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Matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 production in human gingival fibroblasts: the role of protein kinase C

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Background: Matrix metalloproteinase-1 (MMP-1) plays an important role in tissue remodelling and in the pathology of inflammatory diseases including periodontitis. The activity of MMP-1 is firmly controlled by the endogenous tissue inhibitor of metalloproteinase-1 (TIMP-1).

Objective: The aim of the study was to investigate the production and regulation of MMP-1 and TIMP-1 with special regards to the enzyme protein kinase C (PKC) in human gingival fibroblasts.

Methods: Gingival fibroblasts were treated with substances related to PKC such as phorbol 12-myristate 13-acetate (PMA), interleukin-1β, Ca^{2+}-ionophore A231817 and inhibitors of PKC, p38 mitogen-activated protein kinase (p38 MAPK) and tyrosine kinase.

Results: The PKC activator PMA stimulated the production of MMP-1 and TIMP-1 at both the transcriptional and the translational level. The production of MMP-1 and TIMP-1 stimulated by PMA was abolished by the PKC inhibitor bisindolylmaleimide. Treatment of the cells with interleukin-1β or A23187 synergistically increased the stimulatory effect of PMA on MMP-1 production. In contrast, TIMP-1 production was unaffected by interleukin-1β and reduced by A23187. Tyrosine kinase inhibitor herbimycin A reduced MMP-1 production induced by PMA, whereas the p38 MAPK-inhibitor SB 203580 synergistically increased the stimulatory effect of PMA on both MMP-1 and TIMP-1 production.

Conclusion: The present study shows that MMP-1 and TIMP-1 production is regulated differently by interleukin-1β and calcium in human gingival fibroblasts and that this difference is markedly amplified in the presence of the PKC-activator PMA. Taken together, the discrepancy in the production of MMP-1 and TIMP-1 in gingival fibroblasts may contribute to tissue destruction in periodontal diseases.

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Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteolytic enzymes that mediate extracellular matrix degradation (1, 2). The MMP family contains at least 20 members, which are grouped according to their substrate specificity (3) and to their structure (4). Collagenase-1 (MMP-1) serves as an initiator of extracellular matrix destruction and cooperates with other MMPs in the degradation of, *inter-alia*, collagen. The activity of MMP-1 is firmly controlled by its inhibitors, tissue inhibitors of metalloproteinases (TIMPs), mainly TIMP-1 (5).

The balance between MMP-1 and TIMP-1 is an important control point in tissue remodelling and an imbalance may promote tissue destruction. Enhanced protein levels and mRNA expression of MMP-1 have been demonstrated in inflammatory diseases, including periodontitis (6–8). The increased levels of MMP-1 observed in periodontitis may be a result of an alteration in the regulation of MMP-1 by the most predominant cell in the gingivae, i.e. the gingival fibroblast (9, 10), which participates in inflammation and bone resorption (11). We have previously shown that MMP-1 production in human gingival fibroblasts is stimulated by cytokines such as interleukin-1β and tumour necrosis factor-α, as well as partly regulated by p38 mitogen-activated protein kinases (p38 MAPK) and tyrosine kinase (12). The protein kinase C (PKC), a serine/threonine kinase that is known to cooperate with MAPK and tyrosine kinase (13–15), has previously been suggested to be involved in the production of MMP-1 in dermal fibroblasts (16). Although much work has been performed regarding MMP-1 and TIMP-1, comparatively little is known about the regulation and signal transduction pathways utilized to confirm the control of MMP-1 and TIMP-1 in human gingival fibroblasts. The aim of this work was therefore to investigate the production and regulation of MMP-1 and its inhibitor TIMP-1 in relation to PKC in human gingival fibroblasts.

Materials and methods

Fibroblast cultures

Cultures of fibroblast cells were established from gingival biopsies obtained from seven healthy individuals between 3 and 18 years of age with no clinical signs of periodontal disease. The Ethics Committee at Huddinge University Hospital, Karolinska Institutet approved the study. Minced pieces of the tissue were explanted to 25-cm^2 tissue culture flasks (Nunc, Naperville, IL, USA) containing 5 ml of Dulbecco's modified Eagle's medium (Gibco, Paisley, Scotland). The fibroblasts were obtained by trypsinization of the primary outgrowth of cells, as described previously (17). The cells were grown in Dulbecco's modified Eagle's medium supplemented with foetal calf serum (5%) (Gibco). The cells were incubated at 37°C in a humidified incubator aerated with 5% CO_2 in air and routinely passaged using 0.025% trypsin in phosphate-buffered saline containing 0.02% EDTA (Gibco). Cells used for the experiments proliferated in logarithmic phase between the 7th and 12th passages.

Production of MMP-1 and TIMP-1 in human gingival fibroblasts

Gingival fibroblasts, at cell density indicated in the legends to Figures and Tables, were seeded in 24-well plates in Dulbecco's modified Eagle's medium containing 5% foetal calf serum and incubated for 24 h at 37°C. The cell layer was rinsed three times with serum-free Dulbecco's modified Eagle's medium and the cells were thereafter incubated in 500 μl medium supplemented with 1% foetal calf serum with or without 0.1–10 nM phorbol 12-myristate 13-acetate (PMA) in the absence or presence of 2 μM bisindolylmaleimide (Sigma, St Louis, MO, USA), 0.1 μM A23187 (Calbiochem, San Diego, CA, USA), 100 pg/ml interleukin-1β (interleukin-1β: R & D systems, Minneapolis, MN, USA), 10 μM SB 203580 (Calbiochem) or 0.1 μM herbimycin A (Sigma). After 24 h of incubation, the medium was withdrawn and stored at −80°C for analysis of MMP-1 and TIMP-1.

The levels of MMP-1 (total MMP-1) and TIMP-1 (free form of TIMP-1) were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits obtained from Amersham Bioscience (Little Chalfont, UK) and R & D systems, respectively. The detection limit was 6.25 ng/ml for MMP-1 and 0.156 ng/ml for TIMP-1.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Gingival fibroblasts, at cell density indicated in the legends to Figures, were seeded in Petri dishes (100 mm) in Dulbecco's modified Eagle's medium supplemented with 5% foetal calf serum and cultured for 24 h at 37°C. The cell layers were then rinsed three times with serum-free Dulbecco's modified Eagle's medium followed by addition of 5.0 ml 1% foetal calf serum Dulbecco's modified Eagle's medium containing PMA and/or bisindolylmaleimide at concentrations indicated in the legends. After 3 h of incubation the culture medium was removed. The cell monolayer was washed twice with ice cold phosphate-buffered saline, frozen immediately in liquid nitrogen and stored at −80°C for isolation of total RNA. Total RNA was isolated from control and treated fibroblasts and quantified spectrophotometrically at 260/280 nm (18). To obtain first-strand cDNA, 1.0 μg of total RNA was reversed transcribed using Superscript II (Invitrogen, Paisley, UK) in a total volume of 20 μl. The obtained cDNA (2 μl) was used in a final reaction volume of 50 μl for PCR amplification (35 cycles). The PCR reactions were performed, using specific primers and positive controls for MMP-1, TIMP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) supplied from R & D systems, according to the manufacturer's recommendation. For each experiment, PCR amplification was also performed without cDNA to check for contamination. Ten microlitres of each PCR reaction product was

run on 2% agarose gel next to 1 kb DNA-ladder standard (Invitrogen) and visualized with ethidium bromide (USB Corporation, Ohio, IL, USA). The product sizes were 262 base pairs (bp) for MMP-1, 396 bp for TIMP-1 and 576 bp for GAPDH, respectively. The positive controls were 360 bp for MMP-1, 340 bp for TIMP-1 and 340 bp for GAPDH, respectively.

Western blotting

Fibroblasts, at cell density indicated in the legends to Figures, were seeded in 24-well plates and grown as described above. After 24 h of incubation, medium was withdrawn and the protein concentrations were determined according to the Bradford method using bovine serum albumin as standard. Equivalent amounts of protein (15 μg) were separated by electrophoresis on a 4–15% gradient sodium dodecyl sulfate–polyacrylamide gel and transferred thereafter to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were then blocked in 5% dry milk for 1 h, incubated at 4°C overnight with polyclonal primary antibody (1 : 5000) for MMP-1 (Chemicon, Temecula, CA, USA) and thereafter with secondary horseradish rabbit peroxidase-conjugated antibody (1 : 5000) (Dako Corporation, Carpinteria, CA, USA). The membranes were developed using enhanced chemiluminescence (ECL) and exposed to hyperfilm-ECL (Amersham Bioscience).

Statistical analysis

All experiments were performed in cells from at least three individuals and reproducible data representing one of three separate experiments is demonstrated. The student's *t*-test was used in the statistical analysis. *p*-values less than 0.05 were considered statistically significant.

Results

To elucidate the involvement of PKC in the production of MMP-1 and TIMP-1 in human gingival fibroblasts, the cells were exposed to the PKC activator PMA. The phorbol ester PMA, at different concentrations (0.1–10.0 nM), significantly ($p < 0.001$) stimulated the production of MMP-1 and TIMP-1 by gingival fibroblasts (Fig. 1).

In contrast to PMA, the phorbol ester phorbol 13-monoacetate (1.0 μM), an inactive analogue of PMA, did not stimulate the production of MMP-1 or TIMP-1 (data not shown). The stimulatory effect of PMA on MMP-1 production was also confirmed by western blot analyses. The results also showed that MMP-1 was not bound to TIMP-1 and that the released MMP-1 was approximately 53 kDa, the proform of MMP-1 (Fig. 2B).

We also studied the mRNA expression of MMP-1 and TIMP-1 in gingival fibroblasts. The RT-PCR results showed that MMP-1 and TIMP-1 mRNA expression was constitutively expressed. Furthermore, treatment of the cells with PMA (10 nM) enhanced both MMP-1 and TIMP-1 mRNA expression, as demonstrated by Fig. 2A and Fig. 3A. The amount of input RNA was controlled by GAPDH and RT-PCR analysis, which confirmed that the level of GAPDH mRNA expression was similar in unstimulated and stimulated cells (Fig. 2A and Fig. 3A). The enhanced mRNA expression of MMP-1 and TIMP-1 was accompanied by increased protein production in gingival fibroblasts (Figs 2B and C and Fig. 3B).

To further elucidate the involvement of PKC on MMP-1 and TIMP-1 production, the fibroblasts were treated with PMA (10 nM) in the presence or absence of the PKC inhibitor bisindolylmaleimide (2.0 μM). In addition, bisindolylmaleimide abolished the mRNA expression of MMP-1 and TIMP-1 that had been induced by PMA (Fig. 2A and Fig. 3A). Furthermore, the inhibitory effect of bisindolylmaleimide on PMA-induced MMP-1 and TIMP-1 mRNA expression was also reflected by diminished MMP-1 (Figs 2B and C) and TIMP-1 protein production ($p < 0.001$) (Fig. 3B).

As PKC can be activated in a Ca^{2+}-dependent manner (19), the effect of calcium on MMP-1 production was mimicked using the Ca^{2+}-ionophore A23187. Treatment of the cells with A23187 (0.1 μM) alone did not affect MMP-1 production but synergistically ($p < 0.01$) up-regulated the production of MMP-1 induced by PMA (10 nM) (Fig. 4). The synergistic up-regulation of MMP-1 induced by the combination of PMA and A23187 was abolished ($p < 0.001$) by the PKC

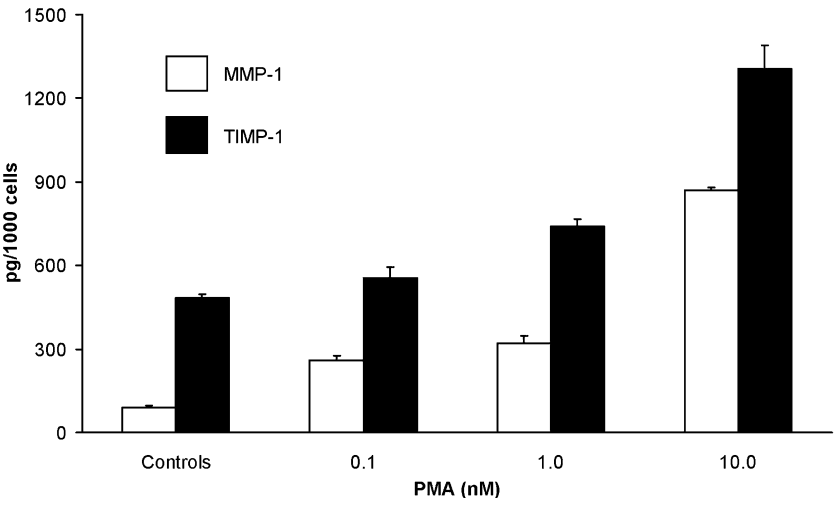

Fig. 1. Effect of phorbol 12-myristate 13-acetate (PMA: 0.1–10.0 nM) on matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) production (24 h) in human gingival fibroblasts. Cell density was 1.5×10^4 cm^{-2}. Mean value of triplicates with SD shown by vertical bars.

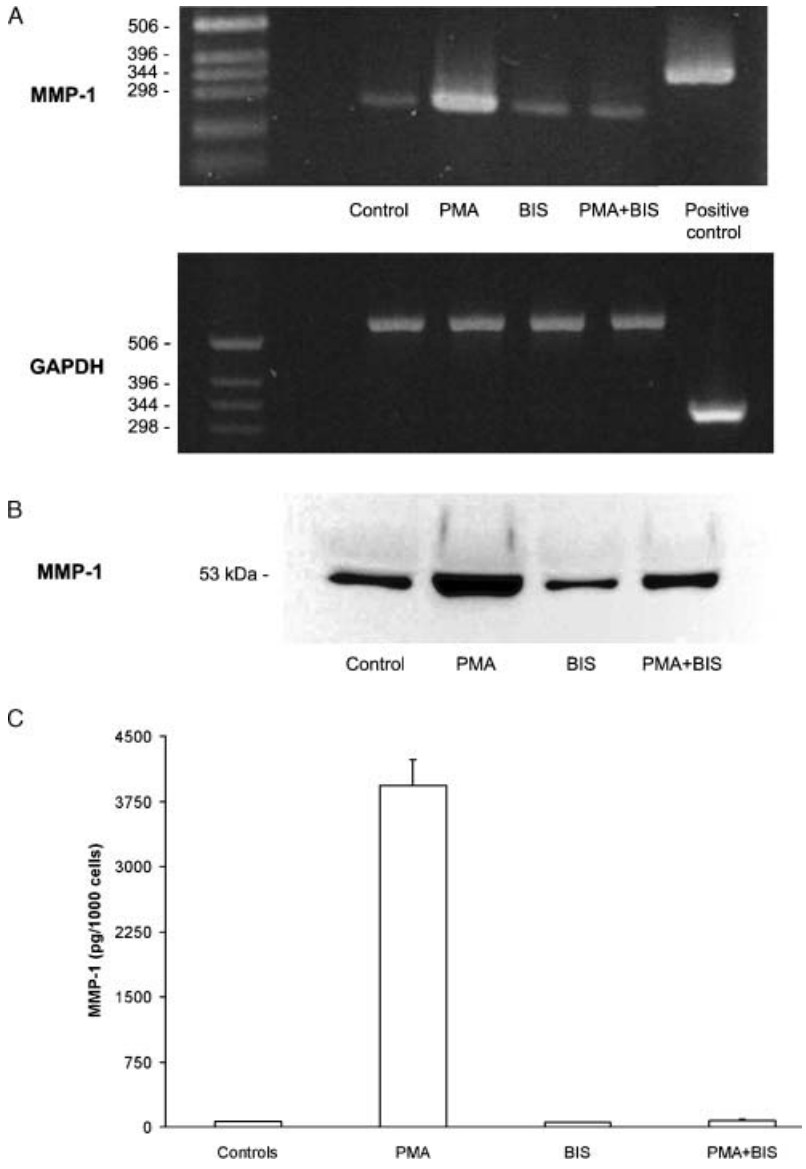

Fig. 2. Effect of bisindolylmaleimide (bisindolylmaleimide: 2.0 μM) on (A) the mRNA expression of matrix metalloproteinase-1 (MMP-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (3 h) and the protein production of MMP-1 determined by western blotting (B) and ELISA (C) (24 h) in the presence or absence of phorbol 12-myristate 13-acetate (PMA: 10.0 nM) in human gingival fibroblasts. Cell density was 1.8×10^4 cm^{-2} for mRNA expression, 1×10^5 cm^{-2} for western blotting, and 1.5×10^4 cm^{-2} for protein production. Vertical bars indicate the mean value of triplicates with SD for protein production.

inhibitor bisindolylmaleimide (2.0 μM) (data not shown). Regarding TIMP-1, treatment of the cells with the Ca^{2+}-ionophore A23187 (0.1 μM) reduced ($p < 0.01$) TIMP-1 production in gingival fibroblasts. Similarly, A23187 also reduced ($p < 0.01$) the stimulatory effect of PMA (10 nM) on TIMP-1 production (Fig. 4). When the fibroblasts were treated with PMA and A23187 in the presence of the PKC inhibitor bisindolylmaleimide, the production of TIMP-1 was significantly ($p < 0.001$) reduced to control levels (data not shown).

The pro-inflammatory mediator interleukin-1β affects intracellular signal pathways including the second messenger PKC (20, 21). The effect of interleukin-1β on MMP-1 and TIMP-1 production was therefore studied. The results showed that interleukin-1β (100 pg/ml), in agreement with previous findings (12), stimulated the production of MMP-1 in gingival fibroblasts. Furthermore, interleukin-1β synergistically ($p < 0.001$) up-regulated the stimulatory effect of PMA (10 nM) on MMP-1 production (Fig. 5). This synergistic up-regulation was abolished in the presence of the PKC inhibitor bisindolylmaleimide (data not shown). Concerning TIMP-1 production, interleukin-1β treatment of the cells neither increased nor up-regulated the stimulatory effect of PMA on TIMP-1 production in human gingival fibroblasts (Fig. 5).

To further elucidate intracellular signal pathways regulating MMP-1 and TIMP-1 production, the involvement of p38 MAPK and tyrosine kinase was also studied. Treatment of the cells with the p38 MAPK inhibitor SB 203580 (10 μM) significantly ($p < 0.01$) up-regulated both MMP-1 and TIMP-1 production induced by PMA (Table 1). However, SB 203580 did not affect the basal production of MMP-1 or TIMP-1 in the control cells (Table 1).

Under the same experimental conditions, we also studied the effect of the tyrosine kinase inhibitor herbimycin A (0.1 μM) in the absence or presence of PMA. Herbimycin A significantly ($p < 0.001$) reduced the MMP-1 production that had been induced by PMA (Table 1). In contrast, the tyrosine kinase inhibitor did not significantly affect the PMA-induced production of TIMP-1 (Table 1).

Discussion

MMP-1 is considered to be a key initiator of collagen degradation in inflammatory responses and elevated levels of MMP-1 have been demonstrated in gingival crevicular fluid and gingival tissue from patients with periodontal disease (6–8). The gingival fibroblast, the most predominant cell in the gingival tissue, participates in the local inflammatory response by its ability to produce MMP-1 *inter alia* (9, 10). In this study, we investigated the regulation of MMP-1 and TIMP-1 with special regards to the enzyme PKC to further explore the signal transduction

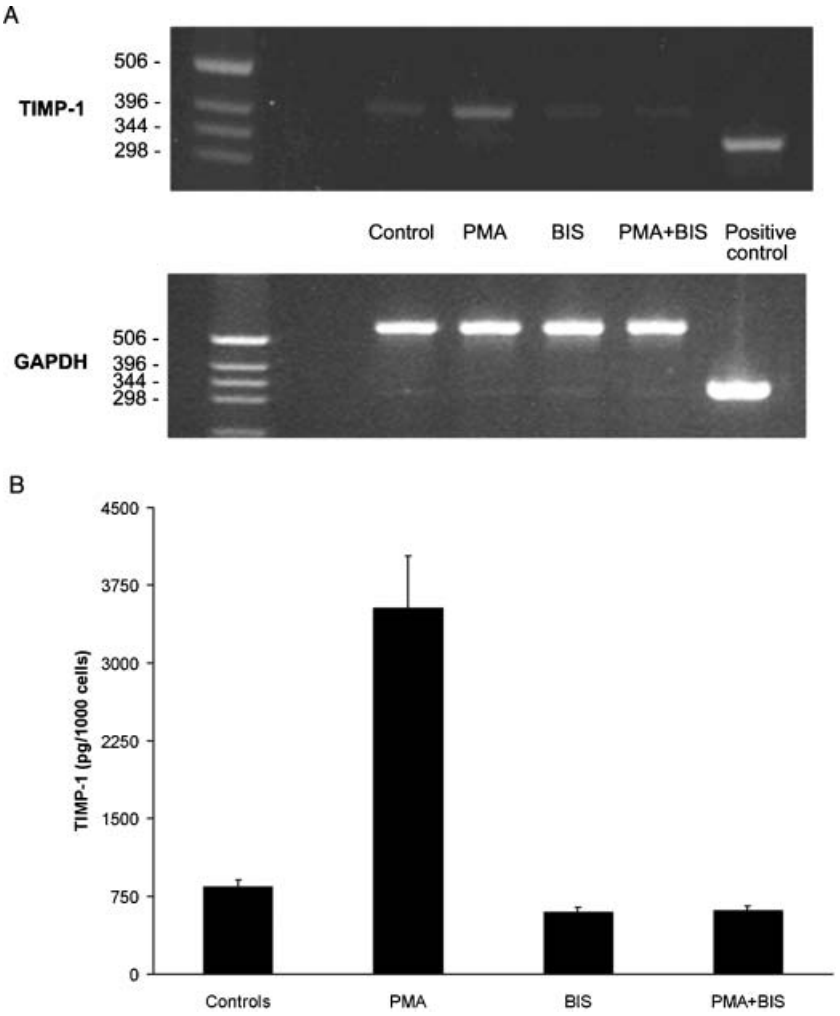

Fig. 3. Effect of bisindolylmaleimide (bisindolylmaleimide: 2.0 μM) on (A) tissue inhibitor of metalloproteinase-1 (TIMP-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression (3 h) and (B) TIMP-1 protein production (24 h) in the absence or presence of PMA (10.0 nM) in human gingival fibroblasts. Cell density was 1.8×10^4 cm^{-2} for mRNA expression and 1.5×10^4 cm^{-2} for protein production. Mean value of triplicates with SD shown by vertical bars.

pathways that regulate the production of MMP-1 and TIMP-1 in human gingival fibroblasts. We here report that the PKC activator PMA stimulates the expression of both MMP-1 and TIMP-1 in human gingival fibroblasts. In addition, MMP-1 production, in contrast to TIMP-1, is stimulated by the inflammatory mediator interleukin-1β and the Ca^{2+}-ionophore A23187, indicating that MMP-1 and TIMP-1 production is regulated differently in human gingival fibroblasts.

Our findings demonstrating that PMA stimulates MMP-1 and TIMP-1 production in human gingival fibroblasts are in line with findings reported in other cell types, including endothelial cells, corneal epithelial cells, and synovial and dermal fibroblasts (22–25). The fact that the PKC inhibitor bisindolylmaleimide abolished both MMP-1 and TIMP-1 mRNA and protein expression induced by PMA indicates that MMP-1, as well as its inhibitor TIMP-1, is mediated by a PKC-dependent pathway at both the transcriptional and the translational levels.

The pro-inflammatory cytokine IL-1β is reported to be involved in the inflammatory process because of its ability to stimulate the production of proteolytic enzymes such as MMP. In gingival fibroblasts, we found that IL-1β stimulated MMP-1 but not TIMP-1 production, indicating that MMP-1 and TIMP-1 production is regulated differently by interleukin-1β. Furthermore, interleukin-1β synergistically up-regulated MMP-1 production stimulated by PMA, whereas TIMP-1 production was unaffected. The synergistic up-regulation of MMP-1 by interleukin-1β and PMA may be explained by the fact that interleukin-1β, similar to PMA, also stimulates PKC in gingival fibroblasts (21). Interestingly, PKC, reported to induce inflammation (26), is an important signal transduction pathway also used by lipopolysaccharides from periodontopathogens (27). Thus, the involvement of PKC in the production of MMP-1 and TIMP-1 in gingival fibroblasts is interesting, since the use of PKC inhibitors as therapeutic agents in the treatment of periodontal diseases has been suggested (28).

Similar to interleukin-1β, the Ca^{2+}-ionophore A23187 synergistically up-regulated the stimulatory effect of PMA on MMP-1 production, indicating that calcium is involved in the regulation of MMP-1, probably by a PKC-mediated pathway. The involvement of calcium is in line with findings in dermal fibroblasts that the Ca^{2+}-ionophore A23187 modestly increases MMP-1 production stimulated by PMA (29). In contrast to MMP-1, TIMP-1 production induced by PMA was reduced in the presence of A23187, which may be because increased intracellular calcium down-regulates TIMP-1 (30, 31). Taken together, the different regulation of MMP-1 and its inhibitor TIMP-1 by gingival fibroblasts, in response to calcium and interleukin-1β, may disrupt the balance between MMP-1 and TIMP-1 and thereby contribute to pathological breakdown of extracellular matrix seen in periodontal diseases (2). This assumption is well compatible with the findings demonstrated by Soell *et al.* (8), showing an increased level of MMPs and a decreased level of TIMPs in periodonal tissue from periodontitis-affected patients.

To investigate the signal pathways involved in MMP-1 and TIMP-1 production induced by PMA, we studied

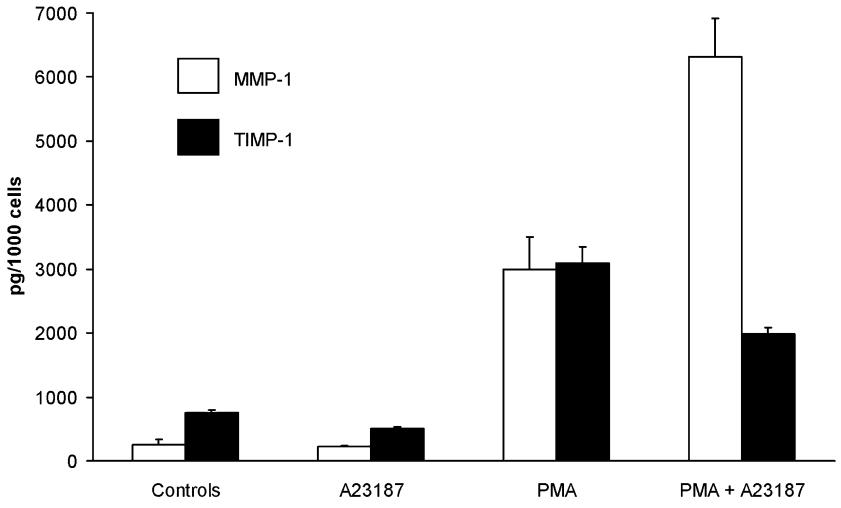

Fig. 4. Effect of the Ca^{2+}-ionophore A23187 (0.1 μM) on the production of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the presence or absence of phorbol 12-myristate 13-acetate (PMA: 10.0 nM) in 24-h cultures of human gingival fibroblasts. Cell density was 1.5×10^4 cm^{-2}. Mean value of triplicates with SD shown by vertical bars.

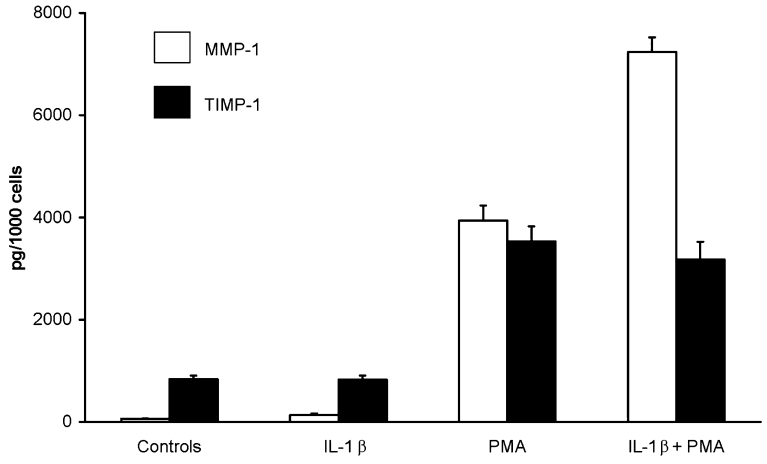

Fig. 5. Effect of interleukin-1β (IL-1β: 100 pg/ml) alone or in combination with phorbol 12-myristate 13-acetate (10.0 nM) on the production of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in 24-h cultures of human gingival fibroblasts. Cell density was 1.5×10^4 cm^{-2}. Mean value of triplicates with SD shown by vertical bars.

p38 MAPK and tyrosine kinase because they have been suggested to be involved in the PKC pathway (13–15). Interestingly, the p38 MAPK-inhibitor SB 203580 synergistically up-regulated the stimulatory effect of PMA on both MMP-1 and TIMP-1 production in gingival fibroblasts, which, to our knowledge, has not previously been demonstrated in any cell types. The stimulatory effect of SB 203580 may suggest that the p38 MAPK signal pathway is downstream of PKC in the regulation of MMP-1. The stimulatory effect of SB 203580 on TIMP-1 production, however, may be a result of feedback mechanisms, since it has been reported that TIMP-1 activates p38 MAPK (32).

The tyrosine kinase inhibitor herbimycin A reduced PMA-stimulated MMP-1 production but did not significantly affect TIMP-1 production, indicating that the involvement of the tyrosine kinase pathway in PMA-stimulated MMP-1 and TMP-1 production seems to be divergent in gingival fibroblasts. In dermal fibroblasts, MMP-1 production induced by PMA is also reduced by herbimycin A (33). The inhibitory effect of herbimycin A on MMP-1 production may be a result of blockage of transient increase in calcium (34).

In summary, our data demonstrate that interleukin-1β and calcium differently influence the production of MMP-1 and TIMP-1 in gingival fibroblasts and that this difference is markedly amplified in the presence of the PKC-activator PMA. Taken together, the discrepancy in the production of MMP-1 and TIMP-1 in the presence of mediators such as interleukin-1β and calcium is important and may con-

Table 1. The effects of herbimycin A (0.1 μM) and SB 203580 (10 μM) in the absence or presence of phorbol 12-myristate 13-acetate (PMA: 10.nM), on the production of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) (pg/1000 cells) in 24-h cultures of human gingival fibroblasts

	MMP-1			TIMP-1		
	–	+ herbimycin A	+ SB 203580	–	+ herbimycin A	+ SB 203580
Controls	163 ± 14	123 ± 5.4	144 ± 23	1126 ± 131	1039 ± 71	1285 ± 64
PMA	1873 ± 204	651 ± 57	2809 ± 102	3364 ± 416	3062 ± 30	6328 ± 506

Cell density was 1.5×10^4 cells/cm^2. Mean value of triplicates ± SD.

tribute to tissue destruction in periodontal diseases.

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