Oral Microbiology Immunology 2008: 23: 320–327
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

CCL20 production is induced in human dental pulp upon stimulation by *Streptococcus mutans* and proinflammatory cytokines

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Takahashi K, Nakanishi T, Yumoto H, Adachi T, Matsuo T. CCL20 production is induced in human dental pulp upon stimulation by Streptococcus mutans *and proinflammatory cytokines.*
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Introduction: Pulpitis is characterized by the marked infiltration of inflammatory cells in response to an invasion of caries-related bacteria. It is well known that chemokines regulate the trafficking of lymphocytes, and CC chemokine ligand 20 (CCL20) has been recently shown to play a crucial role in the recruitment of memory T cells and immature dendritic cells into inflammatory lesions. We previously reported that CCL20 was mainly expressed in microvascular endothelial cells and macrophages that accumulated in inflamed pulp tissues and that its specific receptor, CCR6, was expressed on infiltrated lymphocytes. However, the mechanism of CCL20 expression remains unclear.
Methods and Results: In this study, we investigated the expression of CCL20 in monocytes/macrophages, endothelial cells, and pulpal fibroblasts after stimulation with *Streptococcus mutans*, a representative of caries-related bacteria, or proinflammatory cytokines. CCL20 messenger RNA was detected by reverse transcription–polymerase chain reaction in inflamed pulp, but not in clinically normal pulp. By enzyme-linked immunosorbent assay, *S. mutans* induced a human monocytic cell line, differentiated macrophage-like THP-1 cells, and human umbilical vein endothelial cells (HUVEC) to produce an increased amount of CCL20. Lipoteichoic acid from *S. mutans* also elicited CCL20 production by HUVEC. Moreover, CCL20 production from pulpal fibroblasts was increased by stimulation with inetrleukin-1β and tumor necrosis factor-α.
Conclusion: Our results indicate that CCL20 expression is induced by stimulation with caries-related bacteria that have invaded deeply into the dentinal tubules as well as by proinflammatory cytokines in the inflamed pulpal lesions. It may be involved in the progression of pulpitis via accumulation of inflammatory cells.

Key words: CC chemokine ligand 20; endothelial cells; macrophage; pulpal fibroblastic cells; pulpitis

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Accepted for publication December 4, 2007

Immunocytes including dendritic cells, macrophages, and T lymphocytes constitutively exist in dental pulp tissues (14). It has been considered that bacterial invasion accompanying the advance of dental caries sequentially initiates immune reactions (14, 20, 28, 37, 40). In fact, regarding the initial reactions of pulpitis, a localized accumulation of dendritic cells was found adjacent to odontoblasts beneath superficial dentinal caries lesions (40). As caries-related bacteria invade the deep parts of dentin and come close to the pulp, inflammatory cells such as lymphocytes, macrophages, and neutrophils infiltrate the sites

of the bacteria-invaded area and consequently pulpitis is formed (37). Lymphocytes are key players in the inflammatory infiltrates of the inflamed pulp, and T cells are an especially prominent population of the lymphocytes that play an important role in the pathogenesis of pulpitis (15, 20). In general, it is known that the T cells that infiltrate the inflammatory lesions dominantly express CD45RO (a memory T-cell marker) compared to CD45RA (a naive T cell marker) (36), and it has also been suggested that the infiltration of $CD45RO^+$ cells is predominant in pulpitis (20). Chemokines, which are low molecular weight chemotactic factors, are responsible for this extravasation of memory T cells to inflamed lesions (6).

Chemokines, which are basic proteins that strongly bind to heparin, possess capacities for leukocyte chemotaxis and activation, and are closely involved in various biological processes, such as inflammatory reactions, hematopoietic regulation, and neoangiogenesis (4, 5, 13, 32, 47). Chemokines are mainly classified by the position of cysteine residues, into three subfamilies: CXC, CC, and CX3C. Recently, CC chemokine ligand 20 (CCL20) has been identified to be of the CC chemokine family (17). Expression of CCL20 was found in the gastrointestinal tract (19, 38), skin (31), lymphatic nodes (38), and tonsils (11), and this expression was increased in allergic bronchitis and psoriasis (16, 26). Moreover, it has been reported that CCL20 is capable of attracting memory/activated T cells, and that immature dendritic cells and naive B cells express CCR6, a specific CC chemokine receptor that binds CCL20 (11, 25).

Regarding the expression of chemokines in pulpitis, Huang et al. reported that odontoblasts and mononuclear cells expressed interleukin-8 (IL-8) (18). Levin et al. also reported that expression of messenger RNA (mRNA) for IL-8 was observed in the normal odontoblastic layer (24). In addition, our recent immunohistochemical report showed CCL20 expression in human inflamed pulp but not in normal pulp, and expression was mostly distributed in macrophages and endothelial cells that had accumulated in the area adjacent to carious lesions (30). However, the relationship between chemokine expression and the pathogenesis of pulpitis is still unclear. In particular, the expression patterns and roles of CCL20 in pulpitis lesions remain to be elucidated.

In this study, we determined the expression of CCL20 at the mRNA level in inflamed and normal pulp tissues by reverse transcription–polymerase chain reaction (RT-PCR) and investigated the mechanism of CCL20 expression by stimulation with a caries-related bacterium, *Streptococcus mutans*, and with proinflammatory cytokines in a human monocytic cell line, THP-1, human umbilical vein endothelial cells (HUVEC), and human pulpal fibroblastic cells (HPFC) *in vitro*.

Materials and methods

Clinical pulp tissue samples

At the time of pulpectomy, samples of inflamed pulp tissue were obtained from nine teeth diagnosed as irreversible pulpitis based upon the radiographic diagnosis (radiographs showed the carious lesion coming close to or reaching the dental pulp) and the clinical symptoms, such as spontaneous and continuous pain triggered by cold and hot stimuli. As a control, clinically normal pulp tissue samples were obtained from four non-carious teeth at the time of expedient pulpectomy. All patients (five men and eight women; aged between 19 and 70 years, average 34.8-years-old) were attending the Dental Clinic of Tokushima University Hospital, had no underlying disease, and had no medication history for the past 6 months. This study was approved by the Ethics Committee of Tokushima University Hospital and a written informed consent was obtained from each subject before the procedure.

Bacterial strain and growth condition

S. mutans MT8148 (provided by T. Oohshima, Osaka University, Osaka, Japan) was cultured at 37°C in brain–heart infusion broth (Difco Laboratories, Detroit, MI) for 8 h.

Cell culture

The human monocytic cell line, THP-1 (American Type Culture Collection, Manassas, VA), was cultured in RPMI-1640 medium (Gibco, Grand island, NY) supplemented with 26 mM HEPES (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 10% (volume/volume) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KA) at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. Induction of cell differentiation to macrophage-like cells was followed as described previously (42). In brief, 5×10^5 cells in 1% FBS–RPMI-1640 were seeded in wells of 24-well tissue culture plates with 20 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich, Walkersville, MD) and, cultured for 48 h. Cells were then washed and the medium was replaced to remove PMA for 40 h before stimulation with bacteria or cytokines.

Human umbilical vein endothelial cells (BioWhittaker, Walkersville, MD) were cultured in endothelial cell basal medium 2 supplemented with 2% FBS, 10 ng/ml recombinant human epidermal growth factor, 0.4% human fibroblast growth factor-basic, 0.1% vascular endothelial growth factor, 0.1% ascorbic acid, 0.1% Long R3-insulin-like growth factor-1, 0.1% heparin, 1.0 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 ng/ml amphotericin-B, and 12 μg/ml bovine brain extract (all from BioWhittaker) at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Normal human dental pulp tissues were obtained from non-carious and periodontally healthy third molars or premolars extracted for orthodontic therapy. The minced explants were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS, 1 mM sodium pyruvate, 50 IU/ml penicillin, and 50 μg/ml streptomycin at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. HPFC obtained in this manner were maintained and used within five to nine passages.

Stimulation assay with bacteria or proinflammatory cytokines

S. mutans were collected by centrifugation, washed in phosphate-buffered saline (PBS), and suspended in medium devoid of antibiotics at a concentration of 2.0×10^{10} to 3.0×10^{10} colony-forming units/ml as live bacteria. Bacterial concentrations were determined spectrophotometrically according to a standard curve. Heat-inactivated bacteria were prepared by boiling for 10 min at 100°C. Live or heat-inactivated bacteria were directly added to THP-1 or HUVEC in 24-well tissue culture plates and incubated for the specified periods. In addition to bacteria, lipoteichoic acid (LTA) from *S. mutans* (Sigma-Aldrich) at 1, 10, or 100 μg/ml was used as a stimulant for HUVEC. To eliminate the effects of lipopolysaccharide contamination, polymyxin B, a relatively specific inhibitor of lipopolysaccharide biological activity, was added to the cells and the cells were preincubated for 30 min before stimulation with LTA. HPFC were treated with IL-1β or tumor necrosis factor-α (TNF-α) at 0.1, 1, or 10 ng/ml for 4 h or 12 h. After incubation, the cell culture supernatants were collected and

used to quantify the levels of CCL20 and selected cytokines using an enzyme-linked immunosorbent assay (ELISA), and the attached cells were used for RNA extraction.

Total RNA extraction and RT-PCR

Total RNA from pulpal tissues and cultured cells was extracted using Isogen (Nippongene, Toyama, Japan) or Catrimox-14 (IOWA Biotechnology, Coralville, IA) in accordance with the manufacturers' instructions. Complementary DNA was generated with a TaKaRa RNA PCR Kit (TaKaRa BIO, Shiga, Japan) following the manufacturer's protocol and was subjected to amplification of the target genes by PCR with a Program Temp Control System PC-800 (Astec, Fukuoka, Japan). The amplification reaction was carried out using a PCR Master Mix (ABgene House, Epsom, UK) and target gene-specific primer pairs (Table 1). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control (housekeeping gene). The oligonucleotide primer sequences and amplification conditions used for PCR are shown in Table 1. A sample (10 μl) of each amplified product was subjected to electrophoresis on a 1.5 or 2.0% agarose gel (TaKaRa), stained with ethidium bromide, and visualized by ultraviolet illumination (Cosmobio, Tokyo, Japan).

Table 1. Oligonucleotide sequences of the 5′ and 3′ primers of the target genes

mRNA species	Primer	Sequence	AT[1] (°C)	Size (bp)
CCL20	Sense	5′-TTGCTCCTGGCTGCTTTG-3′	54	367
	Antisense	5′-ACCCTCCATGATGTGCAAG-3′		
IL-8	Sense	5′-ATGACTTCCAAGCTGGCCGTGGCT-3′	60	294
	Antisense	5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′		
IL-1β	Sense	5′-ATGGCAGAAGTACCTAAGCTCGC-3′	60	804
	Antisense	5′-ACACAAATTGCATGGTGAAGTCAGTT-3′		
TNF-α	Sense	5′-TTGCTCCTGGCTGCTTTG-3′	60	446
	Antisense	5′-ACCCTCCATGATGTGCAAG-3′		
ICAM-1	Sense	5′-CGTGCCGCACTGAACTGGAC-3′	60	447
	Antisense	5′-CCTCACACTTCACTGTCACCT-3′		
VCAM-1	Sense	5′-ATTGGGAAAAACAGAAAAGAG-3′	56	642
	Antisense	5′-GGCAACATTGACATAAAGT-3′		
GAPDH	Sense	5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′	60	983
	Antisense	5′-CATGTGGGCCATGAGGTCCACCAC-3′		

[1]AT, annealing temperature.
GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule 1; IL-8, interleukin-8; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule 1.

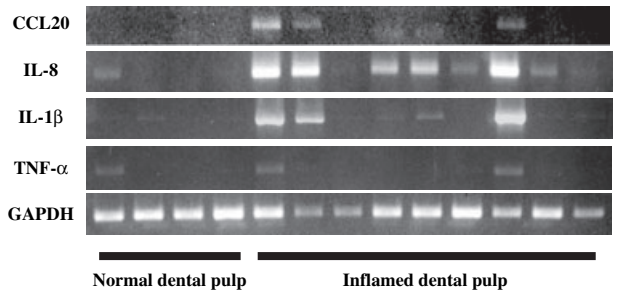

Fig. 1. CCL20, interleukin-8 (IL-8), IL-1β, and tumor necrosis factor-α (TNF-α) gene expression in human dental pulp tissues. RNA was isolated from clinically normal and inflamed pulp tissues. Complementary DNA synthesis and reverse transcription–polymerase chain reaction were performed as described in the Materials and methods. The GAPDH housekeeping gene was used as a control.

ELISA

The concentrations of CCL20, IL-8, IL-1β, and TNF-α in cell culture supernatants were determined using commercially available ELISA kits (Duo Set ELISA Development System, R&D Systems, Minneapolis, MN).

Statistical analysis

All statistical analyses were performed using an unpaired Student's *t*-test. Differences were considered significant when the probability value was less than 5% ($P < 0.05$).

Results

Expression of CCL20, IL-8, IL-1β, and TNF-α mRNA in human pulp tissues

To examine whether human pulp tissue expresses the mRNA of CCL20, IL-8, IL-1β, and TNF-α, we investigated the gene expression of these inflammatory mediators by RT-PCR (Fig. 1). In inflamed pulpal tissues, mRNA expression of CCL20 was detected in three out of nine samples, but it was not detected in any clinically normal pulpal tissue samples. The levels of mRNA expression for IL-8 and IL-1β in inflamed pulpal tissue samples were higher than those in the clinically normal pulpal tissue samples. In contrast, no significant difference of TNF-α mRNA expression level was observed between inflamed and normal pulpal tissue samples.

Production of CCL20 and proinflammatory cytokines from monocytic cells after interaction with *S. mutans*

Recently, we observed by immunohistochemical analysis that CCL20 is expressed in monocytes and microvascular endothelial cells that have infiltrated the inflamed pulp (30). However, it is not yet known how CCL20 expression is up-regulated in the inflamed tissue. In the present study, we determined the levels of CCL20 and of other cytokines produced in monocytic cells (THP-1) after caries-related bacterial stimulation at a multiplicity of infection (MOI) of 400. THP-1 cells did not constitutively express CCL20 mRNA, but the level of CCL20 mRNA in THP-1 cells was up-regulated at 6 h after infection with *S. mutans*, and this increased level was retained up to 24 h after infection (Fig. 2A). The mRNA expression levels of other proinflammatory cytokines (IL-8, IL-1β, and TNF-α) were also up-regulated in THP-1 cells by infection with *S. mutans* (Fig. 2A).

For the evaluation of secreted levels of CCL20 and other proinflammatory cytokines after *S. mutans* infection, THP-1 cells were infected for up to 24 h with *S. mutans* (MOI = 400) and protein levels in the culture medium were then measured by ELISA. The levels of all the tested molecules secreted from *S. mutans*-infected THP-1 cells were increased in a time-dependent manner (Fig. 2B) and these results were in accord with those obtained from RT-PCR.

Next, we determined the levels of CCL20 and other cytokines produced in macrophage-like THP-1 cells, which were differentiated with PMA, after interaction with *S. mutans* at an MOI of 400.

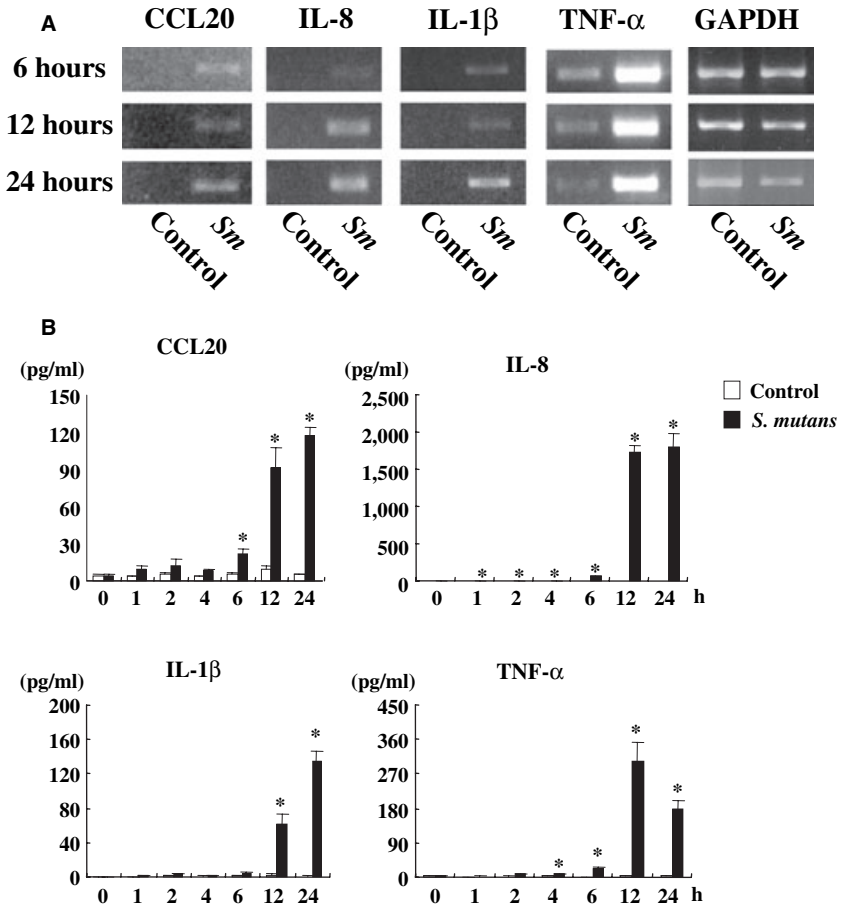

Fig. 2. CCL20, interleukin-8 (IL-8), IL-1β, and tumor necrosis factor-α (TNF-α) gene expression (A) and production (B) in monocytic THP-1 after infection with *Streptococcus mutans*. (A) RNA was isolated from THP-1 cells after bacterial infection (multiplicity of infection 400) for 6, 12 and 24 h. Complementary DNA synthesis and reverse transcription–polymerase chain reaction were performed as described in the Materials and methods. The controls consisted of cells incubated in medium alone. (B) Supernatants were tested at 0, 1, 2, 4, 6, 12, and 24 h, and the protein levels were determined by enzyme-linked immunosorbent assay. Data are the means and SDs of triplicate determinations from three different experiments. Asterisks indicate significant differences ($P < 0.05$) vs. controls values.

Differentiated THP-1 cells constitutively expressed CCL20 mRNA and the mRNA level was up-regulated at 12 and 24 h after infection with live, but not heat-inactivated, *S. mutans* (Fig. 3A). The amount of CCL20 secreted from differentiated THP-1 cells infected with live, but not heat-inactivated, *S. mutans* was significantly increased at 24 h (Fig. 3B).

Production of CCL20 and expression of cell adhesion molecules in HUVEC after infection with *S. mutans* or stimulation with LTA

Next, we determined the level of CCL20 and cell adhesion molecule expression by RT-PCR in HUVEC after *S. mutans* infection. CCL20 mRNA expression was up-regulated in HUVEC after infection with *S. mutans* at an MOI of 4000 for 4 h and this was sustained up to 24 h (Fig. 4A). In addition, the up-regulation of vascular cell adhesion molecule 1 (VCAM-1) was observed at 24 h after bacterial infection at all tested MOI, but up-regulation of intercellular adhesion molecule 1 (ICAM-1) was not observed (Fig. 4A). Previously, Zhou et al. reported that TNF-α is a potent inducer of endothelial adhesiveness and exposure of HUVEC to TNF-α (2 ng/ml) induced strong up-regulation of cell surface expression of ICAM-1 (49). Next, we determined whether *S. mutans* infection induced the up-regulation of TNF-α expression in HUVEC at mRNA and protein levels by RT-PCR and ELISA, respectively. In connection with the finding that there was no increase of ICAM-1 expression after *S. mutans* infection, the up-regulation of TNF-α in HUVEC was not observed up to 24 h at either mRNA or protein level after this bacterial infection (data not shown). Therefore, *S. mutans* infection could not up-regulate the expression of ICAM-1 via autocrine or paracrine mechanisms by TNF-α up-regulation in HUVEC. The protein level of CCL20 in the culture medium was increased by bacterial infection for 24 h, and this induction was dose-dependent (Fig. 4B).

S. mutans had the capacity to induce HUVEC to produce CCL20 at both the mRNA and protein levels. We therefore focused on LTA, a cell-surface component of gram-positive bacteria, derived from *S. mutans* and determined whether this component could activate HUVEC to induce the expression of CCL20, VCAM-1, and ICAM-1. Stimulation with 10 μg/ml LTA augmented CCL20 mRNA expression in HUVEC at 24 h. In the case of stimulation with 100 μg/ml LTA, CCL20 mRNA expression was enhanced at 12 h and the enhancement was retained for up to 24 h (Fig. 5A). Similarly, VCAM-1, but not ICAM-1, mRNA expression, was up-regulated at 12 h after 100 μg/ml LTA stimulation, and this induction was maintained up to 24 h (Fig. 5A). Statistically significant increases in CCL20 protein secretion were also observed in HUVEC after 24 h of stimulation with 1–100 μg/ml LTA (Fig. 5B).

CCL20 and IL-8 production in HPFC

Fibroblasts are a major constituent of the dental pulp and they produce various proinflammatory cytokines in response to stimulation by bacteria and their components. Previous studies showed that levels of the proinflammatory cytokines IL-1β and TNF-α in fibroblasts and pulpal tissues derived from diseased or irreversible pulp were significantly higher than those derived from healthy pulp (3, 35). In the present study, we isolated and cultured HPFC, and then determined the level of CCL20 in response to stimulation by IL-1β or TNF-α. Protein levels of CCL20 and IL-8 secreted from IL-1β-stimulated HPFC were increased in a time-dependent manner (Fig. 6A). Similar tendencies for both inflammatory mediators were observed in TNF-α-stimulated HPFC (Fig. 6B).

Discussion

It has been clarified that CCL20 plays an important role in the infiltration of memory T cells (10, 12). We presumed that CCL20 could contribute to the infiltration of numerous lymphocytes observed in pulpitis. In

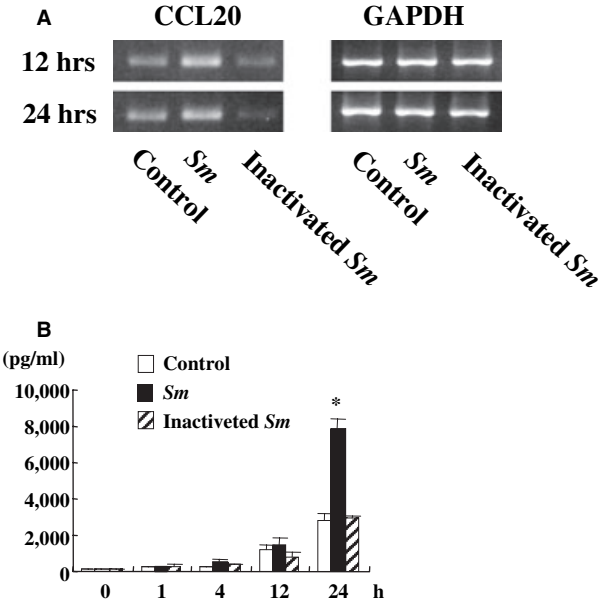

Fig. 3. CCL20 gene expression (A) and production (B) in differentiated THP-1 cells after stimulation with live or heat-killed *Streptococcus mutans*. (A) RNA was isolated from THP-1 cells differentiated with phorbol myristate acetate after 12 h or 24 h of bacterial stimulation (multiplicity of infection 400). Complemetary DNA synthesis and reverse transcription–polymerase chain reaction were performed as described in the Materials and methods. The controls consisted of cells incubated in medium alone. (B) Supernatants were tested at 0, 1, 4, 12, and 24 h, and the CCL20 levels were determined by enzyme-linked immunosorbent assay. Data are the means and SDs of triplicate determinations from three different experiments. Asterisks indicate significant differences ($P < 0.05$) vs. control values.

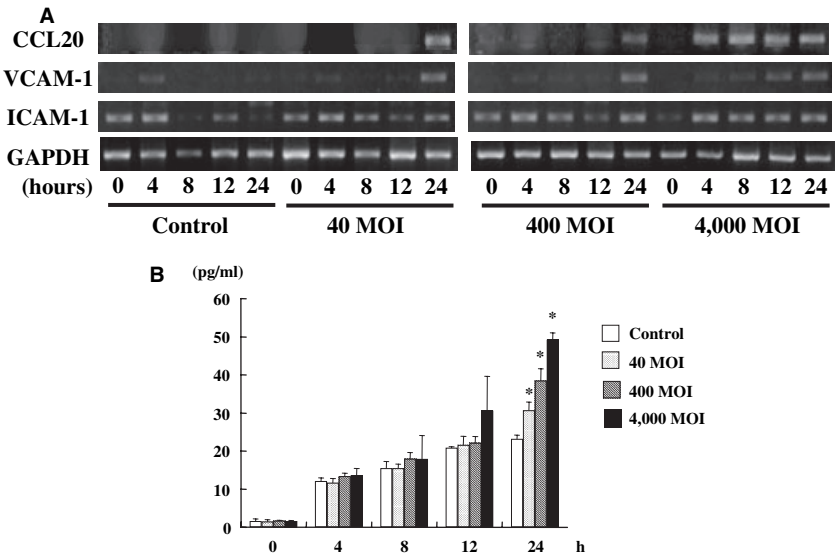

Fig. 4. CCL20, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) gene expression (A) and CCL20 production (B) in human umbilical vein endothelial cells (HUVEC) after infection with *Streptococcus mutans*. Bacteria were added to HUVEC cultures at multiplicity of infection of 40, 400, and 4000 and incubated at 37°C for 0, 4, 8, 12, and 24 h. The controls consisted of monolayers incubated in medium alone. (A) RNA was isolated from HUVEC after bacterial infection. Complementary DNA synthesis and reverse transcription–polymerase chain reaction were performed as described in the Materials and methods. The GAPDH housekeeping gene was used as an internal control. (B) Supernatants were tested at 0, 4, 8, 12, and 24 h, and the CCL20 levels were determined by enzyme-linked immunosorbent assay. Data are the means and SDs of triplicate determinations from three different experiments. Asterisks indicate significant differences ($P < 0.05$) vs. control values.

this study, we demonstrated that the level of CCL20 mRNA expression in inflamed pulp tissues was higher than that in clinically normal pulp tissues (Fig. 1). CCL20 is expressed in psoriasis and allergic dermatitis, and it has been suggested that CCL20 is principally derived from epithelial cells in the inflamed lesions (10, 16, 41). Tanaka et al. also reported that CCL20 expression was induced in intestinal epithelial cells of mice injected with lipopolysaccharide (43). However, dental pulp is deficient in the epithelial layer. Therefore, we previously tried to identify CCL20-expressing cells in the dental pulp and reported that CCL20 was expressed by accumulated macrophages and microvascular endothelial cells in inflamed pulp tissues (30).

To clarify the mechanism of CCL20 production in these macrophages and microvascular endothelial cells, we examined whether these cells could produce CCL20 after infection with caries-related bacteria *in vitro*. At both the mRNA and protein levels, CCL20 expression in monocytic THP-1 cells was induced after infection with live *S. mutans* (Fig. 2A,B). Furthermore, other proinflammatory cytokines (IL-8, IL-1β, and TNF-α) were also up-regulated in monocytic THP-1 cells after *S. mutans* infection (Fig. 2A,B). These results suggest that monocytes, which transmigrated into inflamed sites from the blood, may produce CCL20 as well as IL-8, IL-1β, and TNF-α after caries-related bacterial stimulation. Moreover, we demonstrated that CCL20 mRNA expression was constitutively observed in PMA-differentiated THP-1 cells and that both mRNA and protein levels of CCL20 in these cells were increased after infection with live *S. mutans*. Recently, we have reported that live, but not heat-killed, *S. mutans* had the ability to induce CXCL10 expression in HPFC and assumed that bacterial products from live *S. mutans* are likely to be responsible for CXCL10 expression; once heat-killed, this bacterium did not contain sufficient bacterial components, such as peptidoglycan, to induce CXCL10 production (1). Therefore, in this study, we tried to determine whether heat-killed *S. mutans* could up-regulate the expression of CCL20 in differentiated THP-1 cells and found that stimulation with inactivated *S. mutans* did not induce CCL20 production in these cells (Fig. 3 A,B). These findings suggest that macrophages accumulated directly beneath caries lesions strongly express CCL20 after exposure to caries-related bacteria and may play a principal role in CCL20 production as a compensation for the deficiency of the

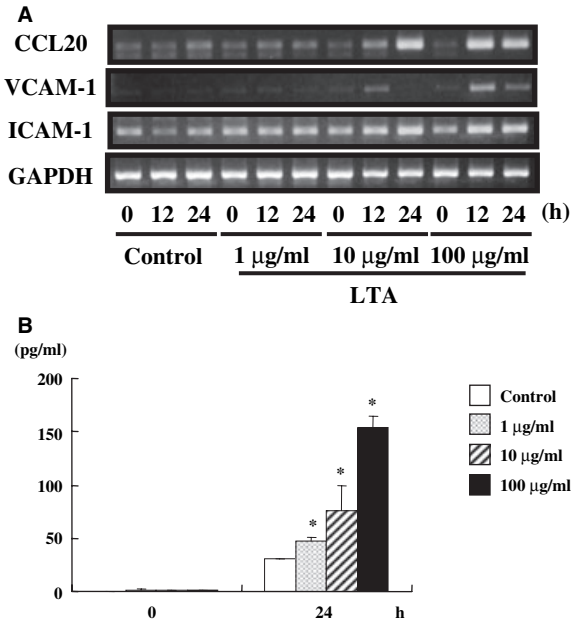

Fig. 5. CCL20, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) gene expression (A) and CCL20 production (B) in human umbilical vein endothelial cells (HUVEC) after stimulation with lipoteichoic acid (LTA) from *Streptococcus mutans*. LTA was added to HUVEC cultures at concentrations of 1, 10, and 100 μg/ml and incubated at 37°C for 0, 12, and 24 h. The controls consisted of monolayers incubated in medium alone. (A) RNA was isolated from HUVEC after stimulation with LTA. Complementary DNA synthesis and reverse transcription–polymerase chain reaction were performed as described in the Materials and methods. The GAPDH housekeeping gene was used as an internal control. (B) Supernatants were tested at 0 and 24 h and the CCL20 levels were determined by enzyme-linked immunosorbent assay. Data are the means and SDs of triplicate determinations from three different experiments. Asterisks indicate significant differences ($P < 0.05$) vs. control values.

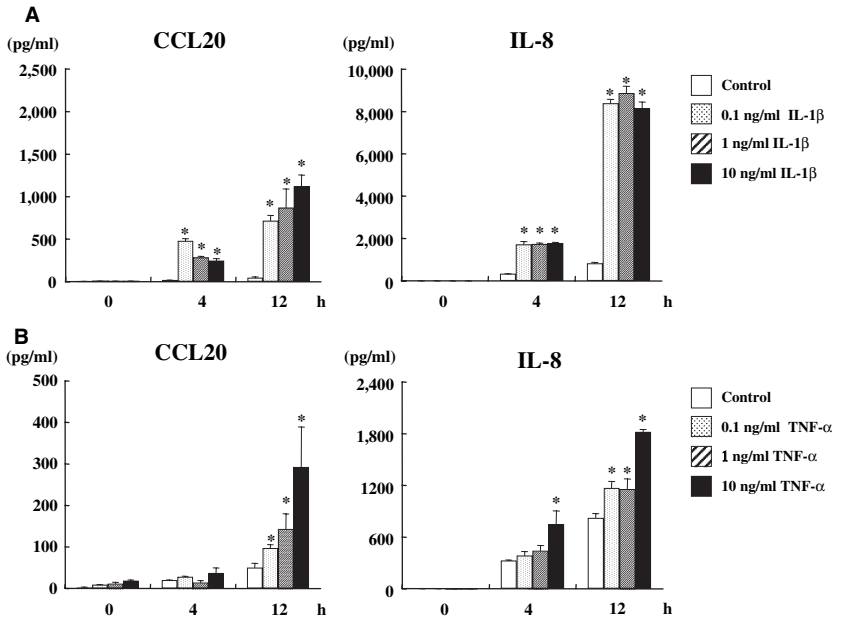

Fig. 6. CCL20 and interleukin-8 (IL-8) protein secretion induced in human pulpal fibroblastic cells (HPFC) upon stimulation by IL-1β (A) or tumor necrosis factor-α (TNF-α) (B). IL-1β or TNF-α were added to HPFC cultures at concentrations of 0.1, 1, and 10 ng/ml and incubated at 37°C for 0, 4, and 12 h. The controls consisted of monolayers incubated in medium alone. Supernatants were tested at 0, 4, and 12 h and the CCL20 and IL-8 levels were determined by enzyme-linked immunosorbent assay. Data are the means and SDs of triplicate determinations from three different experiments. Asterisks indicate significant differences ($P < 0.05$) vs. control values.

epithelial layer. Collectively, our results also suggest that native components from live bacteria have the ability to induce the expression of various proinflammatory mediators and this inductive effect is heat-sensitive.

In general, the accumulation of lymphocytes into the local inflamed lesion is modulated by chemokines and cell adhesion molecules (6, 46), and CCL20 plays an important role in the infiltration of lymphocytes through adhesion of lymphocytes to microvascular endothelial cells (7, 12). Hromas et al. reported that CCL20 was expressed in microvascular endothelial cells stimulated with TNF-α (17), and Dieu-Nosjean et al. also reported that CCL20 in endothelial cells was up-regulated after costimulation with IL-1β and TNF-α (10). Our recent report showed that microvascular endothelial cells in inflamed pulp tissues also expressed CCL20, and this finding suggested that CCL20 might function as an important chemoattractant to supply immunocytes to the inflamed lesions during pulpitis (30). In the present study, we demonstrated that levels of CCL20 expression were slightly increased in HUVEC after *S. mutans* infection (Fig. 4A,B). Furthermore, we found that 24 h stimulation with LTA (1 μg/ml) derived from *S. mutans* induced CCL20 production in HUVEC as much as after live *S. mutans* infection (Fig. 5B). These results indicate that production of CCL20, as well as of cellular adhesion molecules including ICAM-1 and VCAM-1, could be induced in microvascular endothelial cells after activation with bacterial surface components such as LTA, as well as after bacterial infection, which occurrs after invasion of caries-related bacteria into the pulp tissue. Charbonnier et al. reported that CCL20 expression was not induced in interferon-γ-stimulated HUVEC whereas human dermal microvascular endothelial cells (HDMEC) expressed CCL20 regardless of interferon-γ stimulation (9). This finding suggested that the CCL20 production capacity of microvascular endothelial cells isolated from various tissues is different. In future studies, we need to establish an isolation method for microvascular endothelial cells from dental pulp tissues and investigate the reactivity of these microvascular endothelial cells against stimulation by proinflammatory cytokines or bacteria.

In addition to CCL20 expression, we examined the levels of ICAM-1 and VCAM-1 expression in microvascular endothelial cells. VCAM-1 expression at the mRNA level was augmented after

stimulation with LTA as well as after *S. mutans* infection. VCAM-1, which is a cell adhesion molecule and belongs to the immunoglobulin super-family, is expressed by endothelial cells activated with IL-1 or TNF-α, and it participates in infiltration of lymphocytes into inflammatory sites (33). Our results suggest that *S. mutans* infection may induce endothelial cell-mediated accumulation of lymphocytes via VCAM-1 expression at local inflamed sites in pulp tissues.

In this study, it was clarified that HPFC had the ability to produce CCL20 as well as IL-8 after stimulation by proinflammatory cytokines (Fig. 6). It has been previously reported that CCL20 expression was up-regulated under IL-1β or TNF-α stimulation in fibroblasts derived from skin, chronic rheumatism, osteoarthritis, and cornea tissues (8, 16, 27, 29, 39). Our preliminary experiments showed that CCL20 production could not be clearly determined in HPFC after infection with *S. mutans* (data not shown). Dental pulp fibroblasts are major component cells in the dental pulp and they are activated by the inflammatory environment that results from the accumulation of inflammatory cells. Considering this inflammatory process, it is conceivable that fibroblasts produce CCL20 upon stimulation by proinflammatory cytokines secreted from activated macrophages, and that they may participate in $CCR6^{+}$ cell migration.

THP-1 cells were also induced to produce IL-8 upon stimulation by viable *S. mutans* (Fig. 2B). IL-8 is known to be a neutrophil migratory factor, and it has been reported that IL-8 was produced by THP-1 stimulated with *Streptococcus intermedius* (34) or lipopolysaccharide from *Porphyromonas gingivalis* and *Fusobacterium nucleatum* (2). Moreover, it was reported that peripheral blood mononuclear cells stimulated with *P. gingivalis* or *Actinobacillus actinomycetemcomitans* produced higher levels of IL-8 than cells infected with *S. mutans* (21). These findings suggest that monocytes/macrophages may have different degrees of reactivity against various species of oral bacteria. In the future, it will be necessary to determine the ability of macrophages, microvascular endothelial cells, and dental pulp fibroblasts to produce CCL20 after stimulation by other caries-related bacteria, such as *Lactobacillus*.

Our present study showed that macrophages, endothelial cells, and HPFC produced CCL20 after activation. Other recent studies have shown that odontoblasts also express CCL20 mRNA and that the expression level of this chemokine in the odontoblast layer of decayed teeth is significantly higher than that in healthy teeth (45). It has also been reported that other chemokines including CCL3 (CCR5 ligand), CCL4 (CCR5 ligand) and CXC chemokine ligand 10 (CXCR3 ligand), or their receptors are frequently observed in periapical granuloma, one of sequelae of pulpitis (22). Interestingly, Yang et al. identified that 17 chemokines including CCL20 had antimicrobial activity as well as chemotactic activity *in vitro* (48). Future studies are needed to resolve whether CCL20 plays a significant role compared with other chemokines in not only the formation of irreversible pulpitis but also the antimicrobial defense against pulpitis.

Tumor necrosis factor-α expression was low in all pulp samples compared to expression of other cytokines and chemokines (Fig. 1). Kokkas et al. and Pezelj-Ribaric et al. reported that TNF-α gene expression in inflamed human dental pulp tissue is positively associated with the severity of clinical symptoms (23, 35). Moreover, it has been reported that TNF-α concentration decreased as irreversible symptomatic pulpal inflammation progressed. It may be speculated that the decrease in TNF-α concentration represents a point at which tissue is in the late phase of irreversible inflammation, progressing towards total tissue necrosis. Similar findings have been reported by Tani-Ishii et al. who showed that the number of TNF-α-expressing cells increased five times from time zero to the second day, and 10 times from the second to the fourth day, and thereafter decreased until the seventh day of inflammation induced in rats (44). Therefore, it was suggested that this cytokine might be a marker of early inflammation. According to these previous reports, we consider that our clinical pulp tissue samples may have been in the late phase of irreversible pulpitis.

We observed that mRNA expression of CCL20 was detected in three out of nine samples, but was not detected in any clinically normal pulpal tissue samples. In addition to this chemokine, we found up-regulation of IL-8 (seven out of nine samples) and IL-1β (four out of nine) mRNA expression levels in inflamed pulpal tissue samples (Fig. 1). The mRNA expression level of CCL20-positive samples seems to correlate with those of IL-8-positive and IL-1β-positive samples. Moreover, in connection with these results of CCL20, IL-8 and IL-1β expression levels in inflamed pulpal tissue samples, the amount of IL-8 secreted in THP-1 cells after *S. mutans* infection was greater than that of CCL20 (Fig. 2) and the protein levels of CCL20 and IL-8 secreted from IL-1β-stimulated HPFC were increased in a time-dependent manner (Fig. 6). Furthermore, as dental pulp fibroblasts are major component cells in the dental pulp, we consider that HPFC produce CCL20 upon stimulation by proinflammatory cytokines (such as IL-1β) secreted from activated macrophages that have infiltrated the inflamed lesion. In further studies, we also need to obtain the inflamed pulp tissue samples based upon more detailed clinical information and perform more quantitative analysis of CCL20 expression using real-time RT-PCR. These experiments are underway.

In conclusion, CCL20 expression was induced in both monocytes/macrophages and endothelial cells after caries-related bacterial exposure, and HPFC also produced CCL20 in response to proinflammatory cytokines. These findings suggest that CCL20 might play an important role in the progression of pulpitis via recruitment of inflammatory cells into the dental pulp.

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