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ORIGINAL ARTICLE

Collagen degradation by interleukin-1β-stimulated gingival fibroblasts is accompanied by release and activation of multiple matrix metalloproteinases and cysteine proteinases

SW Cox[1], BM Eley[1], M Kiili[2], A Asikainen[2], T Tervahartiala[2], T Sorsa[2]

[1]*Department of Periodontology, Guy's, King's and St Thomas' Dental Institute, King's College London, UK;* [2]*Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital (HUCH); Institute of Dentistry, University of Helsinki; Orton Research Institute, Orthopaedic Hospital of Invalid Foundation, Helsinki, Finland*

OBJECTIVE: Several collagenolytic matrix metalloproteinases (MMPs) have recently been identified in gingival fibroblasts, while secreted cysteine proteinases could also participate in connective tissue destruction in periodontitis. To clarify their involvement, we examined enzyme release during collagen breakdown by cultured cytokine-stimulated fibroblasts.

MATERIALS AND METHODS: Gingival fibroblasts were derived from four chronic periodontitis patients and cultured on collagen gels in serum-free medium for 1–4 days. Collagenolysis was measured by hydroxyproline release into the medium. Proteinases were assessed by electrophoresis and immunoblotting.

RESULTS: Adding interleukin-1β resulted in progressive gel breakdown. This was associated particularly with a shift in MMP-1 band position from proenzyme to active enzyme and the appearance of active as well as proenzyme forms of cathepsin B. There was also partial processing of pro-MMP-13 and increased immunoreactivity for active cathepsin L. In addition, both pro-forms and active forms of MMP-8, membrane-type-1-MMP and MMP-2 were present in control and treated cultures.

CONCLUSIONS: Fibroblast MMP-1 was most likely responsible for collagen dissolution in the culture model, while cathepsin B may have been part of an activation pathway. All studied proteinases contribute to extracellular matrix destruction in inflamed gingival tissue, where they probably activate each other in proteolytic cascades.
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Correspondence: Dr SW Cox, Department of Periodontology, c/o Salivary Research Unit, GKT Dental Institute, Floor 17, Guy's Tower, Guy's Hospital, London, SE1 9RT, UK. Tel: +44 (0)20 7188 7459, Fax: +44 (0)20 7188 7458, E-mail: stephen.cox@kcl.ac.uk
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Introduction

Connective tissue destruction in periodontal diseases could potentially involve the action of several types of host-derived proteinase. Matrix metalloproteinases (MMPs), in particular, can be released initially from inflammatory cells recruited by bacterial plaque chemoattractants (Uitto *et al*, 2003), and then in the established lesion from resident gingival fibroblasts in response to cytokines derived from these infiltrating cells (Reynolds and Meikle, 1997). For fibroblasts, it was shown that interleukin (IL)-1 exposure *in vitro* increased both the activity and synthesis of interstitial collagenase-1 (MMP-1) by cultured gingival cells (Richards and Rutherford, 1990) and that stimulated cells degrade collagen films (Meikle *et al*, 1989).

More recently, it has been shown that gingival fibroblasts from periodontitis patients, either in tissue sections or in culture, can express collagenase-2 (MMP-8), collagenase-3 (MMP-13) and collagenolytic membrane-type 1 matrix metalloproteinase (MT1-MMP; MMP-14), with synthesis of MMP-8 and -13 by cultured cells increasing in response to proinflammatory cytokines (Sorsa *et al*, 1996; Uitto *et al*, 1998; Ravanti *et al*, 1999; Bolcato-Bellemin *et al*, 2000; Tervahartiala *et al*, 2000; Abe *et al*, 2001; Dahan *et al*, 2001). However, much of this study has been based solely on mRNA expression, and protein production of these additional MMPs has not always been confirmed.

Cysteine proteinases also have the capacity to degrade many extracellular matrix molecules, including some collagenous components, and although normally contained within lysosomes, they can be secreted from

cells (Dickinson, 2002). In tissue of periodontitis patients, cathepsin B and L have been localized in gingival fibroblasts and enzyme synthesis demonstrated in cultured cells (Kennett *et al*, 1994; Trabandt *et al*, 1995). In addition, we identified cathepsin B on collagen fibres adjacent to fibroblasts in inflamed gingival tissue (Kennett *et al*, 1997), while others have shown that cytokines can stimulate cathepsin B and L secretion by synovial fibroblasts (Huet *et al*, 1993; Lemaire *et al*, 1997).

To clarify the possible contributions of these various collagenolytic cathepsins and MMPs to substrate degradation by triggered gingival fibroblasts, we used Western blotting to examine their presence, molecular forms and degree of activation in culture media during gel degradation by IL-1β-stimulated cells when compared with untreated controls.

Materials and methods

Gingival fibroblasts

Gingival tissue was obtained from four chronic periodontitis patients (two male and two female, aged 44–64 years) undergoing routine surgery following an initial phase of therapy which included scaling and root planing. Ethical approval was obtained from King's College Hospital Research Ethics Committee and the patients gave informed consent. Tissue was collected, washed and diced in Hank's balanced salt solution with 100 units ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 0.25 $\mu g\ ml^{-1}$ amphotericin C. Tissues were transferred to 25-cm^2 flasks with minimum essential medium (MEM) containing 10% heat-inactivated foetal calf serum (FCS), 2 mM glutamine, 100 units ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. Tissue culture media (Gibco) and plasticware (Nunc) were from Invitrogen (Paisley, UK), unless stated otherwise. Explants were incubated at 37°C in a humidified atmosphere containing 5% CO_2 with regular changes of medium until fibroblast outgrowths were established. Cells were detached with 0.25% trypsin solution and transferred to new 25-cm^2 flasks with fresh medium. Subsequent subcultures were made with a split ratio of 1 : 3 and cells from passage numbers 5 to 8 were used in the experiments.

Cultures on collagen

Collagen gels were produced from an acidic stock solution of rat-tail collagen type I (Collaborative Biomedical Products, supplied by Stratech Scientific, Soham, UK). This was diluted to 1 mg ml^{-1} in ice-cooled phosphate-buffered saline containing sufficient 1 M sodium hydroxide for neutralization; 0.4-ml aliquots were added to 16-mm wells in a 24-well plate and incubated at 37°C for 30 min to generate gels 2-mm deep. Fibroblasts were seeded onto the gels at density of 125 000 cells per well and cultured for 3 days with 1 ml of MEM containing 10% FCS. They were then washed twice for 10 min with serum-free MEM supplemented with non-essential amino acids and insulin–transferrin–selenium (both from Sigma, Poole, UK), before adding 1 ml of this medium to each well. Human recombinant IL-1β (Biogenesis, Poole, UK) at a concentration of 1–20 ng ml^{-1} was included in test wells. After a further 1–4 days, media were collected and frozen at −20°C. All experiments were performed in triplicate.

Hydroxyproline assays

Soluble hydroxyproline in media from collagen cultures was determined by the method of Van der Zee *et al* (1996). 1.0 ml of absolute ethanol was added to 0.5 ml of medium, and the mixture cooled overnight at 4°C to precipitate protein. After centrifugation, the supernatant was collected and the precipitate washed with 0.5 ml of 70% ethanol. Combined supernatants and washings were vacuum dried in a rotary evaporator. Residues were redissolved in 0.5 ml water and 0.5 ml of 12 M hydrochloric acid added. Hydrolysis was carried out overnight in a sealed tube at 110°C. Acid was removed by drying under vacuum in a dessicator with solid sodium hydroxide. Resulting solids were redissolved in 0.5 ml of distilled water and colorimetric reactions conducted by incubation with 0.25 ml of chloramine T reagent for 20 min at room temperature and then 0.25 ml of 4-dimethyl-amino-benzaldehyde/perchloric acid reagent for 20 min at 60°C. Solutions were transferred to a microcuvette and absorbances read at 550 nm. A standard curve was constructed by adding known amounts of hydroxyproline to unused medium and carrying through the analytical procedure as for culture samples.

Electrophoresis and immunoblotting

Protein concentrations in culture media were measured by the Lowry method as modified by Peterson (1977), including the precipitation step to remove interfering components and using Sigma reagents. Prior to electrophoresis, media were freeze-dried and reconstituted at 2–8× concentrations. When loaded onto gels, 10–20 μl of prepared sample per lane contained 5–25 μg of protein for MMPs and 15–50 μg for cathepsin B and L.

The presence and molecular forms of MMP-1, -8, and -13 and MT1-MMP in the culture media were examined by Western immunoblotting as detailed earlier (Kiili *et al*, 2002). Briefly, sample proteins were separated by electrophoresis with 8% polyacrylamide gels containing sodium dodecyl sulphate (SDS-PAGE) under non-reducing conditions and then transferred electrophoretically to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). After use of gelatine to block non-specific binding, membranes were incubated with 0.8 $\mu g\ ml^{-1}$ mouse monoclonal anti-human MMP-1 (Ab-6; Oncogene Research Products, supplied by Merck KGaA, Darmstadt, Germany), 2.0 $\mu g\ ml^{-1}$ rabbit polyclonal anti-human MMP-8, IgG fraction (Hanemaaijer *et al*, 1997), 0.4 $\mu g\ ml^{-1}$ mouse monoclonal anti-human MMP-13 (Ab-1; Oncogene Research Products), or rabbit polyclonal anti-human MT1-MMP, affinity-purified IgG fraction (Biogenesis) diluted 1 : 500. Immunopositive bands were detected by incubation with sheep anti-mouse IgG or donkey anti-rabbit

IgG, each conjugated to horseradish peroxidase and diluted 1 : 800, followed by visualization with enhanced chemiluminescence (ECL) (secondary antibodies and reagents from Amersham Pharmacia Biotech, Buckinghamshire, UK).

Electrophoresis for MMP-2 and cysteine proteinases was conducted with pre-cast 4–12% Bis-Tris gels (Invitrogen) under reducing conditions. After blotting, membranes were treated initially with ProtoBlock (National Diagnostics, Hull, UK). They were then incubated with 0.5 μg ml^{-1} mouse monoclonal anti-human MMP-2 (Ab-8; Oncogene Research Products, supplied by Merck Biosciences, Nottingham, UK), 0.25 μg ml^{-1} mouse monoclonal anti-human cathepsin B (clone CA10; Oncogene Research Products), or 0.5 μg ml^{-1} mouse monoclonal anti-human cathepsin L (Serotec, Oxford, UK). Immunopositive bands were labelled with biotin-conjugated goat anti-mouse/rabbit immunoglobulins (Sigma) diluted 1 : 2000, followed by streptavidin and biotinylated horseradish peroxidase (Duet kit; Dako, Ely, UK). Band visualization was by ECL (Amersham Pharmacia Biotech).

Results

Collagen breakdown by fibroblasts

In initial experiments with one gingival fibroblast line on type I collagen, untreated cells were found to produce some visible but limited shrinkage of gels away from the edges of plate wells, with small amounts of hydroxyproline being released into the medium. Addition of IL-1β caused greater contraction of the gels and corresponding rises in soluble hydroxyproline levels. Both the speed and extent of these changes increased with IL-1β concentration and 20 ng ml^{-1} usually resulted in complete dissolution of the gels after 4 days (Figure 1).

Subsequent experiments were conducted with 10 ng ml^{-1} IL-1β and media were analysed at 3 days. Some variations in response were observed among the four cell lines and between individual cultures

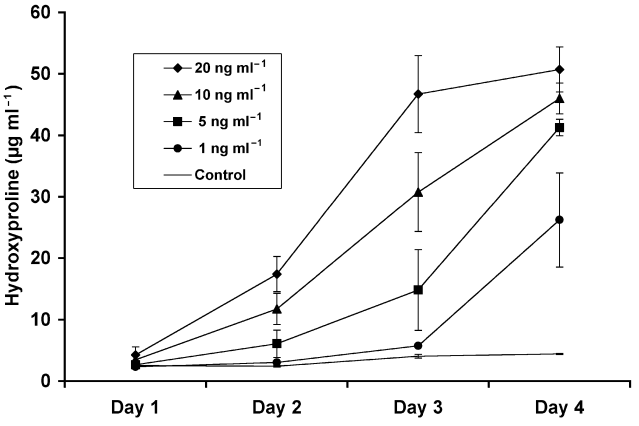

Figure 1 Release of hydroxyproline from collagen by a gingival fibroblast cell line treated with different concentrations of IL-1β over the course of 4 days. Data points and error bars represent the mean ± s.e.m. for three experiments

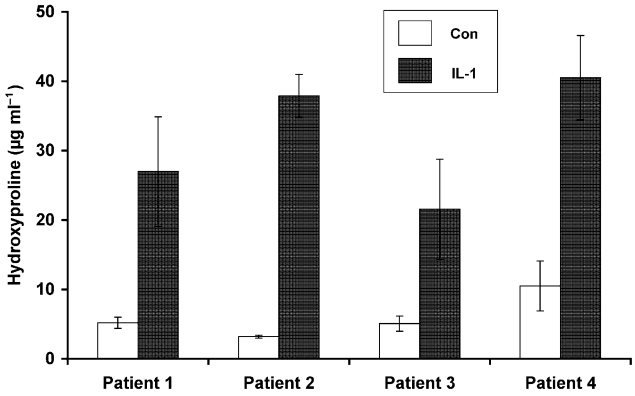

Figure 2 Release of hydroxyproline by four gingival fibroblast cell lines grown on collagen without (Con) and with 10 ng ml^{-1} IL-1β (L-1) for 3 days. Columns and error bars represent the mean ± s.e.m. for three experiments

(Figure 2), but there was a statistically significant difference between the hydroxyproline content of the medium from control cells of 6.0 ± 1.6 μg ml^{-1} (mean ± s.e.m.) when compared with 31.5 ± 5.3 μg ml^{-1} from stimulated cells ($P < 0.05$; paired *t*-test).

MMPs in culture media

In Western blots, MMP-1 was seen very largely as 55-kDa proenzyme in culture media from untreated cells (Figure 3a); after IL-1β stimulation, the great majority of staining was for 45–50 kDa active enzyme (Murphy *et al*, 1992). In both control and IL-1β cultures, MMP-8 appeared in strong 60–65-kDa, moderate 50-kDa and weak 45-kDa bands (Figure 3b). The first band most likely represented the pro-form of the less glycosylated mesenchymal-type enzyme, while the last two corresponded to active forms (Cole *et al*, 1996; Hanemaaijer *et al*, 1997; Kiili *et al*, 2002). High-molecular-weight (>100 kDa) immunoreactivities for MMP-8 probably reflected complexed, dimeric (Prikk *et al*, 2002), or membrane-shed (Owen *et al*, 2004) forms.

MMP-13 immunoblots usually gave only a 60-kDa proenzyme band, but in media from IL-1β-stimulated cultures a partially processed 55-kDa form (Knauper *et al*, 1996) was sometimes present as well (Figure 3c). MT1-MMP showed strong immunoreactivity at 60–65 kDa and also some at 50 kDa (Figure 3d), evidently representing proenzyme shed from the cell membrane and active soluble enzyme respectively (Imai *et al*, 1996; Kazes *et al*, 1998; Li *et al*, 1998; Toth *et al*, 2002); the latter was somewhat stronger after IL-1β stimulation. MMP-2 appeared as a strong, broad band at 60–70 kDa in both control and IL-1β media (Figure 3e), presumably corresponding to a mixture of active and proenzyme forms (Azzam and Thompson, 1992).

The active bands for MMP-1, MMP-8 and MT1-MMP and overall staining for MMP-2 all tended to be stronger in IL-1β cultures with greatest collagen degradation, while the intermediate form of MMP-13 was only seen in such cases.

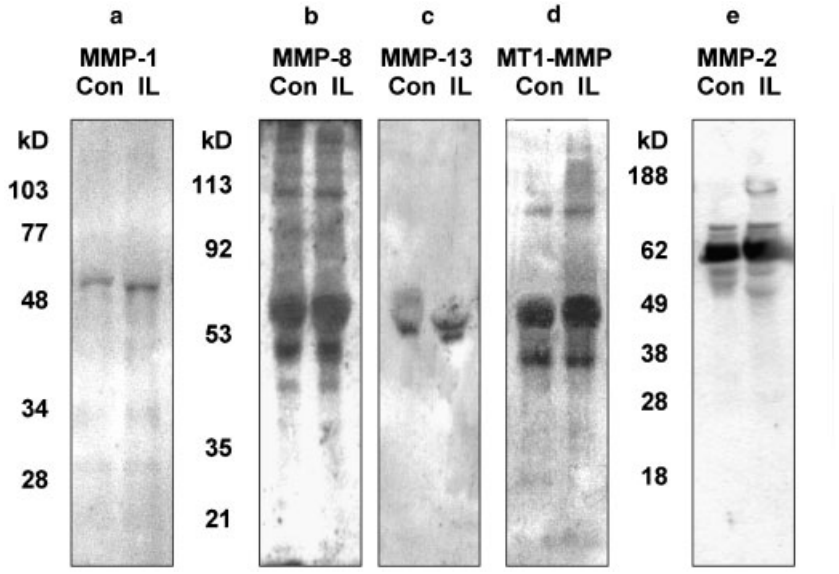

Figure 3 Western blotting for matrix metalloproteinases in 3-day media from control (Con) and IL-1β-stimulated (IL) gingival fibroblasts grown on collagen. (**a**) MMP-1 is seen as a 55-kDa proenzyme band and a 45–50-kDa active enzyme band in the untreated and IL-1β cultures respectively. (**b**) MMP-8 shows bands for 60–65-kDa proenzyme as well as 50- and 45-kDa active forms with both the control and stimulated cells. (**c**) MMP-13 is present as a 60-kDa proenzyme in the untreated culture and additionally as a 55-kDa intermediate form after IL-1β-stimulation. (**d**) MT1-MMP has a strong 60–65-kDa proenzyme and also 50-kDa active enzyme bands in both the control and IL-1β cultures. (**e**) MMP-2 appears as a strong 60–70-kDa band evidently representing a mixture of proenzyme and active forms with both the control and stimulated cells. Positions of molecular weight markers are indicated on the left

Cysteine proteinases in culture media

Immunoblots for cathepsin B showed mostly a 46-kDa proenzyme band in media from untreated cultures (Figure 4a); after IL-1β stimulation, 33-kDa single chain and 27-kDa heavy chain active forms (Hanewinkel *et al*, 1987) were more clearly present. Cathepsin L appeared as a very weak 43-kDa proenzyme band and more obvious 29-kDa single chain and 25-kDa heavy chain active bands (Mason *et al*, 1985; Reilly *et al*, 1989); the active forms were stronger with IL-1β (Figure 4b). For both cathepsins B and L, the relative strengths of the single and heavy chain bands varied from one experiment to another, but overall the association of these active forms with collagen degradation was similar to the MMPs.

Discussion

Media from the collagen cultures of gingival fibroblasts contained, in addition to MMP-1, various forms of other collagenolytic MMPs that were visualized by Western blotting. With cells grown earlier on plastic surfaces, we found a single, weak immunoreactive band for MMP-8 (Sorsa *et al*, 1996), while other studies have reported only mRNA expression for MMP-8 (Abe *et al*, 2001) and likewise for MT1-MMP (Bolcato-Bellemin *et al*, 2000). Here, we demonstrated that cells grown on collagen can release both the proenzyme and active forms of mesenchymal-type MMP-8 (Cole *et al*, 1996; Hanemaaijer *et al*, 1997; Kiili *et al*, 2002) and soluble MT1-MMP (Imai *et al*, 1996; Kazes *et al*, 1998; Li *et al*, 1998; Toth *et al*, 2002). Multiple forms of MMP-8, including a 110-kDa species, have recently been shown to be associated with the cell membrane of neutrophils (Owen *et al*, 2004), and so some of our high-molecular-weight immunoreactivity may have represented enzyme shed from the surface of fibroblasts. With IL-1β, MMP-13 was found not only as proenzyme as previously (Ravanti *et al*, 1999), but also sometimes as an intermediate in its activation process (Knauper *et al*, 1996). Furthermore, the MMP-8, MMP-13 and MT1-MMP species corresponded in part to bands seen in GCF from adult periodontitis patients (Tervahartiala *et al*, 2000; Kiili *et al*, 2002). Active MMP-2 was, as expected (Azzam and Thompson, 1992; Mäkelä *et al*, 1994), also present in the medium from collagen cultures.

Along with MMPs, gingival fibroblasts produced the cysteine proteinases cathepsin B and L. Previous studies have found that fibroblasts stimulated by pro-inflammatory cytokines (Huet *et al*, 1993; Lemaire *et al*, 1997) or grown on collagen (Koblinski *et al*, 2002) secrete cathepsin B and L as proenzymes. Our collagen cultures additionally released active forms of these enzymes (Mason *et al*, 1985; Hanewinkel *et al*, 1987; Reilly *et al*, 1989), especially after treatment with IL-1β. In the case of cathepsin B, this conversion may have reflected processing by urokinase- and tissue-type plasminogen activators (u-PA and t-PA; Dalet-Fumeron *et al*, 1993, 1996), of which t-PA is secreted in particularly high levels by gingival fibroblasts in response to IL-1 and collagen (Lorimier *et al*, 1996; Xiao *et al*, 1998).

Although immunoreactivities for active forms of nearly all metallo- and cysteine proteinases tended to be stronger in IL-1β cultures with greatest collagen degradation, processing of cathepsin B together with activation of MMP-1 were the enzyme conversions most regularly associated with gel dissolution. For MMP-1, our finding was consistent with an earlier report that degradation of collagen films by cytokine-stimulated gingival fibroblasts is linked to release of active collagenase (Meikle *et al*, 1989). Increased collagenase (MMP-1) gene expression and activation has additionally been shown for dermal fibroblasts grown in collagen gels (Mauch *et al*, 1989).

Active forms of MMP-8, MT1-MMP and MMP-2 were also present in both control and IL-1β-stimulated cultures of gingival fibroblasts and cytokine treatment sometimes resulted in partial processing of MMP-13.

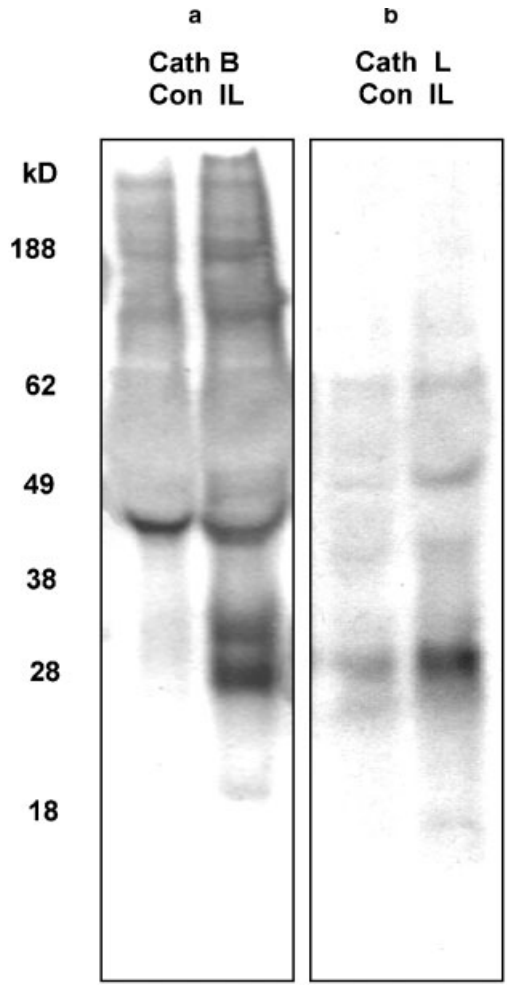

Figure 4 Western blotting for cysteine proteinases in 3-day media from control (Con) and IL-1β-stimulated (IL) gingival fibroblasts grown on collagen. (**a**) Cathepsin B appears mostly as a 47-kDa proenzyme band in the control culture with 33- and 27-kDa active enzyme bands clearly present in the IL-1β-stimulated culture. (**b**) Cathepsin L shows a very weak 43-kDa proenzyme immunoreactivity and 29- and 25-kDa active enzyme bands; the 29-kDa form in particular is stronger after IL-1β stimulation. Positions of molecular weight markers are indicated on the left

MMP-8 and MMP-13 are interstitial collagenases, while MMP-2 and MT1-MMP each possess collagenolytic ability (Aimes and Quigley, 1995; Ohuchi *et al*, 1997). Hence all these MMPs have the potential to contribute to extracellular matrix destruction in periodontal disease. MMP-2 and -13 (as well as MMP-1) have recently been linked to the disappearance of collagen fibres in cultured gingival explants (Ejeil *et al*, 2003) and active MMP-2 identified in periodontally diseased tissue (Korostoff *et al*, 2000). MMP-8, if present on the surface of gingival fibroblasts, could be rendered more stable and resistant to tissue inhibitor of metalloproteinase-1, like the potent, cell membrane-associated enzyme on neutrophils (Owen *et al*, 2004). At the same time, MMP-8 from triggered fibroblasts may possibly play an anti-inflammatory role (Owen *et al*, 2004), protecting against neutrophil-mediated tissue damage in periodontitis.

Cathepsin B can degrade collagen type I (Burleigh *et al*, 1974), while cathepsin L is more potent (Kirschke *et al*, 1982). Both cysteine proteinases, however, act primarily on terminal peptides in intermolecular cross-links (Burleigh *et al*, 1974; Kirschke *et al*, 1982) and while some may have been present in the reconstituted gels (Deshmukh and Nimni, 1972), they were probably not formed in physiological amounts (Siegel, 1976). The two enzymes are therefore unlikely to have been responsible for much collagen degradation in our cell culture system. The same consideration may explain why a cysteine proteinase inhibitor failed to prevent dissolution of dried collagen films by gingival fibroblasts, whereas MMP inactivators did block collagenolysis (Havemose-Poulsen *et al*, 1998).

Cathepsin B and L could, nevertheless, be effective *in vivo* where cross-links occur in mature collagen fibres and other susceptible matrix components are present. Indeed, we have seen cathepsin B on collagen around fibroblasts in inflamed gingival tissue (Kennett *et al*, 1997). Their involvement in soft tissue destruction in periodontitis is, furthermore, supported by the higher cathepsin B- and L-like activities found biochemically in diseased gingival tissue as compared to healthy controls (Eley and Cox, 1991).

Cathepsin B could also contribute to collagen degradation indirectly through activation of MMP-1 (Eeckhout and Vaes, 1977). This is not a very efficient route and the main physiological pathway for MMP-1 activation involves PAs and then plasmin (Murphy *et al*, 1992). However, no plasminogen would have been present in our serum-free cultures and it is possible that its place was taken by cathepsin B that had been processed by fibroblast-derived PAs (Dalet-Fumeron *et al*, 1993, 1996; Lorimier *et al*, 1996; Xiao *et al*, 1998). Conversely, there is some evidence that procathepsin B can be cleaved by active MMPs (Hara *et al*, 1988), while cathepsins B and L are able to process u-PA *in vitro* (Kobayashi *et al*, 1991; Goretzki *et al*, 1992).

Further possibilities centre on MT1-MMP, for which the active form in our culture media could have arisen through direct cleavage by fibroblast u-PA (Kazes *et al*, 1998; Xiao *et al*, 1998). MT1-MMP has an important role in processing pro-MMP-2 (Sato *et al*, 1994) and together MT1-MMP and MMP-2 can activate MMP-13 (Knauper *et al*, 1996). MT1-MMP can additionally cleave pro-MMP-8 (Holopainen *et al*, 2003). Several possible activation mechanisms could therefore operate in parallel, especially in the inflamed tissues where plasminogen is present.

To summarize, we have demonstrated that gingival fibroblasts grown with collagen and stimulated by the proinflammatory mediator IL-1β can produce a number of collagenolytic MMPs and cysteine proteinases. In addition to MMP-1, MMP-8, MMP-13, MT1-MMP and MMP-2 proteins were detected in active or partially processed forms in the media. It was also shown that gingival fibroblasts can release active forms of cathepsins B and L. The MMPs and cysteine proteinases can activate each other in various proteolytic cascades. They have all been identified in fibroblasts and many also in

inflammatory cells in diseased gingival tissue (Kennett *et al*, 1994, 1997; Trabandt *et al*, 1995; Sorsa *et al*, 1996; Uitto *et al*, 1998; Korostoff *et al*, 2000; Tervahartiala *et al*, 2000; Dahan *et al*, 2001) and may well participate in extracellular matrix degradation during periodontitis. MMP-8, as well as its destructive potential, may possibly have a defensive, anti-inflammatory role (Owen *et al*, 2004).

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