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Experimental Oral Pathology

Interleukin-1β induces matrix metalloproteinase-1 expression in cultured human gingival fibroblasts: role of cyclooxygenase-2 and prostaglandin E_2

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OBJECTIVE: Matrix metalloproteinases (MMPs) degrade extracellular matrices and are responsible for excessive connective tissue breakdown in inflammatory disorders. We investigated the mechanism of MMP-1 expression in human gingival fibroblasts in response to the stimulation with interleukin-1β (IL-1β), and the role of inducible-type cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) in the regulation of MMP-1 expression.

MATERIALS AND METHODS: We stimulated cultured human gingival fibroblasts with r(h)IL-1β, and examined the expression of MMP-1 mRNA and protein by quantitative polymerase chain reaction and enzyme-linked immunosorbent assay. The effect of indomethacin, dexamethasone, or cycloheximide (CHX) on the IL-1β-induced expression of MMP-1 was examined. The expression of MMP-1 in gingival fibroblasts stimulated with PGE_2 was also examined.

RESULTS: IL-1β stimulated the expressions of mRNA and protein for MMP-1, in cultured fibroblasts, in time- and concentration-dependent manners. Pretreatment of the cells with indomethacin or dexamethasone inhibited the IL-1β-induced MMP-1 expression. CHX, a protein synthesis inhibitor, also suppressed the MMP-1 expression. IL-1β also induced COX-2 expression in gingival fibroblasts, and PGE_2, a major COX-2 product, was found to enhance MMP-1 expression.

CONCLUSION: The IL-1β-induced MMP-1 expression in gingival fibroblasts may be mediated, at least in part, by COX-2 and its product PGE_2.

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Keywords: MMP-1; IL-1β; PGE_2; human gingival fibroblasts; COX-2; quantitative PCR

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Introduction

Destruction of extracelluar matrix is a pathogenic feature of inflamed connective tissues (Page, 1995). In inflammatory diseases, interstitial collagenases play a key role by initiating the degradation of collagen, the most abundant protein in the extracellular matrix (Fullmer and Gibson, 1966). Periodontal diseases comprise a group of infections that exhibit measurable loss of connective tissue. In patients with progressive lesions, destruction of the collagen fibers that provide the structural support of the teeth results in impaired gingival attachment to the teeth (Goodson *et al*, 1982). Matrix metalloproteinases (MMPs) are a family of enzymes that degrade almost all extracellular matrix components. MMPs play an important physiological role in fetal development, angiogenesis, and tissue repair (Peled *et al*, 2002); however, they are responsible for excessive breakdown of connective tissues in inflammatory disorders (Fullmer and Gibson, 1966). Also degradation of basement membrane and extracellular matrix by MMPs is crucial for invasion and metastasis of tumor cells (Matrisian *et al*, 1994; Westermarck and Kahari, 1999). MMPs are classified, by their substrate specificity, into four broad categories: collagenases (MMP-1, -8 and -13), gelatinases or collagenases type IV (MMP-2 and -9), stromelysins (MMP-3, -10 and -12), and membrane-type MMPs (MT-MMPs).

Matrix metalloproteinases is an important regulator of connective tissue remodeling (Woessner, 1991; Krane, 1995) and present in high concentrations in inflammatory regions (Birkedal-Hansen, 1993). Expression of MMP genes is regulated by a variety of factors including cytokines (Ries and Petrides, 1995). Of the MMPs family, interstitial collagenase (MMP-1) is the key enzyme responsible for degrading type I and III collagen because the initial breakdown of fibrillar collagen network is mediated primarily by MMP-1 (Birkedal-Hansen *et al*, 1993; Krane, 1994). MMP-1 mRNA is increased in adult gingiva affected with periodontitis, as

compared with that in normal gingiva (Kubota *et al*, 1996; Ejeil *et al*, 2003) and decreased after periodontal therapy (Tuter, Kurtis and Serdar, 2002). Interleukin-1β (IL-1β) is known as one of the most potent inducers of MMP-1 and MMP-3 in fibroblasts (Tewari *et al*, 1994); however, the expression of these MMPs is regulated in different manners depending on the subpopulations of fibroblasts (McCulloch and Bordin, 1991). Gingival fibroblasts are under constant aggression of plaque bacteria (Mariotti, 1999) and mechanical forces applied to the gingiva (Theilig *et al*, 2001). Fibroblasts are the predominant cell type in the soft connective tissues of the gingiva and play a pivotal role in gingival pathology. For instance, it is demonstrated that fibroblasts from wounded rabbit oral mucosa express three- to 10-fold higher IL-1 as compared with the cells from normal mucosa (Bronson, Treat and Bertolami, 1989). These findings suggest that MMP-1 and IL-1β are involved in tissue degradation in periodontal lesions.

Prostaglandins (PGs) mediate diverse functions in inflammatory and immunological conditions (DeWitt, 1991). *In vivo* studies have demonstrated that nonsteroidal anti-inflammatory drugs, which inhibit PG production, prevent signs of periodontal destruction such as gingival bleeding, alveolar bone resorption and attachment loss (Williams *et al*, 1989; Heasman *et al*, 1993). The relationship between Prostaglandin E_2 (PGE_2) and the progression of periodontal diseases has been intensively studied. High levels of PGE_2 are detected in the periodontal connective tissues of adult periodontitis lesions (Loning *et al*, 1980; Offenbacher, Heasman and Collins, 1993; Salvi, Beck and Offenbacher, 1998); and PGE_2 levels in the crevicular fluid can serve as a static assessment of ongoing disease activity (Offenbacher, Odle and Van Dyke, 1986; Cavanaugh *et al*, 1998). Unstimulated human gingival fibroblasts were found to produce low levels of PGE_2 (Heath *et al*, 1987), while LPS from periodontal pathogens stimulated PGE_2 production from gingival fibroblasts (Sismey-Durrant and Hopps, 1991). *Campylobacter rectus* (formerly *Wolinella recta*) is a Gram-negative, strictly anaerobic rod that is associated with adult periodontitis (Dzink *et al*, 1985), and it is the most potent enhancer of PGE_2 production by gingival fibroblasts (Dongari-Bagtzoglou and Ebersole, 1996).

However, it is unknown whether the mRNA level of MMP-1 is affected by PGE_2 in human gingival fibroblasts. In the present study, we have studied the mechanism of the IL-1-induced MMP-1 expression in human gingival fibroblasts in culture. Particular reference was made to the role of inducible-type cyclooxygenase-2 (COX-2) and PGE_2 in the regulation of MMP-1 expression.

Materials and methods

Reagents

Cycloheximide (CHX), indomethacin and dexamethasone (DEX) were purchased from Wako (Osaka, Japan). Recombinant human IL-1β [r(h)IL-1β], antibiotic-antimycotic, oligo$(dT)_{12-18}$, Superscript™ II, trizol reagent and α-MEM were from Invitrogen Corporation (Carlsbad, CA, USA). Culture dishes and fetal calf serum (FCS) were from Asahi Techno Glass (Tokyo, Japan). Recombinant ribonuclease inhibitor RNasin™ was from Promega Corporation (Madison, WI, USA). PGE_2 was from Calbiochem (San Diego, CA, USA). LightCycler Fast Start DNA Master SYBR Green I and LightCycler-Primer Set-MMP-1, COX-2 were from Roche Diagnostics (Mannheim, Germany). ELISA kits were from Amersham Pharmacia (Buckinghamshire, UK). The specific primers for porphobilinogen deaminase (PBGD) were synthesized by Hokkaido System Science (Sapporo, Japan).

Cell culture

Human fibroblasts were isolated from healthy gingival tissues of patients who underwent minor oral surgeries at the Hirosaki University Hospital. All the patients gave fully informed consent before providing the samples. Gingival tissues were washed in phosphate-buffered saline, pH7.4, (PBS) and cut into small pieces, which were cultured in α-MEM containing 10% FCS, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and gentamicin (80 μg ml^{-1}) for 2 weeks in an atmosphere of 95% air and 5% CO_2 at 37°C. When cells growing out from the explants had reached confluence, they were subcultured, and experiments were performed on confluent cultures of third to sixth passage. Fibroblasts were stimulated with a series of concentrations (10 pg ml^{-1}–1 ng ml^{-1}) of r(h)IL-1β for up to 24 h. In order to investigate whether anti-inflammatory drugs have a regulatory effect on the MMP-1 mRNA expression, fibroblasts were preincubated with 2 μM indomethacin or 10 μM DEX for 30 min and then stimulated with 5 ng ml^{-1} IL-1β for 8 h. DEX and indomethacin were dissolved in ethanol and the final concentration of ethanol in culture medium was 0.1% (v/v), and control incubation was performed with vehicle only. In experiments on the effects of CHX, fibroblasts were incubated with 500 ng ml^{-1} CHX for 1 h prior to the stimulation with 5 ng ml^{-1} IL-1β for 8 h. In the experiments on the effects of PGE_2, the fibroblasts were incubated with a series of concentrations (1 ng ml^{-1}–10 μg ml^{-1}) of PGE_2 for 8 h at 37°C. PGE_2 was dissolved in ethanol and the final concentration of ethanol in culture medium was 0.1%.

RNA extraction and quantitative polymerase chain reaction

Total RNA was isolated from the cells using a trizol reagent. Single-strand cDNA for a polymerase chain reaction (PCR) template was synthesized from 1 μg of total RNA using a primer oligo$(dT)_{12-18}$ and the Superscript™ II reverse transcriptase under the conditions indicated by the manufacturer.

A glass capillary was filled with 6 μl of water, 2 μl of LightCycler-Primer Set-MMP-1, 2 μl of LightCycler Fast Start DNA Master SYBR Green I, and 10 μl of

reverse-transcribed cDNA (10 ng). Capillaries were closed, centrifuged and placed into the rotor of a LightCycler (Roche Diagnostics, Mannheim, Germany). Denaturation program, amplification, quantification and melting curve program for MMP-1 and COX-2 were defined by the manufacturer's protocol. For the analysis of PBGD, the specific primers were designed according to Grandchamp *et al* (Grandchamp *et al*, 1987). A glass capillary was filled with 14.2 μl water, 0.8 μl $MgCl_2$ (4 mM), 1 μl forward primer (0.5 M), 1 μl reverse primer (0.5 M) and 2 μl LightCycler Fast Start DNA Master SYBR Green I. Then 1 μl of reverse-transcribed cDNA (10 ng) was added as a PCR template. Amplification and detection of target DNA was performed as follows: an initial 10 min incubation at 95°C for FastStart Taq DNA polymerase activation, followed by 40 cycles of denaturation at 95°C for 15 s, primer and probe annealing at 60°C for 10 s and extension at 72°C for 10 s, with monitoring of fluorescence during the annealing phase. This was followed by a melting program of temperature increase from 65 to 95°C at 0.1°C s^{-1} with continuous monitoring of the fluorescence. Data were analyzed by software, LC Run Version 5.23.

ELISA for MMP-1
Samples of fibroblast-conditioned medium or cell lysate were centrifuged briefly, and supernatants were stored at −20°C until use. The level of MMP-1 protein was determined using an ELISA kit.

Statistics
Values were expressed as mean ± s.d., and statistical significance was analyzed by Student's *t*-test or Welch's *t*-test. All probability (*P*) values were based on two-tailed tests, and $P < 0.05$ was considered to be significant.

Results

IL-1β-induced MMP-1 expression in gingival fibroblasts
Time-dependent expression of MMP-1 mRNA in fibroblasts stimulated with IL-1β is shown in Figure 1a. MMP-1 mRNA levels reached the maximum 8 h after the stimulation with 1 ng ml^{-1} IL-1β. The time course of MMP-1 protein production agreed with that of mRNA expression, and the protein levels in conditioned medium and cell lysates increased until 24 h after the stimulation (Figure 1b).

Figure 2a summarizes the quantitative PCR analysis on the concentration-dependent MMP-1 expression. Unstimulated cells expressed a substantial level of MMP-1 mRNA; and IL-1β, at 1 ng ml^{-1}, enhanced the mRNA level by 15-fold. IL-1β also enhanced MMP-1 protein production in a concentration-dependent manner as shown in Figure 2b.

Effects of anti-inflammatory agents and CHX
Effects of indomethacin and DEX on MMP-1 mRNA expression in fibroblasts are shown in Figure 3. Both indomethacin (2 μM) and DEX (10 μM) inhibited the IL-1β-induced MMP-1 mRNA expression to the level similar to that in unstimulated cells.

Pretreatment of the cells with 500 ng ml^{-1} CHX completely inhibited the MMP-1 expression in response to IL-1β (Figure 4).

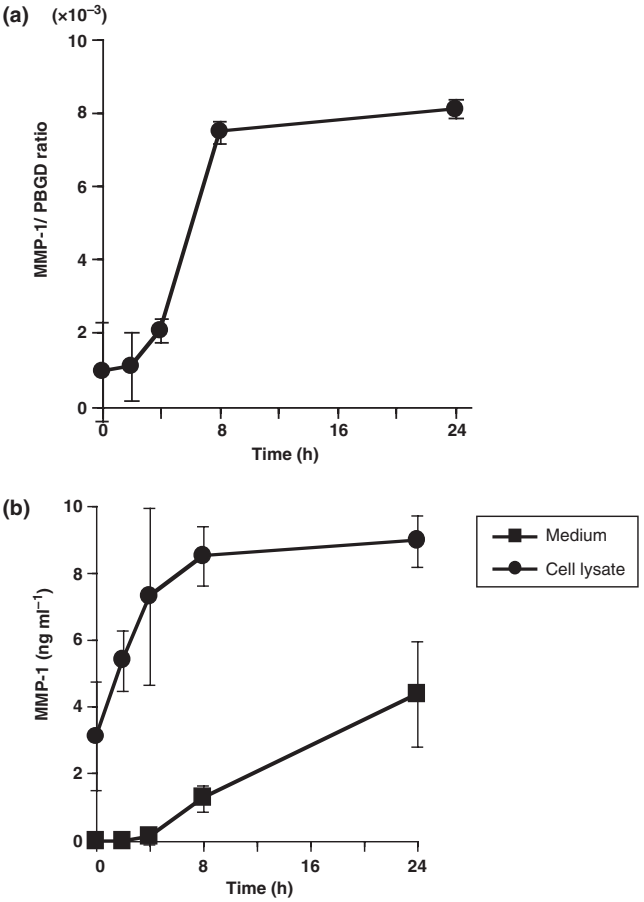

Figure 1 Time course of MMP-1 expression in gingival fibroblasts stimulated with IL-1β. (**a**) Quantification of mRNA for MMP-1. The cells were stimulated with 1 ng ml^{-1} IL-1β for up to 24 h. The relative number of copies of specific cDNA for MMP-1 was quantified using a LightCycler system. The analyses for MMP-1 represent the ratio of MMP-1 to PBGD mRNA. (**b**) Concentration of MMP-1 protein in the medium or cell lysate was determined by ELISA. Means (±s.d.) of three experiments are shown

Expression of COX-2 in gingival fibroblasts
The time course of COX-2 mRNA expression in gingival fibroblasts is shown in Figure 5a. The expression in response to IL-1β reached the maximal level 8 h after the stimulation and decreased thereafter. Figure 5b shows that IL-1β enhanced COX-2 mRNA expression in a concentration-dependent manner in gingival fibroblasts.

Expression of MMP-1 in response to PGE_2
Expression of MMP-1 mRNA in fibroblasts stimulated with PGE_2 is shown in Figure 6. PGE_2 (1 ng ml^{-1}–10 μg ml^{-1}) enhanced the expression of MMP-1 mRNA in a concentration-dependent manner.

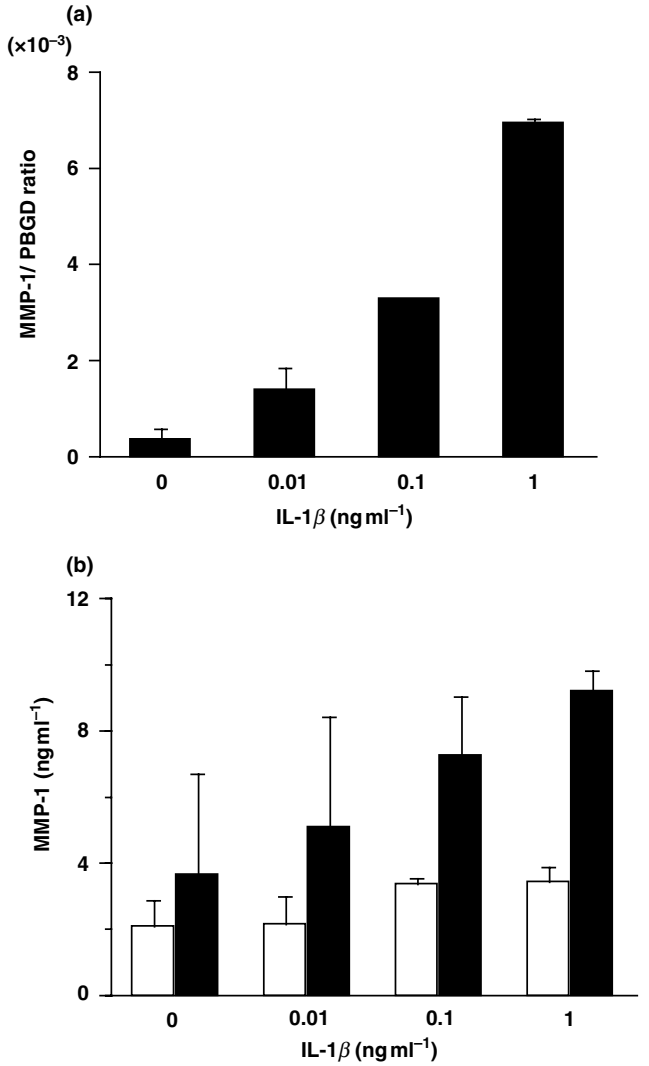

Figure 2 Concentration-dependent induction of MMP-1 in gingival fibroblasts stimulated with IL-1β. (**a**) The cells were stimulated, for 8 h, with IL-1β (0.01–1 ng ml^{-1}), and quantification of mRNA for MMP-1 was performed as described in Figure 1. (**b**) Concentration of MMP-1 protein in the medium (open bars) and cell lysate (black bars) were determined by ELISA as described in Figure 1. Means ($\pm$s.d.) of three experiments are shown

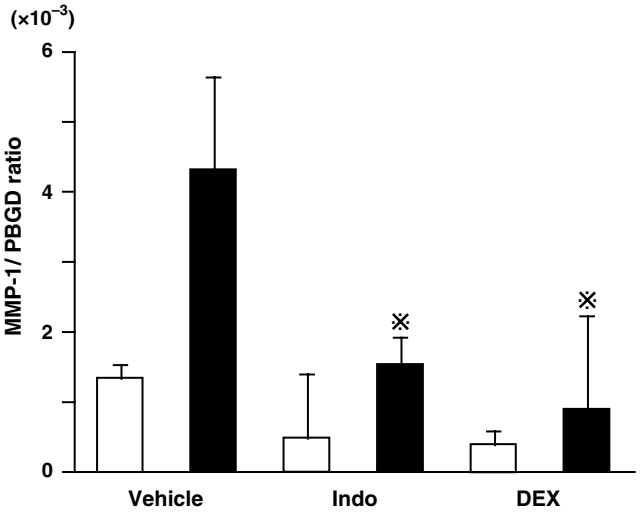

Figure 3 Effect of anti-inflammatory agents on MMP-1 expression in gingival fibroblasts stimulated with IL-1β. Cells were pretreated with 2 μM indomethacin or 10 μM DEX for 30 min, and then incubated with (closed bars) or without (open bars) 5 ng ml^{-1} IL-1β for 8 h. The expressions of mRNA for MMP-1 or PBGD were analyzed by quantitative RT-PCR as described in Figure 1. Each value represents mean $\pm$ s.d. of four experiments. *$P < 0.01$, statistically significant difference compared with vehicle treatment

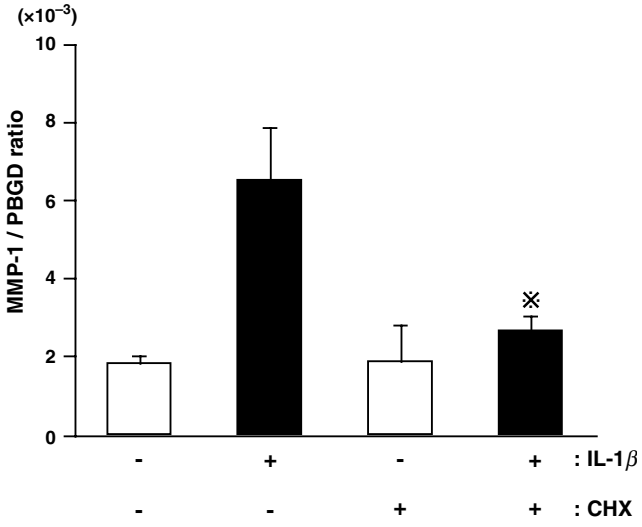

Figure 4 Effect of CHX on the IL-1β-induced MMP-1 expression in gingival fibroblasts. Cells were treated with 500 ng ml^{-1} CHX for 1 h, and then stimulated with 5 ng ml^{-1} IL-1β for 8 h. The analyses for MMP-1 mRNA were performed by quantitative RT-PCR as described in Figure 1. Each value represents mean $\pm$ s.d. of four different experiments. *$P < 0.01$, statistically significant difference compared with vehicle treatment

Discussion

IL-1 has an important role in a variety of processes such as inflammatory reactions (Duff, 1985), cell proliferation and bone destruction (Gowen *et al*, 1983). IL-1 is generated by many types of cells including lymphocytes, monocytes/macrophages and fibroblasts (Kang *et al*, 1996). It stimulates the target cells to synthesize active neutral proteases such as plasminogen activator, MMPs and elastase, which contribute to the increased rate of collagen degradation in inflammatory diseases (MacNaul *et al*, 1990).

Extracellular matrix is regulated by synthesis and organization of its structural constituents and their degradation by metalloendopeptidases that belong to the matrixin family (Woessner, 1991; Birkedal-Hansen, 1995). Fibroblasts synthesize MMPs and play a prominent role in maintaining tissue homeostasis particularly during repair process following tissue injury (Woessner, 1991). Previous reports (Fullmer and Gibson, 1966; Birkedal-Hansen, 1993; Aiba *et al*, 1996) have shown that MMPs are involved in gingival destruction in gingivitis and periodontitis, and that MMP-1 accounts

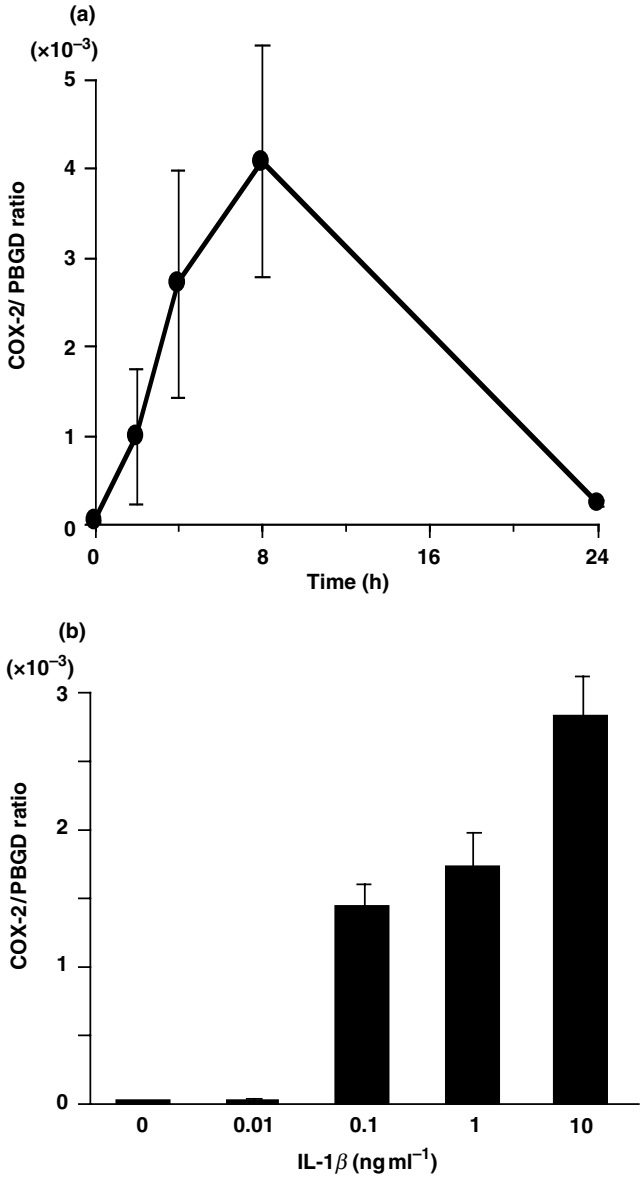

Figure 5 (**a**) Time course of the expression of COX-2 in gingival fibroblasts stimulated with IL-1β. The cells were stimulated with 5 ng ml^{-1} of IL-1β for 2–24 h. The relative number of copies of specific cDNA for COX-2 was quantified using a LightCycler system. The analyses for COX-2 represent the ratio of COX-2 to PBGD mRNA. (**b**) Concentration-dependent stimulation, by IL-1β, of the COX-2 expression in gingival fibroblasts. Cells were stimulated with IL-1β (0.01–10 ng ml^{-1}) for 8 h, and quantification of mRNA for COX-2 was performed. Means ($\pm$s.d.) of three experiments are shown

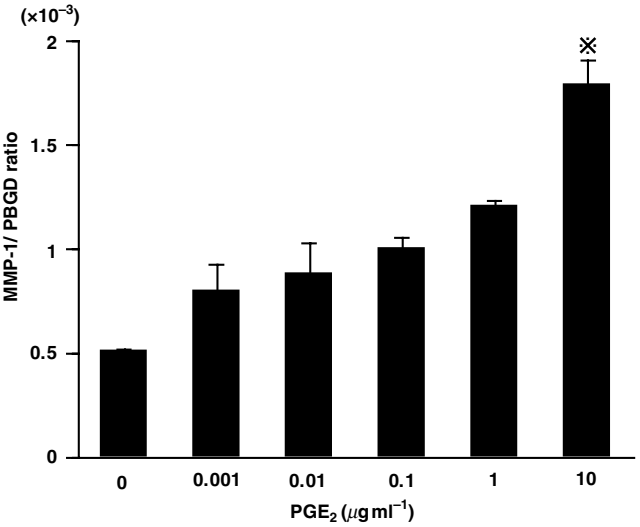

Figure 6 Concentration-dependent stimulation, by PGE_2, of the MMP-1 expression in gingival fibroblasts. Cells were stimulated with PGE_2 (0.001–10 μg ml^{-1}) for 8 h, and quantification of mRNA for MMP-1 was performed as described in Figure 1. Each value represents mean $\pm$ s.d. of four different fields. *$P < 0.01$, statistically significant difference compared with unstimulated control group

for most of the collagenase activities in gingival tissue from periodontitis patients. In the present study, we found the stimulation, by IL-1β, of MMP-1 expression in gingival fibroblasts. IL-1β is known to induce MMP-1 in oral keratinocytes (Wan *et al*, 2001) and chondrocytes (Koshy *et al*, 2002); and gingival fibroblasts may also serve as a potential source of MMP-1 in inflammatory tissue injury.

Pretreatment of fibroblasts with CHX inhibited the expression of MMP-1 mRNA induced by IL-1β, and *de novo* synthesis of certain protein factor(s) may be involved in the MMP-1 expression. IL-1β also enhanced the expression of COX-2 in a time- and concentration-dependent manner; and the COX-2 expression preceded the MMP-1 induction. COX, also known as PGH_2 synthetase, is a key enzyme in PG generation. There are two isoforms of COX: COX-1 is constitutively expressed and COX-2 is readily induced by stimulation with cytokines, mitogens, etc (Kutchera *et al*, 1996). Domeij *et al* (Domeij, Yucel-Lindberg and Modeer, 2002) reported that inhibition of COX-2 resulted in the decreased secretion of MMP-1 protein in culture medium of IL-1β stimulated gingival fibroblasts. The change in expression of mRNA of COX-2, however, was not demonstrated and hence it still remained unclear if the inhibition of MMP-1 was definitely associated with the inhibition of COX-2. In this study we quantified the expression of MMP-1 mRNA by real-time PCR. We found that indomethacin inhibits the expression of MMP-1 mRNA induced by IL-1β. This agrees with previous reports that demonstrated the involvement of COX-2 products in the MMP-1 induction in animal models of inflammatory disorders (Ottino and Bazan, 2001; Lin *et al*, 2002; Long *et al*, 2002). We also found that PGE_2, a major product of COX in fibroblasts, induced MMP-1 mRNA. These results agree with the fact that COX-2 inhibitor indomethacin suppressed the IL-1β induced MMP-1 expression; and the effect of CHX may be explained by the suppression of COX-2 induction.

We conclude that IL-1β stimulates MMP-1 expression in human gingival fibroblasts, and the expression may be mediated, at least in part, through the induction of COX-2 and PGE_2 generation.

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