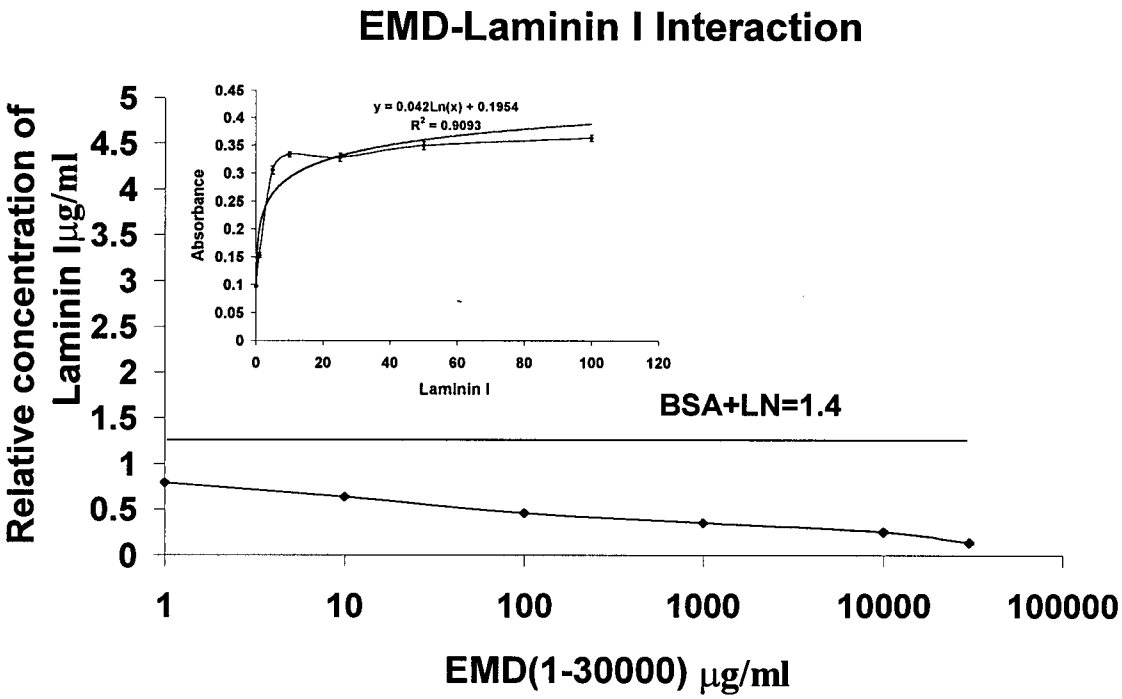

Figure 7. Emdogain-Laminin interaction. Emdogain-coated surface (10 and 30,000 µg/ml) were blocked with BSA 1% and incubated with laminin (10 µg/ml). ELISA was preformed to determine the amount of laminin I bound to each Emdogain concentration. The top graph represents the standard curve for laminin (0-50 µg/ml). Relative concentrations of laminin bounded to Emdogain expressed in µg/ml. (n=4 for each condition; BSA 1% + Laminin I 10 µg/ml= baseline)

3. EMDOGAIN®-CELL INTERACTION

These investigations examined if Emdogain influenced epithelial and periodontal ligament fibroblast cells adhesion, spreading and migration.

3.1. Cell adhesion and spreading

3.1.1. Epithelial cell – Emdogain coated surface interaction:

HaCaT cells plated on Emdogain-coated surfaces (1-30,000 μg/ml) for 2 hours in the presence of cycloheximide to inhibit *de novo* protein synthesis adhered poorly (Fig. 8). Specifically, adhesion was 5% of what was found for adhesion to type I collagen-coated surfaces (positive controls). When the cells were allowed to produce their own matrix or adhesive molecules (i.e. no cycloheximide treatment), the adhesion to Emdogain increased with time. At 2 hours, the adhesion level was 25% of what was found for type I collagen-coated surfaces. While after 4 hours, cell adhesion to Emdogain was significantly improved and reached to a level that was 75% of the positive control. No significant difference in cell-binding activity was found to different Emdogain concentrations (Fig. 8). HaCaT cell spreading on Emdogain (1-30,000 μg/ml) was examined at 2 hours in the presence of cycloheximide. Under these assay conditions Emdogain did not promote cell spreading (5% compared to 98% spreading on the positive control) (Table 2).

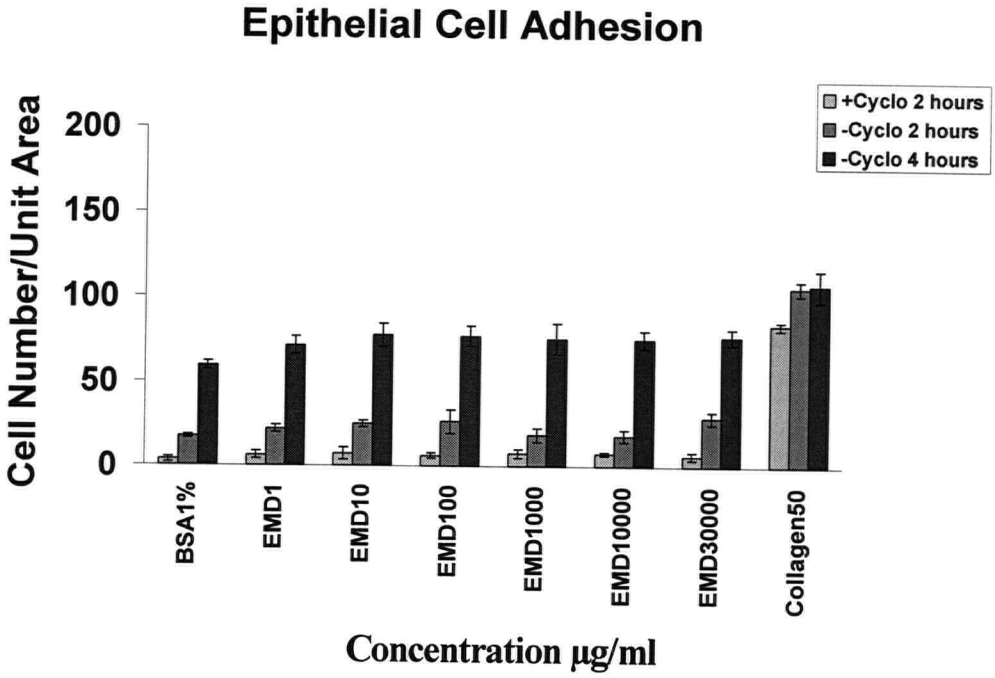

Figure 8. Epithelial cell adhesion on Emdogain. HaCaT cells on Emdogain-coated plastic (1-30,000 µg/ml) were incubated in –Serum (DMEM + 50 µM cycloheximide), or +Serum (DMEM + 10% FBS) conditions. Experiment was stopped at 2 hours in –Serum condition and at 2 or 4 hours in +Serum conditions. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Number of the cells in each condition was counted in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.). EMD (1-30000) significantly different from Collagen50, $p<0.05$

Epithelial Cell Spreading	Concentration, µg/ml		
	BSA1%	EMD 1-30,000	Collagen 50
+Cycloheximide	0%	0%	98%±0.03

Spread Cells/Unit Area

Table 2. Epithelial cell spreading on Emdogain. HaCaT cell on Emdogain-coated plastic (1-30,000 µg/ml) were incubated in the presence of DMEM + 50 µM cycloheximide for 2 hours. Cells were then fixed with 2.5% glutaradehyde followed by staining with 1% crystal violet. The percentage of spread cells of total cell number was calculated in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).

3.1.2. Periodontal ligament fibroblast – Emdogain coated surface interaction:

PDLF adhesion to Emdogain (1-30,000 μg/ml) was concentration dependant with adhesion to Emdogain (10-30,000 μg/m) being significant ($p<0.05$) compared to the BSA 1% (negative control). The maximum adhesion was observed with 10,000 μg/ml Emdogain, both in the presence and absence of cycloheximide. At this maximum level, the adhesion was 40% of the positive control in the presence of cycloheximide after 2 hours. In the absence of cycloheximide, at 10,000 μg/ml Emdogain the adhesion was 25% of the control after 45 minutes and increased to 60% of control after 2 hours (Fig. 9). Spreading of PDLFs on Emdogain-coated surfaces revealed the same pattern as adhesion with the maximum spreading on 10,000 μg/ml Emdogain (± cycloheximide). At this maximum level, cell spreading was 10% in the presence of cycloheximide at 2 hours (100% on positive control). In the absence of cycloheximide, cell spreading was 0% (60% on positive control) after 45 minutes and increased to 21% after 2 hours at its maximum level (100% on control) (Table 3). Evaluation of the spread cells with a Zeiss microscope (25× objective) showed that the cells cultured on Emdogain exhibited a different morphology compared to those plated on type I collagen (positive control). Cells cultured on Emdogain developed several processes, which projected outward from the cell body and sometimes tended to cluster. Cells cultured on collagen formed a monolayer, exhibited well spread cytoplasm surrounding the nucleus and did not cluster (Fig. 10).

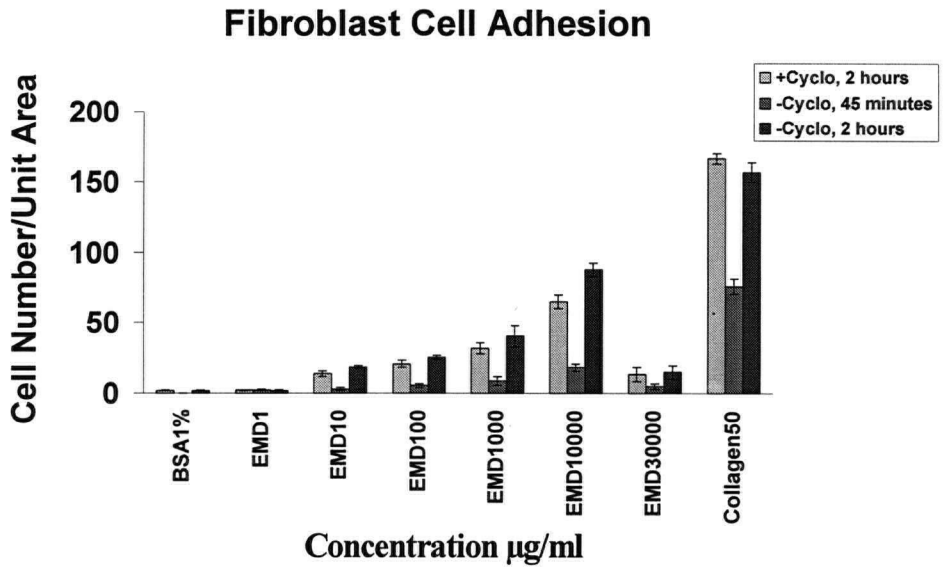

Figure 9. Fibroblast cell adhesion on Emdogain. PDLFs on Emdogain-coated plastic (1-30,000 µg/ml) were incubated in –Serum (DMEM + 50 µM cycloheximide), or +Serum (DMEM + 10% FBS) conditions. Experiment was stopped at 2 hours in –Serum condition and at 45 minutes or 2 hours in +Serum conditions. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Number of the cells in each condition was counted in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).
EMD (1-30000) significantly different from Collagen50, $p<0.05$
EMD (1-30000) significanty different from BSA1%, $p<0.05$
EMD 10000 (±Cyclo, 2 hours) significantly different from other concentrations of EMD, $p<0.05$

	Concentration, µg/ml							
Fibroblast Cell Spreading	BSA1 %	EMDI	EMD10	EMD 100	EMD 1,000	EMD 10,000	EMD 30,000	Collagen 50
+Cycloheximide	0%	0%	0%	0% ±0.01	4.6% ±0.03	9.6% ±0.01	2.0% ±0.02	100.0% ±0.02
-Cycloheximide	0%	0%	0%	4.0% ±0.03	9.0% ±0.01	21.0% ±0.02	3.0% ±0.03	100.0% ±0.02

Spread Cells/Unit Area

Table 3. Fibroblast cell spreading on Emdogain. PDLFs on Emdogain-coated plastic (1-30,000 µg/ml) were incubated in –Serum (DMEM + 50 µM cycloheximide), or +Serum (DMEM + 10% FBS) conditions for 2 hours. Cells were then fixed with 2.5% glutaradehyde followed by staining with 1% crystal violet. The percentage of spread cells of total cell number was calculated in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).
EMD(1-30000) significantly different from collagen $p<0.05$
EMD(100-10000) significantly different between + and – cycloheximide groups, $p<0.05$
EMD 10000 (±Cyclo, 2 hours) significantly different from other concentrations of EMD, $p<0.05$

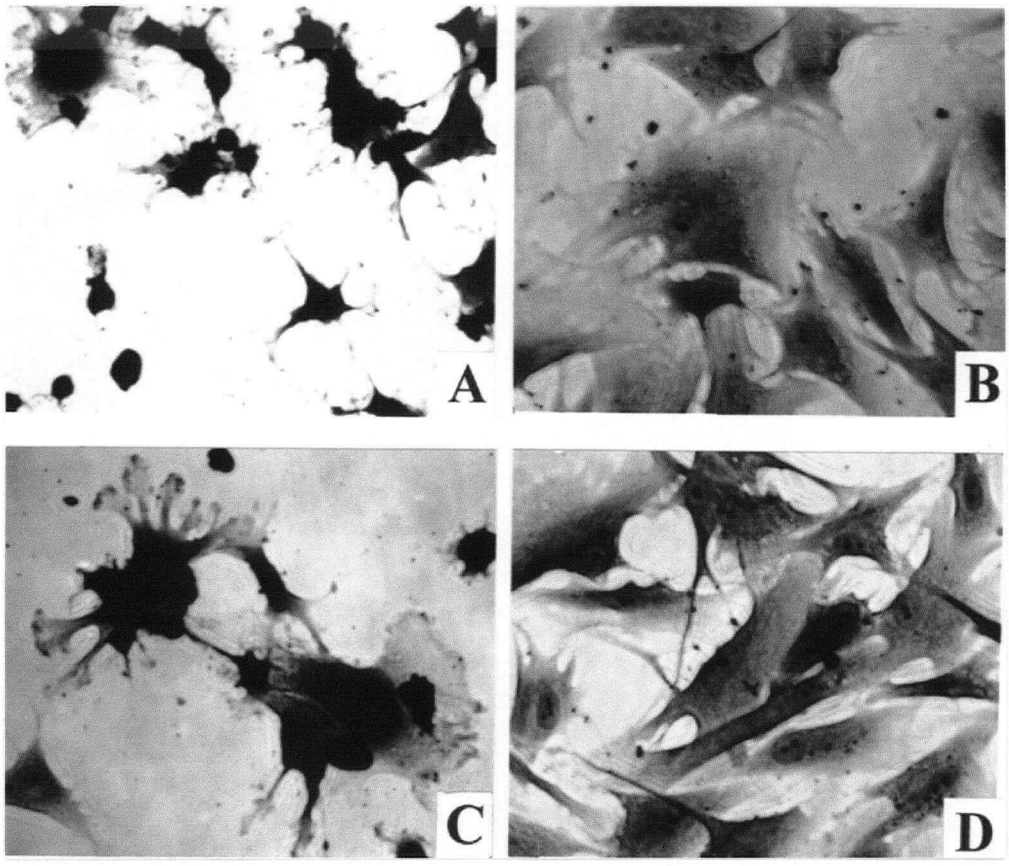

Figure 10. Fibroblast cell morphology. PDLFs on Emdogain-coated plastic (10,000 μg/ml) were incubated in –Serum (DMEM + 50 μM cycloheximide) (A); or +Serum (DMEM + 10% FBS) (C) conditions for 2 hours. Cells were then fixed with 2.5% glutaradehyde followed by staining with 1% crystal violet. Cells grown on collagen type I-coated plastic in –Serum (B) and +Serum (D) conditions were used as controls. Photomicrographs were obtained with a Zeiss microscope (×250).

3.1.3. Integrin blocking assay

Since PDLFs were shown to adhere and spread on Emdogain in the presence of cylcoheximide, we examined if integrins mediated these procedures. Integrins are cell surface glycoproteins that mediate cell-extracellular matrix and cell-cell adhesion (Hynes, 1987) and therefore, play a major role in cell adhesion and spreading. An integrin-blocking assay was performed to investigate if adhesion and spreading of PDLFs on Emdogain-coated surfaces are integrin-dependant. β1 and αv containing integrins play important role in fibroblast binding to the provisional matrix, containing collagen type I and fibronectin (Gailit et al., 1996; Welch et al., 1990; Xu and Clark, 1996; Hakkinen et al., 2000). Therefore, the role of αv and β1 integrin subunits associated with binding of PDLFs that have been seeded on Emdogain was examined. For this purpose, cells were preincubated with monoclonal antibodies L230- 20 μg/ml (that blocks the αv subunit; Houghton et al., 1982) and/or mAb13 - 20 μg/ml (that blocks β1 subunit; Akiyama et al., 1989) and plated on Emdogain-coated surfaces (10,000 μg/ml). This concentration of Emdogain was chosen because maximum adhesion and spreading was found previously (Fig. 9). Since fibronectin is a fundamental component of the provisional matrix, adhesion to fibronectin-coated wells was used as a control. In the fibronectin control group when compared to control with no antibody, preincubation with anti-αv antibody (L230) alone reduced the level of adhesion from 130 cells/unit area to 60 cells/unit area. Spreading was reduced from 100% (no antibody) to 77%. Compared to control with no antibody, pretreatment with anti-β1 antibody (mAb13) alone reduced the adhesion from 130 cells/unit area to 70 cells/unit area. Spreading was reduced from 100% (no antibody) to 31%. Compared to control with no antibody, preincubation with both antibodies

reduced the adhesion from 130 cells/unit area to 10 cells/unit area. Spreading was reduced from 100% (no antibody) to 5% (Fig. 11; Table 4). In the Emdogain group, the reduction in cell adhesion and spreading with antibodies showed the same pattern as in fibronectin group. Compared to control with no antibody (60 cells/unit area), cell adhesion was reduced to 20 and 15 cells/unit area with anti-αv and -β1 antibodies, respectively. Treatment with both antibodies further decreased cell adhesion to 2 cells/unit area. Cell spreading on Emdogain was reduced from 10% (no-antibody) to 0% with anti-αv and/or anti-β1 antibodies (Fig. 11; Table 4).

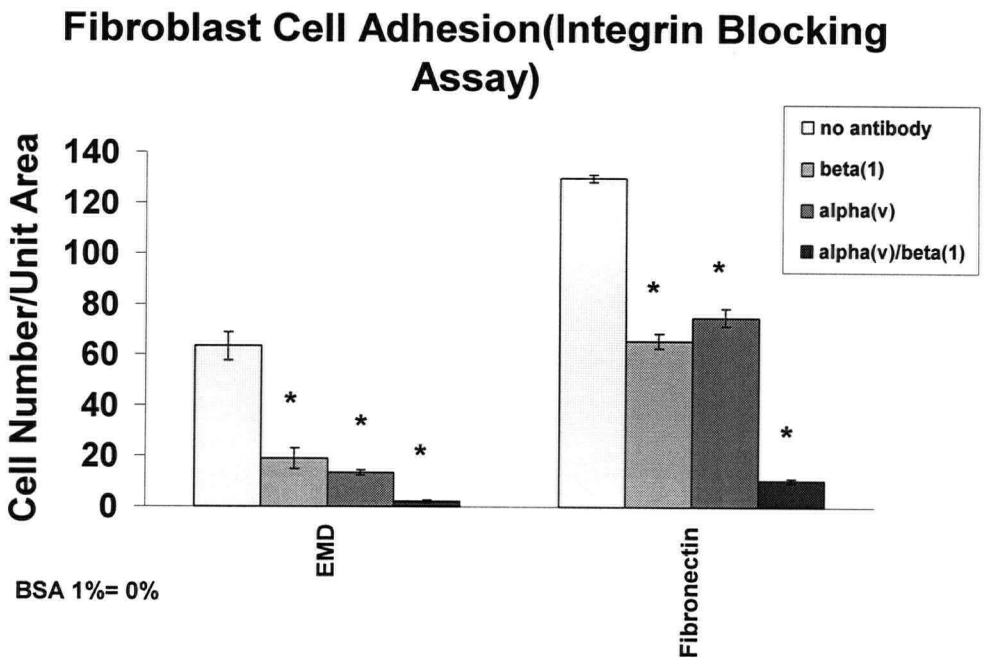

Figure 11. Effect of integrin blocking on fibroblast cell adhesion. PDLFs were preincubated with either no antibody; only anti-αv-integrin antibody; only anti-β-integrin antibody; or both antibodies for 30 minutes and cultured on Emdogain (10,000 μg/ml) or fibronectin (10 μg/ml) in the presence of serum-free DMEM + 50 μM cycloheximide for 2 hours. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Number of the cells in each condition was counted in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).
* = $p<0.05$ Significantly different from no-blocking antibody(control)

Fibroblast Cell Spreading (Integrin Blocking Assay)	No Antibody	Beta (1)	Alpha (v)	Alpha(v) Beta(1)
EMD10,000	10%±0.01	0%	0%	0%
Fibronectin10	100%±0.02	31%±0.02	77%±0.01	5%±0.01

Spread Cells/Unit Area

Table 4. Effect of integrin blocking on fibroblast cell spreading. PDLFs were preincubated with either no antibody; only anti-αv-integrin antibody; only anti-β-integrin antibody; or both antibodies for 30 minutes and grown on Emdogain (10,000 μg/ml) or fibronectin (10 μg/ml) in the presence of DMEM + 50 μM cycloheximide for 2 hours. Cells were fixed with 2.5% glutaradehyde followed by staining with 1% crystal violet. The percentage of spread cells of total cell number was calculated in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).
Antibody-treated groups significantly different from no-antibody groups, $p<0.05$

3.1.4. Emdogain + Fibronectin- or Collagen type I-coated surfaces

Cell adhesion and spreading to ECM proteins that were bound to Emdogain on tissue culture plastic was examined. For this purpose surfaces coated with varying concentrations of Emdogain were incubated with fibronectin (10 μg/ml) or collagen type I (10 μg/ml) before plating the cells. The concentrations of Emdogain used with each protein were chosen based on the previous ELISAs, to achieve the highest interaction between Emdogain/ECM proteins. The equivalent concentrations of the proteins bound to Emdogain was calculated according to the standard curves constructed for each protein using ELISA. These equivalent concentrations were then used to coat tissue culture plastic wells (positive control).

Epithelial cells- HaCaT cells cultured on Emdogain (10-1000 μg/ml) + fibronectin (10 μg/ml), in the presence of cycloheximide, showed a reduction of 60-79% in cell adhesion compared to type I collagen (positive controls). However, compared to surfaces treated with Emdogain alone, cell-binding activity was 2.9-3.4 fold higher. Among the 3 different concentrations of Emdogain that were used for coating, the highest level of adhesion was found with 1000 μg/ml Emdogain + fibronectin (Fig. 12). No cell spreading was observed on Emdogain + fibronectin-coated surfaces compared to 50% on positive controls (Table 5). HaCaT cells cultured on Emdogain (0.1-10 μg/ml) + collagen type I (10 μg/ml) showed a reduction of 60-76% in cell adhesion compared to positive controls. Cell-binding level was 4.7-5.4 fold higher compared to Emdogain-coated surfaces (Fig. 13). Cell-spreading for Emdogain + Collagen type I group was 10-18% compared to 0% for Emdogain group and 70-75% for collagen type I group (positive control) (Table 6).

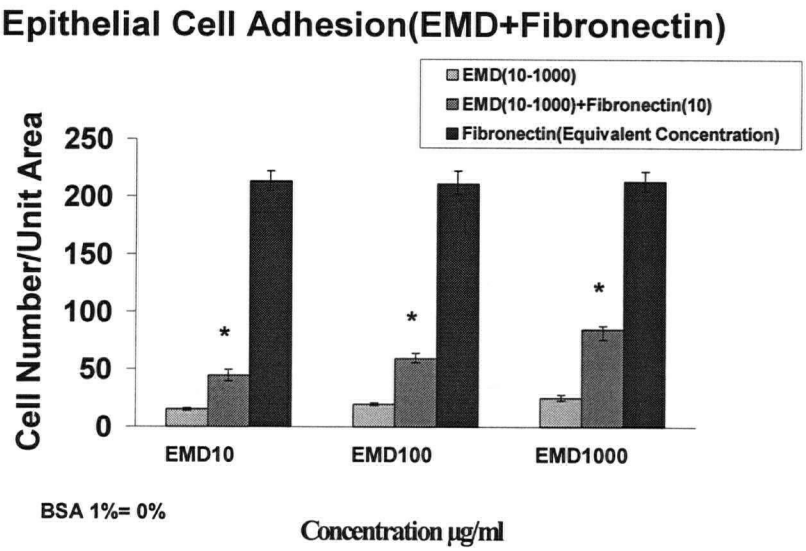

Figure 12. Epithelial cell adhesion on Emdogain + Fibronectin. HaCaT cells were plated on either Emdogain (10-1000 µg/ml); Emdogain (10-1000 µg/ml) + Fibronectin (10 µg/ml); or Fibronectin (equivalent concentration according to ELISA), and incubated in the presence of DMEM + 50 µM cycloheximide for 2 hours. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Number of the cells in each condition was counted in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).

* = $p<0.05$ Significantly different from Collagen50 µg/ml (positive control)

	Concentration, µg/ml			
Epithelial Cell Spreading	BSA1%	EMD 10-1,000	EMD(10-1,000)+ Fibronectin10	Fibronectin (Equivalent Concentration)
+Cycloheximide	0%	0%	0%	50%±0.02

Spread Cells/Unit Area

Table 5. Epithelial cell spreading on Emdogain + Fibronectin. HaCaT cells were plated on either Emdogain (10-1000 µg/ml); Emdogain (10-1000 µg/ml) + Fibronectin (10 µg/ml); or Fibronectin (equivalent concentration according to ELISA), and incubated in the presence of DMEM + 50 µM cycloheximide for 2 hours. Cells were fixed with 2.5% glutaradehyde followed by staining with 1% crystal violet. The percentage of spread cells of total cell number was calculated in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).

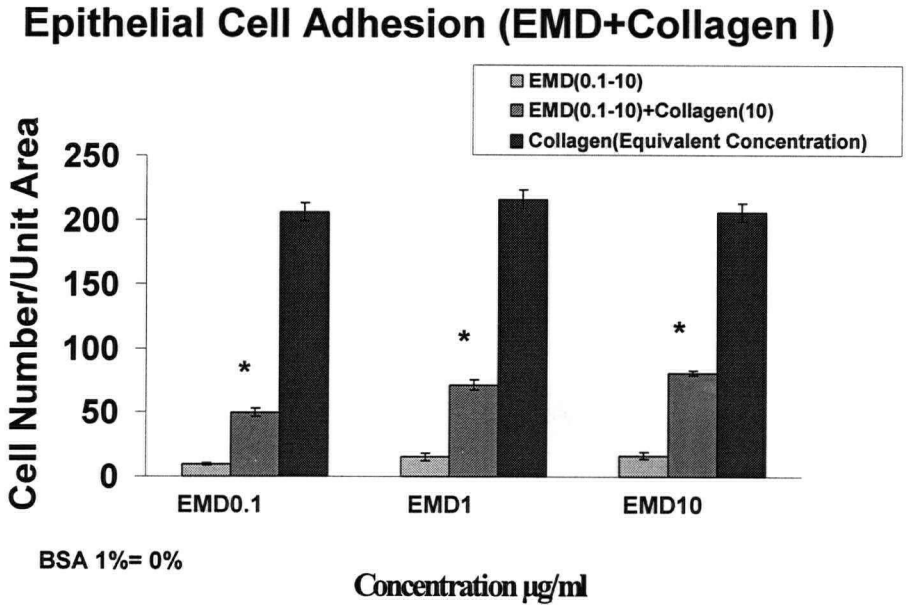

Figure 13. Epithelial cell adhesion on Emdogain + Collagen type I. HaCaT cells were plated on either Emdogain (10-1000 µg/ml); Emdogain (10-1000 µg/ml) + Collagen type I (10 µg/ml); or Collagen type I (equivalent concentration according to ELISA), and incubated in the presence of DMEM + 50 µM cycloheximide for 2 hours. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Number of the cells in each condition was counted in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).
* = $p<0.05$ Significantly different from Collagen50 µg/ml (positive control)

	Concentration, µg/ml					
Epithelial Cell Spreading	BSA1%	EMD 0.1-10	EMD0.1+ Collagen10	EMD1+ Collagen10	EMD10+ Collagen10	Collagen (Equivalent Concentration)
+Cycloheximide	0%	0%	14%±0.01	10%±0.03	18%±0.04	70-75%±0.03

Spread Cells/Unit Area

Table 6. Epithelial cell spreading on Emdogain +Collagen type I. HaCaT cells were plated on either Emdogain (10-1000 µg/ml); Emdogain (10-1000 µg/ml) + Collagen type I (10 µg/ml); or Collagen type I (equivalent concentration according to ELISA), and incubated in the presence of DMEM + 50 µM cycloheximide for 2 hours. Cells were fixed with 2.5% glutaradehyde followed by staining with 1% crystal violet. The percentage of spread cells of total cell number was calculated in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).
Pair-wise comparisons between EMD+Collagen groups and +ve/-ve controls were significantly different, $p<0.05$

Periodontal ligament fibroblast cells- For PDLFs cultured on Emdogain (10-1000 μg/ml) + fibronectin (10 μg/ml), in the presence of cycloheximide, the greatest cell-adhesion was 74% of positive control with 1000 μg/ml Emdogain (Fig. 14). The level of cell spreading was 30% for Emdogain (1000 μg/ml) + fibronectin (10 μg/ml) compared to 75% for positive control (Fig. 15). In Emdogain + collagen type I group, the highest level of adhesion was seen with 10 μg/ml Emdogain that was 90% of positive control (Fig. 16). The level of cell spreading was 33% for Emdogain (10 μg/ml) + collagen type I (10 μg/ml) compared to 62% for positive control (Fig. 17).

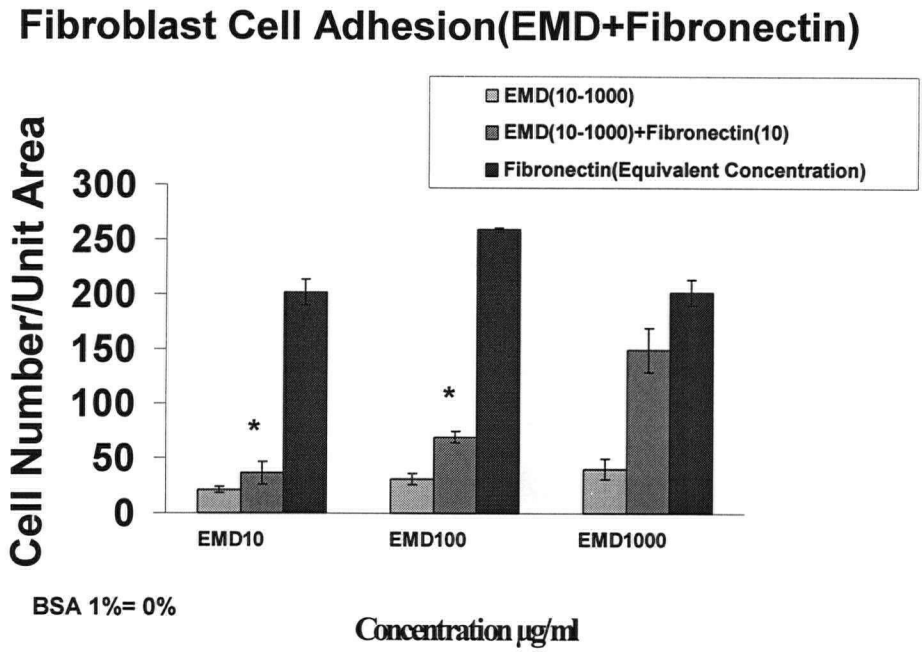

Figure 14. Fibroblast cell adhesion on Emdogain + Fibronectin. PDLFs were plated on either Emdogain (10-1000 µg/ml); Emdogain (10-1000 µg/ml) + Fibronectin (10 µg/ml); or Fibronectin (equivalent concentration according to ELISA), and incubated in the presence of DMEM + 50 µM cycloheximide for 2 hours. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Number of the cells in each condition was counted in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).

* = $p<0.05$ Significantly different from Collagen50 µg/ml (positive control)

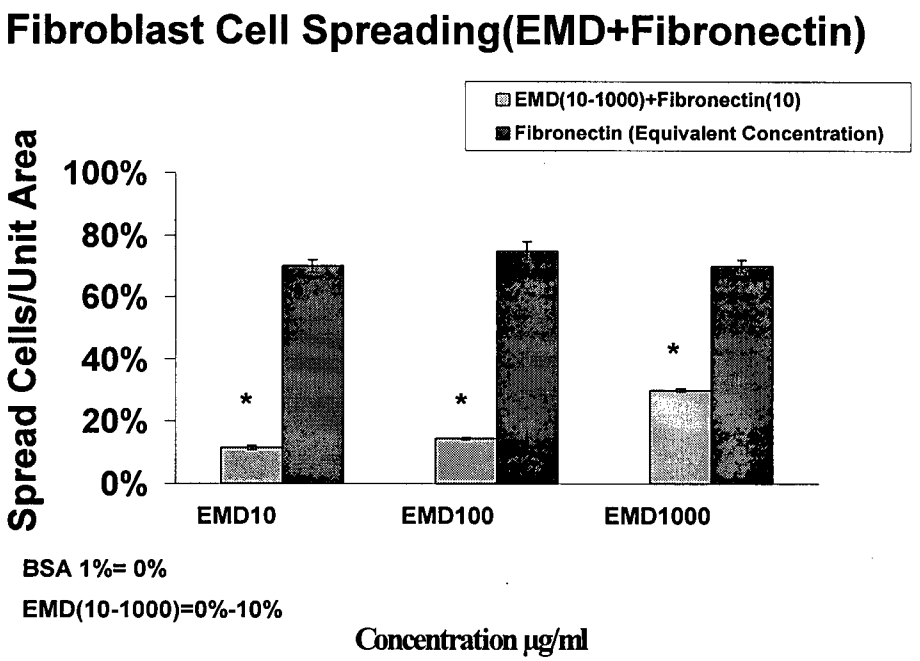

Figure 15. Fibroblast cell spreading on Emdogain + Fibronectin. PDLFs were plated on either Emdogain (10-1000 µg/ml); Emdogain (10-1000 µg/ml) + Fibronectin (10 µg/ml); or Fibronectin (equivalent concentration according to ELISA), and incubated in the presence of DMEM + 50 µM cycloheximide for 2 hours. Cells were fixed with 2.5% glutaradehyde followed by staining with 1% crystal violet. The percentage of spread cells of total cell number was calculated in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).
* = $p<0.05$ Significantly different from Collagen50 (positive control)

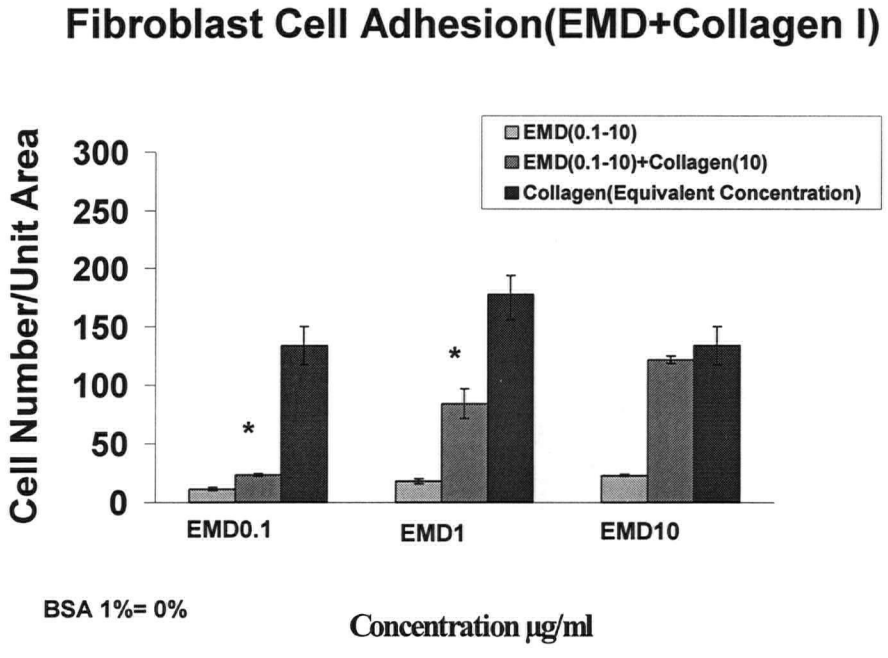

Figure 16. Fibroblast cell adhesion on Emdogain + Collagen type I. PDLFs were plated on either Emdogain (10-1000 µg/ml); Emdogain (10-1000 µg/ml) + Collagen type I (10 µg/ml); or Collagen type I (equivalent concentration according to ELISA), and incubated in the presence of DMEM + 50 µM cycloheximide for 2 hours. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Number of the cells in each condition was counted in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).

* = $p<0.05$ Significantly different from Collagen50 (positive control)

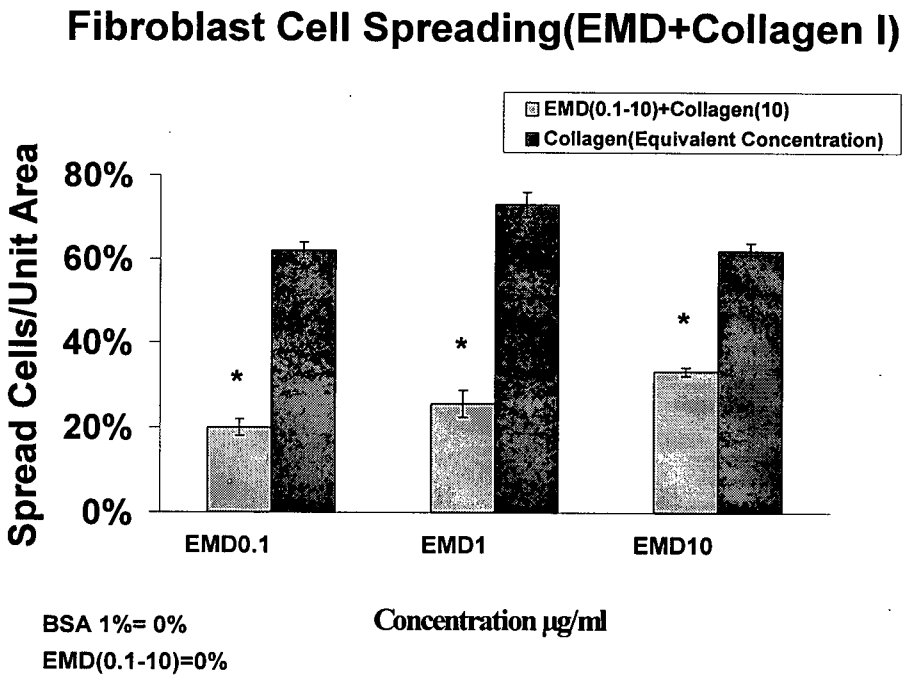

Figure 17. Fibroblast cell spreading on Emdogain + Collagen type I. PDLFs were plated on either Emdogain (10-1000 µg/ml); Emdogain (10-1000 µg/ml) + Collagen type I (10 µg/ml); or Collagen type I (equivalent concentration according to ELISA), and incubated in the presence of DMEM + 50 µM cycloheximide for 2 hours. Cells were fixed with 2.5% glutaradehyde followed by staining with 1% crystal violet. The percentage of spread cells of total cell number was calculated in 3 randomly selected standard fields from 4 independent samples (mean ± s.e.m.).
* = $p<0.05$ Significantly different from Collagen50 (positive control)

3.3. Cell migration

Cell migration on Emdogain-coated surfaces was studied in a lateral cell migration model using stainless steel cylinders.

Epithelial cells- HaCaT cells were cultured on Emdogain (1-30,000 μg/ml)-coated surfaces and allowed to migrate for 72 hours in the presence of 10% FBS (Fig. 18). Cell migration on 10,000 μg/ml Emdogain, showed the highest rate of migration compared to the other concentrations ($p<0.05$). However, this was only significantly different from Emdogain 1 μg/ml. The rate of migration on 10,000 μg/ml Emdogain was significantly different from the negative control (BSA 1%), but not from the positive control (collagen type I) (Fig. 19). To determine the role of TGF-β1 on cell migration, HaCaT cells were allowed to migrate on Emdogain-coated surfaces (10,000 μg/ml) in the presence of 1% FBS + 10 ng/ml TGF-β1 for 72 hours. The results did not show any significant difference in the rate of cell migration on Emdogain between + and – TGF-β1 treated conditions. The same pattern was also seen for collagen type I-coated surfaces. However, on BSA 1% (negative control), TGF-β1 (10 ng/ml) significantly increased the rate of cell migration (Fig. 19). The role of TGF-β1 on proliferation activity of HaCaT cells cultured on Emdogain was determined. The results of the proliferation assay showed a significant TGF-β1 induced increase in HaCaT cell proliferation when grown on Emdogain. The similar pattern was observed for the positive and negative controls (Fig. 20).

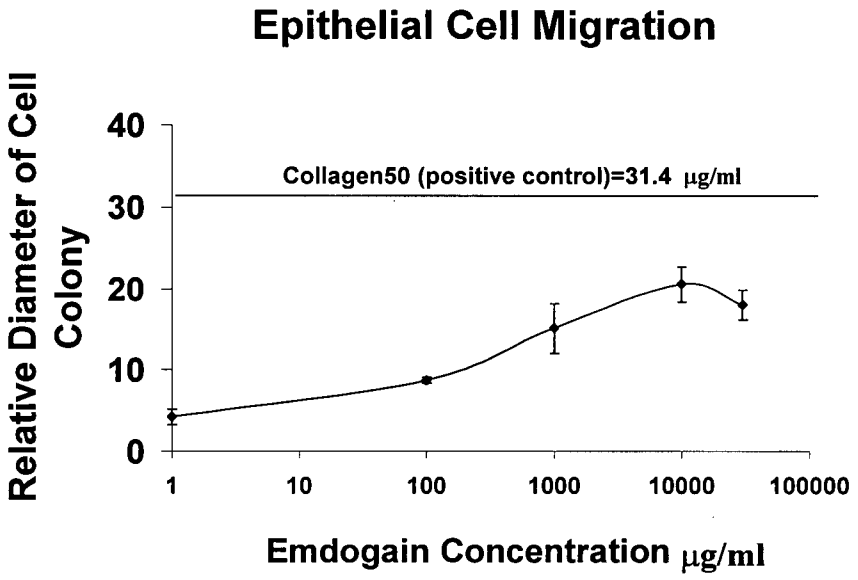

Figure 18. Epithelial cell migration on Emdogain. HaCaT cells on Emdogain-coated plastic (1-30,000 µg/ml) were incubated in the presence of DMEM + 10% FBS for 72 hours. Collagen type I (50 µg/ml) and BSA 1% were used as positive and negative controls, respectively. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Relative cell colony diameter was measured from magnified image of each cell circle. The diameter of the BSA1% cell colony at 3 days was subtracted from this value. The values represent mean ± s.e.m. of 4 independent samples.
EMD 1µg/ml significantly different from other concentrations of EMD, $p<0.05$

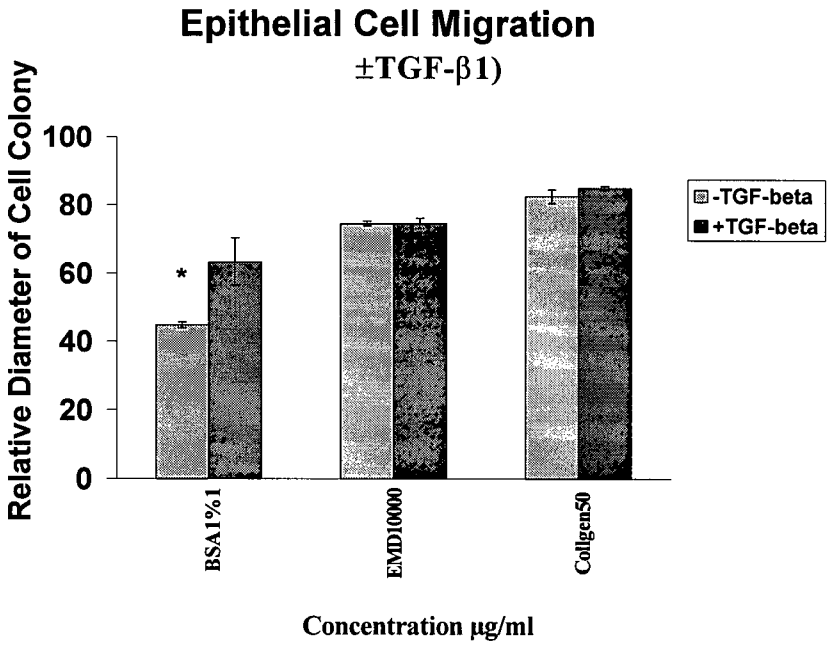

Figure 19. Effect of TGF-β1 on epithelial cell migration on Emdogain. HaCaT cells on Emdogain-coated plastic (10,000 µg/ml) were incubated in DMEM + 1% FBS, in the presence or absence of 10 ng/ml TGF-β1 for 72 hours. Collagen type I (50 µg/ml) and BSA 1% were used as positive and negative controls, respectively. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Relative cell colony diameter was measured from magnified image of each cell circle. The diameter of the original cell colony was subtracted from this value. The values represent mean ± s.e.m. of 4 independent samples.

* = $p < 0.05$ Significantly different from BSA1%+ TGF-β1

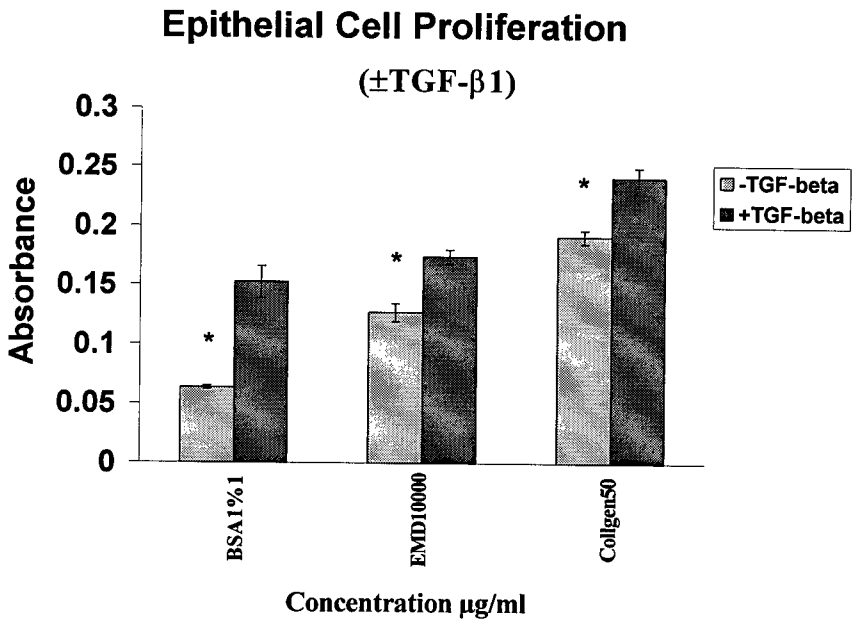

Figure 20. Effect of TGF-β1 on epithelial cell proliferation on Emdogain. HaCaT cells on Emdogain-coated plastic (10,000 µg/ml) were incubated in DMEM + 1% FBS, in the presence or absence of 10 ng/ml TGF-β1 for 72 hours. Collagen type I (50 µg/ml) and BSA 1% were used as positive and negative controls, respectively. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. The dye was dissolved in 10% acetic acid and the color density was measured by a microtiter plate reader set to 595 nm absorbance. The values represent mean ± s.e.m. of 4 independent samples.
* = $p < 0.05$ Significantly different from + TGF-β1

Periodontal ligament fibroblast cells- PDLFs were seeded on Emdogain (1-30,000 μg/ml)-coated surfaces and allowed to migrate for 72 hours in the presence of 10% FBS. Cell migration was slightly higher on 1000 μg/ml Emdogain. This difference was statistically significant when compared to Emdogain 1 μg/ml but not to the other concentrations of Emdogain. The rate of migration on 1000 μg/ml Emdogain was significantly different from the negative control (BSA 1%), but not from the positive control (collagen type I) (Fig. 21). Plating PDLFs on Emdogain (10,000 μg/ml) and in the presence of 1% FBS + 10 ng/ml TGF-β1 resulted in cell death and detachment after 72 hours. This also happened for cells cultured on BSA 1% (negative control). However, the cells cultured on collagen type I (positive control) under the same conditions, continued to survive and there was no significant difference in cell migration between + and – TGF-β1 treated conditions (data not shown).

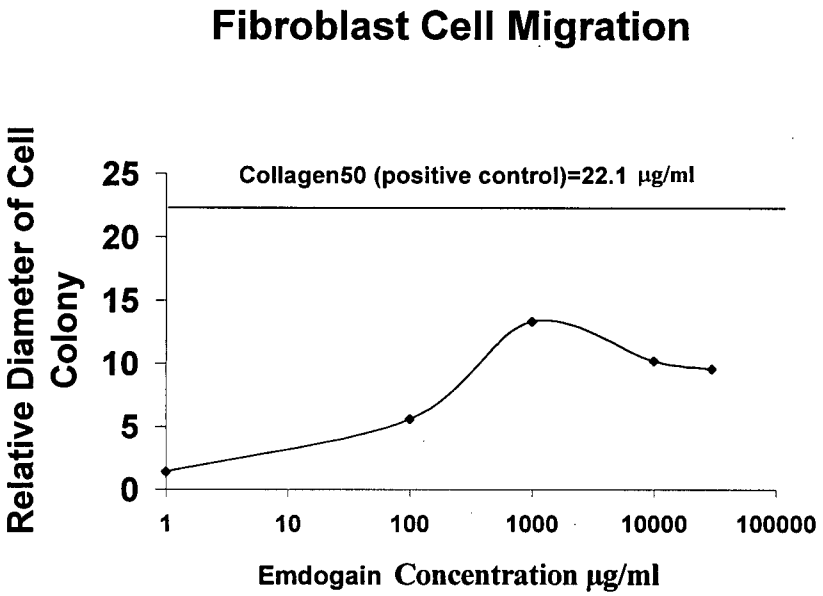

Figure 21. Fibroblast cell migration on Emdogain. PDLFs on Emdogain-coated plastic (1-30,000 µg/ml) were incubated in the presence of DMEM + 10% FBS for 72 hours. Collagen type I (50 µg/ml) and BSA 1% were used as positive and negative controls, respectively. Cells were fixed with 4% formaldehyde followed by staining with 1% crystal violet. Relative cell colony diameter was measured from magnified image of each cell circle. The diameter of the BSA1% cell colony at 3 days was subtracted from this value. The values represent mean ± s.e.m. of 4 independent samples.
EMD 1µg/ml significantly different from other concentrations of EMD, $p<0.05$

CHAPTER FIVE - DISCUSSION

As per the classic description of wound healing, initially there is formation of a clot in the wounded area. Inflammatory cells followed by fibroblasts and endothelial cells then invade the clot to form granulation tissue, while the epithelial cells migrate to cover the denuded surfaces (or form a junction at the tooth surface). Finally, maturation of the healing tissue matrix is seen along with wound contraction. Periodontal therapeutic procedures result in wounding of the periodontal tissues and the consequence of such procedures depend largely on the cellular and molecular events associated with wound healing. Periodontal regeneration is defined as reproduction of the tooth-supporting tissues including cementum, periodontal ligament and alveolar bone (Garret, 1996). Melcher, 1976 suggested that the cells that repopulate the root surface after periodontal surgery determine the nature of the attachment that will form. Following flap elevation, the instrumented root surface can be repopulated by epithelial, gingival connective tissue, bone and periodontal ligament cells. After conventional periodontal treatment, epithelial cells rapidly migrate in an apical direction to reach the most apical part of the instrumentation, forming a long junctional epithelium and preventing the formation of a new attachment (Caton et al., 1980; Listgarten and Rosenberg, 1979). The aim of regenerative procedures is to exclude or delay rapid epithelial cell migration, allowing cells from periodontal ligament and bone to repopulate the root surface and to form a new periodontal attachment. Guided tissue regeneration (GTR) with barrier membranes is effective in preventing epithelial and gingival connective tissue cells from migrating into the blood clot around the instrumented root surface. Histologic studies have shown significantly better results with the use of GTR in treatment of class II furcations and

vertical osseous defects; however, the predictability is not high enough (Nyman et al., 1982a and 1982b; Gottlow et al., 1984 and 1986). More recently, a variety of biological mediators have been used in order to promote repopulation of selected cell populations necessary for periodontal regeneration. Several growth factors such as PDGF, IGF-I, bFGF, TGF-β and BMPs, as single agents or in combinations, have been examined for their periodontal regenerative potential in animal models and in clinical trials (Rutherford et al., 1992 and 1993; Giannobile et al,. 1996). While a solid scientific rational exists for the use of a variety of growth factors in regeneration of periodontal tissues, clinical studies have not led to consistent successful treatment outcomes (Selvig et al., 1994; Howell et al., 1997a and 1997b; Boyne et al., 1997). Another set of therapeutic candidates is extracellular matrix proteins. Fibronectin has been studied for its role in periodontal regeneration in animal models, showing significantly better results compared to conventional treatment (Caffesse et al., 1985; Smith et al., 1987). However the clinical outcome of this treatment modality remains of uncertain benefit (Caffesse et al., 1988; Alger et al., 1990). More recently enamel matrix derivatives, a set of matrix proteins have been studied for their potential role in periodontal regeneration. The rational comes from studies by Slavkin and Boyde, 1975; Slavkin, 1976, proposing that enamel related proteins from Hertwig's epithelial root sheath initiate formation of cementum. Later, studies by Hammarstrom, 1997; Hammarstrom et al., 1997, demonstrated that enamel matrix is involved in the formation of acellular extrinsic fiber cementum during tooth development, and that this matrix has the potential to induce regeneration of cellular cementum in experimental periodontal defects in monkeys. Since the enamel matrix proteins had previously been found exclusively in the developing enamel, they were

ascribed a biological role only in the development of the enamel. With the new finding that the enamel matrix also has a function outside the developing enamel, several studies have been carried out to explore this function. Histological studies in primate models showed that application of enamel matrix proteins in experimentally induced periodontal defects results in formation of periodontal tissues characterized by acellular cementum firmly attached to the underlying dentin, a functionally oriented periodontal ligament and new alveolar bone (Hammarstrom et al., 1997; Araujo et al., 1998; Sculean et al., 2000). Clinical trials have demonstrated significant gain of supporting alveolar bone and clinical attachment in treatment of infrabony defects compared to conventional therapy (Zetterstrom et al., 1997; Heijl et al., 1997b). A few in vitro studies have been conducted to investigate the effect of enamel matrix proteins on behavior of periodontal ligament fibroblasts and osteoblasts (Gestrelius et al., 1997b; Van der Pauw et al., 2000; Hoang et al., 2000; Schwartz et al., 2000). However, the cellular and molecular mechanisms involved in the process of periodontal regeneration induced by enamel matrix proteins has not been extensively examined and is poorly understood.

The present study was undertaken to test the hypothesis that enamel matrix proteins (Emdogain®) promote periodontal regeneration by enhancing the adhesion, spreading and migration of PDL fibroblasts, but not of epithelial cells. Enamel matrix proteins are soluble in acid or alkaline pH and low temperature due to their hydrophobic characteristic. According to a previous study by Gestrelius et al., 1997a, when Emdogain was dissolved under acidic conditions and the solution was neutralized by addition of buffer or alkali about 80% of the protein precipitated as the pH reached 6.5 and practically all of the protein was removed from the solution at the pH of 7.4. In the same

study Emdogain was dissolved in either propylene glycol alginate (PGA) or 10 mM acetic acid and used for adsorption and precipitation experiments. Therefore, in this study the commercially available Emdogain was dissolved in 10 mM acetic acid (pH 5) and used for surface coating. A study on kinetics of enamel matrix proteins have shown that the concentration of Emdogain used in the clinical situations (30,000 μg/ml) significantly decreases shortly after application and for the rest of the healing period the wound environment is exposed to concentrations much lower than the original concentration (Gestrelius et al., 1997a). Therefore, we included a wide range of concentrations in this study, from 30,000 μg/ml which is the concentration used clinically to <1 μg/ml. The surfaces coated with Emdogain in acetic acid were neutralized with PBS before starting the experiments, causing precipitation of the Emdogain that was detected with SEM at the concentrations of ≥1000 μg/ml Emdogain. This finding is in agreement with previous SEM studies, showing that Emdogain 2000 μg/ml under physiological conditions formed aggregates in the shape of spheres or short rods on dentin or model surfaces (Gestrelius et al., 1997a). This concentration dependent precipitation of Emdogain at neutral pH may have a significant impact on Emdogain interaction with ECM.

1. EXTRACELLULAR MATRIX PROTEIN BINDING TO EMDOGAIN®

Granulation tissue deposition is a critical step in wound healing and occurs in an ordered sequence of fibronectin, type III collagen, and type I collagen (Kurkinen et al., 1980). Fibronectin provides a provisional substratum for migration and ingrowth of keratinocytes, fibroblasts and endothelial cells. Types I and III collagen fibrils provide nascent tensile strength for the wound. As the matrix matures, the fibronectin disappears

and collagen bundles grow in size increasing the tensile strength of wounds (Clark, 1996). Re-epithelialization is another major step in wound healing that depends upon a variety of interactions between keratinocytes and the extracellular matrix components. In this process, keratinocytes detach themselves from the basement membrane, migrate into the wound bed and finally regenerate the basement membrane (Woodley et al., 1980; and 1988), including laminin 1, type IV collagen, anchoring filament-associated components (laminin 5), and anchoring fibril collagen type VII (Woodley et al., 1985).

Based on this information it was of interest to examine the binding interaction between Emdogain and the major wound healing associated extracellular matrix proteins; fibronectin, collagen type I and IV and laminin I. Fibronectin and Collagen type I bound to Emdogain, while no binding interaction was observed between collagen type IV or laminin I and Emdogain. The relative concentrations of the ECM protein (fibronectin, collagen type I) that bound to Emdogain had a bell shape with the peak at 100 μg/ml Emdogain for fibronectin and 1 μg/ml Emdogain for collagen type I. This difference may suggest that collagen type I and fibronectin bind to different molecules in Emdogain. When the concentration of fibronectin increased there was an increase in fibronectin binding to Emdogain. This increase was more significant with lower concentration of Emdogain (10 μg/ml). Collectively the results from Emdogain-EMC protein experiments suggest that high concentrations of Emdogain (>1000 μg/ml), do not promote ECM protein binding. A simple explanation could be that insoluble protein aggregates that form with higher concentrations of Emdogain may not bind to ECM proteins. This explanation is also supported by the SEM results, showing that under the conditions used in this study, protein aggregates become detectable as the concentration of Emdogain

reaches 1000 μg/ml and increases with higher concentrations of Emdogain. However, collagen type I binding to Emdogain started to decline at 10 μg/ml Emdogain, which is significantly below the precipitation point. A possible explanation could be that increased concentration of Emdogain used for surface coating masks the binding sites available for collagen type I. As discussed earlier, the study on kinetics of enamel matrix proteins after application onto dental roots in vivo (Gestrelius et al., 1997a) showed enamel matrix proteins dissolved in PGA had a dual elimination curve, with an initial rapid wash-out (24 hours) followed by a much slower phase with a half-life of 50-70 hours and the last percent was not removed until after 2 weeks. This leads to one possible conclusion that the slower Emdogain clearance at lower concentrations (Gestrelius et al., 1997a) may occur because of Emdogain binding to wound ECM proteins (e.g. fibronectin, collagen type I). Therefore, the binding interaction between Emdogain and the ECM proteins may play a critical role in maintaining Emdogain in the wound area. Two different scenarios can be considered for Emdogain binding to ECM proteins. First, ECM proteins (e.g. fibronectin) may bind to precipitations of Emdogain at high concentrations on the root surface. Second, soluble Emdogain at low concentrations may bind to ECM proteins in the wound area. The specific binding site(s) on Emdogain that are involved in the ECM protein interactions is unclear. Enamel matrix proteins (Emdogain) are a complex mixture of several matrix proteins. Although amelogenin isoforms comprise about 90% of this matrix (Uchida et al., 1997), there are other non-amelogenin proteins including tuftelin (Deutsch et al., 1991), sheathlin (Uchida et al., 1991), ameloblastin (Krebsbach et al., 1996), amelin (Fong et al., 1996), sulfated enamel proteins (Smith et al., 1995) and enamelin (Fukae et al., 1996). It should be mentioned that a thorough search of literature

found no previous reports on the interactions between different components of enamel matrix proteins and ECM proteins. (See Table 7. for summary of ECM-Emdogain interactions.)

Table 7. ECM Protein Binding to Emdogain – Precipitation Profiles

	Emdogain Concentrations Tested (μg/ml) 30,000	10,000	1,000	100	10	1	0.1	0.01	0.001
Concentration Dependent Precipitation of Emdogain	++	++	+	–	–	–	–	–	–
Fibronectin 10-μg/ml	–	–	++	+++	++	+	+	–	–
Collagen I 10-μg/ml	–	–	–	–	++	+++	++	–	–
Collagen IV 10-μg/ml	–	–	–	–	–	–	–	–	–
Laminin I 10-μg/ml	–	–	–	–	–	–	–	–	–

+, Significant increase in ECM protein binding to Emdogain.

2. EMDOGAIN®-CELL INTERACTIONS

ECM proteins in a healing wound can influence the biological behavior of keratinocytes and fibroblast such as proliferation, binding to ECM, spreading on provisional matrix and migration (Woodley et al., 1988; and 1990). Epithelium has a remarkable capacity to regenerate following injury and is fundamental to cutaneous and mucosal tissue maintenance (Bartold et al., 2000). Following conventional surgical periodontal therapy the rapid proliferation of epithelial cells leads to the formation of a long junctional epithelium onto the dental root surface and prevents new attachment formation (Caton et al., 1980; Listgarten and Rosenberg, 1979). As previously discussed, the aim of regenerative procedures is to exclude or delay rapid epithelial migration, which in turn allows cells from periodontal ligament and bone to repopulate the root surface and to form a new periodontal attachment. We therefore examined how adhesion, spreading and migration of epithelial and PDL fibroblast cells are regulated by Emdogain.

2.1. Cell adhesion

Epithelial cells cultured on Emdogain-coated surfaces (1-30,000 μg/ml) in the presence of cylcoheximide, showed minimal adhesion (95% lower than the collagen type I positive control,) at 2 hours. However, in the absence of cycloheximide the adhesion to Emdogain was significantly improved (75% and 25% lower than positive control at 2 and 4 hours, respectively). These data indicate epithelial cells adhesion to Emdogain is dependent on the synthesis of cell adhesion proteins. Epithelial cell adhesion was improved when fibronectin or collagen type I (10 μg/ml) which was bound to Emdogain

(compared to Emdogain alone) was used. However, compared to the positive control (fibronectin or collagen type I proteins alone) cell adhesion was still significantly less (Fig. 12 and Fig. 13).

In contrast to epithelial cells, PDL fibroblast adhesion to Emdogain (1-30,000 μg/ml) was concentration-dependent with the maximum adhesion to Emdogain 10,000 μg/ml. The initial adhesion of PDLF to Emdogain required no protein synthesis because there was no statistically significant difference in adhesion at 2 hours to any of the Emdogain concentrations in the presence or absence of cycloheximide (Fig. 9). Adhesion of PDLF to fibronectin or collagen type I (10 μg/ml) bound to Emdogain was improved when compared to Emdogain alone and was comparable to the positive control (fibronectin or collagen type I alone) (Fig. 14 and Fig. 16). An alternative interpretation of the results from cell binding to Emdogain + ECM proteins would be that Emdogain reduced the adhesion of both epithelial cells and PDLFs to ECM proteins. This reduction was more significant for epithelial cells compared to PDLFs.

Cell migration is necessary for epithelial and PDL fibroblast cells to enter the wound provisional matrix. Although cell binding to ECM is required for migration, a very tight binding of the cells to ECM has inhibitory effects on cell migration. Therefore a balance between several variables related to integrin-ligand interactions, including ligand levels, integrin levels, and integrin-ligand binding affinities is required for optimal cell migration on a given substratum (Palecek et al., 1997). As discussed before Emdogain significantly reduced epithelial cell adhesion to ECM proteins. In contrast, PDLF binding to Emdogain + ECM proteins (fibronectin and collagen type I) was

comparable to ECM protein alone. This may affect cell migration into the provisional matrix in vivo, with inhibitory effects on epithelial cell migration.

The mechanism(s) regarding adhesion of epithelial cells and PDLFs to Emdogain is presently not known. One likely family of proteins that regulate adhesion and spreading are integrin receptors. It has been reported that amelin contains the integrin recognition sequence DGEA, suggesting that is may interact with integrins (Cerny et al., 1996). An alternative explanation might be cell-type related differences in production profiles of ECM proteins and growth factors. It has been shown that Emdogain increases release of fibronectin and TGF-β in PDLFs (Van der Pauw et al., 2000, Lyngstadaas et al., 1999). Theoretically, an increased synthesis of fibronection and TGF-β could be associated with a more rapid cell attachment and spreading.

In the connective tissue, fibroblasts are surrounded by a matrix that contains collagen and cellular fibronectin as the major components. Fibroblasts express collagen receptors α1β1 and α2β1 and the major fibronectin receptor α5β1 integrin which they use for adhesion to the matrix (Gailit et al., 1996; Welch et al., 1990; Xu and Clark, 1996). Fibroblasts also express integrin heterodimers of the αv subfamily that can bind to fibronectin (Hakkinen et al., 2000). Based on this information, it was of interest to examine the role of β1 and αv containing integrins in binding PDLFs seeded on Emdogain. For this purpose two monoclonal antibodies were used to block β1 and αv integrin subunits. Fibronectin was used as the positive control since it is a fundamental component of the provisional matrix. The level of adhesion and spreading was reduced by preincubating with either of the antibodies and was further reduced with the addition

of the two antibodies together. The reduction in cell adhesion and spreading on Emdogain followed the same pattern as fibronectin (positive control), suggesting that PDLF adhesion and spreading on Emdogain are integrin dependent and the two integrin subunits ($\beta 1$, αv) associated with fibronectin binding are also involved in PDLF binding to Emdogain. As mentioned earlier, the presence of intergin recognition sequence DGEA has been reported in amelin, a component of enamel matrix proteins (Cerny et al., 1996). Future studies are required to further explore the interaction between Emdogain and intergin receptors associated with wound healing.

2.2. Cell spreading

Spreading of cells and organization of cell cytoskeleton are required before cells are capable of proliferating (Sottile et al., 1998). Emdogain-coated surfaces did not promote epithelial cell spreading in serum-free conditions and in the presence of cycloheximide (0% compared to 98% in positive control). However, cells did spread on collagen type I–Emdogain coated surfaces (18% compared to 75% in positive control) but not fibronectin–Emdogain coated surfaces. Interestingly, PDLF spreading on Emdogain exhibited the same pattern as PDLF adhesion with the maximum response at 10,000 μg/ml Emdogain (10% and 21% in the presence and absence of cylcoheximide, respectively; 100% in positive control). Addition of fibronectin or collagen type I (10 μg/ml) to Emdogain coated surfaces improved cell spreading (compared to Emdogain alone), but this was still significantly lower than positive control (ECM protein alone). Morphology of the PDLFs grown on Emdogain was different than that of positive control (collagen type I), showing several cytoplasmic projections and tending to cluster. This is

aggreement with another in vitro study (Van der Pauw et al., 2000). They showed morphological differences in PDLF cultured on Emdogain and collagen type I.

The PDLF adhesion and spreading on Emdogain that was found in this study differ from a previous study (Gestrelius et al., 1997b). They reported that Emdogain had no significant effect on attachment and spreading of PDLF. This could be due to the differences in experimental designs between the two studies. In the present study Emdogain effect on cell adhesion and spreading was evaluated in both plus and minus serum conditions, as serum contains several adhesion proteins (e.g. fibronectin) that can influence cell binding to Emdogain. Further, cycloheximide was used in serum-free conditions to prevent *de novo* protein synthesis by the cells that can also affect cell binding to the substrate. In the study by Gestrelius et al., (1997b), no comments have been given regarding the growth medium (±serum, ± cycloheximide) used for the experiments. It was reported that Emdogain enhanced cell attachment to a minimal degree at 20 μg/ml, but did not promote cell spreading during this time. It is not clear whether different concentrations of Emdogain were used for coating. In another in vitro study by Van der Pauw et al., 2000, it was reported that Emdogain (1-100 μg/ml) have a stimulatory effect on the attachment and spreading of PDLFs when compared to gingival fibroblasts in the presence of DMEM + BSA 4 mg/ml (6.5 to 48 hours incubation period). Our results did not show significant adhesion and spreading of PDLFs with 1-100 μg/ml Emdogain, however it should be noticed that the incubation period is significantly different between the two studies (2 vs. 48 hours). Finally it should be emphasized that in the present study the highest concentration used is the therapeutic dose that is applied in a

periodontal surgical site (30,000 μg/ml, Emdogain). However in both previous studies concentrations ≤ 100 μg/ml were used for surface coating. (See table 8 A,B for a summary of interactions).

Table 8. Schematic summary of epithelial and PDLF cell adhesion and spreading in presence of cycloheximide

A) Emdogain + Fibronectin

		Emdogain	Emdogain+Fibronectin			Fibronectin
			EMD1,000	EMD100	EMD10	
HaCaT cells	Adhesion	+	+++	++	++	++++
	Spreading	–	–	–	–	++
PDLFs	Adhesion	+	++++	++	++	++++
	Spreading	–	++	+	+	+++

B) Emdogain + Collagen I

		Emdogain	Emdogain+Collagen I			Collagen I
			EMD10	EMD1	EMD0.1	
HaCaT cells	Adhesion	+	+++	+++	++	++++
	Spreading	–	+	+	+	+++
PDLFs	Adhesion	+	++++	+++	++	++++
	Spreading	–	++	++	++	+++

+, Significant increase in ECM protein binding to Emdogain.

2.3. Cell migration

Histologic evaluation of periodontal healing following regenerative therapy with Emdogain showed evidence of periodontal regeneration (Hammarstrom et al., 1997; Yukna and Mellonig, 2000). This requires a delay in re-epithelialization of the wound site, allowing cells from periodontal ligament to repopulate root surfaces. Therefore, we examined cell migration on Emdogain. Unfortunately the results showed that Emdogain at concentrations of 100 to 30,000 μg/ml enhanced migration of both epithelial cells and PDLFs to the level of positive control (collagen type I) which was significantly higher than negative control (BSA 1%). However, low concentration of Emdogain (1 μg/ml) did not promote cell migration (both cell lines). To my knowledge, there is no previous in vitro study on the effect of Emdogain on epithelial cell migration. These data suggest Emdogain effect on epithelial cell migration in vivo is more complicated than a simple inhibitory effect. It might require a series of interactions involving Emdogain, extracellular matrix proteins and different cell types, acting in favor of PDL cells to "win the race". In respect with PDLF migration, a previous study (Gestreslius et al., 1997b), examined the ability of cells to move directionally toward Emdogain (5-100 μg/ml) in DMEM (chemotaxis) that is different from migration evaluated in our study. The results showed that Emdogain produced no enhancement of PDLF migration using a Boyden chamber system.

TGF-β1 is a potent regulator of cell activities, such as cell proliferation and migration (Postlethwaite et al., 1987; Sporn et al., 1987). Therefore, it was of interest to

evaluate the role of TGF-β1 in migration and proliferation of cells seeded on Emdogain (10,000 μg/ml). The results of the migration and proliferation assays indicated that under the conditions used TGF-β1 (10 ng/ml, 72 hours) significantly increased proliferation but not migration of the epithelial cells seeded on Emdogain. It is possible that a combination of different concentrations of Emdogain and TGF-β1 could reveal a concentration dependent effect of TGF-β1 on epithelial cell migration on Emdogain. PDLFs seeded onto Emdogain (10,000 μg/ml) and incubated in the presence of 1% FBS + TGF-β1 attached and spread on Emdogain for 24 hours. However, the cells detached and died at 72 hours. In contrast, cells seeded on collagen type I continued to proliferate and migrate. This suggests that Emdogain, 10,000 μg/ml in the presence of low levels of serum (1% FBS) did not support fibroblast proliferation.

CHAPTER SIX – CONCLUSIONS

This in vitro study was designed to investigate Emdogain's® interact with ECM proteins and its effect on adhesion, spreading and migration of epithelial cells and periodontal ligament fibroblasts. Under the conditions of the study the following was found:

1. Emdogain bound to specific ECM proteins (fibronectin and collagen type I) but not collagen type IV and laminin. The binding was dependent on Emdogain and ECM protein concentration. Further, with Emdogain precipitation at high concentrations no interaction between Emdogain and ECM proteins was observed.

2. PDL fibroblasts adhere to Emdogain and do not require synthesis of adhesive proteins or matrix in order to attach to Emdogain. PDL fibroblast adhesion and spreading on Emdogain is concentration-dependent with maximum adhesion and spreading at 10,000 μg/ml of Emdogain.

3. αv and β1 integrin subunits were required for PDL fibroblast binding to Emdogain.

4. Emdogain did not promote epithelial cell adhesion and spreading. But epithelial cells if allowed to synthesize proteins (- cycloheximide) then they were able to adhere.

5. Addition of ECM proteins (fibronectin or collagen type I) enhanced cell binding to Emdogain that was more significant for PDL fibroblasts compared to epithelial cells.

6. Emdogain supported migration of both epithelial cells and PDL fibroblasts.

CHAPTER SEVEN – FUTURE DIRECTIONS

At the present time very little is known regarding the cellular and molecular mechanisms by which Emdogain regulate periodontal regeneration. There are no reports in the literature in regards to Emdogain interaction with various components of ECM proteins. The in vitro studies have focused on the influence of Emdogain on PDL and gingival fibroblasts and little is known regarding Emdogain effect on epithelial cell behavior. My results from Emdogain-ECM and Emdogain-cell interactions indicate complex interactions between these components rather than a simple stimulatory or inhibitory effect. Moreover, the results from integrin blocking assay have established the involvement of αv and β1 integrin subunits in adhesion and spreading of PDL fibroblasts on Emdogain. Thus it will be of interest to further investigate the role of ECM proteins in Emdogain induced changes in specific cell functions. Further, to achieve better understanding of these interactions more must be elucidated in regards to the role of specific integrin receptors associated with them. Emdogain is a complex mixture of several matrix proteins. Therefore, it will be of interest to investigate what specific proteins in Emdogain interact with ECM to regulate integrin dependent adhesion. In addition it would be of interest to examine if cells plated on Emdogain are induced to express different integrin proteins. Moreover, growth factors such as TGF-β play critical roles in wound healing and at the present there are no reports in the literature regarding how growth factors may affect Emdogain induced changes in cell behavior. Therefore, future research along these lines may help towards understanding in more detail the molecular mechanisms behind Emdogain induced periodontal regeneration.

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