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Increase of CCL20 expression by human gingival fibroblasts upon stimulation with cytokines and bacterial endotoxin

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Summary

We have demonstrated recently that CCL20 was expressed in periodontal diseased tissues and abundant CCR6 positive T cells infiltrated in periodontally diseased tissue. However, it is uncertain which cells can elicit CCL20 production. In the present study, we examined the properties of CCL20 production by human gingival fibroblasts (HGF) culture. Here, we report that interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and *Escherichia coli* lipopolysaccharide (LPS) can significantly induce the production of CCL20 by HGF. We found that TNF-α and *E. coli* LPS enhanced the production of CCL20 by HGF treated with IL-1β. In contrast, interferon-gamma (IFN-γ) dramatically diminished CCL20 production induced by IL-1β. Moreover, we demonstrated that nuclear factor-kappaB (NF-κB), p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases (ERK) play an important role in mediating the production of CCL20 induced by IL-1β and TNF-α. On the other hand, we found that not only NF-κB, p38 MAPK and ERK but also c-Jun NH2-terminal kinase (JNK) are involved in CCL20 production induced by *E. coli* LPS. Finally, we found that HGF express CCR6, CCL20 receptor, and CCL20 induced vascular endothelial growth factor (VEGF) by HGF. Taken together, these findings that HGF will be a source of CCL20 in periodontal tissue, and the CCL20 production will be controlled by proinflammatory cytokine and bacterial LPS in periodontally diseased tissue. Thus, CCL20 by HGF might be involved in inflammatory cells infiltration, and promote the progression of periodontal disease.

Keywords: CCL20, fibroblast, periodontal disease

Introduction

Periodontal disease is characterized as chronic inflammation associated with Gram-negative bacteria in the oral cavity [1,2], resulting in soft tissue destruction and periodontal bone resorption. Although host-immune response to these bacteria has been suggested to be associated with alteration or even progress of this disease [3], the mechanism of leucocyte infiltration in periodontally diseased tissues is still unknown.

It has been reported that some chemokines that are involved in leucocyte migration are expressed in periodontally diseased tissues. Constitutive expression of monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8), along with lesser expression of growth-related gene product gamma (GRO-γ), macrophage inflammatory protein-1 alpha (MIP-1α) and macrophage inflammatory protein-1 beta (MIP-1β) were measured by reverse transcription-polymerase chain reaction (RT-PCR) in healthy gingivae [4]. Up-regulated production of MCP-1 and MIP-1α in diseased tissue was observed to correlate with the degree of inflammation [5,6]. Furthermore, we have reported recently that CCL20 and its receptor, CCR6, positive T cells infiltrated into periodontally diseased tissues [7]. However, it is uncertain which cells can elicit CCL20 production in periodontally diseased tissues.

CCL20 is expected to play a crucial role in trafficking and homing of not only memory T cells but also some kinds of leucocytes, including immature dendritic cells, into inflammatory sites such as atopic dermatitis [8], hepatitis [9], arthritis [10,11] and periodontal disease [7]. CCL20 exerts its activity through binding to CCR6 [12–16], which is not

shared by any other known chemokine, but nevertheless binds a member of the structurally unrelated β-defensins [17]. CCR6 is found to be expressed on immature dendritic cells and memory T lymphocytes as well as on B lymphocytes in various lymphoid organs, and in pancreas [12–15,18,19]. Up-regulation of CCR6 expression on human neutrophils by cytokines can explain the slight chemotactic response of these cells to CCL20 [20].

Fibroblasts were previously considered important connective tissue cells that construct a supporting framework crucial for tissue integrity and repair. Recently, fibroblasts have been suggested to be important sentinel cells in immune systems [21]. Fibroblasts actively define the structure of tissue microenvironments and regulate inflammatory response by the production of cytokines such as IL-1β and IL-8 [22]. However, there is still uncertainty about CCL20 production.

In the present study, we focused on human gingival fibroblasts (HGF). We examined the production of CCL20 by HGF stimulated with proinflammatory cytokines, lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Moreover, we examined the signalling pathways involved in CCL20 production by HGF. Furthermore, we investigated the role of CCL20 on HGF, especially vascular endothelial growth factor (VEGF) production.

Materials and methods

Cells and culture condition

HGF was prepared from the explants of clinically normal gingiva from patients (three females, aged 26–40 years old) during routine distal wedge surgical procedure, with informed consent. The patients had no periodontal disease, and they were systemically healthy with no evidence of known systemic modifiers of periodontal disease (types 1 and 2 diabetes mellitus, osteoporosis and medications known to influence periodontal tissues). Exclusion criteria included those patients who had taken systemic antibiotic, anti-inflammatory, hormonal or other assisted drug therapies in the 6 months prior to the study. Explants were cut into pieces and culture in 100-mm diameter tissue cultured dishes in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KA, USA), penicillin 50 IU/ml and streptomycin 50 μg/ml with a medium change every 3 days for 10–15 days until confluent cell monolayers were formed. The cells were detached with 0·25% trypsin-ethylenediamine tetraacetic acid (EDTA), washed with phosphate buffered saline (PBS) and subcultured in plastic flasks. After three to four subcultures by trypsinization, homogeneous, slim spindle-shaped cells grown in characteristic swirls were obtained. The cells were used as confluent monolayers at subculture levels 5–15. HGF were stimulated with IL-1β (0·1–100 ng/ml; Peprotech, Rocky Hill, NJ, USA), TNF-α (0·1–100 ng/ml; Peprotech), IFN-γ (0·1–100 ng/ml; Peprotech), *Esherichia coli* LPS (0·1–100 μg/ml; serotype 026:B6, purchased from Sigma, St Louis, MO, USA), *Porphyromonas gingivalis* (*Pg*) w83 LPS (1–100 μg/ml), *Staphylococcus aureus* (*Sa*) LTA (1–100 μg/ml; Sigma) and *Streptococcus mutans* (*Sm*) LTA (1–100 μg/ml; Sigma) for the periods indicated in the figure legends. At a predetermined time, cell-free supernatants were harvested and stored at −80°C for cytokine determination. In selected experiments, HGF were cultured for 1 h in the presence or in the absence of SB203580 (0·2–20 μM; Santacruz, Santa Cruz, CA, USA), PD98059 (0·2–20 μM; Calbiochem, La Jolla, CA, USA) and SP600125 (0·2–20 μM; Sigma), MG-132 (0·5–50 μM; Calbiochem) prior to incubation with the various stimuli. The study was performed with the approval and compliance of the Tokushima University Ethical Committee.

Cytokine determination

CCL20 and VEGF concentration in the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). Duoset (R&D Systems, Minneapolis, MI, USA) was used for CCL20 detection. The human VEGF ELISA development kit (Peprotech) was used for VEGF detection. The assay was performed according to the manufacture's instructions. The data were determined by using a standard curve prepared for each assay.

RNA extraction and RT-PCR analysis

Total RNA was prepared from HGF using an RNeasy total RNA isolation Kit (Qiagen, Hilden, Germany). Single-strand cDNA for a PCR template was synthesized from 48 ng of total RNA using primer oligo$(dT)_{12–18}$ (Invitrogen, Carlsbad, CA, USA) and the superscript III reverse transcriptase (Invitrogen) under the conditions indicated by the manufacture. Specific primers were designed from cDNA sequence for CCL20 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each cDNA was amplified by PCR using Hot star *Taq* DNA polymerase (Qiagen). The sequences of the primers were as follows: CCL20-F (5′-TTG CTC CTG GCT GCT TTG-3′), CCL20-R (5′-ACC CTC CAT GAT GTG CAA G-3′), GAPDH-F (5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′) and GAPDH-R (5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′). The conditions for PCR were 1× (95°C, 15 min), 35× (94°C, 40 s, 55°C, 40 s, 72°C, 1 min) and 1× (72°C, 10 min). The products were analysed on a 2·0% agarose gel containing ethidium bromide. The expected size for the PCR products for CCL20 and GAPDH were 367 base pairs (bp) and 985 bp, respectively.

Flow cytometric analyses

Cultured HGF were detached by using PBS-4 mmol/l EDTA and washed twice with PBS. Detached cells were incubated with anti-human CCR6 monoclonal antibody (mAb) (R&D

Systems) or isotype control antibody (Dako, Kyoto, Japan) on ice for 30 min. After washing three times with PBS-1% bovine serum albumin (BSA; Sigma), the cells were incubated with fluoroscein isothiocyanate (FITC)-conjugated rabbit anti-mouse F(ab')$_2$ fragment (Dako) for 30 min on ice. After washing three times with PBS-1% BSA, the cells were analysed immediately by flow cytometry (Epics XL-MCL; Coulter, Hialeah, FL, USA).

Statistical analysis

Data are presented as mean values ± standard deviation (SD). Differences between the two groups were calculated using Student's *t*-test. *P*-values of less than 0·05 were considered significant.

Results

Induction of CCL20 mRNA by IL-1β or TNF-α

As shown in Fig. 1a, CCL20 mRNA was induced by both IL-1β and TNF-α in a dose-dependent fashion. CCL20 mRNA expression was enhanced at 2 h stimulation, peaked at 4 h stimulation and returned weak levels at 24 h stimulation (Fig. 1b).

CCL20 release by stimulated HGF

We examined whether HGF could produce the CCL20 protein by ELISA. We stimulated HGF with proinflammatory cytokines (Fig. 2a), LPS or LTA (Fig. 2b). IL-1β and TNF-α induced a dose dependent increase in CCL20 release. However, IFN-γ could not induce CCL20 (Fig. 2a). *E. coli* LPS could induce CCL20 at low concentrations. On the other hand, *Pg* LPS, *Sa* LTA and *Sm* LTA could not induce CCL20 from HGF significantly (Fig. 2b).

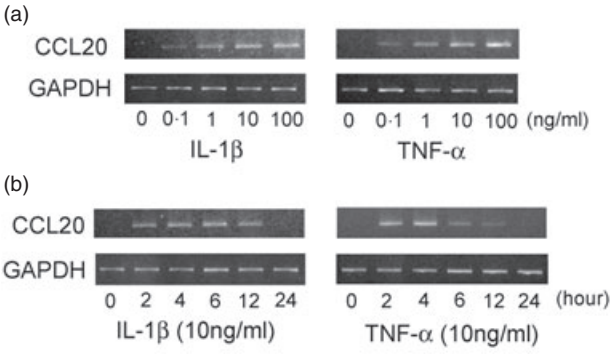

Fig. 1. Induction of CCL20 mRNA by interleukin (IL)-1β and tumour necrosis factor (TNF)-α. (a) Total RNA was prepared from human gingival fibroblasts (HGF) treated without or with IL-1β (0·1–100 ng/ml) or TNF-α (0·1–100 ng/ml) as indicated. After 4 h, reverse transcription-polymerase chain reaction (RT-PCR) analysis was carried out for CCL20 and GAPDH. (b) Total RNA was prepared from HGF treated without or with IL-1β (10 ng/ml) or TNF-α (10 ng/ml) for 2, 4, 6, 12 and 24 h. RT-PCR analysis was carried out for CCL20 and GAPDH. Similar results were obtained in three repeated experiments.

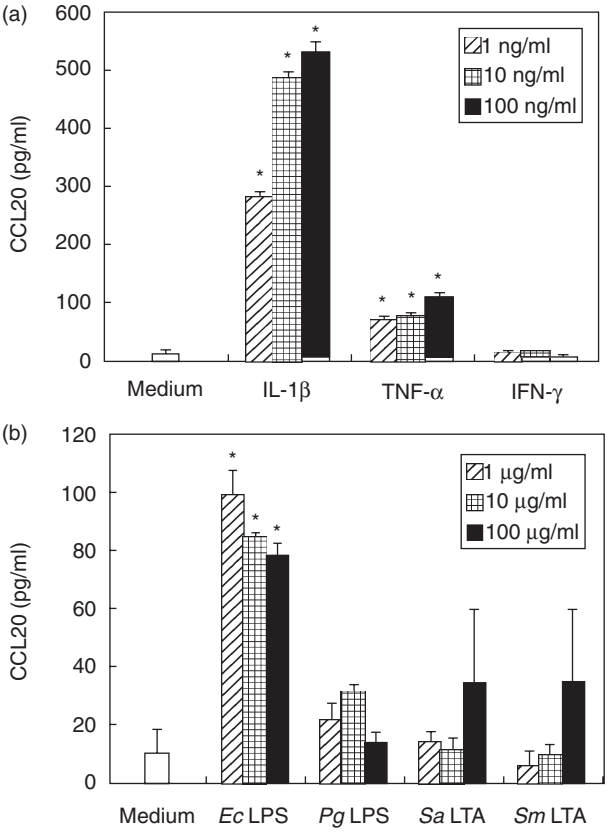

Fig. 2. Release of CCL20 by human gingival fibroblasts (HGF). HGF was cultured in the absence or presence of interleukin (IL)-1β (1–100 ng/ml), tumour necrosis factor (TNF)-α (1–100 ng/ml), interferon (IFN)-γ (1–100 ng/ml), *Escherichia coli* lipopolysaccharide (LPS) (1–100 μg/ml), *Porphyromonas gingivalis* LPS (1–100 μg/ml), *Staphylococcus aureus* lipoteichoic acid (LTA) (1–100 μg/ml) and *Streptococcus mutans* LTA (1–100 μg/ml) for 24 h at 37°C. Medium was removed and assayed for CCL20 release by enzyme-linked immunosorbent assay. Data are presented as the mean ± SD ($n = 3$; by Student's *t*-test, $^*P < 0·05$, stimulated *versus* unstimulated). Similar results were obtained in three repeated experiments.

Effects of TNF-α, *E. coli* LPS and IFN-γ on the production of CCL20 by IL-1β-stimulated HGF

Figure 2 shows that IL-1β, TNF-α and *E. coli* LPS can induce CCL20 by HGF. Next, we examined whether TNF-α, *E. coli* LPS and IFN-γ modulate CCL20 production induced by IL-1β. Figure 3 shows that TNF-α and *E. coli* LPS could enhance IL-1β-induced CCL20 secretion in a dose-dependent fashion. On the other hand, IL-1β-induced CCL20 production was dramatically diminished by IFN-γ treatment (Fig. 3). We examined whether IFN-γ induced cell death by using cell viability assay. IFN-γ treatment did not induce cell death of HGF (data not shown). IFN-γ treatment did not change

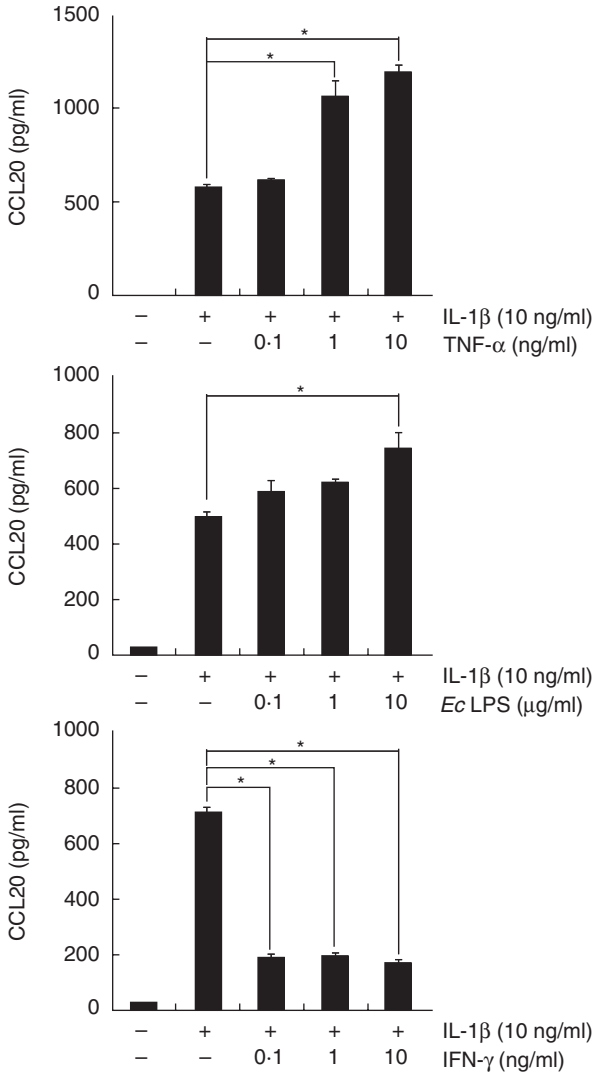

Fig. 3. Effects of tumour necrosis factor (TNF)-α, *Escherichia coli* lipopolysaccharide and interferon (IFN)-γ on CCL20 release by interleukin (IL)-1β–stimulated human gingival fibroblasts (HGF). HGF was stimulated with IL-1β (10 ng/ml) in the presence or absence of TNF-α (0·1–10 ng/ml), *E. coli* LPS (0·1–10 μg/ml) or IFN-γ (0·1–10 ng/ml) for 24 h at 37°C. Medium was removed and assayed for CCL20 release by enzyme-linked immunosorbent assay. Data are presented as the mean ± SD ($n = 3$; by Student's *t*-test, $^*P < 0{\cdot}05$). Similar results were obtained in three repeated experiments.

CCL20 production by HGF stimulated with TNF-α or *E. coli* LPS (data not shown).

Involvement of p38 MAPK, ERK, JNK and NF-κB in induction of CCL20 in HGF stimulated with IL-1β, TNF-α and *E. coli* LPS

To determine whether p38 MAPK and/or ERK and/or JNK and/or NF-κB are required for CCL20 production in response to IL-1β, TNF-α or *E. coli* LPS, the effects of several inhibitors on CCL20 production by HGF were examined. At a concentration of 50 μM, MG-132, a cell-permeable peptide-aldehyde protease inhibitor that blocks NF-κB activation via its effect on the proteasome, completely prevented the induction of CCL20 production stimulated with IL-1β, TNF-α and *E. coli* LPS (Fig. 4). PD98059, a specific inhibitor that binds inactive forms of MEK and prevents their activation and phosphorylation resulting in inhibition of ERK, partially inhibited CCL20 production by HGF stimulated with IL-1β, TNF-α and *E. coli* LPS. SB203580, a selective inhibitor of p38 MAPK, binds with high affinity to p38

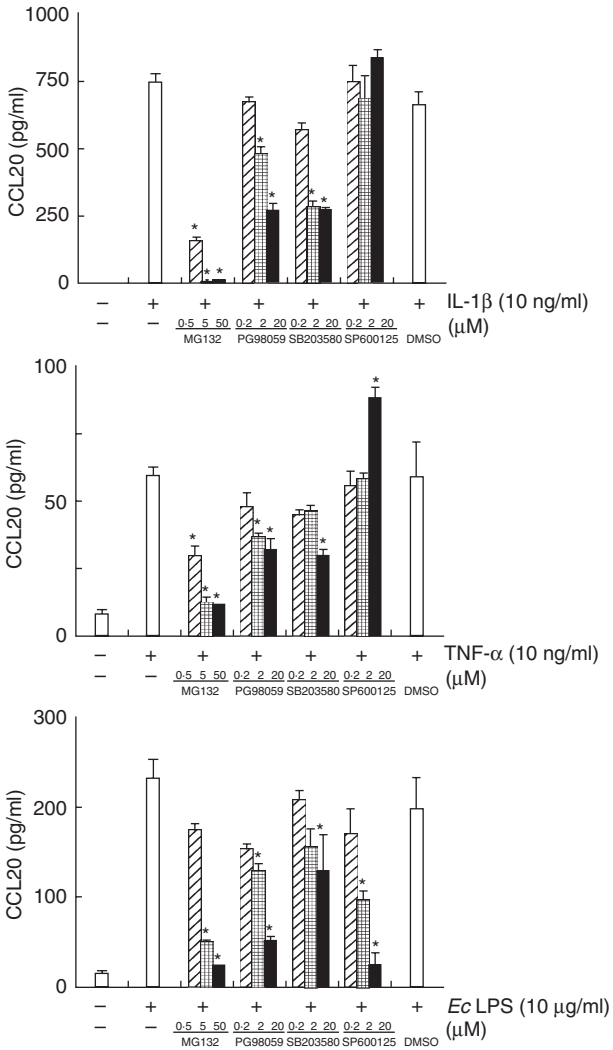

Fig. 4. Effects of signalling pathway inhibitors the production of CCL20 production by stimulated human gingival fibroblasts (HGF). HGF was pretreated for 1 h with or without MG-132 (0·5–50 μM), PD98052 (0·2–20 μM), SB203580 (0·1–10 μM), SP600125 (0·2–20 μM) or dimethylsulphoxide (1 : 2000 dilution) as a control, and treated with interleukin (IL)-1β (10 ng/ml), tumour necrosis factor (TNF)-α (10 ng/ml) or *Escherichia coli* lipopolysaccharide (10 μg/ml) for 24 h at 37°C. Medium was removed and assayed for CCL20 release by enzyme-linked immunosorbent assay. Data are presented as the mean ± SD ($n = 3$, by Student's *t*-test, $^*P < 0{\cdot}05$ *versus* stimulated). Similar results were obtained in three repeated experiments.

MAPK near the ATP-binding site, thus rendering p38 MAPK inactive, partially blocked induction of CCL20 by HGF in response to IL-1β, TNF-α or *E. coli* LPS. SP600125, a selective JNK inhibitor, blocked CCL20 production by HGF stimulated with only *E. coli* LPS. On the other hand, CCL20 production induced by TNF-α was enhanced by SP600125 treatment (Fig. 4).

CCL20 induces VEGF production by HGF

We examined CCR6 expression on HGF using flow cytometry. Flow cytometric analysis revealed that CCR6 was expressed on non-stimulated HGF (Fig. 5a). To determine the effects of CCL20 on HGF, HGF was stimulated with CCL20 and the production of VEGF was analysed by ELISA. Figure 5b shows that VEGF levels were significantly higher in HGF cultures treated with CCL20 (10 ng/ml or 100 ng/ml) compared to the unstimulated HGF culture. At the same time, we examined IL-8 production and ICAM-1 expression by HGF stimulated with CCL20. However, CCL20 treatment did not induce IL-8 production and ICAM-1 expression by HGF (data not shown).

Discussion

It has been reported that CCL20 is a CC chemokine expressed mainly by surface-lining cells, such as mucosal epithelial cells and epidermal keratinocytes [8,23,24]. Its receptor CCR6 is expressed on immature dendritic cells, B cells and memory T cells [13]. Thus, it is likely that CCL20 is an important mediator for both the initiation and effector phases of immune responses. In this study, we examined the mechanism of CCL20 production by HGF. We have shown that CCL20 is highly inducible by proinflammatory cytokines such as IL-1β and TNF-α. Previously, Nakayama *et al.* have also shown that CCL20 is inducible in epidermal keratinocytes by IL-1β and TNF-α [8]. Thus, these proinflammatory cytokines might be universal inducers of CCL20 in human cells. TNF-α was much less effective than IL-1β for induction of CCL20 by HGF. It is reported that the opposite was true in the case of T84 cells (human colon carcinoma cell lines) [25]. Thus, the effects of IL-1β and TNF-α on induction of CCL20 may be dependent on the cellular background.

We showed that *E. coli* LPS could induce CCL20 production by HGF. It is reported that *E. coli* LPS-treated peripheral blood mononuclear cells [26] or neutrophils [27] could produce CCL20. On the other hand, *E. coli* LPS could not induce CCL20 production by epithelial-type cells [25]. This means that the effects of *E. coli* LPS on induction of CCL20 will be dependent upon the type of cells.

We reported that IFN-γ could not induce CCL20 by HGF and inhibited CCL20 production induced by IL-1β. Schutyser *et al.* reported that IFN-γ could not induce

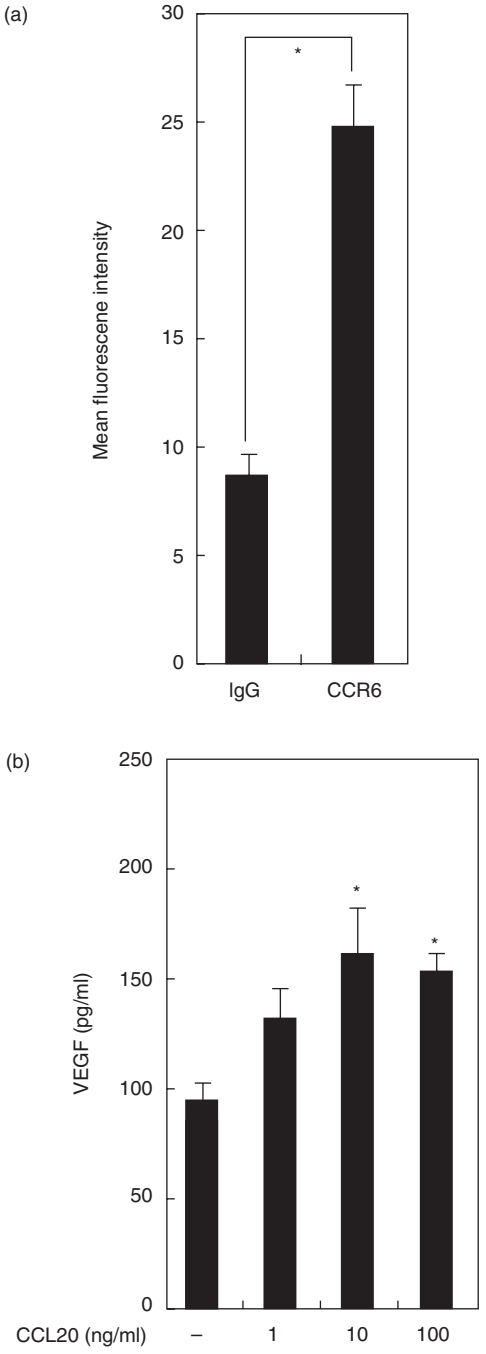

Fig. 5. Release of vascular endothelial growth factor (VEGF) by human gingival fibroblasts (HGF) treated with CCL20. (a) CCR6 expression on HGF. HGF was incubated with human CCR6 mouse antibody or isotype-matched mouse IgG. Cells were then stained with fluoroscein isothiocyanate (FITC)-labelled goat antimouse IgG and analysed with flow cytometry to determine the expression of CCR6. Data are presented as the mean ± SD ($n = 3$; by Student's t-test, $^*P < 0{\cdot}05$). Similar results were obtained in three repeated experiments. (b) HGF was stimulated in the absence or presence of CCL20 (1–100 ng/ml) for 24 h at 37°C. Medium was removed and assayed for VEGF release by enzyme-linked immunosorbent assay. Data are presented as the mean ± SD ($n = 3$; by Student's t-test, $^*P < 0{\cdot}05$, stimulated *versus* unstimulated). Similar results were obtained in three repeated experiments.

CCL20 by epithelial cell, monocyte and skin fibroblasts [26] and Fujiie *et al.* reported that IFN-γ consistently suppressed expression of CCL20 in Caco-2 (human colon carcinoma cell line) and T84 cells [25]. Therefore, our results agree with their studies.

We have demonstrated further that induction of CCL20 by IL-1β, TNF-α and *E. coli* LPS is mediated critically by NF-κB. NF-κB is an important transcriptional factor for inflammatory and immunological responses. NF-κB is known to be involved in expression of various cytokines, acute-phase proteins and adhesion molecules [28]. NF-κB has also been shown to play an essential role in the induction of a number of inflammatory chemokines such as IL-8, monocyte chemoattractant protein-1 (MCP-1)/CCL2, regulated upon activation, normal T-cell expressed and secreted (RANTES)/CCL5 and eotaxin/CCL11 [29–32]. The present results extend its list of targets to CCL20.

Activation of ERK and p38 MAPK pathways by IL-1β, TNF-α and *E. coli* LPS has been well described. Reibman *et al.* reported that ERK and p38 MAPK pathways were related to CCL20 production by airway epithelial cells stimulated with IL-1β or TNF-α [33]. Our results show that HGF use the same signalling pathways to release CCL20 as epithelial cells.

Rhee *et al.* reported that the JNK inhibitor did not modulate CCL20 production induced by flagellin [34]. We report here that CCL20 production induced by *E. coli* LPS was inhibited by the JNK inhibitor; it has been reported that treatment of fibroblasts with *E. coli* LPS caused activation of JNK [35]. The difference between our report and Sang's may be dependent upon the type of cells, because CCL20 production pattern was totally different from each cell type. Further investigation will be necessary concerning the signalling pathway involved in CCL20 production.

Recently, Shiba *et al.* reported that human pulp fibroblasts express CCR6 [36]. Our reports add to the data showing that HGF also express CCR6. We also show that CCL20 induce VEGF. It is reported CCL20 stimulation induced osteopontin expression in human pulp fibroblasts [36]. Fujiie *et al.* reported that CCL20 enhanced the growth of Huh7 cells (hepatocellular carcinoma cells) [25]. CCL20 may be related to tissue remodelling by inducing VEGF production, cell proliferation, extracellular matrix including osteopontin as well as leucocyte infiltration.

In summary, these data describe the synthesis and release of CCL20 by HGF. The wide range of stimuli, including proinflammatory cytokines and LPS, suggest that this chemokine may serve an important role in the progression of periodontal disease. CCL20 released by HGF might be important for CCR6 positive leucocytes, including memory T cells and immature dendritic cells infiltration in periodontal tissues, and might be involved in the pathogenesis of periodontal disease.

Acknowledgements

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