The Effects of Tumor Necrosis Factor-α, Interleukin-1β, Interleukin-6, and Transforming Growth Factor-β1 on Pulp Fibroblast Mediated Collagen Degradation

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Abstract

Dental pulp destruction is believed to be regulated, in part, by the matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs). Cytokines are believed to be important in the pathogenesis of pulpitis. This study examined the effects that TNF-α, IL-1β, IL-6, and TGF-β1 have on the collagen degradation mediated by pulp fibroblasts utilizing a cell-mediated collagen degradation assay. Reverse transcriptase-polymerase chain reaction, Western blot analyses, and zymography were utilized to examine multiple MMPs and TIMPs. The collagen degradation mediated by these cells was stimulated by these cytokines. TNF-α, IL-1β, and IL-6 increased the mRNA and/or protein expression of MMP-1, MMP-2, and MMP-3. TGF-β1 decreased MMP-1 mRNA expression, while only slightly affecting the MMP-2 and MMP-3 mRNA and/or protein. These cytokines did not affect the expression of TIMP-1 or TIMP-2. These results suggest that these cytokines affect pulp destruction, in part, by differentially regulating the MMPs and TIMPs. *(J Endod 2006;32: 853–861)*

Key Words

Collagen degradation, cytokines, dental pulp fibroblasts, matrix metalloproteinases

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Several cytokines have been detected in dental pulp during inflammation. These include interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-10, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β1 (1, 2). These cytokines are believed to be important in the pathogenesis of pulpitis. The production of these cytokines is stimulated by bacteria lipopolysaccharides associated with pulpal diseases. In contrast, some bacteria associated with endodontic treatment failure such as *Enterococcus faecalis* inhibits IL-2 and IL-4 production by activated T lymphocytes (3).

High levels of IL-1β and TNF-α have been detected in inflamed dental pulps and periapical lesions (1, 4). The quantity of IL-1β in diseased dental pulps was reported to be 2.5-fold greater than that detected in healthy pulp tissues (5). It has been demonstrated that the production of IL-1β from human dental pulp cells is stimulated by lipopolysaccharide from *Porphyromonas endodontalis* (6). TNF-α has been shown to induce the expression of tissue plasminogen activator (7) and cyclooxygenase-2 by human pulp cells (8). IL-1 and TNF-α have been shown to stimulate the production of MMP-1 by human pulp cells (9). A previous study also showed that human pulp cells treated with IL-1 or TNF-α stimulated the production of MMP-2 and MMP-9 by human pulp cells in long-term culture (10). However, these cytokines displayed no change in the concentrations of MMP-2 and MMP-9 during short-term culture (11). These studies suggest that IL-1β and TNF-α are involved in the inflammation of dental pulp.

Recently, significant quantities of IL-6 have been detected in the pulp tissue of patient with pulpitis (2) and periapical lesions (12). Several studies have shown that human dental pulp cells are able to produce a significant amount of IL-6 when stimulated with peptidoglycans from *Lactobacillus casei* (13), supernatant from *Porphyromonas gingivalis*, or supernatant from *Prevotella intermedia* (14). The production of IL-6 from human dental pulp cells is also enhanced by proinflammatory cytokines (14) and sensory neuropeptides (15). Moreover, it has been reported that IL-6 increases the plasminogen activator activity of dental pulp cells (16). This suggests that IL-6 may be involved in the extracellular matrix degradation that occurs during pulp inflammation.

TGF-β is the prototypic member of the transforming growth factor superfamily and is expressed by a wide variety of cell types. Three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) have been identified in mammals. TGF-β1, the only isoform detected in human dentin, is known to play a role in tooth development and in modulating the response of the dentin-pulp complex during dental tissue repair by inducing odontoblast and pulpal cell proliferation and differentiation, as well as by stimulating reparative dentinogenesis (17). Recently, an increase in TGF-β1 expression has been detected in the odontoblastic-subodontoblastic layer of irreversible pulpitis when compared to healthy pulp (18). TGF-β1 has been shown to induce the accumulation of dendritic cells in the odontoblast layer, which suggests that TGF-β1 plays a role in the immune response of dental pulp (19). Several studies have also reported that TGF-β1 differentially regulates the expression of MMPs in odontoblasts and dental pulp cells (20, 21).

A characteristic of periapical lesions and suppurative pulpitis is the degradation of the extracellular matrix. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases responsible for the degradation of multiple extracellular matrix components in both physiological and pathological conditions (22). Their activities are regulated, in part, by their interaction with the tissue inhibitors of matrix metalloproteinases (TIMPs). Collagenolytic and elastinolytic activities have been detected in ne-

crotic pulp, while neither enzymatic activities have been detected in healthy pulp (23). Significant levels of MMP-1, MMP-2, and MMP-3 have been detected in acute and chronic inflamed pulps, as well as in periapical lesions when compared with healthy tissues (24).

Inflammatory cytokines are known to play a major role in regulating MMP/TIMP expression in several inflammatory diseases. The stimulatory effect of IL-1α on collagen degradation by dental pulp fibroblasts (DPF) has been reported (25). Moreover, this cytokine alters the balance between the MMPs and TIMPs, as well as increases MMP activation. In this study, the effects of TNF-α, IL-1β, IL-6, and TGF-β1 on cell mediated collagen degradation and their effects on the MMP/TIMP expression from DPF were examined. This expands what is known about the roles that these cytokines play in regulating dental pulp tissue destruction.

Materials and Methods

DPF Culture

DPF were cultured from permanent noncarious teeth extracted from healthy young adults for orthodontic reasons with Institutional Review Board approval as previously described (25). Briefly, the teeth were placed in sterile 0.01-M phosphate buffered saline immediately after extraction and then cut in half horizontally under sterile conditions. The dental pulp tissue was then removed from the teeth, minced, and cultured in Dulbecco's Modified Essential Media (DMEM; Hyclone, Logan, UT) supplemented with 10% bovine growth serum (HyClone), 0.25 μg/ml fungizone (Mediatech, Inc., Herndon, VA), and antibiotics (100 unit/ml Penicillin and 100 μg/ml Streptomycin; Irvine Scientific, Santa Ana, CA). The dental pulp tissue was then incubated at 37°C in a humidified atmosphere of 5% CO_2. DPF cultures were established from the cells growing out of the dental pulp tissue. DPF at early passages were utilized in these studies. The cells used in this study have been defined as "dental pulp fibroblast" on the basis of cell morphology and the ability of these cells to express vimentin as previously described (25).

Cell Mediated Collagen Degradation

Subconfluent DPF were trypsinized (Invitrogen Life Technologies, Carlsbad, CA), resuspended in media, and seeded as single colonies (100,000 cells/150 μl per well) in six-well tissue culture plates coated with 1.5 ml of rat-tail tendon type I collagen (300 μg/ml) per well. After allowing the cells to attach for 2-3 hr, the cells were incubated in serum-free DMEM without or with serial dilutions of recombinant human TNF-α (0.1, 1, 5, 10, or 20 ng/ml; Genentech, Inc., San Francisco, CA), IL-1β (0.025, 0.050, 0.100, 1, or 10 ng/ml; Chemicon, Temecula, CA), IL-6 (0.1, 1, 5, 10, or 20 ng/ml; Chemicon), or TGF-β1 (0.1, 1, 5, 10, or 20 ng/ml; Fitzgerald Industries International, Inc., Concord, MA). The DPF were also incubated with 1 μM of a MMP inhibitor (GM6001; Chemicon) alone or in combination with TNF-α, IL-1β, IL-6, or TGF-β1.

After 4, 6, and 8 days of incubation, the cells were removed with 500 μl of 1% Triton X-100 and 200 μl of 2.5% trypsin. The plates were then stained with Coomassie blue to visualize the collagen degradation. The degradation of the collagen was estimated by the NIH image program (Version 1.62). The results were expressed as a relative ratio between the degraded collagen areas from the TNF-α, IL-1β, IL-6, or TGF-β1 treated cells to that of the untreated cells for each incubation period. The experiments were repeated at least three times to ensure reproducibility.

The effects of cytokine combinations on the collagen degrading ability of the DPF were also examined. The DPF were treated with cytokine combinations of TNF-α (5 ng/ml), IL-1β (1 ng/ml), IL-6 (10 ng/ml), and TGF-β1 (10 ng/ml) with or without 1 μM of GM6001. After 2 days of incubation, the collagen degradation was visualized by staining with Coomassie blue.

Cytokine Treatment and Reverse Transcriptase-Polymerase Chain Reaction

Subconfluent DPF were incubated for 24 h with or without TNF-α (5 ng/ml), IL-1β (1 ng/ml), IL-6 (10 ng/ml), or TGF-β1 (10 ng/ml) in serum-free DMEM. Total cellular RNA was then extracted from the DPF after the RNeasy Mini Protocol (Qiagen Inc., Valencia, CA). The RNA yield was measured by absorbance at 260 nm and stored at −70°C. Reverse transcriptase-polymerase chain reactions (RT-PCR) were performed according to the manufacturer's instructions in a single reaction containing One-step RT-PCR mix (Qiagen Inc.), the mRNA template, and RT-PCR primers as previously described (25). The RT-PCR products were resolved in 1.0% (w/v) agarose gels, stained with ethidium bromide, and photographed under UV illumination. To estimate the amount of the RT-PCR products, the density of the product bands detected was measured using the NIH image program (version 1.62). The density of the band corresponding to a particular MMP or TIMP was expressed as a ratio relative to the density of the band from the cytokine treated samples compared to that from the control sample after standardizing the amount of product with the internal control (cyclophilin) as previously described (25).

Cell Membrane Extraction and Conditioned Media

DPF were grown in a 100 × 20 mm tissue culture dishes coated with 7.5 ml of rat-tail tendon type I collagen (300 μg/ml). Subconfluent DPF were then incubated with or without TNF-α (5 ng/ml), IL-1β (1 ng/ml), IL-6 (10 ng/ml), or TGF-β1 (10 ng/ml) in serum-free DMEM. After 8 days of incubation, the conditioned serum-free media was collected and concentrated 10- to 20-fold by Centripep centrifugal filters (Millipore, Bedford, MA). The cell membrane proteins were extracted from the DPF after the Mem-PER mammalian membrane protein extraction protocol (Pierce, Rockford, IL). The concentrations of the total protein in the concentrated media and cell membrane extracts were determined according to the Bio-Rad Protein Assay protocol (Bio-Rad Laboratories, Hercules, CA). The same amount of total protein of the untreated and TNF-α, IL-1β, IL-6, or TGF-β1 treated samples were used in the following experiments.

Gelatin Zymography

The gelatinolytic activity of the conditioned media was assayed by gelatin zymography. Half of the concentrated media samples were mixed with an equal volume of 2× nonreducing sample buffer. The same amount of total protein of the untreated and treated concentrated media were then resolved in 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis gels copolymerized with 1% gelatin. After electrophoresis, the sodium dodecylsulfate was extracted from the gels with 2.5% Triton X-100 and the gels were then incubated in 0.05 M Tris (pH 7.5), 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ at 37°C overnight. The gels were then stained with Coomassie blue and destained. The gelatinolytic activities were detected as clear bands against the blue background of the stained zymogram.

Western Blot Analyses

The remaining concentrated samples and cell membrane extracts were mixed with 6× reducing sample buffer and boiled at 100°C for 15 min. The same amount of total protein of untreated and TNF-α, IL-1β, IL-6, or TGF-β1 treated concentrated media and cell membrane extracts were resolved in 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis gels and then electroblotted to polyvinylidene fluoride