J Periodont Res 2006; 41; 340–349

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00881.x

Regulation of epithelial cell growth factor receptor protein and gene expression using a rat periodontitis model

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Ekuni D, Firth JD, Putnins EE. Regulation of epithelial cell growth factor receptor protein and gene expression using a rat periodontitis model. J Periodont Res 2006; 41: 340–349. © Blackwell Munksgaard 2006

Background and Objective: Regulation of epithelial cell behavior associated with periodontitis is not well elucidated but many responses will ultimately be regulated by growth factor receptors. Using a rat experimental periodontitis model, protein and gene expression of select growth factor receptors in junctional and pocket epithelium were examined.

Material and Methods: Periodontal disease was induced by daily topical application of lipopolysaccharide using an established protocol. Animals were killed at time 0 (control), and at 2 and 8 wk. Frozen tissue samples were collected from the right palatal gingival soft tissue, and the left periodontal tissues were decalcified and embedded in paraffin. Laser microdissection and quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was used to quantify keratinocyte growth factor receptor (KGFR), hepatocyte growth factor receptor (HGFR), epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor 1 (FGFR1) gene expression, and *in situ* RT–PCR localized these increases to specific epithelial cells. Receptor protein expression was examined immunohistochemically. In cell culture, induction of HGFR and KGFR protein expression by serum, lipopolysaccharide and pro-inflammatory cytokines were examined using flow cytometry.

Results: Eight-week tissue samples exhibited histological changes consistent with periodontitis. KGFR and HGFR gene and protein expression were significantly induced at the 8 wk time point. KGFR expression was significantly up-regulated in basal and parabasal pocket epithelial cells, but HGFR was up-regulated throughout the pocket epithelium. In cell culture serum, lipopolysaccharide and pro-inflammatory cytokines, interleukin-1β and tumour necrosis factor-α significantly induced KGFR protein receptor expression, but HGFR expression was only induced by serum.

Conclusion: KGFR and HGFR are highly up-regulated in this model of periodontal disease and may play a significant role in regulating the proliferation and migration of pocket epithelium.

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Key words: hepatocyte growth factor receptor; keratinocyte growth factor receptor; laser dissection; periodontal disease

Accepted for publication December 19, 2005

Early onset of periodontal diseases is associated with significant epithelial cell proliferation and migration; however, regulation of these processes is poorly understood (1,2). Lipopolysaccharide isolated from periodontal disease-associated gram-negative pathogens is one significant virulence factor inducing many of these cellular responses (3). In response to this bacterial challenge, epithelial cell growth factor receptors probably play a significant role (4,5). Growth factors that bind to the keratinocyte growth factor receptor (KGFR), hepatocyte growth factor receptor (HGFR), epidermal growth factor receptor (EGFR) or fibroblast growth factor receptor 1 (FGFR1) are present in periodontal tissues and signal through their respective receptors.

Keratinocyte growth factor-1 (KGF-1) and hepatocyte growth factor (HGF) are principally expressed by connective tissue cells and classically stimulate epithelial cells through their relatively specific FGFR2-iiib (KGFR) and c-Met (HGFR) receptors, respectively (6–15). Both growth factors are up-regulated in chronic inflammatory conditions (16–21). This up-regulation is probably caused by pro-inflammatory cytokines and bacterial products, such as fimbriae and lipopolysaccharide (8,22,23). In contrast, the regulation and expression of their specific receptors *in vivo* is less clear. To date, an increase in KGFR protein staining was found in tissues collected from advanced disease sites (19). However, the protein and gene expression and regulation with disease onset is yet to be elucidated.

In contrast to the KGF-specific and HGF-specific receptors, EGFR and FGFR1 bind a wide variety of ligands expressed by epithelial cells or adjacent fibroblasts (24–28). Within periodontal tissues, EGFR protein was localized to parabasal cells in oral epithelium and increased in inflamed tissues. In contrast, junctional epithelium stained negative but pocket epithelium stained positive (29,30). FGFR1 is one of four FGFR described to date; it also interacts with a wide variety of FGF ligands, but not KGF-1 or -2, and is expressed by a variety of cells (27,28). One FGFR1-binding ligand, basic FGF (bFGF), was localized to normal and inflamed basal oral epithelium, basement membrane, and within the connective tissue (19,29).

To effectively analyze protein and gene KGFR, HGFR, EGFR and FGFR1 expression and regulation associated with early disease onset, an animal model is required. One model induced histological changes consistent with disease by daily topical application of *Escherichia coli* lipopolysaccharide with protease purified from *Streptomyces griseus* (31–33). Using this model, elongation of rete ridges, apical migration of junctional epithelium, and resorption of alveolar bone – all histological changes consistent with periodontal disease – were first confirmed. Second, KGFR, HGFR, EGFR and FGFR1 gene expression was quantified in junctional and pocket epithelium using quantitative reverse transcription–polymerase chain reaction (qRT–PCR) of RNA purified from laser-dissected junctional and pocket epithelium. Third, receptors up-regulated with disease were localized to specific epithelial cells using *in situ* RT–PCR and associated protein expression was confirmed immunohistochemically. Finally, the regulation of disease-associated receptor protein expression was assayed using an *in vitro* cell-culture model.

Materials and methods

Experimental design

The periodontal disease model utilized in this study was based on a previously established rat animal model (32) with several technical modifications. Briefly, animals were anesthetized daily using Isoflurane (Baxter, Co., Toronto, ON, Canada), then 25 μg/μl of *E. coli* lipopolysaccharide (Serotype O55:B5; Sigma, Oakville, ON, Canada) with 2.25 U/μl of *S. griseus* type XIV proteases (Sigma), resuspended in pyrogen-free water (ICN Biomedical Inc., Aurora, OH, USA), was introduced by micropipette into the left and right palatal gingival sulcus of all three maxillary molars. A total of 21 male Wistar strain rats (6 wk old) were used in this study, with seven animals in each of three groups: time 0 (control); 2 wk of treatment; and 8 wk of treatment. The animal experiments complied with guidelines approved by the Animal Research Committee of The University of British Columbia.

Tissue preparation

Before death, rats were deeply anesthetized using Isoflurane and the right palatal gingival soft tissue was collected by sharp dissection, immediately embedded in OCT (Sakura Finetek USA, Inc., Torrance, CA, USA), frozen in liquid nitrogen and stored at −86°C until required. Sections from these blocks were subsequently used for laser dissection, RT–PCR and qRT–PCR experiments.

After removal of the right palatal biopsy, rats were killed by intracardiac perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under general anesthesia. Following initial fixation, the left maxillary molar regions were resected *en bloc* from each rat. Tissues were decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 wk at 4°C. Paraffin-embedded bucco-lingual 5-μm sections were made and used for morphometric analysis, *in situ* RT–PCR and immunohistochemical analysis.

Morphometric analysis

Sections, of 5-μm thickness, prepared from the paraffin-embedded palatal tissue sections, were stained with hematoxylin and eosin, and the distances from the cemento–enamel junction (CEJ) to the coronal aspect of the connective tissue attachment (extent of apical migration), and from the CEJ to the coronal margin of alveolar bone (bone loss), were measured using a microgrid at 200× magnification. Means of histological data were calculated for each rat. The Mann–Whitney *U*-test was performed using a statistical software package (SPSS version 10.0 J; SPSS, Tokyo, Japan) (32). Induction of periodontitis was defined as statistically significant increases in apical epithelial cell migration from the CEJ and loss of alveolar bone.

Laser dissection and qRT–PCR analysis

Serial, 8-μm-thick cryosections were cut on a cryostat (Cambridge Instruments, Heidelberg, Germany), placed on RNase-free slides (19,34), air-dried for 30 s, rinsed in diethyl pyrocarbonate-treated water (depcH_2O) for 30 s, immediately stained with Histogene (Arcturus, Mountain View, CA, USA) for 20 s and rinsed with depcH_2O for 30 s. Slides were sequentially dehydrated through 75%, 95% and 100% ethanol for 30 s each, transferred into xylene for 5 min and air-dried for 5 min. Immediately after staining, laser-capture microdissection (Arcturus) of junctional epithelium (control), and the equivalent area of apical pocket epithelium (8-wk sample) from 24 serial sections (≈ 100 cells/section and eight sections/rat), were pooled from three healthy or three diseased rats, then samples were collected into 0.2-ml tubes and RNA extracted using the RNeasy Micro Kit (Qiagen, Mississauga, ON, Canada) (19). Reverse transcription was carried out with 15 ng of DNase I-treated total RNA using 15 U of Cloned AMV Reverse Transcriptase and 200 ng of random hexamer primers (Invitrogen, Burlington, ON, Canada) in a 10-μl volume. The resulting cDNA was applied to qPCR using the LightCycler system with the SYBR Green labeling kit (Roche Diagnostics, Laval, QC, Canada). Primer design (Table 1) was based on gene sequence data using website software (http://seq.yeastgenome.org/cgi-bin/web-primer and http://www.geneservice.co.uk/home). For the LightCycler reaction (10.1 μl total volume), 6.1 μl of water, 0.5 μl (500 pmol) of each primer (Table 1), 2.0 μl of Fast Start Master SYBR Green I mix (Roche Diagnostics) and 1 μl of cDNA, were mixed and transferred into capillaries. All samples were prepared as triplicates; no-template controls were included to check for external contamination and for primer–dimer formation. The following program was used for amplification of target and reference genes: after 10 min of denaturation at 95°C, 45 cycles of qPCR with three-segment amplification was carried out, consisting of 10 s at 95°C for denaturation, 5 s at 56°C for annealing and 30 s at 72°C for polymerase elongation. To verify the specificity of the amplification, a melting curve analysis was performed, starting from 65°C with a rate of 0.2°C/s up to 95°C, with continuous measurement of fluorescence. PCR efficiency was calculated from the slope of the standard curves for the target and reference genes. Data analysis was carried out with a relative quantification software tool (REST©) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference gene, and results for target and reference genes were corrected according to their calculated PCR efficiencies (40). Raw data were analyzed by one-way analysis of variance (ANOVA), with p-values of < 0.05 considered significant.

Table 1. Primers

Gene	Size (bp)	Primers (5′–3′)	Reference
KGFR	354	F: CAC TCG GGG ATA AAT AGT TC R: AAC TGT TAC CTG TCT CCG CAG	(35)
FGFR1	211	F: TTT CAA GCA GTT GGT GGA AG R: ATT TGC AAG CTG GGT GGG	(36)
HGFR	162	F: CAG CGG CAA TTC TAG ACA C R: CTG AAG CTG CTT GTC ACT CG	(37)
EGFR	171	F: TGG AGA GAA TCC CTT TGG AG R: TGT TGC TAA ATC GCA CAG C	(38)
GAPDH	600	F: CCACCCATGGCAAATTCCATGGCA R: TCTAGACGGCAGGTCAGGTCCACC	(39)

EGFR, epidermal growth factor receptor; FGFR1, fibroblast growth factor receptor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGFR, hepatocyte growth factor receptor; KGFR, keratinocyte growth factor receptor.

RT–PCR

Frozen sections were air dried within 30 s, fixed in 75% ethanol for 30 s and rinsed in depc-treated water for 30 s. Slides were washed through 75%, 95% and 100% ethanol for 30 s each and dried in a fumehood for 5 min. Control tissue samples from six sections were scraped, using a fresh scalpel blade, into a 0.2-ml tube. RNA extraction was performed using an RNeasy Micro Kit (Qiagen) (19). One step RT–PCR (Invitrogen) 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 a final extension at 72°C for 5 min. An aliquot of the reaction was fractionated on a 1.5% agarose gel prepared in 1× Tris base (50 mM), Boric acid (50 mM), EDTA (1 mM) (TBE) buffer containing 0.1 μg/ml ethidium bromide. The gel was examined using ultraviolet (UV) light and photographed.

In situ RT–PCR

Site-specific receptor mRNA distribution was investigated by *in situ* RT–PCR (34). Paraffin-embedded tissue sections ($n = 3$) from control, 2 wk, and diseased (8 wk) rats were placed on glass slides and incubated at 60°C for 24 h to ensure maximum slide adhesion. Subsequently, sections were dewaxed in xylene for 5 min, washed three times, then immersed in fresh 100% ethanol, rehydrated in 70% ethanol and depcH_2O, incubated in 1% H_2O_2 for 30 min at room temperature and permeabilized with 1 μg/ml Proteinase K for 10 min at room temperature. After inactivating Proteinase K with heating and washing, sections were incubated in 1 U/ml DNase I in RNase-free buffer (40 mM Tris-HCl pH 7.4, 6 mM $MgCl_2$, 2 mM $CaCl_2$) for 30 min in a moist chamber at 37°C. DNase I was inactivated by EDTA solution for 10 min at 65°C, followed by RT–PCR, as described above, using digoxigenin-labeled dNTPs (Roche Applied Sciences). In the positive controls, treatment with DNAse I was omitted, while in the

negative controls, the RT step was omitted. Sections were washed in 2× Nacl (150 mM), Na_3citrate (15 mM), pH 7.0 (SSC), 1× SSC, 0.5× SSC, and phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), for 5 min each, followed by blocking with 2% heated-denatured BSA/PBS at room temperature for 30 min and incubating with anti-digoxigenin peroxidase conjugate (anti-DIG-POD; 1 : 100) (Roche Applied Sciences) for 1 h at room temperature. After three washes in PBS for 15 min, sections were incubated for 5 min in the dark in the presence of 3,3′-Diamino benzidine (DAB)/H_2O_2 solution (Sigma). Slides were mounted and observed under the microscope.

Immunohistochemstry

Representative paraffin-embedded tissue sections from three rats in the control, 2 wk and 8 wk experimental groups were selected and stained with a panel of receptor-specific antibodies. The polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) against KGFR (Sc-122), HGFR (Sc-161), EGFR (Sc-03) and FGFR1 (Sc-121) were diluted at 1 : 200 in PBS/BSA. Receptor protein expression was detected using an avidin–biotin complex detection method (ABC Elix Kits; Vector Laboratories, Burlington, CA, USA), as previously described (19). Digital quantification was carried out to segment the area of interest and determine the ratio of positive expression to area of junctional or pocket epithelium using a Scion Image (Scion Corp., Frederic, MD, USA) computer program, as previously described (41). Means of histological protein data were calculated for each rat and the data were statistically analyzed (Tukey test SPSS Japan 11.5.1; SPSS, Tokyo, Japan).

Flow cytometry

Porcine ligament epithelial cells, from the epithelial rest of Malassez, were isolated and cultured as described by Brunette *et al.* (42). These cells have previously been used as a model for junctional epithelium (42–45). Passage 5–7 cells were seeded at 8×10^3 cells/cm^2 and cultured to 70% confluence in α-minimal essential medium (α-MEM) (StemCell Technologies Inc., Vancouver, BC, Canada) containing 10% fetal bovine serum (FBS) (Flow Laboratories, McLean, VA, USA). For experiments, defined (KBM+) media (46) included keratinocyte basal media (KBM; Clonetics, San Diego, CA, California, USA), antibiotics (100 μg/ml streptomycin sulfate, 100 U/ml penicillin), an antimycotic (amphotericin B; 0.25 μg/ml), 5.0 μg/ml insulin, 0.5 μM hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine and 10 μg/ml fibronectin (Sigma). Cells at 70% confluence were further cultured to quiescence for 8 h in basal KBM+. Cultures were then

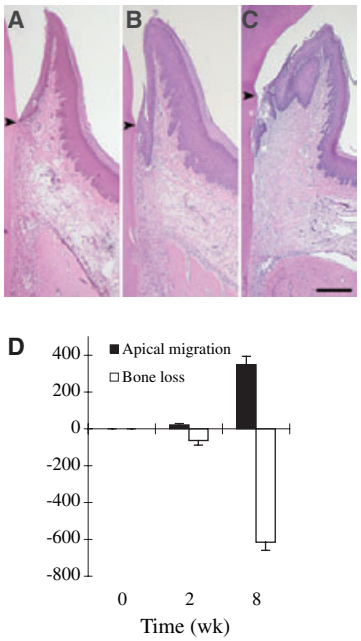

Fig. 1. Induction of histological changes consistent with periodontitis. Lipopolysaccharide with protease was applied daily to the palatal gingival sulcus of maxillary molars and animals were killed by perfusion fixation at time 0 (A), and at 2 wk (B) and 8 wk (C) thereafter. The jaws were removed, decalcified, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Representative sections are shown, and the cemento–enamel junction (CEJ) is designated with black arrows. (D) Mean increase in apical epithelial cell migration past the CEJ and along the root surface was measured in all groups using a microgrid, and the mean alveolar bone to CEJ distance was calculated. Mean bone loss at 2 and 8 wk represented the subtracted differences over the time 0 controls. All values are shown as mean ± standard error (SE) ($n = 7$). Apical epithelial migration and bone loss was significantly induced at 8 wk ($p < 0.001$). Scale bar (black), 200 μm.

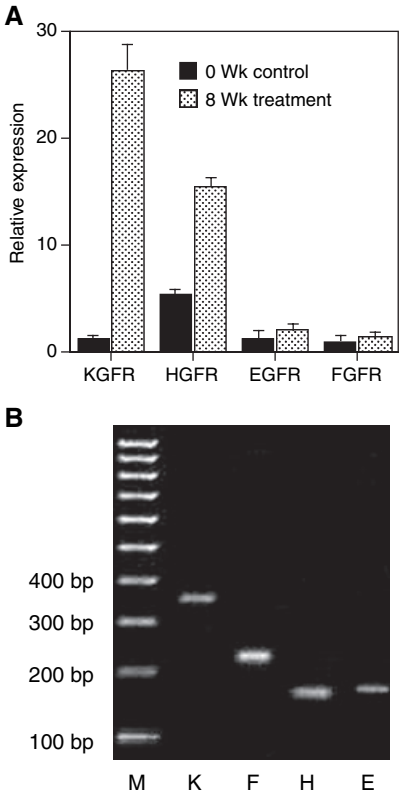

Fig. 2. Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) analysis of receptor gene expression using laser-dissected junctional and 8-wk pocket epithelium. (A) Target epithelium was laser dissected from pooled control and diseased tissues ($n = 3$), RNA extracted and reverse transcribed using random primers. cDNA was subjected to quantitative (q)PCR, and PCR efficiency was calculated from the slope of the standard curves for the target and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and then analyzed using a relative quantification software tool (REST©). (B) Specificity of RT–PCR amplification of target sequences was confirmed by 1.5% agarose-gel electrophoresis using keratinocyte growth factor receptor (KGFR) (K)-, fibroblast growth factor receptor 1 (FGFR1) (F)-, hepatocyte growth factor receptor (HGFR) (H)- and epidermal growth factor receptor (EGFR) (E)-specific primers relative to the molecular weight marker (M).

treated with 50 ng/ml purified *E. coli* lipopolysaccharide (055:B5) or *Porphyromonas gingivalis* lipopolysaccharide (ATCC 33211) (8), or with 5 ng/ml tumour necrosis factor-α (TNF-α) or interleukin-1β (R & D Systems, Minneapolis, MN, USA). Trypsinized cells were washed in PBS, fixed (in PBS containing 2% paraformaldehyde and 5% sucrose), permeabilized (for 4 min in PBS containing 0.5% Triton X-100), then washed five times in PBS. Cells were quenched (in PBS containing fresh 0.05% $NaBH_4$) and blocked (for 30 min in PBS containing 3.0 mg/ml BSA and 1 mg/ml glycine). Cultures were then incubated in the blocking solution with either anti-KGFR or anti-HGFR (Santa Cruz Biotechnology) for 1 h at room temperature. Samples were washed five times (in PBS containing 1 mg/ml BSA) and then incubated in wash solution with a 1 : 100 dilution of Alexa-488 (Molecular Probes, Eugene, OR, USA) secondary antibody and finally washed three times in PBS. Five groups of 10,000 cells were analyzed by flow cytometry at 488 nm. Samples stained only with secondary antibody were included to detect autofluorescence.

Results

Periodontal disease model

Daily application of lipopolysaccharide with protease induced histological changes consistent with the onset of periodontitis (Fig. 1A–C). Specifically, junctional epithelial proliferation apically along the root surface was significantly induced ($p < 0.001$) by 8 wk (Fig. 1C,D). Similarly, loss of alveolar bone was significantly increased ($p < 0.001$) at 8 wk only (Fig. 1C,D). Collectively, the significant increase in apical epithelial cell migration and alveolar bone loss at 8 wk are histological changes consistent with periodontitis and justify it being regarded as a model of periodontal disease.

Quantitative PCR and *in situ* RT–PCR

Epithelial-specific expression of the growth factor receptors KGFR, HGFR, EGFR and FGFR-1 was assayed using qPCR of cDNA synthesized from laser-microdissected pooled samples of junctional and pocket epithelium from control ($n = 3$) and disease ($n = 3$) samples, respectively (Fig. 2A). Quantitative RT–PCR is the most sensitive method for measuring gene expression levels, particularly of low-abundance mRNA, such as growth factor receptors. Monitoring the PCR amplification in real time permits calculation during the exponential phase, when none of the reagents are rate limiting and the reaction is most efficient. Quantification can be achieved using the relative expression software tool (REST©), which compares the relative expression ratio of the target genes based on PCR efficiencies and mean crossing points of test vs. control groups. Differences are normalized using a non-regulated reference gene, such as GAPDH (40). All four receptors were detected and, relative to standardized GAPDH expression, KGFR was the most strongly up-regulated in periodontitis tissues (×25.09). HGFR was more strongly expressed (×4.30) in control tissues than KGFR but was not as strongly induced in the periodontitis tissues (×9.92). EGFR and FGFR were detected in control tissues at approximately the same levels as KGFR, but did not reveal any significant induction in periodontitis samples. To ensure specificity of the amplified products, RNA was extracted from frozen sections, amplified using the same primers

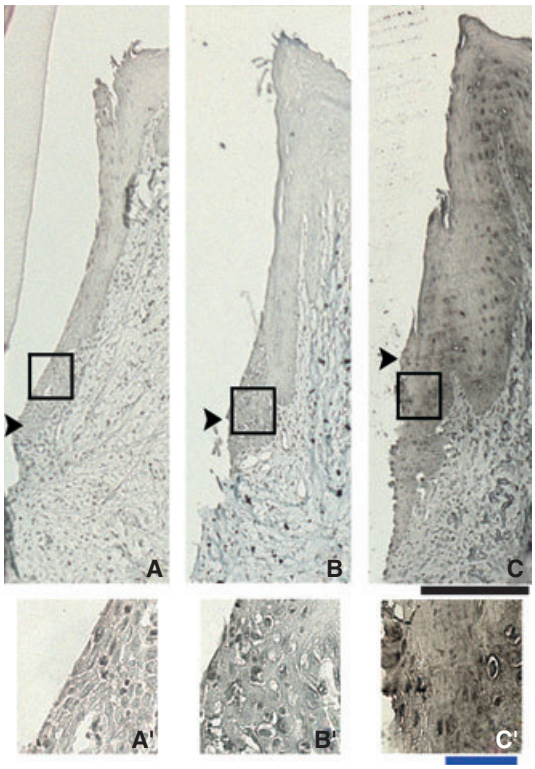

Fig. 3. Significant induction in keratinocyte growth factor receptor (KGFR) gene expression, using *in situ* reverse transcription–polymerase chain reaction (RT–PCR), was localized to basal and parabasal 8-wk pocket epithelium. Paraffin-embedded control (A), 2-wk (B) and 8-wk (C) tissue sections were processed for *in situ* RT–PCR using KGFR-specific primers. Representative tissue sections for each group are shown (A, B, C), with black boxes designating close-ups (A′, B′, C′, respectively). Weak KGFR expression was detected in the cytoplasm of the junctional epithelium (A′), with a minor increase in gene expression identified at 2 wk (B′). In contrast, pocket epithelium associated with 8-wk tissue samples clearly showed significant detectable KGFR gene expression localized to basal and parabasal pocket epithelial cells (C, C′). Arrowheads indicate the cemento–enamel junction. Scale bars: 200 μm (black) and 50 μm (blue).

(Table 1) with RT–PCR and the end products were analyzed by agarose gel electrophoresis. For each of the receptors, a single band of the correct predicted size was found (Fig. 2B).

To specifically localize the increase in KGFR and HGFR expression, *in situ* RT–PCR was utilized. Weak KGFR expression was detected in the time 0 control and 2-wk samples (Fig. 3A/A′,B/B′). In contrast, in diseased tissues, a significant increase was clearly evident in KGFR localized to basal and parabasal tissue associated with pocket epithelium (Fig. 3C/C′). In contrast, HGFR expression was detected at higher levels in control tissues (Fig. 4A/A′) with incremental increases in expression found at 2 and 8 wk (Fig. 4B/B′,C/C′). Moreover, in contrast to KGFR gene expression (Fig. 3C), HGFR gene expression (Fig. 4C) was clearly present through all the layers of the pocket epithelium. Thus, *in situ* RT–PCR was in agreement with qRT–PCR. Omission of DNase I pretreatment showed strong nuclear staining in all tissue types (data not shown). In contrast, omitting the RT step in DNase I-treated samples resulted in no detectable cellular staining (data not shown). Therefore, staining as shown was not associated with residual genomic DNA.

Immunohistochemical analysis of receptor protein expression

KGFR and HGFR protein expression in controls (Fig. 5A,D), 2-wk samples (Fig. 5B,E) and 8-wk periodontitis samples (Fig. 5C,F) were examined. Increased KGFR (Fig. 5A–C) and HGFR (Fig. 5D–F) protein expression from control to 8 wk was clearly seen. The relative increase in protein staining was quantified using Scion image software to calculate the number of pixels that exceeded background threshold values (41). KGFR protein expression in the 8-wk periodontitis samples was significantly increased ×11 over controls ($p < 0.01$) and ×6.6 over the 2-wk group ($p < 0.05$) (Fig. 6). The 8-wk periodontitis-associated HGFR protein expression was significantly increased ×2.8 over the controls ($p < 0.05$) (Fig. 6). Consistent with the *in situ* RT–PCR results (Figs 3C,4C), KGFR protein expression strongly localized in the basal and parabasal epithelial cells of pocket epithelium (Fig. 5C) while HGFR protein expression was strongly expressed in all cells of the pocket epithelium (Fig. 5F). EGFR and FGFR1 proteins were examined, but no significant differences between the time points were found (data not shown).

Regulation of KGFR and HGFR protein expression

To test whether receptor expression was directly induced by lipopolysaccharide or indirectly by disease-associated pro-inflammatory cytokines, KGFR and HGFR protein expression was measured *in vitro* using a porcine periodontal ligament epithelial (PLE) cell culture model. Quiescent cultures in a serum-free defined media were treated with either *E. coli* lipopolysaccharide or *P. gingivalis* lipopolysaccharide, TNF-α or interleukin-1β (proinflammatory cytokines), basal media (negative control) or 10% serum (positive control). Cells were collected, stained with receptor-specific antibodies and analyzed for relative receptor expression levels using flow cytometry. Relative to standardized control levels, KGFR was significantly up-regulated approximately threefold by *E. coli and P. gingivalis* lipopolysaccharide, slightly in excess of the FBS-positive controls (up-regulated ×2.7) (Fig. 7). Induction of KGFR expression by

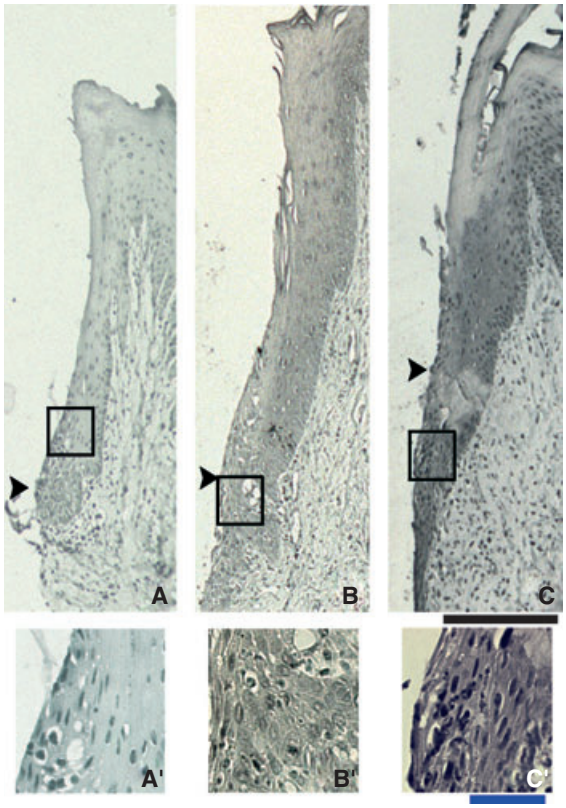

Fig. 4. Significant induction in hepatocyte growth factor receptor (HGFR) gene expression using *in situ* reverse transcription–polymerase chain reaction (RT–PCR) was localized to 8-wk pocket epithelium. Paraffin-embedded control (A), 2-wk (B) and 8-wk (C) tissue sections were processed for *in situ* RT–PCR using HGFR-specific primers. Representative tissue sections for each group are shown (A, B, C), with black boxes designating close-ups (A′, B′, C′, respectively). HGFR-positive expression was detected in the time 0 control junctional epithelium (A′) and increased at 2 wk (B′). In contrast, 8-wk pocket epithelium clearly showed significant detectable HGFR gene expression throughout the pocket epithelium (C, C′). Arrowheads indicate the cemento–enamel junction. Scale bars: 200 μm (black) and 50 μm (blue).

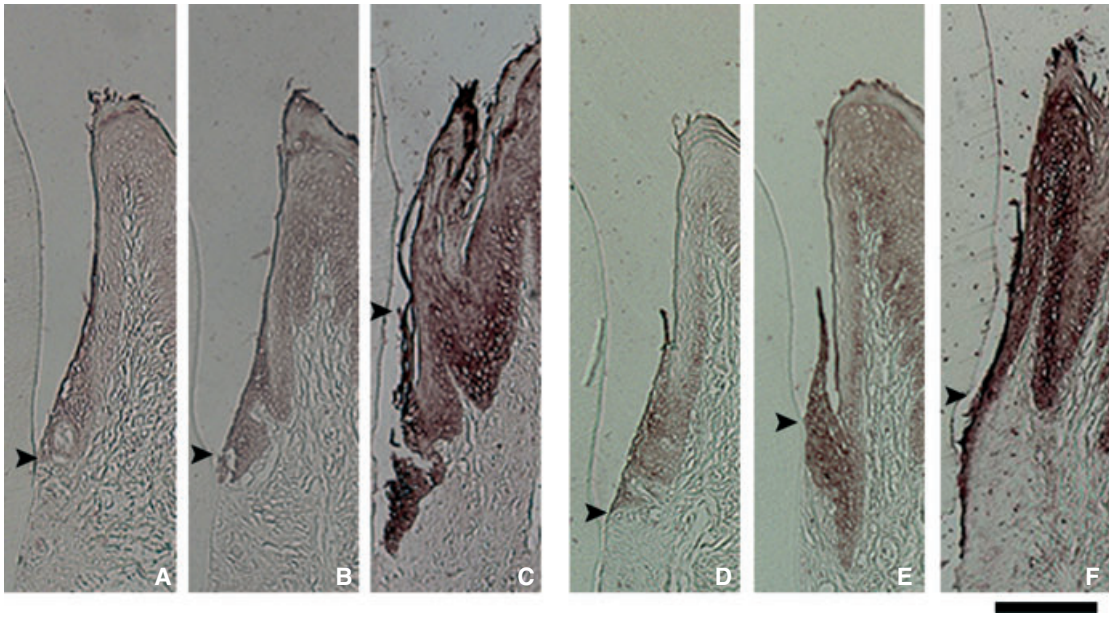

Fig. 5. Receptor-specific protein expression. Paraffin-embedded tissue sections from the control (A and D), 2-wk disease induction (B and E) and 8-wk disease induction (C and F) groups were stained with either anti-keratinocyte growth factor receptor (anti-KGFR)-specific immunoglobulin (A, B, C) or anti-hepatocyte growth factor receptor (HGFR)-specific immunoglobulin (D, E, F) . Expression was detected using an avidin–biotin complex detection method. KGFR (C) and HGFR (F) expression were both significantly increased with the onset of disease. Consistent with the *in situ* reverse transcription–polymerase chain reaction (RT–PCR) results, KGFR protein expression was most clearly associated with basal and parabasal pocket epithelium (C), while HGFR expression was prominently increased throughout the pocket epithelium (F). Arrowheads indicate the cemento–enamel junction. Scale bar, 200 μm.

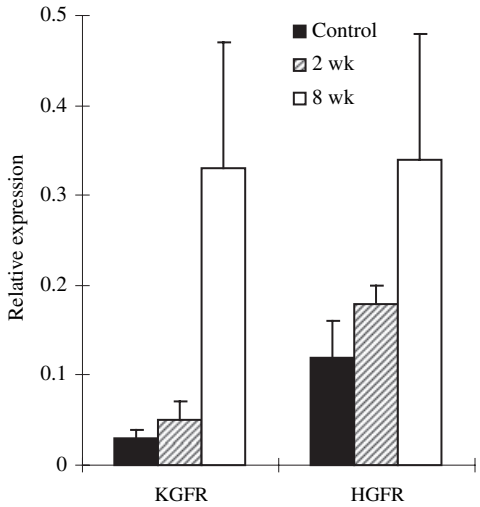

Fig. 6. Keratinocyte growth factor receptor (KGFR) and hepatocyte growth factor receptor (HGFR) protein expression significantly increased with disease onset. Paraffin-embedded tissue sections from each group ($n = 3$) were stained with receptor-specific antibodies, and the relative staining intensity levels were determined using SCION IMAGE software. Generally, staining was variable over the 2- and 8-wk experimental periods. KGFR expression in the 8-wk periodontitis group was significantly greater than in the control group ($p < 0.01$) and the 2-wk disease group ($p < 0.05$). HGFR was significantly increased over the controls ($p < 0.05$). Results are shown as mean ± standard deviation (SD) ($n = 3$).

both pro-inflammatory cytokines (TNF-α and interleukin-1β) were similar to FBS-induced expression levels. In sharp contrast, HGFR protein expression was induced to a lesser extent by FBS (×1.9) but no significant induction in response to lipopolysaccharide or pro-inflammatory cytokines was found. These data suggest that KGFR levels are regulated, at least in part, by a direct epithelial response to lipopolysaccharide as well as disease-associated pro-inflammatory cytokines.

Discussion

Using a periodontal disease model (32), significant bone loss and apical migration of the epithelial attachment was induced. These changes are consistent with periodontitis (1). Virulence factors used to induce disease were not purified from periodontal pathogens; however, they were equally effective at inducing histological changes consistent with periodontitis (32).

KGF-1, which is expressed primarily by fibroblasts, specifically stimulates epithelial cells via their KGFR (6). Previously, this receptor protein was localized to the junctional epithelium and was up-regulated in the pocket epithelium of advanced disease (19) but its regulation in relation to early

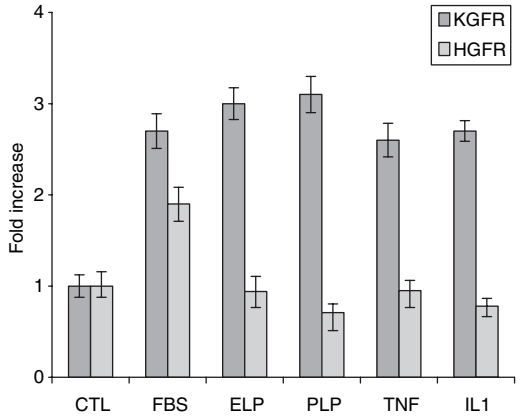

Fig. 7. Regulation of epithelial cell keratinocyte growth factor receptor (KGFR) and hepatocyte growth factor receptor (HGFR) protein expression *in vitro*. Porcine ligament epithelial cells were cultured to 70% confluence, quiesced in defined media and stimulated with 10% fetal bovine serum (FBS), 50 ng/ml *Escherichia coli* lipopolysaccharide (ELP), 50 ng/ml *Porphyromonas gingivalis* lipopolysaccharide (PLP), 5 ng/ml tumour necrosis factor-α (TNF) or 5 ng/ml interleukin-1β (IL1). After 24 h, the cells were trypsinized, washed, fixed and then stained with either anti-KGFR (KGFR) or anti-HGFR (HGFR) immunoglobulin and Alexa-488-labeled secondary immunoglobulin before analysis with flow cytometry ($n = 3$). Data are presented as fold increase over standardized time 0 controls (CTL). The FBS-positive control significantly induced expression of both KGFR and HGFR. However, only KGFR expression was significantly induced by both *E. coli* and *P. gingivalis* lipopolysaccharide and the proinflammatory cytokines, TNF-α and interleukin-1β.

periodontitis and other receptors was not determined. In this study, KGFR was expressed in health; however, gene (×25) and protein (×11) levels were dramatically up-regulated in disease. This KGFR receptor up-regulation may be explained by direct lipopolysaccharide or pro-inflammatory cytokine stimulation of epithelial cells (Fig. 7). This dramatic increase in KGFR expression would enable local epithelial cells to bind elevated KGF-1 ligand expressed by the connective tissue of chronically inflamed periodontal tissues (8,19,22).

As with KGF-1, hepatocyte growth factor is also a paracrine-mediating growth factor (7). HGF is expressed by a variety of oral tissue fibroblasts, and HGFR (c-Met) is expressed primarily by epithelial cells (7,9–13). In this study, HGFR, when compared with KGFR, was expressed at a higher level in healthy individuals, but the relative increase with disease was significantly less (×25 vs. ×10). The HGF concentration in saliva and gingival crevicular fluid are increased in periodontitis patients, and in cell culture, HGF was induced by *P. gingivalis* fimbriae and interleukin-1α (16–18,23). Therefore, an increase in HGFR expression would also enable epithelial cells to respond to increased HGF expressed during disease. In contrast to KGFR, regulation of HGFR expression is less clear. Purified lipopolysaccharide and pro-inflammatory cytokines did not induce HGFR expression, but serum did (Fig. 7). Other HGFR inducers, such as retinoic acid, might be involved (47).

In contrast to the above paracrine mediators, EGFR and FGFR1 expression were not associated with the early onset of disease. Within periodontal pocket epithelium, EGFR was not increased, in contrast to one publication identifying increased EGFR protein in diseased pocket epithelium (30). The fact that our model reflects early disease, while the other represents advanced disease, may explain this difference. FGFR1 is one of four FGFR described to date; it interacts with a wide variety of FGF ligands expressed by a variety of cells but not KGF-1 or -2 (6,27,28). However, FGFR1 was expressed in junctional and pocket epithelium, but was not induced in our model.

In situ RT–PCR effectively localized KGFR and HGFR expression and up-regulation in the epithelium. We have previously established that paraffin-embedded tissues provided excellent tissue morphology, and gene signals could be amplified and localized (34). Using this approach, we localized the epithelial cell KGFR and HGFR gene up-regulation, which had been separately measured using laser microdissection followed by qRT–PCR. Protein expression was also localized to the same respective areas. In disease samples, KGFR localized to basal and parabasal pocket epithelium, and HGFR was expressed throughout the entire pocket epithelium. The possible significance of this up-regulation warrants further study.

Lipopolysaccharide, applied daily to the rat gingival sulcus, was associated with increased basal and parabasal cell proliferation in the junctional and oral sulcular epithelium (31,48). In addition, the migration of epithelial cells along the root surfaces, and the loss of epithelial attachment, ultimately requires an increase in cell motility and the expression of matrix-degrading enzymes, such as metalloproteinases (2). KGF-1 and HGF are potent regulators of all of these processes (7,43,49–53). It is quite conceivable that KGFR and HGFR up-regulation, as shown in the present study, in conjunction with inflammatory up-regulation of connective tissue KGF-1 and HGF, may play a pivotal role in regulating the onset of these processes. In conclusion, we have shown, in an animal model, that two relatively epithelial-specific growth factor receptors – KGFR and HGFR – are significantly induced at the gene and protein levels in our periodontal disease model. The importance of these receptors, and their potential role in driving attachment loss through increased proliferation, migration and matrix degradation, is a critical question requiring further investigation.

Acknowledgements

We are grateful to Ingrid Ellis for editorial assistance in the final preparation of the manuscript. This work was funded by a grant from the Canadian Institutes of Health Research to Edward E. Putnins (#MOP-42371).

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