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Collagenase-3 (Matrix Metalloproteinase-13) Expression Is Induced in Oral Mucosal Epithelium during Chronic Inflammation

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Increased proliferation of mucosal epithelium during inflammation is associated with degradation of subepithelial connective tissue matrix and local invasion of the epithelial cells. Here we have studied, whether collagenase-3 (MMP-13), a collagenolytic matrix metalloproteinase with an exceptionally wide substrate specificity, is expressed in the epithelium of chronically inflamed mucosa. Examination of human gingival tissue sections from subjects with chronic adult periodontitis with *in situ* hybridization revealed marked expression of MMP-13 in basal cells of some epithelial rete ridges expanding into connective tissue. Immunohistochemical staining demonstrated that these cells also expressed strongly laminin-5, suggesting that they are actively migrating cells. A strong signal for MMP-13 mRNA was occasionally also noted in the suprabasal epithelial cells facing the gingival pocket, whereas no collagenase-1 (MMP-1) mRNA was detected in any areas of the epithelium. MMP-13 expression was also detected in fibroblast-like cells associated with collagen fibers of the inflamed subepithelial connective tissue. In organ culture of human oral mucosa, MMP-13 mRNA expression was observed in epithelial cells growing into connective tissue of the specimens. Regulation of MMP-13 expression was examined in cultured normal nonkeratinizing epithelial cells isolated from porcine periodontal ligament. In these cells, MMP-13 expression at the mRNA and protein level was potently enhanced (up to sixfold) by tumor necrosis factor-α, transforming growth factor-β_1, and transforming growth factor-α and by keratinocyte growth factor in the presence of heparin. In addition, plating periodontal ligament epithelial cells on type I collagen stimulated MMP-13 expression (sevenfold) as compared with cells grown on tissue culture plastic. The results of this study show, that expression of MMP-13 is specifically induced in undifferentiated epithelial cells during chronic inflammation due to exposure to cytokines and collagen. Thus, it is likely that MMP-13 expression is instrumental in the subepithelial collagenolysis and local invasion of the activated mucosal epithelium into the connective tissue. *(Am J Pathol 1998, 152:1489–1499)*

Chronic mucosal inflammation is characterized by increased proliferation and migration of epithelial cells associated with inflammatory cell infiltration and degradation of subepithelial connective tissue. Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases collectively capable of degrading essentially all components of the extracellular matrix are primarily responsible for remodeling and degradation of matrix in pathological conditions, such as rheumatoid arthritis, osteoarthritis, autoimmune blistering disorders of skin, dermal photo-ageing, and periodontitis as well as in tumor cell invasion and metastasis.[1,2] At present, the MMP gene family contains 17 members, which are divided into subgroups of collagenases, gelatinases, stromelysins, matrilysin, and membrane-type MMPs (MT-MMPs), according to substrate specificity and structure.[2] The members of the collagenase subfamily, collagenase-1 (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13) are the principal neutral proteinases capable of cleaving native fibrillar collagens in the extracellular space, and they apparently play a key role in the degradation of collagenous matrix.[1,2]

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Human periodontitis provides an excellent model for studies on epithelial cell behavior during chronic inflammation. Gingival connective tissue is lined by two distinct types of epithelium. The visible oral side of gingiva is covered by keratinized mucosal epithelium, whereas the epithelium facing the tooth (junctional or gingival pocket epithelium) is composed of loosely organized nonkeratinizing epithelium, which has a high turnover rate.[3,4] In periodontal inflammation, the gingival pocket epithelium proliferates extensively and grows into the periodontal connective tissue coinciding with extracellular matrix degradation and loss of tooth attachment.[4] It is likely that mucosal epithelial cells actively participate in the connective tissue destruction in this process, as they have the ability to produce several extracellular-matrix-degrading proteolytic enzymes, including collagenase-1 (MMP-1), 72-kd gelatinase (MMP-2), 92-kd gelatinase (MMP-9), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), and a chymotrypsin-like enzyme.[2,5–9] In addition, a collagenolytic enzyme has been detected in proliferating cultures of rat tongue epithelial cells.[10] We have also shown that proliferating mucosal epithelial cells effectively degrade fibrillar collagen in a tissue culture model.[5] Furthermore, inflamed gingival tissue has been found to contain increased collagenolytic activity.[11–13] Both neutrophil collagenase (MMP-8) and collagenase-1 (MMP-1) have been detected in periodontal tissue from patients with periodontitis.[12,15] However, no MMP-1 has been detected in the gingival epithelium by immunohistochemistry, although it is present in the subepithelial stroma in inflamed periodontal mucosa.[14,15]

In this study we have examined the role of collagenase-3 (MMP-13) in matrix degradation during chronic periodontal inflammation. The substrate specificity of MMP-13 is exceptionally wide as compared with other MMPs. In addition to fibrillar type I, II, and III collagens, it degrades type IV, IX, X, and XIV collagens, gelatin, tenascin-C, fibronectin, and proteoglycan core proteins.[16–18] The tissue-specific expression of MMP-13 in humans is limited and has so far been documented only in breast carcinoma tissue,[19] osteoarthritic cartilage,[18,20] rheumatoid synovium,[21] and developing bone.[22,23] Our recent observations show that MMP-13 is expressed in squamous cell carcinomas (SCCs) of the skin, oral cavity, and larynx, mainly by tumor cells in invading margin of the tumor but in some cases also by stromal cells,[24,25] whereas no MMP-13 expression is noted in intact or re-epithelializing epidermis, healthy oral mucosa, or normal keratinocytes in culture.[25–27] In this study we demonstrate that during chronic inflammation MMP-13 mRNA is expressed in gingival pocket epithelium that invades the underlying connective tissue. Furthermore, we show that MMP-13 expression can be induced in normal undifferentiated epithelial cells by growth factors and cytokines present at the site of inflammation and by contact of these cells with collagen. These results suggest an important role for MMP-13 in the degradation of collagenous matrix in chronically inflamed mucosa.

Materials and Methods

Cell Cultures

Porcine periodontal ligament epithelial (PLE) cells were isolated from the rests of Malassez as previously described[28] and cultured in α-minimal essential medium (α-MEM; StemCell Technologies, Vancouver, Canada) supplemented with 15% fetal calf serum (FCS; Flow Laboratories, McLean, VA), 100 IU/ml penicillin G, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B (Gibco, Grand Island, NY). Cells were allowed to grow to approximately 75% confluence and then maintained in the absence of FCS for 48 hours. Thereafter, medium was changed to contain 0.1% FCS, and the cells were incubated with various test substances for 24 hours. In the experiment, in which the effects of keratinocyte growth factor (KGF) and heparin were studied (Figure 5), the PLE cells were cultured on Transwell polycarbonate membranes (Costar, Cambridge, MA) as described previously,[29] as in this culture system the cells respond more potently to KGF than on tissue culture dishes.[6] For studies on the effect of collagen on MMP-13 expression, culture plates were coated with type I collagen as suggested by the manufacturer (Cellon, Strassen, France); collagen was allowed to dry without neutralization, and the plates were washed three times with α-MEM before plating the cells.

Mucosal Explant Culture

Normal masticatory mucosa was obtained from palatinum of a subject (age 14) undergoing an operative liberation of an unerupted maxillary canine for orthodontic reasons. The tissue was cut perpendicularly to the oral epithelium into 1 × 1 × 2 mm pieces that were placed on decalcified dentin matrix so that initially epithelium and connective tissue were in contact with the substratum. The mucosal samples were cultured for 6 days in a Trowell-type tissue culture system, using Eagle's minimal essential medium containing Earle's balanced salt solution, L-glutamine (2 mmol/L), sodium bicarbonate (850 mg/L), streptomycin sulfate (100 μg/ml), penicillin G (100 IU/ml), HEPES buffer (20 mmol/L), and 10% FCS (Flow Laboratories), as described earlier.[5] The collagenous substratum was prepared by cutting extracted human teeth into 200-μm sections with a diamond saw. The sections were then decalcified in 0.5 mol/L HCl at 25°C for 72 hours. Sections of formalin-fixed, paraffin-embedded specimens were processed for *in situ* hybridization analysis.

Cytokines and Growth Factors

Human tumor necrosis factor (TNF)-α was a gift from Dr. Walter Fiers (University of Gent, Belgium). Bovine transforming growth factor (TGF)-β1 was kindly provided by Dr. David R. Olsen (Celtrix Co., Santa Clara, CA). Keratinocyte growth factor (KGF) was from PeproTech EC (Rocky Hill, NJ). Platelet-derived growth factor AB (PDGF) and TGF-α were from Upstate Biotechnology

(Lake Placid, NY), and heparin was from Sigma Chemical Co. (St. Louis, MO).

mRNA Analysis

Total cellular RNA was isolated from cell cultures using the single-step method.[30] Northern blot hybridizations were performed as described previously[31] with cDNAs labeled with [α-^{32}P]dCTP by random priming. Three human MMP-13 cDNA fragments specific for coding and 3′-untranslated region were isolated form plasmids pMMP13HT1, pMMP13HT2, and pMMP13HT3.[25] In addition, a 2.0-kb human cDNA for human MMP-1,[32] a 1.5-kb human cDNA for stromelysin-1 (MMP-3),[33] and a 1.3-kb rat cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[34] were used for Northern blot hybridizations. [^{32}P]cDNA/mRNA hybrids were visualized by autoradiography, and the mRNA levels were quantitated by optical densitometry (Image 1.59, NIH).

In Situ *Hybridization*

Human gingival tissue composed of chronically inflamed connective tissue and proliferating pocket epithelium was obtained from routine periodontal flap surgery of patients with advanced adult-type periodontitis at the Dental Clinic of the University of Turku, Finland. The specimens were obtained with the informed consent of the subjects, and the research was carried out according to the provisions of the Declaration of Helsinki. The patients had undergone conventional periodontal therapy involving scaling of the root surfaces and oral hygiene procedures before surgery.

Sections of formalin-fixed, paraffin-embedded specimens (n = 12) were processed for *in situ* hybridization analysis. *In vitro* transcribed antisense and sense RNA probes were labeled with [α-^{35}S]UTP as described previously.[35] pMMP13HT1 plasmid was linearized within the multiple cloning site with *Xho*I or *Kpn*I to transcribe antisense and sense RNAs, respectively. In addition, a 550-bp *Eco*RV-*Sma*I fragment from the 5′ end of human MMP-1 cDNA[32] was used. The specificity of these probes for the corresponding mRNAs have been shown previously.[22,24–26] Sections were hybridized with probes (2.5×10^4 to 4×10^4 cpm/μl of hybridization buffer) and washed under stringent conditions, including treatment with RNAse, as described previously.[8] After autoradiography for 25 to 35 days, the photographic emulsion was developed and the slides were stained with hematoxylin and eosin. Samples of breast carcinomas known to express MMP-13 mRNA[19] were used as positive controls, and a labeled sense probe was used as a negative control in each experiment.

Immunohistochemistry

Immunostaining for laminin-5 was done on sections adjacent to those used for *in situ* hybridization. The peroxidase/anti-peroxidase technique with diaminobenzidine as chromogenic substrate and Harris hematoxylin as counterstain was used as described earlier.[8] The laminin-5 polyclonal antibody kindly provided by Dr. Karl Tryggvason, Karolinska Institut, Stockholm, Sweden, was used in 1:500 dilution.

Gelatin Zymography and Collagenase Assay

For zymography of gelatinolytic MMPs, conditioned medium samples were subjected to discontinuous SDS-polyacrylamide gel electrophoresis[36] using 7.5% gels containing 1 mg/ml gelatin (G-6650, Sigma). After completion of electrophoresis, the gels were washed twice in 50 mmol/L Tris, 0.02% NaN_3, and 2.5% Triton X-100 buffer (pH 7.5). The second wash was supplemented with 5 mmol/L $CaCl_2$ and 1 μmol/L $ZnCl_2$. The incubation buffer consisted of 50 mmol/L Tris, 0.02% NaN_3, 5 mmol/L $CaCl_2$, and 1 μmol/L $ZnCl_2$ (pH 7.5). After incubation for 20 hours at 37°C, the gels were fixed and stained with 0.2% Coomassie Blue R-250 in 40% methanol and 10% acetic acid and subsequently destained and stored in 7% acetic acid. The gels were photographed using a digital camera, and the negatively stained gelatinolytic bands were analyzed by optical densitometry.

For collagenase assay, aliquots of the culture medium were first incubated with 1 μg/ml trypsin (Sigma) for 1 hour at 37°C to activate the latent collagenase and then for 15 minutes with 10 μg/ml soybean trypsin inhibitor (Sigma). The samples were incubated with 20,000 dpm ^{3}H-labeled soluble type I collagen for 24 hours at 25°C.[11] Thereafter, the samples were heated in the presence of Laemmli's sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The fluorography technique used to visualize the radioactive collagen polypeptides was performed as described earlier.[37] Degradation of the collagen was analyzed by optical densitometry.

Western Blot Analysis

Proteins of the conditioned media were fractionated on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were blocked overnight at room temperature in 5% (w/v) milk powder/0.1% Tween in PBS. They were subsequently incubated for 1 hour with antiserum against recombinant human MMP-13 (1:1000 dilution)[19] or with an antiserum against human MMP-1 (kindly provided by Dr. Henning Birkedal-Hansen, National Institute of Dental Research, Bethesda, MD) in milk/0.1% Tween/PBS. The membranes were washed once for 15 minutes and twice for 5 minutes with 0.1% Tween/PBS, and the bound antibodies were detected using enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK).

Results

Connective Tissue Invading Epithelial Cells Express MMP-13

The principal secreted neutral proteinases responsible for turnover of the collagenous extracellular matrix are

collagenase-1 (MMP-1), neutrophil-derived collagenase (MMP-8), and the more recently discovered collagenase-3 (MMP-13). Of these, MMP-13 shows the widest substrate specificity and a restricted tissue-specific expression pattern. As chronic inflammation of human gingival tissue is characterized by extensive degradation of collagenous matrix we wanted to examine the expression of MMP-1 and MMP-13 in inflamed gingival tissue specimens. In all of these samples, the epithelium forming the lining of gingival pocket had typically proliferated into the underlying connective tissue in the form of a network of finger-like projections. The connective tissue below the epithelium showed extensive loss of collagen as well as distinct areas with dense infiltrates dominated by either plasma cells and lymphocytes or by mononuclear and polymorphonuclear leukocytes. Using *in situ* hybridization, we detected significant expression of MMP-13 mRNA in 5 of 12 inflamed gingival tissue specimens, specifically in the basal cells of some epithelial projections extending into the adjacent connective tissue stroma (Figure 1, A, B, and D). Suprabasal cells of the nonkeratinized epithelium facing the gingival pocket also showed a clear signal for MMP-13 in some areas (Figure 1, E and F). Adjacent areas of epithelium with similar histology were occasionally positive for MMP-13 mRNA, indicating that the expression of MMP-13 is not uniformly distributed but takes place sporadically and apparently under a specific local control.

Signal for MMP-13 mRNA was observed in areas bordering inflamed connective tissue consisting primarily of polymorphonuclear leukocytes and macrophages, whereas the epithelium facing a dense infiltration of lymphocytes and plasma cells did not show the MMP-13 expression. In accordance with our recent observations, no MMP-13 mRNA was observed in keratinocytes of the oral gingival epithelium, although various types of inflammatory cells were found in their vicinity (not shown).

It has been recently shown that tissue invading cancer cells in the migrating front of some human tumors express laminin-5, which is probably an important adhesion protein involved in the motility of the cancer cells.[24,39] We examined therefore whether the MMP-13-expressing epithelial cells of the inflamed gingiva also express laminin-5. In immunohistochemical staining of the adjacent sections, a marked correlation was observed in the expression of MMP-13 and laminin-5. The MMP-13-expressing cells always showed strong cytoplasmic immunostaining for laminin-5 (Figure 1C), whereas weak staining for laminin-5 was found in areas that did not express MMP-13. These findings therefore suggest that the epithelial cells expressing MMP-13 are actively moving into the inflamed connective tissue stroma.

In some tissue sections, cells expressing high levels of MMP-13 mRNA were also detected in the inflamed subepithelial connective tissue (Figure 2). These cells showed either typical fibroblastic morphology or an appearance of macrophages or activated fibroblasts (Figure 2). Interestingly, no collagenase-1 (MMP-1) mRNA was observed in the gingival epithelium, whereas it was occasionally noted in the inflamed connective tissue in fibroblast-like cells different from those expressing MMP-13 (not shown). Expression of MMP-13 in both epithelium and collagen fibril-associated cells strongly suggests an important role for this collagenolytic MMP in the degradation of mucosal connective tissue during chronic periodontal inflammation.

MMP-13 Is Expressed by Undifferentiated Epithelial Cells in Cultured Mucosal Explants

As MMP-13 expression was observed in the unkeratinizing pocket epithelium but not in keratinocytes of the oral epithelium of gingiva, we examined whether the differentiation status is a key factor for priming the epithelial cells for MMP-13 expression. Explants of uninflamed oral mucosa were cultured so that both epithelium and connective tissue were in contact with a collagenous matrix. We have previously demonstrated that the epithelial cells growing between the matrix and the mucosal connective tissue proliferate and express cytokeratin 19, a marker for basal epithelial cells uncommitted for terminal differentiation.[5,43] *In situ* hybridizations of the mucosal explant tissue sections showed expression of MMP-13 mRNA only in the epithelial cells growing into the connective tissue of the explants (Figure 3). These epithelial cells, similar to the MMP-13-positive cells in the gingival sections, were also positive for laminin-5 (not shown). These results, combined with the MMP-13 expression pattern in inflamed gingiva, suggest that MMP-13 expression is related to a specific state of the epithelial cell activation and that the vicinity of inflammatory cells is not required for induction of MMP-13 expression by periodontal epithelial cells.

Growth Factor Control of MMP-13 in Epithelial Cells

To identify factors responsible for stimulating MMP-13 expression in the epithelium we examined the effect of important cytokines and growth factors present in chronic inflammation. For these studies we used normal nonkeratinizing epithelial cells isolated from porcine periodontal ligament. These cells share a cytokeratin profile with the gingival pocket or junctional epithelial cells *in vivo*.[29] Culturing of these cells for 24 hours with TNF-α, TGF-β, PDGF, or KGF in the presence of heparin increased the MMP-13 mRNA signal by 5.6-, 3.4-, 1.3-, and 2.5-fold, respectively (Figure 4A). In contrast to human cells, in which three distinct transcripts of 2.0, 2.5, and 3.0 kb are detected,[19,25,26] only a single MMP-13 mRNA transcript with a size of 2.0 kb was detected in porcine cells, indicating the presence of a single polyadenylation site in the porcine MMP-13 mRNA. No stromelysin-1 (MMP-3) mRNA was detected after any of the treatments (not shown).

As MMP-13 exerts a 50-fold stronger gelatinase activity compared with MMP-1 and MMP-8,[16,18] we examined whether gelatin zymography could be used to estimate the MMP-13 production by PLE cells. As shown in Figure 4B, zymography of the conditioned medium from cultures

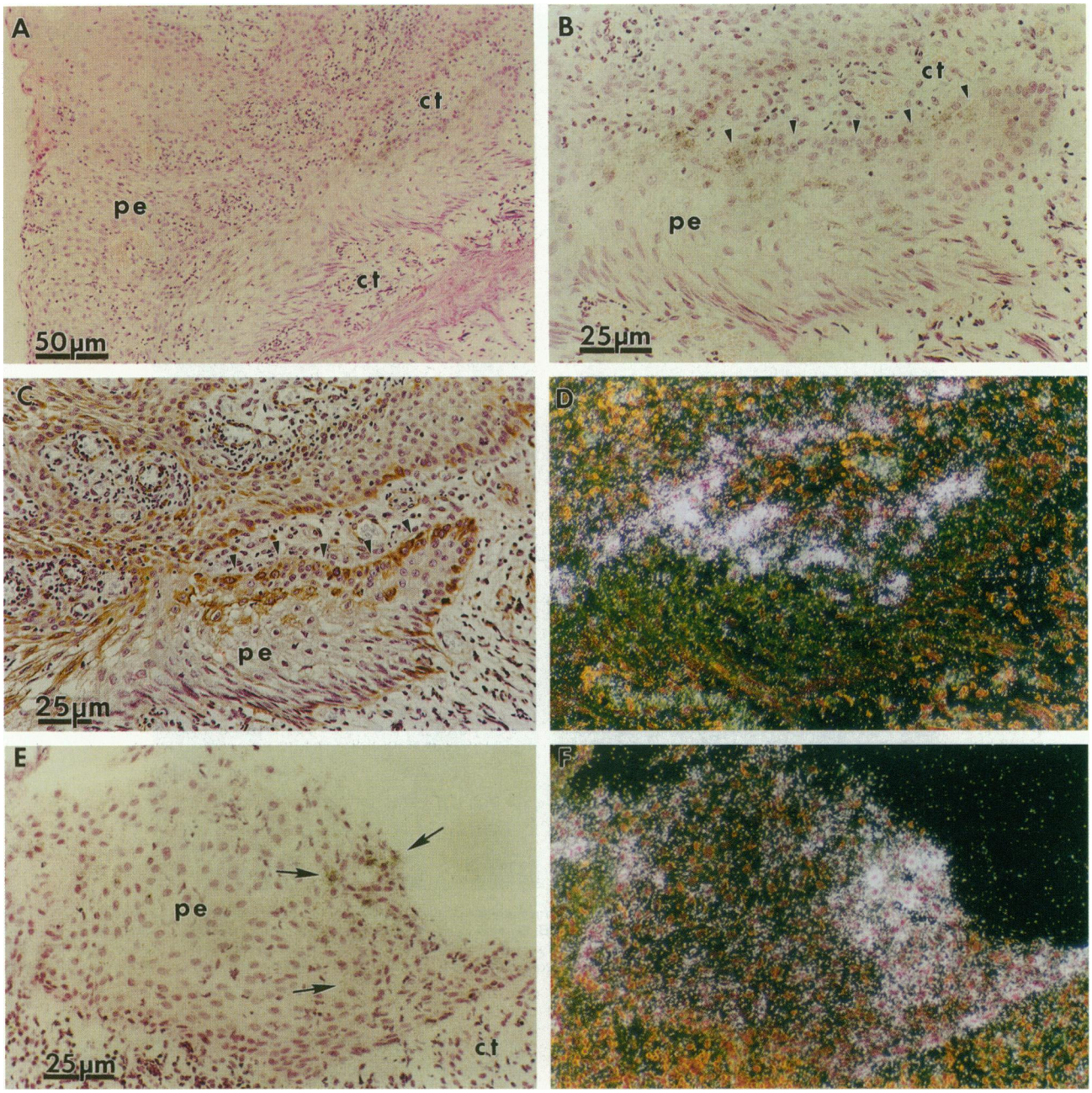

Figure 1. Expression of MMP-13 and laminin-5 in chronically inflamed human periodontium. **A:** Bright-field image of an *in situ* hybridization of gingival tissue showing extensive proliferation of pocket epithelium (pe) and its growth into the adjacent infiltrated connective tissue (ct). **B** and **D:** Bright-field image (**B**) and dark-field image (**D**) of the *in situ* hybridization of **A** at a higher magnification revealing that MMP-13 is expressed in basal cells bordering the epithelium extending into the connective tissue (**arrowheads**). **C:** Immunohistochemical staining demonstrating that laminin-5 is strongly expressed by the cells positive for MMP-13. **E** and **F:** Bright-field and dark-field images of gingival pocket epithelium showing intense MMP-13 signals in both basal and suprabasal (**arrow**) epithelial cells adjacent to the gingival pocket space.

treated with the growth factors for 24 hours showed two gelatinase bands at 92 kd (MMP-9) and 72 kd (MMP-2). In addition, in the medium of cells treated with TNF-α, TGF-β, and a combination of KGF and heparin, a 58-kd gelatinolytic band was detected (Figure 4B). The molecular weight of this gelatinolytic band corresponds to that of the latent form of human MMP-13.[16] Western blot analysis of the same medium samples using an antibody specific for human MMP-13 showed that the amount of 58-kd pro-MMP-13 was enhanced by TNF-α, TGF-β, and KGF plus heparin and correlated well with the levels of 58-kd gelatinolytic proteinase in the same samples (Figure 4C). Analysis of the same samples with an antibody against human MMP-1 revealed low levels of two distinct bands with approximate molecular weights of 52 and 57 kd, corresponding to human MMP-1 (Figure 4C). The levels of MMP-1 were slightly enhanced by TNF-α, TGF-β, and PDGF. The total collagenolytic activity measured as cleavage of soluble radioactively labeled type I collagen was also elevated in medium from cells treated

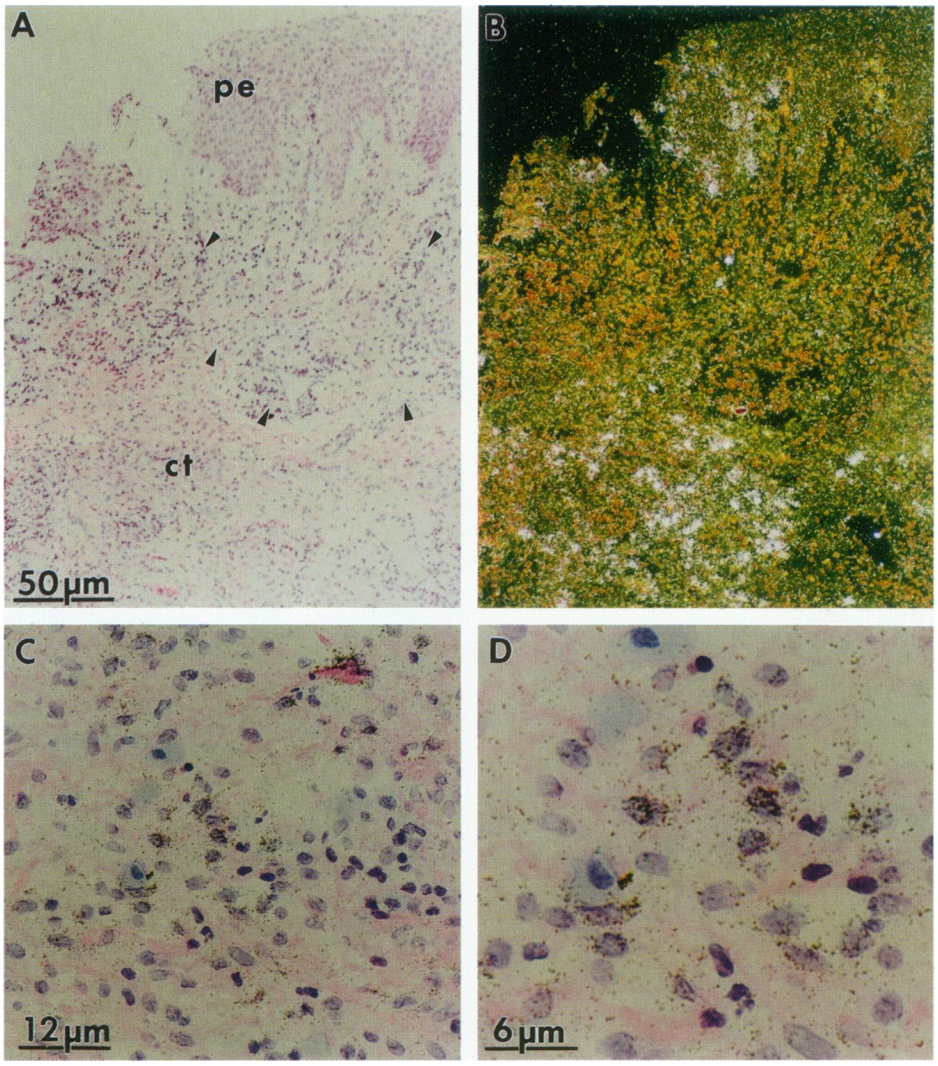

Figure 2. Expression of MMP-13 in connective tissue cells of chronically inflamed human gingiva. **A:** Bright-field image of an *in situ* hybridization showing ulcerating pocket epithelium (pe) and heavily infiltrated connective tissue (ct). **B:** Corresponding dark-field image showing that MMP-13 mRNA can be detected in both basal cells of epithelium and connective tissue cells. Note that MMP-13 expression is absent in the collagen-poor connective tissue area (**arrowheads**) of the strongest inflammatory infiltration. **C** and **D:** Higher magnification of the connective tissue showing that the MMP-13-expressing cells are associated with collagen fibers.

with TNF-α, TGF-β, and KGF in the presence of heparin (not shown). These data strongly suggest that the 58-kd gelatinolytic enzyme is pro-MMP-13 and that MMP-13 is the principal collagenolytic MMP produced by these cells upon the growth factor stimulation. Interestingly, MMP-13 expression was controlled differently from MMP-9 (92-kd gelatinase), as TNF-α up-regulated MMP-9 production by approximately eightfold, whereas it was only slightly affected by TGF-β and KGF. The activity of MMP-2 (72-kd gelatinase) was relatively unchanged by the growth factors. As we have previously noted that KGF in the presence of heparin induces secretion of collagenolytic activity, gelatinase (MMP-9), and urokinase-type plasminogen activator in epithelial cells,[6] we examined in more detail the effects of KGF, heparin, and their combination. Treatment of the cells with KGF in the presence of heparin resulted in a strong induction of MMP-13 mRNA, as demonstrated by Northern blot analysis (Figure 5A). Heparin, which is required for maximal effect of KGF, by itself induced MMP-13 mRNA expression to an approximately 30% lesser extent, whereas KGF alone had no marked effect. Because TGF-α is an effector of KGF action[54] we measured also its effect on MMP-13 expression. As shown by Northern analysis, TGF-α increased MMP-13 mRNA levels in epithelial cells by sevenfold compared with control cells (Figure 5B). Together these data suggest that certain growth factors and cytokines secreted by inflammatory cells, activated connective tissue cells, or epithelial cells are capable of up-regulating MMP-13 expression in undifferentiated epithelial cells.

Contact of the Epithelial Cells with Collagen Triggers MMP-13 Expression

Examination of the explant cultures shown above demonstrate that invading undifferentiated epithelial cells may express MMP-13 mRNA in the absence of inflamma-

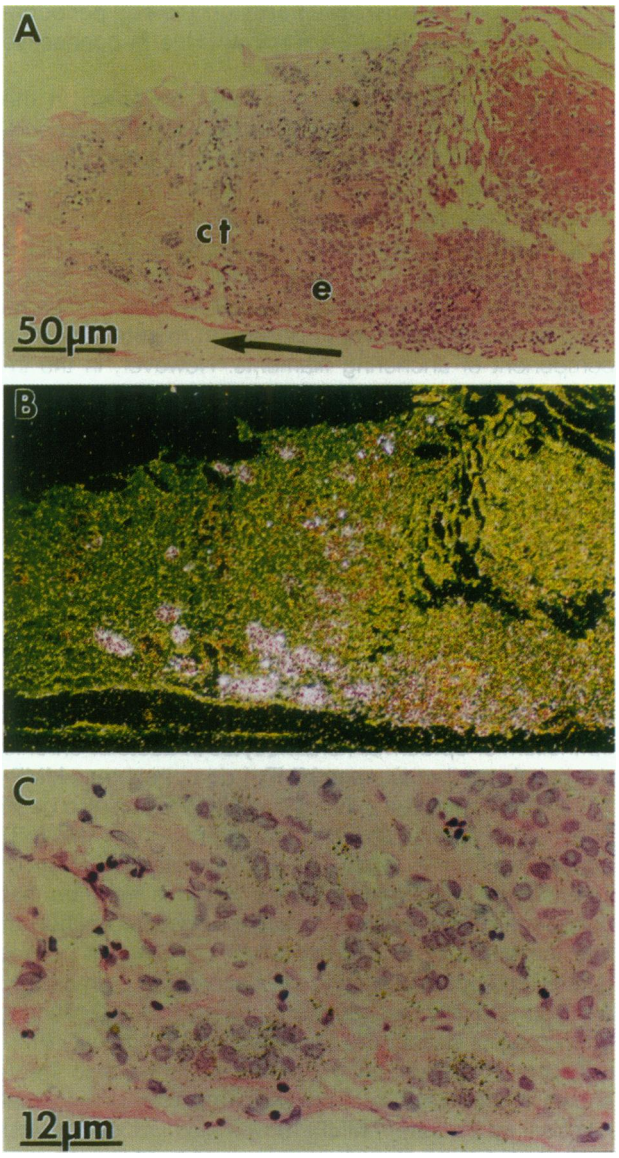

Figure 3. Expression of MMP-13 in epithelium of cultured human oral mucosa. Explants of healthy palatinal mucosa were cultured on decalcified dentin matrix for 6 days. **A:** A section exhibiting an area of epithelial (**e**) growth into the connective tissue (ct). **B:** Dark-field exposure showing a clear signal for MMP-13 mRNA in the proliferating epithelial cells (**arrow**) adjacent to the dentin matrix. **C:** A higher-magnification view from the area indicated by the **arrow** in **A** showing islands of MMP-13-positive epithelial cells within collagen fibers of the explant.

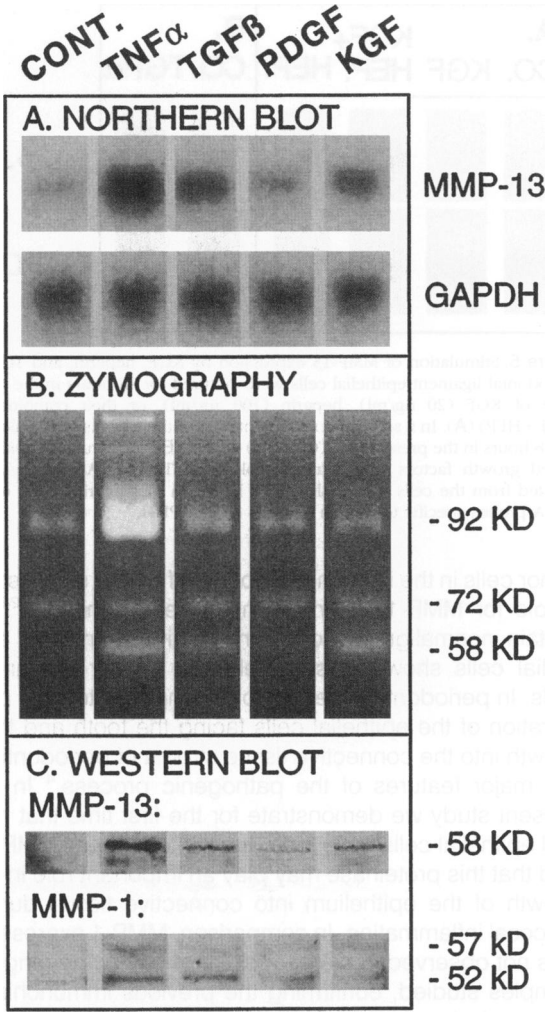

Figure 4. Enhancement of MMP-13 expression by TNF-α, TGF-β, and KGF. Periodontal ligament epithelial cells were cultured for 48 hours in the presence of TNF-α (20 ng/ml), TGF-β1 (10 ng/ml), PDGF (20 ng/ml), or KGF (20 ng/ml) plus heparin (100 μg/ml). Total RNA (20 μg/lane) isolated from the cells was analyzed by Northern blot hybridization using cDNA probes specific to human MMP-13 and GAPDH (**A**). Aliquots of medium were analyzed by gelatin zymography (**B**) and by Western blot using specific MMP-13 and MMP-1 antibodies (**C**), as described in Materials and Methods.

tion. As mucosal epithelial cells migrating into gingival connective tissue in periodontal inflammation most likely get into contact with fibrillar type I collagen, we examined whether culturing periodontal epithelial cells on type I collagen induces MMP-13 expression. As shown in Figure 6, a marked increase (three- and sevenfold) in MMP-13 mRNA abundance was detected in epithelial cells cultured for 24 or 48 hours on collagen as compared with cells cultured on tissue culture plastic. In contrast, collagenase-1 (MMP-1) expression was not markedly affected by contact of epithelial cells with type I collagen. The results indicate that contact with type I collagen is sufficient to induce MMP-13 expression in these epithelial cells in the absence of growth factors or inflammatory cytokines.

Discussion

The human homologue of rat and murine collagenase, collagenase-3 (MMP-13), has been recently cloned and found to show exceptionally wide substrate specificity, as compared with other MMPs.[16–18] This is probably why physiological expression of MMP-13 in humans is restricted to situations in which rapid turnover of fibrillar collagens is required, eg, fetal bone development.[22,23] In addition, MMP-13 is expressed at sites of excessive destruction of collagenous matrix, ie, osteoarthritic cartilage, rheumatoid synovia, and chronic dermal ulcers.[18,20,21,27] We have also recently shown that MMP-13 is expressed by cell lines established from squamous cell carcinomas (SCCs) of the head and neck and *in vivo* by

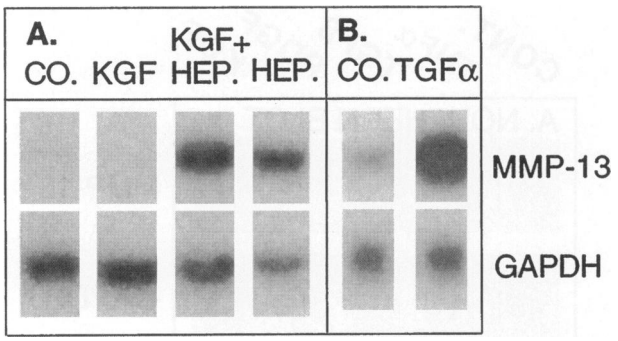

Figure 5. Stimulation of MMP-13 expression by KGF, heparin, and TGF-α. Periodontal ligament epithelial cells were cultured for 48 hours in the presence of KGF (20 ng/ml), heparin (100 μg/ml), or their combination (KGF+HEP) (**A**). In a separate experiment, the epithelial cells were cultured for 48 hours in the presence of TGF-α (20 ng/ml) (**B**). Cells cultured without added growth factors served as control (CO). Total RNA (20 μg/lane) isolated from the cells was analyzed by Northern blot hybridization using cDNA probes specific to human MMP-13 and GAPDH.

tumor cells in the invading periphery of SCCs, suggesting a role for MMP-13 in invasion of these tumors.[24,25] In certain nonmalignant conditions, activated normal epithelial cells show invasive behavior, similar to cancer cells. In periodontal disease, for example, extensive proliferation of the epithelial cells facing the tooth and their growth into the connective tissue stroma of periodontium are major features of the pathogenic process.[4] In the present study we demonstrate for the first time that normal epithelial cells have the capacity to express MMP-13 and that this proteinase may play an important role in the growth of the epithelium into connective tissue during mucosal inflammation. In comparison, MMP-1 expression was not observed in the epithelium of any of the gingival samples studied, confirming the previous immunohistochemical observations.[14,15] As MMP-13 appears to be the primary collagenase in epithelium and is also expressed by connective tissue cells of inflamed periodontium, it is likely to play an important role in connective tissue destruction in this condition.

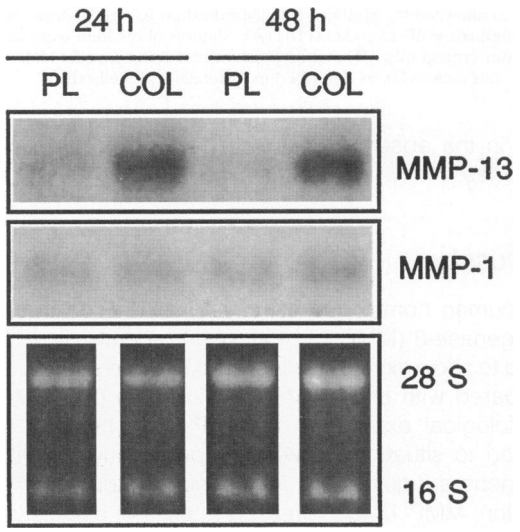

Figure 6. Up-regulation of MMP-13 in epithelial cells by contact with collagen. Periodontal ligament epithelial cells were cultured for 24 or 48 hours on tissue culture plastic plates (PL) or type I collagen-coated plates (COL). RNA of the cells were analyzed by Northern hybridization using specific probes for MMP-13 and MMP-1; rRNA loaded to the agarose gel was visualized by ethidium bromide staining.

Sporadic MMP-13 expression was observed in the basal cells of the projections of pocket epithelium extending into subepithelial connective tissue. In some areas of the epithelium facing the gingival pocket space, the suprabasal cells also expressed MMP-13. The MMP-13-positive cells strongly expressed laminin-5, an adhesion protein associated with keratinocyte migration during wound healing[38] and with cancer cell invasion.[24,39] Laminin-5 is normally present in basement membranes as a component of anchoring filaments. However, in the invading areas of SCCs, the basement membrane is absent.[24] Similar to invading cancer cells, laminin-5 immunostaining in inflamed gingival tissue was mainly cytoplasmic and not arranged extracellularly in a band-like manner, suggesting the absence of an organized basement membrane in these areas. Signal for MMP-13 was not observed in the oral gingival keratinocytes of the same samples even though abundant inflammatory infiltration was often seen in the vicinity of this epithelium. Laminin-5 was present in these areas as a thin extracellular band. These observations are in accordance with our recent observations that keratinocytes in intact oral epithelium or epidermal keratinocytes in acute or chronic dermal ulcers that express MMP-1 do not express MMP-13.[25,27] The selective expression of MMPs has also been observed in studies showing that stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) are expressed in distinct keratinocytes of chronic wounds.[40] It thus appears that only certain populations of cells in mucosal epithelium has the capacity to express MMP-13. Gingival pocket epithelium does not keratinize but expresses both basally and suprabasally cytokeratins 5, 14, and 19, indicating a phenotype of basal cells uncommitted to terminal differentiation.[41] Cytokeratin 19 expression has also been shown to correlate with premalignant changes in oral mucosal epithelium.[42] MMP-13 was also induced *in vitro* in epithelial cells of oral mucosa and in PLE cells that have the same cytokeratin profile as gingival pocket epithelium,[43,29] whereas primary human epidermal keratinocytes do not express the enzyme under the same conditions.[26] Furthermore, we have observed that in SCCs MMP-13 mRNA is expressed by poorly differentiated tumor cells but not by cells undergoing differentiation.[24,25] Therefore, the state of differentiation may be one factor priming the epithelial cells for MMP-13 production.

The expression of MMP-13 in the epithelium in close proximity to inflammatory cells suggests a role for inflammatory cell-derived cytokines in the stimulation of MMP-13 expression. Accordingly, the expression of MMP-13 in periodontal epithelial cells was enhanced by TNF-α and TGF-β. Both of these growth factors have the ability to strongly enhance the expression of MMP-13 in SCC cell lines and transformed human epidermal keratinocytes (HaCaT cells) in culture.[25,26] TNF-α is present in inflamed gingiva,[44] and pathogenic bacteria present in the gingival pocket have been found to trigger TNF-α production.[45] Several components of both gram-positive and gram-negative bacteria, such as lipopolysaccharide,

peptidoglycan, porins, and exotoxins, have the ability to induce production of TNF-α in inflamed tissue.[45] It is also possible that some bacterial products are capable of directly activating epithelial cells to produce MMP-13. In fact, some periodontopathogens have been found to induce secretion of collagenase activity both in fibroblasts and epithelial cells, but whether MMP-1 or MMP-13 is the primary collagenase produced has not yet been established.[46,47] Our results show that cultured porcine periodontal ligament epithelial cells have the capacity to express MMP-1 in addition to MMP-13. However, lack of MMP-1 in human periodontal pocket epithelium strongly suggests that MMP-13 is the primary collagenase in this epithelium.

In addition to cytokines and growth factors released by inflammatory cells, factors resulting from stromal-epithelial cell interactions may regulate MMP-13 expression.[48] KGF is produced by connective tissue cells, but its only target cells are the epithelial cells. During inflammation, cytokines such as interleukin-1 stimulate connective tissue cells to produce KGF.[49] High tissue levels of KGF and interleukin-1 have been found to correlate with the degree of inflammation in Crohn's disease and ulcerative colitis.[50] We have previously reported a marked increase in the secretion of both collagenolytic and gelatinolytic activity by PLE cells treated with a combination of KGF and heparin.[6,51] Heparin, but not KGF alone, also stimulated MMP-13 expression. A combination of KGF and heparin increased MMP-13 mRNA levels more potently than heparin alone, showing that KGF exerts an inductive effect on the MMP-13 expression. Heparin is known to stabilize the tertiary structure of fibroblast growth factors, and it is required for their binding into the high-affinity fibroblast growth factor receptor.[52,53] Based on the 3/4 cleavage of the type I collagen molecules and the reactivity of the enzyme with a polyclonal collagenase antibody, MMP-1 was initially assumed to be responsible for the collagenolytic activity. Our present study using specific cDNA probes to MMP-13 and MMP-1 the MMP mRNAs show that the collagenase induced in KGF-treated epithelial cells is not MMP-1 but MMP-13. An interesting finding of our study is that heparin alone was able to induce MMP-13 expression, raising the possibility that heparin released by mast cells during the inflammatory reaction may play a role in the regulation of MMP-13. Another growth factor that we found to induce MMP-13 is TGF-α, which is the predominant proximal effector of KGF action for epidermal growth.[54]

Contact with extracellular matrix molecules is known to regulate cell functions, such as proliferation, migration, and proteolytic activity. Earlier studies have revealed that the amounts of interstitial collagen, fibronectin, and tenascin are substantially decreased in chronically inflamed gingival tissue.[55,56] Furthermore, basement membrane zone components, such as type VII collagen and the hemidesmosomal integrin $\alpha6\beta4$, are lost in some areas of the epithelium.[56] In these areas of discontinuous basement membrane, epithelial cells may come in contact with interstitial collagen. In the present study we found that culturing epithelial cells on type I collagen induced MMP-13 expression. Type I collagen may therefore play an important role in MMP-13 induction, allowing activated epithelium to grow into connective tissue, similar to invasive tumor cells. Previously, contact with collagen has been found to induce MMP-1 expression in epidermal keratinocytes and fibroblasts.[27,57,58] Interestingly, we did not observe MMP-1 up-regulation in PLE cells cultured on type I collagen, indicating that regulation of MMPs by matrix contact is different for various types of epithelial cells. The MMP-inducing signals from the matrix are transduced, at least partly, through integrins; eg, MMP-1 expression is mediated via integrin receptors for collagen[59] and fibronectin[60]. At present, however, the matrix-directed signal transduction pathways of MMP-13 expression have not been clarified. We have previously observed that the expression of integrins of the $\beta1$ family changes dramatically in chronically inflamed tissue.[56] Focal loss of $\beta1$ integrins ($\alpha2\beta1$ and $\alpha3\beta1$) was found in many areas of the gingival pocket epithelium, whereas other areas were strongly positive for $\beta1$ integrins. These local variations in the tissue composition and integrin expression may be one explanation for marked differences in the MMP-13 expression in different areas of the gingival epithelium.

In conclusion, the results of the present study suggest that collagenase-3 (MMP-13) is involved in the growth of activated undifferentiated mucosal epithelial cells into connective tissue stroma during inflammation. The expression of MMP-13 mRNA was found to be sporadic and therefore under specific regulation that appears to involve signals from both inflammatory cells and peri-epithelial matrix in the affected tissue. Possibly, a combination of certain growth factors and contact with interstitial collagen leads to specific induction of MMP-13 expression and consequently loss of fibrillar collagens in the areas of migrating epithelium. These results thus provide evidence that MMP-13 may play a crucial role in extracellular matrix degradation in chronic mucosal inflammation and consequently imply MMP-13 as a potential target for inhibiting connective tissue destruction in this condition.

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