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Keratinocyte growth factor-1 expression in healthy and diseased human periodontal tissues

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Objectives: Keratinocyte growth factor-1 (KGF-1) is up-regulated 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.

Methods: Tissues were collected from patients with periodontal health or disease, immediately frozen and stained for KGF-1 and KGFR protein expression. Laser capture microdissection of epithelial and connective tissue cells with reverse transcription–polymerase chain reaction (RT–PCR) examined KGF-1 and KGFR gene expression profiles and enzymatic digestion with heparitinase, chondroitinase ABC or pre-treatment with suramin examined epithelial surface molecule interactions with KGF-1.

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 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 pre-treatment, indicating interaction with the KGFR.

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

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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 (1). 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 (1, 2). 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 (3, 4). Keratinocyte growth factor-1 (KGF-1) regulates normal epidermal

homeostasis but is also significantly up-regulated during wound healing and in a variety of chronic inflammatory conditions such as Crohn's disease, ulcerative colitis and psoriasis (5–10). However, little is known about KGF-1 localization in gingival tissues with periodontal disease progression.

KGF-1 [fibroblast growth factor-7 (FGF-7)] belongs to the heparin-binding fibroblast growth factor family (11, 12). KGF-1 represents one of two KGF subfamily members and shares more than 60% sequence identity with KGF-2 (FGF-10) (13). KGF-1 is best described as a paracrine mediator expressed by mesenchymal cells and specifically stimulates epithelial cells (12, 14). Dermal fibroblasts, microvascular endothelial cells, smooth muscle cells and activated γδ T cells all express KGF-1 (14–17). 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 (18, 19). 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 (20, 21).

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 (22–29). Pro-inflammatory cytokines such as interleukin-1α, interleukin-1β, interleukin-6 and tumor necrosis factor-α induced KGF-1 protein and gene expression in gingival fibroblasts *in vitro* (23, 25, 28). In contrast to KGF-1, KGF-2 was only weakly expressed in gingival fibroblasts and this expression was not changed by pro-inflammatory cytokines (28). We recently established that lipopolysaccharide purified from *Escherichia coli* and *Porphyromonas gingivalis* also induced KGF-1 protein and gene expression through a Toll-like receptor signaling pathway (29). KGF-1 expression in gingival tissues and its up-regulation 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.

Material and methods

Sample collection and immunohistochemistry

Oral gingival tissue samples were collected from periodontally healthy patients ($n = 15$) with minimal sulcus depth 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, but who still required open flap debridement to control disease. All samples were collected from patients who had provided informed consent and were not taking prescription medication at the time of sample collection. Tissues were immediately snap frozen in Tissue-Tek O.C.T. compound (Sakura Finetek Inc., Torrance, CA, USA) and sectioned in a cryostat at 8 μm (Cambridge Instruments, Heidelberger, Germany). Tissue sections were acetone fixed, rehydrated and permeabilized in buffer (phosphate-buffered saline + 0.01% Triton X-100 + 0.1% bovine serum albumen), blocked with either goat or horse serum for 20 min at room temperature (20°C) prior to incubation with either 0.25 μg/ml rabbit anti-human KGF-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1 μg/ml rabbit anti-human KGFR (Santa Cruz Biotechnology) antibodies at 4°C overnight in a humidified chamber. Slides were washed [phosphate-buffered saline + 0.1% bovine serum albumin (BSA) + 0.01% Triton X-100] and then incubated with a biotin-labeled secondary antibody for 1 h 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) was used for color development as per 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 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 (30). Semi-quantitative evaluation of staining intensity was based on a previously published method that incorporated the percentage of positively stained cells and color intensity (31). 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.

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 five wells in a 96-well plate was coated with 10 μg/ml (100 μl/well) of KGF-1, KGF-2, basic FGF (bFGF) (Upstate Biotechnology, Lake Placid, NY, USA) or 1% BSA (negative control) proteins for 2 h at room temperature. Wells were washed three times for 5 min (phosphate-buffered saline, pH 7.4, with 0.05% Tween 20) and blocked with 2% heat-denatured BSA/phosphate-buffered saline at room temperature for 1 h and washed three times for 5 min. Rabbit anti-human KGF-1 antibody (1 : 800) was added to the first of each well coated with KGF-1, KGF-2, bFGF or BSA protein for 2 h. Antibodies were transferred to the second equally coated well, incubated for 2 h 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 three times for 5 min prior to the addition of 100 μl/well (1 : 100) anti-rabbit IgG peroxidase conjugated secondary antibody (Santa Cruz Biotechnology), incubated for 1 h, followed by three 5-min washings. Color was developed with ABTS substrate consisting of 4 ml buffer (0.1 M $NaC_2H_3O_2$, 0.05 M NaH_2PO_4), 0.2 ml ABTS (22 mg/ml in distilled water), and 0.04 ml diluted H_2O_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 μl/well) coated wells served as a positive control. Second, 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% sodium dodecyl sulfate–polyacrylamide gel and blotted to hybond-enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham, Buckinghamshire, UK). Filters were blocked with Tris-buffered saline containing 5% skim milk for 90 min at 37°C, washed and incubated with anti-KGF-1 antibody for 3 h 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).

Enzymatic tissue digestion and suramin pre-treatment

To examine the possible interaction of KGF-1 with heparan sulfate or chondroitin/dermatan sulfate proteoglycan tissue sections were digested at 37°C for 1 h with heparitinase (0.04 U/ml, ICN Biomedical, Aurora, USA) in 1 mg/ml BSA in phosphate-buffered saline buffer or chondroitinase ABC (0.7 U/ml, ICN Biomedical) 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 (Sigma, St. Louis, USA) following a pre-established protocol (32). Suramin is a highly anionic naphathalene sulfonic acid derivative that strips KGF-1 from the KGFR. In each case, controls were incubated with phosphate-buffered saline 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) or anti-bFGF (1 : 200, R & D System, Minneapolis, MN, USA) antibodies in a humid chamber at 4°C overnight, followed with biotin-labeled secondary antibodies. Color development was as described previously.

Laser capture microdissection

Frozen sections were air dried for 30 s, rinsed in diethyl pyrocarbonate-treated water (depcH_2O) for 30 s, immediately stained with Histogene Stain (Arcturus, Mountain View, CA, USA) for 20 s and rinsed with depcH_2O for 30 s. Slides were sequentially washed through 75%, 95% and 100% ethanol for 30 s 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) was immediately performed and samples collected into separate 0.2 ml tubes. RNA extractions were performed using Rneasy Mini Kit (Qiagen, Valencia, CA, USA) and RNA was diluted in 30 μl of depcH_2O.

One-step reverse transcription–polymerase chain reaction (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 s, annealing at 57°C for 30 s, 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 × TBE buffer containing 1 μl/100 ml of ethidium bromide (0.1 μg/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.

Results

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 KGF-1, KGF-2, bFGF or BSA proteins, the anti-KGF-1 antibody only bound (high optical density) to wells coated with KGF-1 (Fig. 1A). The relative optical density in wells

Table 1. Primers used for reverse transcription–polymerase chain reaction (RT–PCR) amplification

	Primer (5′–3′)	Size (bp)	Reference
KGF	F: TCTGTCGAACACAGTGGTACCT R: GTGTGTCCATTTAGCTGATGCAT	266	27
KGFR	F: CACTCGGGGATAAATAGTTCC R: AACTGTTACCTGTCTCCGCAG	354	33
K19	F: TCCCGTGACTACAGCCACTACTACACGACC R: CGCGACTTGATGTCCATGAGCCGCTGGTAC	745	34
Type I collagen (Col-I)	F: GGTTCCCCTGGACGAGACGGTT R: AGGAAGCTGAAGTCGAAA	538	35
GAPDH	F: CCACCCATGGCAAATTCCATGGCA R: TCTAGACGGCAGGTCAGGTCCACC	600	36

KGF, keratinocyte growth factor; KGFR, keratinocyte growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

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 (Fig. 1B). Anti-KGF-1 antibody that was not pre-absorbed stained oral basal epithelial cells associated with Rete Ridges (Fig. 1Bi). In contrast, the antibody absorbed against KGF-1 protein retained no positive staining (Fig. 1Bii). However, antibody absorbed against KGF-2 (Fig. 1Biii) or bFGF (Fig. 1Biv) proteins stained in the same pattern as the non-absorbed antibody. Tissues stained with non-immune primary or no secondary antibodies were negative (data not shown). Paraformaldehyde-fixed and paraffin-embedded gingival tissue were negative (data not shown) and therefore all tissues examined were frozen sections. Western blotting with the antibody identified one 21-kDa band consistent with recombinant KGF-1 protein and did not cross-react with KGF-2 protein (Fig. 1c). Therefore, the anti-KGF-1 antibody used in this study was confirmed to be KGF-1 specific.

KGF-1 protein localization in healthy and diseased tissues

Tissues collected from healthy and diseased tissues were stained for KGF-1 protein localization. Clinical indices of the samples collected from healthy patients ($n = 15$) showed a mean ± SD sulcus depth of 2.7 ± 0.62 mm 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 and 62% exhibited bleeding on probing just prior to surgery. The presence of junctional epithelium in health was

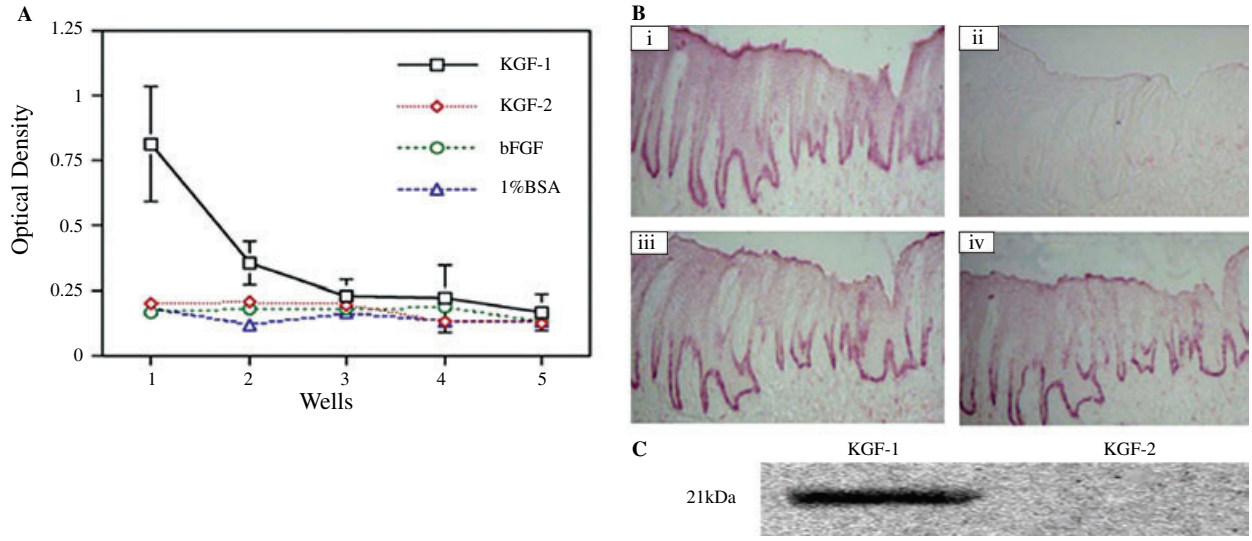

Fig. 1. Anti-keratinocyte growth factor-1 (KGF-1) antibody is specific for KGF-1 protein. (A) Each of five wells of a 96-well plate was coated with 10 μg/ml of KGF-1, KGF-2, basic fibroblast growth factor (bFGF) or 1% bovine serum albumin (BSA) proteins then blocked with 1% BSA and washed. Anti-KGF-1 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, washed and color developed with ABTS substrate. (B) Control non-absorbed antibody (i) and absorbed antibodies against KGF-1 (ii), KGF-2 (iii) or bFGF (iv) proteins were used to stain oral gingival tissues. (C) Recombinant human KGF-1 and KGF-2 proteins (10 ng/lane) were separated in a 12% polyacrylamide gel and blotted. Anti-human KGF-1 antibody was incubated with the blot, washed, followed by a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and detected using chemiluminesence.

confirmed by positive laminin-5 staining on the internal and external basal lamina (30). Only samples with junctional epithelium that showed this positive staining pattern were included in the healthy group (Fig. 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 (Figs 2A and B). In contrast, pocket epithelium staining was generally more intense and all cell layers stained positive (Figs 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 (Figs 2E and F). Oral epithelium collected from patients with disease generally showed more intense pericellular epithelial staining in basal and some parabasal epithelial cells

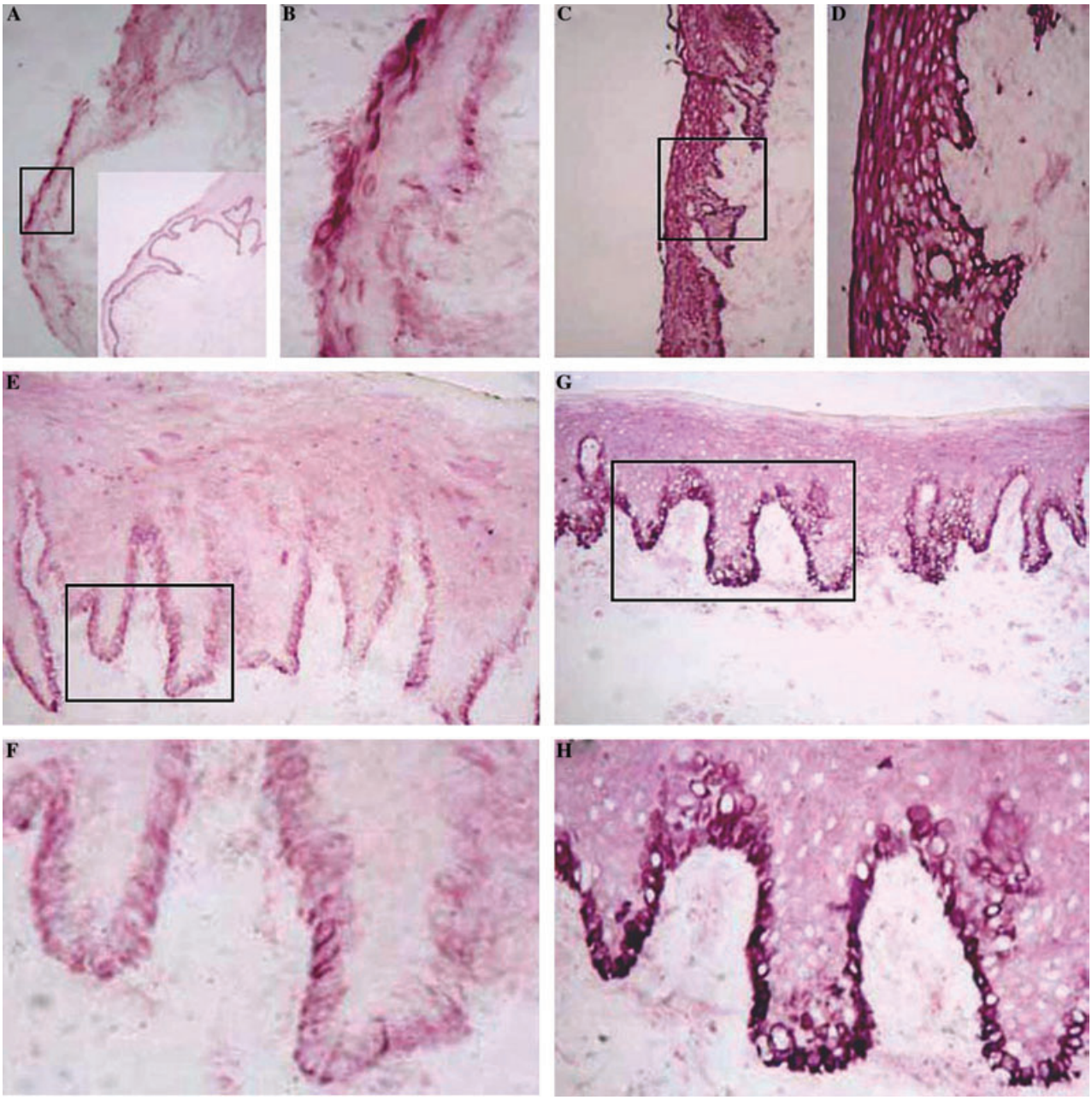

Fig. 2. Keratinocyte growth factor-1 (KGF-1) protein localization to epithelial cells of oral gingival tissues. Tissues collected from healthy patients (A, B, E and F) and patients with advanced periodontal disease (C, D, G and H) were stained with the anti-KGF-1 antibody. Junctional epithelium stained positive for KGF-1 protein (A) and laminin-5 (inset in A). Close-up of black box outlined in (A) is 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.

Table 2. Semi-quantitative evaluation of keratinocyte growth factor-1 (KGF-1) and keratinocyte growth factor receptor (KGFR) staining intensity on healthy and diseased gingival epithelial samples

	Junctional/pocket epithelium		Oral epithelium	
	Healthy $N = 11$	Periodontal disease $N = 10$	Healthy $N = 15$	Periodontal disease $N = 13$
KGF-1 Mean ± SD	6.9 ± 2.1	9.2 ± 2.4**	5.9 ± 1.6	8.6± 2.0**
KGFR Mean ± SD	7.3 ± 1.8	9.0 ± 1.8*	6.5 ± 1.9	7.2 ± 1.7

**$p < 0.01$, *$p < 0.05$.

(Figs 2G and H). In all tissues stained there was minimal staining in the connective tissues (Fig. 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 healthy samples.

KGF receptor localization in healthy and diseased tissues

In healthy gingiva there was positive pericellular KGFR staining in most cells of the junctional epithelium (Figs 3A and B) and this was also found for pocket epithelium (Figs 3C and D). Upon examination, oral epithelium from healthy patients (Figs 3E and F) and diseased patients (Figs 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 healthy or diseased patients (Table 2).

KGF-1 and KGF receptor 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 frozen tissue sections (Fig. 4). RT–PCR amplification was used to examine KGF-1 and KGFR gene expression in the connective tissue and epithelium (Fig. 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.

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 (37, 38). 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 (Fig. 6A, left) and pre-treatment with heparitinase prior to staining effectively removed heparan sulfate from the tissues (Fig. 6A, right). Heparitinase pretreatment was effective in stripping bFGF interaction with heparan sulfate present in the basal lamina as evidenced by loss of bFGF antibody staining of the basement membrane (Fig. 6B, left and right), but was not able to reduce KGF-1 positive staining in the basal epithelial cells (Fig. 6C, left and right). Treatment of tissues with chondroitinase ABC prior to staining with anti-chondroitin sulfate antibody effectively removed chondroitin sulfate (Fig. 6D, left and right) but did not reduce KGF-1 staining intensity (Fig. 6E, left and right). However, pre-treatment of tissue samples with suramin (32) very significantly reduced KGF-1 staining (Fig. 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.

Discussion

Epithelial cell proliferation begins in early periodontal disease and progresses to form pocket epithelium in advanced disease (2). The regulation of this aspect of disease and its significance in disease pathogenesis is not fully understood (1). Pro-inflammatory cytokine expression and lipopolysaccharide from Gram-negative microorganisms associated with disease function to directly or indirectly modulate cellular behavior (3, 4, 39–41). Pro-inflammatory cytokines (interleukin-1α, interleukin-1β, interleukin-6 and tumor necrosis factor-α) and purified lipopolysaccharide from *P. gingivalis* all significantly induced gingival fibroblast KGF-1 gene and protein expression *in vitro* (23, 25, 28, 29). Therefore increased KGF-1 protein expression in periodontal disease

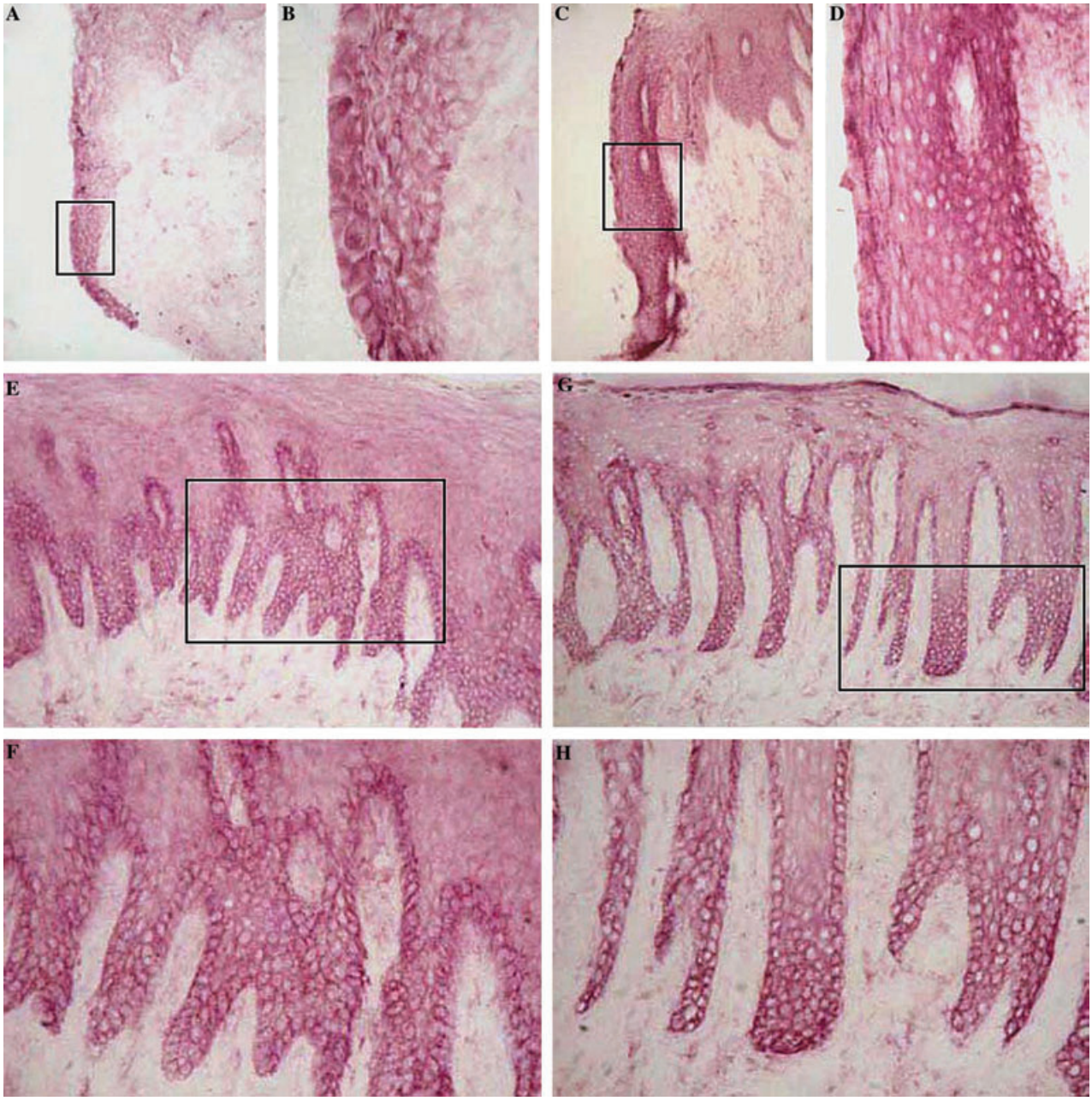

Fig. 3. Keratinocyte growth factor receptor (KGFR) protein localization to epithelial cells of oral gingival tissues. Tissues collected from a patient with periodontal health (A, B, E and F) and a patient with advanced periodontal disease (C, D, G and H) were stained with anti-KGF-1 receptor antibody. Junctional epithelium (A) stained positive for KGFR protein. Close-up of black box outlined in (A) is 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.

as we have described may have been induced by lipopolysaccharide and pro-inflammatory cytokines up-regulated 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 (12, 14–17). However, in ovarian and endometrial tissues, KGF-1 was expressed by epithelial cells (18, 19). Our laser capture with RT–PCR approach established KGF-1 gene expression by connective tissue cells and this supports existing *in vitro* data describing KGF-1 gene expression by gingival connective tissue cells (25, 27–29). The lack of KGF-1 protein staining in the connective tissue may be a reflection of a relatively low protein

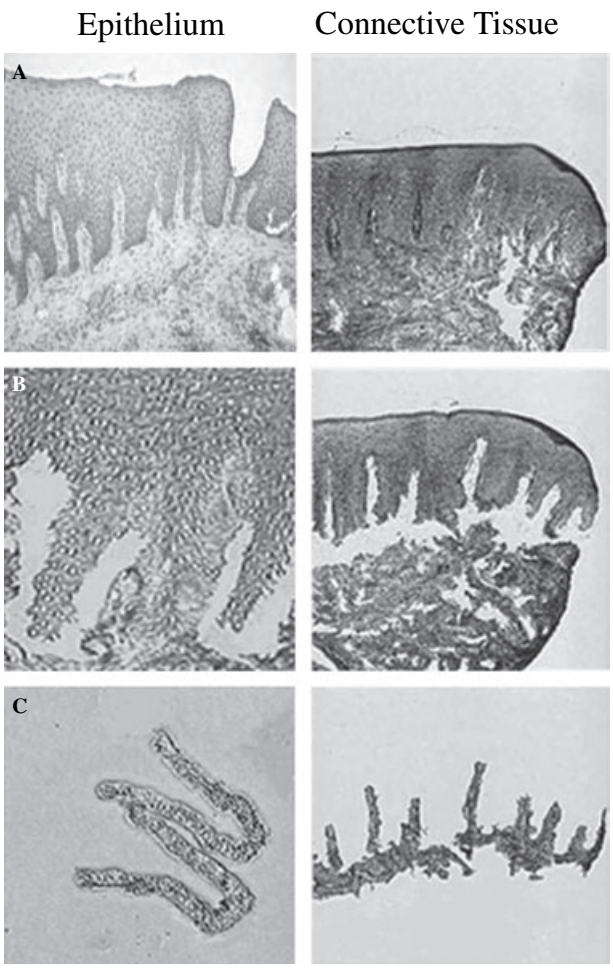

Fig. 4. Laser capture microdissection of specific cells. (A) Oral gingival epithelial tissues were stained with Histogene Stain® to identify dissection areas. (B) Basal epithelial cells (left) and subjacent connective tissue cells (right) were removed with laser capture microdissection. (C) Dissected epithelial (left) and connective tissue (right) cells after capture, which were used subsequently for reverse transcription–polymerase chain reaction (RT–PCR).

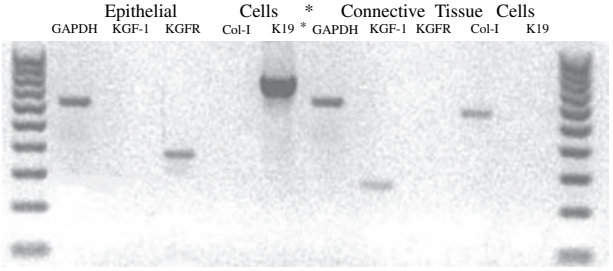

Fig. 5. Reverse transcription–polymerase chain reaction (RT–PCR) amplification of mRNA purified from laser captured epithelial and connective tissue cells. One step RT–PCR kit with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), keratinocyte growth factor-1 (KGF-1), keratinocyte growth factor receptor (KGFR), type I collagen (Col-I) and cytokeratin 19 (K19) specific primers was used to amplify genes present in epithelial and connective tissue cells.

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) (11, 13). An extensive testing protocol (Fig. 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). As 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 (42). 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 (43) 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 (38, 44). For example, glypican, a cell membrane-associated, glycosylphosphatidylinositol-linked heparan sulfate proteoglycan inhibited KGF-1 mitogenic effects, and pre-treatment with heparatinase abolished this effect (45, 46). Conversely, dermatan sulfate (chondroitin sulfate B) augments KGF-1-mediated KGFR activation and cell proliferation (47). 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 heparan sulfate proteoglycan (42), but was ineffective in reducing KGF-1 binding. Pre-treatment of tissues with chondroitinase ABC, which degrades chondroitin (Fig. 6D, left and right) and dermatan sulfate (48), 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

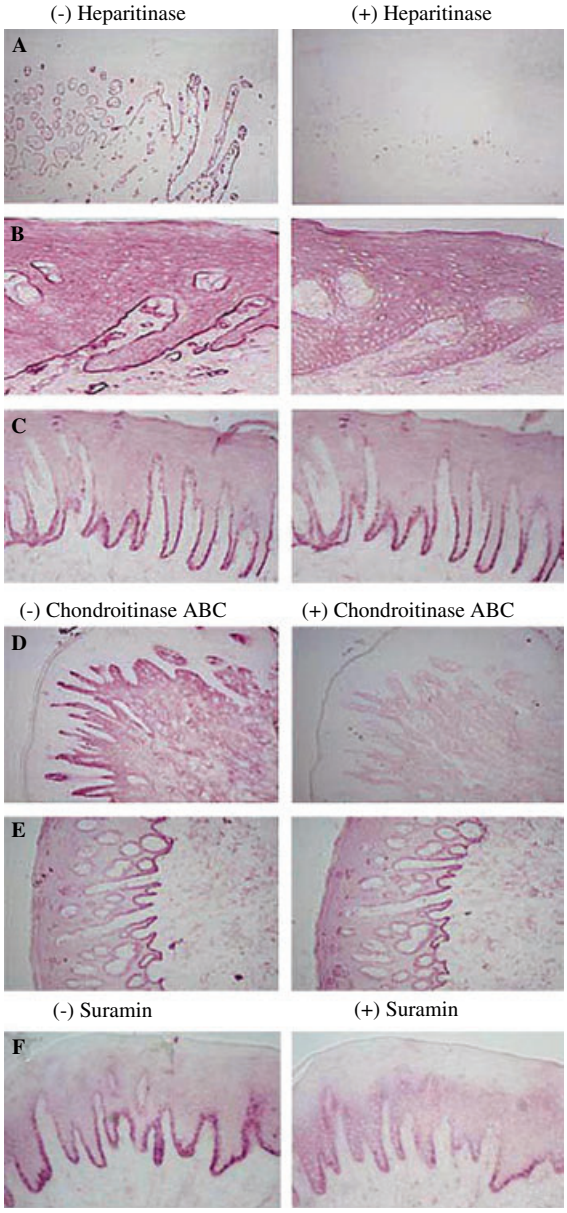

Fig. 6. Keratinocyte growth factor-1 (KGF-1) localization to epithelial cells is mediated through KGF receptor. KGF-1 possible interaction with heparan sulfate was examined in control (left A, B and C) and heparitinase pre-treated tissue samples (right A, B and C). Slides were subsequently stained with 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).

splice variant of fibroblast growth factor receptor-2 and is designated FGFR-2IIIb (20, 21). Pre-treatment of samples with suramin (Fig. 6E, left and right), an agent that strips KGF-1/KGFR interactions (32), 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 may suggest that additional unidentified molecules may interact with KGF-1. KGF-1 low affinity binding interactions with additional unidentified molecules was previously suggested (49).

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 (5–8, 50). 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 (51, 52). These data suggest that periodontal disease 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 (6–8). 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 tumor necrosis factor-α and lipopolysaccharide-induced epithelial cell

apoptosis (53). 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 up-regulation 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 (54–59). All of these cellular processes are associated with pocket formation (1). Therefore, it is interesting to hypothesize that the up-regulation of KGF-1 protein expression that we have identified in periodontitis disease tissues may play a role in regulating onset and progression of epithelial cell proliferation and migration associated with periodontal pocket formation.

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