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Activation of collagen type II expression in osteoarthritic and rheumatoid cartilage*

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Summary. In situ hybridization and immunohistochemical techniques were applied to investigate gene expression and extracellular deposition of collagen type II in normal, osteoarthritic and rheumatoid human articular cartilage. Normal cartilage showed an essentially even extracellular distribution of type II collagen with poly- and monoclonal antibodies, while only a few cells were positive for α1(II) collagen mRNA. In situ hybridization of osteoarthritic and rheumatoid cartilage, however, showed strong enhancement of type II collagen gene expression; transcripts were observed predominantly in the upper middle zone of the articular cartilage while the upper layer was mostly negative and correlated with a zone of reduced proteoglycan staining. The elvated mRNA levels frequently coincided with pericellular immunostaining for type II collagen, indicative for enhanced synthesis of the protein. In two samples, however, pericellular loss of collagen type II staining was found despite positive cytoplasmic signals with the α1(II) RNA probe, suggesting enhanced collagen destruction. Control hybridization with a probe for 18S rRNA revealed very few negative cells throughout both normal and arthritic cartilage samples, ruling out major cell necrosis in the specimens investigated. Thus, our observations identify sites of activated type II collagen synthesis in osteoarthritic cartilage that were predicted by previous biochemical studies and support the notion that damaged cartilage attempts to restore matrix by enhanced synthesis of its components.

Key words: Articular cartilage – Osteoarthritis – Collagentype II – In situ hybridization – Rheumatoid arthritis

Introduction

The outstanding biochemical properties of articular cartilage such as its elasticity, resistance to friction and pressure and an in-built lubricating mechanism reflect the particular composition and architecture of the cartilage matrix. This is made up of a dense, highly crosslinked meshwork of collagen fibrils consisting of type II, IX, and XI collagen (Eyre et al. 1987; Mayne 1989), into which are entrapped proteoglycan-hyaluronan complexes that not only bind or release water, but also protect the collagen network from mechanic and enzymatic erosion. Interwoven with the type II-IX-XI collagen network is a separate meshwork of type VI collagen fibrils (Ayad et al. 1984; Keene et al. 1988).

Degenerative joint diseases are characterized by progressing erosion of articular matrix and destruction of proteoglycans and collagens (McDevitt et al. 1976; Sandy et al. 1976; Dodge et al. 1989) (for reviews see Sokoloff 1983; Poole 1986). Articular chondrocytes are able to respond to destruction by enhanced proteoglycan and collagen type II synthesis up to a certain degree of tissue damage, as shown by biochemical studies on human osteoarthritic cartilage (Mitrovic et al. 1981; Lipiello et al. 1977; Ryu et al. 1984) and in animal models (McDevitt et al. 1976, Eyre et al. 1980). Enhanced synthesis of other cartilage collagens VI, IX, and XI has also been reported (Ronzière et al. 1990), while in healthy adult articular cartilage only low levels or no collagen synthesis can be detected (Repo et al. 1971). Controversially, some sort of switch in chondrocyte synthetic activity to collagen types I and III has been found, indicating a transition to fibroblast-like cells (Gay et al. 1976; Adam and Deyl 1983). In view of the heterogeneity of cartilage erosions in osteoarthritic cartlage, not only between different subjects, but also within one affected joint, we have investigated the extent of extracellular distribution and synthesis of type II collagen in samples of human osteoarthritic and rheumatoid cartilage at both mRNA and protein levels using in situ hybridization and immunohistochemistry. Despite considerable varia-

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tions among samples, in most cases we found enhanced type II collagen synthesis predominantly in the middle zone. The zonal distribution was mainly correlated wth the proteoglycan distribution as estimated after toluidine blue or safranin O staining.

Materials and methods

Tissue preparation. The study was made on a series of 22 specimens from 15 patients undergoing endoprothetic joint replacement for severe osteoarthritic or rheumatoid lesions of the hip or knee joints. Eight macroscopically and histologically normal, and four slightly altered samples were obtained from amputated knee joints and from hip joints obtained no later than 24 h post-mortem. The age of the donors ranged from 53 to 85, 53 to 75, and 17 to 60 years for osteoarthritic, rheumatoid, and normal specimens, respectively.

Cartilage slices were fixed with 4% paraformaldehyde immediately after removal, dehydrated in a RNAse-free series of alcohol, cleared in xylene, and embedded in paraffin wax. Sections (5–8 μm) were cut, mounted on slides, pretreated with 4% 3-triethoxysilylpropylamine-aceton-solution (Rentrop et al. 1986), and stored at room temperature until used.

Histochemistry. In all cartilage samples hematoxylin-eosin, Elastic/van Gieson, safranin O, and toluidine blue staining was performed on parallel sections. The latter two stains were used to estimate the content of proteoglycans (Rosenberg 1971) and showed similar results. All samples were graded histologically according to Mankin et al. (1971). Special care was taken to distinguish original cartilage from newly formed regenerative cartilage at sites of osteophyte formation, and only the former was used for this study.

Immunohistochemistry. Deparaffinized sections were decalcified wiht 0.3 M EDTA (pH 7.2) and incubated with testicular hyaluronidase (2 mg/ml, phosphate buffered saline (PBS), pH 5; 30 min at room temperature) and pronase (1 mg/ml, PBS, pH 7.3, 30 min at RT). Primary antibodies were incubated for 1 h and visualized using alkaline-phosphatase-labeled secondary antibodies and 3-hydroxy-2-naphthylacid 2,4-dimethylanilid as the color substrate. Nuclei were counterstained with haematoxylin. Monoclonal antibodies against type II collagen (CIID3) were kindly provided by Dr. R. Holmdahl (Holmdahl et al. 1986). Rabbit polyclonal antibodies against type II collagen were prepared and affinity purified as described previously (von der Mark et al. 1976).

Preparation of RNA probes. For in situ hybridization a suitable fragment of the collagen type II cDNA was selected and recloned into a pGEM vector (Promega): pHCG 2 contains a ECO RI – Dra I fragment (435 bp, see Fig. 1) from pHCAR 3 (Elima et al. 1985; Sandberg and Vuorio 1987) recloned in Hinc II–Eco RI sites of pGEM 4Z. Another probe, pRNA 1, contains a Xba I–Bam HI fragment (294 bp) of mouse 18S rRNA from pCM 1 (Grummt et al. 1979) recloned in Xba I–Bam HI sites of pGEM 3Z. This probe shows 100% homology to human 18S rRNA and was used as a positive control and for information about cell necrosis prevalent in sections.

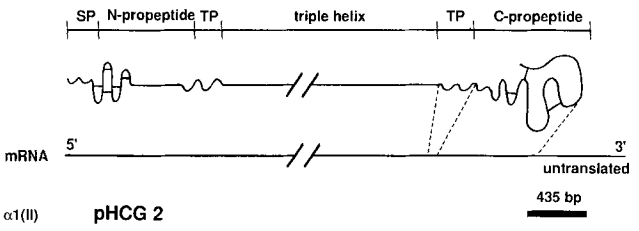


Fig. 1. Diagram showing the position of the RNA probe used for type II collagen relative to the entire mRNA and the different protein domains of α1(II) (SP: signal peptide, TP: telopeptide). The length of the clone is indicated above the bar (according to Sandberg and Vuorio 1987)

The constructs were linearized and transcribed in vitro using T7 and SP6 RNA-polymerase (both Promega) to generate ^{35}S-labeled antisense and sense transcripts, respectively. Unincorporated nucleotides were separated by alcohol precipitation; the quality of the transcripts was controlled using denaturing formaldehyde agarose gels.

In situ hybridization. Immediately prior to hybridization sections were deparaffinized and rehydrated. After 3 min in PBS sections were digested for 7 min with proteinase K (20 μg/ml in 50 mM Tris-HCl (pH 8), 5 mM EDTA, room temperature), postfixed for 5 min with 4% paraformaldehyde (in PBS), washed briefly in double distilled water, acetylated for 10 min in 0.25% acetic acid anhydride (in 0.1 M triethanolamine (pH 8)), washed again in PBS and double distilled water, and dehydrated. Water and PBS were always treated with 0.1% diethylpyrocarbonate overnight and autoclaved. Glassware was baked at 180° C for at least 2 h to inactivate RNAses. The sections were hybridized for 12–16 h at 43° C with riboprobes at a final activity of $3–5 \times 10^7$ cpm/ml depending on their length. The hybridization buffer contained 50% formamide, 10% dextran sulfate, 20 mM DTT, 1 mg/ml t-RNA, 300 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10 mM Na_2HPO_4 (pH 6.4), 5 mM EDTA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidon, and 0.02% bovine serum albumin. After hybridization the tissue sections were washed 15 min in 2 × SSC/0.5% β-mercaptoethanol and 60 min in 0.5 × SSC/0.5% β-mercaptoethanol at 40° C each, treated with RNAses A and T1 for 30 min at 40° C (20 μg/ml RNAse A and 50 U/ml RNAse T1 in 0.5 M NaCl, 10 mM Tris (pH 7.5), 5 mM EDTA), washed briefly in the same buffer, and then for 2 h in 2 × SSC/50% formamide/0.5% β-mercaptoethanol at 45° C, and three times in 0.1 × SSC at room temperature. Finally the slides were dehydrated in a series of ethanol and air-dried.

Autoradiography was performed by dipping slides into Kodak NTB-2 nuclear track emulsion melted at 43° C and diluted 1:1 with double distilled water. After drying slides were exposed for 3–4 days in dark boxes at 4° C. Sections hybridized with the 18S rRNA probe were exposed for 4 to 20 h only. Slides were developed for $1^1/_2$ min (Dektol, Kodak), rinsed in deionized water, fixed for 5 min (Unifix, Kodak) all at 15° C, and rinsed for 5 min in deionized water and for further 10 min in running tap water. The sections were counterstained in 5% Giemsa dye for 1 h. Results were evaluated by dark and bright field microscopy under a Leitz Diaplan microscope.

Results

Pretreatment and washing proecures for in situ hybridization were optimized for maximal specificity and intensity of hybridization signals and minimal background problems. Digestion with a high proteinase K concentration in conjunction with postfixation seemed optimal for our paraformaldehyded fixed paraffin sections, whereas it was detrimental for other tissues and for frozen sections. A combination of intense RNAse A and T1 digestion and subsequent washing with intermediate stringency yielded optimal specificity and intensity of signals and reduced background considerably.

Normal cartilage specimens

Samples ($n=8$) of healthy adult donors were investigated. All specimens were macroscopically and histologi-

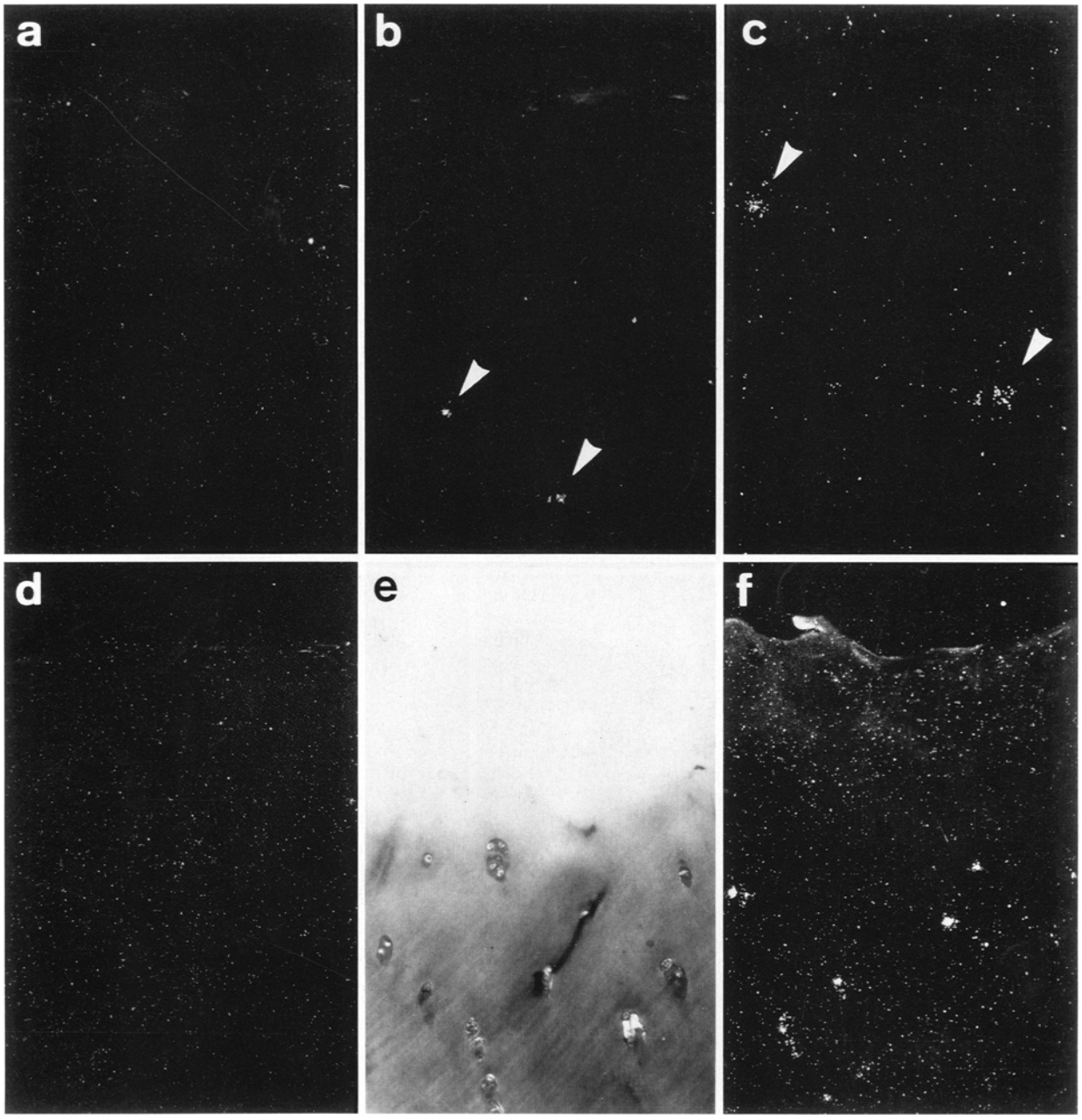


Fig. 2a–f. α1(II) mRNA detection in normal adult articular cartilage samples: no cells (**a, d**) or only few cells (**b, c**: detail) are positive (**a** 49-year-old, m, knee; **b–d** 60 year-old, f, hip). **e, f** α1(II) mRNA is detected in a sample of osteoarthritic cartilage (**f**) compared to toluidine blue staining (**e**). Note the zone of expression of α1(II) starts below the zone of proteoglycan loss (**e, f** 67-year-old, f, knee, OA). (**a, b, d, e, f**: ×130, **c** ×500)

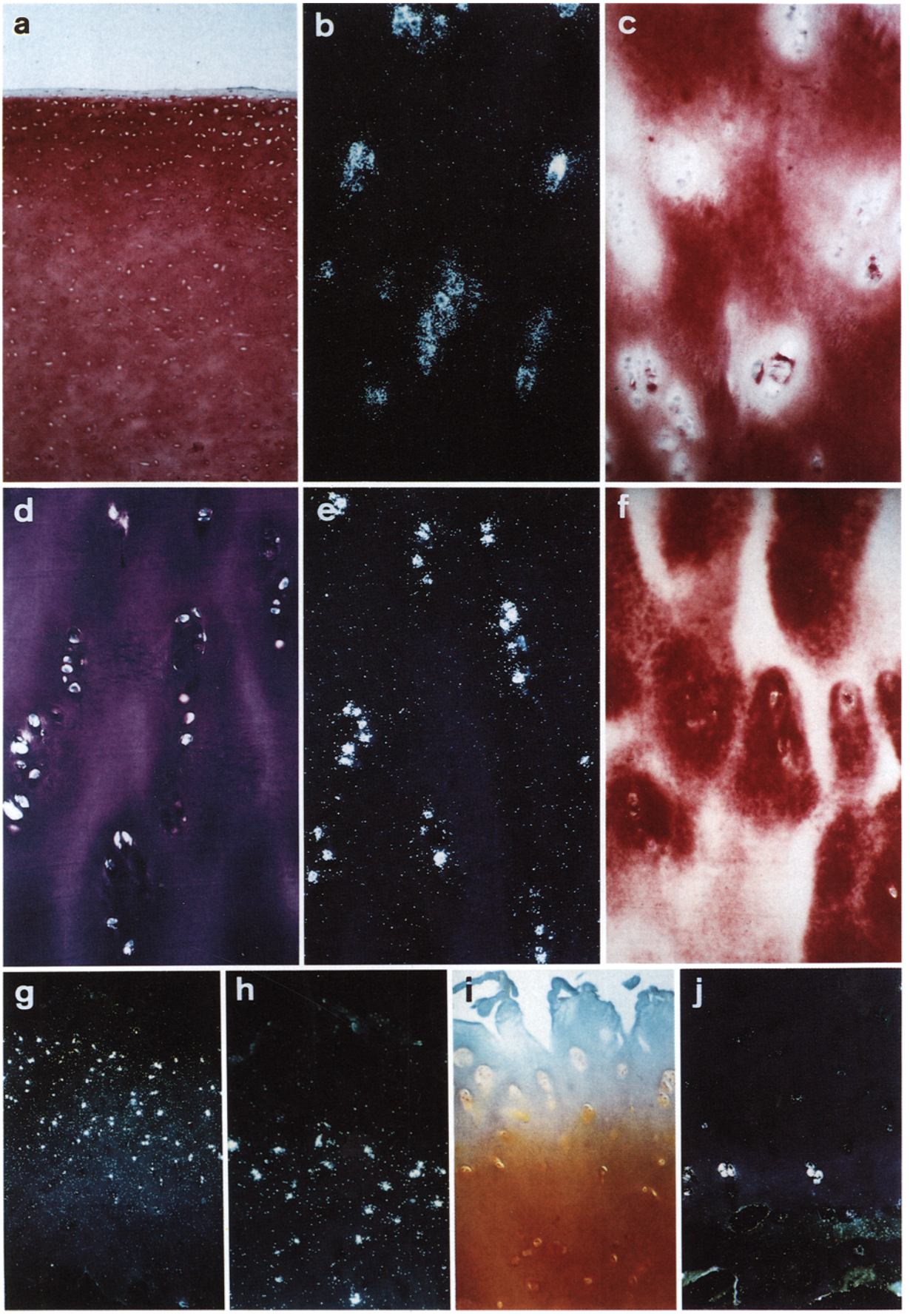
cally normal and showed no major loss of toluidine blue staining. In most samples in situ hybridization with the probe for α1(II) collagen revealed no signals (Fig. 2a, d), but in one sample a few cells in the middle layer showed weak expression of α1(II) mRNA (Fig. 2b, c).

Histochemical and immunohistological staining with a monoclonal antibody to type II collagen of normal cartilage showed an even extracellular staining for type II collagen (Fig. 3a) and proteoglycans (not shown). Only a very thin surface zone of various depth showed no or diminished staining for both matrix components (Fig. 3a).

Osteoarthritic original cartilage

A total of 18 samples from 12 patients undergoing knee arthroplasty ($n=10$) or hip replacement ($n=2$) were examined. All were in the clinically final stages, having areas of complete cartilage erosion. Nevertheless they displayed strikingly different histological features in different areas and showed low to high Mankin-grades (3 to 9).

Most specimens with minor histological abnormalities (low Mankin-grade 3–4; no deeper clefts, no severe proteoglycan loss) showed strongly enhanced collagen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type II expression. In nine of 12 specimens investigated, expression was most pronounced in the middle layers and declined in intensity towards the bone, but mostly did not involve the superficial or the lower deep zones (Fig. 3g, h for schematic repesentation see Fig. 5). Two examples, however, showed even expression over the whole middle and deep zone. In general, the upper border of α1(II) expression matched well with the beginning of loss of toluidine blue and safranin O staining in the surface layer (Fig. 3h, i). In most samples the signals were found in a zonal distribution and only occasionally scattered over the section yielding relatively low signals.

In all sites with more severe histological changes (Mankin-grade 5–9) showing deeper clefts, cell clusters and more intense loss of proteoglycan staining, expression of collagen type II was detected in a zonal distribution similar to specimens with minor grades. In areas of severe loss of proteoglycan staining inlcuding the middle zones, type II collagen expression was absent but started below.

Immunohistochemistry revealed irregular and sometimes diminished interterritorial staining as well as enhanced pericellular deposits of collagen type II (Fig. 3f), coinciding with strong type II collagen expression (Fig. 3e). Intense pericellular staining for toluidine blue and safranin O accompanied that for collagen type II (Fig. 3d) and was absent in areas above the zone of α1(II) expression (Fig. 3i). A precise correlation would, however, require double-detection experiments. In two samples pericellular loss and therefore, presumably, collagen type II degradation was found (Fig. 3c) despite high cytoplasmic α1(II) mRNA levels (Fig. 3b).

Earlier clinical stages of osteoarthritis

Four samples of the earlier clinical stages of osteoarthritis in this series showed signs of minor degenerative lesions (Mankin-grades 3–5, no major fibrillation or cluster forming) with a generally intact articular surface, representing earlier stages of the disease process. All examples showed enhanced type II expression in the middle zone. One sample also showed strong signals in the deep chondrocyte layers, but not in the calcified zone (Fig. 3j). Interestingly, in this sample a blood vessel penetrating from the subchondral bone into the cartilage was observed. Immunohistochemical staining showed an even distribution of type II collagen in these samples.

Rheumatoid cartilage

Examination of six samples of late stage rheumatoid cartilage yielded resutls similar to those described for osteoarthritic specimens. Again, activation of type II collagen was seen depending on the proteoglycan distribution (Fig. 6a–d). Chondroid metaplasia of pannus tissue overlaying one specimen also showed strong type II collagen synthesis (not shown).

Control experiments

Hybridization with control transcripts, e.g. type II collagen mRNA sense strands, never revealed signals above

Fig. 3. **a** Normal cartilage stained with a monoclonal antibody to type II collagen shows even distribution of extracellular type II collagen over the whole section except in the very superficial zone (17-year-old, m, knee). **b, e, g, h** Strong signals for α1(II) mRNA in chondrocyte clusters in osteoarthritic cartilage samples (**b, e**) were mostly accompanied by pericellular intense staining of type II collagen (**f** antibody staining for type II collagen) and proteoglycans (**d** toluidine blue staining). One sample immediately adjacent to a newly-formed osteophyte showed, however, pericellular loss of type II collagen (**c** staining with a monoclonal anti-type II collagen antibody). (**b, c** 79-year-old, f, hip, OA; **d, e, f** 66-year-old, f, knee, OA). **g, h, i** Collagen type II mRNA expression in another osteoarthritic cartilage sample: a zonal distribution starting typically in the middle zone and diminishing towards the bone (bottom); the upper negative zone matches well with the safranin O-negative area (**g, h, i**) (70-year-old, f, knee, OA). **j** One sample of an earlier stage lesion showing strong expression of type II mRNA in deep zone chondrocytes, but not in the calcified cartilage (17-year-old, m, knee, osteosarcoma). (**h, i, j** ×90; **b–f** ×240; **a, g** ×35)

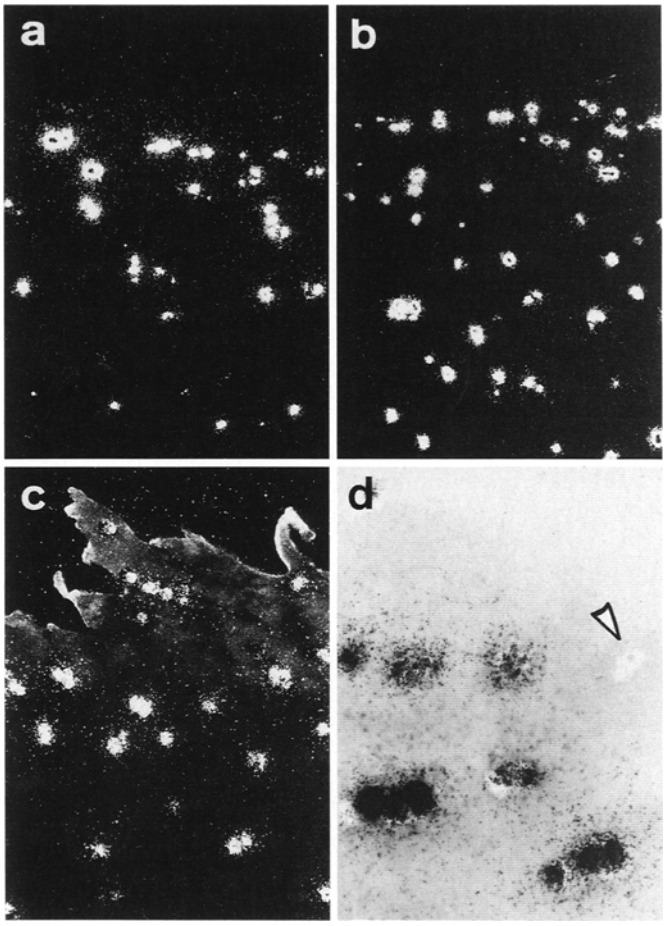

Fig. 4A–d. In situ hybridization with a 18S rRNA probe revealed in normal (**a** 51-year-old, m, hip) and osteoarthritic (**b** 62-year-old, f, knee; **c** 70-year-old, f, knee) articular cartilage samples similar signals over the whole sections, sometimes more accentuated in the superficial areas (**a**). Only very few cells were negative (**d** *arrow*; 62-year-old, f, knee, OA). (**a–c**: ×130; **d** ×600)

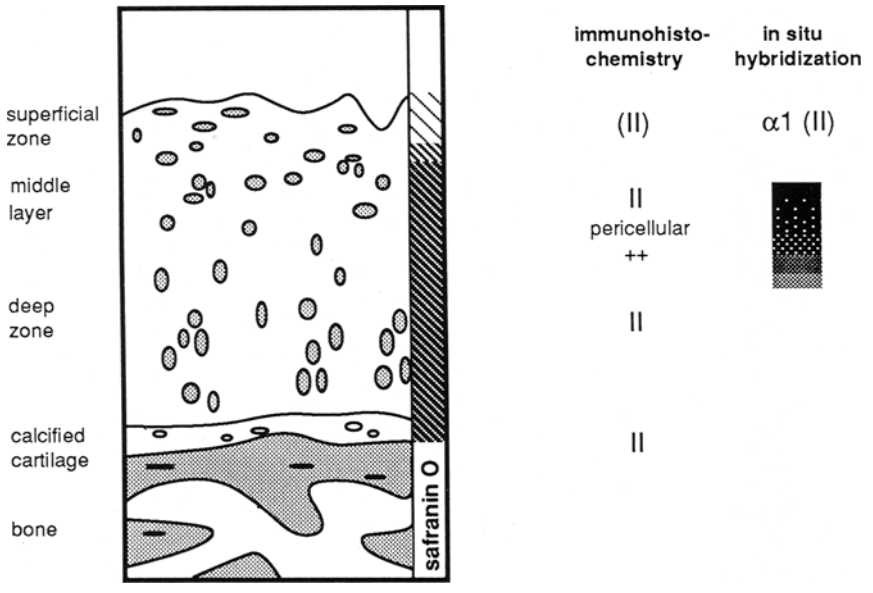

Fig. 5. The diagram shows a typical gradient of type II collagen mRNA distribution found in low Mankin's grade osteoarthritic cartilage samples in relation to immunohistochemical detection of type II collagen and safranin O staining

background (see Fig. 6d). The 18S rRNA probe in all samples showed a positive signal over the whole section, sometimes more accentuated in the superficial and upper middle zones (Fig. 4a–c). This indicates that the cytoplasmic mRNA was retained also in type II collagen-negative samples. Very few cells were seen without any signal at all (Fig. 4d, arrow head).

Discussion

In this study we have demonstrated that articular chondrocytes can respond to cartilage destruction occurring in the early and middle phases of osteoarthritis by enhancing type II collagen gene expression. Strong cytoplasmic signals were obtained with an α1(II) RNA probe predominantly in the middle zone of osteoarthritic cartilage, and, in one sample, also in the deep zone close to subchondral bone lesions and openings to the bone marrow. Thus, our data identify sites of activated type II collagen synthesis and confirm at the mRNA level previous biochemical studies on enhanced collagen synthesis in samples of osteoarthritic human articular cartilage (Lipiello et al. 1977) and in experimental osteoarthritis in dog cartilage (Eyre et al. 1980). Similarly, the synthesis of proteoglycan is significantly enhanced in osteoarthritic cartilage (Collins et al. 1960; McDevitt et al. 1975; Mankin et al. 1981; Mitrovic et al. 1981; Ryu et al. 1984; Sandy et al. 1984). We were able to localize the activation of type II collagen expression to an area in the articular cartilage immediately underneath the zone of proteoglycan loss seen after toluidine blue and safranin O staining (Fig. 5). Major matrix depletions of proteoglycans were not found in areas containing type II collagen-expressing cells. This, and the fact that pericellular enhanced deposition of collagen type II was accompanied by intense pericellular toluidine blue staining indicative of proteoglycan synthesis (Meachim 1972) is consistent with the coordinate expression of collagen type II and proteoglycans in chondrocytes in adult cartilage (Vertel and Dorfman 1979).

In comparison, in most normal adult articular cartilage α1(II) gene expression could not be detected. This was not due to mRNA degradation occurring for technical reasons, as indicated by the strong signal obtained in all areas in these samples with an 18S rRNA probe. The inability to detect cytoplasmic α1(II) mRNA in normal adult articular cartilage by in situ hybridization, however, does not imply complete silence of type II collagen gene activity in this tissue, as very low levels of mRNAs prevalent in cells will not be detected by this method. However, it confirms the presumably very low turnover of collagens in cartilage (Repo and Mitchell 1971).

The even signal distribution found for 18S rRNA is consistent with other studies finding very few necrotic cells in osteoarthritic samples (Roy et al. 1968; Rüttner et al. 1968). The rare 18S RNA-negative cells detected in some of our specimens are probably due to cutting artefacts, but could represent scattered necrotic cells that are known to exist both in normal and degenerated cartilage (Sheldon 1983).

Immunohistochemical staining of consecutive sections of osteoarthritic cartilage with a monoclonal antibody to type II collagen largely confirmed the results obtained by in situ hybridization. We frequently found pericellular intense staining for type II collagen, indicating new synthesis of this protein. However, despite the enhanced type II collagen gene expression and local pericellular deposition of type II collagen, the overall deposition of new type II collagen in osteoarthritic cartilage is often reduced (Burton-Wurster et al. 1982).

Antibody staining of normal adult articular cartilage revealed an even distribution of type II collagen in the matrix. The most superficial layer, however, hardly stained for type II collagen or for proteoglycans and was instead positive for type I and III collagen (Aigner et al. 1992) indicating the different phenotype of cells

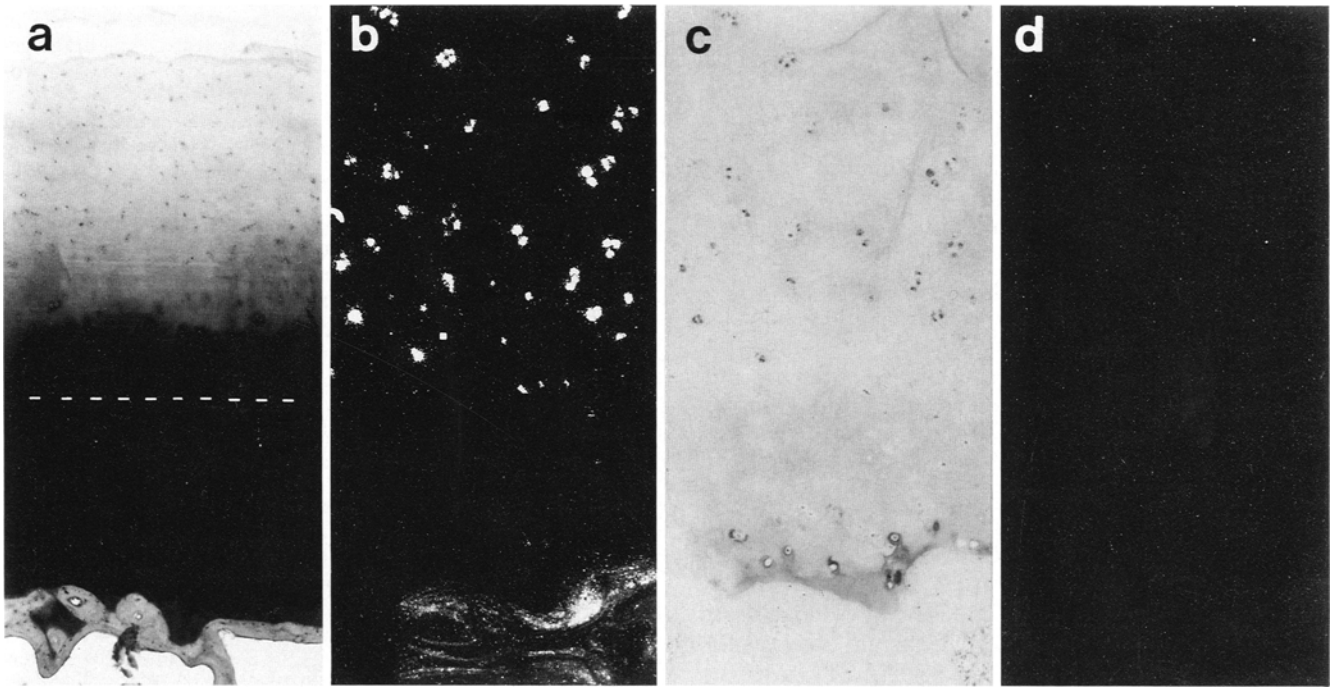

Fig. 6a–d. In situ hybridization with the collagen type II probe of a rheumatoid cartilage sample showing severe proteoglycan loss in the upper and middle zones. **a** overview, low magnification, toluidine blue staining. **b, c** Collagen type II mRNA is expressed by the chondrocytes of the lower middle zone below the dashed line shown in (**a**). **b** bright field, **c** dark field. Hybridization with a control probe gave no signals above background (**d**) (53-year-old, f, knee). (**a** 45×, **b–d** 130×)

at the surface of articular cartilage. These cells could be a remnant of the fetal epichondrium showing significant cytoplasmic signals of α1(I) and α1(III) mRNA after in situ hybridization analysis (Sandberg et al. 1987, 1989).

In some samples of osteoarthritic cartilage, type II collagen-antibody staining and in situ hybridization signals did not correlate. Clusters of deep and middle zone chondrocytes showed strong signals with the α1(II) probe, but complete lack of pericellular staining for type II collagen, suggesting enhanced protease activity (Dean 1991). It remains to be established whether the lack of antibody staining is due to complete destruction of extracellular type II collagen, or only to loss of antigenic epitopes. Using an antibody specific for denatured type II collagen, Dodge and Poole (1989) demonstrated the appearance of denatured collagen chain fragments in osteoarthritic cartilage. Since in our studies similar results were obtained using polyclonal and monoclonal antibodies to type II collagen recognizing native and denatured epitopes on the molecule, it is likely that the pericellular collagen-negative halos around osteoarthritic chondrocytes indicate rather extensive destruction of type II collagen.

The results obtained in the present study provide further evidence that "the osteoarthritic chondrocytes are metabolically hyperactive" (Mankin et al. 1981). Whatever the first stimulus may be – potentially any form of damage of the extracellular matrix – chondrocytes start to restore the matrix by increasing the synthesis of its components such as collagen type II and proteoglycans (Sokoloff 1983). However, our studies show that not all chondrocytes in osteoarthritic cartilage are activated, but that activation occurs in zones, and probably transiently. Similar zones of type II collagen gene activation were also observed in the deeper middle zone in samples of rheumatoid cartilage. Preliminary studies on early osteoarthritic lesions obtained by arthroscopy indicate a more focal activation in contrast to the zonal distribution found in samples investigated in this study (Aigner et al., in preparation).

The more generalized matrix disintegration seen in the later stages is probably initiated by cytokines diffusing from the synovial fluid (for review see Shinmei et al. 1989). In the earlier phases, enhanced matrix degradation may be counteracted by increased synthesis of matrix components, so that the overall content of collagens remains stable for some time (Mankin et al. 1970), with presumably considerable residual damage in the collagen and proteoglycan molecules (Dodge and Poole 1989). In the later clinical stages the ability of chondrocytes to restore the matrix and collagen and proteoglycan synthesis falls (Mankin et al. 1971; Lipiello et al. 1977; own preliminary results). The chondrocytes become necrotic (Weiss and Mirow 1972) and the cartilage undergoes final erosion by mechanical destruction.

The observation that the chondrocytes in the upper layer of osteoarthritic cartilage do not show cytoplasmic αI(II) mRNA may reflect that cells in this area have ceased to express type II collagen after perhaps a phase of transient activation. The fact that this layer has resisted mechanical abrasion despite the lack of new type

II collagen synthesis could be explained by the onset of type III and/or type X collagen synthesis observed in this zone (Aigner et al. 1992; von der Mark et al. 1992), or alternatively that most of the osteoarthritic cartilage samples investigated in this study were for obvious reasons derived from non-weight bearing areas. This should also be taken into consideration when using apparently "normal" samples from morphologically intact areas of the joint as "control" cartilage. Even in such samples there might be a zonal expression of type II collagen, or of type III or type X collagen which are not found in normal specimens.

The general expression patterns presented in this study cannot take into consideration the entire complex variety of collagen alterations found in osteoarthritis. They are influenced by local mechanical factors, age, individual genetic predisposition of the patient, and a number of other factors, which however, end up in a similarly destroyed joint (Meachim et al. 1972).

In conclusion, by using in situ hybridization sites of enhanced synthesis of type II collagen in osteoarthritic and rheumatoid cartilage were localized and these results provide further evidence of the attempt by arthritic chondrocytes to repair cartilagenous injuries.

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