J Periodont Res 2008; 43: 290–304

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.01029.x

Down-regulation of epidermal growth factor receptor-dependent signaling by *Porphyromonas gingivalis* lipopolysaccharide in life-expanded human gingival fibroblasts

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Quinchia-Rios BH, Guerrero M, Abozeid S, Bainbridge B, Darveau R, Compton T, Bertics PJ. Down-regulation of epidermal growth factor receptor-dependent signaling by Porphyromonas gingivalis *lipopolysaccharide in life-expanded human gingival fibroblasts. J Periodont Res 2008; 43: 290–304. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard*

Background and Objective: Human gingival fibroblasts exhibit proliferative responses following epidermal growth factor exposure, which are thought to enhance periodontal regeneration in the absence of bacterial products such as lipopolysacharide. However, lipopolysaccharide challenge activates human gingival fibroblasts to release several inflammatory mediators that contribute to the immune response associated with periodontitis and attenuate wound repair. We tested the hypothesis that *Porphyromonas gingivalis* lipopolysaccharide-activated signaling pathways down-regulate epidermal growth factor receptor-dependent events.

Material and Methods: To study lipopolysaccharide/epidermal growth factor interactions in human gingival fibroblasts, we introduced the catalytic subunit of human telomerase into human gingival fibroblasts, thereby generating a more long-lived cellular model. These cells were characterized and evaluated for lipopolysaccharide/epidermal growth factor responsiveness and regulation of epidermal growth factor-dependent pathways.

Results: Comparison of human telomerase-transduced gingival fibroblasts with human gingival fibroblasts revealed that both cell lines exhibit a spindle-like morphology and express similar levels of epidermal growth factor receptor, CD14 and Toll-like receptors 2 and 4. Importantly, human telomerase-transduced gingival fibroblasts proliferation rates are increased 5–9 fold over human gingival fibroblasts and exhibit a longer life span in culture. In addition, human telomerase-transduced gingival fibroblasts and human gingival fibroblasts exhibit comparable profiles of mitogen-activated protein kinase kinase (extracellular signal-regulated kinase 1/2) activation upon epidermal growth factor or *P. gingivalis* lipopolysaccharide administration. Interestingly, treatment with *P. gingivalis* lipopolysaccharide leads to a down-regulation of epidermal growth

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Key words: epidermal growth factor; life-expanded gingival fibroblasts; mitogen-activated protein kinases; *Porphyromonas - gingivalis* lipopolysaccharide

Accepted for publication June 21, 2007

factor-dependent extracellular signal-regulated kinase 1/2, p38 and cyclic-AMP response element binding protein phosphorylation in both cell types.

Conclusion: These studies demonstrate that human telomerase-transduced gingival fibroblasts exhibit an extended life span and recapitulate human gingival fibroblasts biology. Moreover, this system has allowed for the first demonstration of lipopolysaccharide down-regulation of epidermal growth factor activated pathways in human gingival fibroblasts and should facilitate the analysis of signaling events relevant to the pathogenesis and treatment of periodontitis.

One of the most abundant cellular components of the periodontum (the conjunction of all tissues supporting the teeth) are fibroblasts, which are responsible for the production and degradation of collagen fibers that sustain the structure of the gums and periodontal ligament (1,2). Primary human gingival fibroblasts are used for the study of periodontitis, gingival overgrowth, viral and fungal infections, and several other oral pathologies, including cancer (3–6). Primary human gingival fibroblasts can be obtained by biopsy from healthy individuals during extractions prescribed for orthodontic treatment or from patients with specific conditions such as gingival hyperplasia or diabetes mellitus (7,8). Unfortunately, within 16–20 passages the cells begin to differentiate and their proliferation rate decreases (2,9–12). Because of these features, along with patient-to-patient variation, the use of primary gingival fibroblasts imposes many challenges for the uniform collection of data and for the development of experimental approaches that require prolonged incubation times and/or multiple stimulations.

Aggressive periodontal diseases, such as rapidly progressive periodontitis, are characterized by the presence of highly pathogenic bacteria, such as *Porphyromonas gingivalis*, and the rapid loss of periodontal tissues, including the alveolar bone and periodontal ligament (1,13). Previous reports have shown that the regenerative responses of gingival fibroblasts to growth factors, such as epidermal growth factor, as well as their susceptibility to bacterial toxins, such as lipopolysaccharide, are important determinants of the wound healing processes associated with periodontal regeneration (1,14–16). These studies have also shown that the epidermal growth factor-induced proliferative response of gingival fibroblasts is diminished in the presence of lipopolysaccharide and can be efficiently initiated once the levels of periodontopathogenic bacteria and their products have been removed or reduced (1,15,17,18). Therefore, it has been proposed that the removal of pathogenic factors is imperative for favoring fibroblast-proliferative responses, which subsequently restores the balance of cell death/survival towards regeneration of the periodontum (1).

Gingival fibroblasts respond to bacterial or host-derived factors present during bacterial invasion by producing inflammatory mediators that aid in the immune response (4,14,19–21). In this regard, gingival fibroblasts can detect bacterial invasion through the action of lipopolysaccharide-binding molecules expressed on the cell surface, such as Toll-like receptors 2 and 4, and CD14 (14,20,22–28). Engagement of these lipopolysaccharide-binding molecules stimulates the mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases (ERK)1/2 and p38, and promotes the release of inflammatory mediators, the activation of immune and epithelial cells, the metabolism of collagen fibers, and the resorption of bone (4,16,29–31). These effects, among others, contribute to the pathogenesis associated with rapidly progressive periodontitis.

Gingival fibroblasts also exhibit proliferative responses to epidermal growth factor, which is known to be released by the gingival epithelium (32). Several epidermal growth factor receptor-dependent biological functions, such as proliferation and migration, depend on the initiation of signaling cascades, including MAPK pathways, and the regulation of transcription factors, such as the cyclic-AMP response element binding protein (CREB) (33). Although epidermal growth factor- induced processes have not been well characterized in human gingival fibroblasts, epidermal growth factor receptor ligation in gingival fibroblasts probably induces phosphorylation of the MAPKs ERK1 and ERK2, as these events have been observed in other fibroblasts (34). Interestingly, lipopolysaccharide has been shown to decrease the proliferative rates and migration of gingival fibroblasts (17,35). However, the potential cross-talk between epidermal growth factor and lipopolysaccharide-activated pathways has not been explored. In the present report, we test the hypothesis that *P. gingivalis* lipopolysaccharide-stimulated events down-regulate epidermal growth factor receptor-dependent MAPK activation and phosphorylation of CREB.

Considering the limitations associated with the use of primary gingival fibroblasts, the present studies also focused on generating a stable cell line of human gingival fibroblasts that exhibits improved growth rates and longevity in cell culture in an attempt to test the idea that lipopolysaccharide can modulate epidermal growth factor signaling in these cells. In this regard, because it has been shown that increased telomerase activity can attenuate cell senescence (36), we introduced the catalytic subunit of human telomerase into primary human

gingival fibroblasts to increase their life span in tissue culture and yet preserve their morphological and functional characteristics. In addition, the human telomerase-transduced human gingival fibroblasts serve as a useful model system to test the hypothesis that lipopolysaccharide from the clinically relevant bacteria, *P. gingivalis*, attenuates epidermal growth factor-initiated MAPK and CREB phosphorylation/activation in human gingival fibroblasts.

Material and methods

Materials and tissue culture reagents

Dulbecco's modified Eagle's medium, RPMI medium, penicillin, streptomycin, amphotericin B and sodium pyruvate were purchased from Cellgro Mediatech Inc. (Herndon, VA, USA). Fetal bovine serum, cosmic calf serum and HyQ-CCM5 serum-free medium were purchased from HyClone (Logan, UT, USA). Antibiotic selections of transduced cells were performed using puromycin dihydrochloride from *Streptomyces alboniger* purchased from Sigma-Aldrich (St Louis, MO, USA). *P. gingivalis* (strain 33277) lipopolysaccharide was prepared and repurified (purity: < 0.001% contaminating protein, as determined by the colloidal gold protein stain), as previously described (37). Human recombinant epidermal growth factor was purchased from Upstate (Lake Placid, NY, USA). Human peripheral blood monocytes and eosinophils were separated using reagents from the following sources: Percoll from Sigma-Aldrich; leukocyte separation medium from Cellgro Mediatech Inc.; RosetteSep human monocyte enrichment cocktail from StemCell Technologies Inc. (Vancouver, BC, Canada); and anti-CD16 magnetic bead labeling from Miltenyi Biotech (Auburn, CA, USA). All other chemical reagents used in these experiments were purchased from Sigma-Aldrich.

Cell culture

Human gingival fibroblasts were grown on T-75 vented tissue culture flasks and all other cell lines were grown on 10-cm tissue-culture-treated dishes purchased from Sarstedt (Newton, NC, USA) unless otherwise indicated. Primary human gingival fibroblasts were obtained from the American Type Culture Collection (ATCC) (Hanassas, VA, USA) (CRL-2014, designated human gingival fibroblast-1 (HGF-1) at passage 10) and grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B and 1 mM sodium pyruvate. This medium was used to maintain human telomerase-transduced gingival fibroblasts and normal human dermal fibroblasts (CC2509), which were obtained from Clonetics Corp. (San Diego, CA, USA). The medium was changed every 2 or 3 d and the cells were split at 80% confluency at a 1 : 3 ratio for human gingival fibroblast-1 cells, a 1 : 6 ratio for human telomerase-transduced gingival fibroblasts, or a 1 : 4 ratio for normal human dermal fibroblasts, using 0.1% trypsin (Cellgro Mediatech). The human telomerase-transduced gingival fibroblasts were also grown in the presence of puromycin after transduction, as detailed below under 'Transduction of human telomerase into human gingival fibroblast-1'. The human epidermoid carcinoma cell line, A431, was obtained from the ATCC (CRL-1555) and grown in Dulbecco's modified Eagle's medium containing 10% cosmic calf serum, 100 IU/mL of penicillin, 100 μg/mL of streptomycin and 1 mM sodium pyruvate. The RAW 264.7 murine macrophages were purchased from the ATCC (TIB-71) and grown in RPMI containing 5% cosmic calf serum, 100 IU/mL of penicillin, 100 μg/mL of streptomycin and 1 mM sodium pyruvate. The Toll-like receptor 4-transfected human embryonic kidney cells (HEK) cells were generated as previously described (38) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% cosmic calf serum. Human eosinophils and blood monocytes were obtained from blood donors, as previously outlined (39,40), at the University of Wisconsin Hospital by the Allergy Research Laboratory. The experimental protocol was approved by the Human Subjects Committee of the University of Wisconsin Hospital (Madison, WI).

Transduction of human telomerase into human gingival fibroblast-1 cells

Primary human gingival fibroblasts were infected with a nonreplicative retroviral vector (Moloney murine leukemia virus) containing human telomerase and a puromycin-resistant gene under the control of Moloney murine leukemia virus 5′ long-terminal repeat and Simian virus-40 early promoters (pBABE-human telomerase vector), as described previously (41). Briefly, 60% confluent human gingival fibroblast-1 cells (5×10^5 cells/flask) in a T-75 tissue culture flask were incubated in 2 mL of a solution containing the retroviral vector (multiplicity of infection = 3), and 8 μg/mL of polybrene in serum-free Dulbecco's modified Eagle's medium. The cells were incubated and rocked gently for 2 h at 4°C to facilitate viral adsorption. Next, the viral supernatant was removed and the cells were washed with phosphate-buffered saline (pH 7.4). After this wash, the cells were incubated in a humidified 5% CO_2 atmosphere at 37°C in 10 mL of fresh medium containing 10% fetal bovine serum. The medium was changed every 24 h until the cells reached confluency and were then split at a 1 : 3 ratio. The life-expanded cell line obtained was labeled human telomerase-transduced gingival fibroblasts (QHGF) because they grow more 'quickly' than primary human gingival fibroblasts.

Antibiotic selection of human telomerase-transduced human gingival fibroblast-1 cells

To test human gingival fibroblast-1 survival to puromycin, a dose–response experiment ranging from 0.1 to 50 μg/mL of puromycin was performed. The optimum concentration used for antibiotic selection was the lowest concentration that exhibited a 100% cytotoxic effect within 48 h. According to these results, infected cells

were selected and maintained in 1 μg/mL of puromycin-containing medium (Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate and 0.25 μg/mL of amphotericin B). During antibiotic selection, the medium was changed daily for 7 d.

Telomerase activity assay

To determine telomerase activity of human gingival fibroblast-1 cells and human telomerase-transduced gingival fibroblasts, we used a polymerase chain reaction (PCR)-based telomerase detection assay [telomeric repeat amplification protocol (42)], using the TRAPEZE telomerase detection kit (Chemicon International Inc., Temecula, CA, USA) according to the manufacturer's instructions. Briefly, nuclear extracts were obtained from each sample (1.5 μg of protein) and divided into two aliquots, one of which was heat inactivated at 80°C for 10 min. Next, telomerase template and primers were added to each sample to allow telomere extension for 30 min at 30°C. Telomerase products and an internal PCR control (36 bp) were amplified using the following cycling parameters: 30 s at 94°C, 30 s at 54°C, 60 s at 70°C, for 32 cycles, and a final extension for 10 min at 72°C in an MJ Research MiniCycler (Waltham, MA, USA). Telomerase products were separated by nondenaturing polyacrylamide gel electrophoresis and then stained with 50 μg/mL of ethidium bromide. Telomerase activity was quantified by determining the optical density of the telomere ladder in digital images of gels, which were obtained using the Epi Chemi II Darkroom (UVP Inc., Upland, CA, USA) and the NIH licensed software, online resource available at http://rsb.info.nih.gov.ij). Telomerase activity was normalized to the internal PCR control products and was quantified as total product generated (TPG) using the following equation:

$$\text{TPG units} = \frac{(X_{OD-HI}X_{OD})/{}_{X}C_{OD}}{(\text{TSR8}_{OD} - \text{NT}_{OD})/{}_{\text{TSR8}}\text{C}_{OD}} \times 100$$

where 1 TPG unit represents 1×10^{-3} amol ($\approx$ 600 molecules) of telomere primers extended with at least four telomeric repeats by the telomerase present in the extract following a 30-min incubation; X_{OD} is the experimental sample optical density; ${}_{HI}X_{OD}$ is the heat-inactivated sample optical density; ${}_{X}C_{OD}$ is the experimental sample PCR control optical density; TSR8_{OD} is the quantitation control template optical density; NT_{OD} is the primer dimers/PCR contamination control optical density; and ${}_{\text{TSR8}}C_{OD}$ is the TSR8 PCR control optical density. The linear range of the assay was between 1 and 10,000 TPG units (42). Estimation of processivity: the presence of *n* bands on the gel indicates that the largest telomerase-extended products have $n + 3$ telomeric repeats. This is a measurement of the processivity of the telomerase [i.e. the amount of nucleotides extended before it dissociates from its substrate (43)].

Immunoblotting

Cells were incubated overnight in six-well plates and the following day were stimulated or starved according to each experimental design. At the indicated time points, cells were lysed on ice using lysis buffer, as described for alkaline phosphatase activity. Protein concentration was quantified using the Micro BCA protein assay (Pierce, Rockford, IL, USA), and equivalent levels of protein per sample (50 μg of protein per sample) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and immunoblotted using anti-vimentin clone V9 (1 : 200 dilution; Sigma-Aldrich), anti-cytokeratin 14 clone LL002 (1 μg/mL; Neomarkers/Laboratory Vision Corporation, Fremont, CA, USA), anti-phospho-ERK1/2 pTpY (185/187) (detectable at 44 and 42 kDa, respectively, 1 : 1000 dilution; Biosource International, Camarillo, CA, USA), anti-phospho-p38 pTpY (180/182) (1 : 1000 dilution; Cell Signaling Technology, Inc., Beverly, MA, USA), anti-phospho-cyclic-AMP response element binding protein Ser133 (1 : 1000 dilution; Cell Signaling Technology, Inc.) anti-epidermal growth factor receptor (sc-03, 0.4 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD14 (M-305, 0.2 μg/mL; Santa Cruz Biotechnology), anti-Toll-like receptor-4 (H-80, 0.2 μg/mL; Santa Cruz Biotechnology) and the corresponding horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology). After antibody incubation the peroxidase substrate was added (SuperSignal West Dura Extended Duration Substrate; Pierce Biotechnology Inc., Rockford, IL, USA) and luminescence was captured using the UVP Biochemi-luminescence System (UVP Inc.). Immunoblotting against ERKs 1 and 2 or vimentin was used to generate loading controls for normalizing the signal obtained from the phospho-specific antibodies. Optical densities of the bands were measured using IMAGEJ NIH software, normalized to the loading controls, and the fold increase over the control value was calculated by dividing the normalized phosphoprotein value obtained for each sample by the normalized control value (time zero or untreated sample).

Alkaline phosphatase activity assay

The cell types used in this assay, human telomerase-transduced gingival fibroblasts, human gingival fibroblast-1, normal human dermal fibroblasts and RAW 264.7, were plated at a cell density of 1×10^3 cells/well in 96-well plates, in Dulbecco's modified Eagle's medium containing 10% heat-inactivated-fetal bovine serum for the fibroblast cell lines and RPMI containing 5% cosmic calf serum for the RAW 264.7 macrophages. The cells were incubated overnight, after which the medium was replaced by HYQ serum-free medium (HyClone) containing vehicle or 150 μM ascorbic acid (vitamin C) for 48 h. After ascorbate stimulation, alkaline phosphatase activity was measured as described by Puzas *et al.* (44). The medium was removed, cells were washed with Hank's balanced salt solution, and alkaline phosphatase activity was tested by incubating the cells for

15 min with 5 mM *p*-nitrophenyl phosphate (*p*NPP) as substrate, in 90 μL of assay buffer (0.25 M 2-amino-2-methyl-1-propanol and 2 mM $MgCl_2$, pH 10.1) at 37°C. The reaction was stopped with 10 μL of 2.5 M NaOH, incubated at room temperature for 10 min and the supernatants were transferred to a 96-well plate to quantify *p*-nitrophenol levels by reading the absorbance at 405 nm. Following the removal of the supernatant, the cells were lysed with 30 μL of lysis buffer (2% sodium dodecyl sulfate, 20 mM Tris, pH 8.0, 2 mM EDTA, 1 mM $NaVO_4$, 2 mM dithiothreitol, 20% glycerol) and the protein concentration of the cell lysates was quantified using the Micro BCA protein assay (Pierce). Next, alkaline phosphatase activity was normalized to protein concentration per sample.

Cell proliferation assay

The cells used in these assays were taken from passages 10–12 for the human gingival fibroblasts and from passages 30–40 for the human telomerase-transduced gingival fibroblasts; neither cell population exhibited morphological signs of differentiation. For these experiments, the cells were plated at either 5×10^3 (low cell density) or 1×10^4 (subconfluent monolayer) cells/well in 48-well tissue culture plates (Sarstedt) and incubated in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum. The cell cultures were then synchronized by incubation for 24 h in Dulbecco's modified Eagle's medium containing 1% heat-inactivated fetal bovine serum. At 48 h after plating, the medium were replaced with Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (growing medium) and the number of viable cells was determined every 24 h using the survival/cell proliferation assay Celltiter 96 Aqueous (Promega, Madison, WI, USA). Briefly, at various time points, the medium was aspirated and replaced with 100 μL of fresh medium and 20 μL of 3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS/PMS). The cells were incubated for 1 h at 37°C. During this incubation, the tetrazolium compound is transformed by the mitochondria to soluble formazan. The amount of formazan produced is proportional to the number of cells and was quantified by aliquoting 100 μL of each reaction into a 96-well plate and then determining the absorbance at 490 nm using an ELx800 Universal ELISA Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Two different cell concentrations (10^4 cells/cm^2 and 5×10^3 cells/cm^2) were used within an individual assay to assess the influence of cell density upon proliferation rates. To estimate the proliferation rate for both human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells at each cell density, we calculated the slopes of the overall change on supernatant absorbance throughout the duration of the experiment (by linear regression of the averages at each time point) using Microsoft Excel. Statistical analyses were performed using the Student's *t*-test to compare the differences in overall proliferation rates between human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells at each cell density.

Light microscopy

For morphological analysis, human telomerase-transduced gingival fibroblasts (passage 40) and human gingival fibroblast-1 cells (passage 10) were plated on glass coverslips placed at the bottom of 6-well plates (4×10^4 cells/cm^2). Twenty-four hours after plating, the cells were fixed with formaldehyde-free fixative, Prefer, obtained from Anatech Ltd. (Battle Creek, MI, USA) and then stained with Harris modified hematoxylin and Eosin-Y counterstain (Richard-Allan Scientific, Kalamazoo, MI, USA). Images of the cells were obtained using an Olympus (Leeds Precision Instruments, Minneapolis, MN, USA) BH-2 light microscope at 10× magnification, with a Photometric Coolsnap digital camera from Roper Scientific (Tucson, AZ, USA) and IMAGE-PRO PLUS (Media Cybernetics Inc., Silver Spring, MD, USA) software.

Flow cytometry

Flow cytometric analysis of cell-surface proteins was performed using 5×10^6 cells/sample suspended in 500 μL of Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin (Sigma). Cells were incubated at 4°C with the specified primary antibodies against epidermal growth factor receptor, Toll-like receptor 2 or CD14, for 30 min, as follows: 1 μg/mL of antibody 528 against epidermal growth factor receptor (clone sc-120; Santa Cruz Biotechnology); 1 μg/mL of anti-CD14 (UCH-M1; Santa Cruz Biotechnology); 5 μg/mL of anti-Toll-like receptor 2 (clone TL2.1; Cascade BioScience, Winchester, MA, USA), or the same concentration of isotype control (mouse IgG2A M-5409; Sigma-Aldrich for all antibodies). The cells were washed three times with phosphate-buffered saline and then incubated with 1 μg/mL of either fluorescein isothiocyanate- or R-Phycoerythrin-labeled secondary antibody (Molecular Probes Inc., Eugene, OR, USA) for 30 min at 4°C. The cells were resuspended in 0.1% bovine serum albumin-supplemented Dulbecco's modified Eagle's medium containing 50 μg/mL of propidium iodide (Sigma-Aldrich) to exclude dead cells from the flow cytometry analysis (FACScan flow cytometer; Becton Dickinson, Mountain View, CA, USA). Previous reports have shown that A431 cells express an average of 2×10^6 epidermal growth factor receptors on the cell surface that can be detected by the anti-epidermal growth factor receptor antibody, 528, and these results were validated using an ^{125}I-radiolabeled epidermal growth factor binding assay (45). Therefore, a comparison of epidermal growth factor receptor expression between human gingival fibroblast-1 cells or human telomerase-transduced gingival fibroblasts with A431 cells, as detected with antibody 528, was used to estimate the relative number of receptors expressed on the cell surface of the gingival fibroblasts. Data analysis was performed using

WINMDI 2.8 (NIH licensed software) to obtain histograms of 10,000 live events that were plotted (abscissa) vs. fluorescence (ordinate) and the geometric mean for each histogram was calculated. The geometric means of six experiments were combined using Microsoft Excel to estimate the amount of epidermal growth factor receptor on the cell surface, as described previously (45,46).

Results

Generation of human telomerase-transduced gingival fibroblasts from human gingival fibroblast-1 cells

Because human gingival fibroblasts exhibit proliferative responses following exposure to epidermal growth factor, and lipopolysaccharide challenge activates human gingival fibroblasts to release several mediators that contribute to the inflammatory response associated with periodontitis, we first sought to generate a gingival fibroblast cell line that possessed a more stable phenotype in culture. Recent studies have shown that cells maintaining telomerase activity continue to undergo mitosis, thereby delaying the time at which the cells reach senescence (36,47). Furthermore, the loss of telomerase activity has been directly related to a decrease in cell proliferation rates (48,49). In these assays, the length and the intensity of the DNA ladder produced by each sample was representative of telomerase activity and processivity. The total product generated is a concentration measurement (1 TPG unit $= 1 \times 10^{-3}$ amol or ≈ 600 molecules) of amplified fragments (with at least four telomeric repeats) produced from the given substrate (42). The bands present in each ladder are the different size products extended by the telomerase. A longer ladder will have longer telomeric repeats. Therefore, the number of telomeric repeats indicates the capacity of the enzyme to extend its substrate before disengaging (processivity of the enzyme). A higher number of bands is also indicative of greater telomerase activity. Telomerase extracted from human telomerase-transduced gingival fibroblasts produced an average TPG of 940 units ($\pm$ 90 standard deviation) and 17 ($\pm$ 3 standard deviation) telomeric repeats, which indicated an approximately fourfold increased telomerase activity over human gingival fibroblast-1 cells [average TPG = 210 units ($\pm$ 60 standard deviation) and 8 ($\pm$ 2 standard deviation) telomeric repeats]. No detectable product was found when the telomerase extracts were heat inactivated or in the control reaction with no telomerase added (Fig. 1A). These results are consistent with the idea that telomerase is expressed in both human gingival fibroblast-1 cells

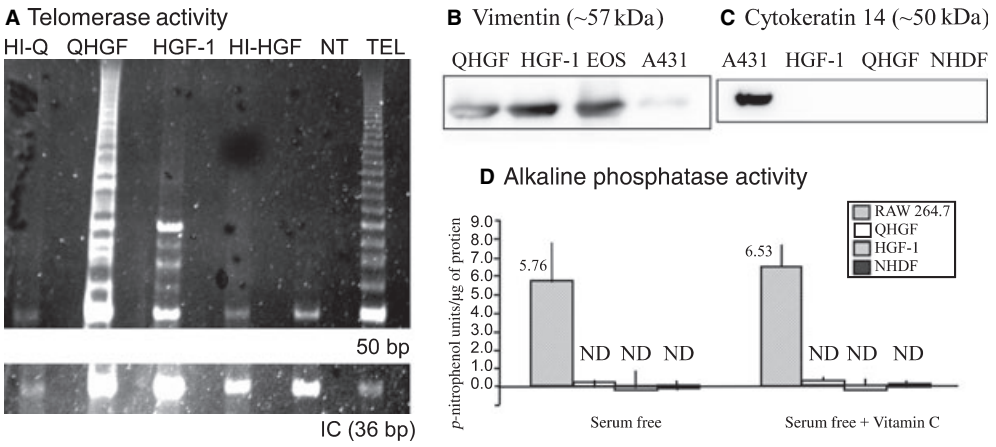

Fig. 1. Telomerase activity in human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells using a telomeric repeat amplification protocol assay and the characterization of fibroblastic markers. (A) The upper panel displays the ladder produced by amplification of a 50-bp telomere template by the telomerase present in the different extracts. HI-Q, heat-inactivated human telomerase-transduced gingival fibroblasts; HI-HGF, heat-inactivated human gingival fibroblast-1 cells. QHGF, HGF-1 and TEL represent human telomerase-transduced gingival fibroblast-, human gingival fibroblast-1- and human telomerase-containing cell extracts; NT, no-telomerase template control (CHAPS buffer). The bottom panel shows a 36-bp polymerase chain reaction amplification product internal control. One representative result out of three separate assays is shown. (B) Human telomerase-transduced gingival fibroblasts, human gingival fibroblast-1 cells, eosinophils and A431 cell lysates were subjected to electrophoresis and immunoblotted using an anti-human vimentin immunoglobulin (reactive with a 57-kDa protein) as a marker for fibroblasts and mesenchymal-derived cells. (C) Human gingival fibroblast-1 cells, human telomerase-transduced gingival fibroblasts and normal human dermal fibroblasts were immunoblotted for cytokeratin 14 (50 kDa), as an epithelial cell marker. (D) RAW 264.7 macrophages, human telomerase-transduced gingival fibroblasts, human gingival fibroblast-1 cells and normal human dermal fibroblast cells were tested for alkaline phosphatase activity (measured as units of *p*-nitrophenol produced/μg of protein; ND, nondetectable) in the absence of serum and with or without vitamin C added for 48 h. The numbers next to the bars are the average values $\pm$ standard deviation obtained from triplicate samples. The bar plot was produced using Microsoft Excel and similar results were obtained in three separate experiments. EOS, eosinophils; HGF-1, human gingival fibroblast-1 cells; IC, polymerase chain reaction amplification product internal control; NHDF, normal human dermal fibroblasts; NT, no telomerase; TEL, human telomerase; QHGF, human telomerase-transduced gingival fibroblasts.

and human telomerase-transduced gingival fibroblasts; however, telomerase activity was enhanced in the human telomerase-transduced gingival fibroblasts.

Expression of fibroblast markers

Human gingival fibroblast-1 are primary cells deposited in the ATCC collection and are obtained from gum tissue that contains multiple cell types. Accordingly, to generate a suitable cell line, it was first necessary to characterize/confirm the identity and characteristics of these cells.

Gingival fibroblasts can be distinguished from epithelial cells, the most abundant contaminant from the original tissue explants, by the expression of mesenchymal markers. Vimentin, an intermediate filament protein, is not normally present on cells from ectodermic origin (such as epithelial cells) (50), whereas the ectodermic marker protein, cytokeratin 14, is not normally expressed in mesenchymal cells, including fibroblasts (51). Accordingly, we tested human gingival fibroblast-1 cells and human telomerase-transduced gingival fibroblasts for the expression of vimentin by immunoblotting. As shown in Fig. 1B, vimentin was detected on cell extracts from human blood eosinophils (mesenchymal origin) and human telomerase-transduced gingival fibroblasts, as well as on cell extracts from human gingival fibroblast-1 cells. As expected, vimentin was not detectable on human epidermoid carcinoma cells (A431). To determine if there were any remaining epithelial cells, the gingival fibroblast cell extracts were immunoblotted for cytokeratin 14. As seen in Fig. 1C, cytokeratin 14 was not detectable in normal human dermal fibroblasts, human gingival fibroblast-1 cells or human telomerase-transduced gingival fibroblasts, but was detected in the human epidermoid carcinoma cell line A431. The presence of vimentin and the absence of cytokeratin 14 in the human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells are consistent with the embryological origin of gingival fibroblasts.

Alkaline phosphatase activity

Alkaline phosphatase activity assays have been used routinely to distinguish between functionally differentiated and nondifferentiated gingival fibroblasts (52). This test is based on the observation that gingival fibroblasts express little alkaline phosphatase activity unless they have acquired a different functional phenotype, under specific host or *in vitro* conditions, such as those associated with osteoclastic-like cells when stimulated with vitamin C (53,54). Thus, an increase in alkaline phosphatase activity indicates that the cells have started to differentiate. To assess if the human telomerase-transduced gingival fibroblasts were functionally differentiated by the process of telomerase transduction and extension of their life span, we tested human gingival fibroblast-1 and human telomerase-transduced gingival fibroblasts for alkaline phosphatase activity following 48 h of stimulation with or without vitamin C (150 μM ascorbic acid). A murine macrophage cell line (RAW 264.7), which possesses alkaline phosphatase activity, was used as a positive control (55). Alkaline phosphatase activity was detected in the RAW 264.7 macrophages, but not in the gingival fibroblasts or dermal fibroblasts (Fig. 1D), even under vitamin C stimulation. These data support the concept that the life-expanded human telomerase-transduced gingival fibroblasts remain functionally undifferentiated and have a phenotype comparable to early passages of their primary counterparts (53,54).

Proliferative profile of human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells

Fibroblast growth and differentiation has been shown to be dependent on cell-to-cell contact (56,57), which, in cell culture, is in part determined by the density of cell plating (cells/area). Previous reports indicate that primary fibroblasts require subconfluent densities (less than 80% of the available surface occupied by adherent cells) in order to proliferate at optimal rates (58,59). To characterize the proliferative profile of human gingival fibroblasts, human telomerase-transduced gingival fibroblasts were grown in parallel with human gingival fibroblast-1 at two different densities: 5×10^3 and 1×10^4 cells/cm^2. As shown in Fig. 2, at time zero, human

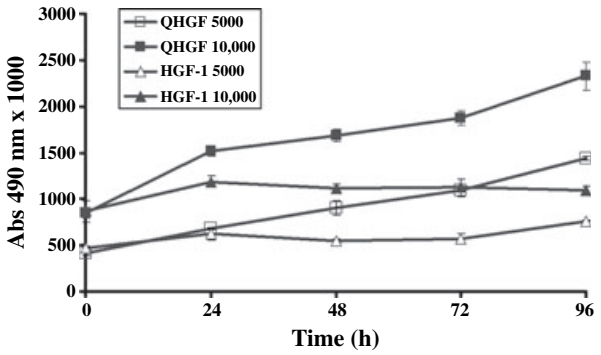

Fig. 2. Proliferation curves for human telomerase-transduced gingival fibroblasts (QHGF) and human gingival fibroblast-1 (HGF-1) cells plated at two different cell densities (5×10^3 or 1×10^4 cells/cm^2) for the stipulated periods of time, as described in the Material and methods. The absorbance of the medium containing MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, which correlates with cell number] was measured at 490 nm, multiplied by 1000 and plotted in the *y* axis against time in the *x* axis. The growth curves described with standard deviations (error bars, $n = 5$) were generated in Microsoft Excel. Slopes were measured by linear regression analysis: human telomerase-transduced gingival fibroblasts, 5000 = 10.2; human telomerase-transduced gingival fibroblasts, 10 000 = 13.8; human gingival fibroblast-1 cells, 5000 = 2.2; human gingival fibroblast-1 cells, 10,000 = 1.6 ($p = 0.02$ at 5×10^3 cells/cm^2; $p = 0.04$ at 1×10^4 cells/cm^2). One representative experiment with five replicates out of three separate assays is shown.

telomerase-transduced gingival fibroblasts, as well as human gingival fibroblast-1 cells, exhibited similar absorbance readings. Over time, human telomerase-transduced gingival fibroblasts continued to grow and displayed a five- to sevenfold increased growth rate (calculated on the slopes), at both cell densities, when compared with human gingival fibroblast-1 cells ($p = 0.02$ at 5×10^3 cells/cm^2, $p = 0.04$ at 1×10^4 cells/cm^2). The increase in MTS activity of the cells at each 24-h time-point was overall higher for human telomerase-transduced gingival fibroblasts, indicating a higher mitotic activity than human gingival fibroblast-1 cells. Generally, human gingival fibroblast-1 cells decreased their growth rate after 48 h in growing medium at both cell densities, and human telomerase-transduced gingival fibroblasts displayed a more consistent profile of cell proliferation that translated into a more efficient yield of new cells.

Cellular morphology

One important phenotypic marker of gingival fibroblasts is their spindle-like morphology (60–62). In this regard, the human telomerase-transduced gingival fibroblasts were monitored for more than 40 passages using a light microscope to assess morphological changes. As illustrated in Fig. 3, bright-field micrographs with 10× magnification of human gingival fibroblast-1 and human telomerase-transduced gingival fibroblast monolayers at subconfluent densities, stained with hematoxylin/eosin for increased contrast, demonstrated that human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells have a similar morphology and both exhibit the spindle-like elongated shape that is characteristic of fibroblastic monolayers.

Expression of epidermal growth factor and lipopolysaccharide receptors

Expression of the epidermal growth factor receptor remains stable in healthy tissues and is regulated during various proliferative disorders and inflammatory conditions of the periodontal tissues (3,63–66). Because the coordinate actions of epidermal growth factor and lipopolysaccharide on human gingival fibroblast behavior are likely to be central to the molecular mechanisms of rapidly progressive periodontitis, information on epidermal growth factor receptor expression in gingival fibroblasts is an important parameter for evaluating normal cell function. Previous studies have shown that epidermal growth factor receptor expression in normal gingival fibroblasts ranges between 150,000 and 170,000 epidermal growth factor receptors per cell (65). As shown in Fig. 4A, comparative fluorescence-activated cell sorter analysis of epidermal growth factor receptor cell-surface expression using A431 cells as a standard [i.e. they express an average of 2×10^6 epidermal growth factor receptors per cell on their surface (45)] revealed that the starting population of human gingival fibroblast-1 cells expressed an estimated average of 180,000 (± 5000 standard deviation) epidermal growth factor receptors per cell. Similarly, human telomerase-transduced gingival fibroblasts expressed an estimated average of 170,000 (± 20,000 standard deviation) epidermal growth factor receptors per cell. In contrast, fibroblasts from other connective tissues express approximately 1×10^6 epidermal growth factor receptors per cell (46). As illustrated in Fig. 4B, comparable levels of epidermal growth factor receptor in human gingival fibroblast-1 and human telomerase-transduced gingival fibroblast cell lysates were also detected by immunoblotting. These data indicated that human telomerase-transduced gingival fibroblasts maintain normal levels of epidermal growth factor receptor

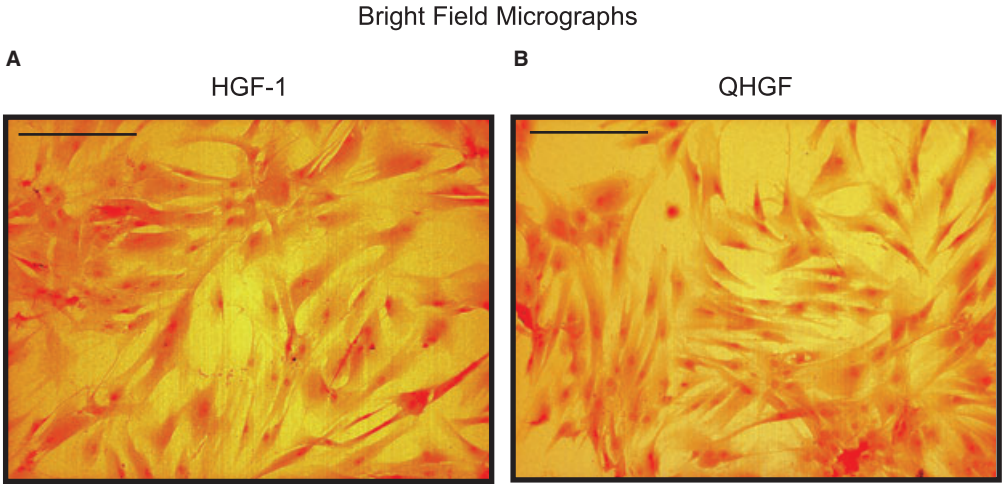

Fig. 3. Fibroblast morphology: bright-field micrographs of human gingival fibroblast-1 and human telomerase-transduced gingival fibroblast monolayers depicting the elongated shape that is characteristic of fibroblasts in subconfluent cultures. (A) Primary human gingival fibroblasts, passage 10 (HGF-1; ATCC CRL-2014), and (B) quick-growing human gingival fibroblasts [human telomerase-transduced gingival fibroblasts (QHGF)], passage 40, plated on glass cover slips and mounted on slides. Pictures were taken at a 10× magnification after 48 h of culture. Scale bar = 10 μm.

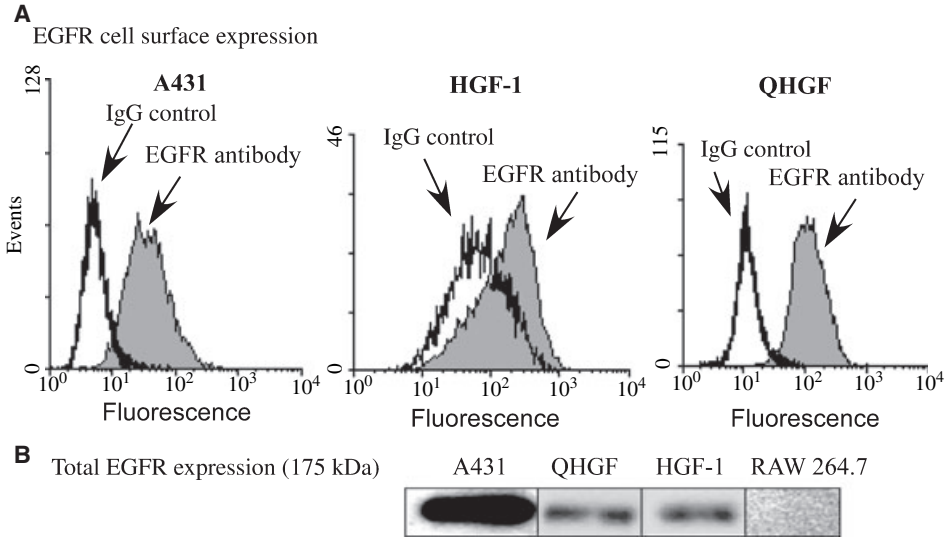

Fig. 4. Expression of epidermal growth factor and lipopolysaccharide-binding molecules. (A) The expression of epidermal growth factor receptor on the cell surface of A431 cells, human gingival fibroblast-1 cells and human telomerase-transduced gingival fibroblasts was measured by flow cytometry using anti-human epidermal growth factor receptor immunoglobulin (528) and compared with bound isotype-control antibody (IgG2a). The fluorescence shift of the histogram over the *x* axis indicates the amount of anti-epidermal growth factor receptor immunoglobulin bound on the cell surface. Human telomerase-transduced gingival fibroblasts expressed a similar level of receptor on the cell surface as human gingival fibroblast-1 cells ($p = 0.4$). (B) Total epidermal growth factor receptor expression was evaluated by immunoblotting 50 μg of protein from A431 cells (positive control), RAW 264.7 macrophages (negative control), human gingival fibroblast-1 cells and human telomerase-transduced gingival fibroblasts using an anti-epidermal growth factor receptor antibody (sc-03). Similar results were obtained in three independent experiments. EGFR, epidermal growth factor receptor; HGF-1, human gingival fibroblast-1; QHGF, human telomerase-transduced gingival fibroblasts.

expression (22) that are not significantly different from those observed with human gingival fibroblast-1 cells ($p = 0.4$). Interestingly, human gingival fibroblasts also express lipopolysaccharide-binding molecules, such as Toll-like receptor 2, Toll-like receptor 4 and CD14, and the co-expression of lipopolysaccharide and epidermal growth factor receptors makes them a unique cell line for using to study epidermal growth factor/lipopolysaccharide signaling pathway interactions. Similarly to the epidermal growth factor receptor, we observed that following expression of human telomerase in human gingival fibroblast-1 cells, the resulting human telomerase-transduced gingival fibroblasts also maintain comparable expression of CD14, Toll-like receptor 2 and Toll-like receptor 4 (data not shown).

Activation of MAPK-associated signaling

Cellular activation by the epidermal growth factor receptor often involves MAPK-dependent pathways that can serve to modulate cell proliferation and migration (33). These same pathways are also rapidly initiated following lipopolysaccharide challenge and are believed to influence the expression and/or release of inflammatory mediators in monocytic cell lines (67). The presence of both the epidermal growth factor receptor and lipopolysaccharide receptor systems in gingival fibroblasts makes them a unique cell line for using to study the potential cross-talk between these signaling pathways. The present studies represent the first analysis of *trans*-modulation between epidermal growth factor and lipopolysaccharide signaling events. Specifically, we tested the hypothesis that *P. gingivalis* lipopolysaccharide, a major contributor to rapidly progressive periodontitis, modifies epidermal growth factor-induced ERK1/2, p38 and CREB phosphorylation in human telomerase-transduced gingival fibroblasts. As shown in Fig. 5A, dose–responses for *P. gingivalis* lipopolysaccharide- and epidermal growth factor-induced ERK1/2 phosphorylation were similar (1 μg/mL of *P. gingivalis* lipopolysaccharide at 5 min of incubation and 10 nM epidermal growth factor at 30 min of incubation promoted maximal activation) for human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells. Figure 5B shows that 1 μg/mL of *P. gingivalis* lipopolysaccharide preparation promoted ERK1/2 phosphorylation starting at 5 min in human blood monocytes. In addition, human gingival fibroblast-1 cells, as well as human telomerase-transduced gingival fibroblasts, were stimulated with 10 nM epidermal growth factor and/or 1 μg/mL of *P. gingivalis* lipopolysaccharide, and the kinetics of ERK1/2 phosphorylation was compared between both cells by immunoblotting (Fig. 5C,D). The induction of ERK1/2 phosphorylation following epidermal growth factor treatment in both human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells was detected after 5 min of stimulation, with a greater

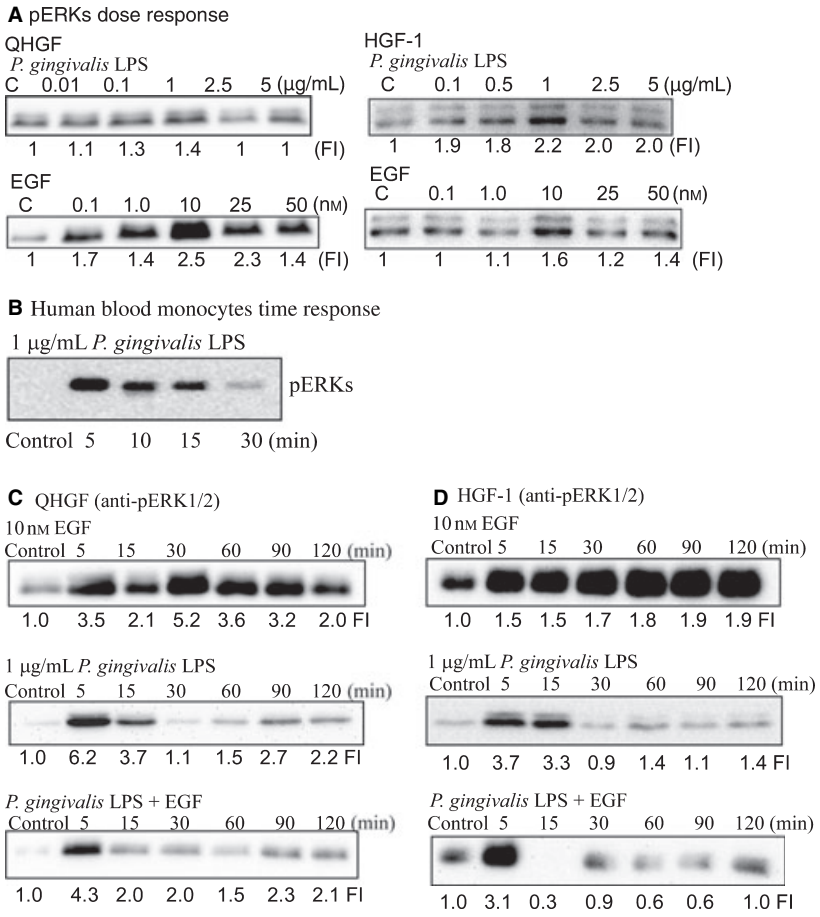

Fig. 5. Kinetics of extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in human monocytes, human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells, upon stimulation with epidermal growth factor and/or *Porphyromonas gingivalis* lipopolysaccharide. (A) Human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells were stimulated with varying concentrations of *P. gingivalis* lipopolysaccharide and epidermal growth factor for 5 and 30 min, respectively, cell extracts were prepared and the samples were immunoblotted with an anti-(phospho-ERK1/2) specific immunoglobulin [the phosphorylated ERKs (pERKs) were present as two bands, namely ERK1 = 44 kDa and ERK2 = 42 kDa]. (B) Human blood monocytes were stimulated with 1 μg/mL of *P. gingivalis* lipopolysaccharide for 5, 10, 15 and 30 min and the phosphorylated forms of ERK1/2 were detected by phospho-specific immunoblotting. (C,D) Human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells were stimulated with 10 nM epidermal growth factor, 1 μg/mL of *P. gingivalis* lipopolysaccharide, or a combination of the two, for the indicated periods of time. Subsequently, sample aliquots containing 50 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-(phosphorylated ERK1/2) specific immunoglobulin. Fold increase values of pERK1/2 optical density (arbitrary units) over control, after normalization to the loading control signal (pan ERK1/2), are depicted under each blot. One representative experiment out of three independent studies is shown.

than twofold increase over basal levels of activation, and this phosphorylation event persisted for at least 120 min. In comparison, human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 challenged with 1 μg/mL of *P. gingivalis* lipopolysaccharide exhibited an increase in ERK1/2 phosphorylation (four- to sixfold over control treatment) that peaked at ≈5 min and was reduced thereafter. In contrast, when human gingival fibroblast-1 cells and human telomerase-transduced gingival fibroblasts were exposed to epidermal growth factor and *P. gingivalis* lipopolysaccharide together, ERK1/2 activation was attenuated at all time points when compared with ERK1/2 activation following epidermal growth factor treatment alone.

A well-recognized marker of lipopolysaccharide-dependent cellular activation is the phosphorylation of MAPK p38 (68). Similarly, epidermal growth factor receptor activation has been shown to contribute to p38 phosphorylation in other cell lines (69), which makes p38 an additional point of intersection for both pathways. As seen in Fig. 6A, treatment of human gingival fibroblast-1 with 10 nM epidermal growth factor or 1 μg/mL of *P. gingivalis* lipopolysaccharide can enhance p38 phosphorylation at 5 and 15 min. Furthermore, *P. gingivalis* lipopolysaccharide and epidermal growth factor co-addition to human gingival fibroblast-1 cells reduced epidermal growth factor-dependent p38 activation by ≈50%.

Next, we evaluated whether *P. gingivalis* lipopolysaccharide administration can also antagonize epidermal growth factor signaling at the level of phosphorylation/activation of the transcription factor CREB (Fig. 6B). Upon epidermal growth factor stimulation, CREB has been reported to be phosphorylated at Ser133 by multiple kinases, including phospho-Akt1 (70), mediating epidermal growth factor-induced mitogenesis. Phosphorylation of CREB is also a downstream target of p38 in lipopolysaccharide-activated cells. Our experiments show that CREB phosphorylation is stimulated by either epidermal growth factor or *P. gingivalis* lipopolysaccharide alone, and that the cotreatment of epidermal growth factor with *P. gingivalis* lipopolysaccharide for 15 min also reduced epidermal growth factor-induced CREB phosphorylation to approximately the same level as observed with *P. gingivalis* lipopolysaccharide treatment alone. These results revealed that *P. gingivalis* lipopolysaccharide challenge inhibits epidermal growth factor receptor-stimulated cell signaling at the level of ERK1/2, p38 and CREB in human gingival fibroblasts (Fig. 6 and data not shown).

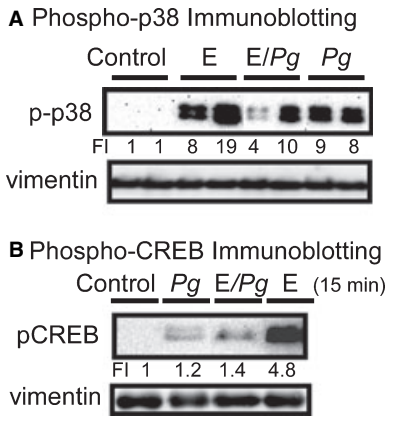

Fig. 6. Transmodulation of p38 and phosphorylation of the transcription factor cyclic-AMP response element binding protein by epidermal growth factor and *Porphyromonas gingivalis* lipopolysaccharide. (A) Upper panel: human gingival fibroblast-1 cells stimulated with 10 nM epidermal growth factor and/or 1 μg/mL of *P. gingivalis lipopolysaccharide* for 5 and 15 min and immunoblotted with an antibody directed against active (dually phosphorylated) p38. Lower panel: equal protein loading between samples was confirmed via immunoblotting with an anti-vimentin immunoglobulin. (B) Upper panel: human telomerase-transduced gingival fibroblasts stimulated with 10 nM epidermal growth factor and/or 1 μg/mL of *P. gingivalis* lipopolysaccharide for 15 min and immunoblotted against phosphorylated cyclic-AMP response element binding protein at Ser133. Lower panel: equal protein loading between samples was assessed by immunoblotting using an anti-vimentin immunoglobulin. The fold increase values of p38 or phosphorylated cyclic-AMP response element binding protein optical density (arbitrary units) over control, after normalization to the loading control (vimentin) signal, are depicted under each blot. Equal amounts of protein were loaded in each lane (50 μg). One representative experiment is shown out of three individual assays. E, epidermal growth factor; FI, fold increase; *Pg*, *Porphyromonas gingivalis* lipopolysaccharide.

Discussion

Primary human gingival fibroblasts grown *in vitro* from gingival biopsies have been used to study the molecular mechanisms of many oral pathologies. However, although epidermal growth factor and lipopolysaccharide are known to be important in rapidly progressive periodontitis, their potential cross-talk in regulating the biological function of human gingival fibroblasts is unknown. To address this issue it was necessary to establish a more stable cell system, given that primary gingival fibroblasts cultures drift with every passage until they functionally differentiate, reach senescence and stop dividing. In the present report, it was found that introduction of the catalytic subunit of human telomerase into human gingival fibroblast-1 cells resulted in an increase in detectable telomerase activity. The gingival fibroblasts thus obtained, human telomerase-transduced gingival fibroblasts, are a stable cell line with suitable growth conditions that include: increased number of passages and life span in culture, and an increased proliferation rate at low cell densities. Conversely, when compared with human gingival fibroblast-1 cells, human telomerase-transduced gingival fibroblasts exhibited no detectable morphological or functional terminal differentiation over 40 passages (compared with an average of 16 passages for the primary human gingival fibroblasts). Increased telomerase activity is correlated with extended life span in primary cells, but it does not necessarily result in immortalization of the cell line (48,49,71). Other strategies, such as introduction of simian virus-40, human papilloma virus 16 or oncogenes have been used to immortalize oral cell lines (72). In some cases, the introduction of oncogenes may alter the functional background and basal activity of immortalized immune cells (73,74). Therefore, it is presently unclear if human telomerase-transduced gingival fibroblasts have been immortalized, but increasing the telomerase activity of human gingival fibroblast-1 cells has preserved their nondifferentiated phenotype for many more passages than usually observed with the primary cells.

Several *in vitro* studies have reported lengthened cell cycle times in primary gingival fibroblasts with increased number of passages and that these parameters varied in response to factors such as the age and health condition of the donor (10,12). Increased proliferative activity of human telomerase-transduced gingival fibroblasts over human gingival fibroblast-1 cells is reflected by their proliferation profiles and this characteristic is preserved at later passages (Fig. 2). The mitotic activity of human telomerase-transduced gingival fibroblasts was the highest during the first 24 h of growth and decreased with time when the cells were plated at a density of 5×10^3 cells/cm^2. This decrease correlated to a reduction in the surface area available to the cells and a concomitant increase in cell density. In the case of human gingival fibroblast-1 at a plating density of 5×10^3 cells/cm^2, there is also a higher mitotic activity during the first cycle, followed by a considerable decrease that may also be explained by a change in the length of the cell cycle. The lengthening of the cell cycle time at either G1 or G2 results in a reduction of the number of cells that divide in a 24-h cycle. At subconfluent densities that are more optimal for fibroblast growth (1×10^4 cells/cm^2), both cell types exhibit higher mitotic activity at the lower cell densities and slower mitotic activity as the cultures become more confluent, especially in the case of human gingival fibroblast-1. Our observations suggest that human gingival fibroblast-1 cells are more susceptible to the effects of increased cell density than human telomerase-transduced gingival fibroblasts, although more detailed studies of the changes in cell cycle times will be required to clarify this event. In general, these results exemplify the major difficulties of working with primary gingival fibroblasts, namely their slow and irregular growth rates. However, the improved growth phenotype of human telomerase-transduced gingival fibroblasts constitutes a promising tool to standardize molecular studies in human gingival fibroblasts, especially considering that the growth conditions of primary cells are challenging for the design of experiments with prolonged stimulations or combinations of stimuli (which demand high yields of cells) that better mimic *in vivo* responses.

We determined that human telomerase-transduced gingival fibroblasts were probably not contaminated with epithelial cells because of their characteristic fibroblast spindle-like morphology when grown in monolayer, the expression of certain fibroblast markers (such as vimentin) and the absence of cytokeratin 14, which is a marker that would appear if there were colonies of contaminating epithelial cells. Although gingival fibroblasts express alkaline phosphatase (72), human telomerase-transduced gingival fibroblasts and human gingival fibroblast-1 cells have little or no alkaline phosphatase activity, and this parameter serves as an important factor in determining the naïve state of the original cells. The activity of this enzyme is a marker for fibroblast differentiation that may appear at later passages and correlates with decreased cellular activation and altered patterns of inflammatory mediator release in primary cells. Human telomerase-transduced gingival fibroblasts are unlikely to be differentiated because of the absence of alkaline phosphatase activity and this feature also distinguishes human telomerase-transduced gingival fibroblasts from fibroblasts originated from the periodontal ligament (75), which are typically positive in alkaline phosphatase assays.

The presence of both the epidermal growth factor receptor and lipopolysaccharide receptor systems in gingival fibroblasts provides a model to examine the cross-talk between these signaling pathways. The presence of lipopolysaccharide from pathogenic bacteria is toxic to the cells in the connective tissues and makes them less responsive to regenerative therapies. The regulatory mechanisms that decide the fate of human gingival fibroblasts and whether they will enter apoptosis or proliferate remain unknown. In this regard, human gingival fibroblasts are one of the few cell types that can respond to both epidermal growth factor and lipopolysaccharide, and the interplay between these two factors is very poorly understood. Therefore, the life-expanded gingival fibroblasts can provide valuable information about the mechanisms used by the cell to select responses to multiple stimuli with antagonistic effects on biological functions, such as cell survival, cell migration and inflammatory mediator release. Additionally, the expression and function of epidermal growth factor receptor and lipopolysaccharide-binding molecules were not affected by the introduction of human telomerase. Cellular activation of human telomerase-transduced gingival fibroblasts by epidermal growth factor and lipopolysaccharide resembles what is observed in the primary cells and will allow advances in the understanding of the mutual regulation of epidermal growth factor and lipopolysaccharide signaling cascades.

Previous studies described that lipopolysaccharide can decrease human gingival fibroblast-1 proliferation and migration. Although the signaling mechanisms mediating these effects have not yet been elucidated, it is known that both epidermal growth factor and lipopolysaccharide activate the MAPKs to mediate cell proliferation, cell survival (70) and the release of inflammatory mediators [nitric oxide and interleukin-1β (67,76)]. We observed that *P. gingivalis* lipopolysaccharide produced a profound down-regulation of epidermal growth factor-dependent signaling, not only at the level of the MAPKs ERK1/2 and p38, but also at the level of phosphorylation of the transcription factor CREB. This transcription factor has been shown to regulate cell cycle progression and is a common focal point of numerous cellular stimuli and signaling events; therefore, multiple factors may participate in the capacity of lipopolysaccharide to attenuate phosphorylation of the epidermal growth factor-induced CREB.

The alterations in epidermal growth factor signaling caused by *P. gingivalis* lipopolysaccharide may be mediated by an array of events, including, among other possibilities, the differential recruitment and altered kinetics of activation of upstream mediators in response to lipopolysaccharide and epidermal growth factor. In this regard, we observed that the co-addition of epidermal growth factor and lipopolysaccharide attenuates the sustained phosphorylation of ERK1/2 and p38 observed with epidermal growth factor alone and that epidermal growth factor-dependent phosphorylation of CREB is inhibited by lipopolysaccharide and results in a response similar to lipopolysaccharide alone. This attenuation of ERK and CREB phosphorylation may result from competition between these two systems (lipopolysaccharide and epidermal growth factor) for the activation or membrane localization of upstream regulators such as Ras, Rac or Raf, and/or it may result from cross-talk between various kinases, phosphatases and other effector molecules that are uniquely regulated by lipopolysaccharide- and epidermal growth factor receptor-dependent processes. Interestingly, previous results have shown that *P. gingivalis* lipopolysaccharide fails to down-regulate tumor necrosis factor-α-induced p38 MAPK activation in endothelial cells (68), indicating that the down-regulation of epidermal growth factor-dependent ERK1/2 and p38 activation by *P. gingivalis* lipopolysaccharide in gingival fibroblasts is cell- and/or pathway-specific.

The complex network of interactions found between lipopolysaccharide and epidermal growth factor signaling in gingival fibroblasts will require further exploration to reveal how to modulate both systems, perhaps at the level of ligand–receptor interaction, in order to favor biological responses necessary for wound healing and regeneration during the pathogenesis of rapidly progressive periodontitis, when the presence of highly pathogenic bacterial products constitutes a persistent aggravating factor.

Acknowledgements

This work was funded by NIH grants GM-53271, AI50500 and CA108467 (to P.J.B) as well as by NIH grants AI054915 and AI034998 (to T.C.), and by the Materials Research Science and Engineering Center on Nanostructured Materials and Interfaces (NSF-DMR-0079983) at the University of

Wisconsin. Many thanks to Dr Greg Weipz, Dr Mary Ellen Bates and Christopher Johnson for many insightful discussions about cross-experimental analyses.

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