Expression of type VI collagen in normal and osteoarthritic human cartilage

BY OLIVER PULLIG, GERD WESLOH AND BERND SWOBODA

Division of Orthopaedic Rheumatology, Department of Orthopaedics, University of Erlangen-Nürnberg, Rathsberger Str. 57, D-91054 Erlangen, Germany

Summary

Objective: This study was undertaken in order to study the expression of type VI collagen in normal and osteoarthritic human knee cartilage.

Methods: Seventy-two osteoarthritic cartilage/bone samples were obtained from 29 patients with primary OA undergoing surgery for a total knee replacement. Normal cartilage was collected from five human knees at the time of autopsy. Type VI collagen protein was localized using a polyclonal anti human type VI collagen antibody, the corresponding mRNA was detected with an 310 base antisense probe, specific for the ß2(VI) collagen chain.

Results: In normal cartilage, type VI collagen protein is concentrated pericellularly around the chondrocytes of all cartilage zones. In the middle and deep zones, type VI collagen was also found in the interterritorial matrix. Type VI collagen mRNA expression was detected in chondrocytes of all cartilage zones. In moderately affected osteoarthritic cartilage, type VI collagen expression was increased. An intensive immunohistological interterritorial staining for type VI collagen was observed in the middle and deep cartilage zones. Specific mRNA signals were also increased especially in the middle and deep cartilage zone. In the superficial zone and calcified cartilage of these samples, type VI collagen mRNA expression was restricted to focal areas. In severe osteoarthritic cartilage, an intensive staining for type VI collagen mRNA was found in clusters of proliferating chondrocytes and in the deep cartilage zone. Type VI collagen was localized pericellularly and in the matrix of chondrocyte clusters. Furthermore, chondrocytes from the deep zone showed a territorial distribution of type VI collagen.

Conclusions: These results demonstrate that in normal and osteoarthritic cartilage, type VI collagen is expressed in a zone specific pattern. The observed increase of type VI collagen expression in osteoarthritis suggests a potential role in the disease process.

Key words: Type VI collagen, Chondrocyte metabolism, Osteoarthritis, In-situ hybridization.

Introduction

A balanced metabolism of the various extracellular matrix components is necessary to maintain the structure and function of articular cartilage. During the process of osteoarthritis (OA), metabolic alterations lead to a finally irreversible loss of the cartilage matrix. Articular cartilage is a highly organized tissue consisting of specific proteoglycans filling the network of collagen fibres which contains type II collagen as major and types IX and XI as minor collagens [1]. Early immunohistological studies by Ayad et al. [2] suggested that hyaline cartilage also contains type VI collagen. Quantitatively, type VI collagen belongs to the minor collagens in cartilage with an approximate content of less then 2% of the total collagens [3, 4]. The molecule forms a heterotrimer, consisting of three genetically different alpha chains (ß1(VI), ß2(VI), ß3(VI)). Type VI collagen monomers are arranged as dimers and tetramers, which form a separate collagenous network [5, 6]. On the amino-terminal and carboxy-terminal ends, two large globular domains are attached to the triple helical domain of the molecule [5, 7]. The globular domains show a variety of sequence homologies to domains of other molecules like the Kunitz-type protease inhibitor, the von Willebrand Factor A domain, fibronectin type III, actin, salivary proteins and other proteins [8, 9] and may be responsible for specific functions of this collagen. In-vitro experiments have shown that type VI collagen has binding capacities to several matrix molecules like type I collagen [10, 11], type II collagen [12] and type XIV collagen [13] and also to noncollagenous proteins like decorin [12, 14],
hyaluronic acid [11, 15], and von Willebrand factor [16]. Interactions of type VI collagen with the cell surface are mediated by α1β1 integrins and α2β1 integrins [17, 18] and also by non-integrin mediated binding mechanisms [19, 20]. Type VI collagen is supposed to play an important role in the organization of the pericellular matrix and to anchor chondrocytes in their surrounding extracellular matrix [21]. In articular cartilage, chondrocytes of the middle and deep cartilage layers are embedded in a pericellular matrix with a high proteoglycan [22] and hyaluronic acid content [23]. The pericellular matrix is surrounded by a pericellular capsule, which consists of fine fibrils forming a basket-weave like network. Outside the pericellular capsule a zone of thicker fibrils and high proteoglycan content forms the territorial matrix [24].

Histomorphological and immunohistochemical investigations have localized type VI collagen fibrils in the pericellular matrix and capsule of the chondrocytes [2, 21, 25, 26]. In the territorial matrix, type VI collagen was detected as randomly distributed deposits [27] and periodic fibrils [25]. Increased levels of type VI collagen were found in experimental models of OA [27, 28] and in normal OA [4, 26]. It is still unclear whether increased concentrations of type VI collagen in cartilage are the result of a continuous deposition of this molecule or also caused by an increased synthesis. In addition, the topographical localization of the protein and gene expression of type VI collagen in the different zones of normal and osteoarthritic cartilage are not yet known. The aim of this study was to systematically characterize and localize the type VI collagen expression in OA by immunohistology and in-situ hybridization to get a better understanding of the metabolic activation of chondrocytes in osteoarthritic cartilage.

Materials and methods

CARTILAGE SAMPLES

Seventy-two osteoarthritic cartilage/bone samples were obtained from 29 patients with primary OA (54–75 year-old donors) undergoing surgery for a total knee replacement. Clinical data were carefully reviewed to exclude any forms of secondary OA and inflammatory joint diseases like rheumatoid arthritis. Normal cartilage was collected from five human knees at the time of autopsy within 18 h after death (34–56 year-old donors). In normal cartilage samples, both the tibia plateaus and femoral condyles were macroscopically normal without any signs of osteoarthritis. The localizations, where the samples were taken from, were carefully monitored by video printouts. Osteoarthritic changes were graded using the grading system of Otte [29] (Table 1). There were five normal samples, 10 samples with slight, 27 samples with moderate, and 35 samples with severe osteoarthritic lesions.

PREPARATION OF TISSUE SAMPLES

One-cm thick cartilage/bone samples with a cartilage surface of about 2.0 cm × 0.5 cm were incubated in freshly prepared paraformaldehyde, 4% (w/v), buffered with 0.01 M sodium phosphate, pH 7.4, containing 0.14 M NaCl (PBS) overnight at 4°C. The tissue samples were decalcified in diethylpyrocarbonate (DEPC)-treated 0.2 M EDTA, pH 8.0 for several weeks at 4°C. The buffer was changed twice per week. Tissue specimens were then embedded in paraffin. Cartilage/bone sections (5 μm) were mounted on glass slides, precoated with 1% (v/v) 3-aminopropyl triethoxysilane and heated for 90 min at 65°C.

PREPARATION OF THE TYPE VI COLLAGEN RNA PROBES

Total RNA was extracted from human knee cartilage. Cartilage (0.5 g) was frozen in liquid nitrogen and pulverized using a stainless steel pestle and mortar. Extraction was performed using acid guanidium isocyanate as described by Chomczynski et al. [30]. One μg of total RNA was transcribed into single-stranded cDNA using AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) and Oligo-p(dT)15 as primer according to the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany). Double-stranded cDNA was generated by PCR using modified primers containing a new HindIII site in the upstream primer (5′-TCC CAA AGG AGA GAA-3′), and a new EcoRI site in the downstream primer (5′-GTC CTT AAG GAC CCC GAG GTA A-3′). The primer pair spans 299 bases coding for the N-terminal area of the triple helical domain of the α2(VI) collagen chain. The PCR product as well as the cloning vector (pT3/T7o18 Life Science, Eggenstein, Germany) were digested with the restriction enzymes EcoRI and HindIII. Following ligation, cloning and amplification the plasmid DNA was checked by multiple restriction digestion as well as by sequencing (Perkin-Elmer, Abiprism, Weiterstadt, Germany). For in-vitro transcription and labelling of antisense and sense riboprobes, the plasmid DNA was digested with HindIII for the antisense probe and...
EcoRI for the sense probe, purified by phenol/chloroform extraction and transcribed using 1 μg of linearized plasmid DNA, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.65 mM UTP and 0.35 mM digoxigenin-11-UTP in 40 mM Tris-HCl, pH 8.0, 6 mM MgCl₂, 10 mM dithiotritol, 2 mM spermidin and 40 U T7 RNA-Polymerase (for the antisense probe) or 40 U T3 RNA-Polymerase (Boehringer Mannheim, Mannheim, Germany) for the sense probe. The components were incubated for 2 h at 37°C. Template DNA was removed with 20 U DNase I (Boehringer Mannheim, Mannheim, Germany). The reaction was stopped by adding 2 μl 0.2 M EDTA, pH 8.0. RNA was further purified by ethanol precipitation and resuspended in 100 μl DEPC-treated H₂O. Transcripts were checked for integrity, length (310 bases) and labeling efficiency by denaturing gel electrophoreses followed by blotting on nitrocellulose membrane and digoxigenin detection using the DIG-nucleic-acid-detection-kit (Boehringer Mannheim, Mannheim, Germany). About 100 ng of a2(VI) collagen antisense or sense riboprobe were used for each tissue section. Immediately before use, riboprobes were incubated at 96°C for 10 min followed by 2 min at 4°C. An Oligo-p(dT)₂₈ primer, 5’-endlabeled with digoxigenin-ddUTP (Biometra, Göttingen, Germany), which detects all polyadenylated mRNAs, was used as a positive control.

CONTROLS

The specificity of the type VI collagen probe was checked by Northern blotting using placenta mRNA as a target (data not shown). The probe was specific for a mRNA of 3.5 kilobases corresponding to the size of the a2(VI) collagen chain. In addition, nucleic acid sequence homologies were tested using the FASTA program at the EMBL Institute in Heidelberg, Germany. Homologies to the α1(VI)-, α3(VI) chain, and to other known collagens and proteins were less then 54%.

In order to exclude a chain specific increased expression of the a2(VI)-chain mRNA, a riboprobe against a3(VI) was generated using the cloning strategy as described above (upstream primer: 5’-GAG AAG CTTGAG GAC CTG GAG A-3’; downstream primer: 5’-CCA GGA ATT CCT TCT CTT TCT T – 3’). The antisense a3(VI) riboprobe (388 bases) has a 99% homology to the triple helical domain of the a3 chain of type VI collagen. Nucleic acid analysis revealed less than 56% homology to the α1(VI)-, a2(VI) chain, and to other known human proteins. Two samples from normal cartilage and cartilage with early, moderate and severe lesions were hybridized. Non-specific staining of the probes to DNA sequences was excluded by an additional RNase treatment of the specimen prior to in-situ hybridization. In all control samples in which the RNA was predigested, no specific cellular staining was observed by in-situ hybridization using the type VI collagen probe or the poly dT-probe.

IN-SITU HYBRIDIZATION

Tissue sections were de-paraffinized in xylene and rehydrated through graded ethanol (100–50%). Sections were incubated with Proteinase K, 50 μg/ml in 100 mM Tris, pH 7.5, with 50 mM EDTA, for 15 min at 37°C and postfixed in 4% (w/v) paraformaldehyde/PBS for 1 min. Sections were briefly washed in DEPC-treated double distilled water and acetylated in 0.1 M triethanolamine by adding 0.3% (v/v) acetic acid anhydride twice for 5 min. Sections were briefly incubated in PBS, dehydrated in increasing concentrations of ethanol (50–100%) and then air-dried under dust-free conditions. Sections were prehybridized with a commercial hybridization buffer including 0.5 M NaCl and blocking reagent (ECL gold hybridization buffer, ECL blocking reagent, Amersham, Braunschweig, Germany). The buffer contained 8 M urea, which is equivalent to 50% (v/v) formamide to reduce the melting temperature of nucleic acid hybrids. Sections were carefully covered with a plastic film (Parafilm, Sigma, München, Germany) and prehybridization was carried out for 1 h at 43°C for a2(VI) collagen probe and at 37°C for the poly dT-probe. For specific hybridization, sections were incubated with 50 μl hybridization buffer containing the a2(VI) or a3(VI) collagen probe (2 μg/ml) or the poly dT probe (1 μg/ml). Again, sections were covered with parafilm and hybridization was carried out in a humidified chamber at 43°C (a2(VI) collagen probe) or 37°C (poly dT probe) overnight. For the hybridization step, the hybridization buffer was completed with 100 ng of the collagen probes, respectively 50 ng of poly-dT probe, 50 μl hybridization solution applied of the specimen, covered with parafilm and incubated in a humidified chamber overnight at 43°C for the type VI collagen probes and 37°C for the poly dT-probe. Sense-strand in-situ hybridization was also performed with a a2(VI) or a3(VI) collagen probe using the same conditions as for the antisense hybridization.

After hybridization, the sections were washed with 1 × sodium chloride, sodium citrate (SSC) for 10 min at room temperature and with 0.3 × SSC at 40°C for 1 h. Non-specifically bound riboprobes were removed by RNase treatment, T1-RNase
25 U/ml combined with RNase A, 5 µg/ml, in 5 mM Tris, 0.5 M NaCl, pH 7.5, 5 mM EDTA for 1 h at 37°C. Sections were briefly washed in 1× SSC at room temperature, stringently washed in 0.1× SSC for 2 h at 45°C (riboprobes) or alternatively at 37°C (oligoprobe) and finally washed in 0.5× SSC at room temperature.

Detection of bound probes was performed using a commercially available nucleic acid detection kit (Boehringer Mannheim, Mannheim, Germany). Briefly, sections were incubated in blocking solution containing 10% (v/v) fetal calf serum. After blocking, an anti-digoxigenin alkaline phosphatase-labelled antibody was added and bound antibodies were detected by adding the substrate solution (X-phosphate/NBT) overnight. Sections were counterstained with 0.5% (w/v) light green (Merck, Darmstadt, Germany) in 5% (v/v) ethanol and mounted in entellan (Merck, Darmstadt, Germany).

IMMUNOLOCALIZATION OF TYPE VI COLLAGEN

Tissue sections were de-paraaffinized in xylene and rehydrated. The sections were then digested with 2 mg/ml hyaluronidase (Merck, Darmstadt, Germany) in PBS, pH 5.5 for 15 min at 37°C and with 1 mg/ml pronase (Boehringer, Mannheim, Germany) in PBS pH 7.4 for 30 min at 37°C. After washing twice in PBS, sections were incubated in 5% (w/v) bovine serum albumin (BSA) buffered in PBS. The polyclonal anti-human type VI collagen antibody (lot no. 2A 0561 Telios Pharmaceuticals, San Diego, CA, U.S.A) was diluted 1:100 in 1% (w/v) BSA/PBS and incubated overnight at 4°C. After three washings in tris-buffered saline (TBS) a biotin-labeled anti rabbit antibody from donkey (Dianova, Hamburg, Germany) diluted 1:80 in 1% (w/v) BSA/TBS was added for 30 min at room temperature. Then, a complex of streptavidin and biotin labelled with alkaline phosphatase was added according to the protocol of the manufacturer (Dako, Hamburg, Germany). After three short washes in TBS, bound antibodies were visualized using Fast Red (Sigma, München, Germany) as a colour substrate. To exclude non-specific background staining sections were incubated with rabbit sera (Dako, Hamburg, Germany) and immunohistochemical detection was followed as described above.

STATISTICAL METHODS

Cartilage zone specific differences in (α2) VI mRNA expression were determined by counting the number of (α2) VI mRNA expressing cells in relation to the number of cells showing a signal for total mRNA. Samples from normal cartilage and cartilage with minor lesions (N=10), moderate lesions (N=10) and severe lesions (N=10) were compared. All samples used by this statistical analysis were from the central weight-bearing area of the lateral femoral condyle. Chondrocytes within a cartilage area of 1 mm in width were counted. For statistical analysis, the percentages of positive cells were compared in different stages of OA using a non-parametric statistic (Mann-Whitney U-test).

Fig. 1. Paraffin sections of normal femoral knee cartilage (54-year old man, original magnifications (1a), (b): 30 × , (c): 325 × , T=tangential zone, M=middle zone, D=deep zone, C=calcified zone). (a). Safranin-0 staining shows a homogenous staining in all cartilage layers with a reduction in the upper superficial layer, which is physiological. (b, c) Staining for type VI collagen using a polyclonal antibody against type VI collagen as described in Material and methods. Type VI collagen concentrates pericellularly around the chondrocytes in all cartilage zones. The interterritorial matrix of the middle layer also stains positive. (c) Higher magnification of the type VI collagen distribution in the middle zones showing the pericellular staining.

Fig. 2. Paraffin sections of a normal femoral knee cartilage (same sample as in Fig. 1), original magnifications 125 × . The upper part of the figures shows the tangential zone, middle part shows the middle zone, and lower part shows the deep zone. (a) Expression of c2(VI) collagen mRNA was shown by in-situ hybridization using a digoxigenin-labelled antisense riboprobe (see Material and methods). Chondrocytes from the tangential, middle and deep zones show expression of c2(VI). (b) Negative control; section was incubated with the sense probe of type VI collagen. (c) In-situ hybridization positive control to detect all polyadenylated mRNA. Hybridization was done using a 5'digoxigenin labeled oligonucleotide specific for the poly-adenosine sites of mRNAs. All sections were counterstained with light green to visualize the contours of matrix and cells.

Fig. 3. Paraffin sections of femoral knee cartilage with moderate OA (71-year old woman, magnifications (a), (b): 30 × , (c): 325 × , T=tangential zone, M=middle zone, D=deep zone, C=calcified zone). (a) Staining of the section with Safranin-0 shows a depletion of proteoglycans in the superficial zone and fissures of the surface (b). Expression of the type VI collagen molecule was visualized using a polyclonal antibody against type VI collagen as described under Material and methods. Interterritorial staining is prominent in the middle zones and areas of the adjacent deep zone. (c) Expression of type VI collagen protein in a higher magnification showing a section from the middle zone with a pericellular and a strong interterritorial staining.
Results
LOCALIZATION OF TYPE VI COLLAGEN IN NORMAL CARTILAGE AND IN CARTILAGE WITH SLIGHT FIBRILLATIONS OF THE ARTICULAR SURFACE

Normal articular cartilage was defined by an intact smooth cartilage surface and an only faint superficial reduction of the proteoglycans in the safranin O staining, which is physiological [31] [Fig. 1(a)].

No significant differences in the number of type VI collagen expressing cells (Mann-Whitney-U test) were found in normal cartilage and cartilage with a slight fibrillation of the articular surface as a minor osteoarthritic lesion. There were also no differences in the distribution of type VI collagen expression. Therefore, these two groups were summarized.

Immunohistologically, chondrocytes of all cartilage layers showed an intensive pericellular staining for type VI collagen. In the subchondral bone, also osteoblasts, osteocytes, yellow bone marrow, and blood vessels stained positive for type VI collagen [Fig. 1(b)]. The staining for type VI collagen in the interterritorial cartilage matrix was zone specific. An interterritorial staining could not be observed in the superficial, lower deep and calcified cartilage zone. However, in the middle cartilage zone and in the adjacent areas of the deep zone a diffuse interterritorial distribution of type VI collagen was observed [Fig. 1(b, c)]. No type VI collagen could be detected in the territorial matrix by immunostaining in all cartilage layers. Negative controls with rabbit serum showed no staining in cartilage and bone (data not shown). In-situ hybridization showed a specific mRNA signal in chondrocytes of the superficial, middle and deep zone, however the signal intensity varied between cartilage zones. Whereas the in-situ hybridization signal had the same intensity in the middle and deep zones, the signal intensity was reduced in the superficial zone [Fig. 2(a)]. Hybridization with the 3(VI) collagen probe revealed no differences in this zone-specific expression (data not shown). The positive control probe for polyadenylated RNAs showed staining in more than 90% of the cells [Fig. 2(c)].

TYPE VI COLLAGEN EXPRESSION IN CARTILAGE WITH MODERATE OSTEOARTHRITIC LESIONS

Cartilage specimens with moderate osteoarthritic lesions were defined by the fibrillation of the articular surface with clefts down to the middle zone. The Safranin O-staining for proteoglycans was markedly reduced [Fig. 3(a)]. The pericellular staining pattern for the type VI collagen molecule was similar to the data observed for normal cartilage, however the staining intensity was higher [Fig. 3(b, c)]. The most striking differences could be observed in the middle cartilage zone where the interterritorial matrix showed a remarkable increase in the staining for type VI collagen. The mRNA expression was most intensive in the middle and deep cartilage layers.
In the upper cartilage zone, the number of α2(VI) mRNA expressing cells varied enormously between different samples. The hybridization signal of the control probe for the α3 chain of type VI collagen corresponded to the signal distribution of the α2(VI) probe.

**TYPE VI COLLAGEN expression in severe osteoarthritic cartilage**

Specimens with progressive matrix destruction showed a loss of the superficial cartilage layers, clefts down to the subchondral bone and the formation of clusters of chondrocytes adjacent to the clefts [Fig. 5(a)]. Chondrocytes of the remaining middle and the deep zones showed the typical pericellular staining. The staining intensity was markedly enhanced compared to normal cartilage or cartilage with minor lesions. Within the remaining zones a territorial deposition of type VI collagen with minor lesions. Within the remaining zones a territorial deposition of type VI collagen was found [Fig. 5(b–c)]. In clusters of chondrocytes a pericellular staining and a diffuse matrix staining was observed. Compared to the pericellular distribution of type VI collagen in normal or early OA the staining intensity was increased. In situ hybridization, these clustering chondrocytes and chondrocytes from the deep cartilage layer showed a strong signal for the type VI collagen mRNA [Fig. 6(a)]. The number of cells expressing type VI collagen mRNA was significantly increased compared to cells from the middle zone of less damaged cartilage (P<0.031) and to cells from the deep zone of cartilage with minor lesions (P<0.03) [Fig. 7]. Hybridization of two samples with the α3(VI) riboprobe also showed prominent expression within the clusters of chondrocytes and chondrocytes from the deep zone [Fig. 6(d)].

**Discussion**

To understand the mechanisms leading to the breakdown of articular cartilage in OA, it is necessary to have detailed information on the matrix metabolism and on putative regulatory interactions between the chondrocytes and the matrix molecules and between the matrix molecules themselves. In the last decades, major matrix components like type II collagen or aggrecan have been investigated extensively. Recent studies also focused on so called minor components of the extracellular matrix like decorin, cartilage matrix protein, cartilage oligomeric matrix protein, and tenascin. This study systematically describes the metabolic activation of type VI collagen in osteoarthritic cartilage.

**Fig. 7.** Distribution of α2(VI) mRNA expressing cells in normal cartilage and cartilage with minor lesions (N=10), moderate lesions (N=10), and severe lesions (N=10). Number of chondrocytes showing expression of the α2(VI) collagen mRNA were counted and set in relation to the number of chondrocytes expressing total mRNA (as revealed by the poly dT probe). Chondrocytes of the superficial, middle, deep and calcified cartilage zone were counted within an area of 1 mm in width. Bars represent means with standard deviations.

Type VI collagen, a minor protein in cartilage, seems to play an important role for the integrity of the cartilage matrix. Matrix interactions have been demonstrated by in-vitro experiments [10, 14] and cell-binding studies. [18–20, 32]. Therefore, a probable physiological function of type VI collagen is to anchor cells to their surrounding matrix [21]. Type VI collagen may function as a bridging molecule between the various components of the extracellular matrix and the chondrocyte surface.

In this investigation, the cartilage/bone samples were divided into four groups: normal cartilage, early-, moderate-, and severe osteoarthritic lesions (Table 1). We used a grading system initially described by Otte [29] that reflects the progressive process of OA. For normal cartilage, our results show a pericellular staining of type VI collagen in all layers of human knee cartilage. This pericellular staining is consistent with ultrastructural and immunohistochemical data of Poole et al. [21, 26] showing that type VI collagen is localized next to the cell membrane. Investigations on isolated chondrons in canine cartilage showed dense aggregates of type VI collagen on the outer margin of the pericellular capsule and less compacted fibrils on the inner margin [26]. Earlier immunohistochemical studies using only hyaluronidase treatment to unmask epitopes found no or only a faint staining for type VI collagen in the interterritorial matrix [27].
additional pronase digestion, normal cartilage sections showed a more intensive and definitely positive staining of the interterritorial matrix in the middle and adjacent deep zone. Electronmicroscopically studies on postnatal human cartilage also showed randomly arranged periodic fibrils of type VI collagen in the interterritorial cartilage matrix without investigating zone-specific distributions [25].

No type VI collagen was localized in the territorial matrix of normal, slight, and moderate affected cartilage samples. Type VI collagen has been shown to be highly resistant against proteolytic degradation mostly due to the high thermal stability of the triple helical domain and the high content of intramolecular disulphide bonds [7]. However, recent in-vitro studies indicate that serine proteinases, lysosomal enzymes, and the matrix metalloproteinase-2 are able to degrade intact type VI collagen [33–35]. A high proteolytic activity in the territorial matrix might explain that this area does not stain for type VI collagen.

Comparing the type VI collagen protein expression in normal articular cartilage and cartilage with minor osteoarthritic lesions, there were no notable differences. All cartilage zones showed a pericellular staining for type VI collagen. In the middle zone and in adjacent areas of the deep zone an interterritorial distribution of type VI collagen was detected. However, in cartilage samples with moderate osteoarthritic lesions the interterritorial staining for type VI collagen was strikingly increased. These data are in accordance with previous data on the type VI collagen content in extracts of normal and osteoarthritic cartilage. McDevitt et al. and Ronziere et al. described a more intensive staining for type VI collagen in Western blots of cartilage extracts from experimentally induced canine OA and from human osteoarthritic cartilage [28, 36]. A significantly higher content of type VI collagen epitopes was quantitated in osteoarthritic human cartilage comparing type VI collagen level in cartilage and normal cartilage using an ELISA assay [4]. These data, however, gave no information on the metabolic activation of the chondrocytes. Therefore, we developed an in-situ hybridization to investigate, if the increased content of type VI collagen in cartilage extracts is caused by an enhanced synthesis of type VI collagen or only the deposition of this molecule in the extracellular matrix. We used a 310 bases antisense probe specific for the human α2(VI) collagen mRNA. Screening of a human sequence database led to a less then 54% sequence homology to other known human proteins. In a control hybridization using a α3(VI) specific ribo-probe a similar distribution pattern was found as for the α2(VI) probe, confirming the in-situ hybridization results for α2(VI) mRNA and suggesting similar stimulatory mechanisms in OA. The expression of type VI collagen mRNA was detectable in cells of the superficial, middle and deep layer. However, in the superficial and deep zones, only about 46% of the chondrocytes showed type VI collagen expression whereas 58% of the middle zone chondrocytes showed a positive type VI collagen mRNA signal (Fig. 7). This may be explained by a zone-specific higher turnover of type VI collagen.

Comparing the in-situ hybridization data on normal and moderate/severe osteoarthritic cartilage, we found an increased signal in osteoarthritic cartilage samples with an increased number of positive cells. These data show that the previously described higher content of the type VI collagen epitopes in cartilage extracts [4] may not only be the result of a continuous deposition of type VI collagen in the extracellular matrix, but is likely to be also caused by an increased synthesis of type VI collagen.

Basal levels of type VI collagen gene expression are detectable in normal cartilage, suggesting, that a continuous synthesis of the protein reflects the physiological cell status. An increased synthesis of type VI collagen in OA could be the result of a specific response to an imbalanced matrix composition as already discussed for other extracellular matrix components [38–40]. We could show that the immunostaining for type VI collagen is increased in severe OA. This was observed for the pericellular staining of all cartilage zones as
well as for the interterritorial staining of the middle and upper deep zone. In in-situ hybridization, α2(VI) collagen mRNA was increased in areas with an enhanced interterritorial staining. Therefore, the interterritorial accumulation of type VI collagen in OA is likely to be the result of an enhanced synthesis of the protein. The high proteolytic residence of type VI collagen may also contribute to the enhanced matrix deposition seen in moderate and severe osteoarthritic cartilage. Since proteolytic enzymes are increased in osteoarthritic cartilage [41], a limited degradation of the protein may occur and smaller fragments are more likely to diffuse into the interterritorial matrix. This hypothesis is supported by biochemical analysis of type VI collagen from osteoarthritic cartilage. Western blot analysis of guanidinium extracts of osteoarthritic cartilage revealed multiple shortened immunoreactive type VI collagen fragments [28].

In cartilage with severe alterations of the extracellular matrix, clusters of proliferating chondrocytes are observed. Chondrocytes of these clusters showed the highest type VI collagen mRNA expression with more than 90% of positive cells. The extracellular matrix surrounding these chondrocytes showed a diffuse staining for type VI collagen. Chondrocytes of these clusters are supposed to represent a metabolically highly activated phenotype. The high amount of newly synthesized type VI collagen may also contribute to the proliferation of chondrocytes in clusters. Recently, in vitro studies have shown that soluble type VI collagen is able to increase the proliferation of mesenchymal cells [20]. Additionally to this possible autocrine pathway, traditional growth factors and/or cytokines may influence the synthesis of type VI collagen in OA. Clusters of chondrocytes are often localized near fissures and clefts of the upper cartilage layer. This shortens the diffusion distance for nutrients as well as for cell mediators from the synovial fluid. The influence of transforming growth factor β (TGF-β) and interleukin-1 (IL-1) on type VI collagen synthesis has been investigated by in-vitro experiments. Increased synthesis of type VI collagen was induced in dermal fibroblasts by TGF-β [42] and in skin fibroblasts by IL-1β [43]. In synovial fibroblasts of patients with OA, IL-1α had the inverse effect on type VI collagen. Cell culture experiments resulted in a decrease of type VI collagen mRNA expression dependent on IL-1α stimulation [44]. In vivo, however, chondrocytes are embedded in their extracellular matrix and regulatory effects are likely to be more complex.

In OA, the biomechanical functions of the extracellular matrix are mainly affected by the disruption of the collagen network and by the loss of proteoglycans leading to a loss of the hydroelastic properties of cartilage [45]. Changes in the metabolic activity of chondrocytes, induced by mechanical impact have been investigated in vivo and in-vitro models [46, 47]. The cascade of mechanotransduction involves proteins, which transduce the extracellular mechanical signal into the cellular compartment [48]. As mentioned before, type VI collagen seems to be a link between cartilage matrix and the chondrocyte surface. It is found in direct contact to the cell membrane, interacts with cellular receptors and with various matrix components. Higher levels of type VI collagen in OA, as demonstrated in this study, may influence the signalling pathways from the extracellular matrix into the cells and contribute to an altered metabolism in osteoarthritic cartilage.

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