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Gingival fibroblasts grown from cyclosporin-treated patients show a reduced production of matrix metalloproteinase-1 (MMP-1) compared with normal gingival fibroblasts, and cyclosporin down-regulates the production of MMP-1 stimulated by pro-inflammatory cytokines

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Sukkar TZ, Thomason JM, Cawston TE, Lakey R, Jones D, Catterall J, Seymour RA. Gingival fibroblasts grown from cyclosporin-treated patients show a reduced production of matrix metalloproteinase-1 (MMP-1) compared with normal gingival fibroblasts, and cyclosporin downregulates the production of MMP-1 stimulated by pro-inflammatory cytokines. J Periodont Res 2007; 42: 580–588. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard

Background and Objective: Cyclosporin-induced gingival overgrowth arises from an alteration in collagen homeostasis and is enhanced by inflammatory changes in the gingival tissues. The aim of this study was to investigate the interaction among interleukin-1, oncostatin M, cyclosporin and nifedipine in promoting the up-regulation of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase by gingival fibroblasts.

Material and Methods: Fibroblast cultures ($n = 5$) were obtained from healthy controls and from patients with cyclosporin-induced gingival overgrowth, and cells were harvested between the fourth and ninth passages. Cells were stimulated with interleukin-1 and oncostatin M, alone or in combination, and with different concentrations of cyclosporin (0–2000 ng/mL) and nifedipine (0–200 ng/mL). MMP-1 and tissue inhibitor of metalloproteinase-1 production was determined using an enzyme-linked immunosorbent assay technique. A CyQuant cell proliferation assay was used to determine the DNA concentration in the sample.

Results: Fibroblasts obtained from patients with cyclosporin-induced gingival overgrowth produced significantly lower levels of MMP-1 than control fibroblasts ($p < 0.001$); tissue inhibitor of metalloproteinase-1 levels were significantly lower ($p < 0.05$), and the ratio of MMP-1 to tissue inhibitor of metalloproteinase-1 was reduced, in the conditioned medium of patients with cyclosporin-induced gingival overgrowth compared with controls. Interleukin-1 and oncostatin M produced a significant increase in the up-regulation of MMP-1, which was reversed when cyclosporin and nifedipine were added to the cell cultures ($p < 0.05$).

Conclusion: Pro-inflammatory cytokines significantly up-regulate MMP-1 in cultured gingival fibroblasts. Up-regulation is attenuated by both cyclosporin and nifedipine. The interaction may account for the synergism between inflammation and cyclosporin-induced gingival overgrowth.

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Cyclosporin is a cyclic polypeptide with potent immunosuppressive activity used widely to prevent graft rejection in organ transplant patients and also in the treatment of autoimmune disease (1). Unfortunately, this drug is associated with several unwanted effects. Of particular interest to the Periodontologist is the problem of gingival overgrowth. The precise mechanism of this unwanted effect remains uncertain. The main feature of drug-induced gingival overgrowth is characterized by an increase in the connective tissue matrix (2); hence, much of the research on the pathogenesis has focused on connective tissue homeostasis.

Previous studies have focused on increases in collagen or proteoglycan metabolism, altered responses of fibroblasts to cytokines and growth factors, and alterations in the levels of matrix-degrading proteinases (reviewed in Trackman & Kantarci) (3).

The synthesis of collagen can be up-regulated by a variety of growth factors, including transforming growth factor-β and connective tissue growth factor, and both transcriptional and post-transcriptional regulatory mechanisms are involved. Some studies have shown that cyclosporin can affect the intracellular degradation of newly synthesized collagen, whereas others have shown that cyclosporin alters the extracellular turnover of collagen (reviewed in Trackman & Kantarci) (3).

In a previous study (4), immunolocalization techniques were used to investigate if the level of matrix metalloproteinase (MMP)-1 and MMP-3 produced by fibroblasts in gingival tissue obtained from drug-free control patients and from patients treated with cyclosporin and with gingival overgrowth is down-regulated. Gingival tissue taken from drug-free control patients showed positive staining for MMP-1 by immunofluorescence and immunoperoxidase staining. However, significantly lower levels of MMP-1 were detected in gingival tissue obtained from patients with cyclosporin-induced gingival overgrowth. This suggested that cyclosporin inhibits the MMP-1 production in human gingival fibroblasts, leading to gingival overgrowth.

Subsequent studies have shown that cyclosporin reduces the levels of MMP-1 mRNA and of MMP-1 protein in both normal and overgrown tissue (5). Some studies have reported that cyclosporin has an effect on the levels of tissue inhibitor of metalloproteinase-1 (6), but others have reported no effect (5). It was suggested that the down-regulation of MMP-1 and the up-regulation of tissue inhibitor of metalloproteinase-1 was dependent on cyclosporin increasing the production of transforming growth factor-β (6). A later study (7) examined the effect of cyclosporin A on cultured gingival fibroblasts from normal tissue. An increased production of type 1 collagen and transforming growth factor-β was reported, but no significant effect on MMP-2, MMP-1 and tissue inhibitor of metalloproteinase-1 production was found. It was concluded that interindividual variations of collagenase levels could predispose some individuals to gingival overgrowth. A further investigation measured the total RNA and cellular proteins in cultured gingival fibroblasts in the presence of cyclosporin (8). Using reverse transcription–polymerase chain reaction techniques, the authors showed that cyclosporin suppressed the expression of MMP-1, tissue inhibitor of metalloproteinase-1 and cathepsin L. Similar effects were shown in both rat and humans on the levels of MMP-1, -2 and -3 using western blotting and zymography (9). Thus, whereas these studies showed a reduction in the levels of MMPs following cyclosporin A treatment, others showed no such effect (10).

The findings from these studies suggest that the effect of cyclosporin on MMP-1 production is equivocal.

Further studies have shown that both cyclosporin and nifedipine affect MMP-1 and tissue inhibitor of metalloproteinase-1 production *in vitro* (11–13), but the mechanism of action is not known. Drug-induced gingival overgrowth is exacerbated by plaque-induced gingival inflammation and/or any underlying periodontal disease (14). Such inflammation will lead to an increase in the production and release of a variety of cytokines, together with an up-regulation of their receptors. The presence of these cytokines will lead to alterations in the response of gingival fibroblasts and in matrix metabolism. Interleukin-1 is known to be present in inflammation and can up-regulate the levels of different matrix-degrading proteinases by a variety of

different fibroblasts, including gingival fibroblasts. It is recognized that cytokines in inflammatory sites do not act alone, but in combination. Studies have shown that both interleukin-1α and interleukin-17 up-regulate the levels of MMP-1 when added to cells in culture. Interleukin-1α, in combination with oncostatin M, induces a synergistic up-regulation of MMP-1 and this leads to a marked increase in collagen breakdown. Oncostatin M alone has been shown to increase collagen synthesis and can induce a marked cellular proliferation of fibroblasts. However, in combination with interleukin-1α, the two cytokines markedly up-regulate the MMPs that are known to degrade collagen. Transforming growth factor-β is known to down-regulate MMP-1 production (11).

In this study we propose to investigate whether there is an interaction between cytokines (oncostatin M and interleukin-1α), drugs (cyclosporin and nifedipine) and mediators of connective tissue homeostasis (MMP-1 and tissue inhibitor of metalloproteinase-1), which may contribute to the development of gingival overgrowth.

Our hypothesis was that there is an interaction between the drugs that cause gingival overgrowth and the mediators that control connective tissue homeostasis (MMP-1 and tissue inhibitor of metalloproteinase-1). We aimed, in this study, to investigate:

- the basal level of production of both MMP-1 and tissue inhibitor of metalloproteinase-1 from human gingival fibroblasts obtained from patients with cyclosporin-induced gingival overgrowth and untreated control patients, from whom gingival tissue was removed for other reasons;
- the effect of pro-inflammatory cytokines and growth factors on cultured human gingival fibroblasts taken from cyclosporin-induced gingival overgrowth patients and control patients with respect to the regulation of MMP-1 and tissue inhibitor of metalloproteinase-1 production; and
- the effect of treating human gingival fibroblasts, stimulated with and without interleukin-lα and interleukin-lα + oncostatin M, with different concentrations of cyclosporin or nifedipine on the levels of MMP-1 and tissue inhibitor of metalloproteinase-1.

Material and methods

Tissues for cell culture were obtained from patients attending the Department of Periodontology, Dental Hospital (Newcastle upon Tyne, UK). Informed written consent was obtained from each patient, and the protocol for this study was submitted to, and approved by, the Local Ethics Committee. Gingival tissue was obtained from five organ-transplant patients medicated with both cyclosporin and nifedipine who had undergone gingivectomy to correct their gingival overgrowth. Control gingival tissue was obtained from five drug-free subjects who had undergone crown-lengthening procedures.

Tissue preparation

Small pieces of gingival tissue were rinsed three times with biopsy medium (500 mL of Eagle's minimal essential medium with Earle's salts, supplemented with 20 mL of penicillin/streptomycin and 1 mL of fungizone; Life Technologies Ltd, Paisley, UK). The gingival samples were placed in a small Petri dish with 5 mL of biopsy medium and gently scraped with two sterile disposable scalpel blades to remove blood. The samples were rinsed with a further 5 mL of biopsy medium, then transferred into a fresh Petri dish. Each sample was cut in half and rinsed again before each half was transferred to new Petri dishes containing 0.5 mL of biopsy medium. Using fresh scalpel blades, the tissue was sliced into very fine pieces (≈ 0.5 mm thick). A further 0.5 mL of biopsy medium was added to each Petri dish. Medium and tissue fragments were drawn up using a 5-mL pipette and transferred to a 25-cm^2 flask (Corning/Costar UK Ltd, High Wycombe, UK). Each Petri dish was rinsed with a further 0.5 mL of biopsy medium and also transferred to the flask. The amount of biopsy medium in the flasks did not exceed 2.0 mL, to encourage the tissue fragments to adhere to the bottom of the flasks. The latter were placed in a 5% CO_2-humidified incubator at 37°C. After 24 h, the biopsy medium was withdrawn from the flasks and replaced with approximately 2 mL of growth medium (500 mL of Eagle's minimal essential medium with Earle's salts supplemented with 6 mL of L-glutamine, 11 mL of penicillin/streptomycin, 60 mL of foetal calf serum, 15 mL of kanamycin and 5 mL of gentamycin; Life Technologies Ltd). After 3–4 d, cultures were fed by withdrawing half of the medium and replacing with fresh growth medium. The feeding was repeated every 3–4 d. When fibroblasts were confluent around some of the tissue fragments, they were transferred to a 25-cm^2 flask and incubated with 0.5 mL of trypsin for 5 min. Cells between the fourth and ninth passages were used for this investigation.

Cytokine stimulation

Cells were seeded at a density of 30,000/cm^2 in 24-well plates and grown to subconfluency (50,000 cells/cm^2) in 1 mL of growth medium. Quadruplicate wells were used for each treatment in all experiments. The plates were incubated overnight at 37°C in 5% CO_2/humidified air to allow equilibration of the cells. The medium was removed from each well and replenished with 1 mL of serum-free medium (500 mL of Eagle's minimal essential medium with Earle's salts supplemented with 6 mL of L-glutamine, 11 mL of penicillin/streptomycin, 25 mL of kanamycin, and 5 mL of acid-treated foetal calf serum; Life Technologies Ltd). The plates were incubated for a further 24 h at 37°C. Serum-free medium was removed from each well and replaced with 1 mL of fresh serum-free medium as the control and with 1 mL of serum-free medium containing the appropriate cytokines. Cells were treated with interleukin-lα (1 μg/mL) (GlaxoSmithKline, Stevenage, UK), oncostatin M (10 μg/mL), interleukin-17 (50 ng/mL) or transforming growth factor-α (20 ng/mL), in serum-free medium. The concentrations were chosen as those giving optimum modulation of MMP-1 in other cell

cultures. For each condition, four wells were used. All cytokines with medium were filtered through a 0.2 μM filter (Pall Gelman Science, Northampton, UK). Plates were then incubated at 37°C for either 24 or 48 h. Supernatants were removed and stored at −20°C after each incubation period. Cell numbers at the end of each incubation period were quantified using the Cy Quant assay. Stock solutions of both cyclosporin (20 mg/mL) and nifedipine (2 mg/mL) were prepared by dissolving the drugs in ethanol. Each solution was then diluted with serum-free medium at a 1 : 10 dilution. The cells were stimulated, as described above, with different concentrations of cyclosporin (0, 500, 1000 and 2000 ng/mL) and nifedipine (0, 50, 100 and 200 ng/mL).

MMP-1 enzyme-linked immunosorbent assay

A double-antibody sandwich enzyme-linked immunosorbent assay technique was used to determine total MMP-1 in conditioned media from gingival fibroblasts (12). Maxisorb 96-well plates were coated with monoclonal antibody to human MMP-1 (RRU-CL1; 2 μg/mL in phosphate-buffered saline) and incubated overnight at 4°C. The plates were washed once with wash buffer and blocked with blocking buffer (200 μL/well) overnight and stored for up to 1 mo. When needed, plates were washed three times in wash buffer. Standard solutions of RRU-CL1 (5–50 ng/mL) in 100 μL were prepared by diluting the stock solution of standard in protein dilutent. Samples diluted in protein dilutent and standard were added to the plates in duplicate (100 μL/well) and incubated for 2 h at room temperature or at 4°C overnight. Plates were then washed three times with wash buffer and incubated for 2 h at room temperature with secondary antibody (nonbiotinylated poloyclonal anti-CL-1, stock concentration 1 mg/mL, diluted 1 : 4000 in protein dilutent, 100 μL/well). Following incubation, plates were again washed three times and incubated for 1 h at room temperature with tertiary antirabbit immunoglobulin horseradish peroxidase (1 mg/mL diluted 1 : 1000 in protein diluent; 100 μL/well). After washing, as described above, *o*-phenylenediamine substrate was applied across the plate at 10-s timed intervals and incubated for ≈ 5–10 min at room temperature. The reaction was ended by the addition of 3 M sulphuric acid (50 μL/well) at the same 10-s intervals. The plates were then read at 490 nm on a Victor 1420 multilabel counter. The concentration of MMP-1 in the samples was calculated from the standard curve. All samples were assayed in duplicate.

TIMP-1 ELISA

A double-antibody sandwich enzyme-linked immunosorbent assay technique was used to determine total tissue inhibitor of metalloproteinase-1 (free tissue inhibitor of metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 complexed to MMPs) (16) in conditioned media from gingival fibroblasts. Maxisorb 96-well plates were coated overnight at 4°C with monoclonal antibody to human tissue inhibitor of metalloproteinase-1 (RRU-T5) at a concentration of 5 μg/mL. The plates were washed once with wash buffer and blocked with blocking buffer (200 μL/well) overnight at 4°C and stored for up to 1 mo. When required, the plates were washed three times with wash buffer. Standard solutions of RRU-T5 (0–50 ng/mL) and samples were diluted in protein dilutent and added in duplicate (100 μL/well) to the 96-well plates for 2 h at room temperature. The plates were then washed three times with wash buffer, and secondary antibody (biotinylated RRU T1) was added (100 μL/well) and incubated for 2 h at room temperature. Again, the plates were washed, as described above, and incubated with streptavidin horseradish peroxidase (100 μL/well) for 30 min at room temperature. Subsequent procedures were identical to the method described for MMP-1. All samples were assayed in duplicate.

CyQuant cell proliferation assay

This assay was used to determine the amount of DNA in each well after each incubation period, and it was assumed to be proportional to the number of cells per well. The standards (0–1000 ng/mL of DNA concentration) were prepared by diluting 1 μg/mL of DNA in CyQUANT GR lysis buffer. The solution was then added to the Maxiosorb Nunc-immuno 96-well plate. The 24 wells were thawed at room temperature, and 200 μL of the CyQUANT GR dye/lysis buffer was added to each well and incubated for 205 min at room temperature. The content of each well was transferred to 96-well plates, which were read at 485 nm on a Victor 1420 multilabel counter. The DNA concentration in the samples was calculated from the standard curve and results were expressed as either ng/mL of MMP-1 or of tissue inhibitor of metalloproteinase-1 per μg of DNA.

Statistical analysis

Experiments to investigate the stimulation of MMP-1 and tissue inhibitor of metalloproteinase-1 with pro-inflammatory cytokines were performed using cells derived from five samples from cyclosporin-induced gingival overgrowth patients and five samples from control patients. Experiments to investigate the effect of cyclosporin and nifedepine on the levels of MMP-1 and tissue inhibitor of metalloproteinase-1 were performed using cells derived from three samples of both cyclosporin-induced gingival overgrowth and control patients. Differences between the different incubation conditions were assessed using a one-way analysis of variance with Bonferroni post hoc analysis. A p-value of < 0.05 was considered statistically significant.

Results

Basal production of MMP-1 and tissue inhibitor of metalloproteinase-1 from control gingival fibroblasts and those obtained from patients with cyclosporin-induced gingival overgrowth

The basal levels of MMP-1 and tissue inhibitor of metalloproteinase-1 were

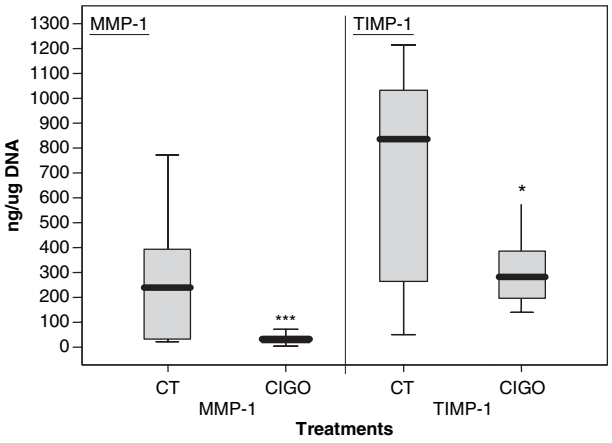

Fig. 1. Basal levels of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 production in control and cyclosporin-induced gingival overgrowth gingival fibroblasts. Gingival fibroblasts derived from either control or cyclosporin-induced gingival overgrowth tissues were cultured for 48 h and the levels of matrix metalloproteinase-1 were measured in the conditioned medium by enzyme-linked immunosorbent assay. CIGO, cyclosporin-induced gingival overgrowth; CT, control; MMP-1, matrix metalloproteinase-1; TIMP-1, tissue inhibitor of metalloproteinase-1. MMP-1, $p = 0.001$; tissue inhibitor of metalloproteinase-1, $p = 0.031$. *, $p < 0.05$; ***, $p < 0.001$.

measured in the conditioned medium derived from the 24-well plates at 24 h. Gingival fibroblasts derived from cyclosporin-induced gingival overgrowth patients produced much less MMP-1 than those derived from control patients. The mean levels were 31 ± 19 ng/mL/μg of DNA in cyclosporin-induced gingival overgrowth patients and 245 ± 216 ng/mL/μg of DNA in control patients (median values are illustrated in Fig. 1). The levels of tissue inhibitor of metalloproteinase-1 in the samples for cyclosporin-induced gingival overgrowth patients had mean levels of 310 ± 147 ng/mL/μg DNA compared with 758 ± 570 ng/mL/μg DNA for control patients (the median values are illustrated in Fig. 1). Measurements were made in duplicate on 20 samples. A much higher ratio of tissue inhibitor of metalloproteinase-1 to MMP-1 was measured in conditioned medium taken from cells derived from cyclosporin-induced gingival overgrowth patients (11–111 tissue inhibitor of metalloproteinase-1/MMP-1) compared with that derived from control patients (3.4–4.6 tissue inhibitor of metalloproteinase-1/MMP-1).

Effect of interleukin-1 and oncostatin M on MMP-1 and tissue inhibitor of metalloproteinase-1 up-regulation in control and cyclosporin-induced gingival overgrowth fibroblasts

Figure 2A illustrates the results at 24 h in cyclosporin-induced gingival overgrowth-derived cell preparations when cultured in the presence of cytokines known (in other cell types) to stimulate maximally the levels of MMP-1 (interleukin-1, 1 μg/mL; oncostatin M, 10 μg/mL; interleukin-17, 50 μg/mL) and tissue inhibitor of metalloproteinase-1 (oncostatin M, 10 μg/mL; transforming growth factor-β, 10 μg/mL). The concentrations used in the study were chosen to elicit the maximum response. Nonsignificant increases were seen when cells were treated with either interleukin-1 or oncostatin M alone. Oncostatin M, which can up-regulate both MMP-1 and tissue inhibitor of metalloproteinase-1 levels, had a consistent, but small, effect on increasing the levels of MMP-1 (Fig. 2A). A significant synergistic response ($p < 0.001$) was seen when interleukin-1 was combined with oncostatin M, and the mean levels of MMP-1 were increased to 120 ± 76 ng/mL. Neither transforming growth factor-β nor interleukin-17 significantly affected the levels of MMP-1 in these cells (Fig. 2A). Similar results were obtained when gingival fibroblasts, derived from control patients, were treated with these cytokine combinations (Fig. 2B). Although the basal levels of MMP-1 were raised in these

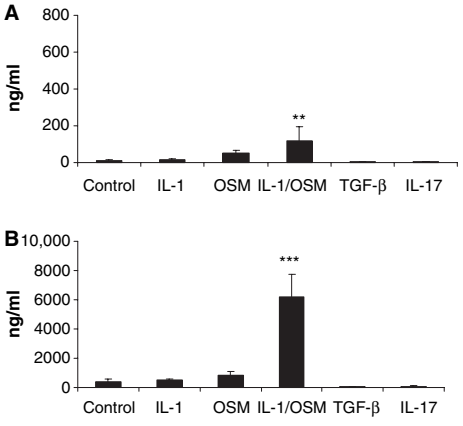

Fig. 2. The effect of cytokines on the levels of matrix metalloproteinase-1 in conditioned medium from gingival fibroblasts derived from control or cyclosporin-induced gingival overgrowth tissues. Gingival fibroblasts derived from either cyclosporin-induced gingival overgrowth (A) or control (B) tissues were cultured in medium for 24 h in the presence or absence of interleukin-1 (1 μg/mL), oncostatin M (10 μg/mL), interleukin-1 + oncostatin M (1 + 10 μg/mL), interleukin-17 (50 μg/mL) or transforming growth factor-β (20 μg/mL), and the levels of matrix metalloproteinase-1 were measured by enzyme-linked immunosorbent assay. IL, interleukin; OSM, oncostatin M; TGF-β, transforming growth factor-β. **, $p < 0.01$; ***, $p < 0.001$.

cells (shown in Fig. 1), the levels of MMP-1 were further increased by stimulation with the combination of the pro-inflammatory cytokines interleukin-1 and oncostatin M ($p < 0.001$) (Fig. 2B), to 6168 ± 1552 ng/mL. When cells were treated with transforming growth factor-β, the levels of MMP-1 were reduced below the basal levels in both cell populations (Fig. 2B). Interleukin-17, which can increase MMP-1 in some cell types (15), had no effect on the levels of MMP-1 in the control cells; these were not raised above those levels measured in control wells and this cytokine was not studied further. Identical results were obtained when the medium samples were analysed at 48 h. No consistent effect of oncostatin M was observed on the levels of tissue inhibitor of metalloproteinase-1 at 24 or 48 h.

These studies thus show that although the basal levels of MMP-1 produced by cyclosporin-induced gingival overgrowth and control fibroblasts were significantly different, both cyclosporin-induced gingival overgrowth and control fibroblasts responded to interleukin-1 and oncostatin M combinations, the most potent stimulant known, by significantly increasing MMP-1 production.

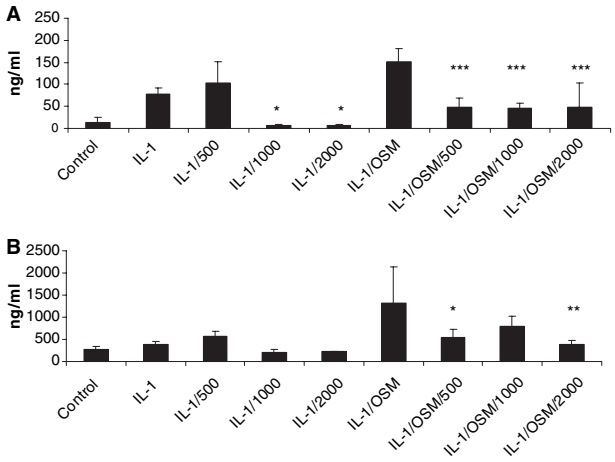

Fig. 3. The effect of cyclosporin on the levels of matrix metalloproteinase-1 in conditioned medium from gingival fibroblasts derived from cyclosporin-induced gingival overgrowth (A) and control (B) tissues. Gingival fibroblasts derived from either control or cyclosporin-induced gingival overgrowth tissues were cultured for 48 h, with and without cytokines, and in the presence and absence of cyclosporin (500–2000 ng/mL), and the levels of matrix metalloproteinase-1 were measured in the conditioned medium by enzyme-linked immunosorbent assay. IL, interleukin; OSM, oncostatin M. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Effect of cyclosporin on interleukin-1 and oncostatin M-induced up-regulation of MMP-1

Gingival fibroblasts were derived from tissue taken from both cyclosporin-induced gingival overgrowth and control patients, as described above. Cells were cultured in the presence and absence of interleukin-1 and interleukin-1 + oncostatin M, cyclosporin was added at 500, 1000 or 2000 ng/mL, and the conditioned medium was removed at 48 h. The levels of MMP-1 were measured in the conditioned medium. No significant reduction was observed in the levels of MMP-1 produced by unstimulated cells at any concentration of cyclosporin tested (data not shown). The results showing the effect of cyclosporin on MMP-1 levels in cyclosporin-induced gingival overgrowth cells following cytokine stimulation are shown in Fig. 3. When MMP-1 was stimulated with interleukin-1, no reduction was seen in the presence of cyclosporin at 500 ng/mL, but the levels were significantly reduced ($p < 0.05$) at the two higher concentrations of cyclosporin. Stimulation with interleukin-1 + oncostatin M increased the levels of MMP-1 above those with interleukin-1 alone, and these were reduced significantly ($p < 0.001$) by all three concentrations of cyclosporin. However, the levels of MMP-1 were not reduced to control levels, even at the highest concentration of cyclosporin. Similar trends were seen with control gingival fibroblasts, but these only reached statistical significance at two concentrations of cyclosporin in interleukin-1 + oncostatin M-treated fibroblasts.

No consistent increase in tissue inhibitor of metalloproteinase-1 levels were found when either cell type was stimulated with transforming growth factor-β or oncostatin M in the presence of cyclosporin (data not shown). When the data for all three experiments were combined, cyclosporin significantly reduced the levels of interleukin-1-stimulated MMP-1. Although the levels of MMP-1 were reduced in the interleukin-1 + oncostatin M-treated conditioned media in all three experiments, this did not reach significance. This was thought to be because large standard errors were obtained in one experiment. Interestingly, although in individual experiments a significant reduction in MMP-1 was seen following treatment with cyclosporin in control cells, overall, when the data were combined, this was not shown to be significant (see Fig. 3).

Effect of nifedipine on interleukin-1 and oncostatin M-induced up-regulation of MMP-1

Gingival fibroblasts were grown from tissue taken from both responders and healthy patients, as described above. Cells were cultured in the presence and absence of interleukin-1 and interleukin-1 + oncostatin M, nifedepine was added at 50, 100 or 200 ng/mL, and the conditioned medium was removed at 48 h. The levels of MMP-1 were measured in the conditioned medium. No significant reduction was observed in the levels of MMP-1 produced by unstimulated cells at any concentration of nifedepine tested (data not shown), except for the highest concentration of nifedepine in control cells where the control levels of 103.0 ± 79.0 were reduced to 31.6 ± 15.3 ($p < 0.01$; data not shown). No effect of nifedepine was

seen on tissue inhibitor of metalloproteinase-1 levels at any concentration, apart from cyclosporin-induced gingival overgrowth cells at the highest concentration of nifedepine where tissue inhibitor of metalloproteinase-1 levels were increased from 155.6 ± 66.5 to 351.0 ± 181 ($p < 0.01$; data not shown). When cyclosporin-induced gingival overgrowth cells were treated with interleukin-1 alone, no reduction in the level of MMP-1 was seen in the presence of nifedepine at 50 ng/mL; MMP-1 levels were reduced at both higher concentrations of nifedepine (Fig. 4), but this did not reach significance. Stimulation with interleukin-1 + oncostatin M increased the levels of MMP-1 above those with interleukin-1 alone, and these were reduced significantly ($p < 0.001$) by all three concentrations of nifedepine. However the levels of MMP-1 were not reduced to control levels, even at the highest concentration of nifedepine. Similar trends were seen with control gingival fibroblasts, although nifedepine only significantly reduced the levels of interleukin-1 stimulated MMP-1.

No consistent effect was seen on the levels of tissue inhibitor of metalloproteinase-1 when either cell type was stimulated with transforming growth factor-β or oncostatin M in the presence of nifedepine (data not shown).

Discussion

We have demonstrated that gingival fibroblasts derived from cyclosporin- and noncyclosporin-treated patients produce markedly different basal levels of MMP-1. Although both cell populations can respond to pro-inflammatory cytokines to up-regulate MMP-1 production, there is a significant difference in the tissue inhibitor of metalloproteinase-1 : MMP-1 ratio in the cyclosporin-induced gingival overgrowth gingival fibroblasts, which would ensure that much less collagen is degraded extracellularly; if the level of collagen synthesis remains unchanged, then the overall result would be deposition of collagen within the tissue, as seen in gingival overgrowth. A much higher ratio of tissue inhibitor of metalloproteinase-1 : MMP-1 was measured in conditioned medium taken from cells derived from cyclosporin-induced gingival overgrowth patients (Fig. 1) compared with that derived from control patients. This suggests that even after the cells have grown out from the tissue and passaged a number of times, they retain characteristics that are heavily in favour of preventing collagen turnover. These findings might offer an explanation for the accumulation of collagen within the overgrowth tissue following cyclosporin treatment.

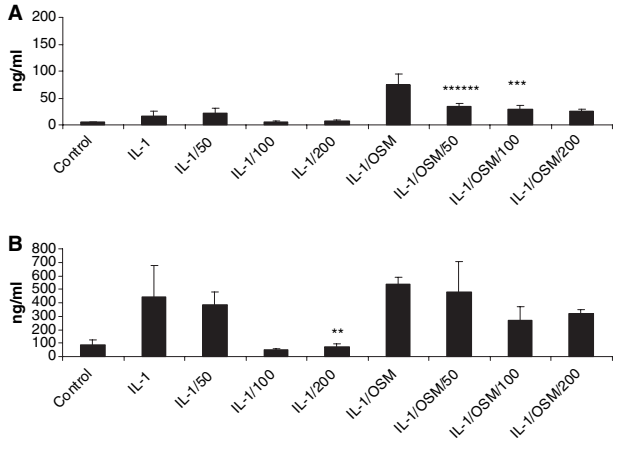

Fig. 4. The effect of nifedepine on the levels of matrix metalloproteinase-1 in conditioned medium from gingival fibroblasts derived from cyclosporin-induced gingival overgrowth (A) or control (B) tissues. Gingival fibroblasts derived from either control or cyclosporin-induced gingival overgrowth tissues were cultured for 48 h, with and without cytokines, and in the presence and absence of nifedepine (50–200 ng/mL), and the levels of matrix metalloproteinase-1 were measured in the conditioned medium by enzyme-linked immunosorbent assay.

We are aware that there are other collagenases (such as MMP-8, MMP-13, MMP-2 and MMP-14) which can also cleave collagen, and other tissue inhibitor of metalloproteinases (such as tissue inhibitor of metalloproteinase-2) which are also present, but the ratio of tissue inhibitor of metalloproteinase-1 to MMP-1 reported in this study explains the overgrowth effect and confirms a previous report which suggested that there was a trend for lower MMP-1 levels in cyclosporin-induced gingival overgrowth cells, although this did not reach significance (16).

In the present study, basal levels were only reduced in cyclosporin-induced gingival overgrowth cells at the highest concentration of cyclosporin with the medium alone. Previous studies have reported different results, with Bolzani *et al.* (9) and Yamada *et al.* (8) reporting reduced levels, but Tipton and coworkers (10) reported no effect. However, in our study there was a clear effect of cyclosporin on MMP-1 levels following stimulation with pro-inflammatory cytokines. It is known that cytokines delivered or regulated by inflammatory cells will interact with gingival fibroblast populations, stimulating the production of a plethora of inflammatory mediators, including MMPs and other proteinases. Cyclosporin is able to down-regulate MMP-1 and so prevent the turnover of collagen within this tissue and promote gingival overgrowth.

Little is known about the effect of nifedipine on gingival fibroblasts with respect to tissue inhibitor of metalloproteinase-1 and MMP-1 production. It has been reported that nifedipine increases collagen deposition by gingival fibroblasts (17). As with cyclosporin, we also showed that nifedipine attenuates MMP-1 and tissue inhibitor of metalloproteinase-1 levels in different populations of fibroblasts stimulated with interleukin-1α and oncostatin M. The mechanism by which nifedipine affects MMP-1 and tissue inhibitor of metalloproteinase-1 production is not well established. It is possible that nifedipine has a similar mechanism to cyclosporin because both drugs participate in preventing Ca^{2+} entry into cells (18,19).

It is interesting that patients medicated with either cyclosporin or nifedipine (or both) exhibit a variable gingival response, such that only 20–60% of patients suffer from this unpleasant side-effect. This variable response has previously been suggested to reflect fibroblast heterogeneity (10). These authors indicated that the effect of cyclosporin on fibroblast collagenase activity was dependent upon many factors, including the cell strain, the presence of appropriate cell subpopulations and the dose of the drug. Other groups have also shown that fibroblast strains derived from different subjects varied in response to cyclosporin, in terms of their synthesis of collagen and total protein production (20). This fibroblast heterogeneity could be an explanation that accounts for the different responses between gingival fibroblasts from controls and subjects with cyclosporin-induced gingival overgrowth.

It is known that the basal levels of MMP-1 and tissue inhibitor of metalloproteinase-1 produced by fibroblasts can vary widely between individuals. There are mutations in the promoter region of the MMP-1 gene that will increase the basal level of transcription, and it is probable that other mutations are present in both MMP-1 and tissue inhibitor of metalloproteinase-1 genes (21). It is possible that the effect of cyclosporin is only seen in those patients who produce low levels of MMP-1. When this is reduced during treatment with cyclosporin, then there is insufficient enzyme to maintain the low level of collagen turnover normally required to maintain connective tissue matrix homeostasis.

Cyclosporin is known to affect a number of different metabolic functions in a variety of cell types. Cyclosporin was reported to exhibit little stimulatory effects on the functions of fibroblasts (cell growth and matrix biosynthesis) obtained from healthy gingival tissues (22). More recently, Chae *et al.* (23) investigated the up-regulation of both interleukin-6 and transforming growth factor-β by cyclosporin, showed that these cytokines induced cell proliferation and concluded that this involved both mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways.

Many cytokines, such as interleukin-1, have been shown to increase MMP-1 gene expression (24,25), and up-regulation is dependent on a proximal activating protein-1 site in the MMP-1 promoter (19) This activating protein-1 site binds a homo or hetro dimer of the oncogene products (C-jun and C-fos), and the transcription of the MMP gene is initiated. However, it is also known that other signalling pathways are also involved and that the mechanism is complex. It has been shown that cyclosporin inhibits MMP-1 by also affecting the Jun N-terminal kinase pathway, although whether this is a direct or indirect effect is not known (26). Jun N-terminal kinase, a member of the mitogen-activated protein kinase family, can phosphorylate c-Jun, which causes an increase in activating protein-1 transcriptional activity, leading to an elevation of the MMP-1 gene expression (27). Other studies have shown that the transcriptional impact of many cytokines is mediated by activating intracellular signalling cascades, including protein kinase C (28), or mitogen-activated protein kinase pathways (29). The mechanism by which cyclosporin suppresses MMP-1 levels could be related to a drug-induced inhibition of Ca^{2+} into cells (19,20). Ca^{2+} activates protein kinase C, which plays a role in regulating the gene expression of MMPs by controlling the transcription factor, activating protein-1 (30–32). Thus, cyclosporin may suppress the high level of MMP-1, resulting from cytokine stimulation in gingival fibroblasts, by one of these mechanisms.

We can conclude, from the present study, that pro-inflammatory cytokines released into the gingival tissues as a consequence of plaque-induced inflammation significantly change the levels of MMP-1 and tissue inhibitor of metalloproteinase-1. Additionally, in cyclosporin-induced gingival overgrowth gingival fibroblasts, both cyclosporin and nifedipine suppress MMP-1 levels and so alter the tissue inhibitor of metalloproteinase-1 : MMP-1 ratio within the tissue. These findings suggest that the effect of cyclosporin and nifedipine is to reduce significantly the level of MMP-1 and so alter the ability of the fibroblasts to remove excess collagen. This action could account for the fibrotic or expanded connective tissue feature that characterizes both the clinical and histological appearances of drug-induced gingival overgrowth.

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