Induction of Keratinocyte Growth Factor 1 Expression by Lipopolysaccharide Is Regulated by CD-14 and Toll-Like Receptors 2 and 4

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Periodontal disease is a chronic inflammatory condition that is associated with increased concentrations of gram-negative pathogenic bacteria and epithelial cell proliferation. Regulation of this proliferation is poorly understood but is most likely controlled by locally expressed growth factors. Keratinocyte growth factor 1, an epithelium-specific growth factor, is expressed by gingival fibroblasts, and its expression is regulated in a concentration-dependent manner by lipopolysaccharide. In this study, induction of keratinocyte growth factor 1 protein expression was dependent on gingival fibroblast expression of membrane CD14 (mCD14) and Toll-like receptors 2 and 4. Lipopolysaccharides from *Escherichia coli* and *Porphyromonas gingivalis* induced membrane expression of CD14 at 1, 3, and 24 h. Specifically, lipopolysaccharide induced low mCD14 expression gingival fibroblasts to express mCD14 at a level consistent with that of high mCD14 expression cells. Functional studies with specific blocking antibodies for CD14 and Toll-like receptors 2 and 4 implicated all of these molecules in signal transduction. The rapid decrease in cell membrane expression of Toll-like receptors 2 and 4 after treatment with lipopolysaccharide was consistent with receptor internalization, and blocking of either of these receptors completely inhibited keratinocyte growth factor 1 protein expression. The transcription factors AP-1 and NF- κ B were involved in lipopolysaccharide induction of keratinocyte growth factor 1 mRNA and protein expression. These results suggest that lipopolysaccharide may induce proliferation of periodontal epithelial cells by upregulating keratinocyte growth factor 1 expression via the CD14 and Toll-like receptor signaling pathway.

Periodontal disease is a chronic inflammatory condition that results from a complex interaction between gram-negative microorganisms associated with disease and the host response that they induce (22). Lipopolysaccharide (LPS), a virulence factor expressed on many periodontal disease-associated pathogens, collectively stimulates a variety of responses in all cells of the periodontal attachment complex (20, 28, 31). One aspect of early periodontal disease onset is significant epithelial cell proliferation and migration. Proliferation and invasion of junctional and sulcular epithelium into the connective tissue starts early in the disease process and may continue to ultimately form a periodontal pocket (33). Topical application of purified *Escherichia coli* LPS to the rat molar gingival sulcus induced significant junctional epithelial basal cell proliferation (46). Regulation of junctional epithelial cell proliferation may occur either directly or indirectly. One such indirect pathway involves LPS stimulation of gingival fibroblasts. This stimulation results in the secretion of growth factors that act in a paracrine manner to subsequently stimulate local epithelial cell proliferation.

Keratinocyte growth factor 1 (KGF-1) and KGF-2 are two members of the current fibroblast growth factor (FGF) family and are classically designated FGF-7 and FGF-10, respectively

(34). These growth factors are expressed primarily by fibroblast cells and specifically stimulate epithelial cells (38, 53). This specificity for epithelial cells occurs because epithelial cells express the FGFR2-iiib receptor variant and KGF-1 and KGF-2 bind only to this receptor variant (5, 24, 29). Not only is KGF-1 significantly upregulated during wound healing and chronic inflammatory diseases such as Crohn's disease, ulcerative colitis, and psoriasis, but it is also involved in the regulation of normal epidermal homeostasis (3, 7, 12, 13, 15, 54). Generally, both KGF family members induce proliferation, migration, and matrix metalloproteinase secretion in a variety of epithelial cells (35–37, 39, 49, 57). Gingival fibroblasts express KGF-1 and -2, but only KGF-1 protein and gene expression was inducible. Serum; the proinflammatory cytokines interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha; and LPSs from *E. coli* and *Porphyromonas gingivalis* significantly induced gingival fibroblast gene and protein expression of KGF-1 (40). The mechanism by which LPS regulates KGF-1 expression in gingival fibroblasts has not been elucidated.

LPS signaling is dependent on membrane CD14 (mCD14) or soluble CD14 (sCD14) and Toll-like receptors (TLRs) (1). Fibroblasts isolated from the skin and lung do not express mCD14, but gingival fibroblasts do (43–45, 51, 52). However, CD14 lacks a transmembrane and cytoplasmic domain and therefore is unlikely to have direct signaling capabilities. Signaling associations occur with members of the TLR family. This receptor family is made up of a least nine members, but TLR4 has been described as the likely receptor involved in LPS recognition. However, evidence suggests that TLR2 may

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also be involved in LPS-induced signaling (21, 27, 30, 58, 59, 61). Membrane expression of TLR is not limited to immune cells. Gingival fibroblasts and epithelial cells, microvascular endothelial cells, cardiac myocytes, T lymphocytes, and intestinal epithelial cells all express TLRs (2, 8, 10, 14, 26, 45, 50, 60).

In this study, LPS purified from *E. coli* was used to stimulate KGF-1 expression in gingival fibroblasts. First, we examined the effects of LPS on CD14, TLR2, and TLR4 expression and compared *E. coli* and *P. gingivalis* LPS effects on membrane expression levels. Second, we examined the signaling pathway that mediates *E. coli* LPS stimulation of KGF-1 mRNA and protein expression. LPS purified from *E. coli* has been extensively used to study cellular responses in several in vitro and in vivo periodontal pathogenesis investigations (44, 46, 52), and we previously established that LPSs purified from *P. gingivalis* and *E. coli* both induced the same qualitative increase in KGF-1 protein expression (40).

MATERIALS AND METHODS

Cell culture. Human gingival fibroblasts were isolated from sites of healthy noninflamed gingiva and passaged in α -MEM Media (Gibco BRL, Rockville, Md.) supplemented with 10% fetal bovine serum (FBS). Cells from passages 5 to 15 were used. Gingival fibroblasts were plated into 96-well plates for enzyme-linked immunosorbent assay (ELISA) or 60-mm-diameter culture dishes for flow cytometry, Western blotting, or Northern analysis. When cultures were 75% confluent, they were switched to α -MEM with 1% FBS for 24 h to bring the cells to quiescence. Cultures were treated with *E. coli* LPS (O55:B; Sigma catalog no. L2880) or *P. gingivalis* LPS (ATCC 33211) (40) at 5 to 100 ng/ml or recombinant human sCD14 (R&D Systems Inc, Minneapolis, Minn.) at 50 ng/ml with α -MEM in the presence and absence of 1% FBS.

In some experiments, cell cultures were preincubated for 30 min with specific blocking anti-human TLR2 (Cascade BioScience, Winchester, Mass.) and/or TLR4 (eBioscience, San Diego, Calif.) monoclonal antibodies at 0.5 μ g/ml. CD14 function was specifically blocked by preincubating cultures with an anti-CD14 polyclonal blocking antibody (R&D Systems) prior to the addition of LPS (50 ng/ml). In addition, 5 to 30 μ M curcumin (Sigma, St. Louis, Mo.) or 2 to 6 nM pyrrolidine dithiocarbamate (PDTC; Sigma) was added in some experiments 30 min prior to the addition of LPS. KGF-1 protein expression levels were assayed at 24 h.

Sandwich ELISA. Plates were coated with the capturing anti-human KGF-1 monoclonal antibody MAB251 (R&D Systems Inc.) at 1.0 μ g/ml, washed, and blocked (2% bovine serum albumin [BSA], 5% sucrose, 0.05% Na₂S₂O₃). Conditioned medium (200 μ l) was centrifuged at 13,000 $\times$ g for 5 min to remove cellular debris, added to the precoated wells, and incubated for 2 h at room temperature. Wells were washed, and 100 μ l of biotinylated anti-human KGF-1 polyclonal antibody (BAF251; 200 ng/ml; R&D Systems Inc.) was added, the wells were incubated for 2 h, and then the wells were washed. Horseradish peroxidase-streptavidin conjugate was added (1:4,000; Zymed, San Francisco, Calif.) with 3,3',5,5'-tetramethylbenzidine (Zymed) substrate. The reaction was stopped with 1 M HCl, and the color intensity was read at 450 nm with an optical density plate reader (Multiskan; Titertek, Huntsville, Ala.). A standard curve was included with KGF-1 protein concentrations of 1 to 750 pg/ml (Upstate Biotechnology, Lake Placid, N.Y.). Examination for statistical significance was done by analysis of variance.

Flow cytometry. Quiescent and LPS (50 ng/ml)-treated cultures at the 1-, 3-, and 24-h time points were washed in phosphate-buffered saline (PBS), lifted with 0.25% trypsin–1 mM EDTA solution, and then neutralized with Trypsin Neutralizing Solution (Clonetics, San Diego, Calif.). Cells were washed in PBS, fixed for 1 h in fresh 1.5% paraformaldehyde, and stored at 4°C in PBS. Samples were blocked (2% BSA, 1% glycine, 0.1% Na₂S₂O₃) for 1 h; incubated with primary anti-human TLR2, anti-human TLR4, or anti-human CD14 antibodies in fresh blocking solution for 2 h at room temperature; and then washed twice with blocking solution. Subsequently, the cells were incubated for 1 h in alexa 488 nm-conjugated secondary antibody (Molecular Probes, Eugene, Oreg.) blocking solution, washed twice in PBS, fixed (1.5% paraformaldehyde–PBS), and stored at 4°C until the time of measurement. Samples were analyzed with a FACScalibre flow cytometer (Becton Dickinson, Franklin Lakes, N.J.). Dot blots were

gated to select viable cells, 2.0×10^4 gated cells were measured per treatment group, and data were plotted as counts versus fluorescence (FL1-H). Data were analyzed with CellQuest software (Becton Dickinson), and the geometric mean of the relative cell fluorescence within the areas of interest was determined. Cells labeled with secondary antibody alone were used to establish background fluorescence.

Northern analysis. Total RNA from control, PDTC, and curcumin triplicate cultures treated as described above in two independent experiments were harvested in TRIzol (Bethesda Research Laboratories, Inc., Rockville, Md.) in accordance with the manufacturer's instructions. Fifteen micrograms per sample was fractionated on formaldehyde gels, transferred to Hybond (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom), hybridized with a ³²P-radio-labeled KGF-1 or GAPD cDNA probe, and analyzed as previously described (40). KGF-1 bands were scanned with NIH Image 1.61 (National Institutes of Health, Bethesda, Md.), normalized to GAPD, and expressed as fold change in expression.

Western blot analysis. Cultures were stimulated for 18 h with LPS (5 and 50 ng/ml), washed with PBS, lysed by addition of ice-cold lysis buffer with sonication twice for 15 s each time, and centrifuged for 30 min at 13,000 $\times$ g (61). Forty micrograms of soluble protein was fractionated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis and blotted to Immobilon P membranes (Millipore Corporation, Bedford, Mass.). The filter was blocked at room temperature for 1 h in 5% (wt/vol) nonfat milk powder in TBS–Tween 20 (25 mM Tris–HCl [pH 8.0], 144 mM NaCl, 0.05% Tween 20). The filter was washed three times, incubated for 2 h with anti-human CD14 antibody (R&D Systems) at 2 μ g/ml in 0.1% BSA–TBS, washed, and incubated for 1 h at room temperature with 1:5,000 rabbit anti-sheep immunoglobulin G (Chemicon International, Inc., Temecula, Calif.) secondary antibody diluted in 0.1% BSA–TBS. After the final washing, bound primary antibody was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). The relative change in the amount of CD14 was calculated by scanning the CD14 band intensity and then determining the total protein by scanning of Ponceau-stained filters. LPS induction of CD14 expression was determined after correction for minor differences in protein loading.

Immunostaining. Human gingival fibroblasts were plated and cultured on radio frequency glow-discharged glass coverslips until they were 70% confluent. Control cultures (α -MEM plus 1% FBS) and cultures treated with LPS (50 ng/ml) for 24 h were fixed (2% paraformaldehyde, 5% sucrose, PBS), washed five times in PBS, quenched (fresh 0.05% NaBH₄, PBS), and blocked (30 mg of BSA per ml, 1 mg of glycine per ml, PBS; 30 min). Cultures were then incubated overnight at 4°C in blocking solution with anti-human CD14 polyclonal antibody. Coverslips were washed five times (1 mg of BSA per ml of PBS) and then incubated in wash solution with a 1:50 dilution of a rhodamine-conjugated secondary antibody (Boehringer, Mannheim, Germany) for 1 h at room temperature in the dark. Cultures were washed two times with PBS and then mounted in Vectashield (Vector Laboratories, Burlingame, Calif.). Preparations were analyzed with an MRC 600 confocal laser fluorescence microscope (Bio-Rad, Hercules, Calif.). Optical sections were taken at 20- μ m intervals at 568 nm (rhodamine), compiled with public domain NIH Image, and then imported into Adobe Photoshop 5.5.

RESULTS

LPS induction of KGF-1 and CD14 protein expression. *E. coli* LPS concentrations of up to 100 ng/ml were tested in quiescent gingival fibroblast cultures with and without FBS supplementation. Concentrations of up to 10 ng/ml induced a concentration-dependent increase in KGF-1 protein expression, with no further increase above 10 ng/ml ($P < 0.0005$) (Fig. 1A). Regardless of the LPS concentration tested, no significant differences were found between serum-free cultures and those supplemented with 1% FBS. Subsequent experiments were carried out with LPS at 50 ng/ml in α -MEM plus 1% FBS.

Since the sCD14 protein may regulate LPS-induced cellular effects, we examined whether the addition of recombinant CD14 protein would further induce KGF-1 protein expression. In either the presence or the absence of serum, the addition of recombinant CD14 had no statistically significant effect on

FIG. 1. LPS purified from *E. coli* significantly induces gingival fibroblast expression of KGF-1 protein in the presence or absence of serum. Gingival fibroblasts were cultured to quiescence in α -MEM supplemented with 1% FBS. Various concentrations of LPS (A) and recombinant human sCD14 (B), with or without LPS (50 ng/ml), were added to serum-free and 1% FBS-supplemented α -MEM. A sandwich ELISA was used to analyze KGF-1 protein levels in 24-h conditioned medium. Mean $\pm$ standard deviation; $n = 4$.

LPS-induced KGF-1 protein expression (Fig. 1B). Collectively, these data suggest that gingival fibroblasts likely express sufficient mCD14 to maximally respond to LPS stimulation. Control cultures were found to express mCD14 protein, and LPS induced a concentration-dependent increase in mCD14, as confirmed by the detection of a 55-kDa protein (Fig. 2A and B). At the highest LPS concentration tested (50 ng/ml), there was a 67% increase in mCD14 protein at 18 h. An increase in mCD14 expression was evident in immunohistochemically stained samples as well. Control cell cultures showed mCD14 expression to be generally quite weak, but cells exhibiting areas of focal staining were evident (Fig. 3A, arrow). In contrast, LPS-stimulated cultures showed a general marked increase in expression of mCD14 (Fig. 3B). This increased mCD14 expression found in the immunohistochemically stained specimens

FIG. 2. LPS induces concentration-dependent induction of total CD14 protein expression. Quiescent gingival fibroblasts were stimulated for 18 h with *E. coli* LPS (50 ng/ml) in α -MEM plus 1% FBS. Whole-cell protein extracts were prepared from control and LPS-treated cultures, and proteins were analyzed by Western blotting with an anti-CD14 polyclonal antibody. (A) LPS (50 ng/ml) induced significant expression of CD14, a 55-kDa protein. (B) In relation to the control (α -MEM plus 1% FBS), LPS (5 and 50 ng/ml) induced a concentration-dependent increase in CD14 total protein expression. Mean $\pm$ range; $n = 2$.

was confirmed by flow cytometry analysis (Fig. 4A to F). Overall, *E. coli* LPS induced mean increases in mCD14 expression (M1 plus M2) of 39, 121, and 18% at 1, 3, and 24 h, respectively. Specifically, for *E. coli* LPS, low (M1 peak) and high (M2 peak) expression gingival fibroblast populations were identified (Fig. 4C to F). At 1 h, the number of gingival fibroblast cells that expressed high levels of mCD14 (M2 peak) increased sharply (Fig. 4D). This rapid increase in high mCD14 expression (M2 peak) began to return to baseline levels at 3 and 24 h (Fig. 4E and F). A representative example of three independent experiments is shown. In addition, stimulation of gingival fibroblast cultures treated with *P. gingivalis* LPS also induced mCD14 levels by 32, 133, and 14% at 1, 3, and 24 h, respectively (data not shown).

Although LPS induction of mCD14 expression was clearly evident, it was unclear whether LPS regulation of CD14 was involved in the regulation of KGF-1 protein expression. To examine this, cultures were preincubated with anti-CD14 blocking antibodies prior to stimulation with LPS. Blocking of mCD14 function inhibited LPS induction of KGF-1 protein expression ($P < 0.005$) (Fig. 5). This inhibition supports mCD14 involvement in the signaling process. CD14 reportedly

FIG. 3. LPS induces significant cell membrane expression of CD14. Quiescent control cultures in α -MEM with 1% FBS (A) and *E. coli* LPS (50 ng/ml)-stimulated gingival fibroblast cultures (B) were fixed at 24 h. Nonpermeabilized cultures were stained with anti-CD14 antibody and a rhodamine-labeled secondary antibody and then photographed by laser microscopy focused on the apical cell surface. (A) Generally, in controls, weak focal cell membrane expression of CD14 was observed, with focal cells exhibiting increased staining (arrow). (B) LPS-treated samples showed a general significant increase in staining.

lacks intracellular signaling capabilities and associates with TLR2 and/or TLR4 in order to initiate intracellular signaling (1, 27, 30, 61). TLR2 and TLR4 involvement in LPS induction of KGF-1 protein expression was therefore examined.

TLR regulation of KGF-1 expression. As measured by flow cytometry, control gingival fibroblast cultures were found to express TLR2 and TLR4 (Fig. 6A). *E. coli* LPS treatment resulted in marked mean percent reductions in cell surface expression of TLR2 and TLR4 of 41 and 51%, respectively, at

FIG. 4. LPS stimulates a significant increase in mCD14. Gingival fibroblasts were treated with *E. coli* LPS (50 ng/ml), collected at various times, fixed, and stained with anti-CD14 antibody without permeabilization and an alexa 488 nm-conjugated secondary antibody prior to flow cytometry analysis. (A) Dot blot identifying the gated region that was examined. (B) Background fluorescence was established by staining cells with the secondary antibody alone. Compared to controls (C), LPS stimulated a significant increase and shift in mCD14 expression (peak shift from M1 to M2) at 1 h (D). By 3 h (E) and 24 h (F), expression began to return to the baseline.

1 h ($P < 0.0001$) (Fig. 6A). By 3 h, the mean percentages of TLR2 and TLR4 expression were reduced 47 and 43%, respectively ($P < 0.0001$). However, by 24 h, the expression of TLR2 and TLR4 was increased over control levels by 12% ($P < 0.0001$) and 4% ($P = 0.0004$), respectively. *P. gingivalis* LPS also induced a rapid decrease in cell membrane expression of TLR2 by 23 and 27% and of TLR4 by 28 and 31% at 1 and 3 h ($P < 0.0001$). Membrane expression levels at 24 h for TLR2 and TLR4 began to return to control levels but were still reduced by 14 and 22% ($P < 0.05$), respectively (Fig. 6A). Preincubation of cultures with specific TLR2 and TLR4 monoclonal blocking antibodies completely inhibited *E. coli* LPS induction of KGF-1 protein expression (Fig. 6B).

To elucidate the intracellular signaling pathway that may regulate LPS induction of KGF-1 expression, we examined whether the transcription factors AP-1 and NF- κ B are involved in this signaling process. When cultures were preincubated with increasing concentrations of curcumin or PDTC, inhibitors of AP-1 (19, 41, 47) and NF- κ B (42), respectively, a dose-dependent reduction in the LPS stimulation of KGF-1 protein expression was found in 24-h medium (Fig. 7A and B). Northern analysis of the same sample treatments at 6 h confirmed

FIG. 5. Addition of CD14-blocking antibody inhibits LPS stimulation of KGF-1 protein expression. Gingival fibroblasts were brought to quiescence in α -MEM supplemented with 1% FBS. In replicate wells, the following treatment groups were tested: α -MEM with 1% FBS (control), α -MEM with 1% FBS plus 50 ng of *E. coli* LPS (LPS) per ml or first pretreatment for 30 min with 0.5 μ g of anti-CD14 blocking antibody per ml with α -MEM and 1% FBS prior to the addition of 50 ng of LPS per ml. KGF-1 protein in the 24-h conditioned medium was measured by a sandwich ELISA. Mean $\pm$ standard deviation; $n = 4$.

that modulation of KGF-1 was occurring at the transcriptional level in a similar dose-dependent manner (Fig. 7C). Scanned bands normalized to GAPD control bands showed that LPS induced a 2.7-fold increase in KGF-1 signal over the control. The LPS-induced KGF-1 signal was reduced in the presence of 2 nM PDTC (1.9-fold) or 5 μ M curcumin (2.1-fold). At the highest inhibitor concentrations tested, the KGF-1 signal fell below control levels. This is in agreement with protein levels that were measured in the same experiment, which indicates the inhibition of constitutive KGF-1 expression levels.

DISCUSSION

Periodontal diseases are chronic inflammatory conditions that result from a complex interaction between host responses and a mixed gram-negative, disease-associated microflora (22). LPS stimulates a myriad of effects in oral epithelia and soft and hard connective tissues of the periodontium (20, 28, 31). *E. coli* LPS has been used in several in vivo and in vitro investigations that examined periodontal cellular responses to LPS (44, 46, 52). For example, topical application of purified *E. coli* LPS to the rat molar gingival sulcus resulted in significant stimulation of junctional epithelial basal cell proliferation (46). The regulation of epithelium-specific growth factor expression by LPS was examined.

Keratinocyte growth factors are paracrine-mediated growth factors that are expressed primarily by fibroblasts and specifically stimulate epithelial cells (38, 53). In contrast to KGF-2, KGF-1 is upregulated by serum and proinflammatory cytokines (4, 6, 9, 48). An increase in KGF-1 expression was found in chronic inflammatory conditions such as Crohn's disease, ulcerative colitis, and psoriasis (3, 7, 12, 13). KGF-1 expression and regulation in periodontal tissues in health and disease have not been as extensively examined. In situ hybridization localized KGF-1 expression in healthy periodontal tissues to the

FIG. 6. LPS downregulates cell membrane expression of TLR2 and TLR4. Gingival fibroblasts were treated with either *E. coli* or *P. gingivalis* (*P. ging*) LPS (50 ng/ml), collected at various times, fixed, stained without permeabilization with either anti-TLR2 (TLR2) or anti-TLR4 (TLR4) monoclonal antibody, and then stained with an alexa 488 nm-conjugated secondary antibody prior to flow cytometry analysis. Mean $\pm$ standard deviation; $n = 4$. (B) Preincubation of cultures with TLR2 and TLR4 blocking antibodies inhibited *E. coli* LPS stimulation of KGF-1 protein expression. Culture groups were treated with α -MEM with 1% FBS (control) or α -MEM with 1% FBS and 50 ng of LPS (LPS) per ml or first pretreated for 30 min with either 0.5 μ g of anti-TLR2 (LPS+aT2) or TLR4 (LPS+aT4) blocking antibody per ml with α -MEM plus 1% FBS prior to the addition of LPS at 50 ng/ml. A sandwich ELISA was used to measure KGF-1 protein expression in 24-h conditioned medium. Mean $\pm$ standard deviation; $n = 4$.

subepithelial rather than the deeper connective tissues of the periodontium, with minimal expression in the vicinity of the junctional epithelia associated with healthy tissues (25). Gingival fibroblasts in cell culture expressed KGF-1 and KGF-2, but only KGF-1 was induced by serum, IL-1, IL-6, tumor necrosis factor alpha, and, in particular, LPSs from *P. gingivalis* and *E. coli* (40). LPS regulation of KGF-1 and its associated signaling mechanism have yet to be elucidated.

Membrane CD14 is involved in LPS-induced signaling (1). However, fibroblast expression of mCD14 is dependent on the tissue type from which the fibroblasts were isolated. Skin and lung fibroblasts do not express mCD14 (43–45, 51, 52). We found that gingival fibroblasts expressed mCD14, its molecular mass of 55 kDa was consistent with previous studies (1, 44), and gingival fibroblasts exhibited an approximate 2-log₁₀ unit distribution in fluorescence. Within the cultures, two cell pop-

FIG. 7. LPS induction of KGF-1 protein expression is regulated by the transcription factors AP-1 and NF- κ B. Quiescent gingival fibroblasts in α -MEM with 1% FBS were preincubated for 30 min with increasing concentrations of the transcription factor inhibitors curcumin (A) and PDTC (B) prior to the addition of *E. coli* LPS (50 μ g/ml). Conditioned medium was collected at 24 h, and the KGF-1 protein level was assayed by a sandwich ELISA. The dotted line represents the KGF-1 protein expression of the negative control (1% serum alone). RNA from cultures was isolated at 6 h for Northern analysis (C). Lanes: 1, negative control (1% serum alone); 2, *E. coli* LPS at 50 ng/ml; 3, LPS plus PDTC at 2 nM; 4, LPS plus PDTC at 4 nM; 5, LPS plus PDTC at 6 nM; 6, LPS plus curcumin at 5 μ M; 7, LPS plus curcumin at 10 μ M; 8, LPS plus curcumin at 30 μ M.

ulations were identified and would support the belief that two cell populations exist, low and high mCD14 expression cells (43, 44). Generally, in our study, LPS stimulated an overall increase in mCD14 but specifically induced mCD14 in low mCD14 expression cells. Addition of recombinant CD14 had no additional inductive effect over that of LPS alone, suggesting that mCD14 was expressed by gingival fibroblasts at a sufficient level to induce maximal KGF-1 protein expression. Classically, binding of LPS to CD14 is significantly enhanced by the presence of lipid binding protein in serum (16). How-

ever, the presence of lipid binding protein in the 1% serum supplement had no significant effect on LPS induction of KGF-1 protein in 24-h culture medium.

CD14 is involved in binding of LPS, and blocking of mCD14 function with specific blocking antibodies inhibits LPS-mediated effects. For example, LPS induction of IL-8 and IL-6 in gingival fibroblasts was inhibited by the addition of CD14-blocking antibodies (1, 44, 51). Blocking of mCD14 function with specific blocking antibody in our study also inhibited LPS induction of KGF-1 protein expression. However, mCD14 is a glycosyl-phosphatidylinositol-anchored surface protein that lacks a transmembrane and cytoplasmic domain and therefore likely recruits additional surface proteins for signal transduction (1). The most likely candidates are members of the TLR family. Our gingival fibroblast lines expressed TLR2 and TLR4 on their cell membranes, which is consistent with previously published data (45). TLR4 has been described as the principal receptor involved in LPS signaling, but evidence also supports TLR2 involvement (21, 27, 30, 58, 59, 61). However, TLR2 may either be a low-affinity LPS receptor or respond to low bacterial lipoprotein contamination that is present in commercial LPS preparations (18, 21, 58, 61). If contaminants stimulating TLR2 were present in our commercial LPS preparation, then blocking of just one TLR should not have fully negated the LPS induction of KGF-1 expression. Blocking of both TLRs with the addition of both blocking antibodies would have been required if both signaling pathways were being stimulated. The complete inhibition of LPS induction of KGF-1 by either blocking antibody and the similar and rapid reduction in cell membrane expression of TLR2 and TLR4 lead to the hypothesis that LPS induction of KGF-1 expression may be mediated by TLR2 and TLR4 heterodimerization prior to internalization. TLR2 heterodimerization with TLR1 or TLR6 has been confirmed (17, 32, 55). Further investigation is required to resolve whether TLR2 and TLR4 heterodimerization is involved in the LPS regulation of KGF-1 protein expression.

Intracellular signaling steps were examined in this study. Preincubation with curcumin inhibits AP-1 binding to its consensus sequence by suppressing the *c-jun* gene (19, 41, 47), but it may also inhibit NF- κ B activity (56). In contrast, PDTC specifically inhibits NF- κ B activation by decreasing the release of the I κ B inhibitory unit from latent NF- κ B (42). The NF- κ B signaling pathway does play a role in LPS-mediated effects, and this same pathway is involved in IL-1 induction of KGF-1 expression (23, 52, 59). Collectively, these data, in conjunction with ours, suggest that LPS induction of KGF-1 expression is mediated through NF- κ B. However, the promoter region of the KGF-1 gene has two AP-1 transcription factor binding sites (11) and curcumin inhibition of AP-1 may have occurred as well.

LPS is one member of a group of molecules, called pathogen-associated molecular pattern molecules, that are recognized by host tissues that express pattern recognition receptors. Recognition of microorganisms by this mechanism forms part of the primitive form of defense called innate immunity (61). However, we showed that LPS regulation of KGF-1 expression is also regulated through this pathway. It is interesting to speculate on the clinical significance of this finding. During the progression of periodontal disease, the epithelial cell barrier can be disrupted, allowing LPS to directly stimulate gingival

fibroblasts to express KGF-1. Expression of KGF-1 and subsequent specific stimulation of epithelial cell proliferation may ultimately serve to reestablish and maintain an effective epithelial cell barrier. This may protect the host from periodontal disease-associated gram-negative pathogens in dental plaque biofilm.

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REFERENCES

1. Antal-Szalmás, P. 2000. Evaluation of CD14 in host defence. *Eur. J. Clin. Invest.* **30**:167–179.
2. Asai, Y., Y. Ohyama, K. Gen, and T. Ogawa. 2001. Bacterial fimbriae and their peptides activate human gingival epithelial cells through Toll-like receptor 2. *Infect. Immun.* **69**:7387–7395.
3. Bajaj-Elliott, M., E. Breese, R. Poulson, P. D. Fairclough, and T. T. MacDonald. 1997. Keratinocyte growth factor in inflammatory bowel disease: increased mRNA transcripts in ulcerative colitis compared with Crohn's disease in biopsies and isolated mucosal myofibroblasts. *Am. J. Pathol.* **151**:1469–1476.
4. Beer, H.-D., C. Florence, J. Dammeier, L. McGuire, S. Werner, and D. R. Duan. 1997. Mouse fibroblast growth factor 10: cDNA cloning, protein characterization, and regulation of mRNA expression. *Oncogene* **15**:2211–2218.
5. Bottaro, D. P., J. S. Rubin, D. Ron, P. W. Finch, C. Florio, and S. A. Aaronson. 1990. Characterization of the receptor for keratinocyte growth factor. *J. Biol. Chem.* **265**:12767–12770.
6. Brauchle, M., K. Angermeyer, G. Hübner, and S. Werner. 1994. Large induction of keratinocyte growth factor expression by serum growth factors and pro-inflammatory cytokines in cultured fibroblasts. *Oncogene* **9**:3199–3204.
7. Brauchle, M., M. Madlener, A. D. Wagner, K. Angermeyer, U. Lauer, P. H. Hofschneider, M. Gregor, and S. Werner. 1996. Keratinocyte growth factor is highly overexpressed in inflammatory bowel disease. *Am. J. Pathol.* **149**:521–529.
8. Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. C. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J. Immunol.* **164**:966–972.
9. Chedid, M., J. S. Rubin, K. G. Csaky, and S. A. Aaronson. 1994. Regulation of keratinocyte growth factor gene expression by interleukin 1. *J. Biol. Chem.* **269**:10753–10757.
10. Faure, E., O. Equils, P. A. Sieling, L. Thomas, F. X. Zhang, C. J. Kirschning, N. Polentarutti, M. Muzio, and M. Arditi. 2000. Bacterial lipopolysaccharide activates NF- κ B through Toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. *J. Biol. Chem.* **275**:11058–11063.
11. Finch, P. W., C. Lengel, and M. Chedid. 1995. Cloning and characterization of the promoter region of the human keratinocyte growth factor gene. *J. Biol. Chem.* **270**:11230–11237.
12. Finch, P. W., F. Murphy, I. Cardinale, and J. G. Krueger. 1997. Altered expression of keratinocyte growth factor and its receptor in psoriasis. *Am. J. Pathol.* **151**:1619–1628.
13. Finch, P. W., V. Pricolo, A. Wu, and S. D. Finkelstein. 1996. Increased expression of keratinocyte growth factor messenger RNA associated with inflammatory bowel disease. *Gastroenterology* **110**:441–451.
14. Frantz, S., L. Kobzik, Y. D. Kim, R. Fukazawa, R. Medzhitov, R. T. Lee, and R. A. Kelly. 1999. Toll4 (TLR4) expression in cardiac myocytes in normal and failing myocardium. *J. Clin. Invest.* **104**:271–280.
15. Guo, L., Q.-C. Yu, and E. Fuchs. 1993. Targeting expression of keratinocyte growth factor to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice. *EMBO J.* **12**:973–986.
16. Hailman, E., H. S. Lichenstein, M. M. Wurfel, D. S. Miller, D. A. Johnson, M. Kelley, L. A. Busse, M. M. Zukowski, and S. D. Wright. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J. Exp. Med.* **179**:269–277.
17. Hajjar, A. M., D. S. O'Mahony, A. Ozinsky, D. M. Underhill, A. Aderem, S. J. Klebanoff, and C. B. Wilson. 2001. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. *J. Immunol.* **166**:15–19.
18. Hirschfeld, M., Y. May, J. H. Weiss, S. N. Vogel, and J. J. Weiss. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like receptor 2. *J. Immunol.* **165**:618–622.
19. Huang, T. S., S. C. Lee, and J. K. Lin. 1991. Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA* **88**:5292–5296.
20. Kent, L. W., F. Rahemtulla, and S. M. Michalek. 1999. Interleukin (IL)-1 and *Porphyromonas gingivalis* lipopolysaccharide stimulation of IL-6 production by fibroblasts derived from healthy or periodontally diseased human gingival tissue. *J. Periodontol.* **70**:274–282.
21. Kirschning, C. J., H. Wesche, T. Merrill Ayres, and M. Rothe. 1998. Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med.* **188**:2091–2097.
22. Kornman, K. S., R. C. Page, and M. S. Tonetti. 1997. The host response to the microbial challenge in periodontitis: assembling the players. *Periodontol.* **2000** **4**:33–53.
23. Li, Y., and C. C. Rinehart. 1998. Regulation of keratinocyte growth factor expression in human endometrium: implications for hormonal carcinogenesis. *Mol. Carcinog.* **23**:217–225.
24. Lu, W., Y. Luo, M. Kan, and W. L. McKeehan. 1999. Fibroblast growth factor-10: a second candidate stromal to epithelial cell andromedin in prostate. *J. Biol. Chem.* **274**:12827–12834.
25. Mackenzie, I. C., and Z. Gao. 2001. Keratinocyte growth factor expression in human gingival fibroblasts and stimulation of in vitro gene expression by retinoic acid. *J. Periodontol.* **72**:445–453.
26. Matsuguchi, T., K. Takagi, T. Musikacharoen, and Y. Yoshikai. 2000. Gene expressions of lipopolysaccharide receptors, Toll-like receptors 2 and 4, are differently regulated in mouse T lymphocytes. *Blood* **95**:1378–1385.
27. Means, T. K., D. T. Golenbock, and M. J. Fenton. 2000. Structure and function of Toll-like receptor proteins. *Life Sci.* **68**:241–258.
28. Meghji, S., W. Qureshi, B. Henderson, and M. Harris. 1996. The role of endotoxin and cytokines in the pathogenesis of odontogenic cysts. *Arch. Oral Biol.* **41**:523–531.
29. Miki, T., T. P. Fleming, D. P. Bottaro, J. S. Rubin, D. Ron, and S. A. Aaronson. 1991. Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. *Science* **251**:72–75.
30. Muzio, M., and A. Mantovani. 2001. The Toll receptor family. *Allergy* **56**:103–108.
31. Nishida, E., Y. Hara, T. Kaneko, Y. Ikeda, T. Ukai, and I. Kato. 2001. Bone resorption and local interleukin-1 α and interleukin-1 β synthesis induced by *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* lipopolysaccharide. *J. Periodontol. Res.* **36**:1–8.
32. Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc. Natl. Acad. Sci. USA* **97**:13766–13771.
33. Page, R. C., and H. E. Schroeder. 1976. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab. Invest.* **34**:235–249.
34. Putnins, E. E. 2001. Keratinocyte growth factor (KGF): its possible use in tissue engineering, p. 129–138. *In* R. E. Horch, A. M. Munster, and B. M. Achauer (ed.), *Cultured human keratinocytes and tissue engineered skin substitutes*. Thieme, Stuttgart, Germany.
35. Putnins, E. E., J. D. Firth, and V.-J. Uitto. 1995. Keratinocyte growth factor stimulation of gelatinase (matrix metalloproteinase-9) and plasminogen activator in histiotypic epithelial cell culture. *J. Invest. Dermatol.* **104**:989–994.
36. Putnins, E. E., J. D. Firth, and V.-J. Uitto. 1996. Stimulation of collagenase (matrix metalloproteinase-1) synthesis in histiotypic epithelial cell culture by heparin is enhanced by keratinocyte growth factor. *Matrix Biol.* **15**:21–29.
37. Putnins, E. E., J. D. Firth, A. Lohachitranont, V.-J. Uitto, and H. S. Larjava. 1999. Keratinocyte growth factor (KGF) promotes keratinocyte cell attachment and migration on collagen and fibronectin. *Cell Adhes. Commun.* **7**:211–221.
38. Rubin, J. S., D. P. Bottaro, M. Chedid, T. Miki, D. Ron, H.-G. Cheon, W. G. Taylor, E. Fortney, H. Sakata, P. W. Finch, and W. J. LaRochelle. 1995. Keratinocyte growth factor. *Cell Biol. Int.* **19**:399–411.
39. Rubin, J. S., H. Osada, P. W. Finch, W. G. Taylor, S. Rudikoff, and S. A. Aaronson. 1989. Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc. Natl. Acad. Sci. USA* **86**:802–806.
40. Sanaie, A. R., J. D. Firth, V.-J. Uitto, and E. E. Putnins. 2002. Keratinocyte growth factor (KGF)-1 and -2 protein and gene expression in human gingival fibroblasts. *J. Periodontol. Res.* **37**:66–74.
41. Sawai, H., T. Okazaki, H. Yamamoto, H. Okano, Y. Takeda, M. Tashima, H. Sawada, M. Okuma, H. Ishikura, H. Umehara, et al. 1995. Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells. *J. Biol. Chem.* **270**:27326–27331.
42. Schreck, R., B. Meier, D. N. Mannel, W. Droge, and P. A. Baeuerle. 1992. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *J. Exp. Med.* **175**:1181–1194.
43. Sugawara, S., R. Arakaki, H. Rikiishi, and H. Takada. 1999. Lipoteichoic acid acts as an antagonist and an agonist of lipopolysaccharide on human gingival fibroblasts and monocytes in a CD14-dependent manner. *Infect. Immun.* **67**:1623–1632.
44. Sugawara, S., A. Sugiyama, E. Nemoto, H. Rikiishi, and H. Takada. 1998.

- Heterogeneous expression and release of CD14 by human gingival fibroblasts: characterization and CD14-mediated interleukin-8 secretion in response to lipopolysaccharide. *Infect. Immun.* **66**:3043–3049.
45. Tabeta, K., K. Yamazaki, S. Akashi, K. Miyake, H. Kumada, T. Umemoto, and H. Yoshie. 2000. Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. *Infect. Immun.* **68**:3731–3735.
 46. Takata, T., M. Miyauchi, I. Ogawa, H. Ito, J. Kobayashi, and H. Nikai. 1997. Reactive change in proliferative activity of the junctional epithelium after topical application of lipopolysaccharide. *J. Periodontol.* **68**:531–535.
 47. Takeshita, A., Y. Chen, A. Watanabe, S. Kitano, and S. Hanazawa. 1995. TGF- β induces expression of monocyte chemoattractant JE/monocyte chemoattractant protein 1 via transcriptional factor AP-1 induced by protein kinase in osteoblastic cells. *J. Immunol.* **155**:419–426.
 48. Tang, A., and B. A. Gilchrist. 1996. Regulation of keratinocyte growth factor gene expression in human skin fibroblasts. *J. Dermatol. Sci.* **11**:41–50.
 49. Uitto, V.-J., K. Airola, M. Vaalamo, N. Johansson, E. E. Putnins, J. D. Firth, J. Salonen, C. Lopez-Otin, U. Saarialho-Kere, and V. M. Kahari. 1998. Collagenase-3 (matrix metalloproteinase-13) expression is induced in oral mucosal epithelium during chronic inflammation. *Am. J. Pathol.* **152**:1489–1499.
 50. Wang, P.-L., M. Oido-Mori, T. Fujii, Y. Kowashi, M. Kikuchi, Y. Suetsugu, J. Tanaka, Y. Azuma, M. Shinohara, and K. Ohura. 2001. Heterogenous expression of Toll-like receptor 4 and downregulation of Toll-like receptor 4 expression on human gingival fibroblasts by *Porphyromonas gingivalis* lipopolysaccharide. *Biochem. Biophys. Res. Commun.* **288**:863–867.
 51. Wang, P.-L., K. Sato, M. Oido, T. Fujii, Y. Kowashi, M. Shinohara, K. Ohura, H. Tani, and Y. Kuboki. 1998. Involvement of CD14 on human gingival fibroblasts in *Porphyromonas gingivalis* lipopolysaccharide-mediated interleukin-6 secretion. *Arch. Oral Biol.* **43**:687–694.
 52. Watanabe, A., A. Takeshita, S. Kitano, and S. Hanazawa. 1996. CD14-mediated signal pathway of *Porphyromonas gingivalis* lipopolysaccharide in human gingival fibroblasts. *Infect. Immun.* **64**:4488–4494.
 53. Werner, S. 1998. Keratinocyte growth factor: a unique player in epithelial repair processes. *Cytokine Growth Factor Rev.* **9**:153–165.
 54. Werner, S., K. G. Peters, M. T. Longaker, F. Fuller-Pace, M. J. Banda, and L. T. Williams. 1992. Large induction of keratinocyte growth factor expression in the dermis during wound healing. *Proc. Natl. Acad. Sci. USA* **89**:6896–6900.
 55. Wyllie, D. H., E. Kiss-Toth, A. Visintin, S. C. Smith, S. Boussouf, D. M. Segal, G. W. Duff, and S. K. Dower. 2000. Evidence for an accessory protein function for Toll-like receptor 1 in antibacterial responses. *J. Immunol.* **165**:7125–7132.
 56. Xu, Y. X., K. R. Pindolia, N. Janakiraman, R. A. Chapman, and S. C. Gautam. 1997–1998. Curcumin inhibits IL1 α and TNF- α induction of AP-1 and NF- κ B DNA-binding activity in bone marrow stromal cells. *Hematopathol. Mol. Hematol.* **11**:49–62.
 57. Yamasaki, M., A. Miyake, S. Tagashira, and N. Itoh. 1996. Structure and expression of the rat mRNA encoding a novel member of the fibroblast growth factor family. *J. Biol. Chem.* **271**:15918–15921.
 58. Yang, R.-B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* **395**:284–288.
 59. Yang, R.-B., M. R. Mark, A. L. Gurney, and P. J. Godowski. 1999. Signaling events induced by lipopolysaccharide-activated Toll-like receptor 2. *J. Immunol.* **163**:639–643.
 60. Zhang, F. X., C. J. Firschnig, R. Mancinelli, X. P. Xu, Y. Jin, E. Faure, A. Mantovani, M. Rother, M. Muzio, and M. Arditi. 1999. Bacterial lipopolysaccharide activates nuclear factor- κ B through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J. Biol. Chem.* **274**:7611–7614.
 61. Zhang, G., and S. Ghosh. 2001. Toll-like receptor-mediated NF- κ B activation: a phylogenetically conserved paradigm in innate immunity. *J. Clin. Invest.* **107**:13–19.

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