

In situ zymographic localisation of type II collagen degrading activity in osteoarthritic human articular cartilage

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Abstract

Objectives—Chondrocytic matrix metalloproteinases (MMPs) are believed to be important in osteoarthritic cartilage degradation. The cartilage lesion of osteoarthritis (OA) is focal and often progressive. During its development chondrocytes differentially up and down regulate production of mRNA for individual MMPs. This observation has potential implications for understanding the disease processes that lead to progressive cartilage loss in OA and designing appropriate targeted treatment. The complex regulation of MMP mediated effects means there is a pressing need to establish whether visualisation of MMP mRNA or protein equates to enzyme activity. The technique of in situ zymography (ISZ) offers a way of examining diseased human tissue for in vivo production of an excess of degrading enzyme over inhibitor. The primary objective of this study was to assess, and if positive follow, collagen II degrading activity in cartilage during development of the OA lesion. A secondary objective was to assess whether there was any correlation between sites of collagen II degrading activity and expression of the collagenase (MMP-13), recently implicated in type II collagen degradation in this lesion.

Methods—Biopsied human normal and osteoarthritic cartilage, showing various degrees of damage, was examined by in situ zymography, with and without enzyme inhibitors, to establish sites of type II collagenase activity. Paired samples were probed for MMP-13 mRNA using ³⁵S-labelled oligonucleotide probes. Comparative analyses were performed.

Results—In situ zymography showed collagen II degrading activity over chondrocytes only in osteoarthritic cartilage. Distribution and amount varied with the extent of cartilage damage and position of chondrocytes, being greatest in deep cartilage and in cartilage lesions where fissuring was occurring. The enzyme causing the degradation behaved as a matrix metalloproteinase. MMP-13 mRNA expression codistributed with the type II collagenase activity.

Conclusion—In OA, chondrocytes can degrade type II collagen. The type II collagen

degrading activity varies in site and amount as the cartilage lesion progresses and throughout codistributes with MMP-13 mRNA expression.

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Accumulating evidence implicates intra-articular synthesis of matrix degrading enzymes, particularly matrix metalloproteinases (MMPs), in cartilage damage in joint disease.¹⁻⁴ Osteoarthritis (OA), a major cause of morbidity in modern societies,⁵ is characterised by cartilage damage. An imbalance between locally synthesised MMPs and their inhibitors (tissue inhibitors of metalloproteinases (TIMPs)), in favour of MMPs⁶⁻⁸ has been causally associated with cartilage loss in OA.^{9, 10} The source of MMPs is debated, but chondrocytes are capable of their production.¹¹⁻¹³ These studies offer unequivocal evidence that in OA articular chondrocytes differentially express MMP mRNA and protein, but there is, to date no direct evidence that in situ the chondrocytes of human OA cartilage can so regulate MMP and TIMP production as to initiate collagen damage. In addition we and others have shown that MMPs are not expressed uniformly, but that expression varies in a highly localised site and stage specific manner as the cartilage lesion progresses.¹⁴⁻¹⁷ Because there is no guarantee that mRNA is translated into protein, that immunohistochemically detected enzyme is in its activated form and that if active enzyme is secreted it is not inactivated by inhibitors, there is a need to establish in situ assays for substrate degrading activity. With advances in applied molecular and cell biology it has become possible to investigate disease processes in situ. Among such techniques is in situ zymography (ISZ), which has the advantage of enabling the production of matrix degrading enzymes to be evaluated and related to site within the cartilage and accepted parameters of cartilage degradation, such as morphology or histochemical staining.

In this study we have used the technique of ISZ, which to our knowledge has not been previously used in this setting, to examine the hypothesis that "chondrocytes from OA, but not normal human cartilage are capable of degrading type II collagen in situ".

As our ISZ study shows the presence of type II collagen degrading activity over chondrocytes, as a first step to defining the nature of the collagen II degrading activity, we have used the in vivo technique of in situ hybridisation (ISH) to examine whether the expression of MMP-13

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(recently proposed as the collagenase responsible for type II collagen degradation in this setting¹⁹⁻²³) codistributes with enzyme activity.

Methods

SPECIMENS

Samples of cartilage and underlying bone were obtained from the medial femoral condyle of 26 patients undergoing knee replacement surgery and six from cadavers within six hours of death. None of the patients had a history of inflammatory joint disease and all had symptomatic OA restricted to the one joint. The medial femoral condyle was chosen because the spectrum of histological cartilage changes in joint replacement specimens is greatest in this site. The central region (approximately 1 cm²) was cut from each with a jeweller's saw. This was then divided into four vertical slabs. Adjacent slabs were paired (giving two pairs per specimen). One slab from each pair was prepared for ISZ and one for ISH.

MICROSCOPIC ASSESSMENT

Processing protocol

ISH—Slabs incorporated articular cartilage and underlying subchondral bone. They were fixed in ice cold (+4°C) 10% v/v neutral buffered formalin for exactly 24 hours and decalcified in 20% EDTA (pH 7.2) under radiological control (mean of 10.35 days (range 9–14 days)). After decalcification the tissue was processed routinely into paraffin wax and 7 µm serial sections mounted onto Vectorbond (Vector Laboratories, 16 Wulfric Square, Bretton, Peterborough PE3 8RF) coated slides for ISH.

ISZ—Cartilage was carefully dissected from underlying calcified cartilage and bone, mounted in OCT compound, and snap frozen in isopentane cooled with liquid nitrogen. Ten µm sections were taken for staining and histological examination.

Staining and grading

Haematoxylin and eosin, and toluidine blue stained sections were examined histologically. These were used to select cartilage specimens for study and grade the cartilage changes.

The selection process had three phases:

- (1) Sections from the decalcified formalin fixed slabs were examined. If the section showed evidence of an origin from a central osteophyte or subchondral fractures the slab and its snap frozen pair were excluded.
- (2) The cartilage changes in all remaining slabs were then graded according to the following scheme independently by two observers. Grade 0—histologically normal cartilage (including normal cadaveric cartilage and morphologically normal cartilage from patients undergoing knee surgery); grade 1—cartilage surface irregularities and loss of metachromasia adjacent to superficial chondrocytes; grade 2—fibrillation of cartilage, extension of fissures to the mid-depth of the cartilage and the formation of some chondrocyte clusters, with, at worst, minor loss of surface

cartilage; grade 3—gross cartilage abnormalities including loss of superficial cartilage, extension of fissures close to subchondral bone and a large number of chondrocyte clusters. If the grade in each member of the pair was the same the pair went forward to the third phase.

- (3) Assessment of total messenger RNA (tmRNA)—as is standard in this laboratory, to ensure that processing (and, in case of cadaveric material, the period between death and harvesting of tissue) had not resulted in loss of RNA, sections of the fixed decalcified slab of each pair were probed for evidence of polyadenylated mRNA with a commercial poly-dT probe (Pharmacia, Birmingham, England) 3' end labelled with digoxigenin-11-dUTP using terminal transferase. Alkaline phosphatase tagged anti-digoxigenin antibody was used to detect hybridisation with fast red as the disclosing agent. Briefly the technique was as follows. Dewaxed sections were rehydrated in DEPC water, then treated with 0.2 N HCl and 2 × standard saline citrate (SSC). Sections were permeabilised with 5 µg/ml proteinase K (Sigma, Fancy Road, Poole, Dorset). Slides were then immersed sequentially in: 0.2% w/v glycine in phosphate buffered saline (PBS), and post-fixed in 0.4% w/v paraformaldehyde in PBS pH7. Immediately before hybridisation sections were immersed in a solution consisting of 0.6 M NaCl, 0.05 M TRIS HCl pH 7.5, 0.1% sodium pyrophosphate, 0.2% polyvinyl pyrrolidone, 0.2% Ficoll, 50 mM EDTA, 0.1 mg/ml sheared salmon sperm DNA, and 20% v/v polyethylene glycol 6000. The hybridisation solution was identical to the pre-hybridisation solution except that it contained 1 µg/ml digoxigenin labelled poly d(T) probe. Sections were incubated at 37°C overnight in this solution and then washed in 2 × SSC at 37°C for 30 minutes followed by 0.1% w/v triton-X-100 in TRIS buffered saline pH 7.4 at room temperature for 15 minutes. The presence of binding of digoxigenin labelled probe was shown with anti-digoxigenin antibody diluted 1:250. The endogenous alkaline phosphatase blocking and staining solution containing 20 mg levamisole, 50 mg fast red TR and 50 mg naphthol AS-BI phosphate in veronal acetate buffer containing 0.002% v/v dimethyl formamide. This gives an insoluble red precipitate only at sites of probe binding. The sections were counterstained with haematoxylin. All sections reacted for tmRNA.

FINAL TISSUE SAMPLES

In all, 29 pairs of slabs (six graded 0, eight graded 1, eight graded 2, and seven graded 3) from 21 patients (mean age 64.3 (range 54–72), men = 9, women = 12), and nine pairs of slabs (all grade 0) from six cadavers (mean age 66.0 (range 41–77), men = 3, women = 3)

were selected for further study. χ^2 Analysis has failed to reveal male/female bias in any grade group.

ISH FOR MMP-13

Sections from the formalin fixed paraffin wax embedded blocks in which tmRNA were probed for collagenase 3 (MMP-13) activity using radioactively labelled oligonucleotide probes. The technique was identical to that described previously²⁴⁻²⁶ and that described above for tmRNA. Two sets of paired oligonucleotide probes were synthesised against published and fully characterised sequences of the human MMP-13 gene. The first pair ((5' to 3') TCATGACCTCATCTTC and GAACAGCTGCACTTAT) were the sequences used as PCR primers in the original isolation of MMP-13¹⁹ and correspond to nucleotides 1030-1045 and 1148-1163. The second pair ((5' to 3') GACTTCACGATGCGATTGCTG and GCATCAACCTGCTGAGGATGC) have been used previously for MMP-13 ISH²³ and correspond to nucleotides 468-488 and 938-958. Probes were synthesised by Oswel DNA Service (University of Southampton, Southampton, SO16 7PX, UK) as were their complementary sense sequences. Both antisense and sense probes were 3' end labelled with ³⁵S-dATP using terminal transferase.

The antisense oligonucleotides showed minimal homology with the other MMP sequences. Use of two probes is a standard method for increasing both the label delivered to each molecule of mRNA and the specificity of the technique.²⁷ Hybridisation was performed as described above (but substituting the radiolabelled oligonucleotide for the poly d(T) probe) with the addition of 50% formamide and the concentration of probe regulated to give 10⁶ cpm per section.^{25, 26} High stringency post-hybridisation washes (2 × SSC for 10 minutes, 1 × SSC for 10 minutes, and 0.5 SSC for 10 minutes) were then conducted at 15°C below the melting temperature. Autoradiography was performed with Ilford K5 emulsion diluted 1:1 with distilled water. Slides were exposed for 35 days, developed in Kodak D-19 developer, fixed and counterstained with Harris's haematoxylin and eosin.

CONTROLS

Controls were of three types. The first (later to be used as "noise") were probe controls consisting of the sense oligonucleotides, used at the same specific activity (or, in the case of tmRNA assessment labelled in the same way) as the antisense oligonucleotides. In the second, during prehybridisation 10 mg/ml of crude bovine RNase was applied to the sections for 30 minutes at 37°C after the immersion in glycine step. In the third, ISH was performed but the probe omitted.

ASSESSMENT OF ISH SIGNAL

Data were recorded over four cartilage zones. Zone 1 was the most superficial and recognised by the lack of polarisable fibres and flattened chondrocytes with their long axes parallel to

the articular surface. Zone 4 was calcified cartilage and zones 2 and 3 represented, respectively, the outer and inner halves of the residuum of the cartilage.

Quantitation of signal was undertaken using automated grain counting. A single observer, using the dot counting function of a Magiscan 600 image analyser, carried out the analysis of signal intensity. "Signal" for each MMP was recorded as the mean number of grains over cells in individual zones in the antisense oligonucleotide probed sections minus the number of grains over cells in serial RNase pre-treated sections. "Noise" was defined as the mean number of grains over cells in serial sections probed with the sense oligonucleotides minus the signal over RNase treated serial sections. Data were expressed as (signal-noise)/noise ratios. (In this way, were signal to equal noise, the result would be 0). A minimum of 60 (median =96, range 60-183) chondrocytes were analysed in each zone of each section and the ratio (S-N)/N was calculated from the average grain number over cells in four serial sections.

ISZ

Fifteen μ m sections were used for ISZ. The technique was a modification of that described by Galis *et al* for use in vascular tissue.²⁸ The sections were mounted on slides precoated with a gel consisting of FITC conjugated bovine type II collagen (Sigma). The collagen type II gel (product number C1188) is gelatin free. The gels were made by mixing equal quantities of melted 1% agarose type IV (37°C) with 1 mg/ml bovine collagen type II and 20 μ l of 20 mg/ml FLUOS reagent. The mixture was spread as a thin film onto a microscope slide and allowed to dry. To assess that during the production of the gel the collagen II had not been denatured the coated slides were subjected to trypsin digestion. For this the 10 coated slides randomly selected from the batch used for the ISZ experiment were immersed in a 1 mg/ml solution of trypsin (Sigma). Tablets containing buffer and trypsin (T-7168) was dissolved according to the manufacturer's instructions. The slides were then incubated with the solution for at 37°C for two hours. The luminosity of the gel on the slide was assessed using a Quantimet 600 before and after trypsin treatment. The overall reduction in luminosity was 3.9% with a range of 1.6 to 5.5%. In the experimental system the sections were moistened with TRIS-HCl buffer pH 7.4 in a moist box and the zymographic reaction allowed to proceed for 48 hours at 35°C. At time 0 (immediately after placing the sections on the slides) 24 and 48 hours, the sections were viewed in a fluorescence microscope attached to a Quantimet 600 image analyser. The image of the cartilage was captured and the number and area of regions of digestion were measured in each of the three zones. The microscope was then switched to Nomarski phase optics, which allows cells to be visualised in unstained sections and an image captured. The two images were superimposed and the

distribution of the areas of digestion compared with the cell distribution.

As controls, serial sections were incubated with inhibitors of proteolytic enzymes:

(1) As a broad spectrum inhibitor of proteases (BSIP)—TRIS buffer (pH 7.4) containing 200 μ M phenylmethyl sulphonyl fluoride ((PMSF), serine protease inhibitor (Sigma)), 1 μ M leupeptin (serine and cyteine protease inhibitor (Sigma)), 100 μ M EDTA (MMP inhibitor, Sigma), and 1 μ M pepstatin (aspartate protease inhibitor (Sigma))

(2) A series of "protease sparing inhibitors" in which one of the components of the above cocktail was omitted.

(3) 100 μ M EDTA in TRIS buffer.

The inhibitor solutions were placed on the sections immediately after the sections were mounted on the gel, and renewed twice each day. These sections were analysed in the same way as the test sections. Sections of rheumatoid synovium were used as positive control tissue.

DATA COMPARISONS

The data from the ISH studies were expressed as grain counts per cell and the data from the ISZ as number of cells over which digestion was seen and as the total area of the zone over which digestion was seen. The (S-N)/N ratios from the two pairs of ISH probes were compared using a paired *t* test. They showed no statistical difference in signal intensity or distribution. The grain number data from the two ISH studies were amalgamated and the pooled data used to compare the ISH and ISZ results. Before comparisons were made the data were normalised by converting the largest measurement to 100% and then expressing all other measurements as a percentage. The data were then compared using a paired *t* test to examine the hypothesis that there was a difference between data sets, having first established whether the data were normally distributed using a Kolmogoroff-Smirnov analysis.

Results

The results are summarised in table 1 and figures 1 and 2.

Table 1 ISH and uninhibited ISZ data from this study

| Zone | Technique | Assay | Unit | Grade | | | |
|------|-----------|-------------|-----------|------------|-------------|-------------|-------------|
| | | | | 0 | 1 | 2 | 3 |
| 1 | ISH | MMP-13 | (S-N)/N | 0 | 0.8 (0.1) | 0.4 (0.2) | — |
| | ISZ | collagenase | cells (%) | 10.5 (4.6) | 41.5 (10.1) | 23.5 (12.6) | — |
| 2 | ISH | MMP-13 | (S-N)/N | 0 | 3.8 (0.4) | 5.1 (1.8) | — |
| | ISZ | collagenase | cells (%) | 12.6 (4.0) | 66.6 (9.5) | 90.5 (6.2) | 71.2 (15.4) |
| 3 | ISH | MMP-13 | (S-N)/N | 0 | 8.3 (3.5) | 16.7 (5.9) | 7.6 (1.7) |
| | ISZ | collagenase | cells (%) | 10.2 (2.8) | 84.6 (17.9) | 89.3 (21.9) | 56.2 (19.8) |
| 4 | ISH | MMP-13 | (S-N)/N | 0 | 17.8 (7.3) | 9.1 (3.9) | 6.7 (2.3) |
| | ISZ | collagenase | area (%) | 0.6 (0.2) | 18.4 (6.7) | 12.2 (5.9) | 3.5 (1.1) |
| | | | cells (%) | XX | XX | XX | XX |
| | | | area (%) | XX | XX | XX | XX |

The ISH data for MMP-13 mRNA are given as the ratio (S-N)/N. The ISZ data for type II collagen degrading activity are given in two forms—the proportion of cells in the zone (cells) with associated areas of matrix breakdown and the proportion of the area of gel beneath the section (areas) digested within the 48 hours of the study. These data are for the sections that have received no inhibitor. No data are available for zone 1 of grade 3 changes (—), as this region is lost as part of the disease process, or for the calcified cartilage (zone 4 (XX)) in the ISZ experiments as this is removed before the technique. Data for cadaveric grade 0 and surgical grade 0 cartilage specimens have been pooled. Data shown as mean (SD).

ISZ

Rheumatoid synovium

Areas of gel digestion, consisting of separate and overlapping circles were seen with type II collagen gels (fig 2E).

Osteoarthritic cartilage

Grade 0 cartilage (morphologically normal) showed minimal evidence of gel digestion. There was no significant difference between the data obtained in cadaveric and surgical grade 0 lesions. In other grades of cartilage lesion, round areas of gel digestion appeared in the test sections corresponding to cells. Not all cells overlay areas of gel digestion. The size of the areas of digestion and their number varied independently of the number of cells and with the zone and grade. Although the area of digestion was greater over chondrocyte clusters than individual cells the mean area per cell was less. Activity was greater over cells adjacent to fissures and areas of cartilage loss than elsewhere within the zone. Of all the inhibitor combinations only those containing EDTA eliminated digestion of the film.

ISH

Controls

In both RNase treated and sense oligonucleotide probed sections the number of silver grains (that is, hybridisation signal) was much lower than sections treated with specific probe (the signal over sense oligonucleotide probed sections was generally greater than RNase treated sections (mean sense oligonucleotide/mRNase signal ratio = 1.33; range 1–2.09)).

Expression of MMP-13 mRNA in articular chondrocytes

Joint replacement specimens—The measured signal over antisense oligonucleotide probed sections varied with the different grades of cartilage damage and the zone. When the mean signal over each set of four serial sections was measured the variation between individual sections and the mean of the four sections was never greater than 21% of the mean and typically was less than 10%. The data for the mean (S-N)/N ratios for each probe, in each

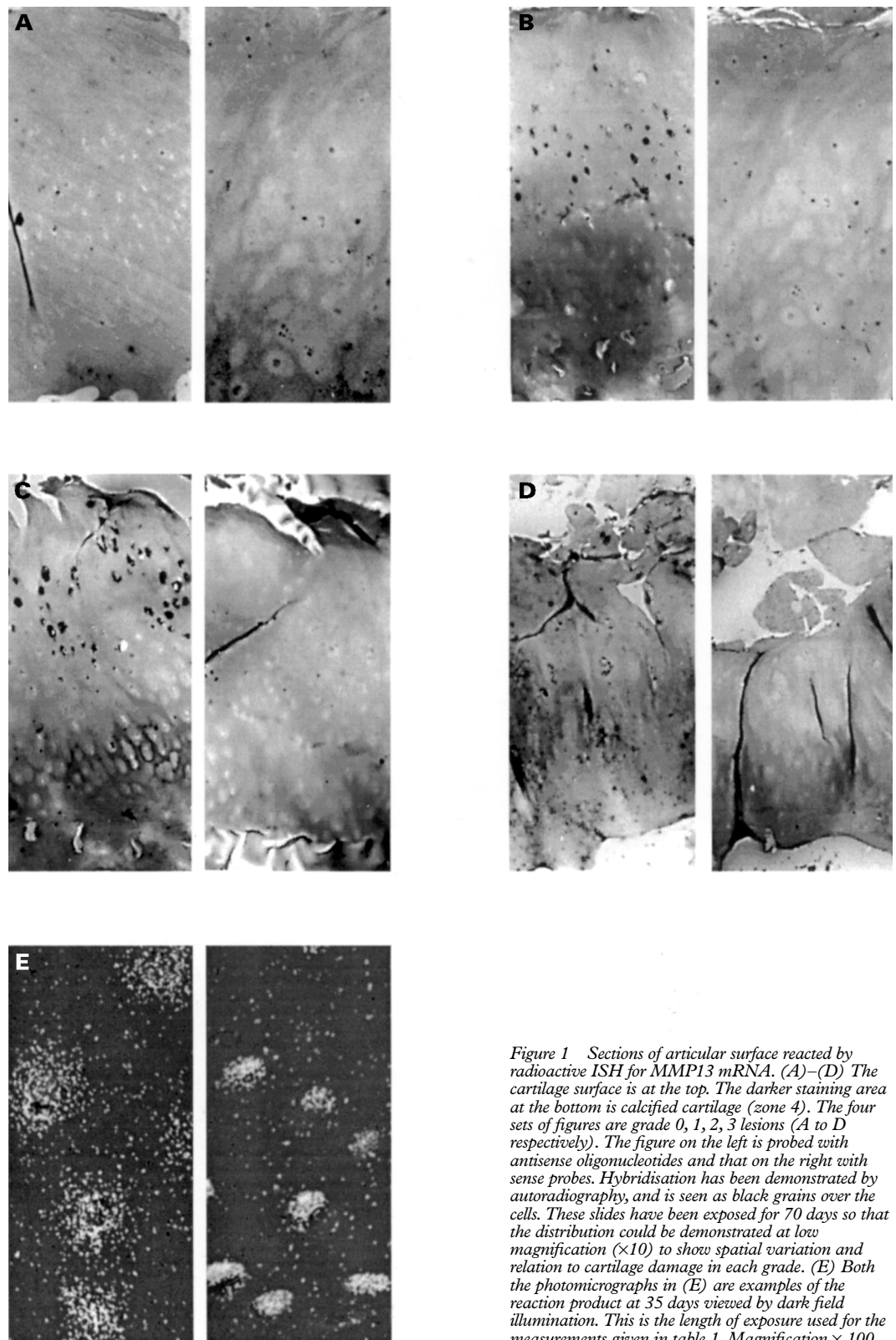


Figure 1 Sections of articular surface reacted by radioactive ISH for MMP13 mRNA. (A)–(D) The cartilage surface is at the top. The darker staining area at the bottom is calcified cartilage (zone 4). The four sets of figures are grade 0, 1, 2, 3 lesions (A to D respectively). The figure on the left is probed with antisense oligonucleotides and that on the right with sense probes. Hybridisation has been demonstrated by autoradiography, and is seen as black grains over the cells. These slides have been exposed for 70 days so that the distribution could be demonstrated at low magnification ($\times 10$) to show spatial variation and relation to cartilage damage in each grade. (E) Both the photomicrographs in (E) are examples of the reaction product at 35 days viewed by dark field illumination. This is the length of exposure used for the measurements given in table 1. Magnification $\times 100$.

zone and each grade of cartilage lesion are detailed in table 1, and examples of the reaction product are illustrated in figure 1. No data are available for zone 1 in grade 3 disease because this zone was uniformly lost during the progression of the cartilage lesion of OA. In general terms the signal for MMP-13 mRNA was greatest in zones 2 and 3 of the cartilage and in grade 1 and 2 cartilage lesions.

Single cells and chondrocyte clusters reacted in a similar way. However, the signal over clusters was generally greater per visible nucleus,

but the difference did not reach statistical significance (data not shown). When signal over cells or clusters close to fissures was compared with signal at a distance from fissures in the same sections the signal was greater close to the fissures. This reached statistical significance at $p < 0.01$. The signal did not reproducibly exceed noise (that is, $(S-N)/N=0$) in the calcified zone (zone 4), or within normal cartilage (either cadaveric or the histologically normal cartilage from patients undergoing joint replacement).

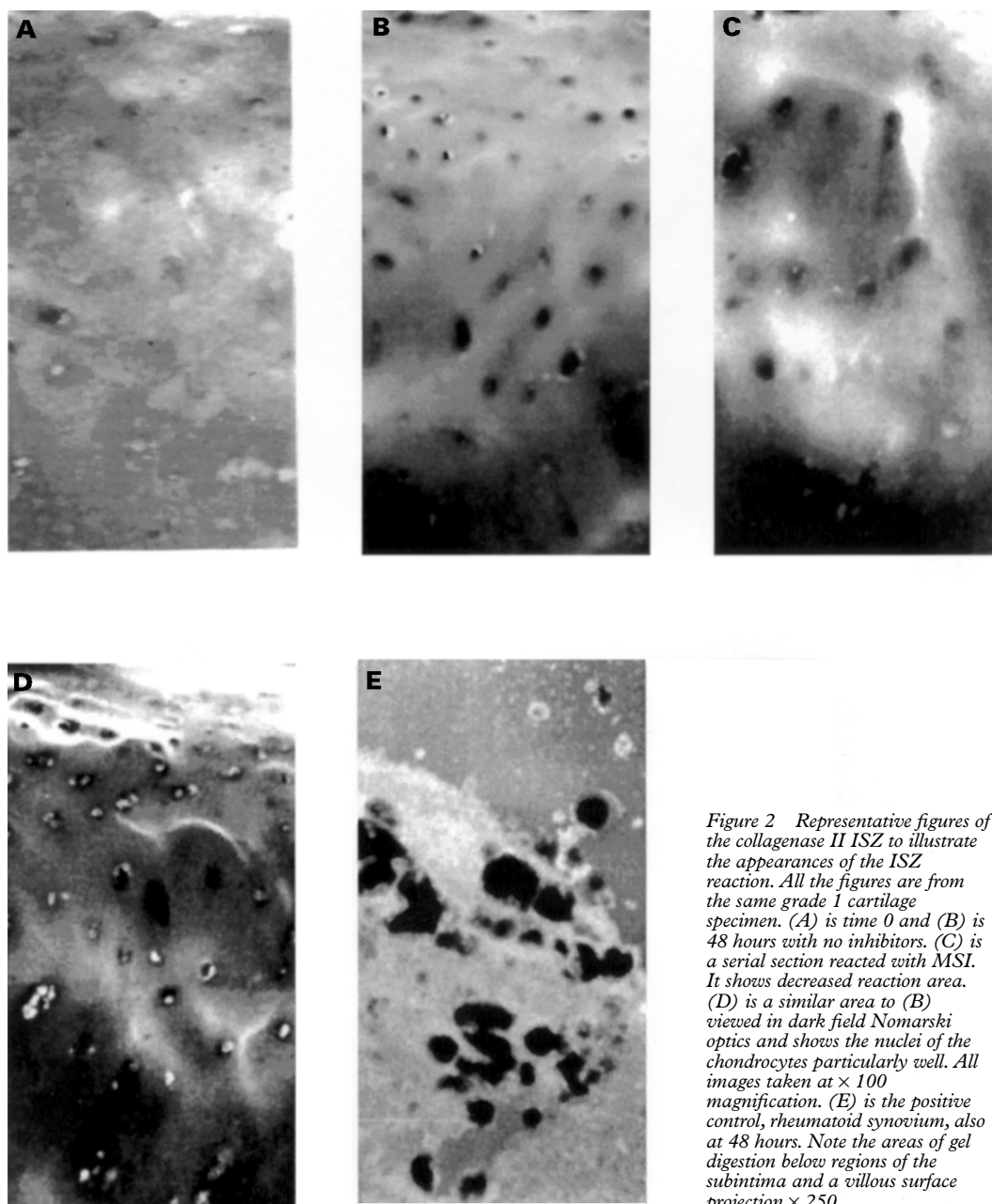


Figure 2 Representative figures of the collagenase II ISZ to illustrate the appearances of the ISZ reaction. All the figures are from the same grade 1 cartilage specimen. (A) is time 0 and (B) is 48 hours with no inhibitors. (C) is a serial section reacted with MSI. It shows decreased reaction area. (D) is a similar area to (B) viewed in dark field Nomarski optics and shows the nuclei of the chondrocytes particularly well. All images taken at $\times 100$ magnification. (E) is the positive control, rheumatoid synovium, also at 48 hours. Note the areas of gel digestion below regions of the subintima and a villous surface projection $\times 250$.

Cadaveric material—Despite having previously selected these specimens on the basis of confirmed tmRNA expression none had detectable MMP-13 gene expression. This was in every way identical to the grade 0 areas from patients with OA.

COMPARISON BETWEEN ISH AND ISZ DATA

When normalised, ISH and ISZ data from paired slabs were compared there was found to be no significant difference between the relative values of ISH and ISZ (values typically $p > 0.5$) in each section.

Discussion

Cartilage integrity relies on coordinated matrix remodelling by chondrocytes. OA is a disorder characterised histologically by focal, often progressive, cartilage degradation and loss. Mechanisms leading to cartilage damage are incompletely understood. A working hypothesis is that in OA, cartilage breakdown is

caused by up regulation of normal MMP production by articular chondrocytes^{11–13 17} without an equivalent rise in production of inhibitors (TIMPs).^{6–8} There is evidence causally implicating abnormal synthesis of synovial cytokines and growth factors^{29–32} in the initiation of MMP production.

There are a number of studies that have suggested that chondrocyte production of MMPs in OA cartilage is focal and varies with the extent of the cartilage damage.^{14–17} This raises fundamental mechanistic questions about OA cartilage damage and has important implications for targeted treatment.³³ The study of spatial and temporal changes in chondrocyte MMP production *in vivo*, in humans imposes practical constraints. Advances in *in situ* biology techniques enable exploration of some of these processes in tissue sections. *In situ* demonstration of the presence of matrix degrading enzymes is usually performed either with ISH for mRNA, or immunohistochemistry for protein. Unfortunately, the

biology of MMPs is such that claiming biological activity based on demonstrating either mRNA or immunoreactive product *in vivo* is open to criticism, as translation of mRNA to protein can never be assured and many antibodies to MMPs react with both the latent and active forms of the enzyme. Even if it were possible to demonstrate that the enzyme disclosed by immunohistochemistry was active, it is impossible to exclude that it might be neutralised, *in vivo* by TIMPs. We have used a different technique, ISZ, to look for evidence of uninhibited type II collagenase activity. Our data show type II collagen degrading activity over chondrocytes in OA cartilage that varies in amount and site of production with the progression of the cartilage damage, as assessed by morphological criteria. The activity is predominantly over chondrocytes in zones 2 and 3, is initiated before morphological evidence of fibrillation, and increases as the typical lesion develops, falling only in the end stage lesion.

This is the first report of ISZ being used to examine synthesis of type II collagen degrading activity by human articular chondrocytes; our description of the production by chondrocytes of collagen II degrading activity and its spatial variation during the progression of the OA cartilage lesion is therefore novel. Our data give direct evidence to confirm the inferences of others. Immunohistochemistry studies of collagen cleavage sites^{34, 35} have shown a difference between normal and OA cartilage. In normal cartilage collagen damage was noted within the superficial zone, becoming more apparent and extending into the upper mid zones in normal cartilage of older people.³⁴ The difference between aging normal and OA cartilage was the presence in the latter of immunodetectable collagen denaturation extending into the lower mid and deep zones, a process that increased with cartilage damage.³⁴ Expression of cleavage sites was most prominent in pericellular regions.^{34, 35} Interestingly in a study of the valgus osteotomy model of OA in the beagle Panula *et al*³⁶ recorded an early (before fibrillation) increase in the thickness of the cartilage. When they analysed the relative contribution of the individual layers of the cartilage to the overall swelling, they found that the greatest swelling occurred in the deep layers (in some parts of the joint by as much as twofold). One possible mechanism that might link these observations with our data showing collagen II degrading activity maximal in the deeper zones of cartilage is that, early in OA—that is, before the onset of overt fibrillation, (our grade 1 lesions), there is reduced integrity of the collagen framework deep within the cartilage caused by chondrocyte production of collagenases. This manifests as detectable collagen cleavage sites, unopposed swelling pressure of the saccharide rich matrix elements, and expansion of the cartilage.

The nature of the enzyme leading to cartilage damage is a matter of some debate. In our inhibitor studies the enzyme behaved as a MMP. Recent interest has focused on the possible role of MMP-13 in cartilage collagen damage in OA. Of the three non-surface bound

MMPs, MMP-13 has been implicated as a candidate because it has a greater cell specificity^{19, 20} and collagen type II degrading efficiency^{20–22} than either MMP-1 (interstitial collagenase, collagenase-1) or MMP-8 (neutrophil collagenase, collagenase-2). It also has gelatinase activity²⁰ and, of the collagenases, is the most efficient peptide degrading enzyme. MMP-13 was first isolated from breast carcinomas in 1994.¹⁹ In that study normal human breast, liver, placenta, ovary, uterus, prostate, and parotid gland tissue did not contain MMP-13 mRNA by northern blot or RNA polymerase chain reaction (PCR) analysis. In another study MMP-13²² could not be detected in normal brain, heart, kidney, liver, lung, skeletal muscle, small intestine, spleen, testis, or thymus after 30 cycles of PCR but was found in cartilage (although the species was not mentioned). Together these studies indicate expression of MMP-13 in normal tissues is limited. In addition to breast cancer and normal cartilage, MMP-13 has been described in humans in mesenchymal cells of normal umbilical cord, hypertrophic chondrocytes, and osteoblasts during fetal bone formation, certain causes of postnatal bone remodelling,³⁷ squamous cell carcinoma of the larynx (but not adjacent normal epithelium)³⁸ and joint disease.^{21–23, 37, 39, 40}

The data presented here show MMP-13 mRNA codistributing with sites of production of collagen II degrading activity. The data for involvement of MMP-13 in arthropathies come predominantly from immunohistochemical^{33, 39–41} and gene amplification studies^{22, 23, 42} of cells and tissue samples extracted from synovium^{37, 40, 42} and cartilage²¹ of normal and diseased joints. Recent studies^{22, 23, 39, 41} have emphasised the potential importance of MMP-13 in human OA. Ours is one of only a few studies to examine MMP-13 in OA human cartilage. Shlopov *et al*,²³ Mitchell *et al*,²² and Reboul *et al*²¹ in 12, six, and eight OA cartilage specimens respectively identified MMP-13 mRNA in cultures of extracted chondrocytes after 30–35 cycles of PCR. Chondrocytes from normal cartilage also expressed MMP-13 (detected after PCR) but at much lower levels. We have demonstrated MMP-13 mRNA in OA but not normal cartilage, the difference between our data for normal cartilage and the PCR studies probably being attributable to the effects of PCR amplification on detection sensitivity. Two previous studies localised MMP-13 in OA cartilage. They gave contradictory results. Shopolov *et al*²³ conducted an ISH based study of MMP-13 in OA cartilage. The only published result is a single photomicrograph of ISH reaction product in one case of OA, in which signal for MMP-13 was distributed throughout the full thickness of a section of cartilage. This contrasts with an immunohistochemical study of MMP-13 protein distribution in 10 OA cartilage specimens and eight normal controls,³⁹ in which expression was less in the superficial layer (our zone 1 and upper zone 2) than the deep (our lower zone 2 and zone 3). In this respect our data support the findings of the

latter study. In the RT-PCR component of their study Shopolov *et al*²³ showed that the level of MMP-13 mRNA was greater in lesional than in normal cartilage from the same OA joint, and increased with cartilage damage. These are similar findings to our own. Moldovan *et al*⁹ related the number of chondrocytes immunoreactive for MMP-13 to fibrillation in OA cartilage, but found no difference. This seems to contradict our data, however the data sets are not comparable. Firstly, they quote data for fibrillated regions of both normal and OA cartilage. We do not recognise fibrillation in normal cartilage and cannot, therefore, produce comparable data. Secondly, they do not specify the morphology of the lesions they describe in OA cartilage; it is not clear, therefore, whether we are comparing like degrees or regions of cartilage damage. Thirdly, they express their data as numbers of stained cells, whereas we look at both the number of reactive cells and the amount of signal over each cell.

Perhaps because ours is a much larger study, our data help clarify the contradictory results from other studies. We agree with Moldovan *et al*⁹ that MMP-13 expression is greatest deep within cartilage and with Shopolov *et al*²³ that MMP-13 expression varies with the morphological extent of the cartilage lesion. A significant finding, not emphasised, but reported by other authors²³ and ourselves is that morphologically normal cartilage within an OA joint and cartilage from a normal joint have very similar (and very low) MMP-13 expression.

We have no data that prove the type II collagen degrading activity we have demonstrated is caused by MMP-13 production. We have used three elements, ISZ, ISH, and cartilage morphology, to make within and between sample correlations of chondrocytic MMP-13 expression, type II collagen degrading activity in situ, site within the cartilage (zones 1–4) and lesional grade (grade 0–3). Our data show no significant difference in the relative patterns of distribution of MMP-13 mRNA and type II collagen degrading activity, which is a necessary prerequisite were MMP-13 to be involved in collagen degradation in OA cartilage.

To summarise, our study has produced novel data demonstrating that the chondrocytes of human OA cartilage are capable, in situ, of degrading type II collagen. The agent causing type II collagen degradation behaves as an MMP. The type II collagen degrading activity is not exhibited by chondrocytes in normal cartilage, either in normal joints or morphologically normal areas of osteoarthritic joints, and varies in both cellular distribution and amount in a manner that can be related to the extent of local cartilage damage. Intriguingly the activity codistributes exactly with expression of MMP-13, a matrix metalloproteinase, implicated, in a number of recent studies, as a possible key mediator of collagen degradation in OA cartilage.

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