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Interleukin-6 Released from Fibroblasts Is Essential for Up-regulation of Matrix Metalloproteinase-1 Expression by U937 Macrophages in Coculture

CROSS-TALKING BETWEEN FIBROBLASTS AND U937 MACROPHAGES EXPOSED TO HIGH GLUCOSE[*]

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Matrix metalloproteinases (MMPs) play a key role in periodontal disease. Although it is known that macrophages and fibroblasts are co-localized and express MMPs in the diseased periodontal tissue, the effect of interaction between these two cell types on MMP expression has not been well elucidated. Furthermore although it is known that diabetes is associated with accelerated periodontal tissue destruction, it remains unknown whether hyperglycemia, a major metabolic abnormality in diabetes, regulates MMP expression by affecting the cross-talking between fibroblasts and macrophages. In this study, human gingival fibroblasts and U937 macrophages were cocultured in a two-compartment transwell culture system, and the cells were treated with normal or high glucose. We found that coculture of fibroblasts and U937 macrophages led to an augmentation of MMP-1 expression by U937 macrophages, and high glucose further enhanced this augmentation. Similar observations were also made in the coculture of fibroblasts and human primary monocytes. We also found that interleukin 6 (IL-6) released by fibroblasts was essential for the augmentation of MMP-1 expression by U937 macrophages. Furthermore our results showed that high glucose, IL-6, and lipopolysaccharide had a synergistic effect on MMP-1 expression. Finally our study indicated that MAPK pathways and activator protein-1 transcription factor were involved in the coculture- and high glucose-augmented MMP-1 expression. In conclusion, this study demonstrates that IL-6 derived from fibroblasts is essential for MMP-1 up-regulation by cross-talking between fibroblasts and U937 macrophages exposed to high glucose, revealing an IL-6-dependent mechanism in MMP-1 up-regulation.

Periodontal disease is characterized by inflammation of periodontal tissues, eventually leading to degeneration of the periodontium (1–3). Matrix metalloproteinases (MMPs),[3] a family of proteolytic enzymes that degrade collagen and other matrix proteins including elastin, fibronectin, proteoglycan, and laminin, play an essential role in the periodontal tissue destruction (4, 5). MMPs are expressed in inflamed periodontal tissue by inflammatory cells including monocytes, macrophages, lymphocytes, and polymorphonuclear cells and also by resident cells such as fibroblasts, epithelial cells, and endothelial cells (6, 7). Lipopolysaccharide (LPS) derived from Gram-negative bacteria, the major pathogens involved in periodontal disease, is a potent stimulator for MMP expression (1).

It has been well established that patients with either type 1 or type 2 diabetes have increased prevalence and severity of periodontal disease (8). Considering the crucial role of MMPs in periodontal disease, it was believed that the periodontal MMP expression was increased in patients with diabetes, leading to an increase in tissue destruction (8). Indeed studies have shown that the periodontal MMP expression is higher in patients with both diabetes and periodontal disease than in those with periodontal disease alone. For example, it was reported that MMP-8 and MMP-9 expression was significantly increased in the gingival tissue of diabetic patients with chronic periodontitis (9). Our recent study showed a trend of increase in MMP-1 expression in periodontal tissues across patients with neither diabetes nor periodontal disease, patients with periodontal disease alone, and patients with both diseases (10). In our effort to understand the mechanisms involved in diabetes-promoted MMP expression, we demonstrated that elevated glucose concentration (high glucose) augmented LPS-stimulated MMP expression in macrophages by enhancing LPS-triggered signaling and transcriptional activity (11, 12). We further demonstrated that lactate, which is associated with hyperglycemia and increased in plasma and saliva of diabetic patients (13, 14), also

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[3] The abbreviations used are: MMP, matrix metalloproteinase; IL, interleukin; AP, activator protein; MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; TIMP, tissue inhibitor of metalloproteinases; cDNA, complementary DNA; siRNA, short interfering RNA; EMSA, electrophoretic mobility shift assay; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; STAT, signal transducers and activators of transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

had a synergistic effect with LPS on MMP expression (15). These studies revealed a molecular mechanism potentially involved in periodontal disease in diabetic patients.

Both macrophages and gingival fibroblasts are present in periodontitis-inflamed periodontal tissue (16, 17), and their interaction has been shown to increase MMP expression (18, 19). However, the underlying mechanism has not been investigated. Furthermore it is unclear whether hyperglycemia alters MMP expression regulated by the interaction between macrophages and fibroblasts. In the current study, we demonstrated that coculture of U937 human histiocytes (resident macrophages) and human gingival fibroblasts in a two-compartment transwell coculture system led to an augmentation of MMP-1 expression, and IL-6 released by fibroblasts played an essential role in the augmentation. We also demonstrated that high glucose further enhanced the augmentation of MMP-1 expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment with LPS—Human monocytes were isolated as described previously (20) from blood obtained from healthy donors. U937 histiocytes (21) and human gingival fibroblasts were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in a 5% CO_2 atmosphere in RPMI 1640 medium (Invitrogen) containing normal glucose (5 mM) or high glucose (25 mM), 10% fetal calf serum, 1% minimum Eagle's medium non-essential amino acid solution, and 0.6 g/100 ml HEPES either independently or in Corning Transwell plates (Sigma) that have two compartments separated by a polycarbonate membrane with 0.4-μm pores. Fibroblasts were grown to 80% confluence in the lower compartment, and U937 cells or human monocytes were grown (0.5×10^6 cells/ml) in the upper compartment. For cell treatment, LPS from *E. coli* was used (Sigma). The LPS was highly purified by phenol extraction and gel filtration chromatography and was cell culture-tested. We compared the potency of this LPS with that of LPS isolated from *Aggregatibacter actinomycetemcomitans*, a kind gift provided by Dr. Edward Lally, University of Pennsylvania, in the stimulation of MMP-1 secretion by U937 cells. Results showed that *A. actinomycetemcomitans* LPS was more potent than *E. coli* LPS at concentrations of 5, 10, and 50 ng/ml but had no difference at 100 ng/ml (Fig. 1).

Enzyme-linked Immunosorbent Assay (ELISA)—MMPs, tissue inhibitors of metalloproteinases (TIMPs), and IL-6 in conditioned medium were quantified using sandwich ELISA kits according to the protocol provided by the manufacturer (R&D Systems, Minneapolis, MN).

Real Time PCR—Total RNA was isolated from cells using the RNeasy minikit (Qiagen, Santa Clarita, CA). First strand complementary DNA (cDNA) was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad) using 20 μl of reaction mixture containing 0.25 μg of total RNA, 4 μl of 5× iScript reaction mixture, and 1 μl of iScript reverse transcriptase. The complete reaction was cycled for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C using a PTC-200 DNA Engine (MJ Research, Waltham, MA). The reverse transcription reaction mixture was then diluted 1:10 with nuclease-free water and used for PCR amplification of MMP cDNA in the presence of the primers. The Beacon designer software (PREMIER Biosoft International, Palo Alto, CA) was used for primer designing. Primers for MMP-1 (5′ primer, CTGGGAAGCCATCACTTACCTTGC; 3′ primer, GTTTCTAGAGTCGCTGGGAAGCTG) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and real time PCR was performed in duplicate using 25 μl of reaction mixture containing 1.0 μl of reverse transcription mixture, 0.2 μM both primers, and 12.5 μl of iQ™ SYBR Green Supermix (Bio-Rad). Real time PCR was run in the iCycler™ real time detection system (Bio-Rad) with a two-step method. The hot start enzyme was activated (95 °C for 3 min), and cDNA was then amplified for 40 cycles consisting of denaturation at 95 °C for 10 s and annealing/extension at 53 °C for 45 s. A melt curve assay was then performed (55 °C for 1 min and then the temperature was increased by 0.5 °C every 10 s) to detect the formation of primer-derived trimers and dimers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5′ primer, GAATTTGGCTACAGCAACAGGGTG; 3′ primer, TCTCTTCCTCTTGTGCTCTTGCTG) served as a control. Data were analyzed with the iCycler iQ™ software. The average starting quantity of fluorescence units was used for analysis. Quantification was calculated using the starting quantity of the cDNA of interest relative to that of GAPDH cDNA in the same sample.

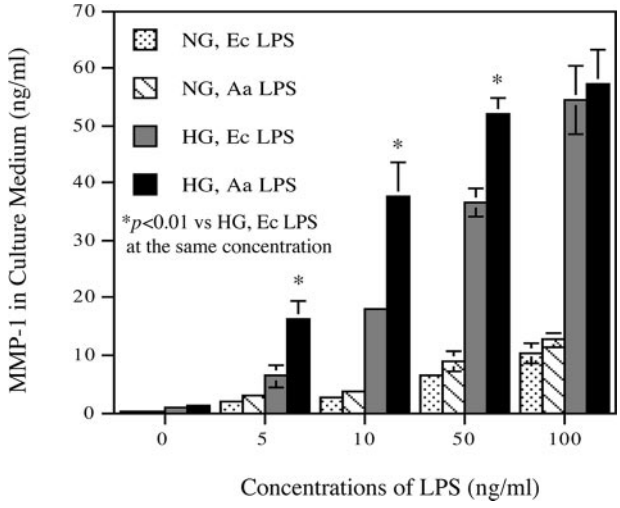


FIGURE 1. **Comparison of the potency of LPS isolated from *E. coli* (*Ec*) with that isolated from *A. actinomycetemcomitans* (*Aa*) in stimulation of MMP-1 secretion.** U937 cells preexposed to normal glucose (*NG*) or high glucose (*HG*) were treated with different concentrations of LPS isolated from *E. coli* or *A. actinomycetemcomitans* for 24 h. After the treatment, the conditioned medium was collected for ELISA to quantify the secreted MMP-1. Data presented are representative of three experiments with similar results. *Error bars* represent mean ± S.D.

IL-6 siRNA Transfection—Human gingival fibroblasts were transfected with 200 nM stealth siRNA directed against IL-6 (CAGACAGCCACUCACCUCUUCAGAA) or control siRNA (CAGGACCCACUCAUCUCCUACAGAA) (GenBank accession number NM 000600; Invitrogen) using Lipofectamine 2000 (Invitrogen) as transfection reagent according to the manufacturer's instruction. After the transfection for 24 h, cells were cultured independently or cocultured with U937 cells in the presence or absence of LPS.

PCR Array—The first strand cDNA was synthesized from RNA using the RT^2 First Strand Kit (SuperArray Bioscience Corp., Frederick, MD). Human inflammatory cytokines and

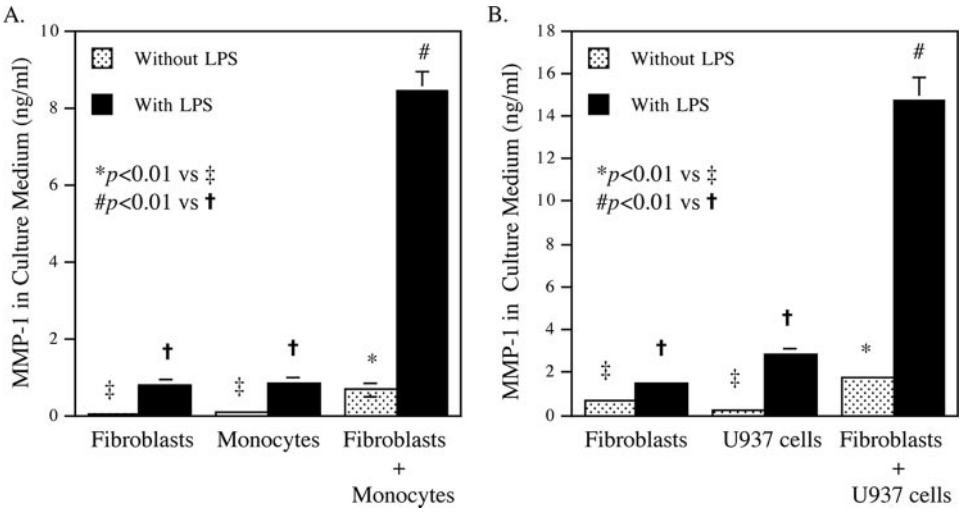


FIGURE 2. **The similarity between the coculture of human monocytes/fibroblasts and U937 macrophages/fibroblasts in the augmentation of MMP-1 secretion.** Human monocytes (*A*) or U937 cells (*B*) were cultured independently or cocultured with human fibroblasts in the absence or presence of 100 ng/ml LPS for 24 h. After the treatment, culture medium was collected for quantification of MMP-1. Cell numbers per well for human monocytes and U937 cells were the same (1×10^6/well). The data (mean ± S.D.) are from one of three independent experiments with similar results. *Error bars* represent mean ± S.D.

receptor PCR array (catalog number PAHS-011, SuperArray Bioscience Corp.) was used to profile the cytokine expression by fibroblasts in the coculture by following the instructions from the manufacturer.

Extraction of Nuclear Proteins—Nuclear protein was extracted using the NE-PER™ nuclear and cytoplasmic extraction kit (Pierce). The concentration of protein was determined using a protein assay kit (Bio-Rad).

AP-1 and NFκB Activity Assays—Two micrograms of nuclear protein of each sample was applied to the assay for AP-1 and NFκB activities using TransAM kits produced by Active Motif (Carlsbad, CA) according to the protocol provided by the manufacturer. These kits contain a 96-stripwell plate to which the consensus-binding site oligonucleotides were immobilized. TransAM assays are up to 100 times more sensitive than gel mobility shift assay and detect transcription factors with specific antibodies.

Electrophoretic Mobility Shift Assay (EMSA)—Ten micrograms of nuclear proteins was used for EMSA to determine AP-1 DNA binding activity. DNA-protein binding reactions were performed at room temperature for 20 min in a buffer containing 10 mM Trizma (Tris base), pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 μg of poly(dI-dC), 5% (v/v) glycerol, and ~0.3 pmol of AP-1 oligonucleotide (Promega, Madison, WI) labeled with digoxigenin-ddUTP using terminal deoxynucleotidyltransferase (Roche Applied Science). Protein-DNA complexes were resolved from protein-free DNA in 5% polyacrylamide gels in 50 mM Tris, pH 8.3, 0.38 M glycine, and 2 mM EDTA at room temperature and electroblotted onto positively charged nylon membranes. The chemiluminescence detection of digoxigenin-labeled probes was conducted by following the instructions provided by the Roche Applied Science. For competition studies, unlabeled AP-1 oligonucleotides that were in 30-fold excess of the labeled AP-1 oligonucleotides were added to the reaction mixture. Supershift assays were performed by adding 1 μg of anti-c-Jun antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) to the reaction mixture. Nuclear factor of activated T-cells antibody (Santa Cruz Biotechnologies) was used as a control antibody.

Statistic Analysis—Data are presented as mean ± S.D. Student's *t* tests were performed to determine the statistical significance of MMP expression among different experimental groups. A value of $p < 0.05$ was considered significant.

RESULTS

The Coculture of Fibroblasts and U937 Macrophages Was Similar to That of Fibroblasts and Human Monocytes in Its Capacity of Stimulating MMP-1 Secretion—It was reported previously that when fibroblasts were cocultured with human primary monocytes MMP-1 secretion was stimulated (18). Thus, we first determined whether the coculture of fibroblasts and U937 cells also stimulated MMP-1 secretion. Results showed that the coculture of fibroblasts and U937 cells in the absence or presence of LPS was similar to that of fibroblasts and human monocytes in stimulation of MMP-1 secretion (Fig. 2, *A* and *B*). The amount of MMP-1 secreted by the coculture of fibroblasts with either human monocytes or U937 cells was much more than the sum of MMP-1 released by independent culture of fibroblasts and that of monocytes or U937 cells.

High Glucose Enhances Fibroblast and U937 Macrophage Interaction-stimulated MMP-1 Expression in the Absence or Presence of LPS—In these experiments, we studied the effect of high glucose on the fibroblast/U937 cell coculture-stimulated MMP-1 secretion. Results (Fig. 3*A*) showed that in the absence of LPS the coculture exposed to normal glucose led to a significant augmentation of MMP-1 secretion as compared with the independent culture of fibroblasts or U937 macrophages (1.71 *versus* 0.67 ng/ml for fibroblasts and 0.22 ng/ml for U937 macrophages). Although the coculture exposed to high glucose also had a marked augmentation of MMP-1 secretion compared with the independent culture of fibroblasts or U937 macrophages (5.49 *versus* 0.58 ng/ml for fibroblasts and 0.98 ng/ml for U937 cells) (Fig. 3*A*), it is noteworthy that the coculture exposed to high glucose had a 3-fold increase in MMP-1 secretion when compared with that exposed to normal glucose (5.49 *versus* 1.71 ng/ml) (Fig. 3*A*).

LPS stimulated MMP-1 secretion from both fibroblasts and U937 macrophages, although the stimulatory effect was more potent in U937 macrophages (Fig. 3*A*). In the presence of LPS, the coculture of fibroblasts and U937 macrophages exposed to normal glucose also markedly augmented MMP-1 secretion as compared with the independent culture of fibroblasts or U937 macrophages (14.71 *versus* 1.47 ng/ml for fibroblasts or 2.8 ng/ml for U937 cells) (Fig. 3*A*), and high glucose further increased MMP-1 secretion by about 3-fold when compared

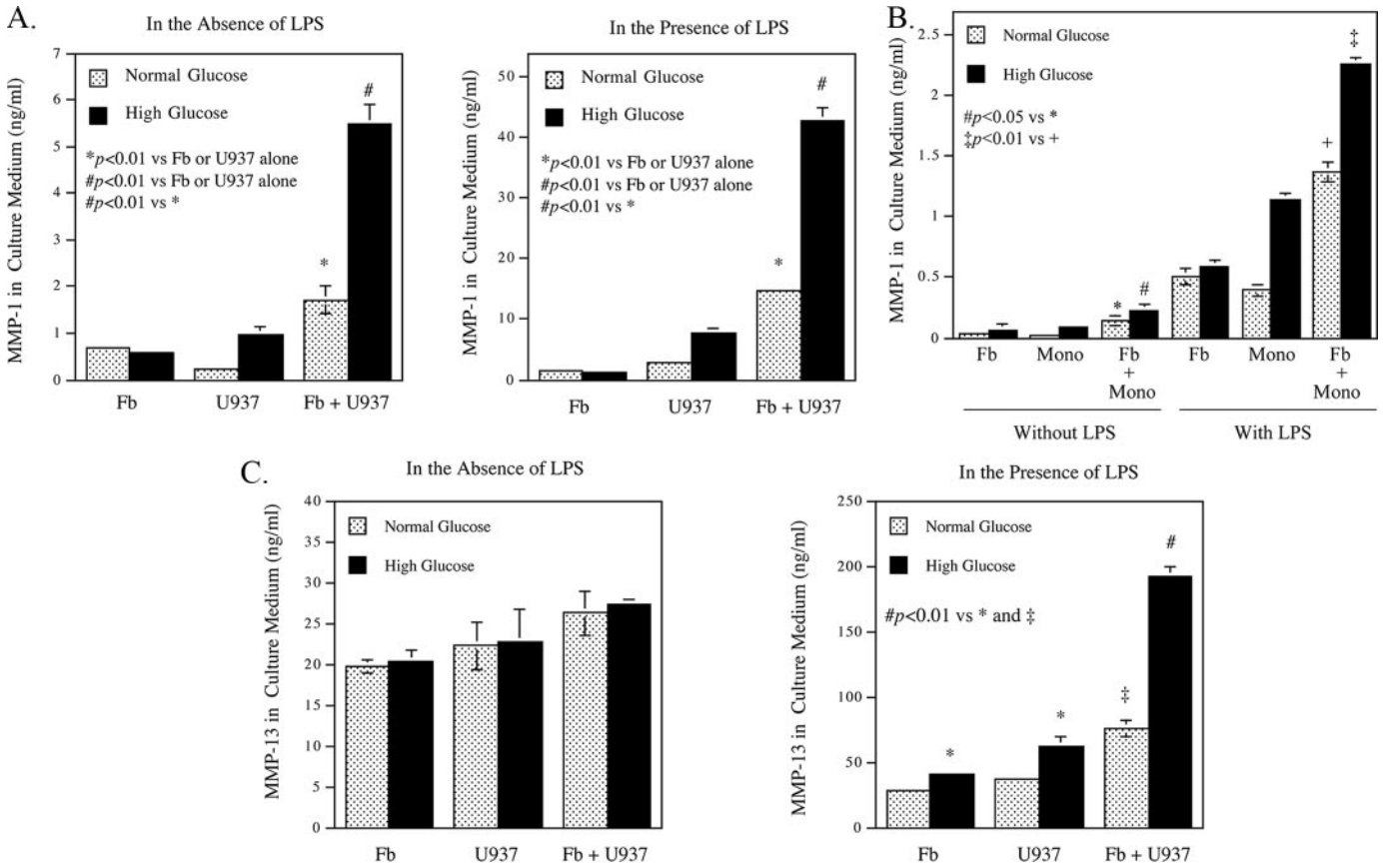


FIGURE 3. **The increased MMP-1 secretion by coculture of fibroblasts with U937 macrophages and further enhancement of the coculture-increased MMP-1 secretion by high glucose.** Human gingival fibroblasts and U937 macrophages in the independent cultures or cocultures preexposed to normal (5 mM) or high glucose (25 mM) were challenged with or without 100 ng/ml LPS. After 24 h, medium was collected to quantify MMPs using ELISA. *A*, the stimulatory effect of the coculture and high glucose on MMP-1 secretion in the absence or presence of LPS. *B*, the stimulatory effect of the coculture of human monocytes and fibroblasts and high glucose on MMP-1 secretion in the absence or presence of LPS. An experiment similar to that described above was performed using human monocytes. *Mono*, monocytes, *Fb*, fibroblasts. *C*, the effect of the coculture and high glucose on MMP-13 secretion in the absence or presence of LPS. The data (mean ± S.D.) are from one of three independent experiments with similar results. *Error bars* represent mean ± S.D.

with normal glucose (42.75 *versus* 14.75 ng/ml). These results showed that although the coculture augmented MMP-1 secretion high glucose further increased the augmentation of MMP-1 secretion by the coculture.

The effect of high glucose on MMP-1 secretion was also examined in the coculture of human monocytes and fibroblasts. Results showed that, similar to the coculture of U937 cells and fibroblasts, high glucose significantly increased MMP-1 secretion from the coculture of monocytes and fibroblasts (Fig. 3*B*). Furthermore results showed that the independent culture of monocytes and U937 cells had similar responses to high glucose in MMP-1 secretion (Fig. 3, *A* and *B*).

The effect of the coculture and high glucose on MMP-13, MMP-2, TIMP-1, and TIMP-2 secretion was also investigated. Results showed that the coculture in the absence of LPS did not increase MMP-13 secretion as compared with the independent culture of fibroblasts or U937 macrophages, and the coculture in the presence of LPS increased MMP-1 secretion only when the cells were exposed to high glucose (Fig. 3*C*). The coculture or high glucose did not increase MMP-2, TIMP-1, and TIMP-2 secretion (data not shown). These results indicate that the genes targeted for up-regulation by the coculture are highly selective.

Fig. 4*A* shows glucose concentration-dependent stimulation of MMP-1 secretion by the coculture of fibroblasts and U937 cells. In the presence of LPS, glucose at 10, 15, and 25 mM increased MMP-1 secretion by 1.4-, 1.8-, and 3.1-fold, respectively, as compared with cells treated with 5 mM glucose. A similar effect of high glucose on MMP-1 secretion was observed in the coculture of human monocytes and fibroblasts (Fig. 4*B*). Glucose at 15 mM increased MMP-1 secretion from the coculture of monocytes and fibroblasts by 2-fold as compared with glucose at 5 mM either in the absence or in the presence of LPS. Fig. 4*C* shows that 5 mM glucose plus 20 mM mannitol had no effect on MMP-1 secretion from the coculture of fibroblasts and U937 cells as compared with 5 mM glucose, suggesting that the stimulation of MMP-1 secretion by 25 mM glucose is not due to increased osmolarity.

U937 Macrophages, but Not Fibroblasts, Had Increased MMP-1 mRNA Expression in Coculture—To determine which type of cells, fibroblasts or U937 macrophages, had increased MMP-1 expression that contributed to the increased MMP-1 level in the coculture system, MMP-1 mRNA in both fibroblasts and U937 cells was quantified using real time PCR. Results showed that as compared with the independent fibroblast culture fibroblasts in the cocultured exposed to either normal or

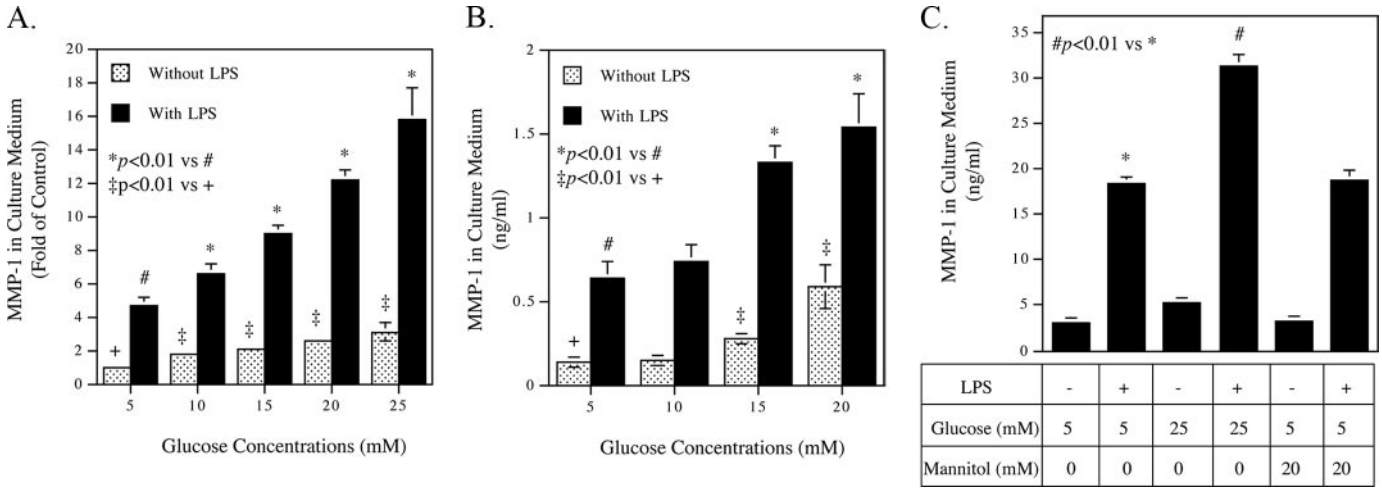


FIGURE 4. **The effect of increasing glucose concentrations and mannitol on MMP-1 secretion by coculture of fibroblasts and U937 macrophages or human monocytes.** *A*, the cocultures of fibroblasts and U937 cells were treated with increasing concentrations of glucose (5–25 mM) in the absence or presence of 100 ng/ml LPS for 24 h. After the treatment, culture medium was collected for quantification of MMP-1 using ELISA. The coculture treated with 5 mM glucose without LPS was designated as control. All data are presented as -fold of the control. *B*, an experiment similar to that described above was performed in the coculture of fibroblasts and human monocytes. *C*, the cocultures of fibroblasts and U937 macrophages were treated with 5 mM glucose, 25 mM glucose, or 5 mM glucose plus 20 mM mannitol in the absence or presence of 100 ng/ml LPS for 24 h. After the treatment, culture medium was collected for quantification of MMP-1. The data (mean ± S.D.) are from one of two independent experiments with similar results. *Error bars* represent mean ± S.D.

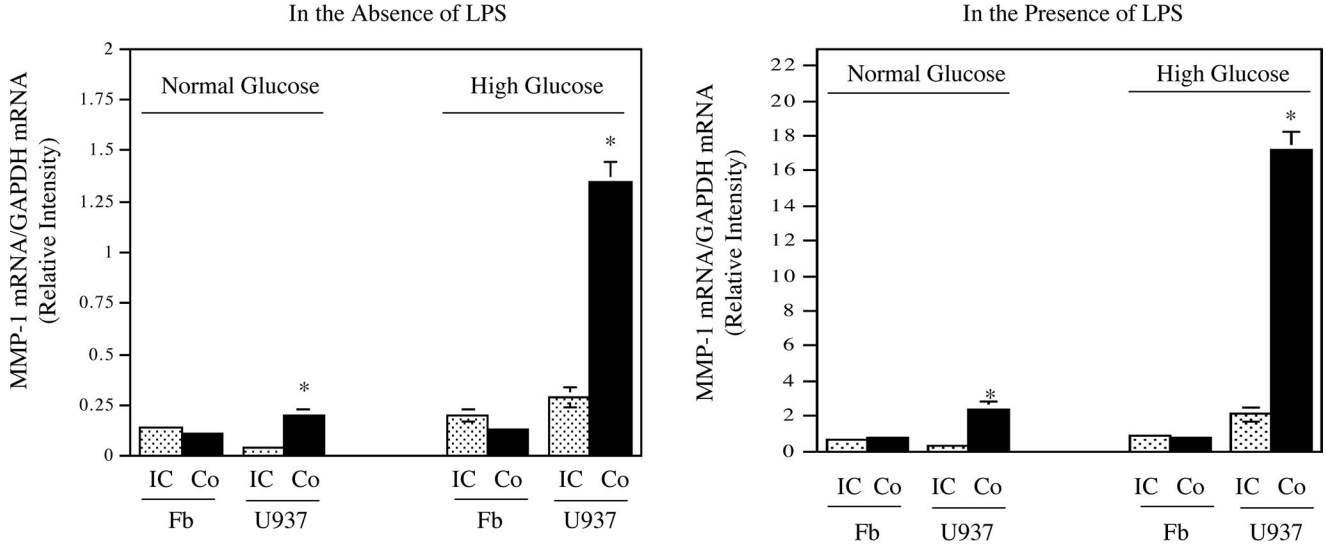


FIGURE 5. **Increased MMP-1 mRNA level in U937 cells, but not fibroblasts, in the cocultures.** Total RNA was isolated from fibroblasts and U937 cells in the experiments presented in Fig. 3 after the treatment. RNA was used in real time PCR to quantify MMP-1 mRNA. *Fb*, fibroblast; *IC*, independent culture of either fibroblasts or U937 macrophages; *Co*, coculture. *, $p < 0.01$ *versus* independent culture of U937 cells. The data (mean ± S.D.) presented are from one of three independent experiments with similar results. *Error bars* represent mean ± S.D.

high glucose did not have an increase in MMP-1 mRNA (Fig. 5). In contrast, U937 macrophages in the coculture had markedly increased MMP-1 mRNA expression when compared with the independent U937 macrophage culture, and high glucose further increased MMP-1 mRNA expression (Fig. 5). After exposure to both high glucose and LPS, the MMP-1 mRNA level in U937 cells in the coculture system was 8-fold higher than that in the independent U937 cell culture (Fig. 5). These results suggest that a soluble factor(s) derived from fibroblasts may stimulate MMP-1 expression in U937 cells and thus augment the MMP-1 level in the coculture.

To provide further evidence that a fibroblast-derived soluble factor augments MMP-1 expression in U937 macrophages, we collected fibroblast-conditioned medium and applied it to U937 cell culture. Indeed we found that the addition of fibroblast-conditioned medium to U937 cell culture increased MMP-1 secretion in a concentration-dependent manner (Fig. 6). Conversely we also collected U937 cell-conditioned medium and applied it to fibroblast culture. Results showed that the addition of U937 cell-conditioned medium to fibroblasts did not increase MMP-1 secretion by fibroblasts (data not shown). MMP-1 expression analysis using quantitative PCR also showed no change in the MMP-1 mRNA level in fibroblasts after incubation with U937 cell-conditioned medium (data not shown). Thus, these data strongly suggested that a soluble factor(s) derived from fibroblasts is essential to stimulate MMP-1 expression in U937 cells and hence augments MMP-1 level in the coculture.

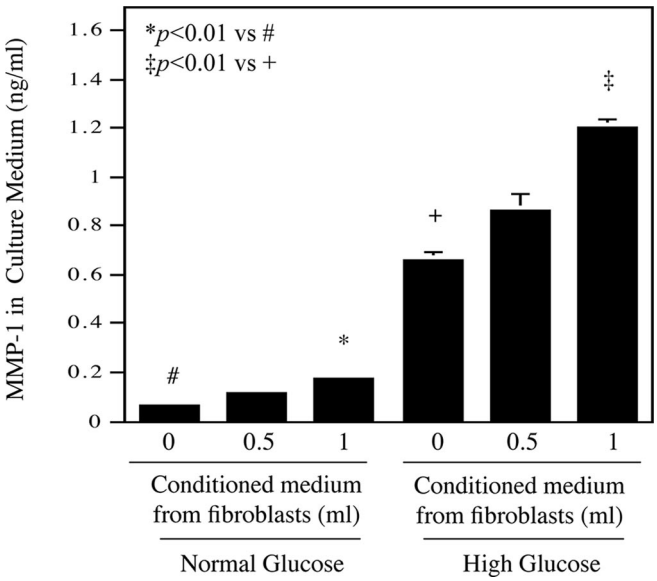


FIGURE 6. **Increased MMP-1 secretion from U937 macrophages by exposing cells to fibroblast-conditioned medium.** The conditioned medium from fibroblast culture was collected after 18 h of incubation. U937 cells were incubated with 2, 1.5, or 1 ml of fresh medium plus 0, 0.5, or 1 ml of fibroblast-conditioned medium (total 2 ml for each well) for 24 h. The secreted MMP-1 in culture medium was quantified using ELISA. The data (mean ± S.D.) presented are from one of two independent experiments with similar results. *Error bars* represent mean ± S.D.

TABLE 1

The expression of major inflammatory cytokines by fibroblasts in coculture of fibroblasts and U937 macrophages treated with normal or high glucose and LPS

Fibroblasts cocultured with U937 macrophages in normal or high glucose-containing medium were treated with 100 ng/ml LPS for 24 h. After the treatment, total RNA was isolated from fibroblasts and used for PCR array analysis of cytokines. The mRNA expression of IL-6, IL-1α, IL-1β, and TNFα was compared with that of GAPDH that was designated as 100%. The numbers outside the parentheses are percentage of GAPDH expression, and the numbers inside the parentheses are threshold cycles (Ct). NG, normal glucose; HG, high glucose. The data presented are from one of two independent experiments with similar results.

	NG	NG + LPS	HG	HG + LPS
IL-6	0.73% (28.6)	35.36% (23.0)	0.63% (28.8)	61.56% (22.2)
IL-1α	0.26% (30)	0.11% (31.2)	0.32% (29.7)	0.32% (29.7)
IL-1β	0.32% (30.1)	0.21% (31.8)	0.06% (30.6)	0.15% (30.4)
TNFα	0.08% (31.8)	0.06% (32.1)	0.09% (31.6)	0.14% (31.0)
GAPDH	100% (21.5)	100% (21.4)	100% (21.2)	100% (21.5)

IL-6 Released by Fibroblasts Is Essential for the Augmentation of MMP-1 Expression by Fibroblast and U937 Cell Coculture—To identify the fibroblast-derived soluble factor involved in the augmentation of MMP-1 expression by U937 cells, we analyzed the inflammatory cytokine expression by fibroblasts in the coculture using an inflammatory cytokine array. From the array analysis, we found that IL-6 was the major cytokine released by fibroblasts and up-regulated by LPS and high glucose (Table 1). In contrast, the expression of IL-1α, IL-1β, and TNFα was low and not stimulated by LPS and high glucose. We also found that IL-6 secretion by the coculture of fibroblasts and U937 cells was increased when compared with that by the independent cultures of fibroblasts or U937 cells and was further increased by high glucose (Fig. 7*A*). High glucose also increased IL-6 secretion from the coculture of human monocytes and fibroblasts (Fig. 7*B*). Because IL-6 is known to stimulate MMP expression (22, 23), we considered fibroblast-derived IL-6 as the soluble factor essential for the augmentation of MMP-1 expression by U937 cells in the coculture system. To confirm this hypothesis, we took two experimental approaches. First, we used anti-IL-6 antibody to block the interaction between IL-6 and U937 cells cultured in high glucose-containing medium. Results (Fig. 8) showed that in the absence of LPS anti-IL-6 antibody completely abolished the augmentation of MMP-1 secretion by the coculture. In contrast, anti-TNFα antibody had no effect. In the presence of LPS, anti-IL-6 antibody inhibited MMP-1 secretion by 78%, whereas anti-TNFα antibody only exerted a 15% inhibition. Although the inhibition of MMP-1 secretion by anti-TNFα antibody was also statistically significant, it was significantly less than that by anti-IL-6 antibody. Second, we transfected fibroblasts with IL-6 siRNA to inhibit IL-6 expression before fibroblasts were cocultured with U937 macrophages. Results showed that the transfection of fibroblasts with IL-6 siRNA markedly inhibited IL-6 expression (Fig. 9*A*). When transfected fibroblasts were cocultured with U937 cells in the absence or presence of LPS, the augmentation of MMP-1 secretion by the coculture was inhibited by 60–63% (Fig. 9*B*). These results strongly indicate that IL-6 plays a major role in the augmentation of the MMP-1 level in the coculture.

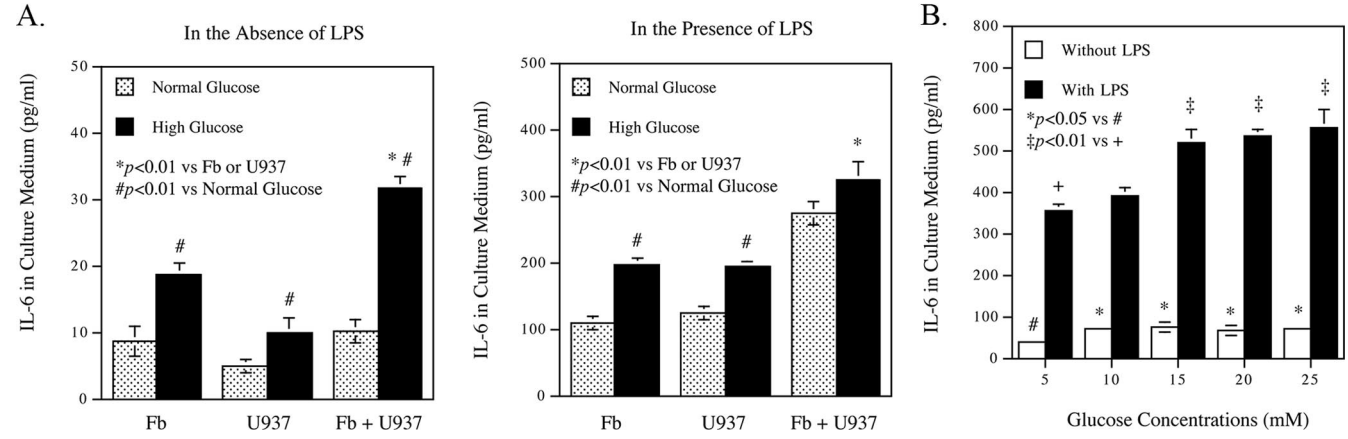
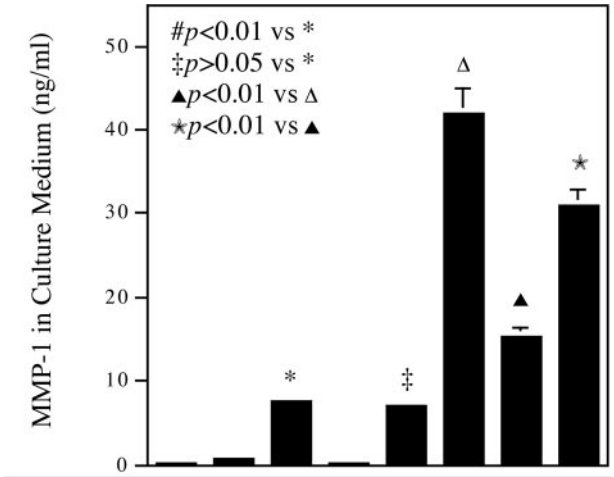


FIGURE 7. **The effect of high glucose on IL-6 secretion by coculture of fibroblasts and U937 macrophages or human monocytes.** *A*, human gingival fibroblasts and U937 macrophages in the independent cultures or coculture preexposed to normal (5 mM) or high glucose (25 mM) were challenged with or without 100 ng/ml LPS. *Fb*, fibroblasts. *B*, the coculture of fibroblasts and human monocytes was preexposed to different concentrations of glucose and treated with or without 100 ng/ml LPS. After 24 h, medium was collected for quantification of IL-6 using ELISA. The data (mean ± S.D.) presented are from one of two independent experiments with similar results. *Error bars* represent mean ± S.D.

To provide more evidence that IL-6 is capable of stimulating MMP-1 expression by U937 macrophages, we compared the stimulatory effects of IL-6 and LPS on MMP-1 expression by U937 macrophages. Results showed that IL-6 was more potent than LPS at the concentrations of 1 and 10 ng/ml and had a synergistic effect with high glucose on MMP-1 secretion (Fig. 10*A*). Furthermore IL-6 and LPS also had a synergistic effect on MMP-1 secretion (Fig. 10*B*).



Fibroblasts	+	-	+	+	+	+	+	+
U937 cells	-	+	+	+	+	+	+	+
LPS	-	-	-	-	-	+	+	+
IL-6 Ab	-	-	-	+	-	-	+	-
TNFα Ab	-	-	-	-	+	-	-	+

FIGURE 8. **Inhibition of the coculture-boosted MMP-1 secretion by neutralizing anti-IL-6 antibody.** High glucose-exposed coculture of fibroblast and U937 cells was incubated with or without 100 ng/ml LPS in the absence or presence of 5 μg/ml anti-IL-6 or anti-TNFα antibody for 24 h. After the incubation, MMP-1 in culture medium was quantified using ELISA. The data (mean ± S.D.) presented are from one of three independent experiments with similar results. *IL-6 Ab*, anti-IL-6 antibody; *TNFα Ab*, anti-TNFα antibody. *Error bars* represent mean ± S.D.

The Involvement of MAPK Pathways and AP-1 Transcription Factor in the Augmentation of MMP-1 Expression by Cell-Cell Interaction—Because it is known that MAPK pathways, which include the ERK, JNK, and p38 MAPK cascades, and STAT3 signal transduction pathways mediate IL-6-stimulated gene expression (22, 23), we determined which pathway is involved in the coculture-augmented MMP-1 expression by applying specific inhibitors of these pathways to U937 cells incubated with fibroblast-conditioned medium. Results showed that PD98059 and SP600125, the inhibitors for ERK and JNK, respectively, significantly blocked basal and fibroblast medium-augmented MMP-1 secretion (Fig. 11). In contrast, AG490, a specific inhibitor for STAT1 and STAT3 pathways, had no effect. In addition, SB203580 and Bay11-7085, the specific inhibitors for p38 MAPK and NFκB pathways, respectively, also failed to block MMP-1 secretion (Fig. 11). These results suggest that the ERK and JNK pathways mediate IL-6-augmented MMP-1 expression. Moreover because it is known that activation of ERK and JNK pathways leads to activation of AP-1 that is known to be a key transcription factor for MMP-1 expression (12), we determined the AP-1 transcriptional activity in U937 cells in the coculture exposed to high glucose using both an ELISA-type transcription factor activity assay and EMSA. Results from the transcription factor activity assay showed that AP-1 activity was increased by the coculture or LPS and further increased by the combination of the coculture and LPS (Fig. 12*A*). In contrast, although LPS increased NFκB (p50 and p65) activity in either U937 cell culture or coculture as expected, the coculture of fibroblasts and U937 cells did not augment NFκB activity (Fig. 12*B*), suggesting that NFκB is not involved in coculture-stimulated MMP-1 secretion. Our EMSA also showed a similar increase in AP-1 activity by the coculture or LPS and a further increase by the combination of coculture and LPS (Fig. 12*C*). The specific interaction between AP-1 and AP-1 probes was determined by adding unlabeled AP-1 probes and anti-c-Jun antibody. Results showed that the addition of unlabeled AP-1 probes resulted in a much lesser amount of the

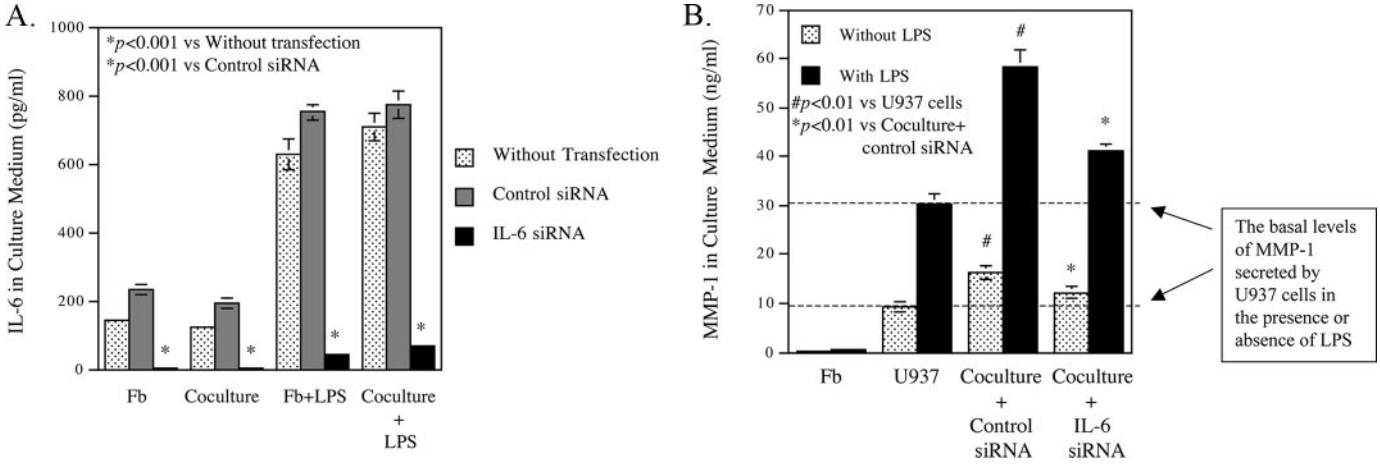


FIGURE 9. **The role of fibroblast-derived IL-6 in the coculture-augmented MMP-1 expression.** *A*, inhibition of IL-6 secretion from independent fibroblast cultures or cocultures of fibroblasts and U937 macrophages by transfection with IL-6 siRNA. High glucose-exposed fibroblasts were transfected with 200 nM IL-6 siRNA or control siRNA. After transfection, cells were either cultured independently or cocultured with U937 macrophages for 24 h in the absence or presence of 100 ng/ml LPS. After the treatment, IL-6 in culture medium was quantified using ELISA. *Fb*, fibroblasts. *B*, inhibition of MMP-1 secretion from coculture of U937 cells and fibroblasts that were transfected with IL-6 siRNA. Fibroblasts were transfected with 200 nM IL-6 siRNA or control siRNA. After transfection, cells were cocultured with U937 macrophages in the absence or presence of 100 ng/ml LPS for 24 h. MMP-1 in culture medium was then quantified using ELISA. The data (mean ± S.D.) presented are from one of three independent experiments with similar results. *Error bars* represent mean ± S.D.

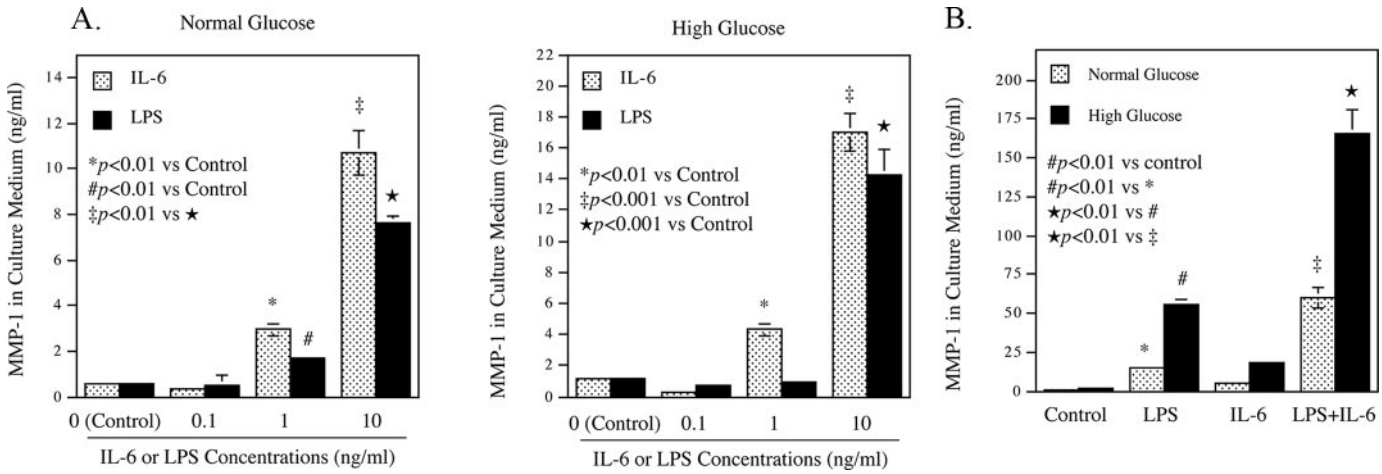


FIGURE 10. **MMP-1 secretion is stimulated by IL-6 and further augmented by LPS and high glucose.** *A*, U937 cells exposed to normal or high glucose were challenged with increasing concentrations of IL-6 or LPS for 24 h. After the treatment, MMP-1 in culture medium was quantified using ELISA. *B*, U937 macrophages pretreated with normal or high glucose were challenged with 10 ng/ml IL-6, 100 ng/ml LPS, or both for 24 h. MMP-1 in culture medium was then quantified using ELISA. The data (mean ± S.D.) presented are from one of three independent experiments with similar results. *Error bars* represent mean ± S.D.

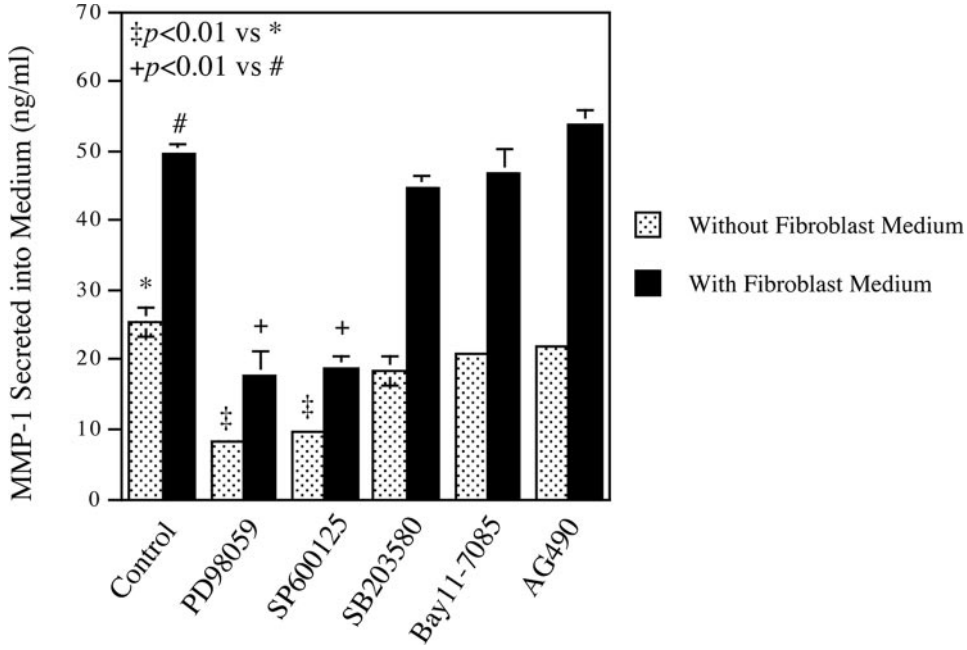


FIGURE 11. **The role of MAPK signaling pathways in the augmentation of MMP-1 secretion from U937 macrophages treated with fibroblast-conditioned medium.** The conditioned medium from fibroblast culture was collected after 18 h of incubation. U937 cells were incubated with 2 ml of fresh medium (control) or with 1 ml of fresh medium plus 1 ml of fibroblast-conditioned medium in the presence or absence of 10 μM PD98059, 10 μM SP600125, 10 μM SB203580, 1 μM Bay11-7085, or 10 μM AG490 for 24 h. After the incubation, the secreted MMP-1 in culture medium was quantified using ELISA. The data (mean ± S.D.) presented are from one of two independent experiments with similar results. *Error bars* represent mean ± S.D.

shifted AP-1 probes, suggesting a competition between labeled and unlabeled AP-1 probes for binding to the transcription factors in the nuclear extracts. The addition of anti-c-Jun antibody led to a supershift of AP-1 probes (Fig. 12*D*). Overall these results indicate that the transcription factor AP-1 is involved in the augmentation of MMP-1 expression by the coculture system.

DISCUSSION

It has been well established that MMPs, especially MMP-8 and MMP-1, play a critical role in periodontal tissue destruction (24, 25). Although MMP-8, which is mainly released by neutrophils, has been considered to be the major collagenase for the collagen degradation (25), studies have shown that MMP-1 also plays an important role in periodontal tissue destruction (24, 26, 27). A study demonstrated that polymorphism in the promoter region of MMP-1 gene, which leads to increased MMP-1 production, is associated with severe chronic periodontitis phenotype in non-smokers (28). MMP-1 mRNA expression was found to be correlated with decreased gingival collagen in patients with periodontal disease (26). Furthermore MMP-1 protein level in gingival crevicular fluid collected from patients with chronic periodontitis was found to be higher than that from periodontally healthy individuals (27). Thus, MMP-1 is likely to act in cooperation with MMP-8 as collagenases to degrade collagen and other matrix proteins. Our current study showed that among MMP-1, MMP-13, and MMP-2, the coculture of fibroblasts and U937 macrophages only augmented MMP-1 expression, suggesting that the gene up-regulated by the coculture is specific. Moreover the coculture did not augment TIMP-1 and TIMP-2, suggesting that the interaction between fibroblasts and U937 macrophages may tip the balance between MMPs and TIMPs to MMPs, leading to an increase in collagen degradation.

It was demonstrated that the coculture of gingival fibroblasts and human monocytes led to an increase in MMP-1 production (18). However, no study was conducted to investigate the

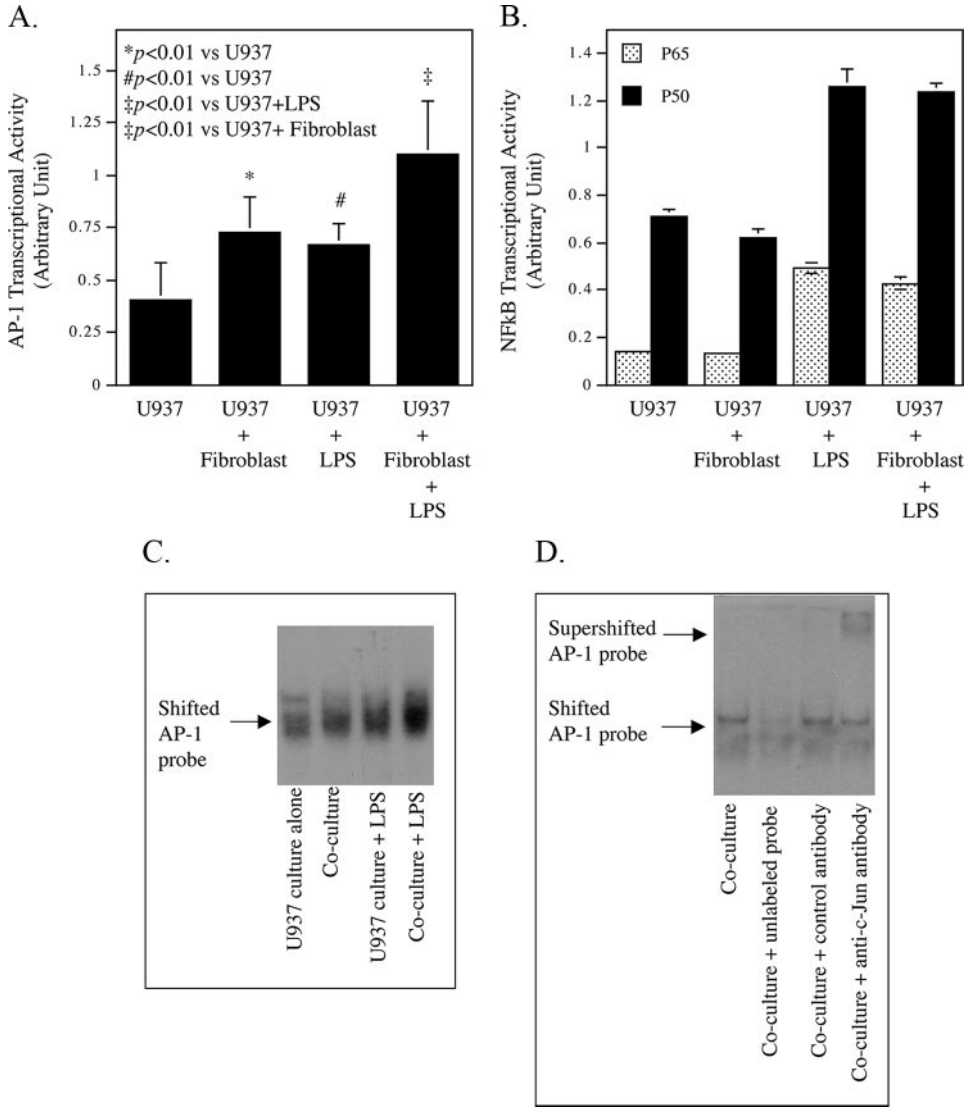


FIGURE 12. **Enhanced AP-1 transcriptional activity in cocultures of U937 cells and fibroblasts in response to LPS.** *A* and *B*, U937 cells cultured independently or cocultured with fibroblasts in high glucose-containing medium were treated with or without 100 ng/ml LPS for 6 h. After the treatment, nuclear protein was isolated and used for AP-1 (*A*) and NFκB (*B*) transcriptional activity assays. NFκB subunits p65 and p50 were detected in the NFκB transcriptional activity assay. *Error bars* represent mean ± S.D. *C* and *D*, nuclear proteins used in the above experiments were applied for EMSAs. The unlabeled AP-1 probe that was in 30-fold excess of labeled probe was used in the competition study. The anti-c-Jun and anti-nuclear factor of activated T-cells (control) antibodies were used for supershift assay. The data presented are from one of two independent experiments with similar results.

underlying mechanism. In our current study, we demonstrated that the coculture of human gingival fibroblasts and U937 macrophages led to a marked augmentation of MMP-1 expression, which is consistent with the report by Domeij *et al.* (18), and IL-6 released by fibroblasts is essential for the augmentation of MMP-1 expression. We also demonstrated that high glucose further enhanced this augmentation. These findings revealed an interesting cross-talking between fibroblasts and U937 macrophages through IL-6 for MMP-1 up-regulation. It is likely that the total amount of IL-6 in the coculture system, which is derived from both fibroblasts and U937 cells, is sufficient to trigger a signaling that acts in concert with the signaling elicited by high glucose and LPS to up-regulate MMP-1 expression by U937 macrophages, leading to a remarkable increase in MMP-1 secretion in the coculture system. This study also suggests that in periodontitis-inflamed tissue LPS derived from bacteria stimulates IL-6 secretion, and IL-6 then acts with LPS synergistically to stimulate MMP-1 expression by macrophages. Because high glucose has a synergistic effect with LPS and IL-6, this up-regulation of MMP-1 expression is likely to be further enhanced by hyperglycemia in diabetic patients.

Our real time PCR showed that when compared with the independent culture MMP-1 expression in the coculture system was increased in U937 macrophages but not fibroblasts. This is an interesting finding. As our results showed that IL-6 plays a key role in MMP-1 up-regulation in the coculture (Figs. 8 and 9), this finding would suggest that IL-6 released by the coculture stimulates MMP-1 expression by U937 cells but not fibroblasts. Actually this notion is consistent with the report by Irwin *et al.* (29) showing that the addition of IL-6 up to the concentration of 1,000 ng/ml to gingival fibroblasts had no effect on MMP-1 expression unless the soluble IL-6 receptor was used. It is most likely that lack of soluble IL-6 receptor in our coculture system is the reason that no MMP-1 up-regulation in fibroblasts was observed. In contrast to fibroblasts, IL-6 effectively stimulated U937 macrophages without soluble IL-6 receptor (Fig. 10*A*). Interestingly our results showed that IL-6 was more potent than LPS at the concentrations of 1 and 10 ng/ml in the stimulation of MMP-1 secretion by U937 macrophages (Fig. 10*A*), indicating that IL-6 is a major player in MMP-1 up-regulation. In our recent investigation on the expression of IL-6, TNFα, IL-1β, MMP-1, and MMP-8 in periodontal tissue, we observed a trend of increase in IL-6 mRNA expression across patients with neither diabetes nor periodontal disease, patients with periodontal disease alone, and patients with both diseases (10). Fascinatingly the same study also demonstrated a trend of increase in MMP-1 expression. Considering the potent stimulatory effect of IL-6 on MMP-1 expression, it is possible that IL-6 released by fibroblasts in periodontitis-inflamed tissue in response to LPS stimulated MMP-1 expression.

LPS is known to be a potent stimulator for MMP expression by macrophages (11, 12). Besides MMPs, LPS also stimulates

the expression of inflammatory cytokines such as IL-6. Given the fact that IL-6 is also a powerful stimulator for MMP-1 expression, it is likely that LPS up-regulates MMP-1 expression via both IL-6-dependent and -independent pathways. Moreover our study showed that LPS and IL-6 had a synergistic effect on MMP-1 expression by U937 macrophages (Fig. 10*B*). Therefore, IL-6 appears to act as an "enhancer" for LPS in the stimulation of MMP-1 expression. Because both LPS and IL-6 stimulate gene expression via MAPK signaling pathways and AP-1 transcription factor, it is possible that LPS and IL-6 have a synergistic effect on MAPK signaling and AP-1 activity, leading to an enhanced stimulation of MMP-1 expression. Further studies are necessary to investigate the coordination of signaling and transcriptional activation between LPS and IL-6 on MMP-1 expression.

Another interesting finding from our current study is that high glucose and IL-6 had a synergistic effect on MMP-1 expression by U937 macrophages (Fig. 10, *A* and *B*). In addition to IL-6, our previous study (12) and the current data (Fig. 10*B*) showed that high glucose also has a synergistic effect with LPS on MMP-1 expression. Thus, we expect that high glucose would augment the stimulatory effect of the combination of LPS and IL-6 on MMP-1 expression. Indeed our results showed that after U937 macrophages were preexposed to high glucose the treatment with IL-6 and LPS led to a 2.5-fold increase in MMP-1 secretion as compared with those preexposed to normal glucose (Fig. 10*B*). Considering that LPS and IL-6 play a critical role in periodontal disease, these findings indicate that diabetic patients with poor glycemic control may have a significant increase in periodontal MMP-1 expression and subsequent tissue destruction as compared with nondiabetic patients during the progression of periodontal disease.

It is known that STAT3 and MAPK pathways are the major cascades mediating IL-6-stimulated gene expression by macrophages (19, 20). In our experiments to determine which pathway is responsible for the coculture-augmented MMP-1 expression, we found that inhibitors for ERK and JNK pathways, but not the STAT3 cascade, blocked MMP-1 expression augmented by conditioned medium from fibroblasts (Fig. 11), suggesting that ERK and JNK pathways, but not the STAT3 cascade, are involved in the MMP-1 up-regulation by cross-talking between fibroblasts and U937 macrophages. This finding is different from the previous reports that both STAT3 and MAPK pathways were involved in IL-6-stimulated MMP-1 expression in bovine chondrocytes (30) and mouse skin epithelial cells (31). It is possible that the cells from different species and different tissues may have different signaling control mechanisms in regulating MMP-1 expression: ERK and JNK MAPK pathways are sufficient in mediating MMP-1 expression for human macrophages, whereas both STAT3 and MAPK pathways are required for bovine chondrocytes and mouse skin epithelial cells. In the investigation of which transcription factor was involved in the coculture-stimulated MMP-1 expression, our study demonstrated that the activity of transcription factor AP-1, which is known to be activated as a result of ERK and JNK MAPK activation (32), was stimulated by the coculture and further enhanced by LPS (Fig. 12, *A* and *C*). Considering that AP-1 transcription factor is critical for MMP-1 expression (33–35), our results indicate that IL-6 stimulates MMP-1 expression through ERK and JNK pathways and subsequent AP-1 transcriptional activation. Because high glucose enhances AP-1 activity as shown by our previous study (12), it is likely that high glucose augments IL-6-stimulated MMP-1 expression via AP-1 activation.

IL-6 has been well documented as a key inflammatory cytokine involved in several inflammation-associated diseases such as coronary artery disease, diabetes, and rheumatoid arthritis (23). In this study, we demonstrated that IL-6 mediated the up-regulation of MMP-1 expression by the coculture of fibroblasts and U937 macrophages exposed to high glucose. Thus, this study strongly suggests that IL-6 is a key player in diabetes-associated periodontal disease and should be considered as a major target for the treatment of periodontal disease.

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