Metalloproteinase and tissue inhibitor of metalloproteinase expression in the murine STR/ort model of osteoarthritis

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Summary

Objective: To study the temporal expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the STR/ort mouse model of osteoarthritis, using in situ hybridization with oligonucleotide probes and specific antisera for each protein.

Methods: In situ hybridization and immunolocalization experiments were performed on serial cryosections of knee joints from STR/ort and control CBA mice. The mRNA was localized using digoxygenin-labeled probes.

Results: MMP2, MMP3, MMP7, MMP9, MMP13, MT1-MMP and TIMP2 mRNA was detected in the tibial articular chondrocytes of STR/ort mice at all ages (12, 18, 24, 30 and 35 weeks). Levels were always higher than in age-matched CBA mice. Neither MMP8 nor TIMP1 mRNA was detected in murine cartilage. The location and distribution of each of the MMP mRNA transcripts varied within the tibial plateau. Immunolocalization consistently detected MMP3 and MT1-MMP in articular cartilage and MMP13 in calcified cartilage. Other proteases and their inhibitors were not detected in either of these cartilages but MMP2 and MMP9 were immunolocalized in bone marrow cells and growth cartilage respectively.

Conclusion: Expression of all the detected MMPs and TIMP-2 is up-regulated in STR/ort mice at the mRNA level. However, failure to detect protein expression for MMPs 2, 7, 9, 13 and TIMPs 1 and 2 in murine chondrocytes by immunohistochemistry indicates that the changes in mRNA levels in STR/ort mice must be interpreted with caution. © 2002 OsteoArthritis Research Society International. Published by Elsevier Science Ltd. All rights reserved.

Key words: Osteoarthritis, Metalloproteinase, Cartilage, Animal model.

Introduction

The limited availability of normal human joint tissues and of tissues from patients with early osteoarthritis (OA) impede study of the disease in man. What causes the disease is unknown. Thus many animal models have been developed to investigate mechanisms underlying the disease. For example, degenerative lesions can be induced chemically, by the injection of toxins or enzymes, or by surgical disruption of the joint mechanics. In marked contrast to these models, STR/ort mice are genetically predisposed to develop an OA-like lesion in the medial tibial cartilage. More than 85% of male STR/ort mice show signs of cartilage degeneration by 6 months of age. Usually, fibrillation of articular cartilage starts on the medial tibial plateau adjacent to the attachment site of the anterior cruciate ligament and progresses to erosion, fissures and cartilage loss. The cartilage on the lateral tibial plateau sometimes develops lesions, but these are usually less severe and less frequent than those on the medial plateau. Changes in chondrocyte expression of cytokine, growth factors and matrix components have been identified early in the disease.

Previous studies identified an increase in the amount of proteoglycan in the tibial cartilage of 16–19 week old STR/ort mice and in the expression of aggrecan mRNA, compared to CBA mice which do not develop OA lesions. This is followed by a decrease in proteoglycan content which is also detected by loss of alcin blue staining of the cartilage matrix. Matrix metalloproteinases (MMPs) are thought to be the major mediators of cartilage degradation and we have detected MMP cleavage of aggrecan and collagen in developing OA in the STR/ort mouse. Whether or not specific MMPs and their inhibitors undergo changes in expression at particular times and sites in the cartilage during development of the disease is unknown. However, such information could be used to develop new forms of therapy. Thus the aim of the present study was to map the expression of mRNA and protein for MMP2, MMP3, MMP7, MMP8, MMP9, MMP13, MT1-MMP, TIMP1...
digested with proteinase K (20 min. They were washed twice in PBS for 5 min and formaldehyde in phosphate-buffered saline (PBS) for room temperature and fixed in freshly prepared 4% paraformaldehyde in deionized water (dH2O). The sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine HCl, pH 8 for 10 min, washed twice in 2×SSC and 0.1×SSC for 10 min each at room temperature. The sections were rinsed briefly in a maleic buffer with pH 7.5) and then blocked with 0.5% blocking solution (standard saline citrate, pH 7) and then in 2×SSC, 50% formamide at 50°C. This was followed by sequential washes in 2×SSC and 0.1×SSC for 10 min each at room temperature. The sections were rinsed briefly in a maleic acid buffer (0.1 M maleic acid, 0.15 M sodium chloride, pH 7.5) and then blocked with 0.5% blocking solution (Roche Diagnostics Ltd, Welwyn, U.K.) in maleic buffer for 45 min at room temperature prior to detecting digoxigenin by reaction with an anti-digoxigenin alkaline phosphatase antibody (1:100) (Roche Diagnostics) in blocking solution for 60 min at 20°C. The inclusion of 1% Triton X-100 at this stage helped to block any non-specific antibody binding. This was followed by two washes in maleic acid buffer with 0.3% Tween-20 and then a single wash in dH2O. Hybridization signal in the sections was detected with 5-bromo-4-chloro-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) (Sigma) which was allowed to develop in darkness for 6 h at 37°C for MMP-13 and overnight for MMP2, MMP3, MMP7, MMP8, MMP9 and TIMP2 (Table I).

The probes were prepared commercially (VH Bio Ltd, U.K. or Genosys Ltd, Cambridge, U.K.) to contain one molecule of digoxigenin-dUTP at both the 3’ and 5’ ends. All pairs of antisense and sense probes were checked by dot blots with anti-digoxigenin antibody to ensure equivalent labeling with digoxigenin. Probes were stored at −20°C. A digoxigenin-labeled oligo dT probe (R and D Systems Ltd, Abingdon, U.K.) was used in control hybridizations to detect poly (A) mRNA. The probes were used at a concentration of 2.5 ng μl−1 in hybridization buffer (Amerham International PLC, Amersham, U.K.). Hybridization solution was applied to each section prior to hybridization overnight in an Omnislide humidity chamber (Hybaid Ltd, London, U.K.) or a Boekel slide moist (GRI Ltd, U.K.) at 42°C. Control sections were exposed to hybridization solution with no probe, or were RNase treated prior to hybridization with antisense or sense oligonucleotide. Sections were then washed, initially in 5×SSC (standard saline citrate, pH 7) and then in 2×SSC, 50% formamide at 50°C. The sections were then hybridized to digoxigenin. Probes were stored at −20°C. A digoxigenin-labeled oligo dT probe (Roche Diagnostics Ltd, Welwyn, U.K.) in maleic buffer for 45 min at room temperature prior to detecting digoxigenin by reaction with an anti-digoxigenin alkaline phosphatase antibody (1:100) (Roche Diagnostics) in blocking solution for 60 min at 20°C. The inclusion of 1% Triton X-100 at this stage helped to block any non-specific antibody binding. This was followed by two washes in maleic acid buffer with 0.3% Tween-20 and then a single wash in dH2O. Hybridization signal in the sections was detected with 5-bromo-4-chloro-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) (Sigma) which was allowed to develop in darkness for 6 h at 37°C for MMP-13 and overnight for MMP2, MMP3, MMP7, MMP8, MMP9, MT1-MMP, TIMP1 and TIMP2. The development was stopped by briefly rinsing the section and TIMP2 in tibial articular cartilage during development of OA in the STR/ort mouse. All of these MMPs and TIMPs have been implicated in the degeneration of cartilage in human OA12–17.

Materials and methods

IN SITU HYBRIDIZATION

All glassware was cleaned in 2% absolve (DuPont Ltd, U.K.) and autoclaved before use. Groups of six male STR/ort mice were analysed at age 12, 20, 25, 30 and 35 weeks. Two age- and sex-matched CBA mice were used as controls at each age. Tissues from each mouse were prepared and processed as above. Tissues from each mouse were analyzed. Serial unfixed and undecalcified sections of the knee joint were cut through the whole knee joint using a cryostat with an automatic drive to ensure sections of constant thickness7. The sections were flash-dried on to electrostatically charged glass slides (BDH Ltd, Poole, U.K.) and baked at 200°C. Solutions were prepared using deionized water treated with 0.1% diethylpyrocarbonate.

Table I

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Sequence (5′–3′)</th>
<th>Probe position</th>
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<td>MMP2</td>
<td>CTTGTACATGGATTCTTGGTAAGCTTACTTGG</td>
<td>1503–1531</td>
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<td>CCAATATACCCGCTTCTGAAGAGAAGA</td>
<td>(NM 008610)</td>
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<td>(NM 010810)</td>
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<tr>
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<td>(NM 010809)</td>
</tr>
<tr>
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<tr>
<td>TIMP1 antisense</td>
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<td>TIMP2</td>
<td>AAGCCTCGCGGACACGACCGATTGAC</td>
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<tr>
<td>TIMP2 antisense</td>
<td>GGCATCAAGGGATAGGGGTTCTGGTGTTACCTT</td>
<td>(NM 010809)</td>
</tr>
</tbody>
</table>

Target mRNA Sequence (5′–3′)

Complement of the antisense nucleotide sequence (MMP3, MMP13, MT1-MMP and TIMP1), while for other probes it was a scrambled version of the antisense nucleotide sequence (MMP2, MMP7, MMP8, MMP9 and TIMP2) (Table I).

In situ hybridizations with one or, at most, two different mRNA probes (see below) so overall sections from 250 STR/ort mice and 50 CBA mice were analysed. Serial unfixed and undecalcified sections of 10 μm were cut through the whole knee joint using a cryostat with an automatic drive to ensure sections of constant thickness7. The sections were flash-dried on to electrostatically charged glass slides (BDH Ltd., Poole, U.K.) and stored dry at −70°C. Sections were thawed at room temperature and fixed in freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min. They were washed twice in PBS for 5 min and digested with proteinase K (20 μg ml−1) in 50 mM Tris HCl buffer, 5 mM EDTA, pH 8, for 10 min at 37°C, after which they were washed in PBS, refixed in 4% paraformaldehyde for 20 min and rinsed in distilled water (dH2O). The sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine HCl, pH 8 for 10 min, washed twice in PBS and air-dried prior to hybridization. Oligonucleotide sense and antisense probes for gelatinase A (MMP2), stromelysin (MMP3), matrilysin (MMP7), neutrophil collagenase (MMP8), gelatinase B (MMP9), collagenase-3 (MMP13), membrane type-1 metalloproteinase (MMP14/MT1-MMP), TIMP1 and TIMP2 were designed from the murine gene sequences using Lasergen software (DNAStar Inc, Madison, U.S.A.) to avoid cross-hybridization with other MMPs18. In some cases the sense probe was the reverse complementary of the antisense nucleotide sequence (MMP3, MMP13, MT1-MMP and TIMP1), while for other probes it was a scrambled version of the antisense nucleotide sequence (MMP2, MMP7, MMP8, MMP9 and TIMP2) (Table I).
twice with dH2O, drying in air and mounting in aqueous
mountant.
Hybridization signals were graded independently by two
observers using the following scale; nil (−), weak (+/−),
positive (+) or strongly positive (++). The sections were
also graded histologically for evidence of osteoarthritis
damage to the articular cartilage as described previously by
(7). Grade 0=no change in cartilage; 1=roughened articu-
lar surface and minor fibrillations; 2=fibrillations down to
zone 2 and some loss of surface lamina; 3=loss of surface
lamina and fibrillations extending down to the calcified
cartilage; 4=major fibrillations and cartilage erosion down
to the subchondral bone; 5=major fibrillations and erosion
of up to 80% of the cartilage; 6=greater than 80% loss of
cartilage; NC=no cartilage present.

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In preliminary experiments approximately 10 STR/ort
mice (aged 12–50 weeks) were analysed for MMP2, 3, 7, 9,
MT1-MMP and TIMP1 and 2 by immunohistochemistry. For
MMP13, a total of 32 STR/ort mice (aged 6 days to 52
weeks) and 14 CBA mice (aged 12–50 weeks) were
examined. However, due to generally low expression levels
of MMPs and TIMPs, a further series of experiments was
conducted in which knee joints were treated with monensin
after 28, 38 (×2), 40 (×1) and 46 (×1) weeks were evaluated (total
38 (×2), 40 (×1) and 46 (×1) weeks were evaluated (total
weeks) and 14 CBA mice (aged 12–50 weeks) were
analysed for MMP2, 3, 7, 9,

Results

Both the STR/ort and CBA strains of mice showed in situ
hybridization signals for MMP2, MMP3, MMP7, MMP9,
MMP13, MT1-MMP and TIMP2 mRNA in chondrocytes of
the medial and lateral tibial cartilage, whereas mRNAs for
MMP8 and TIMP1 were not detected. Expression was
always much more intense in lesion-free cartilage of STR/
ort mice than in normal cartilage in CBA mice. Signal was
not observed when sections were incubated with hybridiz-
ation buffer in the absence of any antisense oligonucleotide
probe, or when the sections were treated with ribonuclease
prior to hybridization. Some sections hybridized with sense
oligonucleotides gave a weak signal but in every case this
was much lower than that observed with the corresponding
antisense probe. Chondrocytes in the cartilage of all STR/
ort and CBA mice hybridized with an oligo DT probe (results
not shown).

MMP2

MMP2 mRNA is expressed throughout the full depth of
the medial and lateral tibial cartilage of both CBA and
STR/ort mice, with high expression in the superficial and
transitional zones. However, expression is always much
stronger in the STR/ort cartilage [Fig. 1(a),(b)]. There
appears to be no difference in the level, or pattern of MMP2
expression in areas with early or late histopathological
lesions [e.g. Fig. 1(c)]. Hybridization with an MMP2 sense
probe [Fig. 1(d)], or with the antisense oligonucleotide after
RNase treatment (not shown) did not give a signal. No
MMP2 protein was detected in the tibial plates of either
CBA or STR/ort mice, irrespective of whether cartilage
lesions were present or not [Fig. 2(a)]. The immunostained
sections were indistinguishable from control sections incu-
bated with sheep IgG [Fig. 2(b)]. Positive staining for
MMP2 was always seen in bone marrow cells of both
strains of mice, indicating that the immunodetection system
worked [Fig. 2(c)].

MMP3

MMP3 mRNA is expressed in areas of intact STR/ort
cartilage and in CBA cartilage at all ages. Expression is

a concentration of 50 μg ml−1. Normal sheep serum used at
the same concentration acted as a control. After washing
with PBS, sections were incubated for 1 h with a swine anti
sheep FITC-conjugated secondary28 antibody diluted 1 in
100 in PBS containing 5% swine serum. Sections were
then washed in PBS, mounted in Vectorshield containing
propidium iodide (Vector Laboratories, CA, U.S.A.) to coun-
terstain the nuclei, and viewed using an Olympus Provis
microscope. The same protocol was used for the goat
polyclonal antibody to MMP13 but rabbit serum was
substituted for swine serum and detection was with a
rabbit antigen FITC conjugated secondary antibody
(Dako). Preimmune goat serum was used as a control.

MT1-MMP protein was detected using a mouse mono-
clonal antibody (100 μg ml−1) and the Vectorlab MOM
kit with visualization by diaminobenzidine (Vector
Laboratories). Preabsorption of the antibody with the
immunogenic peptide (1 mg ml−1) was used as a control.

Sections were viewed using an Olympus Provis
microscope and brightfield illumination.
Fig. 1. MMP2 expression in murine medial tibial cartilage detected by in situ hybridization with an antisense oligonucleotide probe (a)–(c). (a) 24-week CBA, (b) 24-week STR/ort with no OA lesions, (c) 24-week STR/ort with grade 3 lesions, (d) Adjacent section to that in frame B/C but hybridized with MMP2 sense oligonucleotide probe. AC=articular cartilage. Original magnification ×50.

stronger in both lateral and medial cartilage of STR/ort mice than in CBA mice. The highest level of MMP3 transcripts is found generally throughout the full depth of articular cartilage in the central region of the tibial plateau [Fig. 3(a)]. Damaged cartilage usually exhibits a strong hybridization signal at the surface where the tissue is intact, although the occurrence of this varies considerably between animals [e.g. Fig. 3(b) and (c)]. In contrast MMP3 mRNA expression is frequently reduced in the cells surrounding an OA lesion even though transcripts are observed in other cells throughout the depth of the cartilage [Fig. 3(b)].

MMP3 protein is detected in most medial and lateral tibial cartilages of both STR/ort and CBA mice at all ages [Fig. 2(d)]. The strongest MMP3 immunostaining is associated with middle to upper zone chondrocytes in both strains of mice. However, no staining for MMP3 protein is seen in STR/ort chondrocytes adjacent to OA lesions [Fig. 2(e)], correlating with loss of mRNA in these cells. Interestingly, chondrocytes located 50 μm distant from lesions express MMP3 protein [Fig. 2(f)] as in mice with no OA, demonstrating the focal nature of the disease.

MMP7

Both CBA and STR/ort chondrocytes throughout the depth of the cartilage express MMP7 mRNA at all ages. Again expression levels are higher in STR/ort [Fig. 4(a)] than in CBA mice (not shown). The level of mRNA expression decreases in areas surrounding histopathological lesions [Fig. 4(a),(b)]. MMP7 protein was not detected in any medial cartilages and only in one lateral cartilage in a 38-week-old STR/ort mouse. Here, as with the mRNA, it was expressed throughout the full depth of the cartilage [Fig. 2(g)].

MMP8

No MMP8 mRNA was detected in tibial chondrocytes in either STR/ort or CBA strain mice of any age. Thus MMP8 protein expression was not investigated.

MMP9

MMP9 mRNA is expressed at low levels at all ages, throughout the full depth of the cartilage in CBA and STR/ort mice [Fig. 5(a)]. Signal with the antisense probe was greater than with the sense probe [Fig. 5(b)]. However, the level of mRNA expression is decreased in chondrocytes adjacent to histopathological lesions compared to that in cells in areas of intact cartilage from the same tibial plateau [Fig. 5(c)]. MMP9 protein was not detected in the tibial articular cartilage of any of the mice studied [Fig. 2(h)] but it was present in both resting and hypertrophic chondrocytes of the growth plate [Fig. 2(i)] and in bone marrow cells (not shown) of both strains, confirming that the antibody reacts with the protein in the cryosections.

MMP 13

There is only weak expression of MMP13 mRNA in CBA tibial cartilage [Fig. 6(a)]. In marked contrast MMP13 is expressed strongly in the mid and deep zone chondrocytes of lesion free medial cartilage of STR/ort mice at all ages from 12–35 weeks. Expression is particularly intense in the central region of the medial tibial plateau cartilage [Fig. 6(b)]. Generally the lateral tibial plateau chondrocytes of STR/ort mice also show high levels of MMP13 expression in lesion free medial cartilage [Fig. 6(c)], although the signal is always stronger in the medial cartilage. With the development of histopathological lesions in the medial cartilage there are very distinct decreases in MMP13 mRNA expression, as in chondrocytes in damaged areas [Fig. 6(d)]. mRNA levels increase progressively in cells the further away they are from the lesion [Fig. 6(e)]. Similarly MMP13 mRNA levels were decreased in the proximity of any lesions present in the lateral cartilage.

MMP13 protein is always detected in the cells just below the tidemark in both strains of mice at all ages studied [Fig. 2(j)]. Exactly the same pattern of immunostaining was seen in the calcified cartilage with all three MMP13 antibodies used. In contrast, in the 25 STR/ort mice examined, immunostaining was detected in hyaline articular cartilage of only one 38-week-old animal. This was the same one that was positive for MMP7. However, while there was positive staining in the lateral cartilage (OA grade=0) of this mouse, there was none in the medial cartilage (OA grade=4). Moreover, the MMP13 immunostain was mainly in the lateral plateau chondrocytes just below the superficial zone [Fig. 2(k)] and did not therefore colocalize with the general pattern of elevated mRNA expression in the mid and deep zone chondrocytes in STR/ort mice. Eight other STR/ort mice aged 35–46 weeks showed no immunopositive staining for MMP13 in either the lateral or the medial tibial cartilage. With the exception of one mouse that was unaffected by OA lesions, these animals had grade 3–5 OA lesions in the medial tibial cartilage and one had a grade 1
lesion in the lateral cartilage. No MMP13 immunostaining was observed in the hyaline cartilage of any of the CBA mice.

MT1-MMP

MT1-MMP mRNA is expressed at all ages throughout the full depth of the articular cartilage in STR/ort [Fig. 7(a)] and CBA mice (not shown). STR/ort expression was stronger than in CBA mice in both medial and lateral cartilage. Transcript levels are decreased in regions of cartilage damage in STR/ort mice [Fig. 7(b)], but are prominent in the intact cartilage in the same tibial plateau. MT1-MMP protein was readily detected and is distributed throughout the full depth of the cartilage in both strains of mice [Fig. 7(c)]. Immunostaining on the surface of the cartilage is artifactual since it is still present after pre-absorption of the antibody with the immunogenic peptide [Fig. 7(d)]. MT1-MMP staining is reduced in areas adjacent to OA lesions in STR/ort mice [Fig. 7(e)], while sections distant from lesions show a pattern of staining similar to that seen in animals without lesions [Fig. 7(f)]. The protein expression of MT1-MMP correlates exactly with the mRNA expression pattern.

TIMP1

TIMP1 mRNA and protein were not detected in either STR/ort or CBA mice at any age.
Both CBA and STR/ort mice expressed low levels of TIMP2 mRNA at all ages, throughout the full depth of the medial and lateral tibial plateau [Fig. 8(a)]. Interestingly there are no differences in the TIMP2 mRNA level in chondrocytes surrounding early or late histopathological OA lesions [Fig. 8(b)]. TIMP-2 protein could not be detected at any age in either strain of mice (not shown).

The results of the in situ hybridization and immunolocalization of all MMPs and TIMPS investigated are summarized in Table II.

Discussion

The principal matrix degrading proteinases in articular cartilage are thought to be matrix metalloproteinases (MMPs)\textsuperscript{17}. MMPs cleave the core protein of aggrecan and the helical region of type II collagen in characteristic positions leaving new N- and C-terminal amino acid sequences either side of the cleavage site. These constitute neoepitopes\textsuperscript{29}. We have shown that MMPs, including collagenase, are active in the tibial articular cartilage of CBA and STR/ort mice because such neoepitopes can be detected in the tissue\textsuperscript{10,11}. The C-terminal neoepitope created by MMP cleavage of aggrecan between VDIPEN\textsubscript{341–342}FFGVF of the core protein is present in the pericellular matrix of deep zone tibial chondrocytes of normal cartilage in both strains of mice, indicating a physiological role for these enzymes in the tissue. However, with the development of OA lesions in the STR/ort mouse, marked increases in the level of VDIPEN neoepitope were observed in the pericellular matrix of chondrocytes throughout the full depth of the cartilage and in matrix more distant from cells but adjacent to the lesions themselves\textsuperscript{10}. A similar distribution of aggrecan neoepitopes has been...
reported in normal and osteoarthritic human articular cartilage\textsuperscript{30}. While cleavage of the helical region of type II collagen could not be detected in normal murine tibial cartilage with an antibody recognizing the neoepitope resulting from collagenase activity, this neoepitope was present in the extracellular matrix adjacent to osteoarthritic lesions\textsuperscript{11}. Similarly, collagen cleavages generated by collagenase activity have been demonstrated in human osteoarthritic articular cartilage\textsuperscript{31}. Thus, there can be little doubt that members of the matrix metalloproteinase family play a significant role in both normal cartilage matrix turnover and in accelerated breakdown in OA. Whether all or only some members of this large family of proteinases are involved in the latter process is clearly an important question for the design of new therapeutic approaches in OA, but there is little information available about this. One approach to answering it is to map the expression of known MMPs in articular cartilage before and at various stages during the development of OA. The main conclusion from the present study is that mRNA levels for each of the MMPs investigated are increased in the tibial chondrocytes of the STR/ort mouse compared with those in CBA mice. However, using immunohistochemistry we were unable to correlate this finding with corresponding changes in protein levels for
these enzymes, so the mRNA results must be necessarily interpreted with caution. We discuss the failure to detect enzyme protein changes below, particularly with respect to MMP13, which showed very marked increases in its mRNA pool in STR/ort mice.

Three MMP collagenases have been identified\(^{32}\) and collagenase activity has been reported in osteoarthritic cartilage\(^{33}\). They are collagenase 1 (MMP1), collagenase 2 or neutrophil collagenase (MMP8), and collagenase 3 (MMP13). MMP13 has a higher level of activity against type II collagen, characteristically found in cartilage, than MMP1.\(^{34}\) Rodents appear to express MMP13 predominantly, although a recent report also shows that murine MMP1 is expressed during embryogenesis\(^{35}\). The oligonucleotide probe for MMP13 used in our \textit{in situ} hybridization studies does not show homology with the murine MMP1 sequence. In the present \textit{in situ} hybridization studies we found much higher expression of MMP13 in the mid and deep zone tibial chondrocytes of STR/ort mice than in CBA mice. In contrast, the earliest evidence of collagen cleavage in the development of STR/ort OA was detected in areas of roughening of the articular cartilage surface\(^{11}\). The high level of MMP13 mRNA in STR/ort cartilage was present from the earliest age investigated and before histopathological lesions of OA developed. It decreased in chondrocytes immediately adjacent to well-developed lesions. Transcript levels for several other MMPs also decreased in these cells and at least some of them are apoptotic\(^{36}\). MMP13 mRNA has been detected by \textit{in situ} hybridization in the deep layer of human articular cartilage and expression increased in OA and rheumatoid arthritis\(^{37}\). The enzyme was also located immunohistochemically in the deep zone tibial plateau cartilage of dogs in which OA had been induced experimentally\(^{38}\). Moreover, a synthetic collagenase inhibitor, Ro32-3555, was reported to protect against breakdown of cartilage and bone in STR/ort mice, suggesting involvement of a collagenase in this model of OA\(^{39}\). However, despite our data indicating a much higher level of MMP13 gene expression in pre-lesional STR/ort cartilage than in the CBA strain and the other evidence suggestive of a role for this enzyme in the development of OA, we detected its protein only by immunohistochemistry in the chondrocytes of the lateral cartilage of one STR/ort mouse. Since eight other STR/ort mice of similar age had no immunohistochemically detectable MMP13 in either the lateral or medial tibial cartilage (where OA lesions occur most frequently), the one immunopositive lateral cartilage does not constitute convincing evidence for the involvement of the enzyme in the disease process. It is noteworthy that two of our anti-MMP13 antibodies (K73/6 and 1.29/6) detected MMP13 protein by immunohistochemistry in pig articular cartilage after wounding the latter\(^{40}\). Interestingly, all three anti-MMP13 antibodies used in the present study bound to a ligand in the cells of the calcified zone in which no MMP13 mRNA was evident by \textit{in situ} hybridization.

There could be several explanations for the failure to detect MMP13 protein in STR/ort articular cartilage. It may be synthesized in hyaline cartilage chondrocytes, but in amounts below the detection level of the immunohistochemical method, even though three different anti-MMP13 antibodies were used. It is noteworthy that we were also unable to detect either MMP2 or MMP9 in murine articular cartilage by immunohistochemistry but PBS-extracts of microdissected tibial plateau cartilage from either STR/ort or CBA mice showed evidence for the presence of each enzyme on SDS-PAGE Western blots and gelatin zymograms (Flannelly \textit{et al.}, unpublished observations). Thus immunohistochemistry is of lesser sensitivity for detecting MMPs than these other methods. However, we were unable to detect MMP13 in such extracts by either Western blot or by heparin-enhanced gelatin zymography, a sensitive technique for revealing this enzyme\(^{41}\).

One speculation for failure to detect extracellular MMP13 immunohistochemically is that after synthesis it may bind to other proteins, preventing its subsequent detection with antibodies. Barmina \textit{et al.}\(^{40}\) have reported that MMP13 binds to, and is internalized by, a variety of cells, including osteoblasts and fibroblasts. The binding involves a 170 kDa protein which may be a specific receptor for the enzyme. This receptor, Endo-180, and MMP13 are co-expressed during osteogenesis\(^{41}\). Moreover, Endo-180 protein is expressed in tibial articular cartilage chondrocytes of both CBA and STR/ort mice, so secreted MMP13 may be removed rapidly\(^{42}\). However, the current experiments should have detected intracellular MMP13 accumulated during the monensin block, as well as pre-existing extracellular MMP13. Since there was no evidence of intracellular enzyme, none may have been synthesized during the block. MMP13 is known to be only transiently expressed in some situations, for example, murine ossification\(^{43}\), potentially complicating its detection in tissues.

We also considered the possibility that the antibodies used in immunohistochemistry may not be able to access antigenic sites in articular cartilage. However, this seems unlikely because tissues were routinely digested with chondroitinase ABC and, in pilot experiments, were also treated with testicular hyaluronidase, proteinase K and Triton X-100. Thus, if MMP13 mRNA is translated to protein, the monensin block used in our immunohistochemistry should retain the newly synthesized protein in the Golgi, where it should be detectable. Moreover, the anti-MMP13 antibody K73/6 was able to detect MMP13 protein in chondrocytes in wounded pig articular cartilage and in growth plate of gp130 (delta STAT) mice (Ernst, M. and Hembry, R.M., unpublished work) which develop...
Table II

Summary of in situ hybridization and immunohistochemical detection of MMPs and TIMPs

<table>
<thead>
<tr>
<th>MMP or TIMP</th>
<th>mRNA expression</th>
<th>Protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Difference between normal STR/ort and CBA</td>
<td>Difference between normal and post-lesional STR/ort cartilage</td>
</tr>
<tr>
<td>MMP2</td>
<td>Increase in STR/ort superficial and transitional zones</td>
<td>No differences</td>
</tr>
<tr>
<td>MMP3</td>
<td>mRNA increased in STR/ort throughout full depth</td>
<td>Strong signal at intact surface Decreased mRNA by lesions</td>
</tr>
<tr>
<td>MMP7</td>
<td>mRNA increased in STR/ort throughout full depth</td>
<td>mRNA decreased by lesions</td>
</tr>
<tr>
<td>MMP8</td>
<td>No mRNA detected in either STR/ort or CBA</td>
<td>No mRNA detected</td>
</tr>
<tr>
<td>MMP9</td>
<td>mRNA increased in STR/ort throughout full depth</td>
<td>mRNA decreased by lesions</td>
</tr>
<tr>
<td>MMP13</td>
<td>Very strong mRNA in STR/ort</td>
<td>mRNA decreased by lesions</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>mRNA increased in STR/ort throughout full depth</td>
<td>mRNA decreased by lesions</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Not detected in either CBA or STR/ort</td>
<td>No mRNA detected</td>
</tr>
<tr>
<td>TIMP2</td>
<td>Slight increase in STR/ort</td>
<td>None</td>
</tr>
</tbody>
</table>

nd=not detected.
severe joint degeneration. Thus there can be no doubt about this antisierum’s ability to detect the enzyme in hyaline cartilage when it is present. We also detected MMP13 with this antisierum in Western blots of culture medium of equine articular explant cartilage stimulated with IL1β.

We were able to detect collagen cleavage neoepitopes in the STR/ort cartilage from the time of development of very early OA lesions (surface roughening)17. Thereafter, as fibrillations developed, further collagen degradation was evident around the edge of the lesion and in the adjacent extracellular matrix. The mice in the present study included animals up to 50 weeks of age with OA lesions ranging from mild to severe. Thus it seems likely that the failure to detect MMP13 protein in the STR/ort articular cartilage despite the presence of OA lesions and an apparent increase in the level of its mRNA pool is due either to one of the reasons discussed above, or to collagen fibril cleavage being due to other proteases, as discussed previously17.

mRNAs were detected for several other MMPs in articular chondrocytes, notably 2, 3, 7, 9 and MT1-MMP. In each case expression was higher in the STR/ort strain before and after development of lesions than in CBA cartilage, except in those chondrocytes immediately adjacent to OA lesions. mRNA expression of most MMPs occurred throughout the full depth of both CBA and STR/ort articular cartilage. MMP2 (gelatinase A) expression occurs predominantly in the upper zones whereas MMP9 (gelatinase B) is expressed throughout the full depth. MMP2 is reported to have collagenase activity44,45, although this is disputed by others46. As discussed above, we detected this enzyme in murine cartilage extracts by techniques other than immunohistochemistry. It is noteworthy that the prominent MMP2 mRNA expression in the upper zones fits more closely with the observed pattern of collagen cleavage in early OA in the STR/ort mouse17,18, than does the strong expression of MMP13 mRNA in the mid and deep zones of the cartilage.

MMP3 (stromelysin-1) is a possible candidate for degradation of aggrecan in the early stages of STR/ort OA. It is expressed as a protein in the superficial zone of articular cartilage where MMP-generated aggrecan neoepitopes appear10, and where the earliest lesions develop13. MMP3 has a wide range of substrates, and is considered a key enzyme in cartilage catabolism17. As well as degrading matrix macromolecules it participates in the activation of other MMPs such as proMMP9 and MMP1312,24,48. Although the level of MMP3 protein in CBA and STR/ort articular cartilage did not differ significantly in this study, it could, potentially, participate in the osteoarthritic process if its activity was inhibited in normal cartilage but not so in osteoarthritic tissue. However, no changes were detected in either of the two TIMPs (1 and 2) investigated in the study (see below).

MT1-MMP, which has been implicated in the activation of other proteases9, is expressed as a protein throughout the tibial cartilages of both CBA and STR/ort mice. The level of expression appears to be similar in both strains so if it plays a role in the development of OA it is likely to be by activation of proenzymes in the diseased cartilage. However, as the disease progresses and lesions develop, neither MMP3 nor MT1-MMP proteins are found in chondrocytes adjacent to the lesion. Similarly these fail to express mRNAs for a number of MMPs which are transcribed in chondrocytes distant from the lesion.

Protein expression alone, or even changes in the level of protein expression for an MMP, do not in themselves necessarily indicate enzyme activity. The control of MMP activity is complex since these enzymes are secreted as latent proenzymes and must be activated at the cell surface or in the matrix to act on extracellular substrate17,49. Even after activation, MMPs are bound by tissue inhibitors of matrix metalloproteinases (TIMPs), which inactivate them17. It is believed that a change in the ratio of MMP:TIMP may occur in disorders such as OA50 where accelerated matrix catabolism occurs. We assessed the expression of TIMP1 and TIMP2 in the murine articular cartilage. TIMP1 was not detected at either the mRNA or protein level, while TIMP2 transcripts were expressed throughout the full depth of both CBA and STR/ort articular cartilage. The level of expression appeared unchanged with the development of lesions. However, no TIMP2 protein was detected in any murine cartilage. This may have been for the same reasons discussed for the failure to detect MMP13 protein. If TIMP2 protein is present but undetectable in the murine cartilages, its level is unlikely to change during the osteoarthritic process since mRNA levels did not change. TIMP3 and 4 were not investigated in this study.

The expression of MMPs and TIMPs is regulated by growth factors and cytokines51. We have reported previously that the expression of IL1, IL6, TGFβ and IGF1 mRNA is markedly elevated in the tibial articular cartilage of STR/ort mice compared with CBA strain cartilage where transcripts of these genes could not be detected by in situ hybridization. Moreover, mRNAs for cytokines and growth factors were detected in chondrocytes of STR/ort cartilage before the development of histopathological OA lesions52. It seems very likely that the different cytokine environment of the STR tibial cartilage may be a stimulus for the increased expression of MMP2, MMP3, MMP7, MMP9, MMP13 and MT1-MMP, which we detected in this tissue by in situ hybridization. The factor or factors responsible for promoting a high level of cytokine expression by STR/ort chondrocytes remains unknown.

References


