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Enhancement of gingival inflammation induced by synergism of IL-1β and IL-6

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ABSTRACT

Internleukin-1 (IL-1) and IL-6 are the most potent proinflammatory cytokines being involved in inflammatory diseases such as periodontitis. The objective of this study was to examine the synergistic effects of IL-1β and IL-6 on gingival inflammation by targeting cultured human gingival fibroblasts (HGFs). HGFs were treated with IL-1β or IL-6/soluble IL-6R (sIL-6R), and total RNA and total cell lysate were collected to examine expression of gp130 known as a signal transducer of IL-6 using qRT-PCR and Western blotting. IL-1β-mediated IL-6 productivity in HGFs was examined using ELISA method. Likewise, after HGFs and THP-1 macrophages were treated with IL-1β, TNF-α and IL-6, sIL-6R productivity was examined. Next, HGFs were treated with IL-6/sIL-6R after pretreatment of IL-1β, and the intracellular signals were examined using Western blotting. Finally, various mRNA/protein expressions in HGFs treated with IL-6/sIL-6R after pretreatment of IL-1β were examined using qRT-PCR and ELISA method. IL-1β increased significantly both gp130 and IL-6 expression in HGFs. IL-6 increased significantly sIL-6R production in THP-1 macrophages but not HGFs. Co-stimulation with IL-1β and IL-6/sIL-6R induced dramatically the phosphorylation of Stat3, ERK and JNK in HGFs. Interestingly, expression of various inflammation-related molecules such as MMP-1, MCP-1, IL-1ra, bFGF and VEGF were enhanced by co-stimulation with IL-1β and IL-6/sIL-6R in HGFs. Gingival inflammation is regulated by HGFs affected by both IL-1β and IL-6/sIL-6R synergistically through induction of gp130 expression, resulting in progression of periodontitis.

Interleukin-1 (IL-1) is an important pro-inflammatory cytokine that elicits a wide variety of biologic activities in various cell types (14), and has been implicated in the progression of chronic inflammatory disease such as rheumatoid arthritis and periodontitis (1). It is well known that IL-1 induces osteoclastogenesis by targeting osteoclasts, resulting in bone loss (14). IL-6 is also a pleiotropic cytokine that regulates immune and inflammatory responses (14, 26). Although IL-6 has been reported to be a principal regulator of acute phase proteins (16), other cytokines such as IL-1 and tumor necrosis factor (TNF)-α also participate in induction of a broad subset of acute phase proteins (16, 25). Furthermore, IL-6 may promote osteoclastogenesis indirectly by increasing release of RANKL derived from osteoblasts and synovial cells (26). Thus, IL-1 and IL-6 corporate each other, and form complicate network to regulate the inflammatory process. However, in spite of extensive studies on both IL-1 and IL-6 over the past two decades (1, 14, 16, 25, 26), the relationship by these cytokines remains unclear in inflammatory lesions, especially periodontitis.

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Periodontitis is a bacterial infectious disease, and many inflammatory cytokines regulate the pathophysiology (27). Previously, many researchers have shown the effects of inflammatory cytokines such as IL-1 and IL-6 on periodontal cells, *e.g.* fibroblasts, epithelial cells and macrophages (20). In periodontal inflamed tissues, IL-1 and IL-6 may be responsible to the tissue destruction by increasing matrix-metalloproteinases (MMPs) (2, 20). The evidence for the role of MMPs in periodontal destruction has been reported, and it is now recognized that an imbalance between activated MMPs and their endogenous inhibitors, *i.e.* tissue inhibitors of MMPs (TIMPs) leads to pathologic breakdown of the extracellular matrix seen in periodontitis (22). Therefore, the imbalance induced by inflammatory cytokines should be important to understand the mechanism of tissue destruction.

IL-1 activates mitogen-activated protein kinase (MAPKs) or NF-κB pathway via phosphorylation of IL-1 receptor-activated protein kinase (IRAK) in several cell types (24). Whereas, after IL-6 binds to the IL-6 receptor gp80, these proteins build a complex with IL-6 signal transducer gp130, which in turn results in the intracellular dimerization of these glycoproteins (26). This event triggers activation of Janus kinases which phosphorylates distinct intracellular tyrosine residues of the gp130. They serve as docking sites and activate the downstream pathways, at least two distinct signaling cascades: signal transducer and activator of transcription (Stat) 3; and MAPKs (26, 27). Thus, since intracellular molecules activated by IL-1 and IL-6 should be overlapped in many cell types, investigation of the intracellular signals following receptor activation might be required to clarify the potential mechanisms of synergism of IL-1 and IL-6. In addition, it is well known that soluble form of IL-6R (sIL-6R) has agonistic functions of IL-6 and requires cells expressing the gp130 (27). This pathway allows cells that do not express surface IL-6R such as human gingival fibroblasts (HGFs) to respond to the presence of IL-6 (19), resulting in vascular endothelial growth factor (VEGF) production seen in periodontitis lesions (18). Exactly, we previously reported that IL-6 signals in the presence of sIL-6R are initiated in the phosphorylation of gp130 in human gingival fibroblasts (HGFs) (19).

Previously, we have reported that a periodontal pathogen *Porphyromonas gingivalis* infection significantly enhanced gp130 expression in human umbilical vein endothelial cell (HUVEC), and both intracellular MAPKs and NF-κB were activated by the infection (11). Based on these findings, we hypothesized that IL-1β also might induce gp130 expression in HGFs, leading to synergistic effects of IL-1β and IL-6. In the present study, we investigated the possible mechanisms of gingival inflammatory regulation by the synergism of IL-1β and IL-6 by targeting HGFs.

MATERIALS AND METHODS

Reagents. Recombinant human (rh) IL-1β, IL-6 and rhsIL-6R were obtained from R&D Systems (Minneapolis, MN). Antibodies against phospho-Stat3, phospho-ERK and phospho-c-*jun* NH_2-terminal kinase (JNK) were obtained from Cell Signaling Technology (Beverly, MA). Antibody against cathepsin L was purchased from BD Biosciences (San Jose, CA). Antibody against β-actin and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (St. Louis, MO).

Cell culture. HGFs were isolated from the fresh gingival tissue biopsy samples from volunteers as previously reported (19), and were maintained in a medium consisting of DMEM (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and streptomycin (Invitrogen). Informed consent was obtained from each volunteer before participation, and the Ethics Committee of Iwate Medical University approved the research protocol (approval No.1126). The human monocytic leukemia cell line THP-1 cells (a gift from Dr. M. Taira, Iwate Medical University) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS and antibiotics (Invitrogen). THP-1 cells were differentiated by 100 nM phorbol 12-myristate 13-acetate (PMA). Experimental culture designs have been shown in Fig. 1.

Quantitative RT-PCR. To investigate gp130 mRNA expression, HGFs or THP-1 macrophages were treated for 6 h with or without rhIL-1β (1 ng/mL) or IL-6/sIL-6R (50 ng/mL each). Culture design has been shown in Fig. 1A. Next, to investigate the changes of various genes expression, cells were pretreated for 24 h with or without IL-1β (1 ng/mL) and then treated with or without IL-6/sIL-6R (50 ng/mL each) for 6 h (Fig. 1B). Total RNA was extracted with ISOGEN reagent (Nippongene, Tokyo, Japan), and first strand cDNA was synthesized by using the PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan). Quantitative RT-PCR (qRT-PCR) was performed using the Thermal Cycler Dice Real time

System (Takara Bio) with SYBR Premix EX Taq II (Takara Bio) on cDNA generated from the reverse transcription of purified RNA. The mRNA expression of target genes was normalized against β-actin mRNA expression using the comparative cycle threshold method. Primer sequences of target genes for PCR reactions have been shown in Table 1.

Cell proliferation assay. Cell proliferation activity was examined using MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide] assay as described previously (14). Culture design has been shown in Fig. 1B. In short, cells were seeded in each well of a 96-well plate (Corning Costar, Cambridge, MA) in a final volume of 100 µL of DMEM medium supplemented with 0.5% FBS. After cells reached subconfluence, the cells were treated with 1 ng/mL of IL-1β for 24 h and then IL-6/sIL-6R (50 ng/mL each) were added. At the end of the treatments, MTT (final concentration: 0.5 µg/mL) was added to each well and incubated for 4 h in a humidified atmosphere prior to the addition of 100 µL of acid solution (0.04 N HCl in isopropanol) into each well. The reaction mixture on each well of the 96-well culture plate was measured fluorometrically using a micro plate reader (iMARK™; Bio-Rad, Hercules, CA; excitation at 570 nm).

Western blotting. To examine the gp130 expression, HGFs were treated with IL-1β (1 ng/mL) or IL-6/sIL-6R (50 ng/mL each) for 24 h (Fig. 1A). In addition, cells were treated with IL-1β or IL-6/sIL-6R for 10 min to examine the intracellular signaling (Fig. 1B). Total cell lysate was extracted with a lysis buffer [10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1% NP-40 and protease inhibitor cocktail (Complete™; Roche Diagnosis, Berkeley, CA)]. The total proteins

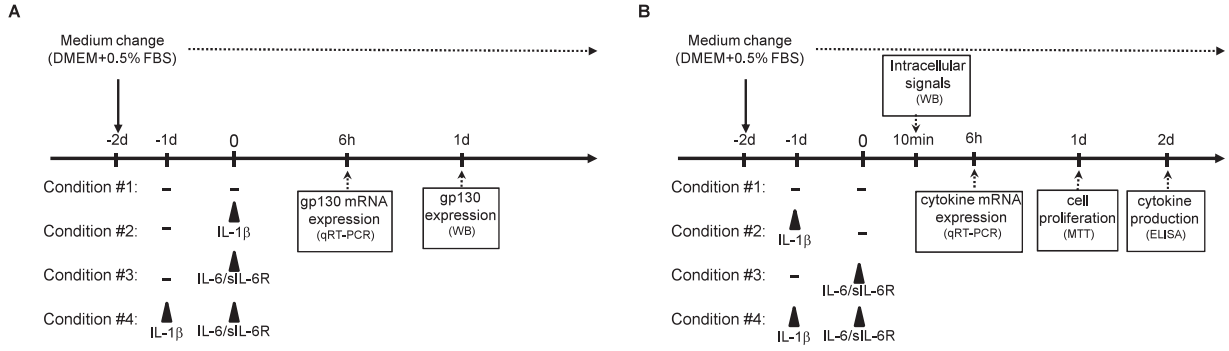

Fig. 1 Culture designs and protocol.

Table 1 *Primer sequences for qRT-PCR*

Target gene	Accession #	Primer
gp130**	-	F: AATGGCAGCATACACAGATGAAGG
		R: ATGCTAAGCAAACAGGCACGACTA
MMP-1	NM_032006.3	F: AAGCAGCCCAGATGTGGAGTGCCT
		R: TGTGTTTGCTCCCAGCGAGGGTTCC
MMP-2**	-	F: GATAACCTGGATGCCGTCGTG
		R: CAGCCTAGCCAGTCGGATTTG
MMP-3	NM_002422.3	F: TACAAGGAGGCAGGCAAGACAG
		R: GGATAGGCTGAGCAAACTGCCA
MMP-13	NM_002427.3	F: CCAGTTTGCAGAGCGCTACCT
		R: TTGCCAGTCACCTCTAAGCCG
MMP-14*	-	F: GGAACCCTGTAGCTTTGTGTCTGTC
		R: TGAGGGTCCTGCCTTCAAGTG
TIMP-1	NM_003254.2	F: TGAAAAGGGCTTCCAGTCCCG
		R: TGGACACTGTGCAGGCTTCAG
TIMP-2	NM_003255.4	F: TGTGCCCTCCCAGGCTTAGTGT
		R: GCTGGCGTCACATGCAGAAAGC
TIMP-3	NM_000362.4	F: GGCTTCACCAAGATGCCCCAT
		R: ACAGCCCCGTGTACATCTTGC
Cathepsin B	NM_001908.3	F: AGCGCTGGGTGGATCTAGGA
		R: GTTGACCAGCTCATCCGACAGG
IGF-1	NM_001111285.1	F: AGACCCAGAAGTATCAGCCCCC
		R: TCTGTCCCCTCCTTCTGTTCCC
IL-1ra	NM_173842.2	F: CCTTGCTGCAGTCACAGAATGG
		R: GGATTTTCTCCCAGAGGGTCGG
IL-33	NM_033439.3	F: GCTCCGCTCTGGCCTTATGAT
		R: TTGACAGGCAGCGAGTACCAG
β-actin*	-	F: ATTGCCGACAGGATGCAGA
		R: GAGTACTTGCGCTCAGGAGGA

MMP, matrix metalloproteinase; TIMP, tissue inhibitors of matrix metalloproteinases; IGF-1, insulin-like growth factor-1. *, purchased from Sigma; **, purchased from Takara Bio.

(10 μg each) were separated in a denaturing 7.5–12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) as described elsewhere. The membranes were then blocked with 5% skim milk in TTBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and subsequently incubated with antibodies against phospho-JNK or phospho-ERK. Immunoreactive proteins were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and Super Signal kits (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. β-actin was used as an internal control of loading proteins. Finally, to examine the cathepsin L production, cells were treated with IL-6/sIL-6R after pretreatment of IL-1β for 48 h, and the culture supernatants were collected and stocked at −80°C until use. To ensure whether the equivalent protein levels of supernatants were loaded in each lane, blotted membrane was stained with coomassie-brilliant blue.

ELISA. To investigate the IL-6 secretion, HGFs were treated for 24 h with or without IL-1β (1 ng/mL). Next, to investigate the sIL-6R secretion, THP-1 macrophages and HGFs were treated for 24 h with or without IL-1β (1 ng/mL), TNF-α (5 ng/mL) and IL-6 (10 ng/mL). Thirdly, to investigate the changes of various proteins secretion, cells were pre-treated for 24 h with or without IL-1β (1 ng/mL) and then treated with or without IL-6/sIL-6R (50 ng/mL each) for 48 h. Culture design has been shown in Fig. 1B. Supernatants were collected and stocked at −80°C until use. The amount of target proteins was measured using sandwich ELISA kits (R&D Systems) according to the manufacturer's instruction.

Statistical analysis. Statistical significances were determined by Student's *t*-test or Turkey-Kramer ANOVA. P value less than 0.05 was considered statistically significant. All analyses were performed with JMP 8.0.2 software (SAS Institute, Cary, NC).

RESULTS

Induction of gp130 expression and IL-6 secretion in HGFs treated by IL-1β

IL-1β but not IL-6/sIL-6R, induced gp130 mRNA expression in HGFs (IL-1β: $P = 0.0062$; IL-6/sIL-6R: 0.4827, Student's *t*-test, vs. untreated cells) (Fig. 2A). Corresponding to the mRNA expression, IL-1β but not IL-6/sIL-6R induced gp130 protein expression in HGFs ($P = 0.0043$, Student's *t*-test, vs. untreated cells) (Fig. 2B). In this experiment, THP-1 macrophages were used as a control of HGFs. Inversely, IL-6 but not IL-1β induced gp130 mRNA expression in THP-1 macrophages ($P = 0.0061$, vs. untreated, Student's *t*-test) (Fig. 2A). In THP-1 macrophages, both TNF-α and TGF-β did not enhance

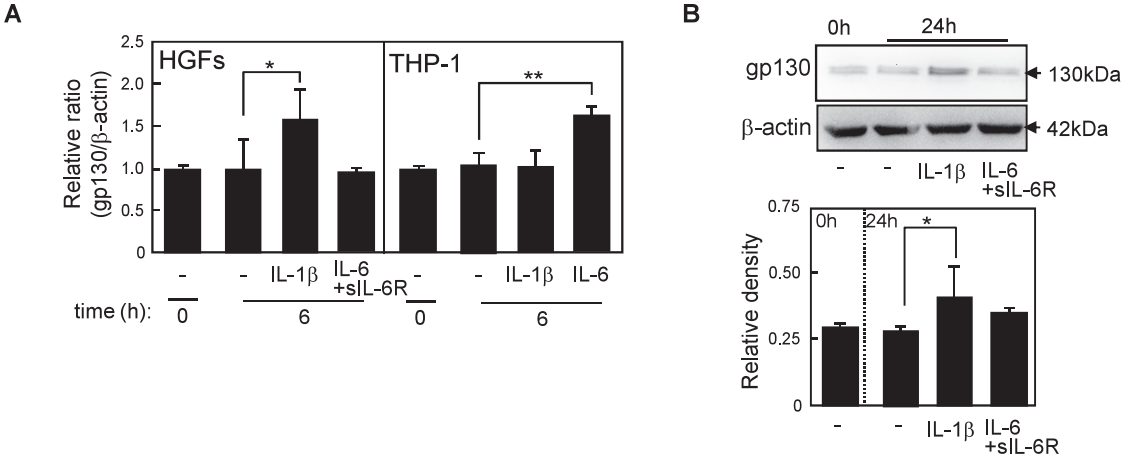

Fig. 2 Effects of IL-1β on gp130 expression. (**A**) Quantitative RT-PCR analysis of gp130 mRNA expression in HGFs and THP-1 macrophages. At 6 h after IL-1β (1 ng/mL) or IL-6/sIL-6R (50 ng/mL each) treatment, total RNA was extracted for qRT-PCR. Results are representative of 3 independent experiments. Relative values of gp130 mRNA were normalized to that of β-actin levels and expressed as relative ratio. *, $P < 0.05$; **, $P < 0.01$, Student's *t*-test. (**B**) Western blot analysis of gp130 protein expression in HGFs (Upper panel). At 24 h after IL-1β (1 ng/mL) or IL-6/sIL-6R (50 ng/mL each) treatment, total cell lysate was extracted for Western blotting. Results are representative of 3 independent experiments. Quantitation of the protein levels in lysates was performed by densitometric scanning of each band using Image J software (NIH, Washington DC, USA), and densitometry values were normalized to β-actin levels and expressed as relative induction (Lower panel). *, $P < 0.05$, Student's *t*-test.

the gp130 mRNA expression (data not shown). IL-1β induced significantly IL-6 secretion in HGFs ($P < 0.0001$, Student's *t*-test, vs. untreated cells) (Fig. 3A).

Induction of sIL-6R production in THP-1 macrophages but not HGFs

IL-6 increased significantly sIL-6R production in THP-1 macrophages ($P < 0.0001$, Student's *t*-test) (Fig. 3B). Whereas there were no significant differences between untreated and IL-1β or TNF-α treated THP-1 macrophages in sIL-6R production (IL-1β: $P = 0.221$; TNF-α: $P = 0.189$, Student's *t*-test). We could not detect sIL-6R in culture supernatants of HGFs treated with any cytokines.

Effect of IL-1β on IL-6/sIL-6R signals in HGFs

As shown in Fig. 4A, pretreatment of IL-1β promoted dramatically the phosphorylation of Stat3, ERK and JNK by IL-6/sIL-6R (Fig. 4B: $P < 0.0001$, Turkey-Kramer ANOVA) in HGFs, probably due to induction of gp130 expression.

Effect of IL-1β or IL-6/sIL-6R on cell proliferation in HGFs

IL-1β promoted significantly cell proliferation of HGFs, whereas IL-6/sIL-6R did not promote the cell proliferation (IL-1β: $P = 0.0063$; IL-6/sIL-6R: $P = 0.1733$, vs. untreated, Student's *t*-test) (Fig. 5).

Synergistic effects of IL-1β and IL-6/sIL-6R on gene expression of inflammation-related molecules

IL-1β but not IL-6/sIL-6R increased significantly both MMP-1 and MMP-3 mRNA expression ($P < 0.0001$, vs. untreated, Student's *t*-test), and IL-6/sIL-6R increased synergistically the MMP-1 and -3 mRNA expression in HGFs pretreated with IL-1β ($P < 0.0001$, vs. IL-1β or IL-6/sIL-6R, Student's *t*-test) (Fig. 6A). IL-1β but not IL-6/sIL-6R increased significantly MMP-2 mRNA expression ($P < 0.0001$, vs. untreated, Student's *t*-test), and the MMP-2 mRNA expression was not enhanced by co-stimulation of IL-1β and IL-6/sIL-6R in HGFs (Fig. 6B and 6C). MMP-13 and -14 mRNA expression was enhanced synergistically by IL-6/sIL-6R in HGFs pretreated with IL-1β ($P < 0.0001$, vs. IL-1β or IL-6/sIL-6R, Student's *t*-test) (Fig. 6D and 6E). Inversely, we observed the tendency that TIMP-1, -2 and -3 mRNA expressions were suppressed in HGFs treated by IL-1β and IL-6/sIL-6R (Fig. 6G, 5H and 6I). Thus, tissue degradation by MMPs might be promoted in inflamed periodontal tissues. Whereas, cysteine protease cathepsin B mRNA expression was not changed (Fig. 6F). IL-1β but not IL-6/sIL-6R decreased significantly IGF-1 mRNA expression ($P < 0.0001$, vs. untreated, Student's *t*-test), however IL-6/sIL-6R did not affect synergistically the IGF-1 mRNA expression in HGFs pretreated with IL-1β (Fig. 6J). IL-6/sIL-6R increased synergistically the IL-1ra and IL-33 mRNA expression in HGFs pretreated with IL-1β ($P < 0.0001$, vs. IL-1β or IL-6/sIL-6R, Student's *t*-test) (Fig. 6K and 6L).

Synergistic effects of IL-1β and IL-6/sIL-6R on protein secretion of inflammation-related molecules

Both IL-1β and IL-6/sIL-6R increased significantly proMMP-1 production (IL-1β and IL-6/sIL-6R: $P < 0.0001$, vs. untreated, Student's *t*-test), and the proMMP-1 production was enhanced significantly by IL-6/sIL-6R in HGFs pretreated with IL-1β ($P < 0.0001$, vs. IL-1β or IL-6/sIL-6R, Student's *t*-test) (Fig. 7A). IL-6/sIL-6R but not IL-1β increased significantly cathepsin B production (IL-1β: $P = 0.1352$, IL-6/sIL-6R: $P = 0.0207$, vs. untreated, Student's *t*-test), and the cathepsin B production was

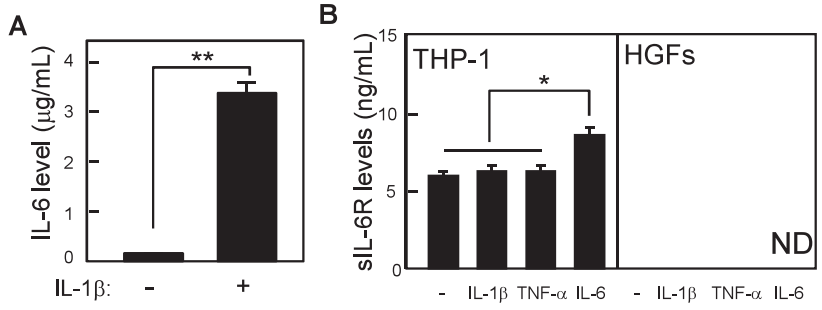

Fig. 3 Induction of IL-6 and sIL-6R. (**A**) Secretion of IL-6 in HGFs stimulated by IL-1β. Cells were stimulated by IL-1β (1 ng/mL) for 24 h and the supernatants were collected. (**B**) Secretion of sIL-6R in THP-1 macrophages and HGFs stimulated by IL-1β, TNF-α and IL-6 (10 ng/mL). Cells were stimulated by I IL-1β (1 ng/mL), TNF-α (5 ng/mL) and IL-6 (10 ng/mL) for 24 h and the supernatants were collected. Both IL-6 and sIL-6R levels were measured using ELISA kits. Data represents as the mean ± SD from 3 independent experiments. **, $P < 0.01$, *, $P < 0.05$ as compared with control (Student's *t*-test).

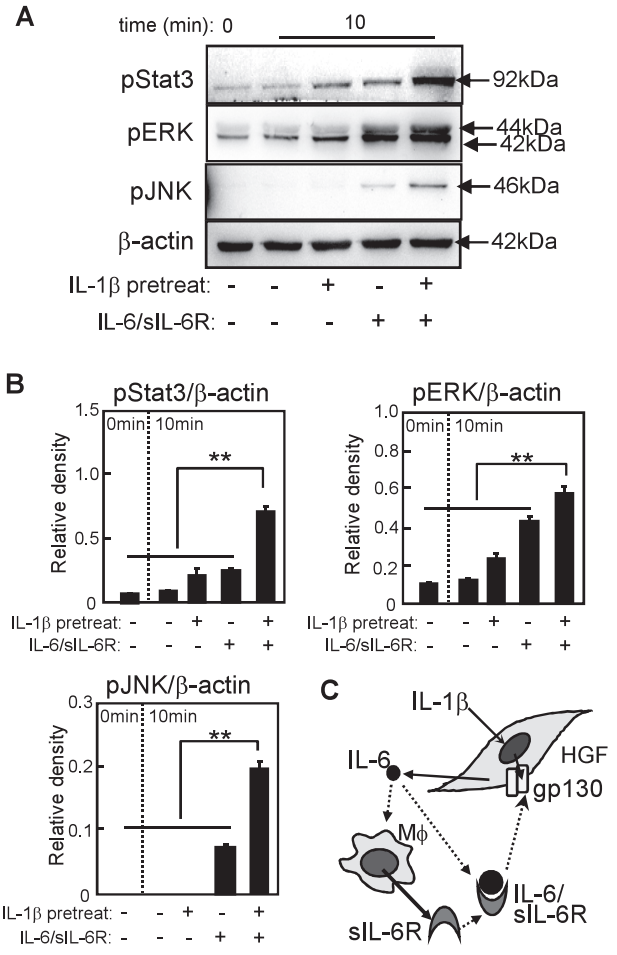

Fig. 4 Induction of IL-6 signals by IL-1β. (**A**) Effects of IL-1β pretreatment on Stat3, ERK and JNK phosphorylation. After HGFs were treated with IL-1β (1 ng/mL) for 24 h, IL-6/sIL-6R (50 ng/mL each) was added into culture plate for 10 min. The phosphorylated proteins were examined by Western blotting. Results are representative of 3 independent experiments. (**B**) Relative levels of phosphorylated proteins (Stat3, ERK and JNK). Quantitation of the protein levels was performed by densitometric scanning of each band (Stat3: 92 kDa, ERK: 42 kDa, JNK: 46 kDa) using Image J software (NIH, Washington DC, USA), and densitometry values were normalized to β-actin levels and expressed as relative induction. **, $P < 0.01$, Turkey-Kramer ANOVA. (**C**) Schematic representation of HGF-macrophage cross-talk. IL-1β might up-regulate functionally IL-6 responsiveness of HGF by autocrine/paracrine loops.

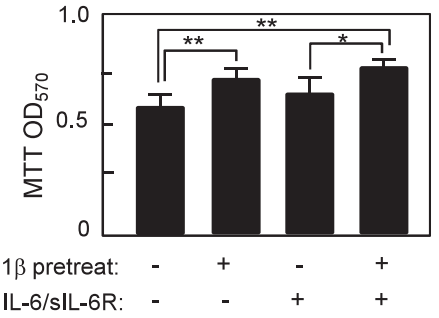

Fig. 5 Cell proliferative activity. After HGFs were treated with IL-1β (1 ng/mL) for 24 h, IL-6/sIL-6R (50 ng/mL each) was added into culture plate for 24 h. The cell proliferative activity was determined by MTT assay. Data represents as the mean ± SD from 3 independent experiments. **, $P < 0.01$, *, $P < 0.05$ as compared with control (Student's *t*-test).

not enhanced by IL-6/sIL-6R in HGFs pretreated with IL-1β ($P = 0.3694$, vs. IL-6/sIL-6R, Student's *t*-test). Thus, we did not find the synergistic effect of IL-1β and IL-6/sIL-6R on cathepsin B production in HGFs (Fig. 7B). On the other hand, cathepsin L production increased synergistically in HGFs treated with IL-1β and IL-6/sIL-6R (Fig. 8). Both IL-1β and IL-6/sIL-6R increased significantly IL-1ra and MCP-1 production (IL-1β and IL-6/sIL-6R: $P < 0.0001$, vs. untreated, Student's *t*-test), and the both IL-1ra and MCP-1 productions were enhanced significantly by IL-6/sIL-6R in HGFs pretreated with IL-1β ($P < 0.0001$, vs. IL-1β and IL-6/sIL-6R, Student's *t*-test) (Fig. 7C, 7E). IL-1β but not IL-6/sIL-6R increased significantly IL-8 production (IL-1β: $P < 0.0001$, IL-6/sIL-6R: $P = 0.9712$, vs. untreated, Student's *t*-test) (Fig. 7D). As shown in Fig. 4F, both IL-1β and IL-6/sIL-6R did not induce IL-12 production in HGFs. Interestingly, strong synergistic effects between IL-1β and IL-6/sIL-6R were observed in the production of growth factors. IL-6/sIL-6R but not IL-1β increased significantly bFGF production (IL-1β: $P = 0.5982$, IL-6/sIL-6R: $P < 0.0001$, vs. untreated, Student's *t*-test), and the bFGF production was enhanced significantly by IL-6/sIL-6R in HGFs pretreated with IL-1β ($P < 0.0001$, vs. IL-6/sIL-6R, Student's *t*-test) (Fig. 7G). Both IL-1β and IL-6/sIL-6R increased significantly VEGF production (IL-1β: $P = 0.0416$, IL-6/sIL-6R: $P = 0.0057$, vs. untreated, Student's *t*-test), and the VEGF production was enhanced significantly by IL-6/sIL-6R in HGFs pretreated with IL-1β ($P < 0.0001$, vs. IL-1β and IL-6/sIL-6R, Student's *t*-test) (Fig. 7H).

DISCUSSION

Cytokine biology regulating inflammatory diseases has been discussed for the last several decades (9, 13). Increased levels of IL-1β have been reported in cases of osteoporosis, rheumatoid arthritis and periodontitis (33). Effects of IL-1β on osteoclasts have been examined *in vitro*, and IL-1β has been found to increase the mature formation as well as the resorptive capacity of osteoclasts (14). In general,

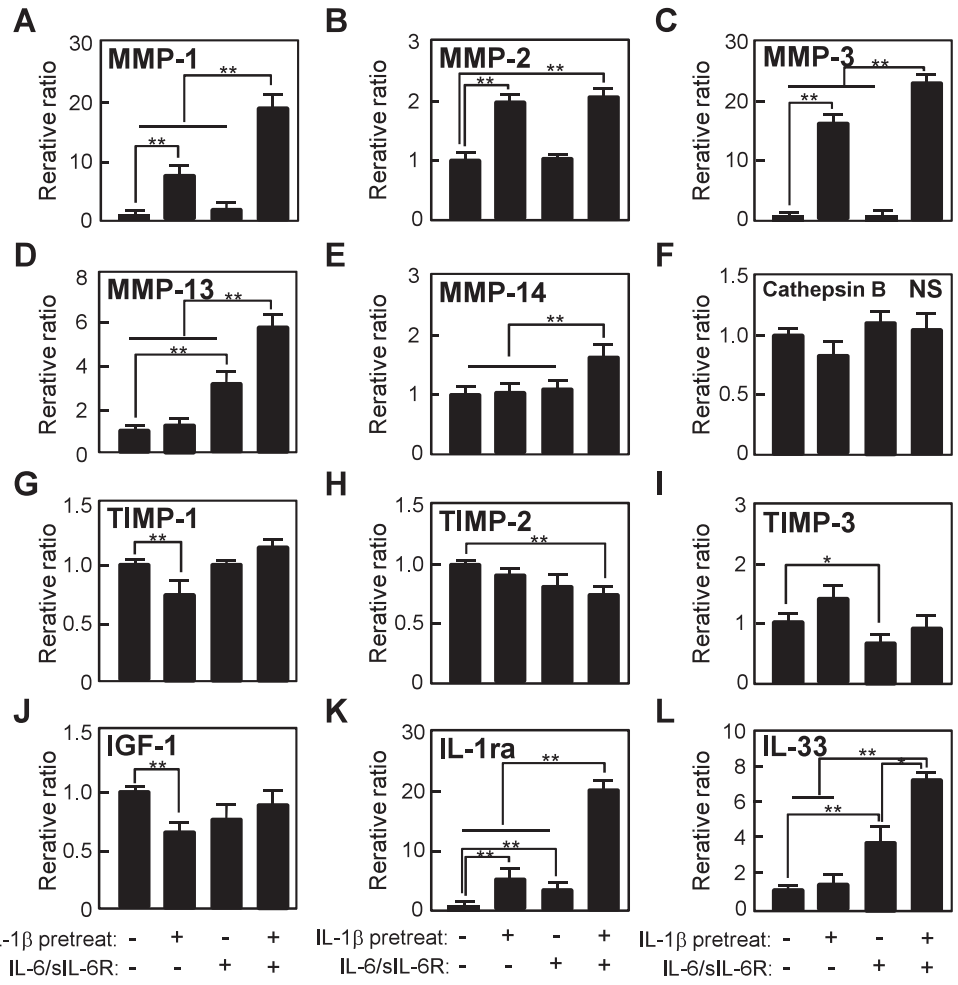

Fig. 6 Synergistic effects of IL-1β and IL-6/sIL-6R on mRNA expression of various inflammation-related molecules in HGFs. After HGFs were treated with IL-1β (1 ng/mL) for 24 h, IL-6/sIL-6R (50 ng/mL each) was added into culture plate for 6 h. Expression of mRNAs was examined by qRT-PCR. Relative values of each mRNA was normalized to that of β-actin levels and expressed as relative ratio. *, $P < 0.05$; **, $P < 0.01$, Student's *t*-test.

IL-6 is well known as a multifactorial cytokine that plays major roles in the pathogenesis of rheumatoid arthritis and periodontitis (27). Thus, although IL-1β must correlate with IL-6 in periodontitis lesion, the relationship between IL-1β and IL-6 remains unknown in pathophysiology of periodontitis. HGFs are the most abundant cells in gingival connective tissues and play an important role in the control of inflammation in inflamed gingiva (27). Responses of HGFs to elevated inflammatory cytokines are thought to contribute to the development of periodontitis (29), although the precise mechanism remains unknown. In the present study, we investigated the regulation of inflammation-related molecules by synergism between IL-1β and IL-6/sIL-6R by targeting HGFs.

We have reported previously that IL-6 signals in the presence of sIL-6R are initiated in the phosphorylation of IL-6 signal transducer gp130 in HGFs (19). At least two distinct signaling cascades, the Stat3 and the MAPKs pathways, have been activated and lead to various responses such as VEGF secretion in HGFs (18). In the present study, we demonstrated for the first time that IL-1β but not IL-6/sIL-6R induces gp130 expression in HGFs at mRNA and protein levels. On the other hand, IL-6/sIL-6R but not IL-1β induces gp130 mRNA expression in THP-1 macrophages. These differences of gp130 induction by inflammatory cytokines might depend on the role of each cell in inflammatory microenvironment. Next, IL-1β also increased significantly IL-6 secretion in HGFs. IL-1β might upregulate functionally IL-6 responsiveness of HGF by autocrine loops. However, IL-6 cannot act to HGFs without sIL-6R, because HGFs do not express suffi-

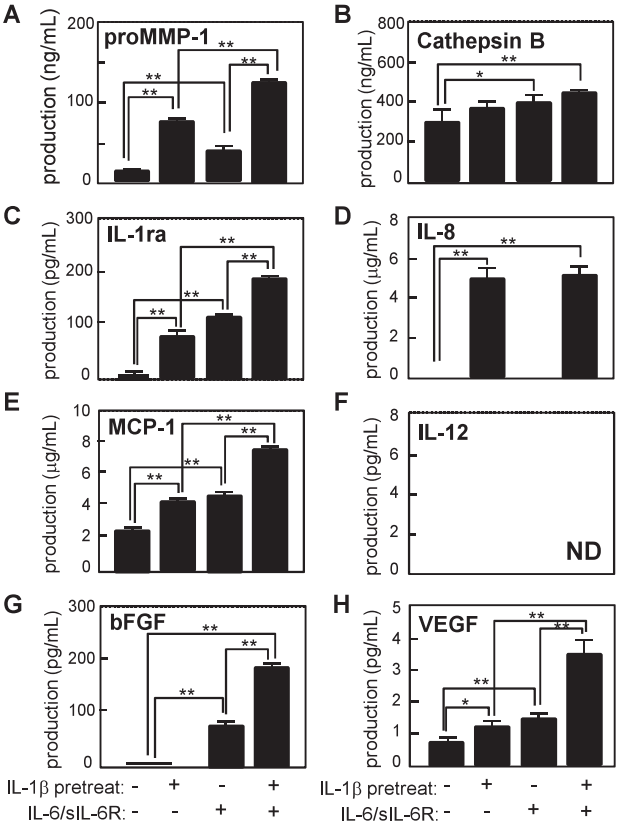

Fig. 7 Synergistic effects of IL-1β and IL-6/sIL-6R on secretion of various inflammation-related molecules in HGFs. HGFs were treated with IL-1β (1 ng/mL) for 24 h, and IL-6/sIL-6R (50 ng/mL each) was added into culture plate for 48 h. The levels of target molecules were measured using ELISA Kits. Data represents as the mean ± SD from 3 independent experiments. **, $P < 0.01$, *, $P < 0.05$ as compared with control (Student's *t*-test). ND, not detected.

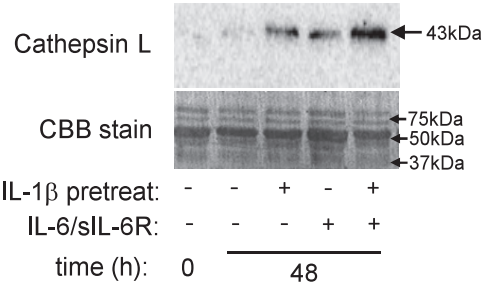

Fig. 8 Cathepsin L production in HGFs. HGFs were treated by IL-1β (1 ng/mL) and IL-6/sIL-6R (50 ng/mL each), and culture supernatants were collected. Secreted cathepsin L levels were analyzed using Western blotting probed with antibodies against cathepsin L. To confirm the equivalent levels of loaded proteins in culture supernatant, blotted membrane was stained with coomassie-brilliant blue.

cient cell surface IL-6R to bind appreciable levels of IL-6 (19). Therefore, we examined whether sIL-6R production is induced by other cell types stimulated by inflammatory cytokines. As shown in Fig. 3B, PMA-differentiated THP-1 macrophages treated with IL-6 but not IL-1β and TNF-α induced significantly the sIL-6R production, whereas HGFs did not produce sIL-6R as expected. Macrophages existing in periodontitis lesions might be a candidate of sIL-6R-producing cells. A series of results might be possible to explain the IL-1β-mediated promotion of IL-6 function by HGF-macrophage cross-talk. To confirm the enhancement of IL-6 function by IL-1β in HGFs, the intracellular signals were investigated. The phosphorylation of Stat3, ERK and JNK induced by IL-6/sIL-6R was enhanced dramatically in HGFs pretreated with IL-1β. The induction of phosphorylation might depend on increase of gp130 by IL-1β in HGFs. Whereas, cell proliferation of HGFs pretreated with IL-1β was not enhanced by IL-6/sIL-6R, thus we could not find the synergism between IL-1β and IL-6 in the cell proliferative activity.

Periodontitis is an infectious inflammatory disease (2, 20, 27). Several inflammatory mediators, cytokines, hormones and growth factors have been detected in periodontitis lesions (20). In the present study, we examined whether several inflammation-related molecules are regulated by IL-1β and IL-6/sIL-6R synergistically by targeting HGFs.

Proteases

Multiple proteases are considered to be involved in the degradation of extracellular matrix seen in periodontitis. These proteases include MMPs, cysteine proteases such as cathepsin B and L, and serine proteases such as urokinase and others (15). MMP-1 plays an important role in degradation of gingival collagen fibers because type I collagen is dominant in gingival connective tissues (8). As expected, production of MMP-1 increased dramatically in HGFs co-stimulated with both IL-1β and IL-6/sIL-6R at mRNA and protein levels. MMP-1 is extracellularly released into the inflamed periodontal tissue, and may be involved in the destruction of connective tissue by directly degrading collagens or by activating the fibronolytic protease cascade. As shown in Fig. 7B, IL-6/sIL-6R induced significantly cathepsin B secretion in HGFs in agreement with our previous report (30). Cathepsin B degrades directly collagen fibers, and the protease contributes to collagen degradation indirectly through activation of MMP-1 (3, 4). Therefore, these results encourage that MMP-1 and cathepsin B released from HGFs co-stimulated with both IL-1β and IL-6/sIL-6R might act coopera-

tively in degradation of periodontal tissues. In addition, since mRNA of TIMP-1 known as MMP-1 inhibitor, was not changed in HGFs treated with the co-stimulation. These findings indicate that tissue degradation by MMP-1 might be promoted, since imbalance between MMP-1 and TIMP-1 should be expressed in the inflamed periodontal tissues.

Chemokines

Chemokines such as IL-8 and MCP-1 may be related to periodontitis severity (7, 28). Previously, several investigators have shown that chemokine levels in gingival crevicular fluid decrease after periodontal therapy (7, 12). Thus, chemokines seem to be attractive therapeutic targets for periodontitis management. As shown in Fig. 7E, MCP-1 secretion was induced significantly and synergistically in HGFs co-stimulated by IL-1β and IL-6/sIL-6R. On the other hand, IL-8 secretion was enhanced by IL-1β but not IL-6/sIL-6R in HGFs, thus no synergistic effect between IL-1β and IL-6/sIL-6R was observed in IL-8 secretion. Secretion of IL-1ra, antagonist of IL-1β, was enhanced significantly and synergistically in HGFs co-stimulated by IL-1β and IL-6/sIL-6R. However, since PMA-differentiated THP-1 macrophages produced over 100-fold IL-1ra compared to that of HGFs (data not shown), it remains unclear whether IL-1ra produced by HGFs down-regulates sufficiently the IL-1 function in periodontitis lesions. HGFs did not secrete IL-12 known as a strong IFN-γ inducer in HGFs treated with IL-1β and IL-6/sIL-6R. Function of IL-1β and IL-6/sIL-6R might be limited in the HGFs-mediated immune responses. In addition, mRNA expression of IL-33, a member of IL-1 family cytokine inducing host defense, was enhanced dramatically in HGFs co-stimulated with both IL-1β and IL-6/sIL-6R. Further experiments will be needed in clinical significance of IL-33 induced by both IL-1β and IL-6/sIL-6R.

Growth factors—possible mechanisms of induction of angiogenesis

Angiogenesis consists of multiple processes such as degradation of vascular basement membranes and surrounding extracellular matrix, and migration and proliferation of endothelial cells as seen in periodontitis lesions (23). Several mediators have been reported as angiogenic factors that are important for these processes (6). VEGF is the most powerful regulator of angiogenesis, and plays an important role in the regulation of inflammatory periodontitis (6). Our previous reports have shown that IL-6/sIL-6R induced VEGF secretion in HGFs (18). As shown in Fig. 7H, co-stimulation with IL-1β and IL-6/sIL-6R enhanced synergistically VEGF secretion in HGFs, possibly resulting in severe angiogenesis of periodontitis lesions. Regarding angiogenesis, it has been reported that murine cathepsin B in vasculature was remarkably up-regulated during the degradation of vascular basement membrane associated with tumor angiogenesis (17). Furthermore, Yanamandra *et al.* has reported that cathepsin B knockdown in glioma cell lines inhibited tumor-induced angiogenesis by modulating the expression of VEGF (31). Therefore, VEGF and cathepsin B released from HGFs co-stimulated by both IL-1β and IL-6/sIL-6R might induce the angiogenesis cooperatively in periodontitis lesions by affecting angiogenesis-inducing cells such as endothelial cells. Basic FGF (bFGF) is also well-known as a strong angiogenic inducer, and can stimulate migration and proliferation of endothelial cells and promote formation of capillary tubes *in vitro* (32). Interestingly, IL-6/sIL-6R induced dramatically bFGF secretion in HGFs, whereas the bFGF was not detected in culture supernatant of HGFs treated by IL-1β. Furthermore, co-stimulation with IL-1β and IL-6/sIL-6R enhanced synergistically bFGF secretion in HGFs. The synergistic effect might depend on IL-1β-induced increase of gp130 on cell surface of HGFs. The common cytokine receptor chain, gp130, controls the activity of a group of cytokines such as IL-6, IL-11, IL-27, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor, oncostatin M, cardiotrophin-1, cardiotrophin-like cytokine and neuropoietin (5). These cytokines are involved in multiple different biological processes, including inflammation, acute phase response, immune responses and cell survival (10). Therefore, increase of gp130 by IL-1β in HGFs will be attractive insight for inflammatory regulation of periodontitis.

HGFs functions are well regulated by various cytokines in periodontitis lesions. Taken together, HGF-mediated synergistic effects of IL-1β and IL-6/sIL-6R may regulate the periodontal inflammation through induction of gp130 expression, leading to progression of periodontitis (Fig. 4C). To clarify the cytokine cascade might contribute to create a new drug for regulation of gingival inflammation.

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REFERENCES

1. Barksby HE, Lea SR, Preshaw PM and Taylor JJ (2007) The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. *Clin Exp Immunol* **149**, 217–225.
2. Birkedal-Hansen H (1993) Role of matrix metalloproteinases in human periodontal diseases. *J Periodontol* **64**, 474–484.
3. Collier SA (2001) Is the corneal degradation in keratoconus caused by matrix-metalloproteinases? *Clin Experiment Ophthalmol* **29**, 340–344.
4. Creemers LB, Hoeben KA, Jansen DC, Buttle DJ, Beertsen W and Everts V (1998) Participation of intracellular cysteine proteinases, in particular cathepsin B, in degradation of collagen in periosteal tissue explants. *Matrix Biol* **16**, 575–584.
5. Demyanets S, Huber K and Wojta J (2012) Vascular effects of glycoprotein130 ligands—part I: pathophysiological role. *Vascul Pharmacol* **56**, 34–46.
6. Ferrara N (2000) Vascular endothelial growth factor and the regulation of angiogenesis. *Recent Prog Horm Res* **55**, 15–35.
7. Gamonal J, Acevedo A, Bascones A, Jorge O and Silva A (2000) Levels of interleukin-1beta, -8, and -10 and RANTES in gingival crevicular fluid and cell populations in adult periodontitis patients and the effect of periodontal treatment. *J Periodontol* **70**, 1535–1545.
8. Hassell TM (1993) Tissues and cells of the periodontium. *Periodontol 2000* **3**, 9–38.
9. He G and Karin M (2011) NF-κB and STAT3—key players in liver inflammation and cancer. *Cell Res* **21**, 159–168.
10. Hirano T, Ishihara K and Hibi M (2000) Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors, *Oncogene* **19**, 2548–2556.
11. Ho YS, Lai MT, Liu SJ, Lin CT, Naruishi K, Takashiba S and Chou HH (2009) *Porphyromonas gingivalis* fimbriae-dependent interleukin-6 autocrine regulation by increase of gp130 in endothelial cells. *J Periodontal Res* **44**, 550–556.
12. Jin J, Söder B and Corbet EF (2000) Interleukin-8 and granulocyte elastase in gingival crevicular fluid in relation to periodontopathogens in untreated adult periodontitis. *J Periodontol* **71**, 929–939.
13. Kishimoto T (2005) Interleukin-6: from basic science to medicine—40 years in immunology. *Annu Rev Immunol* **23**, 1–21.
14. Kwan Tat S, Padrines M, Théoleyre S, Heymann D and Fortun Y (2004) IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev* **15**, 49–60.
15. Lijnen HR (2001) Elements of the fibrinolytic system. *Ann N Y Acad Sci* **936**, 226–236.
16. Mackiewicz A (1997) Acute phase proteins and transformed cells. *Int Rev Cytol* **170**, 225–300.
17. Nalla AK, Gorantla B, Gondi CS, Lakka SS and Rao JS (2010) Targeting MMP-9, uPAR, and cathepsin B inhibits invasion, migration and activates apoptosis in prostate cancer cells. *Cancer Gene Ther* **17**, 599–613.
18. Naruishi K, Nishimura F, Yamada-Naruishi H, Omori K, Yamaguchi M and Takashiba S (2003) C-*jun* N-terminal kinase (JNK) inhibitor, SP600125, blocks interleukin (IL)-6-induced vascular endothelial growth factor (VEGF) production: cyclosporine A partially mimics this inhibitory effect. *Transplantation* **76**, 1380–1382.
19. Naruishi K, Takashiba S, Nishimura F, Chou HH, Arai H, Yamada H and Murayama Y (2001) Impairment of gingival fibroblast adherence by IL-6/sIL-6R. *J Dent Res* **80**, 1421–1424.
20. Okada H and Murakami S (1998) Cytokine expression in periodontal health and disease. *Crit Rev Oral Biol Med* **9**, 248–266.
21. Omori K, Naruishi K, Yamaguchi T, Li SA, Yamaguchi-Morimoto M, Matsuura K, Arai H, Takei K and Takashiba S (2007) cAMP-response element binding protein (CREB) regulates cyclosporine-A-mediated down-regulation of cathepsin B and L synthesis. *Cell Tissue Res* **330**, 75–82.
22. Reynolds JJ (1996) Collagenases and tissue inhibitors of metalloproteinases: a functional balance in tissue degradation. *Oral Dis* **2**, 70–76.
23. Rosenkilde MM and Schwartz TW (2001) The chemokine system—a major regulator of angiogenesis in health and disease. *APMIS* **112**, 481–495.
24. Schweighofer B, Schultes J, Pomyje J and Hofer E (2007) Signals and genes induced by angiogenic growth factors in comparison to inflammatory cytokines in endothelial cells. *Clin Hemorheol Microcirc* **37**, 57–62.
25. Sipe JD (1995) Acute-phase proteins in osteoarthritis. *Arthritis Rheum* **25**, 75–86.
26. Taga T and Kishimoto T (1997) Gp130 and the interleukin-6 family of cytokines. *Annu Rev Immunol* **15**, 797–819.
27. Takashiba S, Naruishi K and Murayama Y (2003) Perspective of cytokine regulation for periodontal treatment: fibroblast biology. *J Periodontol* **74**, 103–110.
28. Taylor JJ (2010) Cytokine regulation of immune responses to *Porphyromonas gingivalis*. *Periodontol 2000* **54**, 160–194.
29. Wang PL and Ohura K (2002) *Porphyromonas gingivalis* lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. *Crit Rev Oral Biol Med* **13**, 132–142.
30. Yamaguchi T, Naruishi K, Arai H, Nishimura F and Takashiba S (2008) IL-6/sIL-6R enhances cathepsin B and L production via caveolin-1-mediated JNK-AP-1 pathway in human gingival fibroblasts. *J Cell Physiol* **217**, 423–432.
31. Yanamandra N, Gumidyala KV, Waldron KG, Gujrati M, Olivero WC, Dinh DH and Rao JS (2004) Blockade of cathepsin B expression in human glioblastoma cells is associated with suppression of angiogenesis. *Oncogene* **23**, 2224–2230.
32. Yuan K, Jin YT and Lin MT (2000) The detection and comparison of angiogenesis-associated factors in pyogenic granuloma by immunohistochemistry. *J Periodontol* **71**, 701–709.
33. Ziebolz D, Pabel SO, Lange K, Krohn-Grimberghe B, Hornecker E and Mausberg RF (2011) Clinical periodontal and microbiologic parameters in patients with rheumatoid arthritis. *J Periodontol* **82**, 1424–1432.