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Matrix Metalloproteinase-8 Is Expressed in Rheumatoid Synovial Fibroblasts and Endothelial Cells

REGULATION BY TUMOR NECROSIS FACTOR-α AND DOXYCYCLINE*

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Neutrophil collagenase (matrix metalloproteinase-8 or MMP-8) is regarded as being synthesized exclusively by polymorphonuclear neutrophils (PMN). However, *in vivo* MMP-8 expression was observed in mononuclear fibroblast-like cells in the rheumatoid synovial membrane. In addition, we detected MMP-8 mRNA expression in cultured rheumatoid synovial fibroblasts and human endothelial cells. Up-regulation of MMP-8 was observed after treatment of the cells with either tumor necrosis factor-α (10 ng/ml) or phorbol 12-myristate 13-acetate (10 nM). Western analysis showed a similar regulation at the protein level. The size of secreted MMP-8 was 50 kDa, which is about 30 kDa smaller than MMP-8 from PMN. Conditioned media from rheumatoid synovial fibroblasts contained both type I and II collagen degrading activity. However, degradation of type II collagen, but not that of type I collagen, was completely inhibited by 50 μM doxycycline, suggesting specific MMP-8 activity. In addition, doxycycline down-regulated MMP-8 induction, at both the mRNA and protein levels. Thus MMP-8 exerts markedly wider expression in human cells than had been thought previously, implying that PMN are not the only source of cartilage degrading activity at arthritic sites. The inhibition of both MMP-8 activity and synthesis by doxycycline provides an incentive for further studies on the clinical effects of doxycycline in the treatment of rheumatoid arthritis.

Extracellular matrix degradation is fundamental to connective tissue remodeling during physiological processes as well as during the progress of several pathological phenomena. Matrix turnover is regulated by a delicate balance among the production, activation, and inhibition of proteolytic enzymes. The matrix metalloproteinases (MMPs)[1] form a gene family of at least 14 enzymes participating in extracellular matrix remodeling. MMPs, together with the factors associated with their regulation, are reported to be highly implicated in various diseases such as rheumatoid arthritis, osteoarthritis, corneal ulceration, atherosclerosis, and tumor invasion and metastasis (for reviews, see Refs. 1–3). Previous studies have demonstrated that neutrophil-derived MMPs such as collagenase (MMP-8) and gelatinase B (MMP-9, 92-kDa type IV collagenase), play a key role in the degradation of extracellular matrix constituents *i.e.* during the course of inflammatory diseases (4–7).

Collagenases exist as three distinct molecules, namely the fibroblast type (MMP-1, collagenase-1) (8), the neutrophil type (MMP-8) (9), and collagenase-3 (MMP-13) (10). They all are able to degrade specifically the fibrillar collagen types I, II, and III as well as type VII and X collagens (11, 12), serpins (4, 13), β-casein, and human α_2-macroglobulin (14). Among collagenases, MMP-8 most effectively hydrolyzes the native type I and II collagens, whereas MMP-1 prefers type III collagen. MMP-8 is a considerably more efficient enzyme than MMP-1 with respect to almost all substrates except for type III collagen (9). MMP-1 is transcribed and expressed by human fibroblasts, keratinocytes, endothelial cells, monocytes, and macrophages, and collagenase-3 by some human malignant breast tumors (10), whereas MMP-8 has been shown to be stored in subcellular specific granules of mature human peripheral blood PMN after synthesis during PMN maturation in bone marrow (15). Previous studies indicate that PMN MMP-8 transcription is completed before PMN emigrate from bone marrow (15) and that MMP-8 activity is regulated by factors that affect the release of MMP-8 by degranulation and not its biosynthesis (4). However, by using *in situ* hybridization, Cole and Kuettner (16) have shown that MMP-8 mRNA is also found in peripheral PMN. More recent observations (17, 18) showed MMP-8 mRNA expression in human cartilage, primarily in chondrocytes. In this study we investigated the expression and regulation of MMP-8, particularly in human rheumatoid synovial fibroblasts (RSF) and endothelial cells. It was shown that MMP-8 is also expressed in cell types other than those belonging to the PMN

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[1] The abbreviations used are: MMP(s), matrix metalloproteinase(s); PMN, polymorphonuclear neutrophil(s) RSF, rheumatoid synovial fibroblasts; TNF-α, tumor necrosis factor-α; APMA, 4-aminophenylmercuric acetate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); DTPA, diethylenetriaminepentaacetic acid; kb, kilobase(s).

lineage. In these cells MMP-8 is secreted as a nonglycosylated protein. The expression of MMP-8 in RSF and endothelial cells was up-regulated by tumor necrosis factor-α (TNF-α) and phorbol 12-myristate 13-acetate (PMA). Doxycycline inhibited not only the catalytic activity of MMP-8, but also the expression of MMP-8 mRNA and protein.

EXPERIMENTAL PROCEDURES

Reagents—Medium 199 supplemented with 20 mM Hepes was purchased from Flow Laboratories (Irvine, Scotland, U. K.). Ham's nutrient mixture F-12 was from Northumbria Biologicals Ltd. (Cramlington, Northumberland, U. K.). Dulbecco's modified Eagle's medium, keratinocyte-SFM, as well as other cell culture reagents were obtained from Life Technologies, Inc. 4-Aminophenylmercuric acetate (APMA), CHAPS, PMA, endoglycosidase F, *N*-glycosidase F, polyoxyethylene 23 lauryl ether (Brij-35), and doxycycline were purchased from Sigma. Native type I and II collagen were isolated from bovine skin and articular cartilage and analyzed for purity by cyanogen bromide cleavage peptide analysis (5). Human recombinant TNF-α (specific activity 2.45×10^7 units/mg) was a gift from J. Tavenier (Biogent, Gent, Belgium). Hydrocortisone was from Diosynth, (Oss, The Netherlands). The avidin-biotin-horseradish peroxidase kit and peroxidase-anti-peroxidase (rabbit) were from Dako A/S (DK-2600 Glostrup, Denmark).

Cell Cultures and Tissue Samples—RSF were isolated from freshly dispersed tissue of patients with rheumatoid arthritis as described by Unemori *et al.* (19). Culture medium was replaced every 2–3 days. Subcultures were obtained by trypsin/EDTA treatment at a split ratio of 1:3. The cells were used after two or six passages. Conditioned media were obtained by incubating the cells in 10-cm^2 dishes for 24 h with 1.5 ml of medium 199 supplemented with 0.1% human serum albumin, 50 μg/ml streptomycin, and 50 IU/ml penicillin, to which the appropriate concentration of test compound was added (10 ng/ml TNF-α or 10 nM PMA). The conditioned media were centrifuged for 4 min at $13,000 \times g$ in a microcentrifuge to remove cells and cellular debris, and samples were frozen at −20 °C until used. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously (20, 21). Gingival fibroblasts were established from healthy oral mucosa taken from the biopsies of gingiva during operations on maxillary canines and cultured as described earlier (22). Human periodontal ligament fibroblasts were obtained and cultured by a technique described earlier by Oikarinen and Seppä (23). Human oral mucosal keratinocytes were cultured as described in detail by Salo *et al.* (22). Human squamous cell carcinoma cells (ATCC number CRL 1628) were cultured as described previously (24). Bone marrow samples ($n = 5$) were obtained from patients (ages 4–13 years) being operated on for orthopedic reasons. PMN were isolated and purified from blood of healthy volunteers (25, 26). The permission for all of these protocols was approved by the Ethical Committee of the University of Oulu. For collecting the media for Western blot analysis, the cells were washed with phosphate-buffered saline (PBS) and incubated for 24 h with medium 199 without serum.

RNA Analysis—For RNA analysis total cellular RNA was extracted from cultured cells: RSF, squamous cellular carcinoma, gingival fibroblasts, periodontal ligament fibroblasts, HUVEC, and keratinocytes. In addition, RNA was isolated from human bone marrow, alveolar bone, and radicular jaw cyst specimen and from purified PMN immediately frozen in liquid nitrogen after purification. RNA extraction and purification were performed as described (27). Northern blot analysis was carried out as described previously (28). For reverse transcription (RT), 0.1–20 μg of RNA was reverse transcribed as described previously (21), using oligo(dT) as a primer. Cycles (denaturation step at 95 °C for 30 s, annealing step at 55 °C for 30 s, and extension step at 72 °C for 30 s) were performed using a thermal reactor. Specific primers for MMP-8 (not recognizing MMP-1 or other MMPs) were designed, based on the published DNA sequence (9): MMP-8 sense primer 5′-AAGGCAACCAATACTGGG, and MMP-8 antisense primer 5′-ATTTTCACGGAGGACAGG. The size of the expected PCR product was 522 bp (29). Primers for the constitutively expressed housekeeping gene β-actin were as described previously (30): β-actin sense primer, 5′-AAGATGACCCAGATCATGTTTGAG; antisense primer, 5′-AGGAGGAGCAATGATCTTGATCTT; expected product size 652 bp. PCR analysis was also carried on human genomic DNA (28). The 522-bp PCR products from human bone marrow, peripheral blood PMN, and human gingival fibroblasts were isolated, subcloned, and sequenced (28). The probe for MMP-8 was obtained by PCR of mRNA from bone marrow and was characterized by sequencing.

Western Blot Analysis—For Western blot analysis, serum-free cell culture medium (200 μl) or purified PMN MMP-8 (5–10 ng) was freeze-dried and resuspended in 100 μl of 10 mM Tris-HCl, pH 7.8, run on 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter paper. Nonspecific binding was eliminated by incubating with PBS supplemented with 5% non-fat dry milk for 90 min at 37 °C. The blots were incubated either with rabbit polyclonal antibodies (affinity-purified IgG fractions) against human MMP-8 (13, 31) diluted 1:1,000 or with nonimmune control serum diluted 1:100 for 1 h at 20 °C. After washings, the blots were incubated with biotinylated goat anti-rabbit immunoglobulins (1:500) for 1 h at 20 °C. After washing, the blots were incubated for 30 min with the avidin-biotin-horseradish peroxidase kit, and the color was developed using diaminobenzidine.

MMP-8 Immunofluorometric Assay—MMP-8 levels were determined by a time-resolved immunofluorescence assay. The monoclonal MMP-8-specific antibodies 8708 and 8706 were used as a catching antibody and a tracer antibody, respectively. The tracer antibody was labeled using europium-chelate (32). The assay buffer contained 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide, and 20 mg/liter DTPA. Samples were diluted in assay buffer and incubated for 1 h, followed by incubation for 1 h with tracer antibody. Enhancement solution was added, and after 5 min fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). MMP-8 was purified from human neutrophil extracts according to Sorsa *et al.* (7). The specificity of the monoclonal antibodies against MMP-8 corresponded to that of polyclonal MMP-8 (data not shown).

Glycosylation Analysis—Endoglycosidase digestions of MMP-8 purified from PMN and from culture media of PMA-stimulated RSF and HUVEC were carried out using a modification of the method described by Mallya *et al.* (33). Briefly, endoglycosidase F digestion was carried out in 20 mM potassium phosphate, pH 7.2, containing 50 mM EDTA, 0.1% SDS, 1% 2-mercaptoethanol, and 1% CHAPS at 37 °C; the reaction with *N*-glycosidase F was done in 100 mM phosphate buffer, pH 7.2, containing 25 mM EDTA, 0.1% SDS, 1.0% 2-mercaptoethanol, and 1% CHAPS. In the deglycosylation reaction of both 0.05–0.2 mg/ml purified PMN MMP-8 or culture media of RSF and HUVEC, 0.05 unit of endoglycosidase F or 0.2 unit of *N*-glycosidase F was used. Before the addition of endoglycosidase, samples were denaturated by heating at 100 °C for 5 min in the presence of 0.1% SDS. The reactions were terminated after overnight incubation by addition of SDS-polyacrylamide gel electrophoresis sample buffer and boiling for 5 min.

Immunohistochemical Staining—Antiserum to neutrophil type MMP-8 collagenase used for immunohistochemical staining was the same as that used for immunoblotting (13, 31). 6-μm-thick cryostat sections of synovial membrane from six rheumatoid arthritis patients were mounted on gelatin-coated slides and fixed in acetone at 4 °C for 5 min and washed in 0.1 M PBS, pH 7.4, at 22 °C before the inhibition of endogenous peroxidase by immersing the sections in 0.3% hydrogen peroxide in methanol for 20 min. The slides were placed in a humid chamber, and the sections were incubated sequentially with 1) rabbit anti-human antiserum against MMP-8, diluted 1:100–1:400 in PBS and 0.1% w/v BSA for 1 h; 2) biotinylated horse anti-rabbit IgG, diluted 1:100 in PBS with 0.1% w/v bovine serum albumin for 30 min; and 3) avidin-biotin-horseradish peroxidase complex, diluted 1:200 in PBS for 30 min. Finally, the sections were incubated for 5 min in a chromogen solution of 3,3′-diaminobenzidine (50 mg/150 ml of PBS) and hydrogen peroxide (final concentration 0.03%). All incubations were performed at 22 °C, and slides were washed twice in PBS between each step. All slides containing consecutive sections were processed further with and without counterstaining with hematoxylin before dehydration in ethanol, clearing in xylene, and mounting. Omission of primary antiserum and use of normal rabbit serum (diluted 1:100–1:400) were included as controls.

Measurement of Collagenase Activity against Soluble Type I and II Collagens—RSF were incubated for 24 h with 10 ng/ml TNF-α. Media were collected, treated with 1 mM APMA, and incubated for 12 h at 22 °C with 1.5 μM native soluble type I or II collagen in 50 mM Tris-HCl, 0.15 M NaCl, 1 mM $CaCl_2$, pH 7.8, in the presence or absence of 50 μM doxycycline. The collagen degradation products were separated by 8% SDS-polyacrylamide gel electrophoresis. The amount of type I or type II collagen cleaved by collagenases (MMP-1 and/or MMP-8) was analyzed by scanning the bands on gel at 595 nm using an ISCO gel scanner. Collagenase activity is expressed as a percentage of collagen degraded (5, 34).

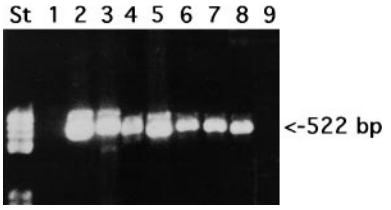

FIG. 1. **Human neutrophil collagenase (MMP-8) RT-PCR from various cellular sources.** RNA samples were transcribed into cDNA by RT, and MMP-8 transcripts were amplified by PCR and subjected to agarose gel electrophoresis. *Lane 1*, negative control in which no cDNA was added; *lanes 2–9*, RT-PCR of RNA from bone marrow (*lane 2*), peripheral blood PMN (*lane 3*), cultured squamous carcinoma cells (*lane 4*), mucosal fibroblasts (*lane 5*), periodontal ligament fibroblasts (*lane 6*), alveolar bone specimen (*lane 7*), radicular cyst specimen (*lane 8*), and cultured oral mucosal keratinocytes (*lane 9*). DNA molecular mass standard (*St*) V (821705) was used as a size marker.

RESULTS

MMP-8 Expression in Several Cell Types and Tissues—Using a sensitive RT-PCR method we analyzed whether the expression of MMP-8 mRNA could be detected in tissues other than the bone marrow. Indeed, a 522-bp MMP-8 transcript amplified from total RNA was detected in peripheral blood PMN; fibroblastic cell lines derived from mucosa (gingival fibroblasts), periodontal ligament, or rheumatoid synovia (RSF); cultured squamous cell carcinoma cells of the tongue (squamous cellular carcinoma); and HUVEC, whereas no amplified transcript was observed in cultured human oral keratinocytes (Figs. 1–3). The specificity of PCR amplification of the RSF mRNA was verified by Southern blot analysis using a specific MMP-8 cDNA probe (Figs. 2 and 3). Using the same MMP-8 primers as those used for MMP-8 RT-PCR, PCR amplification was carried out on human genomic DNA, resulting in a 1,400-bp product, indicating that the area of genomic MMP-8 DNA corresponding to the 522-bp mRNA RT-PCR product contains noncoding introns of the MMP-8 gene (not shown). In addition, the 522-bp PCR products were isolated, subcloned, and sequenced, which revealed 100% homology with the published human MMP-8 sequence (15).

Regulation of MMP-8 Expression in RSF and HUVEC by TNF-α and PMA—Regulation of MMP-8 expression in RSF and HUVEC was studied by treating the cells with 10 ng/ml TNF-α or 10 nM PMA. After isolation RNA was analyzed by using semiquantitative RT-PCR followed by Southern analysis, or by Northern analysis. RT-PCR of RSF RNA showed a 522-bp fragment, and the amount of the amplified product was increased 5-fold after the treatment of TNF-α, and 3–4-fold after PMA treatment (Fig. 2*B*). For HUVEC also nonspecific bands were observed after RT-PCR (Fig. 3*A*). However, Southern blotting revealed results similar to those obtained for RSF (Fig. 3*B*). The amount of amplified β-actin DNA was constant in control, TNF-α-, or PMA-treated cells (Fig. 3*C*). The presence of MMP-8 mRNA was also confirmed by Northern blot analysis (Fig. 2, *C* and *D*). The 3.3-kb MMP-8 mRNA was observed in TNF-α- and PMA-treated cells, whereas using this method no MMP-8 mRNA could be detected in untreated cells (Fig. 2*C*). Thus, both in fibroblasts and in endothelial cells MMP-8 is expressed and regulated by inflammatory mediators or phorbol ester.

Expression of MMP-8 Protein in RSF and HUVEC—To analyze MMP-8 protein expression and secretion, Western blot analysis was carried out on culture supernatants of RSF. A 50-kDa immunoreactive band was observed by using MMP-8-specific polyclonal antibodies (Fig. 4). The MMP-8-specific antibody used in this study did not recognize the related fibroblast collagenase (MMP-1) or other MMPs (MMP-2, MMP-3, MMP-9) because excessive amounts of these proteins showed

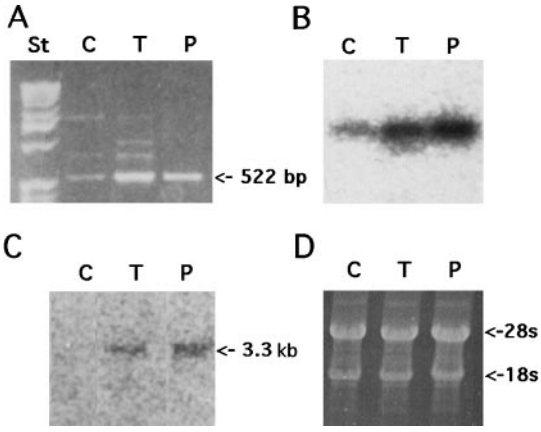

FIG. 2. **MMP-8 mRNA and protein expression in human RSF.** Cultured RSF were incubated for 24 h with 10 ng/ml TNF-α (*T*), 10 nM PMA (*P*), or without mediator (*C*), and RNA was isolated. *Panel A*, MMP-8 RT-PCR. RNA samples of control, TNF-α-stimulated, or PMA-stimulated RSF were transcribed into cDNA by RT. MMP-8 transcripts were amplified by PCR and subjected to agarose gel electrophoresis. DNA molecular mass standard (*St*) V (821705) was used as a size marker. *Panel B*, Southern blot analysis of the PCR-amplified DNA. Amplified transcripts, such as those described in *panel A*, were transferred to nylon filters and hybridized with ^{32}P-labeled MMP-8 DNA probe. *Panel C*, MMP-8 RNA analysis using Northern blotting. RNA samples (7.5 μg) were subjected to Northern blotting and hybridized using a ^{32}P-labeled MMP-8-specific DNA probe. *Panel D*, ethidium bromide-stained agarose gel showing the ribosomal RNAs (18 S and 28 S) demonstrating equal RNA loading.

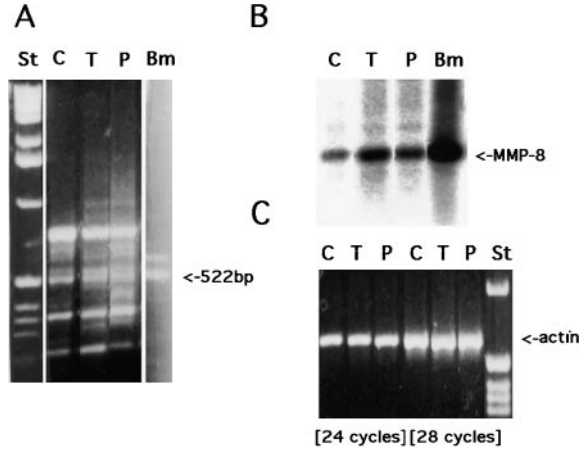

FIG. 3. **MMP-8 mRNA expression in human endothelial cells.** Cultured HUVEC were incubated for 24 h with 10 ng/ml TNF-α (*T*), 10 nM PMA (*P*), or without mediator (*C*), and RNA was isolated. *Panel A*, MMP-8 RT-PCR. RNA samples of control, TNF-α-stimulated, or PMA-stimulated HUVEC were transcribed into cDNA by RT. MMP-8 transcripts were amplified by PCR and subjected to agarose gel electrophoresis. Bone marrow RNA (*Bm*) was used as a positive control. DNA molecular mass standard (*St*) V (821705) was used as a size marker. *Panel B*, Southern blot analysis of the PCR-amplified DNA. Amplified transcripts, such as those described in *panel A*, were transferred to nylon filters and hybridized with ^{32}P-labeled MMP-8 DNA probe. *Panel C*, RT-PCR of β-actin RNA using specific oligonucleotides. RNAs were the same as described in *panel A*.

no cross-reactivity in Western blotting (Fig. 4*A*). At the protein level the induction of MMP-8 was seen also, both in TNF-α- and PMA-treated cells (Fig. 4*B*, *lanes 3* and *4*). Only very weak immunoreactivity was found in the culture media of untreated RSF (Fig. 4*B*, *lane 2*). MMP-8 expression levels in RSF were measured by enzyme-linked immunosorbent assay. In conditioned media of RSF (passage 0 and passage 2 from two different isolations) an MMP-8 production of 6.7 ± 4.0 ng/ml/24 h was determined.

We assume that the 50-kDa band most likely represents the

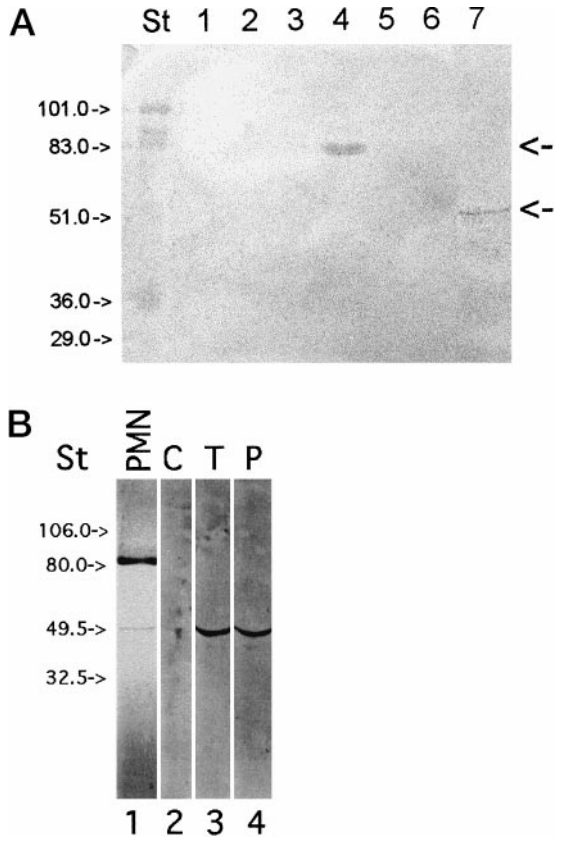

FIG. 4. **MMP-8 protein expression in RSF.** Cultured RSF were incubated for 24 h with 10 ng/ml TNF-α (*T*), 10 nM PMA (*P*), or without mediator (*C*). *Panel A*, 200 μl of the culture media of TNF-α-stimulated cells was freeze-dried and subjected to Western blotting (*lane 7*). In addition 2 μg of different recombinant MMPs were applied to validate the specificity of the MMP-8 monoclonal antibody: MMP-1 (*lane 1*), MMP-2 (*lane 2*), MMP-3 (*lane 3*), MMP-9 (*lane 5*), MMP-13 (*lane 6*). As a control 5 ng of purified PMN was used (*lane 4*). Mobilities of the molecular mass markers (*St*) are indicated. *Panel B*, cultured RSF were incubated for 24 h with 10 ng/ml TNF-α, 10 nM PMA, or without mediator. 200 μl of the culture media was freeze-dried and subjected to Western blotting. As a control 5 ng of purified PMN was used.

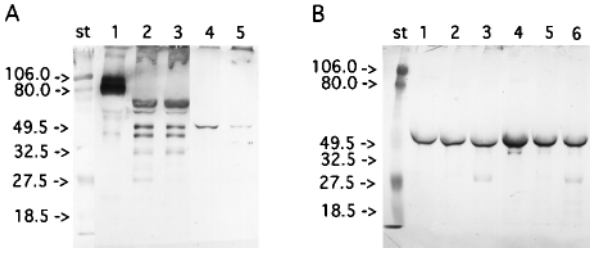

FIG. 5. **Deglycosylation of MMP-8 protein in PMN extract and culture media from RSF and endothelial cells.** MMP-8 purified from PMN (*panel A*) or from culture media of RSF or HUVEC incubated for 24 h with 10 nM PMA (*panel B*) were treated with *N*-glycosidase F or endoglycosidase F and subjected to Western blotting using MMP-8-specific polyclonal antibodies. *Panel A*, *lane 1*, untreated purified MMP-8 (10 ng) from PMN; *lanes 2* and *3*, purified MMP-8 was incubated with 0.1% SDS, boiled for 5 min, and treated with *N*-glycosidase F or endoglycosidase F respectively; *lanes 4* and *5*, culture media from HUVEC and RSF, respectively. *Panel B*, culture media from RSF and HUVEC were treated with *N*-glycosidase F or endoglycosidase F and subjected to Western blotting using MMP-8-specific polyclonal antibodies. *Lanes 1* and *4*, culture media from untreated RSF and HUVEC, respectively; *lanes 2* and *3*, culture media from RSF were incubated with 0.1% SDS, boiled for 5 min, and treated with *N*-glycosidase F or endoglycosidase F, respectively; *lanes 4* and *5*, culture media from HUVEC were incubated with 0.1% SDS, boiled for 5 min, and treated with *N*-glycosidase F or endoglycosidase F, respectively. Mobilities of the molecular mass markers (*st*) are indicated.

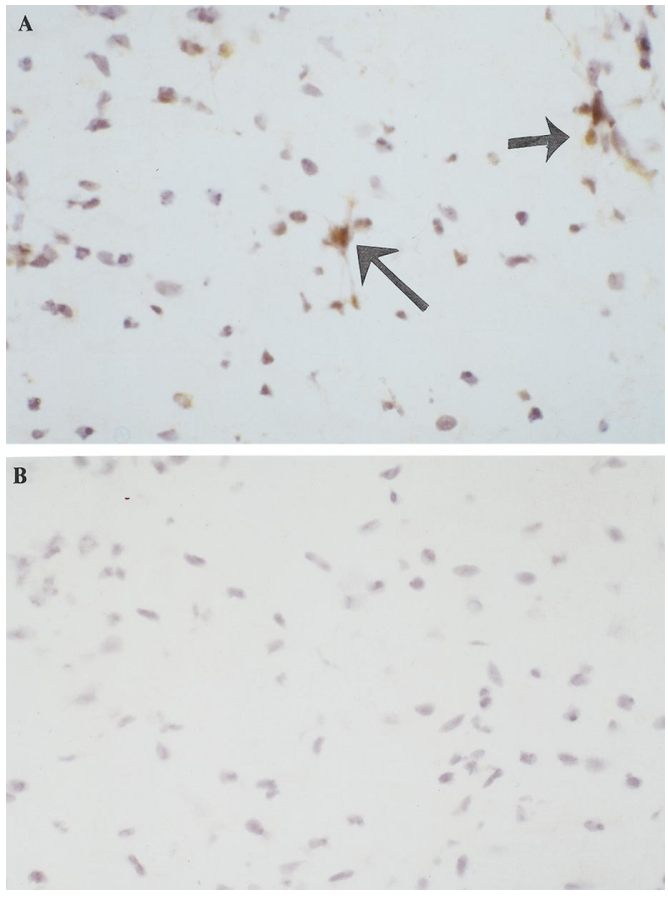

FIG. 6. **Immunolocalization of MMP-8 in the human rheumatoid synovial membrane.** *Panel A*, cryostat sections of synovial membrane from rheumatoid arthritis patients were stained by using polyclonal MMP-8 antibodies (1:100 dilution). *Arrows* indicate mononuclear cells with positive staining. *Panel B*, control section stained with normal rabbit serum (magnification $\times$ 400).

nonglycosylated form of MMP-8 (9, 31). Deglycosylation of PMN MMP-8 (about 80 kDa) by incubation of purified neutrophil MMP-8 with *N*-glycosidase F or endoglycosidase F revealed on Western blot four bands of about 70, 65, 50, and 45 kDa in size (Fig. 5*A*), whereas the treatment of media from PMA-treated HUVEC or RSF did not show any clear changes in the molecular mass of secreted 50 kDa MMP-8 (Fig. 5*B*).

In Vivo Expression of MMP-8 in Rheumatoid Synovial Tissue—Detection of MMP-8 by immunohistochemistry showed MMP-8 expression in mononuclear fibroblast-like cells in the human rheumatoid synovial membrane (Fig. 6*A*), whereas no immunostaining was observed using nonimmune sera (Fig. 6*B*). Similar *in vivo* results were found in tissues from chronically inflamed gingiva and oral mucosa (data not shown).

Inhibition of RSF MMP-8 Activity by Doxycycline—Collagenase activity in culture media of RSF, treated with TNF-α, degraded efficiently both native type I and II collagens into the characteristic αA(3/4) cleavage products (Fig. 7, *lanes 2* and *5*). Previous studies (34) have shown that doxycycline is a strong inhibitor of MMP-8 activity compared with MMP-1 activity (IC_{50} of 30 and 300 μM, respectively). Preincubation of culture media from RSF with 50 μM doxycycline completely prevented the specific degradation of type II collagen (Fig. 7, *lane 6*) but had no effect on the degradation of type I collagen (Fig. 7, *lane 3*). Thus, RSF do produce MMP-8 which, as shown before (9), prefers type II collagen to type I collagen as a substrate, and its catalytical activity is specifically inhibited by doxycycline. Similar results were found in culture media of endothelial cells (not shown).

Effects of Doxycycline on MMP-8 Expression in RSF and

HUVEC—We investigated further whether doxycycline affects the level of MMP-8 expression. Northern blot analysis of RSF, treated with TNF-α or PMA in the presence of doxycycline, showed a clear reduction in the amount of 3.3-kb MMP-8 mRNA (Fig. 8, *A* and *B*). A similar result was obtained using RT-PCR (Fig. 8, *C* and *D*). In addition, MMP-8 protein was reduced in cells treated with TNF-α or PMA in the presence of doxycycline (Fig. 8, *E* and *F*). Obviously, doxycycline not only inhibits the collagenolytic activity of MMP-8 but also affects the induced expression of the 50-kDa MMP-8 protein in synovial fibroblasts.

DISCUSSION

Human neutrophil collagenase (MMP-8) is regarded as a PMN-specific matrix metalloproteinase that is stored in granules and released upon cell activation. This study shows that in addition to peripheral PMN, MMP-8 is expressed in RSF and human endothelial cells. This was demonstrated by RT-PCR, Northern blotting (showing the MMP-8-specific mRNA size of 3.3 kb), Western blotting, immunohistochemistry, substrate specificity, and specific inhibition of enzyme activity by doxycycline. Upon MMP-8 synthesis, which is regulated in these cells by the inflammatory mediator TNF-α, the protein is secreted. MMP-8, interstitial collagenase (MMP-1), and collagenase-3 (MMP-13) are the main proteases that can degrade different types of native collagen. MMP-8 has the highest activity toward cartilage collagen type II. In pathophysiological conditions MMP-8 is regarded as playing a central role at sites of matrix degradation; MMP-8 activity was shown in chronic bronchiectasis (35), cystic fibrosis, in which the level of activity correlated to the disease state (36), and rheumatoid arthritis (37). In addition, MMP-8 is thought to be the only collagenase that acts in cartilage aggrecan degradation (38). Because its synthesis and storage were considered to be restricted to maturating PMN only, MMP-8 activity at sites of tissue destruction was thought to be mediated by the degranulation of PMN (4, 9, 15). In contrast to previous studies, we show that the regulation of tissue destruction by MMP-8 is most likely more complicated, and MMP-8 expression comprises various cell types and tissues. On the basis of our results we hypothesize that as with other members of the MMP family, the expression of MMP-8 is regulated by inflammatory mediators such as TNF-α and interleukin-1 (19, 21). Recently, Cole and Kuettner (16) reported the expression of MMP-8 mRNA in circulating PMN. In addition, the demonstration of MMP-8 expression in cartilage, in particular in human articular chondrocytes (16–18), is of interest and supports our conclusion that MMP-8 transcripts are also present in various types of mesenchymal cells, especially in those collected from chronically inflamed tissues, such as found in rheumatoid arthritis and inflamed gingiva.

The molecular mass of the MMP-8 protein published varies between 85 and 50 kDa, even though forms as small as 20 kDa have been reported by different research groups (7, 12, 39). The variance probably reflects a different degree of MMP-8 glycosylation and whether the enzyme is found in a latent or an active form. In addition, degraded forms of MMP-8 protein may have been detected as well. In this study we observed that MMP-8 was present as a 75–85 kDa protein in PMN. However, only the 50-kDa form was seen in RSF as well as in HUVEC. After deglycosylation of the 75-kDa MMP-8 purified from PMN, the enzyme appeared as four different size bands, one of which

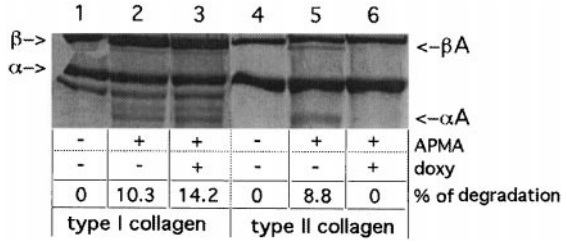

FIG. 7. **Effect of doxycycline on the type I and II collagen degradation by RSF.** RSF were incubated for 24 h with 10 ng/ml TNF-α and the culture media collected. MMPs in the media were activated by incubation for 2 h at 37 °C with 1 mM APMA. *Lane 1*, control: 1.5 μM type I collagen incubated for 12 h at 22 °C with TNC buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.8); *lane 2*, same as *lane 1* after incubation with 1 mM APMA-treated RSF culture medium; *lane 3*, same as *lane 2*, but in the presence of 50 μM doxycycline (*doxy*); *lane 4*, 1.5 μM type II collagen incubated for 12 h at 22 °C with TNC buffer; *lane 5*, same as *lane 4*, but after incubation with 1 mM APMA-treated RSF culture medium; *lane 6*, same as *lane 5* but in the presence of 50 μM doxycycline. α indicates intact type I and II collagen polypeptides, and αA indicates characteristic 3/4 cleavage products resulting from collagenase cleavage of intact collagen.

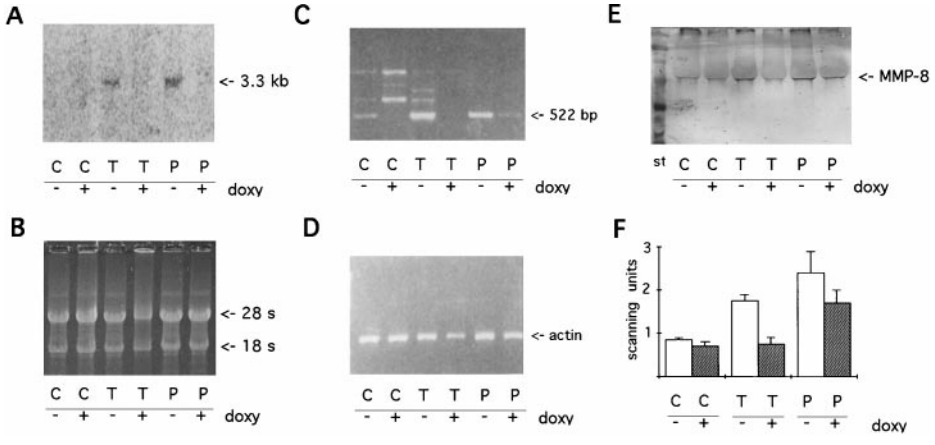

FIG. 8. **Effects of doxycycline on MMP-8 expression in RSF.** RSF were incubated for 24 h with 10 ng/ml TNF-α (*T*), 10 nM PMA (*P*), or without mediator (*C*) in the presence (+) or absence (−) of 50 μM doxycycline. MMP-8 mRNA expression was analyzed by Northern analysis (*panels A* and *B*) and RT-PCR (*panels C* and *D*), and MMP-8 protein expression was analyzed by Western blotting (*panels E* and *F*). *Panel A*, total RNA was isolated, and RNA samples (7.5 μg) were subjected to Northern blotting and hybridized using ^{32}P-labeled MMP-8 specific DNA probe. The size of the 3.3-kb MMP-8 is marked by an *arrow*. *Panel B*, ethidium bromide-stained agarose gel of the same RNA samples as in *panel A*, showing the ribosomal RNAs (18 S and 28 S) and demonstrating equal RNA loading. *Panel C*, RT-PCR analysis. RNA samples were transcribed into cDNA by RT; then, using specific MMP-8 oligonucleotides, MMP-8 transcripts were amplified by PCR and subjected to agarose gel electrophoresis. *Panel D*, RT-PCR of β-actin RNA using β-actin-specific oligonucleotides. RNA samples were the same as described in *panel A*. E. Western blot analysis. 200 μl of the corresponding culture media was freeze-dried and subjected to Western blotting. The antibody was specific for human neutrophil collagenase (MMP-8) and did not recognize human fibroblast collagenase (MMP-1). Mobilities of the molecular weight markers (St) are indicated. F. The bands of two Western blots (RSF passages 2 and 6) were scanned for intensities and are shown as a graphic representation.

corresponded to the size of MMP-8 expressed by RSF. Deglycosylation of the 50-kDa MMP-8 from RSF or HUVEC showed that this secreted form of MMP-8 is glycosylated significantly less or hardly at all compared with the PMN MMP-8. Therefore, it can be speculated that subgranularly stored MMP-8 in PMN requires carbohydrate moieties, whereas a low or nonglycosylated form is secreted as a latent enzyme, similar to other secreted MMPs.

Doxycycline, a commonly used broad spectrum antibiotic, inhibits MMP-8 activity at low concentrations but not that of MMP-1 (7, 34, 40). We confirmed these studies showing that the activity of MMP-8, including the nonglycosylated form secreted by RSF and HUVEC, is inhibited efficiently by 50 μM doxycycline. Lauhio *et al.* (40) have shown that long term doxycycline treatment reduced MMP-8 serum levels in reactive arthritis. The mechanism of this reduction is not known. Therefore, we investigated whether doxycycline may affect MMP-8 synthesis. It was observed that after preincubation with 50 μM doxycycline in RSF as well as in HUVEC both MMP-8 mRNA and MMP-8 protein expression was down-regulated, indicating that doxycycline inhibits not only MMP-8 activity but also its synthesis.

With respect to the demonstrated anti-arthritic effects of doxycycline (7, 40, 41) and the related chemically modified tetracyclines (42), our present findings are relevant to the recent observation that MMP-8 uniquely exerts "aggreganase activity" (17, 38). On the basis of the present and previously published *in vivo* and *in vitro* results (7, 40, 41), this inhibition of degrading activity can be realized by doxycycline concentration attainable *in vivo* (41), at the level of both proteolytic activity and synthesis. Because it has become evident that human cytokine-stimulated synovial fibroblasts, endothelial cells (this study), and joint chondrocytes (18) produce mesenchymal-type MMP-8 species, doxycycline and probably also tetracyclines non-antimicrobial chemically modified (CMTs) may be potential drugs for counteracting the degradation of both articular collagen and proteoglycans *in vivo*.

In summary, previously MMP-8 was considered to be uniquely expressed by neutrophils during their development in bone marrow, and thus MMP-8 was referred to as neutrophil collagenase. The results of the present study show that in addition to peripheral blood PMNs several non-PMN lineage cells, such as fibroblasts and endothelial cells, are able to express MMP-8 mRNA and protein both *in vivo* and *in vitro*. Therefore, we suggest that in future human neutrophil collagenase (MMP-8) should be referred to as collagenase-2 (Cl-2).

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REFERENCES

1. Woessner, J. F. (1991) *FASEB J.* **5,** 2145–2154
2. Matrisian, L. M. (1992) *BioEssays* **14,** 455–463
3. Murphy, G., and Docherty, A. J. P. (1992) *Am. J. Respir. Cell Mol. Biol.* **7,** 120–125
4. Weiss, S. J. (1989) *N. Engl. J. Med.* **320,** 365–376
5. Konttinen, Y. T., Lindy, O., Suomalainen, K., Ritchlin, C., Saari, H., Vauhkonen, M., Lauhio, A., Santavirta, S., and Sorsa, T. (1991) *Matrix* **11,** 395–403
6. Tschesche, H., Bakowski, B., Shettler, A., Knäuper, V., and Reinke, H. (1991) *Biomed. Biochem. Acta* **50,** 755–761
7. Sorsa, T., Ding, Y., Salo, T., Lauhio, A., Teronen, O., Ingman, T., Ohtani, H., Andoh, N., Takeha, S., and Konttinen, Y. T. (1994) *Ann. N. Y. Acad. Sci.* **732,** 112–131
8. Goldberg, G. I., Wilhelm, S. M., Kronberger, A., Bauer, E. A., Grant, G. A., and Eisen, A. Z. (1986) *J. Biol. Chem.* **261,** 6600–6605
9. Hasty, K. A., Jeffrey, J. J., Hibbs, M. S., and Welgus, H. G. (1987) *J. Biol. Chem.* **262,** 10048–10052
10. Freije, J. M., Diez-Itza, I., Balbin, M., Sanchez, L. M., Blasco, R., Tolivia, J., and Lopez-Otin, C. (1994) *J. Biol. Chem.* **269,** 16766–16773
11. Schmid, T. M., Mayne, R., Jeffrey, J. J., and Linsenmayer, T. F. (1986) *J. Biol. Chem.* **261,** 4184–4189
12. Seltzer, J. L., Adams, S. A., Grant, G. A., and Eisen, A. Z. (1981) *J. Biol. Chem.* **256,** 4662–4668
13. Michaelis, J., Vissers, M. C. M., and Winternbourn, C. C. (1990) *Biochem. J.* **270,** 809–814
14. Sottrup-Jensen, L., and Birkedal-Hansen, H. (1989) *J. Biol. Chem.* **264,** 393–401
15. Mainardi, C. L., Pourmotabbed, T. F., and Hasty, K. A. (1991) *Am. J. Med. Sci.* **302,** 171–175
16. Cole, A. A., and Kuettner, K. E. (1995) *Acta Orthop. Scand.* **26,** (suppl.) 98–102
17. Chubinskaya, S., Huch, K., Mikecz, K., Cs-Szabo, G., Hasty, K. A., Kuettner, K. E., and Cole, A. A. (1996) *Lab. Invest.* **74,** 232–240
18. Cole, A. A., Chubinskaya, S., Schumacher, B., Huch, K., Cs-Szabo, G., Yao, J., Mikecz, K., Hasty, K. A., and Kuettner, K. E. (1996) *J. Biol. Chem.* **271,** 11023–11026
19. Unemori, E. N., Hibbs, M. S., and Amento, E. P. (1991) *J. Clin. Invest.* **88,** 1656–1662
20. Jaffe, A. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) *J. Clin. Invest.* **52,** 2745–2752
21. Hanemaaijer, R., Koolwijk, P., le Clercq, L., de Vree, W. J. A., and van Hinsbergh, V. W. M. (1993) *Biochem. J.* **296,** 803–809
22. Salo, T., Lyons, J. G., Rahemtulla, F., Birkedal-Hansen, H., and Larjava, H. (1991) *J. Biol. Chem.* **266,** 11436–11441
23. Oikarinen, K. S., and Seppä, S. T. (1987) *Endod. Dent. Traumatol.* **3,** 95–99
24. Kylmäniemi, M., Oikarinen, A., Oikarinen, K., and Salo, T. (1996) *J. Dent. Res.* **75,** 919–926
25. Boyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21,** 77–89
26. Hibbs, M. S., Hasty, K. A., Kang, A. H., and Mainardi, C. L. (1984) *Collagen Relat. Res.* **4,** 467–477
27. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Tonetti, M. S., Freiburghaus, K., Lang, N. P., and Bickel, M. (1993) *J. Periodont. Res.* **28,** 511–513
30. Ballagy-Pordany, A., Ballagy-Pordany, A., and Funa, K. (1991) *Anal. Biochem.* **196,** 89–94
31. Bergmann, U., Michaelis, J., Oberhoff, R., Knäuper, V., Beckmann, R., and Tschesche, H. (1989) *J. Clin. Chem. Clin. Biochem.* **29,** 351–359
32. Hemmilä, L., Dakubu, S., Mukkala, V.-M., Siitari, H., and Lövgren, T. (1984) *Anal. Biochem.* **137,** 335–343
33. Mallya, S. K., Mookhtiar, K. A., Gao, Y., Brew, K., Dioszegi, M., Birkedal-Hansen, H., and van Wart, H. E. (1990) *Biochemistry* **29,** 10628–10634
34. Suomalainen, K., Sorsa, T., Golub, L. M., Ramamurthy, N., Lee, H. M., Uitto, V. J., Saari, H., and Konttinen, Y. T. (1992) *Antimicrobiol. Agents Chemother.* **36,** 227–229
35. Sepper, R., Konttinen, Y. T., Ding, Y., Takagi, M., and Sorsa, T. (1995) *Chest* **107,** 1641–1647
36. Power, C., O'Connor, C. M., MacFarlane, D., O'Mahonney, S., Gaffney, K., Hayes, J., and FitzGerald, M. X. (1994) *Am. J. Respir. Crit Care Med.* **150,** 818–822
37. Matsuki, H., Fujimoto, N., Iwata, K., Knäuper, V., Okada, Y., and Hayakawa, T. (1996) *Clin. Chim. Acta* **244,** 129–143
38. Fosang, A. J., Last, K., and Neame, J. (1994) *Biochem. J.* **304,** 347–351
39. Hasty, K. A., Hibbs, M. S., Kang, A. H., and Mainardi, C. (1986) *J. Biol. Chem.* **261,** 5645–5650
40. Lauhio, A., Salo, T., Ding, Y., Konttinen, Y. T., Nordström, D., Tschesche, H., Lahdevirta, J., Golub, L. M., and Sorsa, T. (1994) *Clin. Exp. Immunol.* **98,** 21–28
41. Lauhio, A., Salo, T., Tjäderhane, L., Lähdevirta, J., Golub, L. M., and Sorsa, T. (1995) *Lancet* **346,** 645–646
42. Golub, L. M., Suomalainen, K., and Sorsa, T. (1992) *Curr. Opin. Dent.* **2,** 80–90

Matrix Metalloproteinase-8 Is Expressed in Rheumatoid Synovial Fibroblasts and Endothelial Cells: REGULATION BY TUMOR NECROSIS FACTOR- α AND DOXYCYCLINE

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