Cloning of the gene for interstitial collagenase-3 (matrix metalloproteinase-13) from rabbit synovial fibroblasts: differential expression with collagenase-1 (matrix metalloproteinase-1)

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Cartilage, bone and the interstitial stroma, composed largely of the interstitial collagens, types I, II and III, are remodelled by three members of the metalloproteinase (MMP) family, collagenase-1 (MMP-1), collagenase-2 (MMP-8) and collagenase-3 (MMP-13). MMP-1 and MMP-13 may contribute directly to disease progression, since they are induced in patients with rheumatoid arthritis and osteoarthritis. The study of MMP-1 and MMP-13 gene regulation in models of arthritic disease has been problematic because mice and rats, which are typically used, only possess a homologue of MMP-13. Here we show that in contrast with mice and rats, rabbits possess distinct genes homologous to human MMP-1 and MMP-13. Furthermore, rabbit MMP-13 is expressed simultaneously with MMP-1 in chondrocytes and synovial fibroblasts in response to the cytokines interleukin-1 and tumour necrosis factor-α, or the phorbol ester PMA. The time course of MMP-13 induction is more rapid and transient than that of MMP-1, suggesting that distinct mechanisms regulate the expression of these two collagenases. We have cloned the rabbit MMP-13 gene from synovial fibroblasts and demonstrated that the rabbit gene shares greater homology with human MMP-13 than does the mouse interstitial collagenase. Together with the fact that mice and rats do not possess a homologue to human MMP-1, our data suggest that the rabbit provides an appropriate model for studying the roles of interstitial collagenases in connective-tissue diseases, such as rheumatoid arthritis and osteoarthritis.

INTRODUCTION

In rheumatoid arthritis (RA) and osteoarthritis (OA), articular cartilage and bone, which consist of interstitial collagens types I, II and III, are degraded, resulting in joint deformation and pain [1]. Consequently, the enzymes that most effectively degrade interstitial collagens play an important role in the progression of such diseases. The matrix metalloproteinases (MMPs) are zinc- and calcium-dependent enzymes, which together are capable of degrading all components of the extracellular matrix [2]. Collagenase-1 (MMP-1) was the first member of the MMP family to be characterized, and is expressed by most connective-tissue cells and degrades collagens I, II and II at neutral pH [3,4]. Neutrophil collagenase (MMP-8) [5] also degrades collagens I–III, and is expressed in polymorphonuclear phagocytes as stored granules that are released upon cell activation [2]. A recently described enzyme, collagenase-3 (MMP-13) [6,7], also degrades collagens I-III, but is more effective at degrading collagen II than MMP-1 [7]. Unlike MMP-8, MMP-1 and MMP-13 are secreted as soon as they are synthesized [4].

Of the three human interstitial collagenases described, MMP-1 and MMP-13 are most related with respect to nucleic acid/protein sequence and tissue distribution. MMP-1 and MMP-13 share 86% identity at the amino acid level [6], while MMP-8 shares only 57% identity with MMP-1 [5]. Furthermore, in contrast with the tissue-specific expression of MMP-8, MMP-1 and MMP-13 are expressed in a variety of connective tissues. MMP-1 is expressed in fibroblasts, macrophages and chondrocytes, as well as certain tumour cells [4]. MMP-13 is also expressed in chondrocytes [7], although it was originally cloned from a breast carcinoma [6]. The genes for both of these enzymes are induced by inflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF) [3,7–11], which may explain their proposed roles in the pathogenesis of OA and RA [7,11–13].

While it is clear that inflammatory cytokines induce MMP-1 and MMP-13 in the cells of the joint, the role of each enzyme in the progression of disease has not been defined. This is due, in part, to the lack of an appropriate animal model in which to study expression of the two enzymes. Several laboratories have employed models of experimental arthritis in mice and rats to study arthritic disease. However, in both animals, there is only one interstitial collagenase that has been described and it shares 86% homology with human MMP-13 [6,14,15], but not with the human or rabbit MMP-1 [15]. This suggests that the mouse and rat models may not accurately depict the role of all of the interstitial collagenases in cartilage and bone degradation.

As an alternative to mice and rats, the rabbit has been a powerful model for the study of rheumatoid and osteoarthritic arthritic disease [16–18]. Moreover, because the human and rabbit MMP-1 genes share greater than 87% homology [19], important information about MMP-1 gene expression has been derived from studies *in vitro* using rabbit synovial fibroblasts [19–24] and chondrocytes [25–28]. However, the rabbit homologue to human MMP-13 has not been described previously. Here we demonstrate that rabbit synovial fibroblasts contain a gene homologous to human MMP-13 that is distinct from MMP-1. Rabbit MMP-13 is inducible in synovial fibroblasts at

Abbreviations used: DMEM, Dulbecco's modified Eagle's media; FCS, fetal-calf serum; IL-1, interleukin-1; MMP, matrix metalloproteinase; OA, osteoarthritis; RA, rheumatoid arthritis; RAGE, rapid amplification of genomic ends; RF, rabbit synovial fibroblast; RTPCR, reverse-transcriptase PCR; TNF, tumour necrosis factor-α.

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the mRNA and protein levels by inflammatory cytokines or phorbol esters, and this induction profile is distinct from that of MMP-1. Cloning of the cDNA for rabbit MMP-13 demonstrates that the rabbit gene has greater homology with the human gene than does the mouse gene, suggesting that the rabbit may be a more appropriate animal model for the study of interstitial collagenase gene expression in disease.

EXPERIMENTAL

Cell culture and reagents

Rabbit synovial and dermal fibroblast cultures were prepared by digesting synovium and skin with bacterial collagenase A (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) and plating in 20% fetal-calf serum (FCS) in Dulbecco's modified Eagle's media (DMEM), supplemented with PenStrep and glutamine (Gibco/BRL, Gaithersburg, MD, U.S.A.). Cultures were maintained in 10% FCS in DMEM, supplemented with PenStrep and glutamine, and used between the third and seventh passage. Porcine chondrocytes were prepared by collagenase digestion of cartilage derived from pig knees collected from the slaughter house, and plated in DMEM supplemented with 10% FCS. Recombinant human IL-1β and TNF were purchased from Genzyme Corporation (Cambridge, MA, U.S.A.). Phorbol myristate acetate was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Southern-blot analysis of human and rabbit genomic DNA

Genomic DNA from human peripheral blood mononuclear cells and rabbit synovial fibroblasts was digested with restriction endonucleases (*Eco*RI, *Hin*dIII, *Bam*HI, *Sac*I, *Nco*I and *Eco*RV) and subjected to Southern-blot analysis as previously described [29]. The resulting blots were probed with an 800 bp human MMP-13 cDNA fragment [6] using the Renaissance chemiluminescent detection system (New England Nuclear, Boston, MA, U.S.A.) according to the manufacturer's protocol. The blots were then stripped and probed with a 1700 bp human MMP-1 cDNA fragment [23].

Northern-blot analysis of rabbit synovial fibroblast and porcine chondrocyte mRNA

Confluent cultures of synovial fibroblasts and chondrocytes were placed in serum-free media and stimulated as described. Cultures were washed with Hank's buffered salt solution and homogenized in 1 ml of Triazol Reagent (Gibco/BRL). Total RNA was extracted as per the manufacturer's instructions, and 10 μg were run on a 1% agarose gel containing 12% formaldehyde [30]. Gels were stained with ethidium bromide and photographed, blotted on to GeneScreen membranes (New England Nuclear) and probed with the cDNAs described above using the Renaissance chemiluminescent detection system according to the manufacturer's protocol. RNA from explant cultures of human osteoarthritic cartilage and normal rabbit articular cartilage was prepared and subjected to Northern-blot analysis for MMP-1 and MMP-13 as described previously [7].

Western-blot analysis of MMP-13

Cells were cultured to confluence, washed with Hank's buffered salt solution and placed in serum-free media. Following stimulation, media was removed and precipitated with 10% trichloroacetic acid at 4 °C. Protein pellets were resuspended in 2 × reducing sample buffer [30] and run on SDS/PAGE gels. Gels were transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) and probed with a rabbit anti-human MMP-13 antibody (provided by Pfizer Inc., Groton, CT, U.S.A.). This antibody was titred against 10 ng of MMP-13, which was readily detectable using a 1:10000 concentration. At dilutions between 1:5000 and 1:80000, very strong reactivity for MMP-13 (100 ng), and faint-to-undetectable reactivity to MMP-1(100 ng) was observed. MMP-13-specific bands were then visualized on X-ray film by chemiluminescence (ECL; Amersham Life Sciences, Bucks, U.K.).

PCR cloning of the rabbit MMP-13 gene

The majority of the putative rabbit MMP-13 open reading frame was obtained by reverse-transcriptase PCR (RTPCR) using a 5′ primer (5′-TCCTCTTCTTGAGCTGGACTC-3′) corresponding to nt +30–+50, and a 3′ primer (5′-GTTCCAGCCA-CGCATAGTCAT-3′) corresponding to nt +1570–+1590 of the human MMP-13 gene. The product was sequenced using the ABI Prism Cycle Sequencing kit (Applied Biosystems, Inc., Warrington, Cheshire, U.K.) and analysed on an ABI DNA sequencer. The first 14 nucleotides of open reading frame and the putative 5′ untranslated region were obtained by rapid amplification of genomic ends (RAGE). Briefly, genomic fragments were cloned into pBluescript (SK-) (Stratagene, La Jolla, CA, U.S.A.) and then amplified using an SK-specific primer and a 3′ primer (5′-CAGGATTCAGAGGATGGTAGT-3′) corresponding to nt +164–+184 of the rabbit MMP-13 gene.

A second RTPCR reaction was performed, using a 5′ primer (5′-TCAAGATGCAGCCAGGTGTC-3′) and a 3′ primer (5′-AACTAAGCTTTGCCCTGAA-3′) that corresponded to nt +24–+34 and +1531–+1549 of the rabbit MMP-13 gene, respectively. A second RAGE reaction was performed using the SK-specific primer and a 3′ primer (5′-CAAATCATCATC-CTCGTTACT-3′) corresponding to nt +100–+120 of the rabbit gene. The sequence derived from these reactions confirmed the sequence derived from the first round of amplifications.

The putative 3′ untranslated region of the rabbit MMP-13 gene was obtained by RTPCR using a 5′ primer (5′-CTGTTA-CGAGAAAAATGGTTA-3′) corresponding to nt +1325–+1346 of the rabbit gene and an oligo-dT 3′ primer. The sequence was confirmed by two additional amplifications of this product using two separate internal 5′ primers (nt +1366–+1885, 5′-CCCATACAGTTTGAATACAG-3′; nt +1592–+1612, 5′-CTGTATGACTATGCGTGGCTG-3′) and the oligo-dT primer.

RESULTS

Regulation of MMP-13 gene expression in rabbit synovial fibroblasts

To determine if, in addition to the MMP-1 gene, the rabbit genome contains a homologue of MMP-13, rabbit and human genomic DNAs were probed with a 800 bp fragment of the human MMP-13 cDNA [6]. This probe detected restriction fragment patterns that were shared (*Bam*HI, *Sac*I and *Eco*RV) and divergent (*Eco*RI, *Hin*dIII and *Nco*I) between human and rabbit (results not shown). Further analysis using a cDNA probe for human MMP-1 [22] demonstrated that the rabbit sequences recognized by the MMP-13 probe were unique and not due to cross-hybridization with rabbit MMP-1 (results not shown). Thus, there is a rabbit homologue to human MMP-13 that is distinct from the previously described interstitial collagenase, MMP-1.

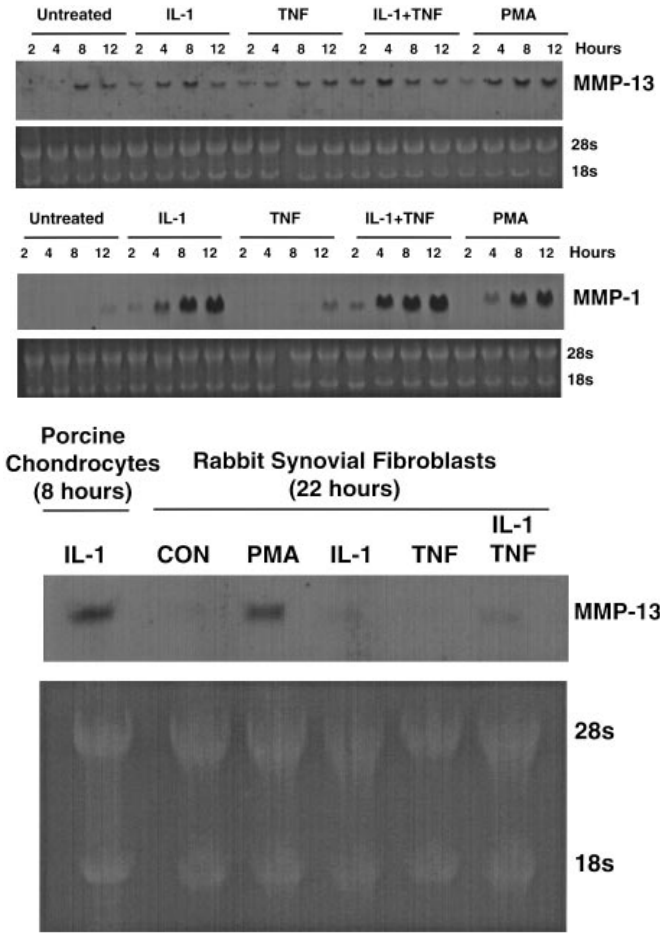

Figure 1 Expression of MMP-13 and MMP-1 mRNA in RFs

RFs were cultured for the indicated times in the absence or presence of IL-1β (10 ng/ml), TNF (10 ng/ml), IL-1 plus TNF, or PMA (10 nM). Total RNA (10 μg) was isolated and analysed by Northern blot using cDNA probes for human MMP-13 [6] (top and bottom panels) and rabbit MMP-1 [35] (top panel). Total RNA from monolayer cultures of porcine chondrocytes that were stimulated for 8 h with IL-1α were included as a positive control for MMP-13 gene expression (bottom panel). Equal loading of total RNA is demonstrated by ethidium bromide staining of 28 S and 18 S rRNA. CON, negative control.

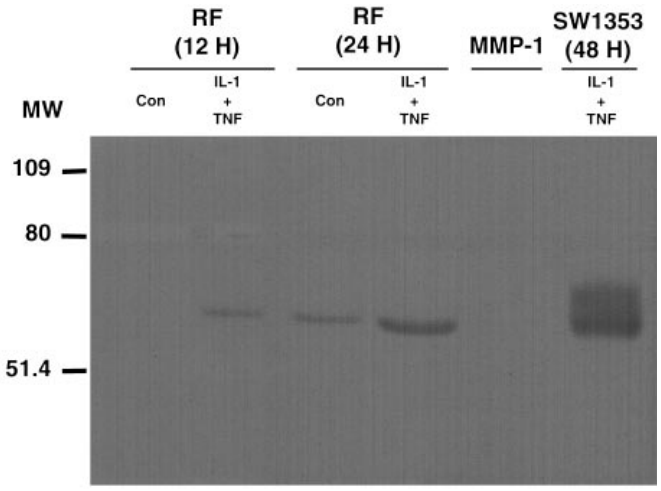

Figure 2 Secretion of MMP-13 protein from RFs

RFs were cultured in the absence or presence of IL-1 plus TNF (10 ng/ml each) for 12 and 24 h. Culture supernatants were then harvested, precipitated with cold 10% trichloroacetic acid, and analysed by Western-blot analysis with a polyclonal anti-human MMP-13 antibody. As a negative control (Con), an equal volume of culture supernatant from baby hamster kidney cells, programmed to constitutively express high levels of MMP-1 [36], was prepared in the same way. As a positive control, culture supernatant from a human chondrosarcoma cell line (SW1353), treated for 48 h with IL-1 and TNF, was also assayed.

Next we examined the expression of interstitial collagenase mRNAs in monolayer cultures of rabbit synovial fibroblasts (RFs). We found that untreated cells do not express detectable levels of MMP-13 mRNA initially, however, MMP-13 levels rise after 8 h of culture in serum-free conditions (Figure 1, top panel), possibly due to the stress of serum deprivation. Treatment of RFs with IL-1 increased MMP-13 mRNA levels at 4 and 8 h, followed by a decline at 12 h (Figure 1, top panel). In contrast, MMP-1 mRNA levels were strongly induced at 8 and 12 h (Figure 1, top panel), confirming our previous findings in RFs [23]. TNF was less effective than IL-1 at inducing MMP-13 or MMP-1 mRNA, but could enhance IL-1-induced collagenase expression (Figure 1, top panel). Cytokine induction of MMP-13 mRNA in RFs was transient, and not detectable above control levels at 22 h (Figure 1, bottom panel). Compared with RFs, MMP-13 was more potently induced in cultured rabbit cartilage, with mRNA levels remaining elevated after 24 h of IL-1 treatment (results not shown). Similarly to IL-1-treatment, MMP-13 mRNA levels increased after 4 h of treatment with the phorbol ester PMA, and this increase preceded the PMA-induced increase of MMP-1 by approximately 4 h (Figure 1, top panel). In contrast with cytokines, PMA induced a sustained time course of mRNA expression, with levels observed at 22 h comparable to those of IL-1-treated primary chondrocytes (Figure 1, bottom panel). Thus, MMP-13 gene expression is induced in RFs by inflammatory cytokines or phorbol esters with a time course that differs from that of MMP-1, suggesting differential mechanisms of regulation for these two collagenase genes. Furthermore, as has been reported for the induction of MMP-1 in RFs [23], the mechanisms controlling PMA- and cytokine-induction of MMP-13 also differ in these cells.

Western-blot analysis of culture supernatants demonstrated that cytokine-treated, but not untreated RFs, secreted detectable MMP-13 protein after 12 h (Figure 2). Low basal MMP-13 gene expression resulted in the accumulation of detectable MMP-13 protein after 24 h, which was enhanced by IL-1 and TNF, confirming the observed increases in mRNA (Figure 1, top panel). Cross-reactivity of the polyclonal rabbit anti-human MMP-13 antibody with recombinant rabbit MMP-1 was minimal (Figure 2), and only detectable with longer chemiluminescent exposures (results not shown). Rabbit MMP-13 protein co-migrated with MMP-13 isolated from human chondrosarcoma cells, although the human cells produced a second, larger $\sim$ 60 kDa species, which may be the result of glycosylation (Figure 2). Thus, MMP-13 protein is secreted by RFs in response to inflammatory cytokines.

Cloning of the rabbit MMP-13 cDNA

The rabbit MMP-13 gene was cloned through RTPCR and RAGE of total RNA and genomic DNA from rabbit synovial fibroblasts with primers designed from the published human sequence [6]. The resulting sequence, which encompasses 645 bp 5′ of the putative transcriptional start site, the 5′ untranslated region, open reading frame and 3′ untranslated region, is depicted in Figure 3 (top panel). Using the ATG start codon located at +29 bp, the open reading frame was translated and aligned with

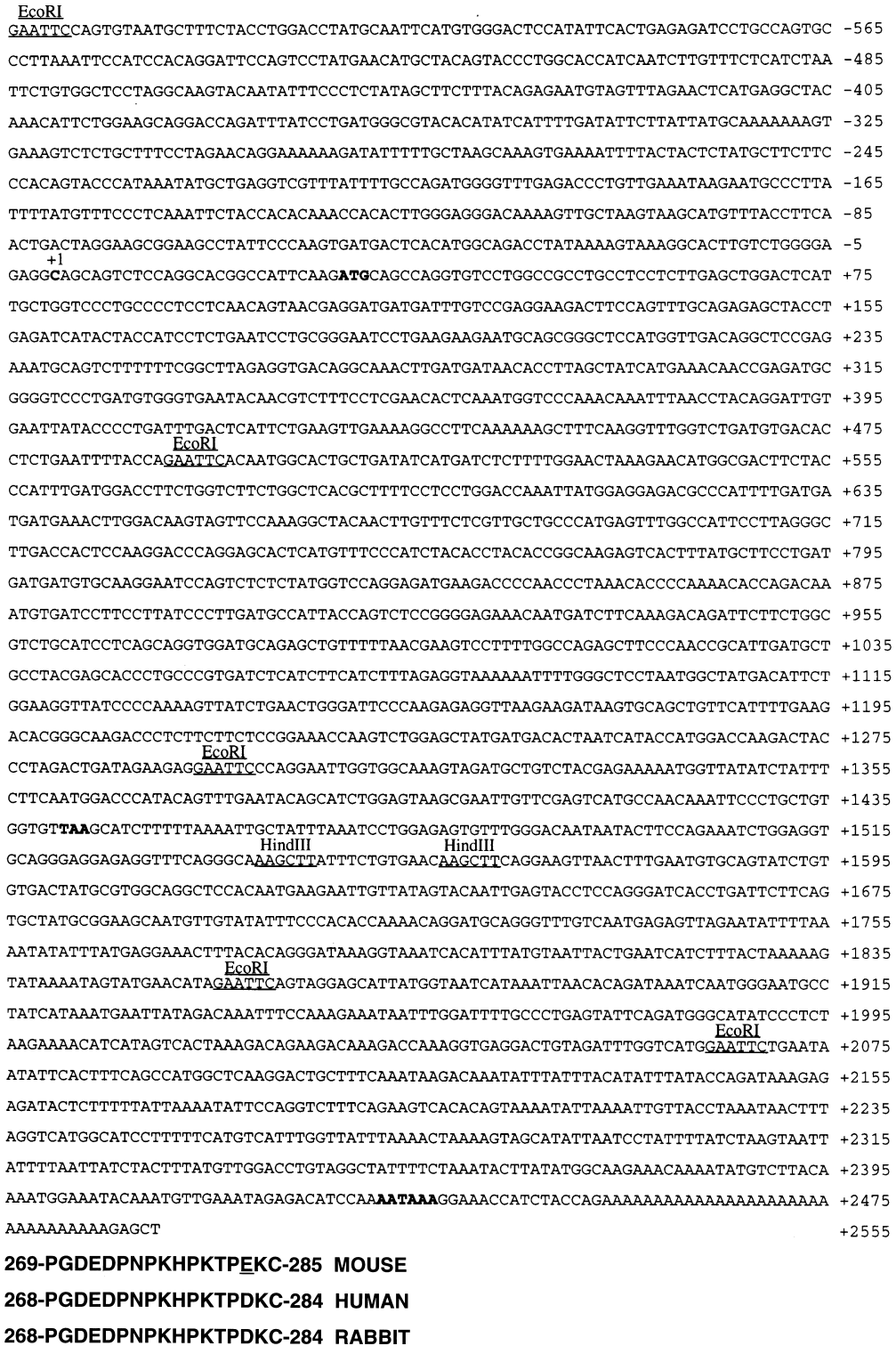

Figure 3 DNA sequence and amino acid homology analysis of the rabbit MMP-13 gene

Top panel. The nucleotide sequence of the rabbit MMP-13 5′ flanking region and cDNA are depicted. The transcriptional start site, translational start codon, translational stop codon and polyadenylation sequence that are homologous to those described in the human gene [6] are shown in bold. Bottom panel. The open reading frame of rabbit MMP-13, and an amino acid sequence alignment of the mouse [14], human [6] and rabbit genes, was created with the Gene Works Sequence Analysis Software. The consensus sequence for these three species is shown within the interstitial collagen substrate specificity domain [6]. The single amino acid substitution present within this region of the mouse gene is underlined.

the published sequences for human [6] and mouse [14] MMP-13 using the Gene Works Sequence Analysis software (Oxford Molecular Group, Campbell, CA, U.S.A.). This analysis demonstrated a high degree of homology among the three species, especially within the 17 amino acids of the human gene (268–284), which are required for the catalytic specificity of interstitial collagenases [6] (Figure 3, bottom panel). Whereas the mouse gene contains one amino acid change in this region relative to the human, the rabbit gene is 100 % homologous to the human gene. Indeed, when overall amino acid identity is assessed, the rabbit gene is 90 % identical to the human, while the human and mouse share only 86 % identity. Unweighted pair-group method, with arithmetic mean (UPGMA), analysis [31] was performed with the Gene Works software and the calculated genetic distance between rabbit and human (0.051 ± 0.008) was significantly less than the distance between human and mouse (0.072 ± 0.009). Thus, the rabbit genome contains homologues to the two inducible interstitial collagenases, MMP-1 and MMP-13. Furthermore, the rabbit MMP-13 gene has greater homology with human MMP-13 than with the gene in mice and rats.

DISCUSSION

The gene for human MMP-13 was originally cloned from a breast carcinoma [6] and then found to be expressed at elevated levels in some samples of osteoarthritic cartilage, as well as IL-1-stimulated cultured chondrocytes [7]. In this study, we have described the cloning and cellular expression of the rabbit homologue of human MMP-13. We found that in addition to MMP-1, MMP-13 can be induced by inflammatory cytokines in both rabbit articular cartilage and synovial fibroblasts, but not in rabbit skin fibroblasts. Of particular interest is our finding that the rabbit MMP-13 shares greater identity with human MMP-13 than does the mouse gene. Considering that MMP-13 is the only interstitial collagenase expressed in the mouse [14,15], our results suggest that the rabbit, which like the human, expresses both MMP-1 and MMP-13, may be a more appropriate animal model for studying the mechanisms regulating the expression of the interstitial collagenase genes.

Indeed, the antigen-induced arthritis model in the rabbit has been used successfully to describe the pathological changes of RA [17,18]. In this model rabbits are sensitized to ovalbumin through intradermal immunizations, then challenged with an intra-articular injection of ovalbumin. Within one day, the joints of these animals express MMP-1 in the cells of the synovium [18] and liberate proteoglycans into the synovial fluid [17,18], two pathological changes characteristic of RA. A role for MMP-13 in both OA and RA has been proposed based on the presence of MMP-13 mRNA and protein in human cartilage and synovium [7,11–13]. With our demonstration of MMP-1 and MMP-13 co-expresson in rabbit cartilage and synovial fibroblasts in response to inflammatory cytokines, the antigen-induced arthritis model in the rabbit may help define the relative roles of interstitial collagenases in the progression of arthritic diseases. This model may also help define additional factors that promote the relatively intense expression of MMP-13 observed in human osteoarthritic cartilage [7].

The expression of MMP-13 in synovial fibroblasts is more temporally restricted than MMP-1. In contrast with MMP-1, which is maximally induced by cytokines within 12–24 h, MMP-13 expression peaks within 4–8 h, and is reduced to basal levels after 22 h. Perhaps this restricted temporal expression of MMP-13 in synovial fibroblasts may explain why it was not detected by one group in human rheumatoid synoviocytes [11]. Furthermore, the more restricted expression may reflect the fact that MMP-13 has a 10-fold greater catalytic activity towards type II collagen than MMP-1 [7]. Type II collagen is a major component of articular cartilage, and unregulated expression of MMP-13 by synovial fibroblasts would have dire consequences for the integrity of the joint. Perhaps in arthritis this strict regulation of MMP-13 is subverted. We found that human osteoarthritic cartilage expressed greater amounts of MMP-13 in response to IL-1 than normal rabbit cartilage (results not shown). Whereas the lower level of expression in the rabbit cartilage could be due to reduced sensitivity to human IL-1, or to lower hybridization efficiency with the human MMP-13 probe, it is also conceivable that MMP-13 is more readily induced in arthritic tissues by the mixture of inflammatory cytokines present in OA.

The expression of MMP-13 is also restricted among fibroblastoid cells. In keeping with previous studies [8], we found no expression in response to cytokines or phorbol esters in rabbit or human dermal fibroblasts (results not shown). Thus to date, RFs are the only primary cell type other than chondrocytes that express both MMP-1 and MMP-13. Given the technical difficulties associated with culturing primary chondrocytes, the RF model should prove a useful tool for characterizing the differential regulation of these interstitial collagenases. Our results also suggest that MMP-13 is not required for remodelling of the interstitial collagens of the dermis. In support of this, the dermis is composed entirely of type I and III collagens, which are substrates that are effectively degraded by MMP-1 [32]. Furthermore, stromal fibroblasts, such as those found in the dermis, secrete elevated amounts of MMP-1 in response to various trauma, including UV-B irradiation [33] and mechanical injury [34]. Thus the extracellular matrix and other cell types that surround fibroblastoid cells may help dictate their interstitial collagenase expression profile.

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