Wound healing at the tooth-gingiva interface

by

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ABSTRACT:
Healing at the junctional epithelium has been studied at the histological level since early animal and human gingivectomy studies were done in the 1970s (Listgarten et al., 1972, Stahl et al., 1972). Relevance of healing in this area relates to regeneration of the periodontal complex, a goal which has, for the most part, eluded clinical periodontology since that time. On a molecular level there has been little research at the tooth-tissue interface and the present study attempts to shed some light on the similarities and differences in this area compared to other areas in the mouth and rest of the body. Examination of several cell adhesion molecules and their respective ligands, as well as the expression of cytokeratins in the area of the junctional epithelium was investigated between 1 and 60 days post wounding. Cytokeratins 16 and 19 were the earliest molecules expressed. Upregulation of these cytokeratins was evident from the earliest time point, at 1 day post wounding. Expression of CK 19 was also observed to a wider extent in the oral epithelium of periodontitis specimens as well as 1 to 7 day post wounding. Expression of integrins β1, β4, and β6 was most intense between 3 and 7 days post wounding and this paralleled the expression of their respective ligands. In addition, expression of these molecules was usually most intense at a distance from the migratory tip suggesting increased activity coronal to the migrating cells. Finally, the expression of β6 was evident at the junctional epithelium of all specimens examined including healthy tissue samples. Taken together this study provides novel information at a molecular level of the cells of the human junctional epithelium during wounding.
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denotes epithelium and CT denotes connective tissue; arrows denote areas of increased staining intensity; arrowheads denote migrating tip of epithelium.
Abbreviations:
ADP    adenosine diphosphate
BP 180 bullous pemphigoid antigen 180
Ca     calcium
CK     cytokeratin
DAT cells directly attached to the tooth cells
EBL    external basal lamina
ECM    extracellular matrix
EGF    epidermal growth factor
FBS    fetal bovine serum
FC     fibrin clot
FGF    fibroblast growth factor
FMLP   N-formyl-methionyl-leucyl-phenylalanine
GAG    glycosaminoglycan
GP     glycoproteins
H&E    hematoxylin and eosin
IL     interleukin
IBL    internal basal lamina
JE     junctional epithelium
KGF    keratinocyte growth factor
LM 5   laminin 5
Mg     magnesium
MMP    matrix metalloproteinase
PDGF   platelet derived growth factor
PG     proteoglycan
PMN    polymorphonuclear neutrophils
TGF    transforming growth factor alpha, beta
TN C   tenascin C
TNF α  tumour necrosis factor alpha
VEGF   vascular endothelial growth factor
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INTRODUCTION:

The ability of the body to repair itself after injury is essential to the survival of the species. There has been a great deal of research interest aimed at the mechanisms by which this occurs in humans, and this has been examined at the clinical, histological and molecular levels. Wound healing is not the same in all parts of the body. The gingival and mucosal barriers of the oral cavity demonstrate unique features in tissue healing. To date there has been little research (Worapamoran et al., 2002; Matsuura et al.1995) addressing the specific cell and molecular characteristics with respect to gingival wound healing. In the present study, the characteristics of wound healing at the tooth gingiva interface will be examined in individuals with healthy gingiva and those with periodontal disease. It is expected that certain extracellular matrix proteins, will have a unique expression pattern during healing at the junctional epithelium compared to healing in wounds in other parts of the body. In order to appreciate the complexity of this system, detailed descriptions of the structure of the gingival epithelium will be presented. In addition, the pathogenesis of periodontal disease will be briefly described. Finally, wound healing will be discussed in detail covering general aspects of healing as well as more specific aspects related to healing at the tooth gingiva interface.
CHAPTER I

Review of the Literature

1.1. Normal structure of the gingival epithelium and the junctional epithelium

1.1.a Epithelium

There are several different types of epithelia within the oral cavity. Gingival epithelium alone can be categorized into three different types, namely the oral epithelium, the sulcular epithelium and the junctional epithelium (Schroeder et al., 1971). Oral epithelium is located coronal to the mucogingival junction and continues to the gingival crest, the sulcular epithelium lines the sulcus and the junctional epithelium lies at or above the CEJ and extends apically to a point approximately 2 mm from the level of the alveolar bone. These 3 types of epithelia differ not only in location but also in terms of histological features (Bartold et al., 2000). Cytokeratin expression is different between the three tissue types as is cell surface molecules and integrin expression (Hormia et al., 1992; MacKenzie et al, 1991; Hormia et al., 1989).

Oral epithelium is orthokeratinized stratified squamous epithelium. This epithelium can be divided into four layers: 1. Stratum basale 2. Stratum spinosum 3. Stratum granulosum and 4. Stratum corneum (Garant, 2003). Stratum basale is in contact with the basement membrane and the cells within this layer divide continuously providing a source of cells for the differentiating layers that lie above (Garant, 2003). Moving closer to the surface of the epithelium is the stratum spinosum which forms the largest of all the layers of differentiating cells (Garant, 2003). Above this layer the cells become more flattened and keratohyalin granules are present in cytoplasms of these cells with these layers of cells forming the stratum
granulosum and the corneum. Between the epithelium and connective tissue lies the basement membrane. It is composed of a lamina densa, a lamina lucida and a reticular layer (Briggaman, 1971). Anchoring fibers composed of type VII collagen can be seen extending from the lamina densa into the connective tissue under an electron microscope (Sakai et al., 1986). Although microscopically lucent for the most part, the lamina lucida does contain electron dense zones which are hemidesmosomes attaching the epithelium to the basement membrane (Sakai et al., 1986).

In health, sulcular epithelium is approximately 0.5 mm deep and extends from the internal crest of the gingiva to the base of the sulcus (Schroeder et al., 1971). Structurally, keratinized sulcular epithelium is similar in many ways to the oral epithelium, being composed of several different layers of cells and attached to the underlying connective tissue by way of a basement membrane (Pollanen et al. 2003).

Although in close proximity to the oral and sulcular epithelium, the junctional epithelium shares few structural similarities. Some of these differences begin during the development of the tissue itself (Bartold et al., 2000). Tooth eruption is essential in the formation of the junctional epithelium. Prior to eruption the enamel of the erupting tooth is covered by a reduced enamel epithelium, which is composed of basal lamina and cells from the outer enamel epithelium (Bartold et al., 2000). As the tooth erupts through the oral epithelium it fuses with the reduced enamel epithelium and the junctional epithelium is formed (Bartold et al., 2000).

The junctional epithelium consists of a single basal layer of cells attached to the underlying connective tissue via hemidesmosomes and above this basal layer are several suprabasal layers containing elongated cells running in a direction that is parallel to the tooth.
Attachment of the junctional epithelium to the tooth is termed the epithelial attachment apparatus (Schroeder et al., 1971). The internal basal lamina (IBL) forms the interface between the tooth and epithelium, and the external basal lamina (EBL) is interposed between the epithelium and the connective tissue via hemidesmosomes (Borradori et al., 1996). Cells attached to the tooth are termed directly attached to tooth cells, DAT cells (Salonen, 1994). DAT cells are attached to the tooth by hemidesmosomes which are composed of bullous pemphigoid antigen 230 protein and plectin and these two proteins are responsible for binding to the transmembrane proteins of the hemidesmosomes which include BP 180 and \( \alpha_6\beta_4 \) integrin which extend into the IBL (Pollanen et al., 2003). IBL is interposed between the DAT cells and the enamel of the tooth. Production of the IBL is by the DAT cells and include laminin-5 and type VIII collagen (Pakkala et al., 2002, Pollanen et al., 2003). Unlike typical basement membranes, the IBL doesn’t contain type IV collagen, laminin-1 and 10, type VII collagen or perlecan (Hormia et al., 1998, Shimono et al., 2003). In fact, it has been suggested that the IBL is not a true basement membrane rather a simple ECM (Pollanen et al., 2003).

Several differences between cells of the junctional epithelium and the oral/sulcular epithelium are apparent (Pollanen et al., 2003). Unlike the consistently stratified layers of cells in the oral epithelium, cells of the junctional epithelium range from a 1-3 cells thick apically to 15-30 cells thick coronally. There are no rete ridges in the junctional epithelium and cells are relatively larger with wider intercellular spaces. Relatively smaller numbers of desmosomes are present in the junctional epithelium and only two "morphotypes" of differentiation exist, namely the cells of the basal layer and cells attached to the tooth surface itself (Pollanen et al., 2003, Bartold et al., 2000).
1.1b. Connective Tissue

Within the context of healing and regeneration of periodontal tissues, epithelial tissue has an important role, as does the underlying connective tissue. Major components of the gingival connective tissue are the fibroblasts, collagen fibers and other matrix components, nerves and vessels. Cells present within the connective tissue during health include (in low numbers): mast cells, macrophages, polymorphonuclear granulocytes, lymphocytes and plasma cells (Garant, 2003). However, fibroblasts form the majority of cells within the connective tissue. The main function of fibroblasts is the maintenance and repair of the extracellular matrix proteins (Bartold et al., 2000). Fibers produced by the fibroblast include collagen fibers, reticulin fibers, oxytalan fibers and elastic fibers and these all form the matrix of the gingival connective tissue. In addition to the function of matrix production, fibroblasts also remodel the matrix surrounding them by phagocytosis and collagenase production. Structurally, fibroblasts are elongated in shape and have extensive protein producing organelles allowing them to maintain and repair the surrounding connective tissues. Several different phenotypes of fibroblasts exist: quiescent, proliferative, migratory and contractile (Hakkinen et al., 2000; Sempowsky et al., 1995). Stationary fibroblasts have few intracellular microfilaments and have a relatively slow rate of proliferation as well as a slower metabolism (Hakkinen et al., 2000). Migratory fibroblasts, on the other hand, express microfilaments, myosin and different types of integrins allowing them to move efficiently. Heterogeneity amongst this population of cells becomes very important in the context of wound healing and regeneration. Quiescent fibroblasts exposed to serum undergo rapid changes in phenotype with large changes in gene expression and subsequent stimulation of cell proliferation (Hakkinen et al., 2000; Iyer et al., 1999). In addition to exposure to serum, differentiation of the fibroblast also appears to be
influenced by interaction with various matrix molecules. Migratory fibroblasts express a variety of integrins and matrix proteins affecting their function and phenotype. Finally, fibroblast differentiation to myofibroblasts coincides with wound contraction and therefore it has been postulated that these sub-populations are involved in this part of wound healing (Hakkinen et al., 2000; Gabbiani et al., 1971).

The matrix surrounding the fibroblasts is another important feature of the gingival connective tissue and has several important functions including: regulation of cell adhesion and gene expression, transportation of essential nutrients, electrolytes, water and metabolites between cells. Collagen is the major component of the extracellular matrix, and a variety of different types are found. Most commonly types I, III and VI fibers are present (Garant, 2003). Type I collagen forms bundles and a fibrillar arrangement within the matrix (Garant, 2003). It is one of the major cell adhesion molecules involved in regulation of cell function (Alimohamad et al., 2005). Type III collagen fibers are also found in the matrix, specifically, near the basement membrane at the junctional epithelium (Narayanan et al., 1985). Like type III collagen, type IV collagen is associated with the basement membrane but is arranged in a sheet like pattern (Garant, 2003). Additional collagens within the extracellular matrix include types VI and V (Garant, 2003).

Other proteins found within the extracellular matrix include proteoglycans and glycoproteins. Proteoglycans contain a protein core to which one or more glycosaminoglycans are attached (Hardingham et al., 1992). There are three different types of proteoglycan families within the extracellular matrix: small leucine rich proteoglycans (SLRPs), hyalectins and basement membrane proteoglycans (Matheson et al., 2005; Iozzo, 1998). Small leucine rich proteoglycans include decorin, biglycan, fibromodulin, and lumican (Matheson et al., 2005;
SLRPs are important in the assembly of the ECM as well as regulation of cell functions such as adhesion, proliferation, differentiation and cell death (Matheson et al., 2005). Hyalectins, another group of proteoglycans, include aggrecan and versican, and are associated with hyaluronan and lectin in the ECM (Iozzo, 1998). The third group of proteoglycans include those that make up the basement membrane proteoglycans such as perlecan (Iozzo, 1998).

Glycoproteins have a central protein core attached mono or oligosaccharides (Bartold, 1991). Functions of glycoproteins include: cell adhesion, migration, embryonic development, wound healing, organization and assembly of the matrix as well as the regulation of blood clotting. Glycoproteins involved in wound healing of the gingival tissues include fibronectin, vitronectin, elastin, and tenascin.

In order for the cells of the connective tissue as well as the cells of the epithelium to receive information about their extracellular environment and the surrounding cells molecules termed integrins are essential.

1.1c Integrins

Integrins are heterodimeric transmembrane glycoproteins that attach cells to ECM or ligands on other cells (Miyamoto et al., 1998). Integrins contain large (α) and small (β) subunits and function in direct cell to cell recognition and interactions with ECM (Albelda et al., 1990). Integrins have binding sites for divalent cations Mg$^{++}$ and Ca$^{++}$ and these are necessary for their adhesion and migration functions. Integrins bind to matrix proteins, such as fibronectin, vitronectin, collagens, laminin and fibrinogen (Hakkinen et al., 2000).

Integrins are expressed by both epithelial and connective tissue cells. Integrins expressed by resting epithelial cells include β1 (associated with α2, α3, α6), and β4 (α6) integrin subunits.
(Larjava et al., 2000). Quiescent fibroblasts within the connective tissue express different integrins including α1β1, α2β1, α5β1, and αv integrins such as αvβ3 (Hakkinen et al., 2000). During wound healing a variety of different integrins are expressed by these cells and will be discussed in detail subsequently.

1.2 Formation of the periodontal pocket leading to changes in the junctional epithelium and connective tissue

In healthy, there are macroscopic and microscopic differences between the types of gingival epithelia. Under conditions of disease these differences become even more exaggerated and further changes occur. With increasing bacterial loads adjacent to the sulcular and junctional epithelium the cells within these structures respond by producing pro-inflammatory cytokines, thereby producing a cascade of events leading to inflammation. Cytokines are soluble proteins secreted by cells that serve as signaling molecules for cell to cell communication. They are important in the regulation of the immune response as well as in growth and differentiation of cells. Cytokines with potent mitogenic activities are termed growth factors. Because of their central role in inflammation they can be detected in early wound healing as well as in periodontal disease.

Page and Schroeder have described the stages of early inflammation in great detail both histologically and clinically in 1976. Early gingivitis is characterized by PMN infiltration. PMNs are chemotactically attracted to the site of "invasion" by gradient of cytokines such as IL-8 and IL-1 as well as other factors such as microbial proteins (for example, formyl methionyl leucyl phenylalanine) as well as neutrophil produced leukotriene B4, and
complement factors (Page 1999; Page et al., 1997). Recently it has been suggested that polymorphisms in IL1 gene clusters contribute to increased susceptibility to gingivitis (Tatakis et al., 2005; Scapoli et al., 2005). During early inflammation, fewer adhesion molecules, such as intercellular adhesion molecule 1 and E selectin, are present in the cells of the junctional epithelium compared to the situation in healthy tissues. Additionally, as cytokines are expressed, the intercellular spaces between cells widen and inflammatory exudate passes into the sulcus (Pollanen et al., 2003). Specific regulation of cytokines by the immune system remains largely unclear although it is thought that there is a large genetic component to the regulation (Seymour, 2004). PMNs begin to pass into the gingival sulcus and start phagocytosing bacteria, helped in this function by antibodies and complement factors (Page 1999; Page et al., 1997). Although PMNs and macrophages aim to eliminate bacteria they do this at the cost of host tissue destruction (Altman et al., 1992). PMN hyper-responsiveness is thought to predispose some individuals to greater risk of progression to periodontitis (Kantarci et al., 2003). Changes in the connective tissue are evident 3-4 days after plaque accumulation, with destruction of collagen bundles near the source of infection (Page et al., 1997; Page et al., 1976). Unlike the collagen loss, there is little net change in the amount of proteoglycans in the area. This is thought to be due to the increased expression of chondroitin sulfate around the inflammatory area with a concomitant loss of dermatan sulfate. As the gingivitis lesion progresses to the "established lesion" there is increased neutrophil migration to the area through the junctional epithelium; however, plasma cell infiltration predominates (Page et al., 1997; Page et al., 1976). Also observed in the established lesion is an increased proliferation of the junctional epithelial cells. Thickening of the junctional epithelium in this way appears to be an attempt by the host to maintain a barrier against the
bacterial insult. Additionally, there is increased collagen loss and as this occurs, the pocket epithelium is no longer attached to the tooth surface (Page et al., 1997; Page et al., 1976). Compared to the original junctional epithelium this pocket epithelium is more permeable and in certain areas can be ulcerated.

Characteristics of the advanced lesion include apical migration of the junctional epithelium. It has been hypothesized that the apical migration is partly mediated by the secretion of collagenases by the basal cells leading to the destruction of the underlying matrix followed by rapid migration of the epithelial tissue (Page et al., 1997). What stimulates the changes from gingivitis to periodontitis is a question that has yet to be answered, although contributions to this disease process appear to be multifactorial in nature. In a 5 year study by Cullinan it was shown that a combination of bacteria, environmental factors (specifically smoking) and host factors (IL-1 positive genotype) contributed to the presence of periodontal disease in a population. Therefore, the end result of the interplay between host response, bacteria and environmental factors leads to a situation of a chronic wound in the region of the junctional epithelium with continued epithelial migration and destruction of surrounding host tissues over time.

1.3 Stages of gingival wound healing: general description

Wound healing occurs in a number of overlapping stages these include blood clot formation, inflammation, granulation tissue formation and tissue remodeling (Clark 1996). There are variations in each of these stages depending on the tissue examined, however a basic
overview will be presented here. Although each of the phases will be presented separately, there is overlap between each.

Initial healing occurs with the formation of a clot. Clotting is a result of activation of several factors including: Hageman factor, tissue procoagulant factor, surface membrane coagulation factors and phospholipids expressed on activated platelets and endothelial cells (Clark 1996; Furie et al., 1988). Subsequently, a blood clot forms which functions to provide hemostasis as well as a scaffold for further cell recruitment (Legrand et al., 1986).

Inflammation can be divided into early and late stages where early inflammation begins immediately after tissue injury and is associated with neutrophil rich infiltrate, and late inflammation which is associated with mononuclear cell rich infiltrates (Clark, 1996). Neutrophils, characteristic of early healing, arrive at the site of injury initially and function to debride the wound (Klebanoff, 1992). They are attracted to the site by fibrin, plasmin degradation products, C5a, leukotriene B4, platelet activating factor, and PDGF (Gimbrone et al., 1984; Tonnesen et al., 1984; Smith et al., 1979). A few days after the injury neutrophils can be found trapped within the wound clot and they persist in the area until epithelial coverage is re established. Cessation of early inflammation occurs at around three days when the neutrophils become trapped within the clot (Clark 1996), at which point any remaining neutrophils in the viable tissue outside of the clot are phagocytosed by macrophages during late inflammation (Newmann et al., 1982).

One day after wounding, the late inflammatory phase of wound healing occurs. This phase overlaps the early phase and is characterized by monocyte rich infiltrate (Clark, 1996). Monocytes are involved at the site of injury and similar to neutrophils, they are attracted to the site via chemoattractants such as TGFβ, fragments of collagen, fibronectin and elastin.
Monocytes change their phenotype to macrophages by adhering to extracellular matrix proteins. Once activated, macrophages release collagenase thereby debriding the wound site (Campbell et al., 1987). These cells are important in the transition between inflammation and repair of the injured site as well as an important source of growth factor in the later wound healing phases. Hours after wounding, epidermal cells first migrate and subsequently begin to proliferate, the stimulus for this action is thought to be derived from the free wound edges and the release of growth factors (EGF, TGFα, heparin-binding epidermal growth factor-like growth factor, and bFGF) and their receptors (Clark 1996). In order for epidermal cells to migrate they dissolve desmosomal and hemidesmosomal connections, retract intracellular tonofilaments and form actin filaments (Stenn et al., 1992). Once this is accomplished the epithelial cells are able to migrate across the wound. Migration is thought to be stimulated by the release of several growth factors included among these are TGFβ, EGF, bFGF and requires degradation of ECM proteins by proteases secreted by the migratory cells. There are two different theories describing the migration of cells across a wound. The leapfrog theory describes epithelial cells at the leading edge of the wound migrating a short distance but eventually become stationary at which time the successive epidermal cell passes over the stationary cell and continues migration in a chain like fashion (Stenn et al., 1992). Another theory that has been proposed involves epidermal cells moving across the wound in a sheet, with the cells at the leading edge pulling the cells behind them as they go (Stenn et al., 1992). Healing of wounds can differ depending on the depth of the wound (Clark, 1996). Those which destroy the basement membrane require that a provisional matrix be established before
re-epithelialization occurs. The provisional matrix is composed of fibrin, fibronectin, tenascin and vitronectin, all of which regulate migration of keratinocytes across the wound (Clark, 1996; Stenn, et al., 1992). If the basement membrane is intact then fibronectin infiltrates the existing basement membrane allowing for the migration of epidermal cells over the wound (Clarke, 1996; Stenn et al., 1992). The source of the fibronectin and fibrin in the provisional matrix is originally from the circulation. However, a few days following injury, wound fibroblasts, macrophages and migrating epidermal cells are able to produce cellular fibronectin (Clark, 1996).

Keratinocytes in particular, produce cellular fibronectin EIIIA and tenascin-C, both of which modulate cell movement (Hakkinen et al, 2000). Fibronectin is an important component of the provisional matrix in the wound and provides a means for cellular migration. There are different isoforms of cellular fibronectin EIIIA, B and V, each of which has different expression depending on the situation. For example EIIIB is expressed strongly in embryonic development and wounding but not in normal tissue (Hakkinen et al., 2000). Vitronectin is another ECM protein that is expressed in wound healing, its functions include adhesion and migration as well as mediation of cell invasion (Preissner et al., 1991). Tenascins are ECM proteins found beneath the epithelium that are upregulated during fetal development as well as wound healing (Mackie et al. 1988).

Granulation tissue is the first stage in the repair of the dermis and occurs approximately four days after injury (Clark, 1996). Induction of granulation tissue formation occurs via growth factors at the site of injury such as PDGF, TGFα, TGFβ and EGF like peptides (Singer et al., 1999). Fibroplasia and neovascularization are the two processes that lead to the formation of granulation tissue (Clark, 1996). Fibroplasia is the process by which fibroblasts produce
components of the ECM leading to the formation of granulation tissue. Fibroblasts migrate into the wound space producing fibronectin, vitronectin (Clark, 1996). Attachment of fibroblasts occurs with expression of $\alpha_4\beta_1$ integrin and occurs by way of the RGDS site (Chan et al., 1992) similarly expression of $\alpha_5\beta_1$ integrin impedes movement of the fibroblast along the matrix (Giancotti et al., 1990). Vitronectin, another component of the blood clot serves as a ligand for $\alpha v \beta 3$ and $\alpha v \beta 5$ expressed by wound fibroblasts, thus allowing them to adhere to the clot (Hakkinen et al., 2000, Gailit et al., 1996). Fibronectin and vitronectin therefore provides direction to the migration of the fibroblasts and they move along this surface by extending lamellipodia toward the wound site (Carter, 1970). Proteolytic enzymes such as plasminogen activator, collagenase MMP1, gelatinase MMP2, and MMP3 stromelysin, also aid in the migration of the fibroblasts by cleaving a path (Mignatti, et al., 1996). Once at the wound, the fibroblasts switch phenotype, become stationary, and begin to produce collagens. The stimulus for this activity is thought to be a result of TGF$\beta 1$ and IL-4 released by macrophages and other cells in the vicinity, resulting in the production of type I and III collagen as well as fibronectin (Clark et al., 1995). Collagen production eventually ceases, as apoptosis of fibroblasts begins to occur by day ten. There is a decreased cellularity of the scar tissue at this time (Clark, 1996). Neovascularization is the second process by which granulation tissue is formed at the wound site and occurs approximately two days following injury (Clark, 1996). Endothelial cells begin to rapidly proliferate and cells enter the perivascular space. This process is dependant on the expression of $\alpha v \beta 3$ by the endothelial cells that binds to fibronectin allowing the cells to migrate into the wound site (Clark, 1996). Tissue remodeling is the final stage of wound healing and is the transition from the provisional matrix to the scar tissue. Similar to the previous phases of wound healing there is
overlap in the time frame of tissue remodeling with the previous phases of repair. As previously mentioned the initial provisional matrix is a plasma derived fibrin clot. Degradation of the fibrin clot by granulation tissue occurs by means of proteolysis at the leading edge of the granulation tissue (Saarialho-Kere et al., 1992). As the fibroblasts of the granulation tissue migrate into the wound site they produce fibronectin and are surrounded by hyaluronan (Clark, 1996). The fibronectin can be considered a second order provisional matrix and serves as a scaffold on which collagen deposition and fibril organization occurs. Hyaluronan is an important part of early granulation tissue and is thought to promote cell movement and adhesion. In addition to functions of cell migration, hyaluronan is also thought to promote cell division and the hyaluronan receptor is expressed on proliferating epithelial cells. The replacement of hyaluronan and fibronectin of the early granulation tissue by proteoglycans and collagen in the late granulation tissue indicates a transition from a matrix that promotes migration and proliferation to one that promotes increased wound strength and resiliency (Clark, 1996).

Proteoglycans are essential in the wound process, initially involved in the regulation of blood coagulation via heparin and heparin sulfate side chains of syndecan and serglycine (Clark, 1996). Subsequently, two weeks into wound repair, fibroblasts begin to produce chondroitin sulfate and dermatan sulfate containing proteoglycans. Chondroitin sulfate containing proteoglycans are thought to accelerate collagen monomer polymerization, and this proteoglycan is present in the granulation tissue but not in the mature scar. ECM proteoglycans are also important in the regulation of cell proliferation and some, such as versican, are thought to modulate cell migration. Levels of syndecan are also increased at the edges of the wound during granulation tissue formation, and are thought to modulate cell
movement. Recently, a study in rats done by Worapamoran (2002), found that the levels of syndecan-1 increased in cells of the fibrin clot, in fibroblasts and in cells close to the junctional epithelium. Later stages of healing exhibited increases in the level of syndecan-2 particularly in areas along the root surface.

Collagen is present primarily in the form of type III collagen in the granulation tissue, its presence in the wound is similar to that found during embryonic development and it is not found in such quantities in normal dermal tissue. There is a sequence of matrix deposition in the granulation tissue and this goes from fibronectin to type III collagen to type I collagen; with deposition of types III and I collagen increase toward day fourteen (Clark, 1996). Additional collagen types that are present in wound healing include types V collagen and VI collagen (associated with angiogenesis).

The final step in wound healing includes the process of wound contraction that follows the deposition of collagen. Wound contraction is mediated by the action of myofibroblasts that express α-smooth muscle actin filaments in their cytoplasm (Darby et al., 1990). Myofibroblasts are connected across the wound by gap junctions and with the contraction of the actin filaments they are able to initiate contraction across the wound, bringing the wound edges closer together (Singer et al., 1984). Myofibroblasts are attached to the matrix by means of the α2β1 integrin, in addition, the collagen is cross linked thereby providing a means by which traction can be applied across the wound (Hakkinen et al., 2000).

1.4 Molecules involved in wound healing

At the molecular level, integrins are central to wound healing. As previously mentioned these
signaling mediators (Hakkinen et al., 2000). These receptors function in cell adhesion during re-epithelialization, cell migration and growth as well as activation of growth factors such as TGFβ (Larjava et al., 2002). Integrins mediate cell adhesion on components of the ECM such as adhesion of keratinocytes and fibroblasts to fibronectin. The ECM of the wound is much different than that of the normal tissue proteins. For example, proteins such as fibronectin, vitronectin, tenascin, and type III collagens are found at the wound site where they would normally not be found or only found in limited quantities (Larjava et al., 1996). Additionally, growth factors and cytokines released during healing regulate integrin expression. Hence, different integrins are found in wound healing than would be found in normal tissues.

Keratinocytes express a number of new integrins including α5β1, α9β1, ανβ1, ανβ6 and ανβ5. Each of these integrins has a different ligand in the wound healing process (Hakkinen et al., 2000).

The ability of the keratinocytes to rapidly re-epithelialize the wound following injury depends on interactions with the ECM via integrins. Keratinocytes express integrins as they migrate along the provisional matrix of the wound. Some of these integrins are unique to wound healing and this might be partly related to the unique components of the basement membrane and provisional matrix upon which they travel. Laminin-5 is deposited by the keratinocytes and forms the provisional basement membrane (Larjava et al., 1993). Cells interact with laminin-5 by α3β1 and α6β4 integrins. These integrins are important in joining the basal keratinocytes to the basement membrane (Neissen et al., 1994).

Integrins with the β1 subunit are strongly expressed in wound healing. Integrin, α3β1, for example, is able to bind ligands in the provisional matrix. Unlike some integrins, α3β1 has the
ability to bind several different ligands including, type I and IV collagens, laminin, fibronectin, laminin-5 and nidogen (Graber et al., 1999).

The interaction of migrating keratinocytes with collagen is mediated through the α2β1 integrin, that interacts with fibrillar collagen types I, III, V, and VI (Hakkinen et al., 2000, Graber et al., 1999). In addition to its primary role in binding collagen it also serves to bind laminin (Graber et al., 1999). α2β1 has additional roles in migration by stimulating the expression of MMP-1 providing proteolytic activity needed for the keratinocytes to move easily through the matrix (Pilcher et al., 1997).

There are two keratinocyte integrins that have important interactions with fibronectin and these are α5β1 and αvβ6. Neither is expressed in normal tissue and each has separate functions in the process of wound healing. Integrin α5β1 is expressed by migrating keratinocytes and is thought to be involved in cell adhesion and migration along fibronectin as well as matrix production (Akiyama et al., 1989). Integrin αvβ6, on the other hand, is thought to be involved in the re-organization of the basement membrane as well as regulation of TGFβ activation (Hakkinen et al., 2000).

Vitronectin, is another ligand present in the provisional matrix which binds the αv integrins. It is thought that this particular ligand integrin interaction mediates the migration of cells during wound healing. Tenascins are another ligand in the wound matrix and integrins that bind this protein include α2β1 and αvβ6. Each of the integrins and their respective ligands used in the present study are outlined and discussed in detail.
1.4a Laminins and associated integrins

Laminins are a group of basement membrane proteins composed of 3 subunits: α, β and γ (Shimono et al., 2003). These proteins are found in the epithelial basement membrane and are thought to be involved in anchoring functions of the cell. Laminins contain a C-terminal domain that binds to cell surface receptors and is involved in cell signaling and anchorage. The N-terminal domain binds to extracellular matrix proteins securing the cell to the ECM (Aumailley et al. 2003). Specifically laminins anchor the keratinocytes to the basement membrane and in the case of the internal basal lamina acts as a link to the tooth surface (Hakkinen et al., 2000). It is important to note that there are two forms of laminin-5 that have apparently opposite functions, one form is the precursor type of molecule and one is the mature molecule (Nguyen et al., 2000). The precursor form is responsible for migratory functions and is deposited into the basement membrane by keratinocytes at the wound edge (Nguyen et al., 2000). In addition, this form of laminin is deposited by leading keratinocytes into the provisional basement membrane (Nguyen et al., 2000). This precursor form is thought to inhibit interactions between integrins which serve as receptors such as α6β4 and α3β1 thereby becoming an essential component of wound closure (Nguyen et al., 2000). The mature form of laminin-5 acts in adhesion of keratinocytes to the underlying basement membrane via its interaction with integrins α6β4 and α3β1 (Hakkinen et al., 2003). It is thought that α6β4 binding to laminin is responsible for hemidesmosome formation and this has been shown in animals and humans who have defects in one of the integrin subunits which results in epithelial blistering and neonatal death (Nguyen et al., 2000). Hemidesmosomes are responsible for binding of the quiescent epithelial cells to the basement
membrane (Nguyen et al., 2000). In addition, to the interaction between laminins and integrins at the epithelial basement membrane hemidesmosomes also serve an adhesive function at the tooth gingiva interface. There are similarities in the ligand and the receptor with respect to the attachment to the tooth surface. Specifically, the hemidesmosomal attachment is mediated by laminin-5, located in the internal basal lamina and integrin α6β4 located within the cell membrane of the DAT cell (Pollanen et al., 2003; Hormia et al., 1998). α3β1 integrin also binds to laminin and serves in cell-to-cell adhesion, specifically it has been shown to be involved in various types of gap junctions (Nguyen et al., 2000) including those between the cells in the junctional epithelium (Shimono et al., 2003). Also related to its function in cell adhesion α3β1 integrin is expressed by fibroblasts in the blood clot 3-5 days after wounding allowing them to adhere to fibronectin within the wound matrix (Larjava et al., 1993).

1.4b. Fibronectins and associated integrins

There are two different forms of fibronectin, one is produced by local cells and one is plasma derived. It is the cellular derivative that is of interest with respect to wound healing as well as fetal development (Yamada et al., 1996). Cellular fibronectin also has different splice variants including EIIIA, EIIIB and (VIIICS) (Yamada et al., 1996). Plasma derived fibronectin is an essential part of early clot formation, it also has importance in granulation tissue formation and the different splice variants of the cellular fibronectin have separate roles in wound healing (Hakkinen et al., 2000). Fibronectin EIIIA is deposited by migrating keratinocytes whilst they migrate through the wound matrix. In addition, it also triggers the release of
TGFB which in turn leads to the differentiation of fibroblasts into myofibroblasts ultimately leading to wound contraction (Hakkinen et al., 2000). Conversely, fibronectin EIIIB is expressed in granulation tissue formation and not expressed during migration of epithelial cells (Hakkinen et al., 2000).

α5β1 is an important receptor for fibronectin which is expressed by fibroblasts in the blood clot. This integrin binds the RGD sequence of fibronectin and is responsible for keratinocyte attachment to fibronectin as well as cell migration on fibronectin (Clark et al., 1996). To date it has not been associated with the junctional epithelium (Shimono et al., 2003). αvβ6 is another receptor for both types of cellular fibronectin and is thought to have a similar function to the α5β1 integrin (Clark et al., 1996).

1.4c. Tenascin

Tenascin-C is a large hexameric glycoprotein that is widely expressed in the extracellular matrix (Chiquet-Ehrismann et al., 2003). Several possible roles have been suggested for these proteins most of which center around regulation of stromal epithelial interactions (Chiquet-Ehrismann et al., 2003). Consequently, it has been suggested to have a critical role in wound healing events as well as neural crest morphogenesis and tumor metastasis (Mackie et al., 1999). Its key role in several functions was further suggested by its highly conserved sequence through vertebrates (Chiquet-Ehrismann et al., 1994a). However, initial tenascin-C knock out studies failed to provide support for this theory, with apparently normal development of knock-out mice (Mackie et al., 1999). After these initial knock-out studies took place a redundant role for tenascin-C was suggested (Erikson et al., 1993b). However, upon closer examination of these animals several abnormal behavioral characteristics were
noted (Fukamauchi et al., 1998b) as well as abnormal glial scarring which suggested a role for this protein in wound healing (Steindler et al., 1995). Further studies reported abnormalities in matrix organization in epidermal and corneal wounds from these knockout mice, and the suggestion has been made that tenascin-C influences either fibronectin expression or its maintenance in the wound matrix (Mackie et al., 1999). Also of interest is the observation that tenascin-C is strongly expressed in inflammatory skin conditions (Mackie et al., 1999). αvβ6 is a receptor for tenascin and fibronectin and its expression peaks in 7-14 days after healing and continues for at least 28 days (Hakkinen et al., 2000, Larjava personal communication).

1.4d. Cytokeratins

Cytokeratins participate in formation of intracellular skeleton of epithelial cells. Specifically the present investigation will focus on CK 16 and 19 expression at the junctional epithelium during healing. CK 16 is of interest in wound healing since it is thought play a role in keratinocyte activation following wounding of the dermis (Paladini et al., 1998). CK 16 is expressed at low levels constitutively in various different stratified epithelia including human oral epithelium (Moll et al., 1982). During wound healing CK16 is thought to play a role in suprabasal keratinocytes and its function is related to its COOH terminal domain (Paladini et al., 1998). Expression of CK 16 is observed between 4-6 hours post injury, making it one of the earliest detectable molecular changes in wounds (Paladini et al., 1998). Therefore it is induced well before cell migration begins (Wawersik et al., 2001). There has been some controversy as to the function of cytokeratin expression in wound healing with some authors
arguing that it has a role in keratinocyte activation (Paladini et al., 1998) and others stating that it appears to delay epithelialization, inhibit mitotic activity, and inhibit migration of keratinocytes (Wawersik et al., 2001). There have been reports of CK 16 expression in the human primary junctional epithelium (Shimono et al., 2003). Differences in expression of CK 16 between inflamed and "healthy" tissue varies from study to study with some studies finding no differences between the two tissue types (Sculean et al., 2001) and others finding increased expression following inflammation (and MacKenzie et al., 1993; Ouhayoun et al., 1990). In a study which included human and monkeys subjects, wounding of the junctional epithelium followed by a five month healing period resulted in no differences in cytokeratin expression between the normal and healed tissues (Sculean et al., 2001). Overall, however, CK16 expression is consistently observed with enhanced cell proliferation (O'Guin et al., 1993).

CK 19 is unique in that it is considered a consistent differentiation marker of the junctional epithelium (Shimono et al., 2003). In a human study examining wounding of the junctional epithelium CK 19 was found to be expressed similarly 5 months post wounding compared to normal junctional epithelium (Sculean et al., 2001). Inflammation can upregulate CK 19 expression (Mackenzie et al., 1997).

1.5 Healing at the tooth gingiva interface

Most of the studies which examined healing at the tooth tissue interface have focused on early healing events and consequently these will be discussed in the greatest detail (Wijesko et al., 1991a; Polson and Proye, 1983). Events of healing at the root surface although unique
in some ways, possess many similarities to those mentioned in dermal wound healing, such as initial fibrin clot formation. Taylor (1972) performed one of the first animal studies, using monkeys, examining the reattachment of the gingiva to the tooth. The following sequence of events was noted in this study: ten minutes after wounding, bacteria and debris were noted in the wound, after one day this debris and bacteria was reduced. Two days later the wound site filled with leukocytes and the beginnings of hemidesmosomes were found on the epithelial side of the wound. Three days after wounding, there is attachment at the apical portion of the wound in which hemidesmosomes are present. Attachment of the epithelium to the tooth occurs by five days. In a study by Polson and Proye (1983), early healing events were examined at the tooth gingiva interface by extracting teeth from monkeys and subsequently reimplanting them following debridement and citric acid treatment of the root surface. Healing was then observed at various time points. Formation of a fibrin clot was observed after one day that adhered to the root surface, and upon re-examination at three days this clot was disrupted and replaced by a long junctional epithelium. However, in the specimens which had been demineralized with the citric acid solution there was no junctional epithelium, instead the fibrin clot was still adherent to the root surface and there was no apical migration of the junctional epithelium. Based on the results of this study it would appear that two possible healing outcomes are possible at this site, one that involves healing by repair with a long junctional epithelium and the other which involves a tendency towards regeneration of the original attachment between the tooth and the gingiva. More in depth examination of early healing was performed in a study by Wijesko et al. (1991a), in which dentinal blocks were implanted into alveolar ridges of beagle dogs. These studies looked at specimens ten minutes after implantation and found plasma precipitate at the dentin-gingiva-interface
followed by a fibrin clot and neutrophils at six hours and maturation of the clot by three days. Neutrophils present migrated toward the dentin surface and there were strands within the fibrin clot that bound to the connective tissue as well as the dentin surface. Granulation tissue formation began at three days with the presence of macrophages and fibroblasts, and by seven days connective tissue began to replace the fibrin clot and attached to the surface of the dentin. The new collagen which formed at day seven was oriented parallel to the surface of the dentin and the authors suggest that the proximity of the collagen to the dentin surface and the orientation of the fibers serves as a means of adherence of the tissue to the tooth. In a follow up study (also using beagle dogs by Wijesko et al., 1991b), heparin was applied to the experimental sites in an attempt to disrupt the fibrin clot formation. This resulted in the formation of a long junctional epithelium as well as tissue recession. Other means of repair in a periodontally involved tooth have been proposed by Melcher (1976). Accordingly, the type of repair depends on the cells that populate the root surface during healing. As previously discussed, if cells from the gingival epithelium populate the root surface then a long junctional epithelium will result, where as if periodontal ligament cells are able to populate the wound then new connective tissue attachment occurs. Other proposed means of repair include connective tissue adhesion with root resorption and root resorption and ankylosis both of which occur if bone cells occupy the wound site. Animal studies (Selvig et al., 1988; Cole et al., 1980) which follow healing for more prolonged times suggest that new cementum formation occurs at three weeks after wounding. Microscopically, the connective tissue attachment is first observed parallel to the tooth and there is speculation that this adhesion is mediated in some way by fibronectin secreted by fibroblasts in the area (Grevstad, 1990). This collection of studies seem to suggest that healing at this site can occur by either repair or
regeneration and this is dependant on whether or not the fibrin clot is disturbed during the initial stages of healing. In most cases of the periodontally involved tooth, however, it would appear that repair rather than regeneration occurs (Caffesse et al., 1993).

In terms of wound healing at the junctional epithelium relatively little at the molecular level is known. Histological studies have characterized the cells that reform the junctional epithelium after gingivectomy in animal studies (Listgarten et al., 1973; Frank et al., 1972; Listgarten et al., 1972 a, b) and human studies (Stahl et al., 1972). However, little is known of the parallels that may exist between healing at the tooth surface and other areas of the oral cavity and body. Consequently, the expression of various integrins and their ligands at the junctional epithelium following wounding, would present novel information in the area of oral wound healing.
CHAPTER II

Aim of the study

The goal of the present investigation is to investigate the cellular mechanisms during the healing of periodontally diseased tissue at the tooth gingival interface. This would include investigation of the expression of cell adhesion molecules and their respective ligands as well as cytokeratins. It is hypothesized that healing at the tooth interface contains unique features with specific molecular markers different than those in other areas of the oral cavity and the body.
CHAPTER III
MATERIALS AND METHODS

3.1 Subjects
21 subjects (15 males and 6 females) were included in the present investigation and a total of 35 teeth were sampled. Age of the subjects ranged from 31 to 70 years of age with an average age of 50. Subjects were excluded if systemic conditions were such that wound healing may be compromised. Exclusion criteria included uncontrolled diabetes, heavy smoking, HIV, Hepatitis A, B, C, any hematological condition affecting healing, or any systemic condition related to poor wound healing. For a list of subjects and their demographic data see Table 1. Subjects were included in the study if they presented with hopeless teeth requiring extraction that could be feasibly extracted with gingival tissue attached. Two groups were included, a negative control group consisting of individuals with “healthy” periodontium and probing depths less than 3 mm, and a test group consisting of subjects who presented with probing depths and attachment loss greater than 4 mm.

3.1 i) Control subject protocol
Hopeless teeth were anaesthetized using 2% lidocaine with 1:100 000 epinephrine administered either through block anesthesia or through infiltration depending on the site of extraction. Using a number 15 blade a small portion of the buccal, lingual, mesial and/or distal gingiva was outlined to include the sulcular epithelium as well as a portion of the attached gingiva (Figure 1). Extraction of the tooth with the tissue sample attached
Table 1 Demographics of patient pool included in the investigation. NB: subject codes in bold print represent those who have more than one time point of healing assigned.
<table>
<thead>
<tr>
<th></th>
<th>Patient Code</th>
<th>Sex</th>
<th>Age</th>
<th>Group</th>
<th>Tooth number</th>
<th>Area harvested</th>
<th>Probing depths of the area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PK1 and PK2</td>
<td>M</td>
<td>52</td>
<td>Healthy sample</td>
<td>16 and 14</td>
<td>Both Buccal</td>
<td>3-4 mm</td>
</tr>
<tr>
<td>2</td>
<td>CC38</td>
<td>M</td>
<td>46</td>
<td>Healthy sample</td>
<td>38</td>
<td>Disto buccal</td>
<td>3 mm</td>
</tr>
<tr>
<td>3</td>
<td>SD 1-4</td>
<td>F</td>
<td>48</td>
<td>Healthy sample</td>
<td>14</td>
<td>Palatal</td>
<td>3 mm</td>
</tr>
<tr>
<td>4</td>
<td>JOC14</td>
<td>M</td>
<td>53</td>
<td>Healthy sample</td>
<td>14</td>
<td>Buccal</td>
<td>3 mm</td>
</tr>
<tr>
<td>5</td>
<td>GF26B</td>
<td>M</td>
<td>66</td>
<td>Healthy sample</td>
<td>26</td>
<td>Buccal</td>
<td>3-4 mm</td>
</tr>
<tr>
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<td>F</td>
<td></td>
<td>Healthy sample</td>
<td>25</td>
<td>Buccal</td>
<td>1-3 mm</td>
</tr>
<tr>
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<td>JW22B</td>
<td>M</td>
<td>38</td>
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<td>22</td>
<td>Buccal</td>
<td>1-3 mm</td>
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<td>M</td>
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<td>67</td>
<td>0 Day</td>
<td>21, 24, 33, 34</td>
<td>21, 33, 34 were buccal; 24 was palatal</td>
<td>6-9 mm</td>
</tr>
<tr>
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<td>M</td>
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<td>0 Day</td>
<td>46</td>
<td>Distal and buccal</td>
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</tr>
<tr>
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<td>F</td>
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<td>1 Day</td>
<td>27</td>
<td>Buccal</td>
<td>5 mm</td>
</tr>
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<td>M</td>
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</tr>
<tr>
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<td>F</td>
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</tr>
<tr>
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<td>5 mm</td>
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<td>M</td>
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<td>Mesial and buccal</td>
<td>6-8 mm</td>
</tr>
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<td>M</td>
<td>49</td>
<td>7 Day</td>
<td>22</td>
<td>Buccal and palatal</td>
<td>5 mm</td>
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<td>M</td>
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<td>26</td>
<td>Buccal and palatal</td>
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<td>AR27B</td>
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<td>40</td>
<td>28 Day</td>
<td>27</td>
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</tr>
<tr>
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<td>F</td>
<td>76</td>
<td>61 Day</td>
<td>43</td>
<td>Mesiobuccal, mesiobuccal</td>
<td>5 mm</td>
</tr>
<tr>
<td>AS23B, AS12B</td>
<td>AS23B, AS12B</td>
<td>F</td>
<td>49</td>
<td>Periodontal control</td>
<td>23 and 12</td>
<td>Buccal</td>
<td>5 mm</td>
</tr>
<tr>
<td>JS12B</td>
<td>JS12B</td>
<td>M</td>
<td>45</td>
<td>Periodontal control</td>
<td>12</td>
<td>Buccal</td>
<td>5 mm</td>
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</tbody>
</table>
to the tooth was carried out. Both tissue and tooth were then placed in tissue culture medium and labeled according to tooth number and area of tooth from where the gingival tissue was harvested (i.e. buccal, lingual mesial or distal). The site was curetted and post operative instructions were provided both written and verbally to the patient.

3.1 ii) Test subject protocol

Several different treatment groups were included in the test subject group. Patients were randomly assigned to each of these groups. However, circumstances in some cases did not allow for random selection and this was due to patient availability for appointment times. Based on the objectives of the study various healing time points were selected as well as positive controls (periodontitis group).

Subjects assigned to the periodontitis group had hopeless teeth with probing depths and attachment loss greater than 4 mm. A similar clinical protocol to the negative control group was performed. This included anesthesia of the tooth, outlining a portion of gingival tissue associated with the tooth, followed by extraction of the tooth with the attached tissue sample.

Subjects assigned to the 0 day wounding group had hopeless teeth that had probing depths and attachment loss greater than 4 mm. Teeth and associated tissues were anesthetized; followed by excision and curettage of the sulcus. Extraction of the tooth and attached tissue was then performed and the sample was placed in tissue culture medium. Individuals were assigned to one of the following test groups: 1 Day, 3 Day, 5 Day, 7 Day, 14 Day, 28 Day, or 61 Day post wounding. Two appointments were required for individuals selected to these groups. During the first appointment the tooth for extraction
Figure 1. Wound preparation and biopsy technique. A, removal of sulcular lining using a Nordland IB6900 blade; B, debridement of the tooth and pocket lining; C, Following healing period, outline of biopsy; D, removal of tissue fragment with tooth attached. Healing period of 0, 1, 3, 5, 7, 14, 28 or 60 days assigned randomly.
was anaesthetized. Excision of the sulcus lining was performed with a number 15 blade, followed by curettage of the sulcular tissues using a universal curette (Figure 1). There was an interval between the first and second appointment that corresponded to the healing time point group to which the subject was assigned. During the second appointment, extraction of the tooth and associated tissue sample was performed, as described by the protocol listed above. For each time point there were at least 2 subjects with the exception of the 61 day point in which there was only one. Often there was more than one tooth per patient per time point (Table 1). All of the procedures were approved by the Ethics Committee for Clinical Human Experimentation at the University of British Columbia.

3.2 Tissue Processing

Once tissues were stored in tissue culture medium they were processed as soon as possible following the extraction, with a maximum interval of six hours between harvesting and processing. Tissue was removed from the tooth using a chisel under a dissecting microscope in an attempt to preserve junctional epithelium. Tissue samples were then placed in Tissue TeKT™ (OCT compound, Sakura Finetek USA Inc., Torrance, California, USA) and snap frozen in liquid nitrogen. Care was taken with each sample to ensure orientation was consistent between different samples. Once frozen in liquid nitrogen, tissues were then placed in a −80°C freezer until sectioning of the tissues could be performed. Tissue specimens were sectioned between 6 μm and 8 μm using a cryostat. Sectioned specimens were mounted on coated microscope slides and stored at −80°C until staining could be commenced.
3.3 Staining Procedures:

Hematoxylin and Eosin (H&E) staining: Following sectioning of each sample one in every ten slides was stained with H&E and mounted using Entellan mounting medium (Harleco EM science, Newark, New Jersey, USA). Sections were then examined using a light microscope (Axiolab E, Zeiss, North York, Ontario) equipped with 4x, 10x, and 20x objective. Selected images were photographed using a Nikon Coolpix digital camera series 995 (Torrance, California, USA) that was attached to a Nikon Eclipse microscope (Torrance, California, USA) equipped with a 4x, 10x, and 20x objective. This allowed for evaluation of the general histology of the specimen and orientation of the sample. Appropriate orientation of the specimen, visualization of the junctional epithelium, absence of tearing or damage to the specimen, were all criteria used to further select slides for immunohistochemical staining.

Immunohistochemical staining and analysis: Localization of cytokeratins 16, 19, laminin-5, integrins β1, β4, β6, α5, α3, type IV collagen, tenascin-C and fibronectin EDA was undertaken (for a comprehensive list of the antibodies and their specifics see Table 2). Frozen sections were fixed with acetone (-20°C) for 5 minutes and individual sections were circled with grease pens and rinsed. Sections were then incubated in normal blocking serum (Vectastain; Vector Laboratories Inc., Burlingame, California) in a humidified chamber for 30 minutes at room temperature, then rinsed and incubated with the primary antibody of interest (Table 2). Negative controls were used for each antibody type and consisted of sections incubated with normal blocking serum without primary antibody. Sections were incubated with primary antibody diluted in phosphate...
Table 2: List of antibodies used, their dilution and their source.
<table>
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<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Animal</th>
<th>Mono or Polyclonal</th>
<th>Dilution used</th>
<th>Company or Source</th>
<th>Secondary Antibody used</th>
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<tbody>
<tr>
<td>Cytokeratin 19</td>
<td>A53-B/A2:sc-6278</td>
<td>Mouse</td>
<td>Monoclonal IgG1</td>
<td>1:800</td>
<td>Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA)</td>
<td>Vectastain biotinylated</td>
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<td>Laminin 5</td>
<td>GB3:ab1207</td>
<td>Mouse</td>
<td>Monoclonal IgG1</td>
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<td>Abcam Ltd. (Cambridge, Cambridgeshire, UK)</td>
<td>Vectastain biotinylated</td>
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<tr>
<td>Collagen type IV</td>
<td>MAB 1430</td>
<td>Rabbit</td>
<td>Polyclonal IgG1</td>
<td>1:50</td>
<td>Chemicon International Inc. (Pittsburgh, PA, USA)</td>
<td>Vectastain biotinylated</td>
</tr>
<tr>
<td>Fibronectin EDA</td>
<td>1 ST-9</td>
<td>Mouse</td>
<td>Monoclonal IgG1</td>
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<td>Accurate Chemical &amp; Scientific Corporation (San Diego, California, USA)</td>
<td>Vectastain biotinylated</td>
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<td>Tenascin C</td>
<td>TN2</td>
<td>Mouse</td>
<td>Monoclonal IgG1</td>
<td>1:400</td>
<td>Life Technologies (Gaithersburgh, MD, USA)</td>
<td>Vectastain biotinylated</td>
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<tr>
<td>Cytokeratin 16</td>
<td>CBL273</td>
<td>Mouse</td>
<td>Monoclonal IgG1</td>
<td>1:100</td>
<td>Chemicon International Inc. (Pittsburgh, PA, USA)</td>
<td>Vectastain biotinylated and Alexa Fluor 488 goat anti-mouse</td>
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<td>αvβ6 integrin</td>
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<td>Rabbit</td>
<td>Monoclonal IgG1</td>
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<td>MAB 16</td>
<td>Rat</td>
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<td>Donkey anti-rat</td>
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<td>MAB integrin 13</td>
<td>Rat</td>
<td>Monoclonal</td>
<td>1:800</td>
<td>Gift from Dr Yamada (Bethesda, Md, USA)</td>
<td>Donkey anti-rat</td>
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<td>β4 integrin</td>
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<td>Chemicon International Inc. (Pittsburgh, PA, USA)</td>
<td>Vectastain biotinylated</td>
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buffer saline (PBS) and bovine serum albumin (BSA, 1 mg/ml) 0.01% Triton X-100 overnight (16 hours minimum), and then rinsed and incubated with biotinylated secondary antibody for one hour. This reaction was followed by incubation with ABC avidin/peroxidase reagent (Vectastain Elite Kit, Vector Laboratories Inc., Burlingame, CA, USA). Sections were then rinsed and reacted with Vector VIP substrate peroxidase for a length of time between 30 seconds and 12 minutes. This reaction time was dependant on detection of color development in the specimen under light microscopy. The reaction time for each antibody was standardized during a staining session as follows: one antibody was stained at a time (usually the periodontitis specimen) and observed under the light microscope, once a reaction had occurred the timer was stopped and then other time points of that same antibody were stained for the same amount of time.

In order to stop the reaction the sections were rinsed with distilled water for a period of 10 minutes. Sections were then allowed to air dry and mounting of the sections took place using Vectamount permanent mounting medium (Vector Laboratories, Burlingame, California, USA). Stained and mounted sections were viewed under a light microscope (Axiolab E Zeiss, North York, Ontario) equipped with a 4x, 10x, and 20x objective. Select images were recorded using a Nikon Coolpix digital camera series 995 that was attached to a Nikon Eclipse microscope equipped with a 4x, 10x, and 20x objective. Immunofluorescent staining was also undertaken for cytokeratin 16 detection. Frozen sections were rinsed with acetone (-20°C) for 5 minutes, rinsed and incubated with a blocking solution of phosphate buffer saline (PBS, 10 mg/ml) and bovine serum albumin (BSA) 0.01% Triton X-100 solution for 30 minutes at room temperature. Sections were then incubated overnight at 4°C with a monoclonal antibody against cytokeratin 16.
Table 3: Staining intensity of each of the antigens as observed in various healing time points. +, mild intensity; ++, moderate intensity; +++ , strong intensity; - no staining.
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<th>L5</th>
<th>CK19</th>
<th>F-EDA</th>
<th>TN-C</th>
<th>Col IV</th>
<th>β1</th>
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7 Day Time Point

| Migrating tip           | +++| +++| +++| +++| ++  | +++  | ++  | ++  | +++| 
| Suprabasal epithelial cells | - | +  | -  | -  | +    | +    | +   | +   | +  | +  |
| Basal epithelial cells adjacent to the tissue | + | +++| -  | -  | +    | +++  | +   | +   | +  | +  |
| Basal epithelial cells adjacent to the tooth | + | +++| -  | -  | +    | +++  | +   | +   | +  | +  |
| EBL                     | +++| -  | +++| ++ | +++  | +++  | ++  | -   | +  | +  |
| IBL                     | +++| -  | +++| +++| +++  | +++  | +   | +   | +  | +  |
| Blood vessels           | -  | -  | ++ | +  | +    | -    | -   | -   | -  | -  |
| Intracellular staining of epithelial cells | - | +  | -  | -  | -    | +    | -   | +   | -  | -  |

14 Day Time Point

| Migrating tip           | +++| +++| ++ | +++| +++  | +++  | +++  | +++  | +++| 
| Suprabasal epithelial cells | - | +++| -  | +  | ++   | +    | ++   | ++   | ++ | ++ |
| Basal epithelial cells adjacent to the tissue | + | +++| -  | +  | ++   | +++  | +    | +    | +  | +  |
| Basal epithelial cells adjacent to the tooth | + | +++| -  | -  | +    | +++  | +    | +    | +  | +  |
| EBL                     | +++| -  | +++| ++ | +++  | +++  | +    | +    | +  | +  |
| IBL                     | +++| -  | +++| +++| +++  | +++  | +    | +    | +  | +  |
| Blood vessels           | -  | -  | ++ | +  | +    | -    | -   | -   | -  | -  |
| Intracellular staining of epithelial cells | - | +  | -  | -  | -    | +    | -   | +   | -  | -  |

28 Day Time Point

| Migrating tip           | +++| +++| ++ | +++| +++  | +++  | ++   | +++  | +++| 
| Suprabasal epithelial cells | - | +++| -  | -  | +    | +++  | +    | +    | +  | +  |
| Basal epithelial cells adjacent to the tissue | + | +++| -  | -  | +    | +++  | +    | +    | +  | +  |
| Basal epithelial cells adjacent to the tooth | + | +++| -  | -  | +    | +++  | +    | +    | +  | +  |
| EBL                     | +++| -  | +++| ++ | +++  | +++  | +    | +    | +  | +  |
| IBL                     | +++| -  | +++| +++| +++  | +++  | +    | +    | +  | +  |
| Blood vessels           | -  | -  | ++ | +  | +    | -    | -   | -   | -  | -  |
| Intracellular staining of epithelial cells | - | +++| -  | -  | +    | +++  | +    | +    | +  | +  |

High intensity staining +++
Moderate ++
Low +
No staining or inflammation -
diluted in PBS/BSA triton X-100 0.01%. Sections were then rinsed and incubated with fluorescent conjugated secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (Invitrogen Detection Technologies, Eugene, Oregon, USA) for 1 hour at room temperature in darkness. Specimens were rinsed again in darkness and then mounted using ImmunoMount™ medium (Thermo Shandon, Pittsburgh, PA, USA). Mounted sections were viewed under fluorescent microscope (Zeiss Axioplan2, North York, Ontario, Canada) and images were captured using a QICam™ camera (Burnaby BC, Canada) mounted to the microscope and fed directly to the Northern Eclipse™ software program (Haverhill, Suffolk, UK). Two separate investigators viewed all immunostained specimens, and staining was assessed as mild, moderate or intense. See Table 3 for detailed results of staining intensities.
CHAPTER IV
RESULTS

4.1 Clinical healing

There were no post operative complications observed in any of the subjects included in the study. Healing at each of the time points recorded appeared uneventful from a clinical perspective. There was no drop out from the study in the test subjects.

4.2 Histology

As described in the materials and methods section samples from each of the specimens were stained with hematoxylin and eosin. Healthy controls were considered “healthy” periodontally and the periodontitis patients were considered to have increased attachment loss beyond which was considered “healthy”, which included probing depths and attachment loss greater than 4 mm. Specimens from normal gingiva contained oral, sulcular, and parts of the junctional epithelium as judged by morphology. Periodontitis specimens contained oral epithelium and some pocket epithelium together with junctional epithelium. Pocket epithelium was defined as epithelium facing the tooth with elongated rete ridges (Figure 2A&B). Specimens representing 0 day time points exhibited no visible sulcular or junctional epithelia (Figure 2C), indicating that the pocket wall was successfully removed by the curettage method. Fibrin clot is present between the sulcular tissue and where the tooth would be in both the 1-and 3-day-wound samples (Figure 2E). By day 5 the fibrin clot was no longer observed between the tooth and the healing tissue and the epithelium showed remarkable migration along the wounded surface (Figure 2F).
Figure 2: Histology of healing wounds at tooth-gingiva interface (H&E staining). A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day-old wound; E, 3-day-old wound; F, 7-day-old wound; G, 14-day-old wound; H, 28-day-old wound; Fibrin clot is denoted as FC; junctional epithelium is denoted as JE; and pocket epithelium is denoted as PE.
From day 1 to 28 there is continued apical migration of the epithelial cells that line the sulcus and tooth (Figure 2D-H arrowheads). Inflammatory cells were present below the epithelium in all wounded specimens (Figure 2 F&G).

4.3 Immunohistochemistry

4.3a) Expression of cytokeratin 19

Healthy control samples stained intensely for CK 19 along the cells of the sulcular and junctional epithelium (Figure 3A). Basal cells of the oral epithelium were moderately stained in random areas (Figure 3A small arrows). Sulcular/junctional epithelium in the periodontitis samples was also CK 19 positive. Furthermore, basal cells of the oral epithelium also expressed CK 19 in generalized pattern. In the 0-day samples CK 19 was expressed in the basal keratinocytes of the tip of the gingival margin where its expression was restricted to a few basal cells at the tips of the rete ridges of the oral epithelium. Basal cells of the oral epithelium within the periodontitis specimen appeared to have a wider expression than that observed in healthy samples (Figure 3B small arrows). This “expanded” expression of CK 19 within the oral epithelium was consistently observed between 1 and 7 days post wounding (Figures D-F arrows) and by 14 days, CK 19 expression was similar to that observed in the healthy sample. In 1-day-old wounds CK 19 expression changed considerably from that of the healthy specimens. Suprabasal staining was mild to absent in most areas of the sulcular epithelium (Figure 3D). Intense staining was observed at the migrating tip as well as in the basal epithelial cells facing the tooth and tissue (Figure 3 D). In 3-day-old wounds, moderate suprabasal staining was
present primarily in the region of the migrating tip of sulcular epithelium (Figure 3E inset). Basal cells facing the tooth and tissue sides in the migrating tip continued to be intensely stained. In 7-day-old wounds the staining intensity of CK 19 along the sulcular/junctional epithelium and basal cells of the oral epithelium had returned to those observed in healthy specimens (Figure 3A&F small arrows). When CK 19 expression was compared to wounded palatal oral epithelium (data not shown) it was noted that in the palatal gingiva, the epithelium had only a few cells in the tips of rete ridges expressing CK 19 suggesting that keratinocyte phenotypes in these two wounds within a few mm apart are considerably different.

4.3b) Expression of β1 integrins

Expression of β1 integrins in the healthy specimen was most intense in the keratinocytes facing the putative IBL (Figure 4A arrows). There was also moderate staining present in the basal epithelial cells facing the EBL as well as the suprabasal cells of the sulcular and oral epithelium (Figure 4A small arrows). There did appear to be intercellular staining visible in some of the basal and suprabasal cells. The most intense staining was present at the apical portion of the sulcular/junctional epithelium. Expression of β1 integrins in periodontitis specimens was similar to that in the healthy gingival sample (Figure 4B). In 0-day specimens, β1 integrins were expressed at the basal surface of basal keratinocytes as well as in endothelial cells surrounding the blood vessels (seen in all samples). In addition, connective tissue fibroblasts showed moderate β1 integrin expression in all specimens. In 1-day old wounds β1 integrin was expressed at the migrating tip of
epithelium (Figure 4D arrows). Most expression was confined to the basal aspect of basal keratinocytes and between the cells (Figure 4D inset). Mild to moderate expression of β1 integrin was also seen in cells in the connective tissue and migrating keratinocytes of the 3-day-old wound continued to express β1 integrin observed at the migrating tip of epithelium (Figure 4E). At a distance from the tip there is intense expression in the basal cells facing both the tissue and the tooth (Figure 4E arrows). Some of β1 integrin expression, especially that in the basal cells closest to the tooth appears to be intracellular (Figure 4E inset). In 7-day-old wounds, β1 integrin expression is largely normalized, with intense staining of the basal cells facing the tooth and tissue (Figure 4F).

4.3c) Expression of β4 integrin

The greatest intensity of β4 integrin expression in the healthy and periodontitis specimens was present in the basal cells facing the tooth or IBL (Figure 5B small arrow). Intense staining was also observed in the cells facing the EBL. β4 integrin was also strongly expressed by the basal keratinocytes of the oral epithelium and endothelial cells surrounding the blood vessels. In each of the healing time points β4 was always present underneath the migrating keratinocytes. The most intense staining for β4 integrin was, however, at a distance from the migrating tip of epithelium (Figure 5D-F arrows). 1 day post wounding, there is moderate staining of the basal cells of the migrating epithelium (Figure 5D). However, there is very little suprabasal staining at the tip of the migrating epithelium unlike the healthy and periodontitis specimens. Similarly at 3 days there is still a portion of the suprabasal cells at the tip of the migrating epithelium in which staining is
Figure 3. Immunohistochemical localization of cytokeratin 19. A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day old wound; E, 3-day-old wound; F, 7-day-old wound; fibrin clot is denoted as FC; connective tissue is denoted as CT; oral epithelium is denoted as OE, pocket epithelium is denoted as PE; sulcular epithelium is denoted as SE; arrowheads denote wound edge; small arrows denote intense area of staining in the oral epithelium.
Figure 4. Immunohistochemical localization of Beta 1 integrin. A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day-old wound; E, 3-day-old wound; F, 7-day-old wound; Arrows denote increased intensity of staining in particular healing time points. Fibrin clot is denoted as FC; connective tissue is denoted as CT; oral epithelium is denoted as E.
Figure 5  Immunohistochemical localization of integrin β4. A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day-old wound; E, 3-day-old wound; F, 7-day-old wound; G, 14-day-old wound; H, 28-day-old wound; Fibrin clot is denoted as FC; connective tissue is denoted as CT; oral epithelium is denoted as E; small arrows indicate areas of intense staining; large arrow in the 1-day-old wound indicates absence of β4 expression.
absent (Figure 5E large arrow). By 28 days the pattern of staining returns to that of the healthy control specimens (not shown).

4.3 d) Expression of β6 integrin

In all specimens with the exception of the 0 and 1 day time points showed β6 integrin showed expression along the cells of the junctional epithelium only (Figure 6A,B,E,F). Suprabasal cells of the junctional epithelium in the healthy specimen and periodontitis specimen expressed β6 integrin moderately, and the greatest intensity of staining is observed in the basal epithelial cells facing the tooth IBL (Figure 6A&B). Additionally, this expression was strongest coronal to the most apical portion of the sulcular epithelium (Figure 6B small arrows). In 1-day-old wounds there is no expression of β6 integrin apparent in the specimen with the exception of non specific staining of the fibrin clot (Figure 6D). In 3-day-old wounds, β6 integrin is expressed by the migrating keratinocytes with the strongest expression in cells facing the wound bed (Figure 6E). In 7-day-old wounds expression of β6 integrin in the junctional/ wound epithelium was returned to the level at the unwounded healthy tissue(Figure 6F).

4.3 e) Expression of α5 integrin

Increased intensity of staining for α5 integrin is observed in the basal epithelial cells of the junctional epithelium facing the tooth in the healthy and periodontitis specimens (Figure 7A&B arrows). Moderate staining of the basal epithelial cells facing EBL is also
present in these specimens. In 1-day-old wounds α5 integrin was expressed coronal to the tip there is basal and suprabasal cells at the very tip of the migrating epithelium were less intensely expressed (Figure 7D arrows). In 7-day-old wounds the expression of α5 integrin had returned baseline levels observed in the healthy and periodontitis specimen (Figure 7F). In all specimens, endothelial cells surrounding the blood vessels also expressed α5 integrin.

4.3 f) Laminin-5 expression

Laminin-5 was intensely expressed in the junctional epithelium in all specimens (except time point 0). Laminin-5 expression in both the IBL and EBL of the junctional epithelium. Migrating keratinocytes in 1-day-old wound expressed laminin-5 against the wound bed (Figure 8C). In 3-day-old wounds laminin-5 was expressed by migrating keratinocytes facing the connective tissue side as well as those facing the fibrin clot (i.e. staining surrounded the entire migrating epithelial tip). In 7-day-old wounds, laminin-5 expression in the regenerating junctional epithelium mimicked that seen in unwounded specimen (Figure 8 F). However, there was some expression of laminin-5 also in the connective tissue underneath the epithelial tip (Figure 8F).

4.3 g) Tenascin-C:

Generalized staining for tenascin-C, was present in the connective tissue of all specimens with most intense staining along the basement membrane of both the oral epithelium as
Figure 6. Immunohistochemical localization of integrin of beta 6. A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day-old wound; E, 3-day-old wound; F, 7-day-old wound; G, 14-day-old wound; H, 28-day-old wound; Fibrin clot is denoted as FC; arrows indicate areas of intense staining; arrowheads indicate wound edge; CT denotes connective tissue and E denotes oral epithelium.
Figure 7. Immunohistochemical localization of integrin α5. A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day-old wound; E, 3-day-old wound; F, 7-day-old wound; Fibrin clot is denoted as FC; connective tissue is denoted as CT; oral epithelium is denoted as E; arrows indicate areas of intense staining; arrowheads indicate wound edge.
Figure 8. Immunohistochemical localization of laminin-5. A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day-old wound; E, 3-day-old wound; F, 7-day-old wound; fibrin clot is denoted as FC; connective tissue is denoted by CT; oral epithelium is denoted by E; arrows indicate areas of intense staining; arrowheads.
Figure 9. Immunohistochemical localization of tenasin-C. A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day-old wound; E, 3-day-old wound; F, 7-day-old wound; fibrin clot is denoted as FC; E denotes epithelium; CT denotes connective tissue; arrows indicate areas of intense staining.
well as the sulcular/junctional epithelium (Figure 9A-F). In the healthy tissue and the periodontitis specimens staining was absent from the suprabasal cells. In all specimens there was moderate to intense staining of the deep connective tissues and intense staining of the blood vessels (Figure 9A-F). In 1-day-old wounds there was no staining at the tip of the migrating epithelium, however just coronal to the tip there was staining of the EBL and adjacent connective tissue (Figure 9D small arrows). In 3-day-old wounds, tenascin-C was expressed underneath the migrating keratinocytes at the tip of the epithelium (Figure 9E small arrows). In 14-day-old wounds, expression of tenascin-C resembled unwounded specimen (Figure 9F). In some areas, tenascin-C was also expressed in keratinocytes facing the tooth (Figure 9F).

4.3 h) Expression of fibronectin EDA

Fibronectin EDA was expressed throughout the connective tissue of all specimens with greatest intensity proximal to the apical portion of the sulcular/junctional epithelium (Figure 10A-F small arrows). Mild staining was observed in the connective tissue side directly beneath the migrating epithelial tip (Figure 10 E arrowhead) in 3-day-old wounds. In 7-14-day-old wounds, fibronectin EDA was expressed in the area just apical to the migrating tip as well as underneath keratinocytes facing the regenerating IBL and EBL (Figure 10 F small arrows). By 14 days the pattern and intensity of staining had returned to that observed in the wounded healthy specimens (not shown).
4.3 i) Expression of type IV collagen

In all specimens type IV collagen staining decorated the basement membrane areas of the epithelia and the blood vessels. There was no expression of type IV collagen in the migrating tip (Figure 11D&E arrowhead) at the 1 and 3-day-old wounds. In 7 and 14-day-old wounds, type IV collagen was expressed by basal keratinocytes at the small distance of the migrating epithelial tip but never by the advancing cells at the tip (Figure 11G). No type IV collagen was found in any specimens facing the tooth.

4.3 j) Cytokeratin 16 Expression

Migrating wound epithelium was CK 16 positive in all specimens suprabasally in all time points (not shown).
Figure 10. Immunohistochemical localization of Fibronectin EDA. A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day-old wound; E, 3-day-old wound; F, 7-day-old wound; fibrin clot is denoted as FC; arrows indicate areas of intense staining; E denotes epithelium and CT denotes connective tissue; arrowhead indicates tip of migrating epithelium.
Figure 11. Immunohistochemical localization of type IV collagen. A, Unwounded clinically healthy gingiva; B, Periodontal pocket tissue; C, gingival specimen immediately after surgical curettage; D, 1-day-old wound; E, 3-day-old wound; F, 7-day-old wound; G, 14-day-old wound; H, 28-day-old wound; FC denotes fibrin clot; E denotes epithelium and CT denotes connective tissue; arrows denote areas of increased staining intensity; arrowheads denote migrating tip of epithelium.
CHAPTER 5

DISCUSSION

Wound healing at the tooth tissue interface has important clinical implications with respect to regeneration of attachment to previously diseased teeth. An understanding of healing at this site will provide mechanisms by which improvements in regenerative capabilities can be achieved. Recently several studies have characterized integrins and ECM components which are involved in re epithelialization of gingival wounds (Hakkinen et al., 2000). Due to the speed of cell migration of keratinocytes compared to other periodontal cells true regeneration of the tooth gingiva interface is difficult. One of the future possibilities in this field would be to target these integrins which are involved in keratinocyte migration and proliferation, with antibodies or other biomolecules in order to delay their function and hence provide time for regeneration of the connective tissue attachment rather formation of long epithelial attachment to the root surface.

Most studies related to wound healing and regeneration of the periodontal attachment have been performed on animal models (Wikesjo et al., 1991a; Polson et al., 1983) and involve teeth which weren’t previously affected by periodontal disease. This is an important consideration since the vast majority of periodontal patients requiring periodontal regenerative treatment have root surfaces which were previously disease affected. Polson and Proye (1983), examined early wound healing of the dentin-connective tissue interface in
monkeys. The protocol involved extraction and reimplantation of teeth (with or without
demineralized with citric acid), and subsequent observation of healing events for 1, 3, 7 and
21 days. After day one, there was evidence of a fibrin clot between the tooth and the tissue
and this was retained until three days in the demineralized specimens, after which point the
clot was disrupted and a long junctional epithelium lined the root surface. It is critical to
realize the differences between wound healing at the tooth gingiva interface and in epidermis
or palatal mucosa. In the epidermis or in the full thickness palatal wound, the fibrin clot
bridges the gap between the two identical wound margins. However, in the case of the
gingival wound at the tooth interface the fibrin clot bridges a gap between two dissimilar
tissues, the tooth and the gingiva. If the fibrin clot is stabilized to the tooth exposing tissue
fibers by acid demineralization it appears to prevent downgrowth of the junctional epithelium
(Polson et al., 1983). However, multiple human studies did not appear to confirm these
animal studies and therefore, demineralization of the root surface is not sufficient to prevent
the formation of a long junctional epithelium. In another study examining early wound
healing events, Wikesjo et al. (1991a) examined healing in beagle dogs after full thickness
flaps were reflected in edentulous sites and dentin blocks were implanted in surgically created
sites in the bone. Tissue was examined 4 and 6 hours as well as 1, 3, and 7 days post
surgically under light and electron microscopy. At the microscopic level healing at the tooth
surface seemed to emulate epidermal healing with fibrin clot bridging the space between the
two tissues between 1 and 3 days, along with the presence of fibroblasts and macrophages in
the area. By 7 days parts of the fibrin clot were replaced by connective tissue which adhered
to the dentinal surface as well. It has been speculated (Polson, 1994) that the stability of the
initial fibrin clot is essential in the regeneration of attachment along the periodontal wound.
In effect, the breakdown of the clot results in apical migration of the epithelium along the tooth surface of the periodontal wound (Polson 1994). In the present investigation, light microscopic views of hematoxylin and eosin stained human periodontal wounds at various time points illustrate what would appear to be the presence of a fibrin clot at 1 day and 3 day time points but not beyond this time point (Figure 2D&E). The presence of a long junctional epithelium was observed at 5 days and beyond (Figure 2). The migration speed of keratinocytes at the tooth gingiva interface seemed to be subjectively faster than in palatal wounds (Larjava et al., 1993; Habijanac et al., unpublished) although no quantitative comparisons were made. In palatal wounds 2mm wounds are re-epithelialized between 5-7 days from both wound edges (Hakkinen et al., 2000; Larjava et al., 1993). In gingival wounds at the tooth interface, epithelial cells show early migration (already by day one) and in 5 days the epithelium has migrated considerably longer than in palatal wounds although migration proceeds from one wound edge only. The reason for the faster migration at the tooth gingiva interface remains to be unraveled but may involve different repertoire of cytokines and also cell phenotypes. Both of these observations support the findings of the animal studies previously mentioned (Wikesjo et al., 1991a; Polson et al., 1983).

\( \alpha v \beta 6 \) is an integrin which is expressed during wound healing and is usually not present constitutively in the non healing epidermis or attached gingiva (Hakkinen et al., 2004, Hakkinen et al., 2000). Interestingly, in the present study \( \alpha v \beta 6 \) integrin was expressed in normal junctional/sulcular epithelium confirming previous findings in our laboratory (Garcia et al, unpublished). Integrin \( \alpha v \beta 6 \) could regulate keratinocyte migration because its expression is upregulated in early wound healing and its capacity to bind both tenascin-c and
fibronectin EDA in the wound bed matrix (Hakkinen et al., 2000). Although αvβ6 is expressed in early wound healing, its strongest expression is observed with basement membrane formation and granulation tissue formation when the edges of the wound have come together (Hakkinen et al., 2000; Haapasalmi et al., 1996). Previous studies examining human wounds have observed upregulation of β6 integrin expression at day 3 in the migrating epithelium, and once the wound edges had approximated at day 14 the expression of β6 by the basal epithelial cells was downregulated (Clark et al., 1996). In the present study, similar to the aforementioned studies, expression of β6 integrin started in early wound healing at three days, in the basal cells (Figure 6E). However, unlike the previous animal and human studies staining continued indefinitely post wounding and also was present through the entire long junctional epithelium (Figure 6F). These findings combined with the presence of β6 integrin expression in the junctional epithelium of the healthy samples, suggests a unique role for β6 integrin wound situation in the epithelium adjacent to the tooth and could mimic a chronic non healing wound. One possible role beyond cell migration and adhesion could be the capacity of αvβ6 integrin to activate TGFβ1 (Hakkinen et al., 2000). By this mechanism, αvβ6 integrin could participate in regulation of gingival inflammation and be involved in IL-1 elicited inflammation. Further studies are necessary to evaluate the exact role of αvβ6 integrin in human junctional epithelium.

Fibronectin is a ligand for αvβ6 and is found in the basement membrane zone in wounds (Stenn et al., 1992). In vitro hypotheses suggest that in early healing serum fibronectin is bound by α5β1 and later in healing αvβ6 binds fibronectin resulting in the ability of keratinocytes to move across the provisional wound matrix. In the present study fibronectin
EDA was expressed underneath the migrating keratinocytes confirming previously published observations from palatal wounds (Larjava et al., 2002). These results suggest that fibronectin EDA could serve as a ligand for αvβ6 integrin in the regenerating junctional epithelium. Fibronectin is also a ligand for α5β1 integrin that is only expressed in keratinocytes during wound healing (Hakkinen et al., 2000; Larjava et al., 1993). A variety of functions have been proposed for α5β1 integrin including matrix assembly of fibronectin as well as migration and adhesion of keratinocytes on fibronectin (Hakkinen et al., 2000; Akiyama et al., 1989).

Presently we examined the expression of α5 as well as β1 and found that these integrin subunits are present in strongest intensity in the epithelial cells adjacent to the tooth during wound healing but also appears to be constitutively expressed by cells of unwounded junctional epithelium (Figure 12B). Previous findings have shown (Larjava et al., 1993) that migrating cells in wounded oral epithelium express α5β1 early in healing. However, the present study does not show strong expression of α5 or β1 early in healing (Figure 12D&E) but does show moderate expression later in healing paralleling the fibronectin expression as well as the β6 expression. Furthermore, the fact that α5 and β1 is expressed by the pocket epithelium in the control specimens (Figure 12B) provides support for the theory that the pocket epithelium acts as a chronic wound site with constant migration of new cells into the area, in part regulated by α5β1 integrin and its ligand fibronectin.

Tenascin-C, a large extracellular matrix glycoprotein thought to be involved in the regulation of epithelial-mesenchymal interactions. The location of tenascin-C in adult tissues is generally in the stromal tissue directly beneath the basement membrane zone (Crossin 1996). Indeed the results of the present study illustrate more intense expression of tenascin-C in the papillary connective tissue below the basement membrane in all samples, compared to other
areas within the connective tissue. During wounding there is increased expression of tenascin (Latijnhouwers et al., 1996; Luomanen et al., 1993; Mackie et al., 1988). Previous studies examining animal tissue (Mackie et al., 1988) and human tissue (Hakkinen et al., 2000b; Haapasalmi et al., 1996) have found deposition of tenascin-C just below the migrating tip in early wound healing. The most intense expression of tenascin-C in the present study was ahead of the migrating tip of epidermal cells in the 3-and 7-day wounds. After this time period expression was intense along the stromal aspect of the basement membrane. Moderate staining intensity was observed adjacent to the tooth side of the long junctional epithelium in the 7 and 14 day wounds (Figure 9F&G). In the present study the expression of β1 integrin appears to occur at similar healing time points to tenascin-C and in similar areas. This finding support results of previous studies examining oral wounds that find patterns of co localization of β1 integrins and tenascin-C (Hakkinen et al., 2000b; Haapasalmi et al., 1996; Larjava et al., 1993). Therefore, tenascin-C may serve as a ligand for β1 integrins on migrating epithelial cells in the junctional epithelium. Another potential integrin which uses tenascin-C as a ligand during wound healing is αvβ6 integrin. As suggested previously for palatal wounds it is possible that tenascin-C could serve as a ligand for αvβ6 integrin during the formation of long junctional epithelium as they are co-expressed spatio-temporally in these wounds. Epithelial β1 integrins could bind a variety of ligands including laminins, tenascin-C, fibronectin (Yamada et al., 1996, Larjava et al., 1996). These integrins are found in the keratinocytes of the skin as well as oral epithelium (Larjava et al., 1996). Among β1 integrins that are expressed by keratinocytes, α2β1 can bind to collagen and laminin-5, α3β1 to collagens, laminin-1 and fibronectin, and α5β1 to fibronectin (Larjava et al., 1996). β1 integrins are strongly expressed in wound healing (Larjava et al., 1993; Hertle et al., 1992)
suggesting their importance in re epithelialization of wounds. Upon wounding, integrin expression changes at the wound margin and there is suprabasal integrin expression as well as production of integrins which specifically recognize proteins of the provisional wound matrix (Hertle et al., 1992). The importance of β1 integrins has been illustrated in knockout studies in which β1 integrin-null mice exhibit delayed re-epithelialization of wounds (Grose et al., 2002). Histologically, β1-null mice exhibited no migrating epithelial tongue. In addition, the keratinocytes were increased in number at the wound edges (Grose et al., 2002). One of the ligands for β1 integrins is laminin-5, and it would appear that β1 is required for correct localization of laminin-5 in the basement membrane during healing (Grose et al., 2002, Brakebusch et al., 2000). In addition, β4 integrin exhibited irregular staining in the β1-null mice 5 days post wounding (Grose et al., 2002). These results suggest that one of the key roles of β1 integrins in re-epithelialization following wounding is that of epithelial migration. In the present study, the pattern of β1 integrin expression possibly reflects the migratory activity of cells at various time points post-wounding in the human pocket epithelium

α6β4 integrin is a major component of hemidesmosomes that attach basal cells to the basement membrane (Sonnennberg et al., 1991; Stepp et al., 1990). α6β4 interacts with laminin-5 as well as bullous pemphigoid proteins to attach keratinocytes to the basement membrane (Uitto et al., 1992; Rouselle et al., 1991). Attachment of the junctional epithelium to enamel is mediated by this complex and therefore individuals with mutations coding this complex have severe cutaneous skin disorders as well as early onset periodontal disease (Uitto et al., 1992). During wounding α6β4 integrin underneath migrating keratinocytes is present. The distribution is stimulated from hemidesmosomes, to diffuse plasma membrane location and is thought to mediate attachment to laminin-5 during migration of epithelial cells
(Kurpakus et al., 1991). In the present study β4 integrin was present underneath the migrating keratinocytes suggesting a similar role for this integrin in wound healing at different times. Furthermore the expression of α6β4 integrin at all time points occurred on both the connective tissue side of the epithelium as well as the tooth side, supporting previous studies which have demonstrated adhesion of the junctional epithelium to the enamel by way of the α6β4 integrin (Hormia et al., 1992).

Laminin-5 is a ligand for keratinocyte integrins during both migration, in wound healing and adhesion in resting epithelium (Nguyen et al., 2000; Kainulainen et al., 1998; Zhang et al., 1996; Larjava et al., 1993). Integrins α3β1 and α6β4 both serve as receptors for laminin-5 during migration of keratinocytes (Niessen et al., 1994; Carter et al., 1991). It has been suggested that laminin-5 is the first extracellular matrix component to be deposited by the keratinocytes as they migrate over the provisional wound (Zhang et al., 1996, Larjava et al., 1993). Expression of laminin-5 during oral wound healing of full thickness wounds (Larjava et al., 1993) was expressed during all phases of wound healing by the migrating keratinocytes, as early as 1 day through to 7 days. Additionally, it is present along the basement membrane of non-wounded epithelium and is specifically part of the anchoring filaments (Larjava et al., 1993). These results in oral epithelium are paralleled in the skin (Martin, 1997). Similarly, results from the present study show laminin-5 expression of the basement membrane zone as early as 1 day post-wounding (Figure 8D). Additionally, laminin-5 expression is present in the stroma facing the tooth at 1 day post-wounding and continues to be expressed through to 28 days (Figure 8 E-H). In support of previous studies examining laminin-5 expression during wound healing (Nguyen et al., 2000; Kainulainen et al., 1998; Zhang et al., 1996; Larjava et al., 1993) the present study also exhibits early
expression of laminin-5 which continues through all phases of wound healing in the junctional epithelium. This indicates the importance of laminin-5 for migration of healing junctional epithelial cells in addition to its importance as a means of attachment of epithelial cells to the tooth and the underlying connective tissue.

Type IV collagen is a classical component of the basement membrane produced by keratinocytes which aids in their attachment to the basement membrane (Stepp et al., 1990). In oral and pocket epithelium type IV collagen is present along the basement membrane although in differing intensities of expression (Haapasalmi et al., 1995). In the chronically inflamed tissue of pocket epithelium type IV collagen is present in a punctate distribution along the basement membrane, unlike the continuous line of expression observed in normal healthy oral epithelium (Haapasalmi et al., 1995). In addition, the relative amount of type IV collagen is reduced at the basement membrane. The present study exhibited similar differences between healthy oral epithelium (Figure 11A) and chronically inflamed pocket epithelium (Figure 11B) with relatively less expression of type IV collagen and relatively higher numbers of type IV collagen positive blood vessels in the inflamed tissue compared to the healthy tissue (Figure 11A&B). In wound healing oral keratinocytes as well as dermal keratinocytes don’t express type IV collagen when they move into wounds (Larjava et al., 1993; Olerud et al., 1988; Clark et al., 1982). Once the epithelial sheets confront each other at 7 days expression of type IV collagen is more pronounced in oral wounds (Larjava et al., 1993). Migration of the keratinocytes in the present study exhibited a similar pattern of type IV collagen expression with no expression observed at the migrating tip of cells (Figure 11D&E) instead expression followed behind the tip of cells similar to that observed in dermal
wounds. Additionally there was a punctate pattern of expression by 7 and 14 days post wounding indicating that the final reconstruction of the basement membrane may take place in more of a simultaneous pattern (Figure 11F&G).

CK 19 expression was observed in the cells of the junctional epithelium of all samples as well as in selected areas of the basal epithelial cells of the oral epithelium. Similar results have been found in previous studies of the junctional epithelium (Shimono et al., 2003). From the results it would appear keratinocytes that advance into the gingival wound are CK 19 positive. This suggests a unique phenotype for gingival keratinocytes at the tooth gingiva interface as keratinocytes of epidermal and palatal wounds do not express CK 19 (Mackenzie et al., 1997). During, the early phase of healing of the JE, the expression of CK 19 also increased in areas other than migrating tip such as the basal cells of the oral epithelium. This increased expression of CK 19 is observed until day 7 post-wounding at which point CK 19 expression in the basal cells of the oral epithelium returns to that of the control specimens. It has been previously shown that expression of CK 19 is up regulated with inflammation (Pritlove-Carson et al., 1997; Mackenzie et al., 1995) that could in part explain the upregulation of CK 19 in migratory as well as oral epithelium. The early high expression of CK 19 cannot, however, be explained by inflammation above as epidermal and palatal wounds contain inflammatory cells. What regulates the CK 19 phenotype of wound keratinocytes in the tooth-gingiva interface remain to be explained.

Cytokeratin 16 has been considered as an early marker of wound keratinocytes in epidermis (Mackenzie et al, 1991). As it is constantly expressed in the unwounded marginal gingiva it does not serve as a good marker of wound keratinocytes in this tissue. General expression of
CK 16 in gingiva does however, support the notion that keratinocyte phenotype migrating into the tooth-gingiva interface is unique.
CONCLUSIONS:

1. Cytokeratins 16 and 19 were expressed as early as 1 day post wounding of the pocket epithelium suggesting that wound keratinocytes at the tooth-gingiva interface have a unique phenotype.

2. Expression of integrins and their ligands by wound keratinocytes resembled that seen in epidermal and palatal wounds.

3. Several integrins of the β1 integrin family as well as αvβ6 integrin were not downregulated even in the late wounds as they are in epidermal and palatal wounds.

4. Re-expression of integrins of normal junctional epithelium occurs early in wound healing and does not require contact with the tooth.
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