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Expression of matrix metalloproteinase-1, matrix metalloproteinase-2 and extracellular metalloproteinase inducer in human periodontal ligament cells stimulated with interleukin-1beta

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Background and Objectives: Matrix metalloproteinases (MMPs), produced by both infiltrating and resident cells of the periodontium, play important roles in physiologic and pathologic events. Both interleukin-1beta and extracellular MMP inducer can stimulate the expression of MMPs, which in turn leads to breakdown of the periodontium. However, it is currently unknown whether interleukin-1beta up-regulates MMPs through stimulating the expression of extracellular MMP inducer. The aims of this study were to investigate the effect of interleukin-1beta on the expression of MMP-1, MMP-2 and extracellular MMP inducer in human periodontal ligament cells and to evaluate whether the regulation of MMP-1 and MMP-2 by this cytokine occurred through an effect on extracellular MMP inducer expression.

Material and Methods: Cultured human periodontal ligament cells were treated with varying concentrations (0.01–10 ng/mL) of interleukin-1beta at for 6, 12 and 24 h. Reverse transcription–polymerase chain reaction, enzyme-linked immunosorbent assay, gelatin zymography and western blotting were performed to measure the mRNA and protein levels of MMP-1, MMP-2 and extracellular MMP inducer.

Results: Basal levels of mRNA and protein for MMP-1, MMP-2 and extracellular MMP inducer were detected in untreated human periodontal ligament cells. Interleukin-1beta significantly up-regulated the expression of MMP-1 and MMP-2 mRNA and protein ($p < 0.05$); however, the levels of mRNA and protein for extracellular MMP inducer were not significantly different ($p > 0.05$). In the culture medium, the concentration of MMP-1 was also increased significantly, but

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the concentration of MMP-1 was not related to the concentration of extracellular MMP inducer ($R^2 = 0.2538$, $p > 0.05$).

Conclusion: Interleukin-1beta up-regulated the levels of MMP-1 and MMP-2, but it did not alter the expression of extracellular MMP inducer. Expression of MMP-1 and MMP-2 might be elevated by interleukin-1beta and extracellular MMP inducer via two different signal pathways.

Periodontitis results in the loss of the connective tissue attachment of the tooth to the supporting alveolar bone (1). The anatomic structure providing this attachment is periodontal ligament. As fibroblasts are the prominent cell type in this tissue, it is likely that periodontal ligament fibroblasts play important roles not only in the physiologic maintenance and turnover of extracellular matrix in the periodontal ligament, but also in the pathologic degradation of extracellular matrix (2). Significant evidence exists to show that matrix metalloproteinases (MMPs) derived from resident cells play an important role in periodontal tissue remodeling and pathological states (3,4). Among them, MMP-1 serves as an initiator of extracellular matrix destruction (5), and MMP-2 is probably another important factor for localizing the enzymes to appropriate sites for matrix degradation (6,7). Tissue extracts and cultured tissue explants of inflamed human gingiva were shown to contain higher levels of activity of MMP-1 and MMP-2 than tissue extracts and cultured tissue explants from healthy human gingiva (8), and the concentrations of MMP-1 and MMP-2 in gingival crevicular fluid were positively correlated with the severity of periodontal disease (9,10). However, the exact mechanism underlying the up-regulated expression of MMPs at the site of extracellular matrix destruction has not been fully elucidated.MMPs are released initially from inflammatory cells recruited by bacterial plaque chemoattactants (11) and then in the established lesion from resident periodontal cells, including gingival fibroblasts and periodontal ligament cells, in response to cytokines derived from these infiltrating cells (12). Among cytokines, interleukin-1beta is thought to play a central role in mediating inflammatory responses (13–16). Recently, another mediator of MMPs, extracellular MMP inducer, was identified. Extracellular MMP inducer is a highly glycosylated transmembrane protein belonging to the immunoglobulin superfamily (17). As a signal molecule and stimulator of MMPs, extracellular MMP inducer plays a role in many physiological and pathological processes, such as tumor invasion and metastasis, tissue development and repair, cardiovascular diseases and inflammation (18–20). Extracellular MMP inducer has two forms, membrane-bound and soluble, and studies have indicated that both are equally capable of inducing MMP production from fibroblast cells (21,22). A recent study has shown that an increased level of soluble extracellular MMP inducer in gingival crevicular fluid was associated with enhanced severity of periodontitis (23); our team also found that the levels of both extracellular MMP inducer mRNA and protein were enhanced significantly in gingival tissues from patients with periodontitis compared with those from clinical healthy individuals (24). However, it is currently unknown whether interleukin-1beta can regulate the expression of extracellular MMP inducer in human periodontal ligament cells, or whether extracellular MMP inducer is involved in the regulation of MMPs by interleukin-1beta. If interleukin-1beta can stimulate the expression of extracellular MMP inducer, then extracellular MMP inducer might play a role in the up-regulation of MMPs stimulated by interleukin-1beta.

These data led us to examine the effect of interleukin-1beta on the expression of MMP-1, MMP-2 and extracellular MMP inducer in human periodontal ligament cells and to evaluate whether extracellular MMP inducer participated in the regulation of MMPs controlled by interleukin-1beta.

Material and methods

Cell culture

Primary human periodontal ligament cells were obtained from the premolars (extracted for orthodontic reasons) of five volunteers. All patients were non-smokers, exhibited a pocket depth of < 3 mm, no clinical attachment loss, no bleeding on probing and no radiographic evidence of bone loss. None of the subjects had a history of systemic disease or had received antibiotics or other medications, or periodontal treatment, in the past 4 mo. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. The purpose of the study was explained to each subject in detail before they agreed to participate, and informed consent was obtained from each subject.

Extracted teeth were washed twice with phosphate-buffered saline (pH 7.4; containing 500 U/mL of penicillin and 500 mg/mL of streptomycin). Periodontal ligament tissues attached to the mid-third of the root were removed using a surgical scalpel, minced and placed in a 25-cm^2 flask (Corning; Corning-Costar Corp., Cambridge, MA, USA) and kept in Dulbecco's modified Eagle's minimal essential medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL), 100 U/mL of penicillin and 100 mg/mL of streptomycin in 5% CO_2 at 37°C in humidified air until cells grew out of the explants. After reaching confluence, cells were subcultured at a ratio of 1:3. For this study, cells from passages six to eight were used.

RNA isolation and reverse transcription–polymerase chain reaction

Passage 6–8 human periodontal ligament cells were seeded into the wells of six-well culture plates (Corning) at 2–3×10^5 cells/well. Prior to treatment, cells were pre-incubated with Dulbecco's modified Eagle's minimal essential medium, without fetal bovine serum, for 24 h and were then treated with recombinant human interleukin-1beta (0.1, 1, or 10 ng/mL; PeproTech Inc., Rocky Hill, NJ, USA) for 6, 12, or 24 h in serum-free medium; 0 ng/mL of recombinant human interleukin-1beta at each time-point served as a control. Samples of conditioned medium were collected and stored at −70°C for enzyme-linked immunosorbent assay (ELISA) and gelatin zymography analyses, and cells were harvested in TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) for RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR).

Total RNA was extracted from cells using TRIzol™, according to the manufacturer's instructions (Invitrogen). RNA concentrations were calculated from the absorbance at 260 nm, and purity was assessed by the 260 nm : 280 nm absorbance ratio. One microgram of total RNA was used in 20-μL RT reactions with TOYOBO Rever Tra Ace (TOYOBO, Osaka, Japan), according to the manufacturer's instructions. Synthesized cDNA was then used for PCR analysis.

PCR amplifications were performed in a 25-μL reaction mix containing 1 μL of cDNA, 2.5 μL of 10× PCR buffer, 50 nM primers and 1 U of Taq polymerase (Takara Biotechnology Co. Ltd, Dalian, China). Beta-actin gene expression served as an endogenous control. Primer sequence, annealing temperature and thermal cycles are listed in Table 1. All RT-PCR reactions were performed in duplicate.

The PCR products were visualized on a 1.5% agarose gel following staining with ethidium bromide and were analyzed densitometrically using the Gene Gnius Bio imaging system (Syn Gene, Cambridge, UK). The relative levels of expression were quantified by comparison with the amplified endogenous control (beta-actin).

Analysis, using ELISA, of extracellular MMP inducer and MMP-1 in supernatant

The amount of MMP-1 secreted into culture medium was measured using a Quantikine Human pro-MMP-1 Immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Soluble extracellular MMP inducer in the culture supernatant was quantified as described by Betsuyaku *et al.*(25). Briefly, the wells of microtiter plates (Corning) were coated overnight by incubation at room temperature with 1 μg/mL rabbit of anti-human extracellular MMP inducer polyclonal antibody (Zymed/Invitrogen, Carlsbad, CA, USA). After three washes with wash buffer (0.05% Tween 20 in phosphate-buffered saline, pH 7.4), the plate was blocked by incubation at room temperature for 2 h with phosphate-buffered saline containing 1% bovine serum albumin. For generation of a standard curve, recombinant human extracellular MMP inducer (Abcam, Cambridge, UK) was diluted with 0.1% bovine serum albumin and 0.05% Tween 20 in phosphate-buffered saline (pH 7.3) in polypropylene tubes to final concentrations of 4000, 2000, 1000, 500, 250, 125, 62.5 and 31.25 pg/mL. The plate was then washed three times with 0.05% Tween 20 in phosphate-buffered saline (pH 7.3), and 100 μL of culture supernatant or standard was added to each well, followed by incubation for 2 h at room temperature. After three washes with 0.05% Tween 20 in phosphate-buffered saline (pH 7.3), the plate was incubated with 100 μL of biotinylated anti-human extracellular MMP inducer immunoglobulin (400 ng/mL; R&D Systems) for 2 h at room temperature. After washing, 100 μL of streptoavidin horseradish peroxidase (diluted 1:1000; Zhongshan Golden Bridge Biotechnology, Beijing, China) was added to each well. After 30 min, the plate was washed three times and incubated with 100 μL of a 1:1 mixture of hydrogen peroxide and tetramethylbenzidine (Jingmei Biotech, Shenzhen, China) in the dark, and the reaction was stopped by the addition of 1 M sulfuric acid within 30 min. The absorbance values were determined at 450 nm using an Ultra microplate reader (Bio-tek Instruments, Inc., Winooski, VT, USA). A linear response was obtained with extracellular MMP inducer standards from 31.2 to 4000 pg/mL. The concentration of extracellular MMP inducer in the culture supernatant was determined according to the standard curve. All assays were performed in duplicate, and the means were used.

Table 1. Primers used for the reverse transcription–polymerase chain reaction

Gene	Primer	Annealing temperature (°C)	Cycle	Product length (bp)	GenBank accession number
EMMPRIN	F: GGC CAG AAA ACG GAG TTC AA R: GCG CTT CTC GTA GAA GA	57.5	28	492	NM_001728.2
MMP-1	F: CAT CCA AGC CAT ATA TGG ACG TTC R: TCT GGA GAG TCA AAA TTC TCT TCG T	59.5	28	611	NM_002421.2
MMP-2	F: ATG ACA GCT GCA CCA CTG AG R: CTC CTG AAT GCC CTT GAT GT	59	28	425	NM_004530.2
Beta-actin	F: GCG AGA AGA TGA CCC AGA TCA TGT T R: GCT TCT CCT TAA TGT CAC GCA AGA T	62	30	300	NM_001101.2

EMMPRIN, extracellular MMP inducer; MMP, matrix metalloproteinase.

Gelatin zymography

MMP-2 activity in the conditioned medium was evaluated using gelatin zymography. The total protein concentration in the medium was determined using a bicinchoninic acid kit (Sangon, Shanghai, China), and 25 μg of protein per lane electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel containing 1 mg/mL of gelatin under nonreducing conditions. After electrophoresis, the gel was gently shaken in renaturing buffer (2.5% Triton-X 100) at room temperature for 30 min to remove sodium dodecyl sulfate, and then incubated in developing buffer (0.2 M NaCl/5 mM $CaCl_2$/50 mM Tris–HCl, pH 7.5/0.02% Brij35) at 37°C for 24 h. The gel was stained with 0.5% (w/v) Coomassie Brilliant Blue and destained with solution containing 50% methanol and 10% acetic acid. Active MMP-2 was detected as clear bands at 62 kDa. Digital photographs of the gels were subjected to the IMAGE PRO PLUS 5.01 software package (Media Cybernetics, Silver Spring, MD, USA) in order to quantify the integrated optical density.

Western blot analysis

Passage 5–8 human periodontal ligament cells were pretreated as described in the section entitled 'RNA isolation and reverse transcription-polymerase chain reaction. Based on our preliminary study, cells were treated with 1 ng/mL of recombinant human interleukin-1beta (Peprotech) for 6, 12 and 24 h. Total protein was isolated using M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, 100 μL of reagent was added to each well of the six-well plate respectively, kept on ice for 5 min; thereafter, lysate was collected in a microfuge tube, which was centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant (protein solution) was collected and used for western blotting analyses. Protein extracted from periodontal ligament cells without treatment was used as a control. The protein concentration was quantified using a bicinchoninic acid kit (Sangon). Each protein sample (25 μg/line) was run on a 10% sodium dodecyl sulphate polyacrylamide gel at 70 V for 2 h. A PageRuler™ Prestained Protein Ladder (Fermentas AB, Vilnius, Lithuania) was included to provide molecular weight standards. The separated proteins were then electroblotted onto a Hybond™ poly(vinylidene difluoride) transfer membrane (Amersham Biosciences, Little Chalfont, UK) using a humidified blotter. The membranes were then washed once with 10 mM Tris–HCl/150 mM NaCl/0.1% Tween 20 and blocked for 2 h in 10 mM Tris–HCl/150 mM NaCl/0.1% Tween 20 containing 5% (w/v) skim milk. Membranes were incubated with β-actin (Pierce), diluted 1:5000, with 2 μg/mL of MMP-1 (Neomarker; Lab Vision Corp., Fremount, CA, USA) or with 2 μg/mL of extracellular MMP inducer (Zymed) antibody in 10 mM Tris–HCl/150 mM NaCl/0.1% Tween 20 overnight at 4°C and were then washed three times, for 10 min each wash, with 10 mM Tris–HCl/150 mM NaCl/0.1% Tween 20 to remove unbound antibody. Membranes were then incubated with a 1:5000 dilution of goat anti-rabbit horseradish peroxidase-conjugated IgG (Pierce) in 10 mM Tris–HCl/150 mM NaCl/0.1% Tween 20 for 2 h at room temperature. Membranes were washed as stated above and antibodies were detected using the enhanced chemiluminescence western blotting system (Santa Cruz Biotechnology, Inc., Island, CA, USA), according to the manufacturer's instructions. The blots were then exposed to photographic films (KODAK; Eastman Kodak Company, Rochester, NY, USA) and the density of each lane was quantified using the QUANTITY ONE® software package (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All experiments were carried out three times, with each treatment performed in duplicate ($n = 6$), and the means with standard deviations were calculated. The statistical significance of differences among each group was examined by one-way analysis of variance using the least significant difference, and the correlation between extracellular MMP inducer and MMP-1 protein in the conditioned medium, measured using ELISA analyses, was analyzed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Results were considered significant when the *p*-value was < 0.05.

Results

Effect of interleukin-1beta on the mRNA levels of MMP-1, MMP-2 and extracellular MMP inducer

Compared with controls, significantly increased levels of MMP-1 and MMP-2 mRNA were observed following treatment with interleukin-1beta ($p < 0.05$) but the expression of extracellular MMP inducer was not altered significantly ($p > 0.05$; Fig. 1).

In untreated human periodontal ligament cells, MMP-1 mRNA was expressed at a relatively low level (Fig. 1B). Treatment with interleukin-1beta up-regulated the mRNA level of MMP-1 in a dose-dependent manner. At 6 h, 10 ng/mL of interleukin-1beta significantly stimulated the expression of MMP-1 mRNA ($p < 0.05$); at 12 h, the mRNA level was 1.56-fold higher than that of the control when treated with 0.1 ng/mL interleukin-1beta ($p < 0.05$), 3.04-fold higher with 1 ng/mL of interleukin-1beta ($p < 0.05$) and 6.04-fold higher with 10 ng/mL of interleukin-1beta ($p < 0.01$); each treatment significantly elevated the MMP-1 mRNA level at 24 h when compared with the control ($p < 0.05$ at 0.1 ng/mL, and $p < 0.01$ at 1 and 10 ng/mL of interleukin-1beta).

In controls, we detected a high level of MMP-2 mRNA (55.6–61.2% relative to beta-actin; Fig. 1C). At 6 h, 1 and 10 ng/mL of interleukin-1beta significantly stimulated the expression of MMP-2 mRNA when compared with the control ($p < 0.05$). At 12 and 24 h, each concentration of intereukin-1beta significantly up-regulated the mRNA level of MMP-2 (at 12 h, $p < 0.05$ for 0.1 and 1 ng/mL of interleukin-1beta, and $p < 0.01$ for 10 ng/mL of interleukin-1beta; at 24 h, $p < 0.05$ for 0.1 and 10 ng/mL of interleukin-1beta, and $p < 0.01$ for

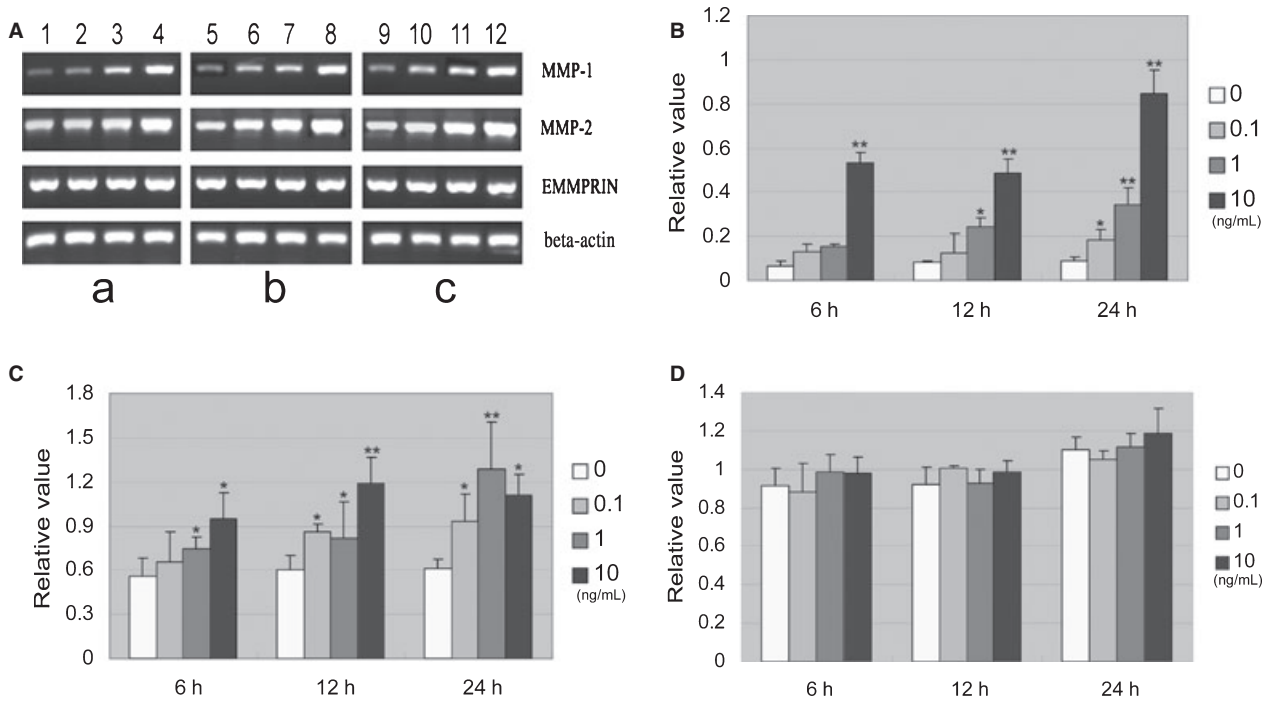

Fig. 1. Effect of interleukin-1beta on the expression of matrix metalloproteinase (MMP)-1 mRNA, MMP-2 mRNA and extracellular MMP inducer mRNA. (A) Image of the reverse transcription–polymerase chain reaction (RT-PCR) product following treatment of human periodontal ligament cells with interleukin-1beta for 6 h (a), 12 h (b) or 24 h (c). Control, lanes 1, 5 and 9; 0.1 ng/mL of interleukin-1 beta, lanes 2, 6 and 10; 1 ng/mL of interleukin-1beta, lanes 3, 7 and 11; and 10 ng/mL of interleukin-1beta, lanes 4, 8 and 12. (B) Bar charts (mean relative value), with standard deviations, of the MMP-1 mRNA level ($n = 6$). At every time-point, interleukin-1beta was found to up-regulate MMP-1 mRNA in a dose-dependent manner. (C) Mean relative value, with standard deviation, of MMP-2 mRNA after treatment with interleukin-1beta ($n = 6$). Except for 0.1 ng/mL of interleukin-1beta at 6 h, every treatment resulted in a significant increase in the mRNA level of MMP-2 when compared with controls. (D) Mean relative value of extracellular MMP inducer mRNA after treatment with interleukin-1beta ($n = 6$). After treatment of human periodontal ligament cells for 6–24 h with varying concentrations of interleukin-1beta, no significant change in the level of extracellular MMP inducer mRNA was observed. *$p < 0.05$; **$p < 0.01$. 67 × 40mm (600 × 600 DPI). EMMPRIN, extracellular MMP inducer.

1 ng/mL of interleukin-1beta), and the increase ranged from 1.35-fold to 2.10-fold. The mRNA level was lower when cells were treated with 1 ng/mL of interleukin-1beta than when treated with 0.1 ng/mL of interleukin-1beta ($p > 0.05$); a similar result was found for 1 and 10 ng/mL of interleukin-1beta at 24 h.

Compared with the mRNA levels of MMP-1 and MMP-2 in untreated human periodontal ligament cells, the level of extracellular MMP inducer mRNA was higher (0.91–1.01-fold higher, relative to expression of beta-actin). After treatment with interleukin-1beta, the level of extracellular MMP inducer mRNA was not significantly different from pretreatment levels ($p > 0.05$; Fig. 1D).

Effect of interleukin-1beta on MMP-1 and extracellular MMP inducer secretion

In supernatant samples of untreated human periodontal ligament cells, the concentration of MMP-1 was 1.37–1.79 pg/mL. Treatment of human periodontal ligament cells with interleukin-1beta increased the MMP-1 protein level in a time-dependent and dose-dependent manner (Fig. 2A). Compared with the control at 6 h, 0.1 ng/mL of interleukin-1beta increased the level of MMP-1 protein by 2.36-fold ($p > 0.05$), 1 ng/mL of interleukin-1beta increased the level of MMP-1 protein by 7.72-fold ($p < 0.05$) and 10 ng/mL of interleukin-1beta increased the level of MMP-1 protein by 11.50-fold ($p < 0.01$); at 12 and 24 h, interleukin-1beta increased the concentration of MMP-1 in a dose-dependent manner, and the increase was 2.54–10.12 fold.

The concentration of extracellular MMP inducer of all supernatant samples was on the standard curve (Fig. 2B). The level of soluble extracellular MMP inducer in controls was much more higher than the level of MMP-1, ranging from 2.11 to 2.57 pg/mL. When compared with controls, interleukin-1beta did not significantly alter the secretion of extracellular MMP inducer into culture medium (Fig. 2C; $p > 0.05$). The correlation of protein concentration between extracellular MMP inducer and MMP-1

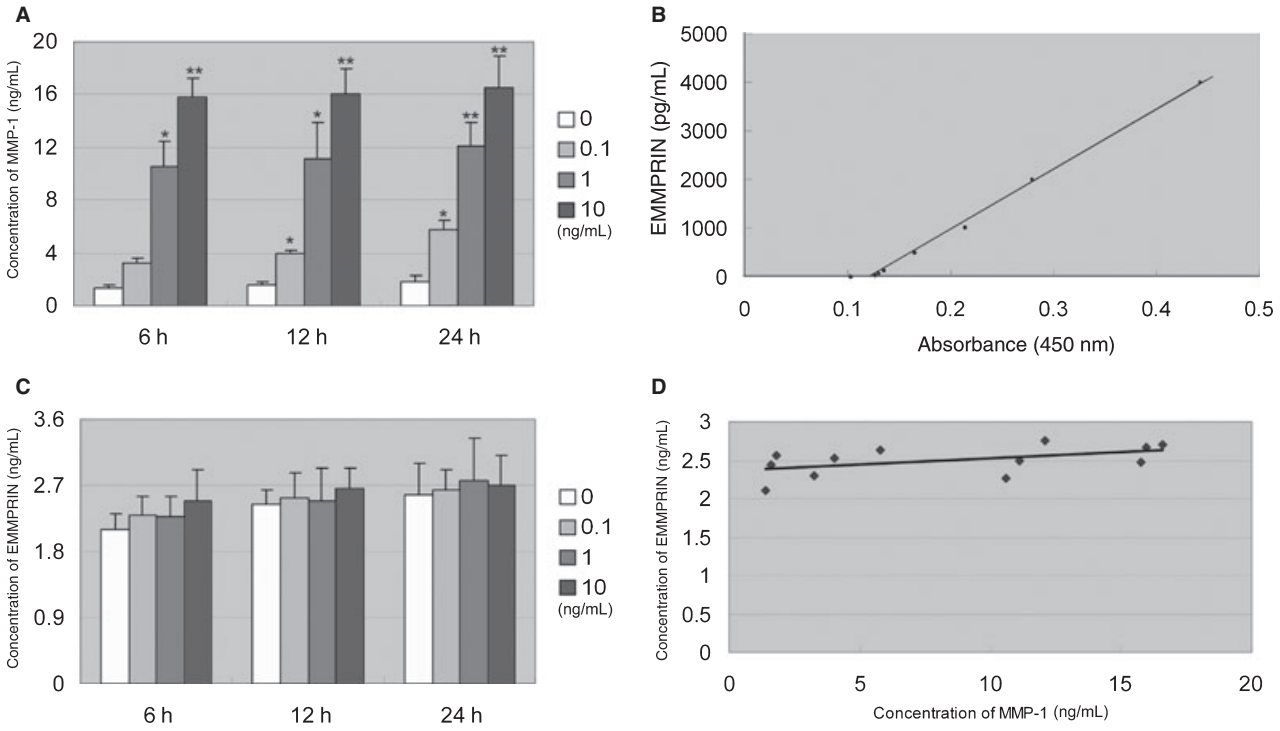

Fig. 2. Effect of interleukin-1beta on extracellular matrix metalloproteinase (MMP) inducer and secretion of MMP-1 protein into the supernatant. (A) Interleukin-1beta was found to up-regulate the secretion of MMP-1 mRNA in a dose-dependent manner. Except for 0.1 ng/mL of interleukin-1beta at 6 h, the MMP-1 concentration following each treatment was significantly higher when compared with the MMP-1 concentration of the respective control ($p < 0.05$). The bars represent as the mean value, and the standard deviation is shown ($n = 6$). (B) Standard curve of extracellular MMP inducer. The concentrations of extracellular MMP inducer in the supernatant samples were all within the range of the curve. (C) Concentration (mean + standard deviation, $n = 6$) of extracellular MMP inducer in the culture medium of human periodontal ligament cells after treatment with interleukin-1beta. No treatment had a significant effect on the amount of soluble extracellular MMP inducer produced ($p > 0.05$). (D) Correlation of the levels of MMP-1 and extracellular MMP inducer in the supernatant: no significant correlation was found ($R^2 = 0.2538$, $p > 0.05$). *$p < 0.05$; **$p < 0.01$. 70 × 43 mm (600 × 600 DPI). EMMPRIN, extracellular MMP inducer.

was not significant (Fig. 2D; $R^2 = 0.2538$, $p > 0.05$).

Effect of interleukin-1beta on the level of active MMP-2 in the culture medium

A 62-kDa (active MMP-2) band was observed using gelatin zymography (Fig. 3A), and the results indicated that interleukin-1beta stimulated the expression of MMP-2 protein (Fig. 3B). At 6 and 12 h, each concentration of interleukin-1beta significantly up-regulated the level of MMP-2 when compared with the control ($p < 0.01$); at 24 h, only 1 ng/mL of interleukin-1beta resulted in a significant increase in the MMP-2 level ($p < 0.05$). At 6 h, a significant difference was observed between 10 and

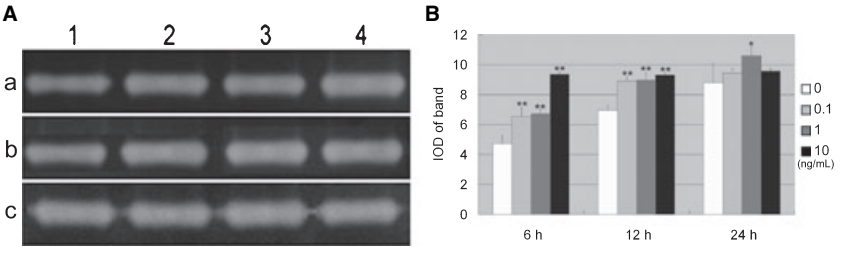

Fig. 3. Effect of interleukin-1beta on the level of active matrix metalloproteinase (MMP)-2, as analyzed using gelatin zymography. (A) A band of 62 kDa was clearly visible when human periodontal ligament cells were treated with 0 (lane 1), 0.1 (lane 2), 1 (lane 3) or 10 (lane 4) ng/mL of interleukin-1beta for 6 h (a), 12 h (b) or 24 h (c). (B) Graph of MMP-2 level after treatment with interleukin-1beta (mean + standard deviation values are shown, $n = 6$). At 6 and 12 h, every concentration of interleukin-1beta significantly increased the level of active MMP-2, and the level of MMP-2 treated with 10 ng/mL of interleukin-1beta for 6 h was higher than that following treatment with 0.1 or 1 ng/mL of interleukin-1beta; at 12 h, no significant difference was observed among the different concentrations of interleukin-1beta. At 24 h, only 1 ng/mL of interleukin-1beta was found to up-regulate the MMP-2 protein level significantly. *$p < 0.05$; **$p < 0.01$. 35 × 11 mm (600 × 600 DPI). IOD, integrated optical density.

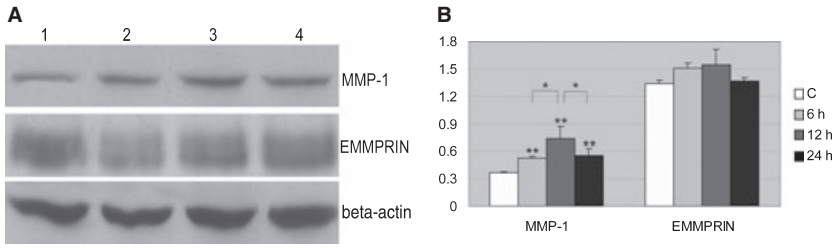

Fig. 4. Effect of interleukin-1beta on the synthesis of matrix metalloproteinase (MMP)-1 and extracellular MMP inducer protein, determined using western blotting. (A) Image of a western blot after treatment of human periodontal ligament cells with 10 ng/mL of interleukin-1beta for 6 h (lane 2), 12 h (lane 3) or 24 h (lane 4). Lane 1 is a control. (B) Bar chart of MMP-1 and extracellular MMP inducer after treatment with interleukin-1beta (mean + standard deviation, $n = 6$). Treatment with interleukin-1beta resulted in a significant up-regulation of MMP-1 protein synthesis in human periodontal ligament cells, but little effect was observed on the level of extracellular MMP inducer protein. The level of MMP-1 protein after treatment with interleukin-1beta for 12 h was higher than that after treatment with interleukin-1beta for 6 or 24 h. *$p < 0.05$; **$p < 0.01$. 35 × 11 mm (600 × 600 DPI). C, control; EMMPRIN, extracellular MMP inducer.

0.1 ng/mL or 1 ng/mL of interleukin-1beta; at 12 and 24 h, no significant difference was found among treatment groups.

Effect of interleukin-1beta on MMP-1 and extracellular MMP inducer synthesis

We detected a band of ~57-kDa conjugated with MMP-1 antibody using western blotting. In the control, MMP-1 protein was synthesized at a low level, about 37% relative to expression of beta-actin (Fig. 4A). When treated with 1 ng/mL of interleukin-1beta, the level of MMP-1 protein increased significantly ($p < 0.05$ at 6 h; $p < 0.01$ at 12 and 24 h). There was no significant difference between 6 and 24 h of treatment with interleukin-1beta ($p > 0.05$), but significant differences were detected between 6 and 12 h or between 12 and 24 h of treatment with interleukin-1beta ($p < 0.05$). The MMP-1 protein level at 24 h was decreased when compared with the MMP-1 protein level at 12 h (Fig. 4B).

In the control, the level of expression of extracellular MMP inducer (a disperse band of about 55 kDa) was a little higher than the expression level of MMP-1 (1.34-fold higher than that of beta-actin); after treatment, synthesis of the extracellular MMP inducer did not change significantly when compared with the control ($p > 0.05$; Fig. 2A,B).

Discussion

Periodontal diseases, especially periodontitis, are chronic infectious diseases characterized by a destructive inflammatory process affecting the supporting tissues of the tooth, resulting in attachment loss, formation of periodontal pockets, resorption of the alveolar bone and eventual tooth loss (26). The local host responses to periodontopathic bacteria include the recruitment of leukocytes and the subsequent release of inflammatory mediators and cytokines, which appear to play crucial roles in the pathogenesis of periodontal diseases (27). Among the pro-inflammatory cytokines, interleukin-1 has been reported to play a crucial role in the pathogenic process of periodontitis (27–29). Interleukin-1 is a multifunctional cytokine capable of medicating both immune and inflammatory responses. The levels of interleukin-1alpha and interleukin-1beta were found to be elevated in the gingival crevicular fluid of patients with periodontitis, and interleukin-1beta was the prominent form present in periodontal tissues (30).

In the present study, we used different concentrations of interleukin-1beta to treat human periodontal ligament cells for different periods of time. The results demonstrated that interleukin-1beta significantly increased the mRNA and protein levels of MMP-1 and MMP-2; the effect on MMP-1 mRNA and protein occurred in a dose-dependent manner. These results were in agreement with results from other studies, which found that interleukin-1beta was able to up-regulate MMP-1 and MMP-2 in a variety of different fibroblasts (31–36). Periodontal ligament cells are the predominant cells in periodontal ligament, a richly collagenous tissue, and given the fact that the concentration of interleukin-1beta is increased in sites with periodontal diseases, up-regulation of MMP-1 and MMP-2 by interleukin-1beta might lead to the breakdown of periodontal ligament collagens, which in turn will result in attachment loss.

Periodontitis is a form of periodontal disease that is clinically distinct from gingivitis, and there is necessarily some unique mechanistic component, evident at a cellular level, which separates these clinical diseases. Birkedal-Hasen summarized the key to understanding why inflammation of the gingiva may, or may not, give rise to tissue destruction and attachment loss, as follows: (i) different cell types express different complements of MMPs; (ii) different cytokines elicit different transcriptional effects on the expression of MMPs; and (iii) different cell types do not necessarily respond in the same manner to a given cytokine (3). Ade *et al.* found that expression of MMP-1 mRNA in human gingival fibroblasts was abundant and quantitatively similar to expression of beta-actin mRNA, whereas mRNA for MMP-2 was present in much smaller amounts, even in the presence of interleukin-1beta, and, moreover, MMP-2 protein was undetectable using ELISA; a low concentration (0.01 ng/mL), interleukin-1beta dose-dependently increased the expression of MMP-1 mRNA, whereas a higher concentration of interleukin-1beta (1 ng/mL) was required to stimulate MMP-2 (37). In the present study, we detected mRNA and protein of MMP-1 and MMP-2 in human periodontal ligament cells. The relative value of MMP-1 mRNA was only 6.6–9.1% of

beta-actin in controls, whereas the level of MMP-2 mRNA was much higher than MMP-1 mRNA; and a low concentration of interleukin-1beta (0.01 ng/mL for 12 h) significantly increased the expression of MMP-1 and MMP-2 mRNA. These contradictory results might be elucidated by the second possibility outlined above (i.e. that different cytokines elicit different transcriptional effects on the expression of MMPs).

In addition to pro-inflammatory cytokines, growth factors and bacterial virulence factors, extracellular MMP inducer is a modulator of MMPs (3,18,38,39). Extracellular MMP inducer was originally identified as a tumor surface protein capable of inducing the expression of MMPs in fibroblasts from tumor stroma, and was initially named 'tumor cell-derived collagenase stimulatory factor' (17,40). Following cloning, tumor cell-derived collagenase stimulatory factor was established as a transmembrane glycoprotein that was highly homologous to proteins of the immunoglobulin superfamily (41,42) and was able to induce the production of several MMPs as a signal molecule (41,43,44). Extracellular MMP inducer has two forms: membrane-bound and soluble. Studies indicated that the membrane-bound and soluble forms of extracellular MMP inducer are equally capable of inducing MMP production from fibroblast cells (21,22,45,46). Emingil *et al*. detected soluble extracellular MMP inducer in gingival crevicular fluid, and also found that the elevated level of extracellular MMP inducer in gingival crevicular fluid was closely associated with the enhanced severity of periodontitis (23). Studies performed by our team suggested that mRNA and protein of extracellular MMP inducer were expressed in healthy gingival tissues, and that the levels of these increased significantly in inflamed gingiva from patients with chronic periodontitis. Moreover, increased levels of MMP-1 and MMP-2 were related to the enhanced expression of extracellular MMP inducer (paper in press). In the present study, we investigated, for the first time, the expression of extracellular MMP inducer mRNA in human periodontal ligament cells. The results demonstrated that interleukin-1beta up-regulated MMP-1 and MMP-2 mRNA in human periodontal ligament cells, but this regulation did not seem to result from enhanced extracellular MMP inducer mRNA. We also detected membrane-bound and soluble extracellular MMP inducer in human periodontal ligament cells. Similarly to the results for extracellular MMP inducer mRNA, the level of extracellular MMP inducer protein was not significantly changed when human periodontal ligament cells were treated with interleukin-1beta. These findings suggest that although extracellular MMP inducer could elevate the expression of MMPs in different fibroblasts, it might not participate in the signaling pathway of MMP-1 and MMP-2 up-regulation by interleukin-1beta in human periodontal ligament cells *in vitro*. However, it is possible that interleukin-1beta might have other effects, such as changing the expression or space structure of receptors for extracellular MMP inducer, altering extracellular MMP inducer signaling, or changing the glycosylation of extracellular MMP inducer (47). It has been reported that the glycosylated extracellular MMP inducer is closely associated with the inducing function of MMPs (48); however, it is still unknown whether the level of glycosylation is related to this function. In the present study, the band detected using the anti-extracellular MMP inducer immunoglobulin during western blotting was wide, possibly because of the different glycosylation level of extracellular MMP inducer. A Similar study has been performed by Braundmeier *et al*., which indicated that extracellular MMP inducer was also not involved in the up-regulation of MMPs by interleukin-1beta in human uterine endometrial fibroblast cells (47,49). The difference was that, in the present study, we detected soluble extracellular MMP inducer in supernatant using ELISA and found membrane-bound extracellular MMP inducer in human periodontal ligament cells using western blotting.

Interestingly, mRNA and protein of MMP-1, MMP-2 and extracellular MMP inducer were detectable in untreated human periodontal ligament cells. These results indicated that, similarly to MMP-1 and MMP-2, extracellular MMP inducer might also play a role in the physiological turnover of periodontal ligament by acting as a stimulator of MMP-1 and MMP-2.

Taken together, the results of the present study suggest that following stimulation with interleukin-1beta, the up-regulated levels of MMP-1 and MMP-2 might be involved in periodontal tissue destruction, and that interleukin-1beta had no effect on the mRNA and protein levels of extracellular MMP inducer. The up-regulation of MMP-1 and MMP-2 by interleukin-1beta might be independent of the extracellular MMP inducer.

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Reference

1. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet* 2005; **366:**1809–1820.
2. Nakaya H, Oates TW, Hoang AM, Kamoi K, Cochran DL. Effects of interleukin-1beta on matrix metalloproteinase-3 levels in human periodontal ligament cells. *J Periodontol* 1997;**68:**517–523.
3. Birkedal-Hansen H. Role of matrix metalloproteinases in human periodontal diseases. *J Periodontol* 1993;**64:**474–484.
4. Reynolds JJ. Collagenases and tissue inhibitors of metalloproteinases: a functional balance in tissue degradation. *Oral Dis* 1996;**2:**70–76.
5. Domeij H, Modeer T, Yucel-Lindberg T. Matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 production in human gingival fibroblasts: the role of protein kinase C. *J Periodont Res* 2004;**39:**308–314.
6. Allan JA, Docherty AJ, Barker PJ, Huskisson NS, Reynolds JJ, Murphy G. Binding of gelatinases A and B to type-I collagen and other matrix components. *Biochem J* 1995;**1:**299–306.
7. Allan JA, Docherty AJ, Murphy G. The binding of gelatinases A and B to type I collagen yields both high and low affinity sites. *Ann NY Acad Sci* 1994;**732:**365–366.

8. Seguier S, Gogly B, Bodineau A, Godeau G, Brousse N. Is collagen breakdown during periodontitis linked to inflammatory cells and expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gingival tissue? *J Periodontol* 2001;**72:**1398–1406.
9. Ding Y, Uitto VJ, Firth J *et al*. Modulation of host matrix metalloproteinases by bacterial virulence factors relevant in human periodontal diseases. *Oral Dis* 1995;**1:**279–286.
10. McCulloch CA. Host enzymes in gingival crevicular fluid as diagnostic indicators of periodontitis. *J Clin Periodontol* 1994; **21:**497–506.
11. Uitto VJ, Overall CM, McCulloch C. Proteolytic host cell enzymes in gingival crevice fluid. *Periodontol 2000* 2003;**31:**77–104.
12. Reynolds JJ, Meikle MC. Mechanisms of connective tissue matrix destruction in periodontitis. *Periodontol 2000* 1997;**14:** 144–157.
13. Boch JA, Wara-aswapati N, Auron PE. Interleukin 1 signal transduction – current concepts and relevance to periodontitis. *J Dent Res* 2001;**80:**400–407.
14. Honig J, Rordorf-Adam C, Siegmund C, Wiedemann W, Erard F. Increased interleukin-1beta (IL-1beta) concentration in gingival tissue from periodontitis patients. *J Periodont Res* 1989;**24:**362–367.
15. Kasasa SC, Soory M. The effect of interleukin-1 (IL-1) on androgen metabolism in human gingival tissue (HGT) and periodontal ligament (PDL). *J Clin Periodontol* 1996;**23:**419–424.
16. Orozco A, Gemmell E, Bickel M, Seymour GJ. Interleukin-1beta, interleukin-12 and interleukin-18 levels in gingival fluid and serum of patients with gingivitis and periodontitis. *Oral Microbiol Immunol* 2006;**21:**256–260.
17. Biswas C, Nugent MA. Membrane association of collagenase stimulatory factor(s) from B-16 melanoma cells. *J Cell Biochem* 1987;**35:**247–258.
18. Gabison EE, Hoang-Xuan T, Mauviel A, Menashi S. EMMPRIN/CD147, an MMP modulator in cancer, development and tissue repair. *Biochimie* 2005;**87:**361–368.
19. Toole BP. Emmprin (CD147), a cell surface regulator of matrix metalloproteinase production and function. *Curr Top Dev Biol* 2003;**54:**371–389.
20. Muramatsu T, Miyauchi T. Basigin (CD147): a multifunctional transmembrane protein involved in reproduction, neural function, inflammation and tumor invasion. *Histol Histopathol* 2003;**18:**981–987.
21. Norgauer J, Hildenbrand T, Idzko M *et al*. Elevated expression of extracellular matrix metalloproteinase inducer (CD147) and membrane-type matrix metalloproteinases in venous leg ulcers. *Br J Dermatol* 2002;**147:**1180–1186.
22. Tang Y, Kesavan P, Nakada MT, Yan L. Tumor-stroma interaction: positive feedback regulation of extracellular matrix metalloproteinase inducer (EMMPRIN) expression and matrix metalloproteinase-dependent generation of soluble EMMPRIN. *Mol Cancer Res* 2004;**2:**73–80.
23. Emingil G, Tervahartiala T, Mantyla P, Maatta M, Sorsa T, Atilla G. Gingival crevicular fluid matrix metalloproteinase (MMP)-7, extracellular MMP inducer, and tissue inhibitor of MMP-1 levels in periodontal disease. *J Periodontol* 2006; **77:**2040–2050.
24. Dong W, Xiang J, Li C, Cao Z, Huang Z. Increased expression of extracellular matrix metalloproteinase inducer is associated with matrix metallo-proteinase-1 and -2 in gingival tissues from patients with periodontitis. *J Periodont Res* 2009; **44:**125–132.
25. Betsuyaku T, Tanino M, Nagai K, Nasuhara Y, Nishimura M, Senior RM. Extracellular matrix metalloproteinase inducer is increased in smokers' bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* 2003;**168:**222–227.
26. Williams RC. Periodontal disease. *N Engl J Med* 1990;**322:**373–382.
27. Okada H, Murakami S. Cytokine expression in periodontal health and disease. *Crit Rev Oral Biol Med* 1998;**9:**248–266.
28. Tatakis DN. Interleukin-1 and bone metabolism: a review. *J Periodontol* 1993; **64:**416–431.
29. Sakai A, Ohshima M, Sugano N, Otsuka K, Ito K. Profiling the cytokines in gingival crevicular fluid using a cytokine antibody array. *J Periodontol* 2006; **77:**856–864.
30. Stashenko P, Fujiyoshi P, Obernesser MS, Prostak L, Haffajee AD, Socransky SS. Levels of interleukin 1beta in tissue from sites of active periodontal disease. *J Clin Periodontol* 1991;**18:**548–554.
31. Alexander MB, Damoulis PD. The role of cytokines in the pathogenesis of periodontal disease. *Curr Opin Periodontol* 1994;**1:**39–53.
32. Brown RD, Jones GM, Laird RE, Hudson P, Long CS. Cytokines regulate matrix metalloproteinases and migration in cardiac fibroblasts. *Biochem Biophys Res Commun* 2007;**362:**200–205.
33. Elias JA, Gustilo K, Baeder W, Freundlich B. Synergistic stimulation of fibroblast prostaglandin production by recombinant interleukin 1 and tumor necrosis factor. *J Immunol* 1987;**138:**3812–3816.
34. Kida Y, Kobayashi M, Suzuki T *et al*. Interleukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts. *Cytokine* 2005;**29:**159–168.
35. Lin SK, Wang CC, Huang S *et al*. Induction of dental pulp fibroblast matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 gene expression by interleukin-1alpha and tumor necrosis factor-alpha through a prostaglandin-dependent pathway. *J Endod* 2001;**27:**185–189.
36. Ohshima M, Otsuka K, Suzuki K. Interleukin-1beta stimulates collagenase production by cultured human periodontal ligament fibroblasts. *J Periodont Res* 1994; **29:**421–429.
37. Abe M, Kawamoto K, Okamoto H, Horiuchi N. Induction of collagenase-2 (matrix metalloproteinase-8) gene expression by interleukin-1beta in human gingival fibroblasts. *J Periodont Res* 2001; **36:**153–159.
38. Chang YC, Yang SF, Lai CC, Liu JY, Hsieh YS. Regulation of matrix metalloproteinase production by cytokines, pharmacological agents and periodontal pathogens in human periodontal ligament fibroblast cultures. *J Periodont Res* 2002; **37:**196–203.
39. Sorsa T, Tjaderhane L, Salo T. Matrix metalloproteinases (MMPs) in oral diseases. *Oral Dis* 2004;**10:**311–318.
40. Ellis SM, Nabeshima K, Biswas C. Monoclonal antibody preparation and purification of a tumor cell collagenase-stimulatory factor. *Cancer Res* 1989; **49:**3385–3391.
41. Biswas C, Zhang Y, DeCastro R *et al*. The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. *Cancer Res* 1995; **55:**434–439.
42. Fossum S, Mallett S, Barclay AN. The MRC OX-47 antigen is a member of the immunoglobulin superfamily with an unusual transmembrane sequence. *Eur J Immunol* 1991;**21:**671–679.
43. Guo H, Zucker S, Gordon MK, Toole BP, Biswas C. Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. *J Biol Chem* 1997;**272:** 24–27.
44. Kataoka H, DeCastro R, Zucker S, Biswas C. Tumor cell-derived collagenase-stimulatory factor increases expression of interstitial collagenase, stromelysin, and 72-kDa gelatinase. *Cancer Res* 1993; **53:**3154–3158.
45. Taylor PM, Woodfield RJ, Hodgkin MN *et al*. Breast cancer cell-derived EMMPRIN stimulates fibroblast MMP2 release through a phospholipase A(2) and 5-lipoxygenase catalyzed pathway. *Oncogene* 2002;**21:**5765–5772.

46. Yan L, Zucker S, Toole BP. Roles of the multifunctional glycoprotein, emmprin (basigin; CD147), in tumour progression. *Thromb Haemost* 2005;**93:**199–204.
47. Braundmeier AG, Nowak RA. Cytokines regulate matrix metalloproteinases in human uterine endometrial fibroblast cells through a mechanism that does not involve increases in extracellular matrix metalloproteinase inducer. *Am J Reprod Immunol* 2006;**56:**201–214.
48. Sun J, Hemler ME. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. *Cancer Res* 2001; **61:**2276–2281.
49. Braundmeier AG, Fazleabas AT, Lessey BA, Guo H, Toole BP, Nowak RA. Extracellular matrix metalloproteinase inducer regulates metalloproteinases in human uterine endometrium. *J Clin Endocrinol Metab* 2006;**91:**2358–2365.