KGF-1 and KGF Receptor Expression in Human Periodontal Disease and in vitro Microwounding-Associated Ligand-Independent KGFR Activation

by

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ABSTRACT

Objectives: Periodontal disease is a chronic inflammation resulting in periodontal attachment loss. Keratinocyte Growth Factor-1 (KGF-1) is upregulated in chronic inflammation and specifically stimulates epithelial cell proliferation by signaling through the epithelial-specific Keratinocyte Growth Factor Receptor (KGFR). First, we examined KGF-1 and KGFR expression and localization in human periodontal tissues. Second, we extended these studies by developing an in vitro mechanical wound model to mimic trauma to the periodontal pocket epithelium and examined ligand independent KGFR activation and cell migration.

Methods: In our study of human gingival tissues, we used immunohistochemistry and laser capture microdissection with RT-PCR to analyze KGF-1 and KGFR expression and localization. To study ligand independent KGFR phosphorylation, KGFR internalization along the wound edge was imaged using immunohistochemical staining and KGFR phosphorylation confirmed using immunoprecipitation with western blotting. Wounding induced oxidative stress was detected using DCFH-DA (2',7'-dichlorofluorescin diacetate) and modulated by pretreatment with an antioxidant. Changes in migration were examined in the presence or absence of pathway specific inhibitors.

Results: KGF-1 protein localized to areas of junctional and basal oral epithelial cells was significantly increased in periodontal pocket epithelium (p<0.01) and oral epithelium (p<0.05) of disease-associated tissues. KGFR localized to the junctional
and the parabasal cells of oral epithelium, and was increased in disease-associated pocket epithelium (p<0.05). Laser capture microdissection with RT-PCR confirmed KGF-1 and KGFR were specifically expressed by connective tissue and epithelium, respectively. In our cell culture model, mechanical wounding induced ligand independent KGFR activation. ROS (Reactive Oxygen Species) generation along the wound edge was associated with KGFR activation and scavenging of ROS reduced KGFR phosphorylation. The c-Src family inhibitor, PP1, significantly inhibited KGFR phosphorylation. Functionally cell migration was reduced by PP1 (82.7%), SU5402 (70%) and PD98059 (57%).

Conclusions: KGF-1 and KGFR proteins are expressed in health but significantly induced in human diseased periodontal tissues. Microwounding associated generation of ROS mediates KGFR phosphorylation via c-Src kinase signaling and induced wound edge cell migration. Therefore, regulation of epithelial cell behavior associated with the onset and progression of periodontal disease may possibly be mediated by two related but distinct mechanisms. (1) Ligand-dependent activation of KGFR due to upregulation of KGF-1. (2) Ligand-independent activation of KGFR due to chronic microwounding.
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Chapter 3 is a version of paper has been published in *The Journal of Periodontal Research*. Chapter 4 is a slightly expended version of a paper submitted for publication to *The Journal of Investigative Dermatology*. The two papers were co-authored with James Firth and Edward Putnins. The division of tasks among three authors is outlined below.

Overall guidance and directions for the research was provided by Edward Putnins; The research experiments and data analysis were carried out by me. Initial writing and development of the manuscripts were done by me with support from Edward Putnins. James Firth provided technical and editorial support.
CHAPTER 1

1. Introduction

1.1 Periodontium and Periodontitis

1.1.1 Introduction of Periodontium

The periodontium provides support for the normal function of teeth and is comprised of gingiva, periodontal ligament, alveolar bone, and cementum (Hassell, 1993). Gingiva, the main focus of my study, includes gingival epithelium and connective tissue. The gingival epithelium is divided into three distinct epithelia: oral gingival epithelium, sulcular epithelium and junctional epithelium (Figure 1.1a). This review will focus on the organization of gingiva and the pathological changes that occurs with the onset and progression of periodontitis.

1.1.1.1 Epithelium of the Gingiva

1.1.1.1.1 Oral Gingival Epithelium

Oral gingival epithelium (OE) (Figure 1.1a) includes both the marginal gingiva coronal to the free gingival groove and the attached gingiva from the free gingival groove to the mucogingival junction. The epithelial-connective tissue interface is demarcated by saw-like projections of epithelium called “rete ridges”. The oral epithelium is a keratinized, stratified squamous epithelium that is comprised of 4 layers: stratum basale (basal layer), stratum spinosum, stratum granulosum and stratum corneum. The stratum basale is comprised of small cuboidal basal cells that lie in contact with the basement membrane. These cells proliferate and migrate continuously from the basement membrane upwards and give rise to mature keratinocytes (Bartold et al.,
Keratins (K), one of the largest groups of intermediate filament proteins, are the major cytoskeleton proteins of epithelial cells. They are attached to the plasma membrane via desmosomes or hemidesmosomes (Yancey, 1995). Expression of keratins in epithelial cells depends on specific cell phenotypes, stages of cell differentiation (keratinization), and/or pathological modifications such as inflammation (Sawaf et al., 1991). For example, K5 and K14 are expressed in the basal proliferative epithelial layer; simple, non-keratinized, and less differentiated epithelial cells express K7, K8, K18 or/and K19; Hyperproliferated cells express K6 and K16; Keratinized (expression of stratum corneum) epithelial cells express K1 and K10 (Sawaf et al., 1991). In oral gingival epithelial cells, keratins are expressed differently in different layers of oral epithelium. Keratin 5 and 14 (K5 and K14) are positive at basal layer while K1 and K10 are positive at stratum spinosum (Presland and Dale, 2000). Keratin 19 (K19), a marker of non-keratinized epithelial cells, is not typically expressed in basal cells of the oral epithelial cells but occasional positive punctuated staining at the tip of rete pegs in keratinized oral epithelial cells has been reported (Csiszar et al., 2007).

1.1.1.1.2 Sulcular Epithelium

The sulcular epithelium (SE) faces the tooth surface and extends from the coronal margin of the gingiva to the coronal margin of the junctional epithelium (Figure 1.1a). In contrast to keratinized squamous oral epithelium, sulcular epithelium is a non-keratinized or para-keratinized squamous epithelium. Its keratin expression is different from OE. In SE, basal cells are K5, K14 and K19 positive; while cells of spinosum
layer are K4, K13 and K16 positive but K1 and K10 negative. This epithelium is thin, with increased internal cellular spaces. This organization suggests this tissue is less resistant to the injurious effect of bacteria or their products such as LPS. In contrast, this organization facilitates the movement of tissue fluids immunoglobin and immune defense cells neutrophils into the gingival sulcus (Bartold et al., 2000; Schroeder and Listgarten, 1997).

1.1.1.3 Junctional Epithelium

The junctional epithelium (JE) forms a collar of epithelial attachment between the gingiva and the tooth enamel and cementum. In health, the normal attachment ends at the cemento-enamel junction (CEJ). Junctional epithelium is about 15-30 cell layer thick coronally and narrows apically to only a few cell layers. At the CEJ, JE is a non-keratinized stratified squamous epithelium and lies adjacent to the SE and has no rete pegs present. The lack of JE cell keratinization and presence of wide intercellular spaces are associated with migration of bacteria and virulence factors towards the CT and conversely, immune cells into the sulcus (Pollanen et al., 2003). In contrast to SE, juntional epithelium express keratins such as K5, K13, K14, and K19, and K8, K16 and K18 are weakly expressed in all layers of JE (Bosshardt and Lang, 2005). Moreover, junctional epithelial cells exhibit rapid turnover, which enables rapid repair of damaged tissue (Genco, 1996). JE turn over rate is about 5 days, twice the rate of the oral gingival epithelium (Skougaard, 1970).

In health, junctional epithelium is firmly attached to the tooth and forms an epithelial
barrier against the bacteria in plaque. JE lies between two basal laminas. First, junctional epithelium attaches to the tooth surface via a unique basal lamina termed the internal basal laminal (IBL). Second, the basal lamina facing the gingival connective tissue is termed the external basal lamina (EBL) (Bosshardt and Lang, 2005).

The external basal lamina (EBL) is continuous with the basement membrane of the sulcular epithelium and attaches the junctional epithelium to the underlying connective tissue. EBL is composed of a lamina lucida, lamina densa and a lamina fibroreticularis (also known as the sub-basal lamina). Typical matrix components of the EBL are collagen IV, and VII, laminin-1, laminin-5, laminin-6, heparan sulfate proteoglycan (HSPG), fibronectin, nidogen, perlecan (Bosshardt and Lang, 2005). Heparan sulfate, the predominant glycosaminoglycan in the gingival epithelium, accounts for 60% of total glycosaminoglycan (Bartold et al., 2000; Bartold et al., 1981). Heparan sulfate PG is localized primarily in the basement membranes of epithelium (Erlinger et al., 1995).

The internal basal lamina (IBL) is synthesized by cells directly attached to the tooth (DAT cells). The IBL has distinct constituents differing from the EBL. It is unique because it is composed in part of laminin-5 and type VIII collagen but lacks the classical EBL components laminin –1, type IV and VII collagen. Therefore, the IBM of junctional epithelium is not considered a true basement membrane (Pollanen et al. 2003; Salonen et al., 1984; Salonen and Santti, 1985; Salonen et al., 1991; Bosshardt and Lang, 2005). JE cell attachment to laminin-5 present in the IBM is mediated by α6β4
integrins present in the hemidesmosome (Pollanen et al., 2003). However, laminin 5 may also interact with α3β1 and α6β1 as well (Colognato and Yurchenco, 2000).

### 1.1.1.2 Connective tissue

Connective tissue (CT), also called lamina propria, is located underneath the epithelial layer, and by volume encompasses a major proportion of the gingival tissues. Connective tissue is composed of cells such as fibroblasts, lymphocytes and endothelial cells, and the extracellular matrix (ECM), containing principally collagen fibers and macromolecules like proteoglycans (Schroeder et al., 1973).

#### 1.1.1.2.1 Cellular components

The cellular component comprises approximately 8% by volume of the CT compartment. Fibroblast cells, the most abundant cells in CT, account for 5% of CT volume. Endothelial cells and immune cells such as leukocytes, mast cells, macrophages account for 3% (Schroeder et al., 1973). The principal function of fibroblasts is to synthesize and maintain the components of the extracellular matrix of the connective tissue. In general, the morphology of fibroblasts is typical elongated or spindle shape, having prominent rough endoplasmic reticulum and Golgi apparatus which is consistent with their high synthetic activity required to maintain tissue homeostasis. Expression of a wide range of matrix metalloproteinases (MMPs) are capable of degrading collagens, proteoglycans and other ECM components (Bartold et al., 2000). Fibroblasts also synthesize and secrete a number of growth factors, cytokines and metabolic products that further regulates cell activity in either an
autocrine or a paracrine manner (Pollanen et al., 2003).

1.1.1.2 Extracelullar Matrix (ECM)

Gingival ECM is composed principally of collagen fibers and other macromolecules such as proteoglycans. Approximately 60-65% of the CT is type I and III collagen. Type I collagen is preferentially organized into denser fibrils in the lamina propria while type III collagen appears to be preferentially localized as thinner fibers in a reticular pattern near the basement membrane at the epithelial junction. Other types of collagen fibers such as type II, V, VI, IX, XI, and XII have also been found in lesser amounts within the gingival CT (Karimbux, et al., 1992; Narayanan and Hassell, 1985). Proteoglycans (PG), such as glypican, biglycan, decorin, syndecan, CD44, composed of a core protein and side chain glycosaminoglycans (GAG) that are covalently linked to the core proteins such as heparan sulfate (HS), dermatan sulfate (DS) and chondroitin-4 sulfate (ChS-4), are ubiquitous constituents of the periodontal tissues. Within the gingival connective tissues, dermatan sulfate and chondroitin sulfate account for approximately 60% and 30% of total glycosaminoglycans, respectively, and the remaining 10% is made of roughly equal proportions of hyaluronan and heparan sulfate (Bartold et al., 2000; Bartold et al., 1981; Hakkinen et al., 1993).
Figure 1.1 Diagram of healthy and diseased periodontal gingiva. (a) Normal periodontium. The gingival epithelium is divided into 3 groups: oral epithelium (OE), sulcular epithelium (SE) and junctional epithelium (JE). (Reproduced with permission from Pollanen et al. 2003) (b) In diseased periodontal gingiva, subgingival calculus is presented in the deepened periodontal pocket, immune cell infiltration is increased, JE permeability is increased, and JE cells migrate apically accompanied by alveolar bone destruction.
1.1.2 Periodontitis

1.1.2.1 Introduction of Periodontitis

Periodontal disease is a chronic inflammatory disease that ultimately results in the loss of the periodontal attachment and alveolar bone. The presence of specific gram negative bacteria is associated with the progression of periodontal disease. With disease onset their numbers can be increased to $10^5$ times higher than those associated with health (Lovegrove, 2004). The bacteria associated with periodontal diseases are predominantly gram-negative anaerobic bacteria and may include *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *S. intermedius* and *Treponema sp.* (Lovegrove, 2004).

On a clean tooth surface, salivary proteins rapidly and selectively adsorb onto the enamel surface to form an acquired enamel pellicle in just a few minutes. It is followed by the adherence of various oral micro-organisms. Within 24 to 48 hours, Gram-positive rod and coccoidal organisms are the first organisms to adhere to the formed enamel pellicle; subsequently, filamentous bacteria gradually dominate the maturing plaque biofilm, and eventually gram negative anaerobic bacteria such as *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *spirochetes* dominate in deeper layer of plaque (Scheie, 1994). Distinctive subgingival microflora are predominantly made up of gram-negative, anaerobic bacteria while supragingival microflora are gram positive (Lovegrove, 2004).
Plaque absorbs calcium and phosphate from saliva for the formation of supragingival calculus and from crevicular fluid for the formation of subgingival calculus (Jin and Yip, 2002). Supragingival and subgingival calculus contain 37% and 58% mineral content by volume, respectively (Friskopp and Isacsson, 1984). During the progress of periodontal disease, the hard and rough surface of subgingival calculus (Figure 1.2b), compared to smooth clean cementum surface (Figure 1.2a), not only provides an ideal host to a complex micro-system of microorganisms that cause inflammation of periodontal tissues, but also creates micro-wounds on the inner surface of the pocket epithelia (PE) (Schroeder and Listgarten, 1997). Consequently, it makes the barrier function of pocket epithelia even weaker to bacteria and toxin attack.

The initiation and progression of periodontal attachment loss includes junctional epithelial cell proliferation and migration apically, dissolution of Sharpey’s fibers with loss of attachment, and ultimately resorption of alveolar bone (Birkedal-Hansen, 1998). The transition from junctional epithelium (JE) to pocket epithelium (PE) is regarded as a hallmark in the development of periodontitis (Bosshardt and Lang, 2005). PE phenotype is similar to JE, however, increased expression of K19 through all layers in periodontitis associated PE has been described (Mackenzie and Gao, 1993). The regulation of JE proliferation and migration associated with periodontitis is poorly understood but local expression of cytokines and growth factors likely plays a significant role.
Figure 1.2 SEM view of smooth surface of cementum (a) and SEM view of rough surface of calculus specimen (b): Holes within the calculus are attributed to spaces previously occupied by microorganisms. (Reproduced with the permission from Rohanizadeh et al. 2004; Garey, 2001).
1.1.2.2 Pro-inflammatory Cytokines in Periodontal Diseases

In periodontal disease, the initial inflammatory infiltration is primarily PMNs and T cells located immediately beneath the SE and JE (Page, 1976). The infiltrated immunocompetent cells, such as B-cell, T-cells, PMNs and macrophages, secret proinflammatory cytokines such as IL-1, IL-6 and TNF-α (Daniel and Van Dyke, 1996; Suchett-Kaye et al., 1998). Stimulated by bacteria and their toxin such as LPS, local epithelial cells and fibroblasts also express cytokines, IL-1α, IL-1β, IL-6, IL-8 and TNF-α (Okada, 1998). These cytokines are expressed in low level in health but significantly increase in periodontitis. Specifically, IL-1 expressed by the epithelial cells widens the intercellular spaces of the junctional epithelium. The increase in intercellular space is associated with cell and inflammatory exudate movement to the gingival sulcus and movement of virulent factors and bacteria from the external surface towards the connective tissue (Fig 1.1b) (Lopez-Otin and Diamandis, 1998; Uitto et al., 1998).

In addition to JE proliferation and migration, onset of periodontal disease is associated with degradation of basement membrane and Sharpey’s fiber (Birkedal-Hansen, 1998). Cytokines can also stimulate immune resident gingival CT cells to secret MMPs and growth factors. In addition, PMNs also release collagenase (MMP-8) and gelatinase B (MMP-9) that contribute to the degradation of ECM in CT (Pilcher et al., 1997). Proinflammatory cytokines, particularly IL-1 and TNFα from PMNs, are potent inducers of MMPs, and stimulate resident host cells such as fibroblasts and keratinocytes to up-regulate their MMP expression (MacNaul et al., 1990). Basal
junctional epithelial cells when stimulated by TNF-α express collagenase-3 (matrix metalloproteinase-13) (Uitto et al., 1998) and this general increase in MMP expression degrades the underlying matrix and facilitates epithelial migration within the gingival connective tissues (Lopez-Otin and Diamandis, 1998).

1.1.2.3 Growth Factors in Periodontal Diseases

Growth factors expressed by resident gingival cells or recruited inflammatory cells are thought to play an important role in regulating the proliferation and migration of cells in the periodontium and the expression and degradation of various ECM components. Excessive or continuous expression of growth factors in inflamed periodontal tissues is believed to regulate periodontal disease pathogenesis (Okada, 1998). Hepatocyte growth factor (HGF) has been shown to be associated with periodontitis (Nagaraja and Pradeep, 2007). HGF protein was increased in gingival crevicular fluid (GCF) and in saliva samples collected from periodontitis patients, and HGF protein increase was positively correlated with disease (Ohshima, 2002; Kakimoto et al., 2002; Nagaraja and Pradeep, 2007). Furthermore, HGF expression was induced by P. gingivalis and IL-1α, suggesting HGF upregulation may be mediated by host and bacterial mechanisms (Sugiyama et al., 2000). Platelet-derived growth factor (PDGF) was also shown increased three times higher in the human inflamed gingiva than healthy gingiva and was localized to pocket epithelia (Pinheiro et al., 2003). Conversely, EGF concentrations in GCF from periodontal patients and healthy control subjects were compared and no significant differences in EGF concentrations were found (Mogi et al., 1999). In addition, in the cell culture studies, KGF-1, not KGF-2, was expressed by
gingival fibroblasts and KGF-1 protein and gene expression was induced by pro-inflammatory cytokines and *P. gingivalis* and *E. coli* LPS (Sanaie et al., 2002; Putnins et al. 2002). Using a rat periodontitis model, KGFR expression by junctional epithelial cells was increased 25 times in periodontitis rat samples compared with that of health (Ekuni et al., 2006). In contrast, EGFR and FGFR1 expression was not induced using this model. The upregulation of KGFR suggests that the regulation of epithelial cell behavior during disease onset may in fact occur via the paracrine mediator, KGF-1

### 1.1.3 Summary

In summary, JE is a non-keratinized stratified squamous epithelium with a relatively weak barrier against bacteria invasion. One early event of periodontitis is characterized as JE proliferation and migration along root surface, followed by detachment coronally and concomitant formation of deepened periodontal pocket. The process of this transition from JE to PE is unknown; however, growth factors have been suggested to play an important role in this transition. KGF-1, a specific mitogen for epithelial cells, and its receptor KGFR are upregulated both at the protein and gene level in an *in vitro* periodontitis cell culture model and in an *in vivo* rat periodontitis model. *P. gingivalis* and *E. coli* LPS stimulation of the KGF-1 upregulation *in vitro* further suggests that KGF-1/KGFR may play an important role in the initiation and progression of periodontitis. Therefore, one focus of this dissertation is the protein and gene expression of KGF-1/KGFR associated with the onset of periodontal diseases.
1.2 FGF and FGFR family

1.2.1 FGF Family

Fibroblast growth factor (FGF) was initially purified in the mid-1970s from bovine brain extracts (pituitary gland) and stimulated NIH3T3 fibroblasts cell growth (Gospodarowicz et al., 1974). Since that time, further members of the family have been subsequently identified. Fibroblast Growth Factors (FGFs) are a family of structurally related polypeptides, composed of 23 members, and are designated as FGF-1 to 23 (Reuss and von Bohlen und Halbach, 2003). Not all 23 FGFs are found in human or mouse. For example, FGF15 is not found in human while FGF19 is not found in mouse (Ornitz and Itoh, 2001). The name of fibroblast growth factor is misleading (Baird and Klagsbrun, 1991). First, some FGFs stimulate fibroblast proliferation, but also induce proliferation of many other cell types such as microvascular endothelial cells (Smola, 1993), smooth muscle cells (Winkles et al., 1997) and activated γδ T cells (Boismenu, 1994) as well. Second, FGF’s actions are more diverse than proliferation and FGFs play a major role in development, wound healing, morphogenesis, cell differentiation, cell migration and cell survival (Thisse and Thisse, 2005).

Across species, FGF proteins are highly conserved and share greater than 90% amino-acid sequence homology. Within species (human), FGFs are highly conserved in both gene and amino-acid sequences. General protein structure is similar in all FGFs with molecular mass from 17 to 34 kDa and sharing 13-71% amino-acid homology. All FGFs consist of two highly conserved core-domains (black), separated by a spacer region of variable length (Figure 1.3a). All FGFs in their core domains contain two
conserved cysteines (Szebenyi and Fallon, 1999) and 28 highly conserved amino-acid residues (Ornitz and Itoh, 2001). FGFs 3 to 8, 10, 15, 17 to 19, and 21 to 23 all possess an amino-terminal signal sequence which allows secretion from cells (Figure 1.3b). In contrast, FGF-1, -2, -9, -16 and -20 lack the signal sequence on the amino-terminal end. Extracellular release of these growth factors is likely to occur upon cell damage (Reuss and von Bohlen und Halbach, 2003).

Some FGFs are expressed exclusively during embryonic development (FGF3, 4, 8, 15, 17 and 19), whereas others are expressed in both embryonic and adult tissues (FGF1, 2, 5-7, 9-14, 16, 18, and 20-23). Collectively, FGFs play major roles in development, morphogenesis and wound healing, often signal directionally and reciprocally across epithelial-mesenchymal boundaries to regulate cell growth, survival, differentiation and migration (Thisse and Thisse, 2005; Ornitz and Itoh, 2001; Hogan, 1999).

1.2.1.1 Keratinocyte Growth Factor (KGF-1): FGF7

Keratinocyte growth factor (KGF-1), also known as FGF-7, was first isolated from M426 human embryonic lung fibroblasts as a monomeric glycoprotein with molecular weight 26-28 KDa (Finch, 1989). The growth factor was termed keratinocyte growth factor because of its specific mitogenic activity on mouse epidermal keratinocytes (Rubin et al., 1989). The KGF-1 molecule is a single chain and its cDNA encodes a 194 amino acid protein (Finch, 1989). When expressed as a recombinant protein its molecular weight is approximately 21 kDa (Ron et al., 1993).
1.2.1.2 KGF-1: A Paracrine Mediator

KGF-1 is best described as a paracrine mediator that is expressed by mesenchymal cells and specifically stimulates epithelial cells through the cell type specific KGFR (FGFR2IIib) (Finch, 1989; Rubin, 1989). KGF-1 is expressed in a broad variety of cells such as dermal fibroblasts, microvascular endothelial cells, smooth muscle cells and activated γδ T cells (Winkles et al., 1997; Boismenu, 1994; Smola, 1993; Finch, 1989). KGF-1 was recently found to be expressed by epithelial cells from airway (trachea and bronchus) (Hicks et al., 2004), ovary (Steele et al., 2001), and endometrium (Ka, 2000). For example, both KGF-1 and KGFR were detected in endometrial epithelia between days 12 and 15 of the estrous cycle and pregnancy using Northern Blot analysis (Ka, 2000). However, questions have been raised that this expression in epithelia might be due to contamination of endometrial epithelial samples by subjacent connective tissue.

1.2.1.3 KGF-2 (FGF10)

FGF-10, also called KGF-2, is most closely related to KGF-1 within the FGF family. It is a glycosylated heparin binding protein with 215 amino acids and a molecular weight of 30 KDa. It shares 60% amino acid homology with KGF-1 and has similar characteristics to KGF-1. It is expressed by mesenchymal cells and stimulates epithelial cell migration and proliferation suggesting it is a paracrine mediator. Therefore FGF-7 and FGF-10 are described as KGF-1 and KGF-2 respectively. KGF-2 (FGF10) is crucial as an essential regulator of lung and limb formation (Sekine et al., 1999; Ohuchi et al., 2000). However, within gingival fibroblasts KGF-2 gene and
protein expression was not induced by LPS or pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNF-α (Sanaie et al., 2002). These data suggest that it may not play a major role in periodontal disease pathogenesis.
Figure 1.3 FGF protein structure features. (a) All FGFs contain two highly conserved core-domains (black), separated by a central space region of variable length, and two highly conserved cysteines in the core-domain (Reproduced with permission from Ruess, 2003). (b) Some FGFs contain a signal sequence (grey) in the N-terminus; FGFs (FGF1 and FGF2) contain the core-domain which comprised 12 β strands labeled 1-12 region (black box): the heparin-binding region (pink box), the region binding to FGFR IgII domain (green box), the region binding to FGFR IgIII N-terminal half domain (blue box), and the region binding to FGFR IgIII C-terminal half alternatively spliced region (red box) (Reproduced with permission from Ornitz, 2001).
1.2.2 Fibroblast Growth Factor Receptor (FGFR) Family

FGFR family is one of the tyrosine kinase growth factor receptor families. To date, 5 members in FGFR family are proposed and designated as FGFR1-5 (Reuss and von Bohlen und Halbach, 2003; Sleeman et al., 2001). The first four known FGFRs, FGFR1 to FGFR4, share 55% to 72% homology at the protein level in human and 98% homology between human and mouse (Johnson and Williams, 1993). FGFR5 is a recently identified FGFR family member. At the protein level, it shares approximately 32% homology with the other four FGFRs (Sleeman et al., 2001).

FGFRs share many common structural motifs (Figure 1.4). These are three extracellular immunoglobulin-like domains (IgI, IgII and IgIII), a signal peptide (SS), an acidic region (A), a transmembrane domain (TM), juxtamembrane domain (JM) with a phosphotyrosine-binding (PTB) site, kinase insert region (KI), and cytoplasmic split tyrosine kinase domains (TK1 and TK2) which are phosphorylated upon FGF binding (Galzie et al., 1997). FGF ligands principally bind to the IgII and IgIII domains of FGFRs, while Ig I and the acid box has an autoinhibitory function (Olsen et al., 2004; Schlessinger, 2003). A unique feature of FGFR5 is that it completely lacks an intracellular tyrosine kinase domain. However, its extracellular N-terminal domain contains a putative secretory signal sequence and has all the conserved residues like three typical immunoglobulin-like domains and an acidic box, all of which are required for ligand binding. FGF2 protein binds to FGFR5 but at a much lower affinity than binding to FGFR2 (Sleeman et al., 2001; Kim et al., 2001).
FGFR1 is preferentially expressed by the mesenchyme (Klint and Claesson-Welsh, 1999) and FGFR2 is widely expressed in mammary epithelial cells, endothelial cells, fibroblasts, vascular smooth muscle and monocytes (Miki et al., 1992); FGFR3 is expressed mainly in brain, spinal chord, cartilage and hematogenous tissues (Hughes, 1997), and FGFR4 expressed mainly in endodermally-derived tissues such as the gastrointestinal tract, pancreas, muscle, and adrenal gland (Givol and Yayon, 1992; Hughes, 1997; Partanen et al., 1991). Lastly, FGFR5 was highly expressed in liver, kidney, heart, brain, and skeletal muscle and expressed at low levels in lung, small intestine and spleen (Sleeman et al., 2001)

FGF ligand specificity to specific FGFRs is due to alternative splicing of FGFR gene. This alternative splicing occurs in the extracellular third Ig-like domain (IgIII) (Miki, et al., 1992; Givol and Yayon, 1992) (Figure 1.5). FGFR2 gene with exon 7 encodes for the N-terminal half of IgIII and is designated ‘IIIa’, an abbreviated, secreted, soluble receptor (Johnson et al., 1991). Genes with either exon 8 or 9 encodes for the C-terminal half of IgIII and are designated as ‘IIIb’ or ‘IIIc’ respectively. For FGFR1 and FGFR2 all three splice variants have been detected; FGFR3 has both IgIIIb and IgIIIc isoforms, and FGFR4 exists exclusively as the IgIIIc isoform (Reuss and von Bohlen und Halbach, 2003). Ligand-binding specificity to FGFRs isoforms is dependent on the C-terminal half IgIII domain. For example, FGFR2IIIb binds FGF1, 3, 7, 10, and 22 but not FGF2, while the FGFR2IIIc isoform binds FGF2 and FGF18, but not FGF7 and FGF10 (Eswarakumar et al., 2005).
1.2.2.1 FGFR2IIIb-KGFR

KGFR is also designated as FGFR2IIIb and is one of the alternatively spliced isoforms of FGFR2 (the other isoform is FGFR2IIIc). KGFR differs from FGFR2IIIc on the carboxy-terminal half of the IgIII loop close to the transmembrane domain. In this region, KGFR and FGFR2IIIc sequences share only 47% homology (Miki et al., 1992). The binding specificity is exclusively dependent on this region (Rubin et al., 1995). The KGFR isoform is exclusively expressed on cells of epithelial origin such as gut epithelial cells, hepatocytes (Housley, Morris et al., 1994), skin keratinocytes (Pierce et al., 1994), alveolar type II cells (Panos et al., 1993), and binds with 5 known ligands: FGF1, FGF3, FGF7 (KGF1), FGF10 (KGF2) and FGF22. In contrast, FGFR2IIIc is expressed exclusively in mesenchymal cells (reviewed by Eswarakumar et al., 2005; Orr-Urtreger et al., 1993). It is clear that mesenchymal and epithelial expression of KGF-1 and KGFR respectively supports the hypothesis that KGF-1 is a mesencymally-derived paracrine mediator of epithelial growth and development (MacDonald and Hill, 2002).
Figure 1.4 Schematic structure of FGFRs, including four members: FGFR1, FGFR2, FGFR3 and FGFR4 (Reproduced with permission from Galzie, 1997).
Figure 1.5 FGFR IIIb and IIIc isoforms generated by alternative splicing of FGFR transcripts of exon 8 and exon 9 respectively (Reproduced with permission from Eswarakumar, 2005).
1.2.2.2 Studies of KGF-1 and KGFR Knockout Mice

KGF-1 knockout mice expressed no abnormalities in epidermal growth or wound healing, however, reduction in hair follicle growth was described (Gao, 1996). In contrast, KGF-1 over-expression in epithelial cell using a keratin 14 promoter induced significant skin wrinkling because of a gross increase in epidermal thickness (Guo et al., 1993).

To further test the role of this growth factor, KGFR knock out mice lacking the FGFR2IIib (KGFR) whilst retaining expression of the FGFR2IIic were generated. The mice developed defects of the limbs, lung, anterior pituitary gland, the salivary glands, inner ear, teeth and skin, as well as minor defects in skull and bone formation (De Moerlooze et al., 2000; Peters, Werner et al., 1994). Generating dominant-negative KGFR transgene targeted to basal keratinocytes of skin was characterized by epidermal atrophy, abnormalities in the hair follicles, dermal hyperthickening and substantially delayed reepithelialization of the wound (Werner et al., 1994b).

A probable explanation why the KGFR knockout mice exhibited significant phenotypic changes while KGF-1 knockout mice exhibited few abnormality is likely due to KGFR signaling by growth factor ligands, such as FGF1, 3, 7, 10 and 22. Therefore, an alternative ligand could function through the receptor to compensate for the absence of the knockout ligand. The most likely candidate for compensating KGF-1 might be FGF10, which is most structurally similar to KGF-1 (Werner, 1998).
1.2.3 Function of KGF-1

In general, FGFs regulate development, wound healing, morphogenesis, tumorogenesis (Thisse and Thisse, 2005). KGF-1 is significantly up-regulated during wound healing (Werner, 1992; Werner et al., 1994b; Marchese, 1995) and chronic inflammation (Bajaj-Elliott et al., 1997; Brauchle et al., 1996; Finch, 1997). Generally, KGF-1 regulates epithelial proliferation (Marchese et al., 1990; Rubin et al., 1989; Sato et al., 1995), migration (Putnins et al., 1999; Tsuboi et al., 1993), differentiation (Hines and Allen-Hoffmann, 1996), and is anti-apoptotic (Firth and Putnins, 2004; Hines and Allen-Hoffmann, 1996).

1.2.3.1 KGF-1/KGFR in Wound Healing

1.2.3.1.1 In vivo Wound Repair Process

Wound repair includes 3 phases: inflammation, tissue formation (including reepithelialization, granulation, neovascularization and matrix formation), and remodeling. Reepithelialization is an important component of wound repair as it serves to restore the barrier function of skin to reduce the chance of infection and loss of fluid from the skin within hours after wounding. Epithelial cells adjacent to a wound undergo a carefully regulated repair process involving loss of cell polarity, changes in morphology of the cells, cell migration and re-establishment of cell-cell junctions (Dieckgraefe et al., 1997; Yates and Rayner, 2002; Krawczyk and Wilgram, 1973). Those events involve detachment of keratinocytes from neighboring cells by removal of intercellular desmosomes. For basal keratinocytes, separation from the underlying
basement membrane is also necessary, which occurs via the breakdown of multiprotein hemidesmosomes (Singer and Clark, 1999).

In addition to cell migration, reepithelialization also involves proliferation. This occurs in epithelial cells located behind the migrating keratinocytes probably by the “free-edge-effect” and under the regulation of local growth factors and increased growth factor receptor expression (Clark, 1996). Growth factors can be expressed by macrophages or dermal cells, as well as by keratinocytes themselves in an autocrine or paracrine or juxtacrine fashion (Clark, 1996). Many growth factors in FGF family such as bFGF and KGF-1 have been shown to stimulate reepithelialization in animal models (Hebda et al., 1990), supporting the idea that these growth factors are active during normal wound repair. Once migration stops due to contact inhibition, keratinocytes reattach themselves to the underlying substrata, reconstitute the basement membrane, and then resume the process of terminal differentiation to generate a new, stratified epithelium (Martin, 1997).

1.2.3.1.2 KGF-1/KGFR in Wound Healing

The significant induction of KGF-1 mRNA expression (more than a 160-fold increase at 24 hours) observed after cutaneous injury was significantly greater than the 2-10 fold increase of FGF1, FGF2 and FGF5 (Werner, 1992). Moreover, topical application of KGF-1 to wounds showed significant acceleration of reepithelialization and increased the epithelial thickness (Staiano-Coico et al., 1993; Pierce et al., 1994). Conversely, reduced KGF-1 expression during wound repair in healing-impaired diabetic mice was
demonstrated and it was hypothesized that this decrease may explain impaired wound healing in these diabetic animals (Werner et al., 1994a; Brauchle et al., 1995). Dominant negative KGFR expression in mice skin substantially delayed reepithelialization of the wound (Werner et al., 1994b). Further in vitro studies established that KGF-1 stimulated epithelial cell proliferation (Takahashi et al., 1996; Sato et al., 1995), and cell migration. Collectively, KGF-1 upregulation upon wounding or delayed healing due to the reduced expression of KGF-1 supports the concept that KGF-1 is essential for normal wound healing.

KGF-1 also appears to regulate epithelial cell differentiation. With exposure to a high Ca\(^{2+}\) concentration in vitro, KGFR expression was upregulated or expression was increased from basal to suprabasal cells (Marchese et al., 1997). However, KGF-1 had no effect on the expression of differentiation-specific keratins in human primary keratinocytes (Latkowski et al., 1995). Using immunohistochemical analysis, distribution of KGFR on human colon tissue was increased on more differentiated cells both in vivo and in vitro (Visco et al., 2004). More interestingly, those differentiating cells are still able to proliferate in response to the KGFR ligands such as KGF-1 and KGF-2, but not in response to the epidermal growth factor receptor (EGFR) ligands TGF-\(\alpha\) and EGF, suggesting that KGF-1 may be required to sustain proliferative activity in partially differentiated cells and KGFR plays a distinct role from EGFRs in the control of epithelial cell proliferation and differentiation (Capone et al., 2000; Visco, et al., 2004; Capone et al., 2000).
**1.2.3.2 KGF-1/KGFR in Chronic Inflammation**

KGF-1 is also significantly upregulated in a variety of chronic inflammatory conditions like Crohn’s disease, ulcerative colitis and psoriasis (Bajaj-Elliott et al., 1997; Brauchle et al., 1996; Finch, 1996; Finch, 1997) as well as in periodontal disease (Sanale et al., 2002; Ekuni et al., 2006). Within the oral cavity, KGF-1 is expressed by a variety of oral tissue fibroblasts isolated from oral buccal mucosa, periodontal ligament (Gron, 2002; Dabelsteen, 1997), and gingiva (Ohshima, 2002; McKeown, 2003; Mackenzie, 2001). These data suggest that KGF-1 is required for normal gingiva and oral bucal mucosa epithelial cell growth and differentiation.

KGF-1 protein and gene expression in gingival fibroblasts *in vitro* was also found significantly induced by different proinflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α (Sanaie et al., 2002). In the same study, KGF-1 was most abundantly expressed while KGF-2 was only weakly expressed in gingival fibroblasts and KGF-2 expression was not induced by proinflammatory cytokine stimulations (Sanaie et al., 2002). In addition, LPS purified from *Escherichia coli* and *Porphyromonas gingivalis* induced KGF-1 protein and gene expression (Putnins et al., 2002). Recently, using a rat periodontitis model, KGFR gene expression was increased more than 25 fold in the periodontitis group when compared to the controls, while other growth factor receptors, such as HGFR increased 10 fold, EGFR and FGFR1 did not increased at all (Ekuni et al., 2006). These data strongly suggested that KGF-1/KGFR play an important role in regulating epithelial cell structure and function and may regulate epithelial cell behavior that is associated with the initiation and progression of periodontal diseases.
1.2.4 FGF/FGFR Signaling Pathway

1.2.4.1 Heparan Sulfate Proteoglycans (HSPG) Interaction With FGF/FGFR Complex

FGFs bind both their principal receptors and heparan sulfate proteoglycans. Regions involved in ligand binding, the high affinity binding sites, are distinct from regions that bind cell surface and ECM heparin or heparan sulfate proteoglycans, the low affinity binding sites (Figure 1.3b) (Ornitz and Itoh, 2001; Reuss and von Bohlen und Halbach, 2003).

HSPGs are ubiquitously distributed in all animal tissues. Within tissues they are mainly on the cell surface and in the extracellular matrix. They are composed of a core protein and a covalently attached glycosaminoglycan chains (GAG) formed by repetitive sulfated disaccharides of uronic acid and glucosamine. Heparan sulfate is a member of the glycosaminoglycan family and is structurally similar to heparin made by mast cells. Both consist of a variably sulfated repeating disaccharide unit by which they interact with diverse proteins such as fibroblast growth factors (FGFs), protease inhibitors and cell adhesion molecules to generate numerous biological activities (Capila and Linhardt, 2002). For example FGF-2 (bFGF) strongly binds to basement membrane heparan sulfate (Friedl, 1997). The discovery of HS’s low affinity binding to FGFs facilitated FGF’s purification and led to the observation that HS/heparin function as an accessory molecule that could stabilize FGF from heat, pH, proteolysis degradation and regulate FGF binding and activation to FGFR (Eswarakumar et al., 2005).
Dimerization of the FGF receptors is a prerequisite for receptor transphosphorylation and activation of downstream signaling molecules. A large body of biochemical and cellular evidence points to a direct role of heparin/HSPG in the formation of an active FGF/FGFR signaling complex. However, the precise mechanism how the HS interacts with FGF/FGFR complex to form the conformation and stoichiometry of molecules is not clear. Two models have been proposed in recent studies to describe the interaction of FGF/FGFR/HSPG. First, a single HS molecule binds 2 FGFs which, in turn, binds 2 FGFRs to induce signal transduction (DiGabriele et al., 1998). Second, a trimeric complex of 1 HS, 1 FGF and 1 FGFR must form first, and then dimerise with a second trimeric complex to form a dimeric 2:2:2 FGF:FGFR:HS/heparin ternary complex to induce signaling (Schlessinger et al., 2000).

The studies on the modulation of heparan/heparin on KGF-1/KGFR activity are controversial. Several reports have proposed that heparin or HSPGs is not required for formation of a KGF-1/KGFR complex, and the presence of heparin or HSPGs is actually inhibitory to the formation of the KGF-KGFR complex (Reich-Slotky, 1994; Ron et al., 1993). This might be caused by the occupancy of the HSPG binding site on the KGFR, which may specifically inhibit KGF-1 binding and consequently receptor activation. In contrast, other reports have suggested that HS or heparin are required for both 2:1 KGF-1/KGFR complex formation and activation of KGF-1/KGFR on cells (Jang et al., 1997; Hsu et al., 1999).
1.2.4.2 FGFRs Signaling Pathway

1.2.4.2.1 Ligand-dependent FGFRs Signaling Pathway

The binding of FGF and HSPG to FGFR induces receptor auto-phosphorylation at multiple tyrosine residues in the cytoplasmic tail of FGFR (at least 7 tyrosine residues), leading to recruitment or/and phosphorylation of intracellular signaling molecules, such as FRS2, Grb2, Gab1, Shp2 and PLCγ (Figure 1.6). The signaling via these pathways regulates cell proliferation, differentiation, migration, cell survival and cell shape (Eswarakumar et al., 2005; Klint and Claesson-Welsh, 1999). The intracellular activation of FGFRs primarily leads to signal transduction down to two independent signaling pathways: First, primarily through adaptor protein FRS2 linking to Ras/Raf/MAPK cascade or PI-3 kinase/Akt signaling pathway. Second, via phospholipase Cγ (PLCγ) signaling and subsequent protein kinase C (PKC) activation.

1.2.4.2.1.1 The FRS2-Ras-MAPK Signaling Pathway

FRS2 is a lipid-anchored docking protein, and is the major downstream substrate of FGFR that links FGFR to Ras-MAPK pathway. As a member of the family of docking proteins, FRS2 contains a phosphotyrosine-binding (PTB) domain that is normally attached to membrane. The PTB domain is capable of interacting with a nonphosphorylated region in the juxtamembrane domain of FGFRs and with tyrosine phosphorylated motif in Grb2 through SH2 domain (Guy et al. 2002). FRS2 interacts directly and constitutively with FGFR1 through its PTB domain and does not need receptor activation (Ong et al., 2000). The region of binding is mapped to the juxtamembrane domain of FGFR1, which is considerably longer than that of other
Figure 1.6 Cell signaling pathway of FGFRs (Modified from Eswarakumar et al., 2005).
receptor tyrosine kinases with a highly conserved sequence throughout the mammalian FGF receptor family. Therefore, it is likely that FRS2 may associate with all FGFR members regardless if they are activated or not (Ong et al., 2000; Eswarakumar et al., 2005).

FRS2 includes highly homologous members FRS2α and FRS2β. FRS2α is ubiquitously expressed and can be detected at every developmental stage of the mouse, while the expression of FRS2β is primarily confined to tissues of neuronal origin (Lax et al., 2002). EGF, PDGF or insulin does not induce tyrosine phosphorylation of FRS2. Unlike EGFR or PDGF-R, phosphorylated FGFR can not directly bind to adaptor protein Grb2. It has to use phosphorylated FRS2 to bind to adaptor protein Grb2 through SH2 domain.

FRS2 has multiple phosphorylation sites, four binding sites for the adaptor protein Grb2 and two binding sites for the Shp2 (protein tyrosine phosphatase). FGF stimulation leads to FRS2 recruiting four Grb2 molecules directly and two indirectly via Shp2. Then Grb2 binds to Sos by SH3 domain. Grb2/Sos complexes are thus recruited directly or indirectly via Shp2 upon tyrosine phosphorylation of FRS2 in response to FGF-stimulation, and concomitantly leading to Ras-MAPK pathway (Lax et al., 2002; Hadari et al., 1998; Ong et al., 2000) (Figure 1.6), which is specifically critical in regulating cell proliferation (Matsui, Brody et al., 1999) and cell migration (Pintucci et al., 2002; Antoine et al. 2006; Kouhara et al., 1997).
1.2.4.2.1.2 PI-3 Kinase/Akt Signaling Pathway

Experiments with embryonic fibroblasts from FRS2α-/- mice demonstrated that FRS2α plays not only a critical role in FGF-induced Ras-MAP kinase stimulation but also PI-3 kinase (PI-3K) stimulation (Eswarakumar et al., 2005) (Figure 6). Following FGFR tyrosine phosphorylation and subsequent FRS2 phosphorylation, Grb2 recruits the docking protein Gabl, which binds constitutively to the C-terminal SH3 domain of Grb2. The assembly of Grb1 with Grb2/FRS2α complex enables tyrosine phosphorylation of Gab1. Activated Gab1 then recruits signaling protein PI3K leading to activation of the PI3K-Akt cell survival pathway (anti-apoptotic pathway) (Schlessinger and Lemmon, 2003) (Figure 6). KGF-1 stimulation of cells caused a fivefold increase in Akt kinase activity and it is important in KGF-1 mediated cell survival (Ray, 2005).

1.2.4.2.1.3 The PLCγ Signaling Pathway

FGF mediated FGFR phosphorylation recruits phospholipase Cγ (PLCγ) through its SH2 domain (Mohammadi et al., 1991). PLCγ is a cytoplasmic membrane-associated protein that cleaves phosphatidyl-inositol-4, 5-bisphosphate to inositol trisphosphate (IP3) and diacylglycerol (DAG). Consequently the released IP3 stimulates the release of calcium from the endoplasmic reticulum. DAG and calcium both activate protein kinase C (PKC). PLCγ pathway is not directly involved in cell migration but regulates cell survival (Landgren et al., 1998; Reuss and von Bohlen und Halbach, 2003).
1.2.4.2.2 Ligand-independent FGFR Signaling Pathway

The functional interaction (reciprocal cross-talk) between integrins and growth factor receptors has become increasingly clear in transducing cell signals of cell proliferation, migration and survival (Cabodi et al., 2004). Accumulated evidence suggests that integrins through their physical association with receptors could directly activate growth factor receptors in the absence of their growth factor ligands (Yamada and Even-Ram, 2002). This association of αvβ3 integrin with PDGFβ receptor (Schneller, 1997), VEGF receptor (Soldi et al., 1999) and β1 integrin with EGFR (Yu et al., 2000) have been observed. Specifically, PDGF-R, EGFR, VEGF-R, HGFR are all transactivated after integrin engagement without the presence of their ligands (Yu et al., 2000; Yamada and Even-Ram, 2002).

Integrin clustering and binding to ECM molecules such as collagen and fibronectin phosphorylates their downstream signal molecule focal adhesion kinase (FAK), a nonreceptor tyrosine kinase, which can activate c-Src by binding its SH2 domain. Consequently this leads to binding of Grb2-Sos complex which subsequently activates Ras-MAPK signaling to promote cell migration and proliferation. FAK is thought to be able to bridge cytoplasmic tails of growth factor receptors and integrins through its binding to c-Src SH2 domain (Cabodi et al., 2004).

Integrins interact directly with the intracellular cytoskeleton. Mechanical stimuli such as distension or compression alter the cell morphology and cytoskeleton. Upon mechanical injury integrins on the cell surface may lose attachment to its ECM
molecules and require immediate re-attachment to their ECM ligands. The dynamic binding of integrins to both ECM extracellular and actin-associated cytoskeletal proteins (e.g. talin, vinculin) intracellularly during the wounding may induce intracellular (Maniotis et al., 1997; Yates and Rayner, 2002) signaling.

Although the association of integrin αvβ3 with FGFR1 upon the stimulation of both fibronectin and FGF2 has been reported by Sahni (2004), few studies have reported the involvement of integrins in ligand-independent FGFR activation. Recently, there are studies indicated that ultraviolet irradiation (UVB) induced reactive oxygen species (ROS) production was able to induce KGFR ligand-independent activation and signal transduction, which was similar to that elicited by the KGF ligand (Marchese et al., 2003). The following chapter will focus on ROS and its regulation of KGFR.

1.2.5 Summary

In conclusion, KGF-1 (FGF-7), a member of the FGF family, is a paracrine mediator expressed by mesenchymal cells and specifically targets epithelial cells. Like other FGFs, KGF-1 activates its only receptor, KGFR, which is expressed exclusively by epithelial cells, and concomitantly signals mainly via the Ras-MAPK, PI-3K and PLCγ signaling pathways to exert their biological effects, regulating cell proliferation, migration, differentiation, and survival in a variety of cell types (Reuss and von Bohlen und Halbach, 2003; Landgren et al., 1998). Collective studies have suggested that KGF-1/KGFR play an important role in wound healing and chronic inflammation, such as periodontal disease. This dissertation focuses on how the KGF-1/KGFR regulates
the epithelial cell behavior in terms of epithelial cell proliferation and migration.
1.3 Oxidative Stress (Reactive Oxygen Species-ROS)

1.3.1 Introduction

Radicals (often referred to as free radicals) are defined as "any molecule capable of independent existence that contains one or more unpaired electrons" (Halliwell, 1991). These unpaired electrons are highly reactive, so the radicals are very likely to rapidly take part in chemical reactions. There are two groups of free radicals namely reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Mo, 2005; Shen and Liu, 2006) (Figure 1.7). They are natural byproducts of the normal metabolism of oxygen and generally expressed in all mammalian cells (Chapple, 1997). ROS includes superoxide anion (O$_2^-$), hydroxyl radicals (OH$^-$), hypochlorous acid (HOCl) and hydrogen peroxide (H$_2$O$_2$). Hydrogen peroxide is not a true radical but is readily capable of generating free radicals such as OH$^-$ and HOCl. Collectively these radicals are capable of reacting with and damaging various molecular targets including DNA, proteins and lipids (Chapple, 1997). RNS are nitrogenous products including nitric oxide (NO), nitrogen dioxide (NO$_2$), and peroxynitrite (ONOO$^-$). Different from ROS, RNS possess distinct chemical and biological properties and can induce nitrosative stress via nitrosylation and nitration of their target molecules (Shen and Liu, 2006; Levonen et al., 2001).

Mitochondria, the main site of oxygen metabolism, are the main sites of ROS and RNS generation. The superoxide radical (O$_2^-$), a product of oxygen metabolism, is produced by NADPH oxidase (phox), cytochrome P-450 and xanthine oxidase in the mitochondria (Ray, 2005). This highly reactive molecule is scavenged by the mitochondrial superoxide dismutase (SOD) to produce hydrogen peroxide (H$_2$O$_2$).
Glutathione peroxidase (GPx), acting on a reduced glutathione (GSH), present mainly in mitochondria and nuclei, or enzyme catalase (CAT), localized largely or entirely in peroxisomes, convert hydrogen peroxide (H$_2$O$_2$) to H$_2$O and O$_2$. Alternatively, H$_2$O$_2$ can produce highly reactive hydroxyl radical (OH$^-$) via fenton reaction with Fe$^{2+}$ or Cu$^{2+}$, or hypochlorous acid (HOC!) through myeloperoxidase. The former is one of the most reactive radicals and can cause extensive damage to proteins, lipids, and DNA (Canakci et al., 2005; Kim et al., 2003) (Figure 1.7). In RNS pathway, free radical nitric oxide (NO) produced from L-arginine with isoenzyme NO synthases (NOS) also reacts with superoxide radical to produce a peroxynitrite (ONOO$^-$), which is a highly reactive radical and can cause extensive tissue damage (Canakci et al., 2005) (Figure 1.7).

ROS and RNS play a major role in the normal physiological function of all cells. For example, both ROS and RNS have the ability to activate a variety of receptor-mediated signaling pathways. Practically all amino acids can serve as targets for oxidative attack by ROS or RNS, while some amino acids such as tyrosine and cysteine are particularly sensitive to them (Droge, 2002). Tyrosine is an important amino acid involved in cell membrane receptor phosphorylation reaction and signal transduction pathways (Ischiropoulos and al-Mehdi, 1995; Stadtman and Berlett 1997). Peroxynitrite modifies proteins in the aromatic ring of tyrosine residues through tyrosine nitration. Peroxynitrite is known to activate the epidermal growth factor receptor (EGFR) as well as platelet-derived growth factor receptors (PDGFR), the latter leading to activation of anti-apoptotic pathway, phosphoinositide-3-kinase (PI3K) and Akt (Klotz et al., 2002; Zhang et al., 2000). On the other hand, peroxynitrite has also been reported to cause inactivation of protein function. For example, the dose-dependent inactivation of
glutathione reductase (GR) by peroxynitrite both in vivo and in vitro conditions was observed (Sahoo et al., 2006). Another study showed nitration by peroxynitrite reduced enzyme prostaglandin H synthase-1 (PGHS-1) (Deeb et al., 2006).

ROS at high levels, or chronically produced, can cause oxidative stress within tissues and result in direct damage to cellular components, such as protein, cell membrane and DNA:

a) Protein: A relative minor structure modification of a single protein molecule can lead to a marked change in its biological activity. Hydroxyl and peroxynitrite generation can effectively react with amino acids to generate denatured or non-functioning proteins by protein fragmentation, cross-linking, and unfolding. Tyrosine nitration may not only compromise protein function, for example, tyrosine nitrosylation of the mitochondrial enzyme (MnSOD) is associated with the loss of enzyme function (Canakci et al., 2005), but also have serious consequence in cellular regulation (Chapple and Matthews, 2007).

b) Lipid membrane: Hydroxyl or peroxynitrite are particularly effective in attacking polyunsaturated fatty acids in cell membranes through lipid peroxidation. It creates cross-linkages and disrupts the membrane structure and function, which leads to an influx of Ca$^{2+}$ and subsequent activation of Ca$^{2+}$-dependent proteases. The accumulation of the peroxidation in cell membranes leads to a profound effect on cell fluidity, which affects the activity of transmembrane enzymes, transporters, receptors and other membrane proteins (Chapple and Matthews, 2007; Canakci et al., 2005).
c) DNA: Substantial DNA modification can be generated by hydroxyl, nitric oxide and peroxynitrite, including DNA strand cleavage, base pair mutations, deletions and insertions. It can lead to gene mutation and, in turn, results in malignant transformation or cell death (Canakci et al., 2005).
Figure 1.7 Pathway of ROS and RNS production and clearance (Modified from Kim et al., 2003).
1.3.2 ROS in Wound Healing

1.3.2.1 Hydrogen Peroxide (H$_2$O$_2$)

Generation of ROS exposes host tissues to oxidative damage and negatively impacts normal cell functions. ROS are implicated in a variety of pathological processes and diseases, but also are necessary for normal host defense (Monteiro and Stern, 1996; Chapple and Matthews, 2007). During wound healing, H$_2$O$_2$ is generated to a large degree by macrophages and PMNs and to the lesser degree it is generated in local fibroblasts, keratinocytes, and endothelial cells (Rhee, 1999; Suchett-Kaye et al., 1998). H$_2$O$_2$ is a weak ROS with limited tissue cytotoxicity unless its concentration exceeds 50uM (Halliwell et al., 2000) or is converted to OH$^-$ by reacting with Fe$^{2+}$ metal ions (Fenton reaction) or ultraviolet light (Chapple, 1997). Intracellular H$_2$O$_2$ or extracellular H$_2$O$_2$ that crosses through the cell membrane by passive diffusion directly react with intracellular molecules to regulate signaling molecules such as tyrosine kinases or indirectly by generation of more reactive oxygen species such as OH$^-$ through fenton reaction (Halliwell et al., 2000; Chapple and Matthews, 2007; Duarte et al., 2007).

At the wound sites, low concentrations of H$_2$O$_2$ supports wound healing. Studies have shown that low concentrations of H$_2$O$_2$ is increased in wound edge cells and accelerated the wound healing process by facilitating in vivo wound angiogenesis (Roy et al., 2006). Mouse skin epithelium, superoxide expression (consequently H$_2$O$_2$ expression) significantly increased in wound edge epithelial cells at 12 hours post wounding. From the same study, p47phox deficient mice with impaired NADPH
oxidase demonstrated reduced endogenous \( \text{H}_2\text{O}_2 \) generation in the wound site and compromised wound angiogenesis and healing (Roy et al., 2006). However, the abnormal wound closure in p47phox deficient mice was completely corrected by low-dose topical \( \text{H}_2\text{O}_2 \) treatment (Whitlock et al., 2002; Roy et al., 2006).

Taken together, it is clear that at low concentrations \( \text{H}_2\text{O}_2 \) at the wound site plays an important role in supporting the dermal wound healing process as a second messenger (Roy et al., 2006).

### 1.3.2.2 Nitric Oxide (NO)

NO is a highly diffusible intercellular signaling molecule implicated in a wide range of biological effects. It is generated by catalyzing the \( L\)-arginine through the enzyme nitric oxide synthetase (NOS). The NO synthetases exist in three distinct isoforms, two constitutives (endothelial-eNOS and neuronal-nNOS) and one inducible isoform (iNOS). They are expressed in a wide range of cells such as macrophages, keratinocytes, endothelial cells and fibroblasts (Luo and Chen, 2005; Curra et al., 2006).

There is increasing evidence for a functional role of NO in wound healing that indicates NO has a positive regulatory effect on wound repair. When rats are fed an arginine-free diet, wound healing is impaired; conversely, when humans and animals are fed an arginine-enriched diet there is improved healing as measured by collagen deposition and wound breaking strength (Barbul et al., 1990). Consequently inhibition
of iNOS by competitive inhibitors decreases collagen deposition and breaking strength of incisional wounds (Schaffer et al., 1999). In addition, use of NO donors (NO application) also improves incisional and excisional wound healing in rats (Shabani et al., 1996).

Many impaired wound healing events associated with medical conditions also show reduced NO production. For example, the wound in diabetic mice is associated with decreased formation of NO metabolites in the wound environment. Addition of L-arginine and NO donors can partially reverse the impaired healing associated with diabetes by restoring wound NO levels to physiologic levels (Efron et al., 1999). Not only can inadequate levels of NO lead to the impaired wound healing, NO production in excess of physiologic concentration also negatively impacts wound healing as well. Induction of sterile inflammation by turpentine generated a sterile abscess and increased NO synthesis with subsequent impaired collagen production. However, iNOS inhibition restores normal collagen synthesis without affecting the overall inflammatory responses (Rizk et al., 2004). The production of NO with superoxide (O$_2^-$), peroxynitrite (ONOO$^-$), is a highly reactive radical and can cause extensive tissue damage (Canakci et al., 2005).

**1.3.3 ROS Involved In Periodontal Disease**

Up-regulation of ROS production in periodontitis has been described. The predominant inflammatory cells associated with periodontitis, neutrophils, are a major source of ROS generation during an inflammatory response, which are regarded as being highly
destructive in nature (Chapple and Matthews, 2007). Besides PMNs, ROS are also produced by other cells associated with periodontal tissues such as gingival fibroblasts and pocket epithelial cells. ROS produced by gingival fibroblasts is responsible for the initial degradation of extracellular matrix components seen in periodontal disease (Misaki et al., 1990). The chronic production of superoxide by epithelium within the crevice/pocket also represents an important source of local ROS production (Chamulitrat et al., 2004).

In an organotypic culture, using normal human oral epithelial cells exposed to H$_2$O$_2$ induced hyper-proliferation. It was suggested that the ROS induced hyperproliferation may explain some of the changes in epithelium associated with onset of periodontal disease (Royack et al., 2000). On the other hand, removal of excessive production of ROS in an animal model by scavenger enzymes, such as SOD and CAT and GSH, decreased the periodontal tissue damage. Local delivery of SOD and CAT in a Beagle dog periodontitis model provided a significant reduction in periodontal pocket depth, attachment loss, and suppressed local inflammation (Petelin et al., 2000). In a clinical study, glutathione-peroxidase level decreased with periodontal pocket depth increase (the progression of periodontal disease), while it increased significantly with significant (p < 0.05) improvement of clinical periodontal parameters after the completion periodontal treatment (Huang et al., 2000; Tsai et al., 2005).
1.3.4 ROS Induce Ligand-independent TK-GFR (Tyrosine Kinase Growth Factor Receptors) Activation via c-Src Kinase

1.3.4.1 ROS (H₂O₂) Is Associated With Phosphorylation of TK-GFR Including KGFR

As a small, diffusible, and ubiquitous molecule, H₂O₂ fulfills the important prerequisites for an intracellular messenger (Rhee, 1999). Extensive evidence exists to establish that H₂O₂ generation mediates cell migration, growth, and gene expression by regulating signaling molecules such as ERK, PI3K, NF-κB, and tyrosine kinases (Ushio-Fukai, 2006; Rojkind et al., 2002; Chapple and Matthews, 2007). Growing evidence suggests that H₂O₂ may serve as a second messenger that activates various tyrosine kinase growth factor receptors. Insulin receptor (Koshio et al. 1988), PDGF receptor (Sundaresan et al., 1995), EGFR (Peus et al., 1998; Peus et al., 1999; Sato et al., 2003; Monteiro and Stern, 1996) and KGFR (Marchese et al., 2003) can all be activated by H₂O₂ in a ligand-independent manner.

Ultraviolet B radiation (UVB) treated keratinocytes or keratinocytes treated with the pro-oxidative agent, cumene hydroperoxide (CUH), a synthetic and stable hydroperoxide, both induced H₂O₂ production and ligand-independent KGFR activation and internalization. Pretreatment of cell cultures by the ROS scavenger N-Acetyl-L-cysteine (NAC, a synthetic precursor for glutathione), in conjunction with glutathione peroxidase enhanced ROS (H₂O₂) scavenging and abolished KGFR activation and internalization (Belleudi et al., 2006; Marchese et al., 2003).
1.3.4.2 c-Src Is Associated With TK-GFR Activation And Acts As The Linker of H₂O₂ And TK-GFR

H₂O₂ also regulates c-Src kinase activation and directly activate c-Src (Abe, 1997; Suzuki et al., 2002; Sato et al., 2003; Saito et al., 2002). Cellular Src (c-Src), a cytoplasmic non-receptor protein tyrosine kinase and a member of Src family tyrosine kinases, serves as a regulator of TK-GFR activation (Martin 2001; Vasant et al., 2003; Griendling and Ushio-Fukai, 2000). c-Src kinase has been shown to modulate the activity and signaling of TK-GFR, such as EGF-R, PDGF-R, and IGF-R (Thomas and Brugge, 1997; Saito et al., 2002; Peterson et al., 1996; Gonzalez-Rubio et al., 1996). c-Src activation induces tyrosine phosphorylation of EGF-R on Tyr845 in vitro (Tice et al., 1999). In addition, dominant negative c-Src kinase mutants or over expression of a negative regulator of c-Src kinase were both associated with inhibition of EGFR tyrosine phosphorylation (Luttrell et al., 1997). Pretreatment of cells with the c-Src inhibitor, 4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1), or the expression of dominant-negative c-Src also blocked integrin dependent ligand-independent EGFR phosphorylation (Moro, 2002; Bromann et al., 2004). Antioxidants (ROS scavenger enzyme) including NAC and ebselen (a glutathione peroxidase mimetic) inhibited c-Src phosphorylation in vascular smooth muscle cells (VSMCs), while in the same study the c-Src kinase inhibitor PP1 or overexpression of kinase-inactive c-Src attenuated EGFR transactivation (Ushio-Fukai et al., 2001). A similar inhibitory effect of NAC on EGF receptor transactivation has been demonstrated in rat cardiac fibroblasts (Wang et al., 2000). It strongly suggests that c-Src is one signaling molecule that links ROS and EGFR phosphorylation.
Taken together, all evidences strongly suggest ROS (H₂O₂) induced ligand-independent tyrosine kinase growth factor receptors (TK-GFRs) activation is linked via the c-Src kinase signaling molecule (Thomas and Brugge, 1997).

1.3.5 Summary

ROS, serving as a second messenger, is involved in wound healing process and, at low concentration, positively facilitate wound healing. The exact molecular mechanism involved in this process is not clear. It may possibly be explained by ROS mediated cell membrane receptor phosphorylations, such as TK-GFR, and their following signal transduction pathways, c-Src kinase and MAPK. This in turn regulates wound edge cell migration and proliferation. This dissertation focuses on the relationship of ROS and ligand-independent KGFR activation during wounding, which has not been addressed in previous studies.
1.4 References


Jang, J. H., F. Wang, et al. (1997). "Heparan sulfate is required for interaction and activation of the epithelial cell fibroblast growth factor receptor-2IIIb with..."


CHAPTER 2

2. Rationale, Hypothesis and Objectives

2.1 Rationale

Periodontal disease is a very common oral disease that affects a large portion of the population. One early event associated with periodontitis is JE proliferation and apical migration along root surface. However, the regulation of JE proliferation and migration associated with the initiation and progression of periodontitis is unclear.

This dissertation has focused on one epithelial specific growth factor, KGF-1, which is expressed by CT and stimulates epithelial cells through the KGFR. To date we have established that KGF-1 is expressed by human gingival fibroblasts and its expression is induced by \textit{P. gingivalis} and \textit{E. coli} LPS and pro-inflammatory cytokines (Sanaie et al. 2002; Putnins et al. 2002). Furthermore, in a rat periodontal disease model, KGFR protein and gene expression were significantly up-regulated in basal and parabasal pocket epithelial cells compared to other growth factor receptors such as HGFR, EGFR and FGFR1 (Ekuni et al. 2006). It is unknown if KGF-1 and KGFR are expressed in human gingival tissues and how this changes with the onset of disease. Therefore, we first examined the expression and localization of KGF-1 and KGFR in JE and PE in healthy and diseased human periodontal tissue samples.

The periodontal pocket is a unique environment. PE and JE are non-keratinized stratified squamous epithelium with wide intercellular junction and this relatively weak epithelial barrier is positioned next to a root surface that is often covered with a rough
and abrasive calculus. Therefore, it seems quite possible that this weak epithelial architecture which is juxtaposed next to an abrasive surface can easily be microwounded. The impact that this local microwounding may have on KGFR activation, events regulating activation of this receptor and the functional significance of this receptor activation mechanism on wound edge epithelial cells will be examined using an *in vitro* scrape wounding model.
2.2 Hypothesis and Objectives

2.2.1 Hypothesis I: KGF-1 and KGFR proteins and genes are expressed in human gingival tissues and their expression is increased in periodontitis tissue samples

Objective 1: To localize KGF-1 and KGFR protein expression in human healthy and periodontal diseased tissues

Objective 2: To localize KGF-1 and KGFR gene expression in both gingival epithelium and connective tissues using Laser Capture Microdissection and RT-PCR

2.2.2 Hypothesis II: KGFR activation associated with in vitro epithelial cell culture microwounding occurs in a ligand-independent manner

Objective 1: To examine if in vitro microwounding is associated with ligand-independent KGFR activation

Objective 2: To examine the mechanism regulating ligand-independent KGFR activation

2.2.3 Hypothesis III: Ligand-independent KGFR activation regulates in vitro wound edge cell apoptosis, proliferation and migration

Objective 1: To examine the downstream signaling pathways activated by ligand-independent KGFR activation

Objective 2: To examine the effect of ligand-independent KGFR activation on wound edge associated epithelial cell apoptosis, proliferation and migration.
CHAPTER 3

3. Keratinocyte Growth Factor-1 Expression in Healthy and Diseased Human Periodontal Tissues

3.1 Abstract

Objectives & Methods: Keratinocyte Growth Factor-1 (KGF-1) is upregulated in chronic inflammation and specifically stimulates epithelial cell proliferation by signaling through the epithelial-specific Keratinocyte Growth Factor Receptor (KGFR). We examined KGF-1 and KGFR protein and gene expression in healthy and diseased periodontal tissues and explored the mechanism regulating KGF-1 localization to gingival epithelial cells using immunohistochemistry and laser capture microdissection.

Results: In tissues collected from healthy patients, KGF-1 protein localized to areas of junctional and basal oral epithelial cells and was significantly increased in periodontal pocket epithelium (p<0.01) and in the oral epithelium (p<0.05) of disease-associated tissues. KGFR localized to the junctional and the basal and parabasal cells of oral epithelium, with the relative staining intensity being increased in disease-associated pocket epithelium (p<0.05). Laser capture microdissection with RT-PCR confirmed KGF-1 and KGFR were specifically expressed by connective tissue and epithelium, respectively. KGF-1 localization to epithelial cells was largely eliminated by Suramin pretreatment, indicating interaction with the KGFR.

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Conclusions: KGF-1 and KGFR proteins are expressed in health but significantly increased in diseased periodontal tissues. We hypothesize that upregulation of KGF-1 and KGFR protein associated with disease regulates epithelial cell behavior associated with onset and progression of periodontal pocket formation.
3.2 Introduction

Three major events are associated with initiation and progression of periodontal attachment loss. These include local epithelial cell proliferation and migration, dissolution of Sharpey’s fibers with loss of attachment and ultimately resorption of alveolar bone (Birkedal-Hansen, 1998). It is known that induction of epithelial cell proliferation and migration begins in “early” disease and ultimately results in lining the pocket as disease progresses (Birkedal-Hansen, 1998; Page, 1976). The regulation of this process and its contribution to disease is poorly understood but local expression of cytokines and growth factors likely play significant roles (Kornman, 1997; Okada, 1998). Keratinocyte Growth Factor-1 (KGF-1) regulates normal epidermal homeostasis but is also significantly upregulated during wound healing and in a variety of chronic inflammatory conditions like Crohn’s disease, ulcerative colitis and psoriasis (Bajaj-Elliott et al., 1997; Brauchle et al., 1996; Finch, 1997; Finch, 1996; Guo et al., 1993; Werner et al., 1992). However, little is known about KGF-1 localization in gingival tissues with periodontal disease progression.

KGF-1 (FGF-7) belongs to the heparin-binding fibroblast growth factor family (review (Ornitz and Itoh, 2001; Rubin et al., 1989). KGF-1 represents one of two KGF subfamily members and shares more than 60% sequence identity with KGF-2 (FGF-10) (Emoto, 1997). KGF-1 is best described as a paracrine mediator expressed by mesenchymal cells and specifically stimulates epithelial cells (Finch et al., 1989; Rubin et al., 1989). Dermal fibroblasts, microvascular endothelial cells, smooth muscle cells and activated γδ T cells all express KGF-1 (Winkles et al., 1997; Boismenu, 1994;
Traditionally, KGF-1 transcripts were exclusively detected in the connective tissue. However, KGF-1 was recently found to be expressed by epithelial cells in some tissues (Steele et al., 2001; Ka, 2000). In these cases an autocrine stimulation pathway is likely to be in place. The almost exclusive specificity of KGF-1 for epithelial cells is due to epithelial specific expression of Keratinocyte Growth Factor Receptor (KGFR). This receptor is a splice variant of Fibroblast Growth Factor Receptor-2 (FGFR-2) and is designated FGFR-2IIIb (Miki, 1991; Bottaro, 1990).

In oral tissues, KGF-1 was expressed in vitro by fibroblasts isolated from oral buccal mucosa, gingiva, periodontal ligament and in the stroma associated with inflamed periapical tissues (Gao et al., 1996; Gron et al., 2002; Ohshima et al., 2002; McKeown et al., 2003; Dabelsteen et al., 1997; Mackenzie and Gao, 2001; Sanaie et al., 2002; Putnins et al., 2002). Pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α induced KGF-1 protein and gene expression in gingival fibroblasts in vitro (Sanaie et al., 2002; Gron et al., 2002; McKeown et al., 2003). In contrast to KGF-1, KGF-2 was only weakly expressed in gingival fibroblasts and this expression was not changed by pro-inflammatory cytokines (Sanaie et al., 2002). We recently established that LPS purified from *Escherichia coli* and *Porphyromonas gingivalis* also induced KGF-1 protein and gene expression through a Toll-like receptor signaling pathway (Putnins et al., 2002). KGF-1 expression in gingival tissues and its upregulation by pro-inflammatory cytokines and lipopolysaccharide support the putative role of KGF-1 in regulating epithelial cell function in periodontal diseases.
The purposes of our study were to first examine and determine if KGF-1 and KGFR proteins and genes were expressed in tissue samples collected from periodontally-healthy patients and patients with disease and if significant differences in protein expression between health and disease were identifiable. In addition, we identified significant KGF-1 protein localization in oral epithelial basal cells and all layers of junctional and pocket epithelium. Therefore, we explored KGF-1 and KGFR gene expression profiles of epithelial and connective tissue cells and examined the cellular binding mechanism involved in KGF-1 protein localization associated with periodontal tissues.
3.3 Material and Methods

3.3.1 Sample Collection and Immunohistochemistry

Oral gingival tissue samples were collected from periodontally-healthy patients (n=15) with minimal sulcus depth (<3 mm) and bleeding on probing who either required surgical crown lengthening or from residual tissues attached to teeth extracted for non-periodontal reasons. In addition, tissue samples were collected from patients with periodontitis (n=13) with advanced attachment loss (pocket depth ≥ 5 mm), but who still required open flap debridement to control disease. All samples were collected from patients who had provided informed consent (Appendix I) and were not taking prescription medication at the time of sample collection. Tissues were immediately snap frozen in Tissue-Tek O.C.T. compound (Sakwa Finetek Inc., Torrance, USA) and sectioned in a cryostat at 8 μm (Cambridge Instruments, Heidelberger, Germany). Tissue sections were acetone fixed, rehydrated and permeabilized in hybridization buffer (PBS + 0.01% Triton X-100 + 0.1% bovine serum albumen [BSA]), blocked with either goat or horse serum for 20 min at room temperature prior to incubation with either rabbit anti-human KGF-1 (Santa Cruz, CA, USA) or rabbit anti-human KGFR (Santa Cruz, CA, USA) antibodies at 4°C overnight in a humidified chamber. Slides were washed (PBS + 0.1% BSA + 0.01% Triton X-100) and then incubated with a biotin-labeled secondary antibody for 1 hr at room temperature, washed, reacted with an avidin biotin peroxidase complex (ABC Elix Kits, Vector Laboratories, Burlingame, CA, USA) at room temperature for 30 min and Vector VIP substrate (Vector Laboratories, Burlingame, CA, USA) was used for color development as manufacturer’s directions. Negative controls consisted of either incubation with non-
immune serum without inclusion of primary antibody or with hybridization buffer minus primary antibody prior to addition of the appropriate secondary antibodies. To ensure that junctional epithelium was present in our healthy patient samples, they were stained with an anti-laminin-5 antibody (Cedarlane Laboratories, Ontario, Canada). Laminin-5 positive staining of the internal and external basal lamina confirmed the presence of junctional epithelial cells (Oksonen et al., 2001). Semi-quantitative evaluation of staining intensity was based on a previously-published method that incorporated the percentage of positively-stained cells and color intensity (Krajewska et al., 1996). To ensure that all slides were developed in an equivalent manner, we included a slide stained for perlecan in each experiment. Positive perlecan staining was similar for all samples and this served as an effective control during the color development step. Statistical differences in relative KGF-1 and KGFR staining intensity between healthy and diseased tissue samples were examined using Student’s t-test.

3.3.2 Anti-KGF-1 Antibody Specificity Experiment

To ensure anti-KGF-1 antibody specificity, we rigorously examined this antibody’s specificity using a three-step approach. First, we utilized ELISA. Each of 5 wells in a 96-well plate was coated with either 10 ug/ml (100 ul/well) of KGF-1, KGF-2, bFGF (Upstate Biotechnology, Lake Placid, WX, USA) or 1% BSA (negative control) proteins for 2 hr at room temperature. Wells were washed 3 X 5 min (phosphate-buffered saline, pH 7.4, with 0.05% Tween 20) and blocked with 2% heat-denatured BSA/PBS at room temperature for 1 hr followed by washing (3 X 5 min). Rabbit anti-
human KGF-1 antibody (1:800) was added to the first of each well coated with either KGF-1, KGF-2, bFGF or BSA protein for 2 hr. Antibodies were transferred to the second equally-coated well, incubated for 2 hr and then transferred and incubated with each of the three similarly-coated wells. These absorbed anti-KGF-1 antibodies were used for subsequent immunohistochemical stainings (see below). Each of the wells was washed 3 X 5 min prior to the addition of 100 ul/well (1:100) anti-rabbit IgG peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA), incubated for 1 hr followed by 3 X 5 min washings. Color was developed with ABTS substrate consisting of 4 ml buffer (0.1 M NaC$_2$H$_3$O$_2$, 0.05 M NaH$_2$PO$_4$), 0.2 ml ABTS (22 mg/ml in distilled water), and 0.04 ml diluted H$_2$O$_2$ (0.2 ml of 30% stock solution in 7 ml distilled water). The optical density of wells was measured with a microtiter plate reader set to 570 nm absorbance (Bio-Rad Laboratories, CA, USA). Anti-KGF-1 antibody (1:800, 75 ul/well) coated wells served as a positive control. Secondly, to ensure antibody specificity, tissue slides were stained with the pre-absorbed antibodies from the above ELISA experiment. Non-absorbed anti-KGF antibody alone or pre-absorbed anti-KGF-1 antibodies were reacted with human gingival tissue sections as described previously. Lastly, Western blotting was utilized to determine if the anti-KGF-1 antibody cross-reacted with KGF-2 protein. KGF-1 and KGF-2 protein (10 ng/lane) were fractionated on a 12% SDS-polyacrylamide gel and blotted to hybond-ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK). Filters were blocked with TBS containing 5% skim milk for 90 min at 37°C, washed and incubated with anti-KGF-1 antibody for 3 hr at room temperature. Filters were then incubated
with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. Detection was performed using the Amersham ECL Kit (Amersham, Buckinghamshire, UK).

3.3.3 Enzymatic Tissue Digestion and Suramin Pretreatment

To examine the possible interaction of KGF-1 with heparan sulfate or chondroitin/dermatan sulfate proteoglycan tissue sections were either digested at 37°C for 1 hr with heparitinase (0.04 U/ml, ICN Biomedical, Aurora, USA) in 1 mg/ml BSA in PBS buffer or chondroitinase ABC (0.7 U/ml, ICN Biomedicals, Aurora, USA) in 0.1 M Tris-HCl + 0.03 M sodium acetate, pH 8.0 buffer, respectively. KGF-1 interaction with KGFR was examined by incubating tissue sections with 100 µM Suramin (a polyanionic drug to dissociate growth factors from their receptors) (Sigma, St. Louis, USA) following a pre-established protocol (Marchese et al., 1995). In each case, controls were incubated with PBS using equivalent experimental conditions. Experimental and control slides were incubated with one of the following primary antibodies: anti-KGF (1:800), anti-KGFR (1:200), anti-heparin sulfate (1:100, Seikagaku, Tokyo, Japan), anti-chondroitin sulfate (1:50, Sigma, St. Louis, USA) or anti-bFGF (1:200, R&D System, Minneapolis, USA) antibodies in a humid chamber at 4°C overnight, followed with biotin-labeled secondary antibodies. Color development was as described previously.

3.3.4 Laser Capture Microdissection

Frozen sections were air dried for 30 sec, rinsed in DEPC-treated water (depCH₂O) for 30 sec, immediately stained with Histogene Stain (Arcturus, CA, USA) for 20 sec and rinsed with depCH₂O for 30 sec. Slides were sequentially washed through 75%, 95%
and 100% ethanol for 30 sec each, transferred into xylene for 5 min and dried in a fumehood for 5 min. Laser capture microdissection of subepithelial connective tissue and basal epithelial cells from serial sections (Arcturus, CA, USA) was immediately performed and samples collected into separate 0.2 ml tubes. RNA extractions were performed using Rneasy Mini Kit (Qiagen, CA, USA) and RNA was diluted in 30 ul of depcH₂O.

One step RT-PCR (Invitrogen, Burlington, Ontario, Canada) was performed following the manufacturer’s protocol using sense and antisense primers (Table 1). The PCR program consisted of an RT step of 50°C for 30 min, initial denaturation at 94°C for 3 min, and followed by 40 cycles of denaturation for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min. An aliquot of the reaction was fractionated on a 1.5% agarose gel (Biorad) prepared in 1 X TBE buffer containing 1 ul/100 ml of ethidium bromide (0.1 ug/ml). The gel was examined using UV light and photographed. Negative controls (without sample mRNA) were also added to each amplification run. In cases where bands were not visualized at first, repeated PCR amplifications were performed using the original PCR reaction in order to ensure that no target sequence was present.
<table>
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<th>bp size</th>
<th>Reference</th>
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<td>266</td>
<td>Mackenzie and Gao, 2001</td>
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<td></td>
<td>GTGTGCTCTTAGCTGATGCAT (R)</td>
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<td>AACTGTTACCTGTCTCCGAG (R)</td>
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<td></td>
<td>TCTAGACGGCGAGTCAGGTCCACC (R)</td>
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Table 1. Primers Used for RT-PCR Amplification.
3.4 Results

3.4.1 Anti KGF-1 Antibody Specificity

The amino acid similarity that exists between KGF-1 and KGF-2 and other FGF family members warranted further investigation to ensure anti-KGF-1 antibody specificity. This was approached in several ways. When ELISA plate wells were pre-coated with either KGF-1, KGF-2, bFGF or BSA proteins, the anti-KGF-1 antibody only bound (high OD) to wells coated with KGF-1 (Figure 3.1A). The relative optical density in wells coated with either KGF-2 or bFGF was the same as BSA coated wells (negative control), indicating no anti-KGF-1 antibody cross-reactivity with these proteins. When the antibody was transferred to the third KGF-1 coated well, the optical density was reduced to control levels, indicating that the recovered antibody should not have retained KGF-1 specificity. To ensure this was the case, the three KGF-1 antibodies absorbed with KGF-1, KGF-2 and bFGF were used to stain oral gingival tissues (Figure 3.1B). Anti-KGF-1 antibody that was not pre-absorbed stained oral basal epithelial cells associated with Rete Ridges (Figure 3.1B-i). In contrast, the antibody absorbed against KGF-1 protein retained no positive staining (Figure 3.1B-ii). However, antibody absorbed against KGF-2 (Figure 3.1B-iii) or bFGF (Figure 3.1B-iv) proteins stained in the same pattern as the non-absorbed antibody. Tissues stained with non-immune primary or only secondary antibodies were negative (data not shown). Paraformaldehyde fixed and paraffin embedded gingival tissues were negative (data not shown) and therefore all tissues examined were frozen sections. Western blotting with the antibody identified one 21kDa band consistent with recombinant KGF-1 protein.
**Figure 3.1 Anti-KGF-1 Antibody is Specific for KGF-1 Protein.** (A) 10 ug/ml of either KGF-1, KGF-2, bFGF or 1% BSA was used to coat 5 wells each of a 96-well plate, then blocked with 1% BSA and washed. Anti-KGF-1 antibody was reacted with each of the first wells before being transferred to each of the successive wells. The wells were washed prior to addition of peroxidase-conjugated anti-rabbit antibody, and color developed with ABTS substrate. (B) Control non-absorbed antibody (B-i) and absorbed antibodies against KGF-1 (B-ii), KGF-2 (B-iii) or bFGF (B-iv) proteins were used to stain oral gingival tissues. (C) 10 ng/lane of recombinant human KGF-1 and KGF-2 proteins were separated in a 12% polyacrylamide gel and blotted. Anti-human KGF-1 antibody was incubated with the blot, washed, followed by blotting with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and detected using chemiluminescence.
and did not cross-react with KGF-2 protein. Therefore, the anti-KGF-1 antibody used in this study was confirmed to be KGF-1 specific.

3.4.2 KGF-1 Protein Localization in Healthy and Diseased Tissues

Tissues collected from healthy and diseased tissues were stained for KGF-1 protein localization. Clinical parameters of the samples collected from healthy patients (n=15) showed a mean ± SD sulcus depth of 2.7 ± 0.62 mm (range from 1mm to 3mm) and 7% exhibited bleeding on probing just prior to surgery. Samples collected from the disease group (n=13) had a mean ± SD pocket depth of 6.7 ± 1.0 mm (range from 5 mm to 11 mm) and 62% exhibited bleeding on probing just prior to surgery. The presence of junctional epithelium in health was confirmed by positive laminin-5 staining on the internal and external basal lamina (Oksonen et al., 2001). Only junctional epithelial samples that showed this positive staining pattern were included in the healthy group (Figure 3.2A inset). KGF-1 positive staining of some junctional epithelial cells was found with the more intense staining of cells in close approximation to the internal and external basal lamina (Figure 3.2A and B). In contrast, pocket epithelium staining was generally more intense and all cell layers stained positive (Figure 3.2C and D). KGF-1 staining in the oral epithelium collected from healthy patients was also positive in basal epithelial cells with generally more intense staining associated with the rete ridges (Figure 3.2E and F). Oral epithelium collected from patients with disease generally showed more intense pericellular epithelial staining in basal and some parabasal epithelial cells (Figure 3.2G and H). In all tissues stained there was minimal staining.
Figure 3.2 KGF-1 protein localization to epithelial cells of oral gingival tissues. Tissues collected from healthy patients (A, B, E and F) and patients with advanced periodontal disease (C, D, G and H) were stained with the anti-KGF-1 antibody. Junctional epithelium stained positive for KGF-1 protein (A) and laminin-5 (inset in A). Close-up of black box outlined in (A) shown in (B). Pocket epithelium stained KGF-1 positive (C) with close-up of black box shown in (D). Oral epithelium collected from a patient with periodontal health (E) with close-up of black box shown in (F) and oral epithelium collected from a patient with periodontal disease (G) with close-up of black box shown in (H) stained positive to varying degrees for KGF-1.
in the connective tissues (Figure 3.2). Using a semi-quantitative rating method, KGF-1 staining intensity was found to be significantly higher in pocket epithelium \((p<0.01)\) when compared to junctional epithelium (Table 2). Interestingly, KGF-1 staining intensity was also significantly increased in the oral epithelium associated with disease samples \((p<0.05)\) when compared to health.

3.4.3 KGF Receptor Localization in Healthy and Diseased Tissues

In healthy gingiva, there was positive pericellular KGFR staining in most cells of the junctional epithelium (Figure 3.3A and B) and this was also found for pocket epithelium (Figure 3.3C and D). Upon examination, oral epithelium from healthy patients (Figure 3.3E and F) and diseased patients (Figure 3.3G and H) generally showed basal and parabasal epithelial staining. The mean KGFR staining intensity was higher \((p<0.05)\) in pocket epithelium when compared to junctional epithelium. However, there was no statistically significant difference in KGFR staining intensity in oral epithelium collected from health or disease (Table 2).

3.4.4 KGF-1 and KGFR Gene Expression Profile

The preponderance of literature supports KGF-1 expression by connective tissues but some published data supports KGF-1 expression by epithelial cells in select situations. To determine the source of KGF-1 gene expression, we used laser capture microdissection with RT-PCR amplification of target genes. We effectively dissected basal epithelial cells and subjacent connective tissue cells into separate tubes from our
Figure 3.3 KGFR protein localization to epithelial cells of oral gingival tissues. Tissues collected from a patient with periodontal health (A, B, E and F) and a patient with advanced periodontal disease (C, D, G and H) were stained with anti-KGFR antibody. Junctional epithelial (A) stained positive for KGFR protein. Close-up of black box outlined in (A) shown in (B). Pocket epithelium stained KGFR positive (C) with close-up of black box shown in (D). Oral epithelium collected from a patient with periodontal health (E) with close-up of black box shown in (F) and oral epithelium collected from a patient with periodontal disease (G) with close-up of black box shown in (H) stained positive for KGFR.
Table 2. Semi quantitative evaluation of KGF-1 and KGFR staining intensity on healthy and diseased gingival epithelial samples. IHC staining of KGF-1 and KGFR from 13 periodontal disease and 15 healthy gingival samples were analyzed using a semi quantitative evaluation method (Krajewska et al., 1996). Statistical significance was determined using an unpaired t-test. KGF-1 staining was significantly increased in the diseased oral epithelial samples (**)P<0.01) and pocket epithelium associated with disease showed significant higher KGF-1 staining (**)P<0.01) too. KGFR staining in the oral epithelium has no difference between healthy and diseased group. However significant higher KGFR expression was found in pocket epithelium from diseased group compared to JE from healthy group (*p<0.05).
Figure 3.4 Laser capture microdissection of specific cells. (A) Oral gingival epithelial tissues were stained with Histogene Stain® to identify dissection areas. (B) Basal epithelial cells (left) and subjacent connective tissue cells (right) were removed with laser capture microdissection. (C) Dissected epithelial (left) and connective tissue (right) cells after capture, which were used subsequently for RT-PCR.
Figure 3.5 RT-PCR amplification of mRNA purified from laser captured epithelial and connective tissue cells. One step RT-PCR kit with GAPDH, KGF-1, KGFR, type I collagen (Col I) and cytokeratin 19 (K19) specific primers was used to amplify genes present in epithelial and connective tissue cells.
frozen tissue sections (Figure 3.4). RT-PCR amplification was used to examine KGF-1 and KGFR gene expression in the connective tissue and epithelium (Figure 3.5). GAPDH served as our positive control. The one predicted band (600 bp) was found in both the epithelial and connective tissues. Controls of no RNA or primers were negative (data not shown). KGFR (354 bp) gene but not KGF-1 was expressed in epithelial cells. Two additional rounds of KGF-1 re-amplification were negative (data not shown). In contrast, KGF-1 (266 bp) but not KGFR was expressed in connective tissue. To ensure no cell cross-contamination between the epithelial and connective tissues, we included cytokeratin 19 (K19) (745 bp) as our epithelial specific gene and Type I collagen (538 bp) as our connective tissue specific gene. No false positives were found, which confirmed that our dissections were cell type specific. These data demonstrate that the KGF-1 gene is expressed by cells of the connective tissue but the protein localizes in the basal epithelial cells.

**3.4.5 KGF-1 Protein Interaction With Epithelial Cells**

We further investigated this KGF-1 protein localization to epithelial cells. KGF-1 does associate with glycosaminoglycan side chains of proteoglycans (Kim et al., 1998) (McKeehan et al., 1998). We first stained tissue samples with an anti-heparan sulfate antibody and positive staining was identified at the basal surface of the epithelial cells and in the connective tissues (Figure 3.6A left) and pre-treatment with heparitinase prior to staining effectively removed heparan sulfate from the tissues (Figure 3.6A right). Heparitinase pre-treatment was effective in stripping bFGF interaction with
Figure 3.6 KGF-1 localization to epithelial cells is mediated through KGFR. KGF-1 possible interaction with heparan sulfate was examined in control (left A, B and C) and heparitinase pre-treated tissue samples (right A, B and C). Slides were subsequently stained with either anti-heparan sulfate antibody (A), anti-bFGF antibody (B) or anti-KGF-1 antibody (C). Additional slides were either controls (left D and E) or pre-digested with Chondroitinase ABC (right D and E) and then stained with either anti-chondroitin sulfate (D) or anti-KGF-1 antibody (E). Control slides (F-left) or Suramin pre-treated slides (F-right) were subsequently stained with anti-KGF-1 antibody (F).
heparan sulfate present in the basal lamina as evidenced by loss of bFGF antibody staining of the basement membrane (Fig 3.6B left and right), but was not able to reduce KGF-1 positive staining in the basal epithelial cells (Figure 3.6C left and right). Treatment of tissues with chondroitinase ABC prior to staining with anti-chondroitin sulfate antibody effectively removed chondroitin sulfate (Figure 6D left and right) but did not reduce KGF-1 staining intensity (Figure 3.6E left and right). However, pre-treatment of tissue samples with Suramin (Marchese et al., 1995) very significantly reduced KGF-1 staining (Figure 3.6F). Pre-treatment of tissues with Suramin had no effect on KGFR staining intensity (data not shown). These data support KGF-1 protein localization on epithelial cells is largely due to the interaction with KGFR.
3.5 Discussion

Epithelial cell proliferation begins in early periodontal disease and progresses to form pocket epithelium in advanced disease (Page, 1976). The regulation of this aspect of disease and its significance in disease pathogenesis is not fully understood (Birkedal-Hansen, 1998). Pro-inflammatory cytokine expression and lipopolysaccharide from Gram-negative microorganisms associated with disease function to directly or indirectly modulate cellular behavior (Kornman and Tonetti, 1997; Takashiba et al., 2003; Okada, 1998; Darveau and Page, 1997; Suchett-Kaye et al., 1998). Pro-inflammatory cytokines (IL-1α, IL-1β, IL-6 and TNF-α) and purified lipopolysaccharide from \textit{Porphyromonas gingivalis} all significantly induced gingival fibroblast KGF-1 gene and protein expression \textit{in vitro} (Sanaie et al., 2002; Putnins et al., 2002; Gron et al., 2002; McKeown et al., 2003). Therefore increased KGF-1 protein expression in periodontal disease as we have described may have been induced by lipopolysaccharide and pro-inflammatory cytokines upregulated in response to disease-associated plaque biofilm.

Classically, KGF-1 is described as a paracrine mediator because it is expressed by mesenchymal cells and specifically stimulates epithelial cells (Finch et al., 1989; Rubin et al., 1989; Smola and Fusenig, 1993; Boismenu, 1994; Winkles et al., 1997). However, in ovarian and endometrial tissues, KGF-1 was expressed by epithelial cells (Steele et al., 2001; Ka, 2000). Our laser capture with RT-PCR approach established KGF-1 gene expression by connective tissue cells and this supports existing \textit{in vitro} data describing KGF-1 gene expression by gingival connective tissue cells (Sanaie et
The lack of KGF-1 protein staining in the connective tissue may be a reflection of a relatively low protein concentration in connective tissue cells and high KGF-1 localization in epithelial cells. This finding raised the possibility that the putative KGF-1 protein localization was a result of antibody cross-reactivity with related proteins. KGF-1 is one of 23 members in the fibroblast growth factor family and shares the highest similarity with KGF-2 (FGF-10) (Ornitz and Itoh, 2001; Emoto, 1997). An extensive testing protocol (Figure 1) established that the anti-KGF-1 antibody did not cross-react with KGF-2, its most related member, nor did it cross-react with bFGF (FGF-2). Since bFGF was previously reported to bind to keratinocytes and the basement membrane of epidermis, we had included this antibody in our specificity analysis to ensure that our antibody was not cross-reacting with bFGF (Friedl et al., 1997). The data established that our antibody was KGF-1 specific and KGF-1 protein was localized to areas of gingival epithelial cells. Previously, KGF-1 protein localization to breast epithelial cell had been described (Palmieri, 2003) but the possible cellular molecules regulating this finding were not explored.

One characteristic of KGF-1, common to all FGF molecules, is the interaction with heparan sulfate proteoglycans, and this interaction may inhibit or augment signaling (McKeehan et al., 1998; Bernfield et al., 1999). For example, glypican, a cell membrane-associated, glycosylphosphatidylinositol-linked heparan sulfate proteoglycan (HSPG) inhibited KGF-1 mitogenic effects, and pre-treatment with heparatinase abolished this effect (Berman et al., 1999; Bonneh-Barkay et al., 1997).
Conversely, dermatan sulfate (chondroitin sulfate B) augments KGF-1-mediated KGFR activation and cell proliferation (Trowbridge et al., 2002). Therefore, interaction of KGF-1 with proteoglycans may account for KGF-1 localization to epithelial cells. However, pre-treatment of tissues with heparatinase effectively eliminated tissue heparan sulfate and stripped bFGF interaction with basement membrane HSPG (Friedl, Chang et al., 1997), but was ineffective in reducing KGF-1 binding. Pre-treatment of tissues with chondroitinase ABC, which degrades chondroitin (Figure 6D left and right) and dermatan sulfate (Mitropoulou et al., 2001), did not reduce KGF-1 protein localization. Collectively, KGF-1 localization in gingival epithelium does not appear to be due to interaction with heparan, chondroitin or dermatan sulfate-containing proteoglycans. The principal high-affinity receptor for KGF-1 is a splice variant of fibroblast growth factor receptor-2 and is designated FGFR-2IIIb (Miki et al., 1991; Bottaro et al., 1990). Pre-treatment of samples with Suramin (Figure 6E left and right), an agent that strips KGF-1/KGFR interactions (Marchese, 1995), very significantly reduced but did not completely eliminate KGF-1 staining. The residual staining may in part be due to internalized KGF-1 that could not be removed or it suggests that additional unidentified molecules may interact with KGF-1. KGF-1 low affinity binding interactions with additional unidentified molecules was previously suggested (Reich-Slotky et al., 1994).

KGF-1 staining in basal oral epithelial cells and junctional epithelium and its increase with disease supports a role for KGF-1 in regulating epithelial cell behavior during onset and progression of periodontal disease. KGF-1 is induced in patients with
chronic inflammatory conditions, Psoriasis, Ulcerative Colitis and Crohn’s disease. In particular, KGF-1 expression was induced more in inflamed areas (Bajaj-Elliott et al., 1997; Finch et al., 1999; 1997; 1996; Brauchle et al., 1996). In agreement with these in vivo non-oral studies, we describe an increase in KGF-1 protein in patients with periodontal disease (mean pocket depth 6.7 mm). However, in health (mean 2.7 mm sulcus depth), weaker but still positive KGF-1 staining was present in areas of basal oral epithelium and all cell layers of junctional epithelium, suggesting a possible role in maintaining normal epithelial homeostasis. The increase in KGF-1 protein expression that extended to the oral gingival epithelium in patients with disease was unexpected. However, K19 expression, which is normally localized to junctional epithelium, was also increased in the oral epithelium of patients with disease (Sawaf et al., 1991; Bosch et al., 1989). These data suggest that periodontal diseases not only induces significant local tissue changes at the immediate site of the disease (i.e. periodontal pocket), but at the cellular level these changes may extend to the oral epithelium as well. In contrast to KGF-1, regulation of KGFR expression in chronic inflammatory conditions is more variable. KGFR expression was either unchanged or reduced in patients with inflammatory bowel disease when compared to controls, but KGFR expression was induced in patients with Psoriasis (Finch et al., 1997; 1996; Brauchle et al., 1996; Firth and Putnins, 2004). In this study, patients with periodontal disease showed a mean increase in KGFR protein levels in pocket epithelium when compared to junctional epithelium; however, this increase did not extend to the oral epithelium.
To our knowledge, this is the first in vivo study to describe KGF-1 and KGFR protein and gene expression and their changes from periodontal health to disease. Their increase may regulate several epithelial cellular processes. KGF-1 is a potent inhibitor of TNF-α and lipopolysaccharide-induced epithelial cell apoptosis (Firth and Putnins, 2004). Therefore, KGF-1 protein in basal epithelial cells and all layers of junctional epithelium may function to inhibit apoptosis and maintain epithelial cell barrier integrity. However, with the transition to a more disease-associated Gram-negative microflora, an increase in pro-inflammatory cytokine expression and upregulation of KGFR protein expression, additional stimulatory effects may occur. Based on several in vitro studies, KGF-1 is a potent oral and non-oral epithelial-specific mitogen, it induces epithelial cell migration and expression of matrix metalloproteinases-1, -9 and -13 (Drugan et al., 1997; Tsuboi et al., 1990; Putnins et al., 1995; Putnins et al., 1996; Putnins et al., 1999; Uitto et al., 1998). All of these cellular processes are associated with pocket formation (Birkedal-Hansen, 1998). Therefore, it is interesting to hypothesize that the upregulation of KGF-1 protein expression that we have identified in periodontal disease tissues may play a role in regulating onset and progression of epithelial cell proliferation and migration associated with periodontal pocket formation.
3.6 References


4. An *in vitro* Analysis of Mechanical Wounding Induced Ligand Independent KGFR Activation

4.1 Abstract

**Objectives:** We have established that the epithelial-specific Keratinocyte Growth Factor Receptor (KGFR) was significantly upregulated in human diseased periodontal tissues. Since periodontal tissues consist of a weak junctional epithelial cell barrier juxtaposed next to very rough and abrasive calculus we hypothesized this may be associated with mechanical wounding induced ligand independent activation of the KGFR. We developed an *in vitro* mechanical wounding model to examine ligand independent KGFR activation and associated intracellular signaling events regulating wound edge apoptosis, proliferation and migration.

**Methods:** Confluent quiescent HaCaT cell line cultures were microwounded on a 1 mm grid. KGFR internalization evident along the wound edge was examined using confocal microscopy and KGFR activation was assayed by immunoprecipitation with phosphotyrosine-specific antibodies followed by Western blotting using KGFR and downstream FRS2 and Src-specific antibodies in the presence and absence of inhibitors. Various ligand-independent signaling pathways were examined but reactive oxygen species (ROS) generation was examined in detail using DCFH-DA with or without the H$_2$O$_2$ scavenger (NAC). Mechanical wounding induced ROS generation...
and its impact on cell migration was examined in the presence and absence of intracellular pathway specific inhibitors.

**Results:** Mechanical wounding induced ligand independent KGFR activation and internalization. KGFR internalization was associated with H$_2$O$_2$ generation along the wound edge and scavenging of H$_2$O$_2$ with NAC inhibited KGFR activation. c-Src family inhibitor, PP1, significantly inhibited KGFR and associated FRS2 phosphorylation. Mechanical wounding did not induce proliferation nor apoptosis but significantly induced wound edge migration and this migration was significantly reduced by the selective inhibitors PP1 (82.7%), SU5402 (70%) and PD98059 (57%).

**Conclusion:** Microwounding associated generation of H$_2$O$_2$, mediates KGFR and FRS2 activation via c-Src kinase signaling and ultimately induced wound edge associated cell migration. These *in vitro* data suggest ligand independent KGFR activation associated with microwounding within a periodontal pocket may regulate to some degree epithelial cell migration associated with the onset of periodontal disease.
4.2 Introduction

KGFR (keratinocyte growth factor receptor), also described as FGFR2IIIb, is exclusively expressed on epithelial cells and binds ligands KGF-1 or KGF-2. These growth factors are primarily expressed by cells present in the connective tissue. Transgenic mice which express a dominant-negative FGFR2-IIIb mutant in basal keratinocytes of the epidermis suffered a delay in wound re-epithelialization (Werner et al., 1994a). In addition, up-regulation of KGFR has been described in a number of chronic inflammatory diseases such as psoriasis (Brauchle et al., 1996; Finch et al., 1996; Finch et al., 1997). In our previous study we established that KGFR was upregulated in human chronic periodontitis and these data agree with a similar increase in KGFR expression identified using an experimental rat periodontal disease model (Li et al., 2005; Ekuni et al., 2006). Ligand-dependent signaling is one common KGFR activation pathway. Receptor activation induces downstream phosphorylation and activation of FRS2, MAPK, PKC, PLCγ, PI3K, which collectively regulate proliferation, differentiation, migration and cell survival (Ceridono et al., 2005). However, ligand-independent signaling has been described for a number of tyrosine kinase receptors. Two alternate ligand-independent signaling pathways are either dependent on integrins or reactive oxygen species (ROS) generation.

Integrins are receptors composed of α and β chains that link the extracellular matrix to the intracellular environments and ultimately regulate cellular behavior by triggering multiple signaling pathways involving Ca²⁺, tyrosine phosphorylation of cytoplasmic proteins and activation of MAPKs (Cabodi et al., 2004). On the one hand, integrins and
growth factor receptors can signal as parallel pathways or synergize such that integrins trigger ligand-independent TK-GFR activation (Comoglio et al., 2003; Miyamoto et al., 1996; Yamada and Even-Ram, 2002). For example, ανβ3 induced ligand independent activation of the PDGFB receptor, insulin like growth factor receptor (Schneller, 1997), VEGF receptor (Soldi et al., 1999) and FGFR1 (Sahni, 2004). In addition, β1 integrin interaction and activation of EGFR have also been described (Yu et al., 2000). Specifically, PDGFR, EGFR, VEGF-R, HGFR are all activated after integrin engagement without the presence of their ligands (Yu et al., 2000; Yamada and Even-Ram, 2002). Numerous studies have demonstrated c-Src may play a intermediary role in TK-GFR ligand-independent activation (Thomas and Brugge, 1997; Bromann et al., 2004). Generation of c-Src kinase dominant-negative mutants or overexpression of a c-Src kinase negative regulator effectively blocked tyrosine phosphorylation of the TK-GFR (Luttrell et al., 1997). Pretreatment of cells with the c-Src inhibitor PP1 or the expression of dominant-negative c-Src blocked integrin mediated ligand-independent phosphorylation of EGF-R (Moro, 2002; Bromann et al., 2004). These data suggest that c-Src phosphorylation plays a role as a signaling intermediate (Moro, 2002; Bromann et al., 2004). To date KGFR activation by integrins has yet to be described.

In addition, generation of reactive oxygen species (ROS) can act as secondary messenger and lead to ligand-independent TK-GFR activation. ROS, such as hydrogen peroxide (H2O2), superoxide radicals, and hydroxyl radical (Chapple, 1997), are generated in response to a variety of external stimuli such as heat, ultraviolet (UV)
light, infection, and mechanical wounding (Canakci et al., 2005). Peroxynitrite (ONOO⁻), a member of reactive nitrogen species (RNS), produced by nitric oxide (NO) reacting with superoxide radical through isoenzyme NO synthases (NOS), is a highly reactive radical and known to have the ability to modify proteins through tyrosine nitration in the aromatic ring of tyrosine residues, which is an important amino acid involved in cell membrane receptor phosphorylation reaction and signal transduction pathways (Ischiropoulos and al-Mehdi, 1995; Stadtman and Berlett, 1997; Canakci et al., 2005). Peroxynitrite has been reported both activate protein function such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptors (PDGFR) (Klotz et al., 2002; Zhang et al., 2000), and inactivated protein function such as glutathione reductase (GR) (Sahoo et al., 2006). Functionally, ROS generation mediates various biological responses such as cell migration (Ushio-Fukai, 2006; Ushio-Fukai and Alexander, 2004). Growing evidence supports that ROS generation activates a variety of tyrosine kinase growth factor receptors. Specifically, the IGF receptor (Koshio, Akanuma et al., 1988), PDGFR receptor (Saito, Frank et al., 2002), and EGFR (Peus et al., 1998) (Peus et al., 1999; Monteiro and Stern, 1996), and Src kinase, a non-receptor tyrosine kinase, (Gonzalez-Rubio et al., 1996; Saito et al., 2002) have all been shown to be activated by ROS generation. Growing evidence supports KGFR may also be activated by generation of ROS. Generation of ROS in UVB treated keratinocytes or increased ROS in cells treated with the pro-oxidative agent CUH (cumene hydroperoxide) all induced ligand-independent KGFR activation and internalization (Marchese et al., 2003; Belleudi et al., 2006). This ligand-independent KGFR activation induced by generation of ROS signaled through similar downstream
pathways used by ligand-dependent KGFR activation (Marchese et al., 2003). Conversely, KGFR activation and internalization was abolished by preincubation with ROS inhibitor NAC (N-Acetyl-L-cystein) (Belleudi et al., 2006; Marchese et al., 2003).

In periodontal diseases the pocket epithelium lies in direct contact with the rough surface of subgingival calculus and likely undergo chronic microwounding on the inner surface of the pocket epithelium. We hypothesize this microwounding may induce ligand independent activation of the KGFR. To examine this possibility we utilized an in vitro microwounding cell culture model (Ellis et al., 2001; Firth and Putnins, 2004). We identified KGFR was internalized and it colocalized with increased ROS along the wound edge. Tyrosine phosphorylation of the KGFR and down stream FRS2 occurred in a ligand independent manner and was dependent on generation of ROS and phosphorylation of c-Src. Functionally, ligand-independent KGFR activation was associated with ERK activation and regulated wound edge cell migration.
4.3 Materials and Methods

4.3.1 Cell Culture

HaCaT keratinocytes, a generous gift from Dr Hubert Fusenig, (German Cancer Center, Heidelberg, Germany) were cultured in a humidified 95% air, 5% CO₂ incubator at 37 °C and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (Gibco, Rockville, MD), 100 mg/ml streptomycin sulfate, 100 U/ml penicillin and 0.25 mg/ml amphotericin B). Subsequently a defined media made up of DMEM plus 100 mg/ml streptomycin sulfate, 100 U/ml penicillin, 0.25 mg/ml amphotericin B, 5.0 ug/ml insulin, 0.5 mM hydrocortisone, 0.1 mM ethanolamine and 0.1mM phosphoethanolamine was used to quiesce the cultures and all post wounding experiments (Ellis et al., 2001; Firth and Putnins, 2004).

4.3.2 Inhibitors And Antibodies

Antibodies and inhibitors used in the experiments are listed below: SU5402 (Calbiochem, United Kindom), wortmannin (Calbiochem, United Kindom), calphostin C and PD98059 (Sigma, St. Louis, USA), PP1 (BIOMOL Research Laboratories), N-Acetyl-L-cystein (NAC), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma, St. Louis, USA), rabbit anti-KGFR (Bek, C-17) and rabbit anti-FGFR1 (Santa Cruz Biotechnology, CA, USA), mouse anti-KGFR Functional blocking antibody (R&D System, Minneapolis, USA), mouse anti-phosphotyrosine (PY) (R&D, Minneapolis, USA), agarose-conjugated mouse monoclonal anti-phosphotyrosine antibody (R&D, Minneapolis, USA), rabbit polyclonal anti-FRS2 (Santa Cruz Biotechnology, CA, USA),
USA), rabbit monoclonal anti-phospho-Src (Tyr416) (Upstate, Lake Placid NY, USA), rabbit anti-ERK1/2 polyclonal antibody (Chemicon international, Inc., Temecula CA, USA), rabbit anti-phospho-ERK1/2 polyclonal antibody (Chemicon international, Inc., Temecula CA, USA), KGF-1 (Upstate, Lake Placid NY USA); secondary anti-rabbit (mouse) IgG-HRP (Santa Cruz Biotechnology, CA, USA); Alexa Fluor 546 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse antibodies (Molecular Probes, Burlington, Ontario, Canada), anti-BrdU (Sigma Missouri US), anti-nitrotyrosine antibody (Upstate, Lake Placid NY USA), anti-β1, β3, β4, β6 antibodies (Chemicon international, Inc., Temecula CA, USA) and β5 (Upstate, Lake Placid NY USA), normal rabbit IgG (Santa Cruz Biotechnology, CA, USA). DSP [Dithiobis(succinimidylpropionate)], DTSSP (3,3’-Dithiobis sullosuccinimidylpropionate) (Pierce Biotechnology, Rockford USA), peroxynitrite (Upstate, Lake Placid NY USA).

4.3.3 In vitro Microwound Models

Single mechanical wound: 5X10⁴ cells were plated on Lab-Tek 8-well glass chamber slides (Nalge Nunc International, Roskilde Denmark, USA) and cultured to confluence prior quiescing in defined medium for 8 hours and wounded once across the maximum diameter with a pipette tip. At select time points cultures were fixed and prepared for immunohistochemical analysis.

Multiple mechanical wound model: HaCaT cells were cultured to confluence in 100 mm cell culture plates and quiesced in defined medium for 8 hrs. Multiple parallel
wounds were generated by a 2 cm portion of a standard plastic hair comb. Non-overlapping wounds across the diameter were made at 0°, 45°, 90° and 135° to the original scrapes. Cells were washed and incubated in defined medium with or without functional blocking antibodies or inhibitors from 0-24 hours. Cell lysates were prepared for immunoprecipitation and western blotting.

4.3.4 Immunostaining And Confocal Laser Scanning Microscopy

Cultures on glass tissue culture chamber slides were fixed with 2% paraformaldehyde + 5% sucrose in PBS, permeabilized with 0.5% Triton X-100, in PBS for 4 min, washed 10 min 5 times in PBS and blocked using 3% bovine serum albumin (BSA) + 1% glycine in PBS for 30 minutes at room temperature. Samples were incubated with either primary rabbit anti-KGFR (1:50) or anti-FGFR1 antibodies followed by mouse anti-phosphotyrosine (1:50) antibody in blocking solution for 1 hour. In addition, samples were fixed and double stained with anti-KGFR and anti-integrin antibodies β1, β3, β4, β5, and β6. Slides were washed with 1mg/ml BSA in PBS 3X5 min prior incubation with anti-rabbit (Alexa Fluor-546, Molecular Probes, Eugene, Oregon USA) and anti-mouse (Alexa Fluor-488, Molecular Probes, Eugene, Oregon USA) secondary antibody for 1 hour at room temperature in the dark. Slides were washed 5X3 min with PBS, mounted with Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL. USA). All samples were analyzed using confocal laser scanning microscope (Nikon D-eclipse, Japan) with multiple z-axis sections taken every 2 μm. Sections representing the mid cell region were used for KGFR internalization while basal sections for integrin and KGFR colocalization.
4.3.5 Immunoprecipitation and Western Blot Analysis

For western blot analysis, multiple wounds were prepared in 100 mm dishes and at time points from 0-60 min post wounding the plates were washed 3 times with ice cold PBS and cells lysed in RIPA buffer (1XTBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide) (Santa Cruz Biotechnology) supplemented with the proteinase inhibitors (1mM PMSF, protease inhibitor cocktail and 1mM sodium orthovanadate, Santa Cruz Biotechnology, CA, USA) at 4°C. Lysates were centrifuged for 20 min at 10,000G at 4°C and the supernatants collected. All samples were standardized to equal protein concentration (100 ug), fractioned under reducing condition on a 7.5% SDS-polyacrylamide gel and transferred to Immobilon PVDF Transer Membrane (polyvinylidene difluoride) (Millipore Corporation, Bedford, MA, USA). The membrane was blocked with 5% skim milk in PBS with 0.1% Tween-20 for 1 hour at room temperature, washed with 0.1% Tween-20 PBS prior to incubation with either anti-KGFR, anti-FRS2, anti-phospho-Src, anti-phospho-ERK1/2 or anti-ERK1/2 antibodies. Proteins were detected with horseradish peroxides-conjugated goat anti-rabbit or anti-mouse secondary antibody, and developed on film (Kodak) using enhanced chemilluminescence (Amersham Corp., Arlington Heights, IL). For immunoprecipitation experiments, cell lysates were prepared as before and equal protein concentrations were incubated with agarose-conjugated mouse anti-phosphotyrosine antibodies (R&D) overnight at 4°C. Beads were centrifuged at 2500 rpm for 5 min at 4°C and washed 2 times with PBS and once with deionized water and reduced with sample buffer and boiled for 5 minutes prior fractionation with PAGE and western blotting with antibodies of interest.
To examine the direct association between β1 or β5 integrins with KGFR we used the crosslinking regents DSP and DTSSP (Pierce Biotechnology, Rockford, USA). DSP [Dithiobis(succinimidylpropionate)], is a water-insoluble, homobifunctional N-hydroxysuccimide ester (NHS-ester), used for cross linking molecules intracellularly; while DTSSP is a water-soluble analog and recommended for cross linking of molecules on cell surface extracellularly. Both prevent molecule separation during cell lysis procedures. A confluent cell monolayer in a 100 mm culture dish was pretreated with 2 mM DST or 1 mM DTSSP for 30 min at RT prior cell culture wounding or KGF-1 treatment. Cell lysis were collected after 0, 5, 15, 30, 60 min of wounding or 15 min after KGF-1 treatment and immunoprecipitated with KGFR and western blotting with either β1 or β5.

### 4.3.6 Assay of Intracellular ROS Generation

A fluorescent dye, 2',7'-dichlorofluorescin diacetate (DCFH-DA), was used to detect intracellular ROS generation. DCFH-DA is a cell-permeable non-fluorescent probe that is de-esterified intracellularly and rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescin (DCF) in the presence of ROS (especially hydrogen peroxide) (Bass et al., 1983). For assays, quiescent confluent monolayer cultures on glass chamber slides were preincubated with DCFH-DA (5µM) with or without N-Acetyl-L-Cysteine (NAC) for 30 min in 37°C humid incubator prior mechanical wounding. NAC can act as a precursor for the antioxidant intracellular glutathione (Figure 1.7 Chapple, 1997). Cultures were fixed from 0 to 60 minutes post-wounding and followed by anti-KGFR
staining (described above). Confocal laser scanning microscope was utilized to detect DCF fluorescence and its colocalization with intracellular KGFR.

Generation of superoxide due to oxidative stress may also react with nitric oxide (NO) to generated Peroxynitrite (ONOO\(^{-}\)). This highly reactive radical can modify protein structure by nitration of tyrosine residues and this may ultimately increase or decrease protein functional activity (Klotz et al., 2002; Zhang et al., 2000; Sahoo et al., 2006). Therefore, we tested if wounding induced wound edge tyrosine nitration and if peroxynitrite induced KGFR activation. First, confluent cultures were wounded and fixed at time 0, 15 min, 1 hr, 2 hrs and 3 hrs post wounding and immunohistochemically stained using anti-nitrotyrosine antibody (Upstate, Lake Placid NY USA). Second, confluent cultures were quiesed for 4 h, washed twice with phosphate-buffered saline (PBS), and subsequently placed in 3 ml of modified PBS (50 mM Na2HPO4, 90 mM NaCl, 5 mM KCl, 0.8 mM MgCl2, 1 mM CaCl2, and 5 mM glucose, pH 7.4) (van der Vliet et al., 1998). This modified PBS provides sufficient buffering capacity to avoid pH changes after the addition of alkaline ONOO\(^{-}\) solutions (Upstate, USA). Control and wounded cultures were washed and ONOO\(^{-}\) was added on the side of the culture dishes and immediately mixed with the incubation buffer by rapid swirling in order to assure optimal exposure of the cells to ONOO\(^{-}\) before decomposition. Fresh peroxynitrite was added every 5 min because of its short half-life. In peroxynitrite control experiments, cells were treated as above but with equal volumes of degraded peroxynitrite (vehicle control) (Upstate, USA) (van der Vliet et al., 1998).
4.3.7 Cell Migration Assay

Quiescent confluent cultures incubated on chamber slide were preincubated with or without inhibitors SU5402 (50uM) (Mohammadi et al., 1997), PD98059 (50uM), PP1 (20uM) (Karni et al., 2003), calphostin C (0.1uM), Wortmannin (1.0uM) for 1 hour in 37°C prior mechanical wounding with a pipette tip. Fresh media plus inhibitors was added back to the cultures. A digital image of the wounded area was identified and photographed at a standardized magnification immediately post wounding (t=0) and this area re-photographed at 24 hours. The reduction in relative wound area was determined using Image J (National Institute of Health, Maryland, USA) and results expressed as percentage of wound closure at 24 hours (Tenaud et al., 2000). Three samples per inhibitor group were averaged and statistical significance determined using a student t-test (p<0.05).

4.3.8 BrdU Proliferation Assay

Bromodeoxyuridine incorporation was used to specifically determine if wounding associated KGFR phosphorylation was associated with wound edge associated proliferation. Confluent cultures were quiesced for 8 hours, wounded and incubated with BrdU (10uM) (Sigma, Missouri US) for 2 hours prior fixation then treated with 2N HCl for 30 min at 37°C and thoroughly rinsed with PBS. BrdU concentration and incubation time were determined through pilot studies. Slides were incubated with prewarmed trypsin solution (100μl-0.1% w/v in PBS) for 20 min at 37°C and rinsed with PBS. After blocking, cultures were incubated with anti-BrdU (1.5ug/ml) (Sigma, Missouri US) for 2 hour at 37°C, followed by secondary anti-mouse Ab at 1:100 for I
hr in the dark at room temperature. BrdU positive cells were counted at 3 locations of wounded area or non-wounded area and averaged to represent the sample. Total 3 samples were averaged as the representative for each group and statistical significance was determined using a student t-test (p<0.05).

4.3.9 Cell Apoptosis Assay

Confluent cultures were quiesced for 8 hours and preincubated with or without the FGFR inhibitor SU5402 for 1 hour at 37°C and cultures were subsequently wounded. Cultures were placed in fresh media with SU5402 and fixed at 1, 3, 6, 12 and 24 hours, permeabilized (Roche Applied Science, Switzerland) for 2 minutes on ice and incubated with TUNEL reaction mixture for 1 hour at 37°C in the dark. The SU5402 inhibitor concentration was determined from pilot experiments in which KGF-1 dependent stimulation of KGFR was blocked. Apoptotic cells were detected using confocal microscope under the wave length of 546 nm. For each sample 3 areas were examined and the number of apoptotic positive cells were counted and averaged to represent this sample mean. A total 3 samples were averaged for each experimental condition.
4.4 Results

4.4.1 Wounding Induced Internalization of KGFR Is Associated With Ligand-Independent KGFR Phosphorylation

To simulate epithelial microwounding we utilized an *in vitro* scrape wound model (Ellis et al., 2001; Firth and Putnins, 2004). Using immunohistochemistry this model effectively allowed us to examine KGFR internalization and overall cell-associated tyrosine phosphorylation (Figure 4.1a-left). Expanding this model to larger cell culture plates increased the number of wound margin associated cells allowing specific examination of KGFR phosphorylation using polyacrylamide gel electrophoresis (PAGE) and Western blotting (Figure 4.1a-right). All experiments were done in serum free conditions using a previously described defined medium (Firth and Putnins, 2004) to ensure no KGFR binding ligands were present in the culture media. To begin confluent HaCat epithelial cell monolayer were wounded and fixed at 3 hr prior to immunohistochemical staining with anti-KGFR (Figure 4.1b) and anti-FGFR1 (Figure 4.1c) antibodies. Using a confocal microscope with the plane of analysis limited to the mid cell region significant KGFR internalization was identified in all wound edge associated cells and extended 2-3 cell layers away from the wound (Figure 4.1b). In contrast weak but positive FGFR1 staining was identified but no relative change on staining intensity at or away from the wound edge was noted (Figure 4.1c). Secondary antibody alone controls were negative in both cases (data not shown). As receptor internalization may be associated with receptor associated tyrosine phosphorylation wounded cell cultures were fixed at various time points prior to
Figure 4.1. KGFR but not FGFR1 intracellular staining in HaCaT cells along the wound edge was increased. Single and multiple in vitro scrape wound model scraped with pipette tip (a-left) or a comb (a-right); Confluent cultures were quiesced in defined medium for 8 hours prior wounding and then fixed followed by staining with anti-KGFR and anti-FGFR1 antibodies. Images were analyzed using confocal laser scanning microscopy and all images represent the middle cell region (b): intense intracellular KGFR was localized to cells at the wound edge; (c) In contrast, staining for FGFR1 was diffuse through all cells and no increased staining along the wound edge.
Figure 4.2 Increased KGFR staining at the wound edge colocalized with phosphotyrosine (PY). Confluent cultures were fixed at 1h, 2h, and 24 hours post wounding followed by double staining with anti-KGFR and anti-phosphotyrosine. Images were analyzed under confocal laser scanning microscope and all images represent the mid cell region.
double staining using anti-KGFR and anti-phosphotyrosine (PY) antibodies (Figure 4.2). Wound edge associated KGFR and phosphotyrosine specific staining significantly increased at 1 hr and 2 hr and colocalized in wound edge associated cells (merge images). Little wound edge associated KGFR and phosphotyrosine intracellular staining nor colocalization was identified at 24 hours (Figure 4.2). These data suggest that wounding induced significant phosphorylation in wound edge cells within 2 hr may possibly be associated with KGFR phosphorylation. As multiple receptors may possibly be phosphorylated an immunoprecipitation and Western blotting approach was utilized to specifically examine the kinetics of KGFR phosphorylation. Using the multiscrape model (Figure 4.1a-right) multiple wounds were made to a confluent quiescent cell monolayer and timed whole cell lysates prepared (Figure 4.3a-top). At time zero (time 0) no KGFR phosphorylation was detected. However, tyrosine phosphorylation of KGFR was detected within 5 min of wounding, peaked at 15-30 min decreased at 60 min and was not detectable at 120 minutes. In all whole cell lysates that were prepared equal KGFR protein was present (Figure 4.3a-bottom). The kinetics of wound induced KGFR activation was compared to ligand dependent KGFR activation. KGF-1 (50 ng/ml) was added to the cell cultures, whole cell lysates prepared and immunoprecipitation and Western blotting done as in Fig 3a. KGF-1 induced KGFR phosphorylation within 5 min, increased from 15-30 min and decreased to baseline levels at 120 min (Figure 4.3b-top). Equal KGFR protein was present in each time point cell lysis sample (Figure 4.3b-bottom). These data support wounding induces KGFR phosphorylation and this induction follows similar kintetics as found for ligand dependant receptor activation.
Figure 4.3 Mechanical wounding induced KGFR phosphorylation is similar to KGF-1 induced KGFR Phosphorylation. (a) Multiple wounds across confluent quiescent cultures were made and cell lysates prepared 0, 5, 15, 30, 60 and 120 minutes post wounding. Equal protein concentrations were used for immunoprecipitation (IP) with phosphotyrosine (PY) and western blotting (WB) with anti-KGFR antibodies (a-top). To ensure the same KGFR concentration was presented parallel gels using the same samples and protein concentrations were blotted with anti-KGFR antibody (a-bottom). (b) As a positive control, 50ng/ml KGF-1 was added to quiescent cultures and cell lysates were prepared after 0, 5, 15, 30, 60, and 120 minutes. Equivalent protein concentration for each sample was used for immunoprecipitation with PY and western blotting with anti-KGFR antibody (b-top). As above to ensure the same KGFR concentration parallel gels with the same samples were blotted using anti-KGFR antibody (b-bottom). Mechanical wounding and KGF-1 both induce significant phosphorylation of KGFR at 15-30 minutes.
Wounding of cells in culture may induce expression of numerous growth factors that may in turn induce receptor activation via an autocrine pathway. Specifically, FGF growth factors FGF1, FGF3, FGF7, FGF10 and FGF22 (Eswarakumar et al., 2005) are all possible ligands for KGFR. Therefore we examined whether wounding had induced autocrine expression of one of these growth factors and in turn one of these growth factors induced KGFR activation (Figure 4.4). Pretreating cultures with a KGFR-specific blocking antibody reduced in a concentration dependent manner KGFR phosphorylation with 10 µg/ml effectively blocking ligand induced KGFR phosphorylation (Figure 4.4a). In contrast preincubation of cultures with 10 µg/ml of blocking antibody did not reduce wound induced KGFR tyrosine phosphorylation (Figure 4.4b). These data suggest KGFR activation is not mediated by autocrine expression of KGFR binding growth factors and may be ligand independent.

4.4.2 Integrins Association With KGFR Do Not Mediate Wound Induced Ligand-Independent KGFR Activation

Integrins are involved in some cases of ligand-independent activation of RTKs (receptor tyrosine kinase) (Yu et al., 2000; Yamada and Even-Ram, 2002). We therefore hypothesized wounding induced ligand-independent KGFR activation may be mediated through an integrin-FAK signaling pathway. We first colocalized KGFR with different β integrin subunits (β1, β3, β4, β5, β6) at the wound edge using an anti KGFR and anti β integrin subunit double staining approach (Figure 4.5). The staining pattern was different for each of the antibodies tested but overall, positive staining was found for β1, β4, β5, and β6 but only β1 and β5 appeared to colocalize
Figure 4.4 Scrape wounding induced KGFR activation was ligand-independent. (a) Quiescent cultures were pretreated with or without functional anti-KGFR blocking antibody at 4, 7, 10ug/ml or normal rabbit IgG at 10ug/ml (negative control) for 1 hour before adding 50ng/ml KGF-1 and cells were lysed at 15 min. The same protein concentration for each sample was used for immunoprecipitation (IP) with anti-phosphotyrosine (PY) and western blotting (WB) with anti-KGFR. The data established that 10ug/ml of KGFR blocking antibody effectively inhibits ligand-dependent KGFR phosphorylation. (b) Subsequently confluent cultures were pretreated with 10ug/ml functional blocking anti-KGFR or 10ug/ml of normal rabbit IgG for 1 hour prior wounding and cells were lysed at 15 min. As above equivalent protein concentrations were used for immunoprecipitation with anti-phosphotyrosine (PY) and western blotting with anti-KGFR. Functional blocking antibody of a ligand binding to KGFR did not inhibit wounding induced KGFR phosphorylation.
Figure 4.5 KGFR colocalized with β1 and β5 integrins at the wound edge. Confluent cultures were quiesced for 8 hours, wounded and fixed at 12 hours. Samples were double stained for anti-KGFR and anti-integrin subunits β1, β3, β4, β5, and β6 antibodies. All integrins were examined with a confocal microscope and images presented represent sections of middle portion of cells. Based on the merged images it suggested that β1 and β5 integrins colocalized with KGFR.
with KGFR at the wound edge while β4 and β6 did not (Figure 4.5).

To test whether protein-protein interactions were present between β1 or β5 integrins and KGFR we immunoprecipitated using an anti-KGFR antibody and blotted with β1 or β5 specific antibodies. To ensure the antibody was β5 specific we also ran a whole cell extract positive control and this was positive for β5 (Figure 4.6a). β5 did not co-immunoprecipitate with KGFR in wounding-induced or ligand-induced KGFR activation (Figure 4.6). No difference in results were found in experiments using either DSP (Figure 4.6a) or DTSSP (Figure 4.6b) cross linking agents. These data suggest β5 is not directly associated with KGFR. In contrast, even without DSP or DTSSP KGFR immunoprecipitation did pull down β1 integrin (Figure 4.7a). However, regardless of using mechanical wounding or KGF-1 stimulation the relative amount of β1 associating with KGFR did not change from 0-60 min post stimulation or wounding.

To further test if integrin β1 or β5 may mediate ligand-independent KGFR activation upon wounding we preincubated with functional β1 and β5 blocking antibodies. Confluent cultures were pretreated with functional blocking β1 or β5 for 1 hour prior wounding and cells lysed 15 min postwounding and tyrosine phosphorylated proteins immunoprecipitated and immunoblotted with anti-KGFR antibody. Functional blocking of integrin activation didn’t inhibit wound-induced KGFR activation. These data suggest β1 and β5 apparent colocalization at the wound edge (Figure 4.5) was unlikely to be directly involved in wound induced ligand-independent KGFR activation (Figure 4.7).
Figure 4.6. β5 integrin does not immunoprecipitate with KGFR. Confluent cultures were wounded and cell extracts prepared at time point of 5, 15, 30, and 60 minutes. A non-scratched negative control (Negative Cont) and a KGF-treated positive control were prepared as well. Prior to lysis cultures were treated with the intracellular cross-linking agent DSP (a) and the extracellular cross-linking agent DTSSP (b). KGFR was immunoprecipitated, fractioned using PAGE and blotted with anti-β5 antibody. Whole cell protein extract was used as a positive control to ensure antibody specificity. Regardless of the cross-linking agents, no direct association of β5 integrin was identified in control, KGF-treated and wounded samples.
Figure 4.7 β1 integrin immunoprecipitates with KGFR but β1 functional blocking antibody does not inhibit scratch induced phosphorylation of KGFR. (a) Confluent cultures were scratch wounded and treated with KGF-1 (positive control) and cell lysates was prepared at 0, 5, 15, 30 and 60 minutes. KGFR was immunoprecipitated from cell lysates, fractioned using PAGE and blotted with anti-β1 specific antibody. Whole cell protein extract was included as a positive control. β1 integrin did immunoprecipitated with KGFR but the relative amount of β1 did not change post wounding. (b) Confluent cultures were pre-treated with β1 and β5 functional blocking antibodies and a non-specific IgG antibody for 1 hour at 37°C and then scratch wounded. Cell lysates were prepared at 15 minutes and tyrosine phosphorylated protein were immunoprecipitated, fractioned and blotted with KGFR antibody. Preincubation of anti-β1 and β5 blocking antibodies prior wounding failed to inhibit KGFR phosphorylation.
4.4.3 Wounding Induced ROS Generation Induces KGFR Phosphorylation

A previous study has shown UV light induced ROS generation does induce KGFR phosphorylation (Marchese et al., 2003). Therefore, we examined if wounding induced KGFR phosphorylation was mediated by ROS generation. First, we examined whether wounding induced ROS at the wound edge. No detectable ROS (i.e. no DCF fluorescence) was detectable immediately post-wounding (Figure 4.8a-time 0). A significant increase in DCF fluorescence from 5 min to 30 minutes post-wounding within 1 to 3 cell layers along the wound edge was present and this reduced to control levels at 60 minutes (Figure 4.8a). No detectable ROS was detected in cells away from the wound edge and pretreatment of cultures with NAC inhibited this increase in ROS generation (data not shown). The most significant induction in ROS generation was noted at 5-15 min and cells exhibiting the greatest fluorescence also exhibited the greatest degree of KGFR internalization (Figure 4.8a-arrows). KGFR phosphorylation was significantly increased by wounding and preincubation of cultures with the antioxidant NAC inhibited KGFR phosphorylation in a concentration dependent manner (Figure 4.8b). These data suggest wound-induced generation of ROS does mediate KGFR phosphorylation.

As generation of peroxynitrite (ONOO\(^{-}\)) by reaction of superoxide with nitric oxide (NO) [see Figure 1.7-Literature review] may impact protein function due to nitration of tyrosine residues we examined if this pathway was involved in KGFR phosphorylation. No significant overall increase in nitration of tyrosine residues was identified immunohistochemically (Figure 4.9a). In addition treatment of control and wounded
Figure 4.8. Generation of ROS along the wound edge colocalized with KGFR and pretreatment of culture with an antioxidant effectively inhibits KGFR phosphorylation. (a) Quiescent cultures were pre-incubated with 2',7'-dichlorofluorescin diacetate (DCFH-DA) at 5 uM for 30 min prior to wounding. Cells were fixed at time 0, 5, 15, 30 and 60 minutes post wounding and followed by immunohistochemical staining with anti-KGFR. Images were analyzed under confocal laser scanning microscope. Generation of fluorescence (DCF) after oxidation of DCFH-DA was detected after 5 to 30 minutes wounding along the wound edge and this colocalized with increased KGFR staining (arrows). Colocalization was most significant at 15 minutes post-wounding. (b) Confluent quiescent cultures in 100mm culture dishes were pre-incubated with or without N-acetylcysteine (NAC) at 10mM, 15mM, 20mM for 30 min and then multiple wounds were applied. Cells were lysed after 15 minutes and equivalent protein concentrations for each sample were used for immunoprecipitation with anti-phosphotyrosine (PY) and western blotting with anti-KGFR antibody.
Figure 4.9 Peroxynitrite (ONOO⁻) does not induce ligand-independent phosphorylation of KGFR. (a) Confluent cultures were wounded and fixed at time 0, 15 min, 1 hr, 2 hrs and 4 hrs post wounding and immunohistochemically stained using anti-nitrotyrosine antibody. Wounding did not induce a increase production of peroxynitrite at wound edge. (b) Confluent cultures were treated with peroxynitrite (ONOO⁻) or degraded peroxynitrite for 15 minutes in modified PBS. In addition one set of samples were scratch wounded and treated with peroxynitrite. Addition of peroxynitrite did not induce KGFR phosphorylation and did not induce a further increase in scratch induced KGFR phosphorylation.
cultures with peroxynitrite had no impact on the relative degree of KGFR phosphorylation in control and wounded cultures (Figure 4.9b). Therefore generation of peroxynitrite (ONOO\(^{-}\)) and subsequent nitration of tyrosine residues does not appear to play a role in wounding-induced KGFR phosphorylation.

Hydrogen peroxide generation has been shown to induce Src kinase phosphorylation (Gonzalez-Rubio et al., 1996; Saito et al., 2002) and Src kinase may regulate RTKs (receptor tyrosine kinase) ligand-independent phosphorylation and activation (Thomas and Brugge, 1997). To test this hypothesis, we treated control and wounded cultures with H\(_2\)O\(_2\) in the presence and absence of NAC (Figure 4.10a). Wounding and pretreatment with H\(_2\)O\(_2\) both induced significant Src kinase phosphorylation and in both treatment conditions this was inhibited by pretreatment with NAC. Subsequently, preincubation with the Src kinase inhibitor PP1, inhibited wounding induced KGFR phosphorylation (Figure 4.10b-top). Furthermore, the KGFR downstream signaling molecule FRS2 was also phosphorylated by wounding (Figure 4.10b-bottom). The large electrophoretic mobility shift (from 77 to 95 kDa) may be due to the fact that FRS2 has multiple tyrosine phosphorylation sites (Lax et al. 2002). Pretreatment of cultures with PP1 inhibited FRS2 tyrosine phosphorylation and associated mobility shift as well (Fig 4.10b-bottom).

4.4.4 Functional Significance of Wounding-Induced KGFR Phosphorylation

A commercially available FGFR inhibitor was purchased. This inhibitor, SU5402,
Figure 4.10 ROS generation is associated with phosphorylation of c-Src, KGFR and FRS2 and the inhibition of c-Src phosphorylation abolished KGFR and FRS2 phosphorylation. (a) Confluent cultures were either wounded or treated with H₂O₂ in the presence and absence of the antioxidant NAC. Cell lysates were prepared, fractioned using PAGE and blotted using anti-phospho-c-Src antibody (p-Src). Both wounding and H₂O₂ induced c-Src phosphorylation were effectively inhibited by preincubation with NAC. (b) Confluent cultures were wounded in the presence and absence of the c-Src inhibitor PP1. Wounding induced phosphorylation of KGFR and downstream FRS2 were effectively inhibited by preincubation with PP1.
Figure 4.11 SU5402 should effectively block tyrosine phosphorylation of several FGFRs. A blast gene comparison was done for the kinase insert region (KI) of FGFR 1 through 4. SU5402 blocks the peptide sequence YASKGN and this sequence is present in FGFR1, FGFR2IIIb, FGFR2IIIc and FGFR3.
binds to the intracellular KI region of FGFRs and blocks their activation. To ensure that this inhibitor could block KGFR activation we completed a blast analysis (Figure 4.11). SU5402 binds to the shared peptide sequence YSKGN (amino acid 566-571) and this is common to FGFR1, FGFR2 and FGFR3 (KI) (Mohammadi et al., 1997; Partanen et al., 1991). Therefore this inhibitor could effectively block KGFR phosphorylation.

4.4.4.1 Migration Assay

As apparent cell migration may be influenced by cell proliferation we first examined whether the apparent KGFR internalization at the wound edge was associated with an increase in wound edge-associated cell proliferation. To specifically examine this cell population we elected to utilize a BrdU incorporation assay. No significant difference in the number of proliferating cells were found at the wound edge when compared to the cell population away from the wound edge (Figure 4.12). These data suggest that KGFR phosphorylation and activation at the wound edge does not appear to induce significant cell proliferation in this area and is not likely to significantly impact cell migration.

As cell migration often signals through the MAP kinase pathway we first examined ERK phosphorylation post wounding (Fig 13a-top). Wounding induced significant ERK phosphorylation and preincubation of cultures with PP1 (Src kinase inhibitor, 20uM), SU5402 (KGFR phosphorylation inhibitor, 50uM), or PD98059 (MAPKK
Figure 4.12. Wounding does not induce an overall increase in cell proliferation at the wound edge. (a) Confluent and quiescent cultures were scratch wounded and 2 hours prior to fixation 10μM BrdU was added. At 24 hours samples were fixed and immunostained with anti-BrdU antibody and the number of BrdU positive cells counted at the wound edge and within the confluent cell layer away from the wound edge (non-wounded area). (b) BrdU positive cells were counted at wounded area or non-wounded area and averaged to represent the sample. Total 3 samples were averaged as the representative for each group. No significant difference in the cell proliferation between wound edge and non-wounded area (p>0.05).
a. Wounding Inhibitor

- Cont
- SU5402 (50uM)
- PD98059 (50uM)
- PP1 (20uM)
- Calphostin C (0.4uM)
- Wortmannin (2uM)

b. Time post-wounding (24h)

- Cont
- SU5402 (50uM)
- PD98059 (50uM)
- PP1 (20uM)
- Calphostin C (0.4uM)
- Wortmannin (2uM)

C. Percentage of wound closure

- Cont
- SU5402
- PD98059
- PP1
- Calphostin C
- Wortmannin

Time post-wounding (24h)
Figure 4.13 Pathway specific inhibitors significantly inhibit epithelial cell migration. (a) The wounding induced ERK1/2 activation was significantly decreased by MAPKK inhibitor PD98059, FGFR inhibitor SU5402 and c-Src inhibitor PP1. (b) Confluent quiescent cultures were incubated without or with SU5402 (50uM), PD98059 (50uM), PP1 (20uM), Calphostin C (0.4uM) or wortmannin (2 uM). Using a pipette tip a single wound was made to the cell monolayer. Wound area was recorded by digital camera immediately post wounding (time 0), and the same location was re-photographed at 24 hours. The images are representative of 3 independent experiments. (c) Percentage of wound closure areas at 24 hours was determined for each sample and the relative change in cell migration in relation to the non-inhibitor treated control were analyzed (t-test). The results show the mean±standard deviation. *p<0.05.
inhibitor, 50uM) all significantly decreased wounding-induced ERK1/2 phosphorylation 15 minutes after wounding. To ensure equal protein loading we probed the blots using an anti-ERK antibody and the relative ERK protein amounts were equal (Figure 4.13a-bottom). By 24hr the relative wound area had significantly decreased in control samples but preincubation of cultures with SU5402, PD9805, and P1 significantly inhibited wound closure at 70%, 57% and 82.7%, respectively (p<0.05) compared to control (Figure 4.13b and c). In contrast preincubation with calphostin C (inhibitor of PKC, 0.4uM) and wortmannin (inhibitor of PI3K, 2uM) had no significant impact on relative wound closure. The results demonstrated wounding induced ligand-independent Src kinase mediated KGFR activation induces significant cell migration.

4.4.4.2 Apoptosis

KGF-1 signalling through the KGFR has previously been shown to inhibit apoptosis (Firth and Putnins, 2004). To further investigate if wounding induced KGFR activation may function to inhibit wound edge apoptosis we utilized a TUNEL assay with and without the KGFR phosphorylation inhibitor SU5402. No statistically significant difference in the number of apoptotic cells between SU5402 treated and untreated groups at all 5 post wounding time point was found (Figure 4.14). The number of apoptotic cells away from the wound edge were generally similar between SU5402 treated and untreated wounds (data not shown). These data suggest that ligand-independent phosphorylation of the KGFR does not mediate apoptosis signaling in this model system.
Figure 4.14 Pre-incubation with SU5402 was not associated with a significant increase in cell apoptosis at the wound edge. Confluent cultures were pre-incubated with or without SU5402 for 1 hour at 37°C prior mechanical wounding and cultures were fixed at 1, 3, 6, 12 and 24 hours and TUNEL stained. Apoptotic cells at these areas of the wound edge were counted and averaged to represent this sample. Total 3 samples were averaged as the representative for each group. No significant difference in the number of wound edge associated apoptotic cells was found when phosphorylation of KGFR was inhibited by SU5402.
4.5 Discussion

KGF-1 and KGFR in Chronic Inflammation and Wound Healing

KGF-1 and KGFR are alternative gene spliced isoforms of FGFR2 (i.e. FGFR2IIIb) and are exclusively expressed on cells of epithelial origin (Pierce et al., 1994). Numerous studies support KGFR mediated signaling as an important receptor regulating epithelial cell behavior during wound healing and chronic inflammation. Knocking out KGFR expression in transgenic mice was associated with skin epidermal atrophy and dermal hyperthickening and reduced the rate of incisional wound healing in these animals (Werner et al., 1994b). Relative expression of KGFR in chronic inflammatory conditions is more variable. KGFR expression was either unchanged or reduced in patients with inflammatory bowel disease (Finch et al., 1996; Brauchle et al., 1996) or KGFR expression was significantly induced in patients with Psoriasis (Finch et al., 1997) and periodontal disease (Sanaie et al., 2002; Putnins et al., 2002; Ekuni et al., 2006; Li et al, 2005). Further support for the importance of KGFR signaling in chronic periodontal disease is provided in a timed rat model. Of the receptors examined, KGFR expression was the most significantly induced receptor (Ekuni et al., 2006).

Periodontal disease is a unique form of chronic inflammation because of the placement of a relatively weak junctional epithelium juxtaposed next to a chronic biofilm that is often associated with rough and abrasive subgingival calculus. This unique biological association does suggest that epithelial microwounding is possible and we hypothesized that this may regulate KGFR activation. To test this hypothesis we
utilized an *in vitro* cell culture model (Ellis et al., 2001) and established that wounding was associated with rapid KGFR phosphorylation.

Wounding in general and microwounding in periodontal tissues may induce autocrine expression of cytokines and growth factors. For example, wounding of rat intestinal epithelial-1 (RIE-1) cell cultures was associated with EGFR activation and this activation was due to autocrine expression of TGF-α which signaled through the EGFR (Myhre, Toruner et al., 2004). Since the KGFR receptor can bind multiple ligands FGF-1, -3, -7, -10 and -22 (Eswarakumar et al., 2005), we tested whether autocrine expression of any of these growth factors may have induced KGFR phosphorylation. However, preincubation with KGFR blocking antibody had no impact on wound induced KGFR phosphorylation (Figure 4.4) suggesting activation occurred in a novel ligand-independent manner.

**Integrin Regulation of Growth Factor Receptor Activation**

Classically, integrins are cell adhesion molecules that mediate cell–cell and cell–extracellular matrix interactions. They transmit signals bidirectionally across the plasma membrane and regulate many biological functions, including wound healing, cell differentiation and cell migration (Luo and Springer, 2006). However, integrin are also able to trigger ligand-independent TK-GFR activation (Comoglio et al., 2003; Miyamoto et al., 1996). Direct physical association with growth factor receptors for αvβ3 integrin with PDGFB receptor (Schneller, 1997), VEGF receptor (Soldi et al., 1999) or FGFR1 (Sahni, 2004) or β1 integrin subunit with EGFR (Yu et al., 2000)
have been shown using immunoprecipitation and western blotting approaches. This
direct linkage was associated with PDGFR, EGFR, VEGFR, HGFR transactivation
after integrin engagement without presence of ligand (Yu et al., 2000; Yamada and
Even-Ram, 2002). No study has addressed the interaction of integrins with KGFR but
FGFR1 has been shown to interact with αvβ3 (Sahni, 2004). Therefore we first
screened if integrins colocalize with KGFR at the wound edge. To date β1 (Parks,
2007), β4 and β5 (Huang et al., 2000) and β6 (Thomas et al., 2006) have all been
demonstrated to be expressed in keratinocytes and up-regulated during wound healing.
Of the β subunits examined only β1 and β5 appeared to colocalize with KGFR at the
wound edge and only β1 coimmunoprecipitated with KGFR. The relative amount did
not change post wounding and preincubation of cultures with β1 and β5 blocking
antibodies did not block wounding induced KGFR phosphorylation. No evidence
supporting an integrin role in mediating KGFR phosphorylation was found but to our
knowledge, this is the first evidence that integrin β1 direct association with KGFR.

**Oxidative Stress and Receptor Activation**

During wound healing and chronic inflammation various ROS and RNS are released
mainly by inflammatory cells such as macrophages and PMNs and to lesser degree by
local fibroblasts and keratinocytes (Rhee, 1999) (Suchett-Kaye et al., 1998). Up-
regulation of ROS production in periodontitis has also been described (Chapple and
Matthews, 2007). At low levels ROS produced within cells are involved in cell
signaling to facilitate wound healing (Roy et al., 2006) but at high levels both ROS and
RNS can generate oxidative damage and negatively impact cellular function.
Generation of the short lived superoxide ($O_2^-$) is a product of oxygen metabolism and is rapidly metabolized to $H_2O_2$ or reacts with NO to form peroxynitrite (ONOO$^-$) (Ray, 2005). Generation of both of these radical may impact receptor activity.

Generation of peroxynitrite and subsequent tyrosine nitration on protein tyrosine residues did activate in a ligand-independent manner EGFR and PDGFR and functionally inhibited apoptosis (Klotz et al., 2002; Zhang et al., 2000). In our model system we were not able to show any evidence of increased protein tyrosine nitration at the wound edge and direct stimulation of cultures with peroxynitrite had no effect on KGFR phosphorylation. These data suggest the NO signaling pathway was not involved in KGFR phosphorylation but generation of $H_2O_2$ did significantly induce KGFR phosphorylation.

Stress induced generation of $H_2O_2$ may serve as a second messenger that activates various tyrosine kinase growth factor receptors (Koshio et al., 1988; Sundaresan et al., 1995; Peus et al., 1998; Peus et al., 1999; Sato et al., 2003; Monteiro and Stern, 1996). In addition UVB radiation or pretreatment of cultures with cumene hydroperoxide both induced KGFR activation and internalization and pretreatment of cultures with the antioxidant N-Acetyl-L-cysteine (NAC) abolished this activation (Marchese et al., 2003; Belleudi et al., 2006). Wounding of our cell cultures was also associated with significant generation of $H_2O_2$ at the wound edge and pretreatment of the cultures with NAC effectively inhibited KGFR phosphorylation (Figure 4.8). Pretreating cultures with $H_2O_2$ also induced KGFR phosphorylation. Collectively these data support a
significant role for H$_2$O$_2$ generation in KGFR activation.

**Intracellular Signaling and Impact of Ligand-Independent KGFR Activation**

ROS (H$_2$O$_2$) regulation of EGFR auto-activation is mediated by c-Src phosphorylation (Ushio-Fukai et al., 2001). In our study preincubation of cultures with the antioxidant NAC reduced wounding induced c-Src phosphorylation. Inhibition of KGFR and downstream FRS2 phsophorylation was achieved by preincubation with the c-Src kinase inhibitor PP1. As was found for the EGFR, our data supports that ROS (H$_2$O$_2$) induced KGFR ligand-independent activation is also mediated via c-Src kinase.

H$_2$O$_2$ generation and stimulation of tyrosine kinase growth factor receptors mediates cell migration, growth, and expression of numerous genes (Ushio-Fukai, 2006; Rojkind et al., 2002; Chapple and Matthews, 2007). Ligand dependent activation of KGFR has been shown to induce epithelial cell migration (Tsuboi, Sato et al., 1993), proliferation (Rubin et al., 1989; Marchese et al., 1990) and exert anti-apoptotic effects (Firth and Putnins, 2004). In our model system ligand-independent activation of KGFR was not associated with significant changes in wound edge proliferation or apoptosis. However, a significant induction in cell migration was found.

Ligand-independent KGFR phosphorylation signaled through the FRS2-MAPK (ERK1/2) pathway. These data are in agreement with a previous study that demonstrated UV treatment induced ligand-independent KGFR with FRS2-ERK1 downstream signaling (Marchese et al., 2003). In our study increased cell migration at
the wound edge was associated with signaling through the KGFR pathway. However, pretreatment of cultures with SU5402 reduced migration up to 70% which suggests additional signaling pathways may have been stimulated as well. Wounding of a rat intestinal epithelial cell culture monolayer induced MAPK activation as well and this activation was mediated by autocrine expression of TGF-α (Ellis et al., 2001; Myhre et al., 2004). Therefore, post wounding migration is significantly regulated by ligand-independent activation of KGFR but additional pathways like EGFR may have been activated as well.

In conclusion, mechanical wounding induced ligand-independent KGFR internalization and phosphorylation (Fig 4.15). Pretreatment of cultures with the antioxidant NAC or pretreatment with the c-Src kinase inhibitor PP1 both effectively inhibited KGFR phosphorylation and established that generation of ROS (H2O2) and c-Src kinase phosphorylation were absolutely critical for KGFR phosphorylation. Downstream of the KGFR, FRS2 was phosphorylated and ultimately induced ERK1/2 phosphorylation. Functionally, ligand independent KGFR activation was not associated with wound edge cell proliferation or apoptosis but did induce significant cell migration. This rapid and ligand independent activation of KGFR may serve to immediately induce cell migration in order to begin the re-establishment of the epithelial barrier. In periodontal
Figure 4.15 Proposed upstream and downstream signaling pathways mediating wounding induced ligand-independent KGFR activation. Generation of ROS by mechanical wounding induced c-Src kinase phosphorylation, which in turn induced KGFR and downstream FRS2 phosphorylation. Ligand-independent activation of KGFR induced ERK1/2 phosphorylation and cell migration.
disease pocket epithelial cells are positioned next to a abrasive calculus surface and over time chronic microwounding may induce KGFR phosphorylation and cell migration that is associated with periodontal pocket formation.
4.6 References


CHAPTER 5

5. Dissertation Discussion, Conclusions and Future Work

5.1 Dissertation Discussion

Periodontal disease is a common chronic inflammatory disease that has associated with junctional epithelial cell proliferation, loss of connective tissue attachment to the tooth and alveolar bone. Microorganisms present in dental plaque clearly are associated with periodontal disease and specifically Gram negative anaerobic bacteria including \textit{A. actinomycetemcomitans}, \textit{P. gingivalis} are strongly associated with disease progression (Lovegrove, 2004). In addition the host defenses induced in response to the bacterial challenge modulates disease onset and progression as well. Specifically, cell mediated immunity, humoral immunity, local expression of cytokines and growth factors by resident and recruited cell populations collectively regulate local epithelial and connective tissue cell behavior (Okada and Murakami, 1998). Recently, the understanding that this chronic inflammatory condition has oxidative stress associated with it has brought another facet to consider when it comes to our understanding of periodontal disease pathogenesis (Chapple and Matthews, 2007). Oxidative stress is a normal outcome of cellular metabolism and a certain minimum level is associated with normal cellular function but excessive oxidative stress due to increased generation of radicals or decreased expression of antioxidants can negatively impact cell function. The degree to which this occurs is just beginning to be fully appreciated.

Periodontal disease progression is associated with junctional epithelial cell proliferation, loss of connective tissue attachment and alveolar bone. These three do
occur temporally and spatially at the same time but it is unclear which one is rate limiting (Birkedal-Hansen, 1998). Since we are principally interested in the regulation of disease onset and of the three significant tissue changes described above it is the proliferation of junctional epithelium that has been shown to occur in “early” disease (Page and Schroeder, 1976). Therefore, we focused our studies on growth factor regulation of epithelial proliferation and migration.

Keratinocyte Growth Factor-1 (KGF-1) is an excellent growth factor to examine because this growth factor and its receptor are associated with wound healing and chronic inflammatory conditions but its association with periodontal disease and its role in human disease pathogenesis has not been extensively examined. This growth factor is expressed primarily by connective tissue cells and specifically stimulates local epithelial cells because they express the KGF-1 specific receptor, KGFR. This dissertation utilized a combination of in vivo and in vitro approaches to examine KGF-1 and KGFR expression and regulation in the context of periodontal disease pathogenesis.

Previously it was established that lipopolysaccharide and proinflammatory cytokines induced gene and protein expression of KGF-1 by human gingival fibroblasts (Putnins et al., 2002; Sanaie et al., 2002). These data had suggested that KGF-1 may in fact regulate junctional epithelial cell proliferation and migration but an in vivo analysis of KGF-1 and KGFR gene and protein expression in healthy and disease associated human tissues was required. This dissertation (chapter 3) established that KGF-1 and
KGFR proteins were expressed in human periodontal tissues and both were significantly increased in diseased tissue samples. The intense positive immunohistochemical localization of KGF-1 protein to basal and parabasal epithelial cells was somewhat unexpected and was examined in more detail. KGF-1 is classically described as being expressed by connective tissue cells; however, there are a few publications that suggested KGF-1 may in fact be expressed by some epithelial cells (Steele et al., 2001; Hicks et al. 2004). To answer this question we utilized laser microdissection of the epithelium and subjacent connective tissues in conjunction with RT-PCR to localize KGF-1 and KGFR expression. We confirmed that in periodontal tissues KGF-1 was expressed by cells within the connective tissue compartment but localized to the epithelium in our immunohistochemical stainings due to interaction with the KGFR. These data strongly support that KGF-1 and KGFR are both expressed and increased in human periodontal disease and may in fact directly regulate local junctional/periodontal pocket epithelial cell migration and proliferation associated with periodontal disease pathogenesis (Li et al., 2005).

KGFR expression and upregulation in human disease (Li et al., 2005) was also confirmed in a timed in vivo rat periodontitis model (Ekuni et al., 2006). However, an increase in KGFR protein expression does not always signify an increase in receptor activity. The periodontal environment is a unique chronic inflammatory situation because there exists a relatively weak juncitonal epithelial cell barrier that is juxtaposed next to an abrasive calculus surface. This significance of this situation and its impact on KGFR phosphorylation was tested. This study would not be possible using in vivo
approaches, therefore, we utilized an *in vitro* scratch wound model. The advantage of using this model is that it separates the epithelial cell interaction from other cell populations and allows us to examine the intracellular signaling pathways regulating KGFR phosphorylation and the outcome(s) of this activation.

Using this *in vitro* approach we established that wounding induced KGFR phosphorylation via a ligand independent manner and was induced by generation of ROS (chapter 4). This finding provides further support for the significant role that ROS generation may play in the regulation of epithelial cell behaviour within periodontal tissues. This rapid and ligand independent activation of KGFR may serve to immediately induce cell migration in order to try to reestablish an epithelial barrier or may support migration of epithelial cells that occurs with the onset and progression of periodontal pocket formation (Li et al., 2007-manuscript in preparation).
5.2 Conclusions

1. KGF-1 and KGFR are expressed in human periodontal tissues and the expression of both was increased in diseased periodontal tissues. KGF-1 protein localization to basal and parabasal epithelial cells was not due to epithelial expression of this growth factor but was due to interaction with the KGFR. This direct ligand and receptor interaction suggests this signaling pathway may possibly be associated with the onset and progression of periodontal pocket formation.

2. In our \textit{in vitro} cell culture model, wounding induced rapid ROS generation at the wound edge. This in turn induced KGFR and downstream FRS2 phosphorylation via c-Src kinase. Functionally this ligand independent signaling was associated with ERK 1/2 phosphorylation and an increase in wound edge associated epithelial cell migration.

3. Therefore, within the context of periodontal disease pathogenesis there exists a potential that increased KGFR expression in human periodontal tissues may in turn be regulated by two mechanisms. First, proinflammatory and lipopolysaccharide induced expression of KGF-1 may in a ligand dependent manner induce KGFR signaling. Second, we identified a novel KGFR regulatory mechanism that may be unique to the periodontal environment. In this model microwounding of junctional/pocket epithelium by the abrasive nature of calculus may via ROS generation induce KGFR activation in a ligand independent manner. Collectively increased KGFR signaling either in a ligand-dependent or a ligand-independent manner may induce epithelial cell proliferation and migration.
5.3 Future Work

1. To determine if direct stimulation of junctional epithelial cell proliferation can regulate the onset and progression of periodontal disease we plan to selectively induce local epithelial cell proliferation. Since KGFR is relatively selective to epithelial cells we will continue to work with this growth factor. Two possible approaches may be utilized. If sufficient recombinant KGF-1 can be secured we will apply it daily and directly to the gingiva using our rat periodontal disease model. Alternatively, we will make a transgenic mouse that expresses KGF-1 in junctional epithelium. This can be accomplished by using the cytokeratin 19 promoter to drive expression in the junctional epithelium. This will establish an autocrine positive feedback loop to drive junctional epithelial cell proliferation. Using either approach we should be able to test whether induced proliferation and migration of junctional epithelium induces subsequent loss of periodontal soft and hard connective tissues.

2. Within the context of periodontal tissues most of the work to date has focused on ROS generation within immune cells and little has been on the impact and functional significance of ROS generation in epithelial cells. Utilizing our cell culture model we will expand our ROS studies to include additional cytokines, growth factors and lipopolysaccharides isolated from periodontal disease associated microorganisms to determine the functional significance of ROS generation in junctional and pocket epithelium.
5.4 Reference


6. Appendices

Appendix I

Informed Consent Form For Human Tissue collection
INFORMED CONSENT FORM:
Healing Responses of Oral Cells during Periodontal Disease

Principal Investigator: Dr. Edward E. Putnins (604-822-1734)

Location: Room 179, Dentistry Bldg., UBC

Purpose

This study aims to examine the way in which oral gingiva (tissue helping to hold teeth in place) is biologically controlled during the onset and progression of periodontal disease ("gum disease"). Tissue samples removed during the surgical procedure will be kept for two reasons. First, cells covering the tissues will be cultured and maintained in the laboratory and will be examined for factors that control cell growth and movement. Second, some tissue will be stained to see if specific factors that regulate their growth are being expressed. Together it is anticipated that this information will help improve our understanding by which periodontal disease initiates and progresses.

Samples from two types of subjects will be collected. One sample set will be collected from subjects that require the surgery to help control the periodontal disease process. The second set of samples will be collected from subjects that are receiving surgery because they require some tissue removal so that the tooth can be properly restored (crown lengthening) or are having a tooth extracted for other reasons (impacted tooth, orthodontics).

Risk

Only tissue that is normally discarded will be kept and no additional participation on the part of the subject is required. Therefore no additional risk or discomfort should be found. If problems are noted then contact emergency number 604-822-3293. Signing this consent form in no way limits your legal rights against the sponsor, investigators, or anyone else.
Confidentiality

You understand that you are encouraged to ask any additional questions that you may have and your participation is voluntary. You may refuse to participate in this study at any time without any consequences to the continuation of your dental treatment. Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, research records and medical records identifying you may be inspected in the presence of the Investigator or his or her designate by representatives of Health Canada, and the UBC Research Ethics Board for the purpose of monitoring the research. However, no records that identify you by name or initials will be allowed to leave the Investigators' offices. Should the results be published, individuals will not be identified by name, initial, or date of birth. Subjects of the study will not receive any results.

The UBC phone number for research subjects to call should they have any concerns about their rights or experience as research subjects is 604-822-8598 and is called the 'Research Subject Information Line in the UBC Office of Research Services'. This information line is not intended to provide urgent service to subjects with immediate needs for medical care for research-related injury.

Subject Consent

The study procedures have been explained to you completely and you acknowledge that you have been given a copy of this consent form. After reading and understanding the above information, you hear by voluntarily consent to participate in this study.

Subject Signature: __________________________ Date: __________________________

Print name: __________________________

Witness Signature: __________________________ Date: __________________________

Print name: __________________________

Investigator Signature: __________________________ Date: __________________________

Print name: __________________________

EEP: October 31, 03
Appendix II

Ethics Approval for User’s Study
Certificate of Expedited Approval: Amendment

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<td>Oral Biological &amp; Medical Sci</td>
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INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

UBC Campus

CO-INVESTIGATORS:

Wiebe, Colin, Oral Biological & Medical Sci

SPONSORING AGENCIES

Canadian Institutes of Health Research

TITLE

Localization, Expression & Functional Analysis of KGF-1 During Periodontal Disease Onset & Progression

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CERTIFICATION:

In respect of clinical trials:

1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of the this Research Ethics Board have been documented in writing.

The amendment(s) for the above-named project has been reviewed by the Chair of the University of British Columbia Clinical Research Ethics Board and the accompanying documentation was found to be acceptable on ethical grounds for research involving human subjects.

The CREB approval period for this amendment expires on the one year anniversary date of the CREB approval for the entire study.

Approval of the Clinical Research Ethics Board by one of:

Dr. P. Loewen, Chair
Dr. A. Gagnon, Associate Chair
Dr. J. McCormack, Associate Chair