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Ultrastructural localization of type VI collagen in normal adult and osteoarthritic human articular cartilage

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Summary

Objective: Type VI collagen is a major component of the pericellular matrix compartment in articular cartilage and shows severe alterations in osteoarthritic cartilage degeneration. In this study, we analysed the exact localization of type VI collagen in its relationship to the chondrocyte and the (inter)territorial cartilage matrix. Additionally, we were interested in its ultrastructural appearance in normal and osteoarthritic cartilage.

Design: Distribution and molecular appearance was investigated by conventional immunostaining, by multilabeling confocal scanning microscopy, conventional transmission, and immunoelectron microscopy.

Results: Our analysis confirmed the pericellular concentration of type VI collagen in normal and degenerated cartilage. Type VI collagen formed an interface in between the cell surface and the type II collagen network. The type VI collagen and the type II collagen networks appeared to have a slight physical overlap in both normal and diseased cartilage. Additionally, some epitope staining was observed in the cell-associated interterritorial cartilage matrix, which did not appear to have an immediate relation to the type II collagen fibrillar network as evaluated by immunoelectron microscopy.

In osteoarthritic cartilage, significant differences were found compared with normal articular cartilage: the overall dimension of the lacunar volume increased, and a significantly increased type VI collagen epitope staining was observed in the interterritorial cartilage matrix. Also, the banded isoform of type VI collagen was found around many chondrocytes.

Conclusions: Our study confirms the close association of type VI collagen with both, the chondrocyte cell surface and the territorial cartilage matrix. They show severe alterations in type VI collagen distribution and appearance in osteoarthritic cartilage. Our immunohistochemical and ultrastructural data are compatible with two ways of degradation of type VI collagen in osteoarthritic cartilage: (1) the pathologically increased physiological molecular degradation leading to the complete loss of type VI collagen filaments from the pericellular chondrocyte matrix and (2) the transformation of the fine filaments to the band-like form of type VI collagen. Both might implicate a significant loss of function of the pericellular microenvironment in osteoarthritic cartilage.

Key words: Collagen Type VI, Osteoarthritis, Cartilage, Ultrastructure.

Introduction

Type VI collagen is an heterotrimer of three different α chains with short triple helical domains and rather extended globular termini[1,2], which assembles inside the cell to dimers or tetramers and are subsequently secreted into the extracellular matrix. It forms its own fine fibrillar network in virtually all connective tissues except bone[3–5]. Type VI collagen fibrils appear on the ultrastructural level as fine filaments, microfibrils or segments with faint crossbanding of 110 nm periodicity as previously observed in articular cartilage and other tissues[3,6–10], though not all fine filaments represent type VI collagen e.g. in the pericellular cartilage matrix[11–15].

In hyaline articular cartilage, type VI collagen is concentrated within the pericellular matrix[10,16–18] 3;3;12;31;31;40, which has been shown to be differently composed in many respects compared to the territorial and interterritorial cartilage matrices[11–13,19–22]. The function of type VI collagen is so far rather unclear. Type VI collagen has been shown to bind to integrins of the chondrocyte membrane via RGD-sequences in the α1 and α2 chains[23–26], though the exact interaction remained unclear. Additionally, type VI binds to other proteins of the pericellular matrix such as other collagens, decorin, fibromodulin, hyaluronan, and fibronectin[27–29]. Thus, type VI collagen most probably acts as an interface between the rigid interterritorial cartilage matrix and the chondrocyte and is presumably involved in cell anchoring as well as matrix cell signaling[9].

Hallmarks of osteoarthritic cartilage are the degradation of extracellular matrix components and the destruction of the pericellular microenvironment. The latter was suggested to be a central step occurring very early in the osteoarthritic degeneration process[30–33]. Studies have shown increased amounts of soluble type VI collagen in osteoarthritic cartilage[10], potentially corresponding to the significant interterritorial staining seen in a previous study[34]. The consequences of the derangement of the pericellular matrix are unknown. As type VI collagen is

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thought to bind integrins it might impede proper cellular signaling. On the other hand, the cell might lose its protective basket against compression forces. Alterations in the type VI collagen microenvironment might also influence the synthetic activity of cells[35] and, thus, modulate e.g. proteoglycan synthesis[36]. The type VI microenvironment might also be a central factor for the formation of chondrocyte clusters[32], as the pericellular matrix might be an important factor for proliferation of chondrocytes[32,37], which is one characteristic feature of osteoarthritic cartilage. The loss of the matrix, e.g. by enzymatically isolating chondrocytes, is a well known strong metabolic activating event for articular chondrocytes.

The aim of the present study was to exactly localize type VI collagen by confocal scanning microscopy and on the ultrastructural level in normal and osteoarthritic cartilage in order to elucidate the interaction in between the type VI collagen fibrils and the main type II-IX-XI collagen cartilage fiber network on the one side and the cell surface on the other.

Material and methods

TISSUE SAMPLING, HISTOCHEMISTRY, AND TISSUE GRADING

The study was performed on a series of 15 cartilage specimens from 15 patients undergoing endoprosthetic joint surgery for severe osteoarthritic lesions of the hip or knee (femoral condyles) joints. All cases satisfied the ARC classification criteria for osteoarthritis (OA) of the hip and knee[38]. Cases of rheumatoid origin were excluded from the study. Seven macroscopically and histologically normal samples were obtained from knee (femoral condyles) and hip joints obtained not later than 24 h post mortem. The age of the donors ranged from 28 to 79 years and 52 to 78 years for surgical and normal specimens respectively.

Cartilage slices were fixed and processed as described previously[39] and for all samples toluidine blue and safranin O stainings were performed in order to estimate the proteoglycan content. The samples were graded according to Mankin *et al*. 1971[40].

CONVENTIONAL IMMUNOHISTOCHEMISTRY

Conventional immunohistochemical studies were performed using a streptavidin-biotin-complex technique (Biogenex, Mainz, Germany) with alkaline phosphatase as detection enzyme as described previously[34].

Antibodies against the N-terminal domains of the α3-chain of type VI collagen were kindly provided by Dr R. Timpl (MPI, Munich)[29] and used at a dilution 1:5000. Monoclonal antibodies against β1-integrin were purchased from Biohit (Helsinki, Finland) and used at a dilution of 1:500. Monoclonal antibodies against type II collagen were kindly provided by R. Holmdahl (Uppsala, Sweden[41]) and monoclonal antibodies against vimentin were purchased from Dakopatts (Denmark). Both were used at a dilution of 1:50.

FLUORESCENCE IMMUNOHISTOCHEMISTRY

For fluorescence immunohistochemistry, sections were pretreated with hyaluronidase (2 mg/ml in PBS, pH 5) and protease XXIV (0.2 mg/ml in TB, PBS 7,3) and incubated with the antibodies at a ten times higher concentration than in conventional immunohistochemistry. The signals were detected subsequently using Cy-5-labeled secondary antibodies (Dianova, FRG). Nuclear DNA was counterstained with DAPI or propidium iodide.

DOUBLE-LABELING EXPERIMENTS FOR TYPE VI COLLAGEN AND β1 INTEGRIN OR TYPE II COLLAGEN

For double immunodetection using mouse monoclonal anti-β1-integrin or anti-collagen type II and rabbit polyclonal antitype VI collagen antibodies, histological sections were pre-treated with hyaluronidase (2 mg/ml in PBS, pH 5) and protease XXIV (0.2 mg/ml in TB, PBS 7,3) and incubated with a mixture of both antibodies at a ten times higher concentration than in conventional immunohistochemistry. The signals were detected subsequently using FITC-labeled goat antirabbit and Cy-5-labeled goat antimouse antibodies (Dianova, FRG). The sections were evaluated by confocal laser scanning microscopy.

CONFOCAL SCANNING MICROSCOPY

Immunofluorescence analysis and confocal scanning microscopy was performed using a Biorad 1000 microscope on conventional histological and 50–100 μm thick vibratome sections as described previously[34]. Fluorochrome-labeled secondary antibodies (FITC, Texas Red, and Cy-5, Dianova, FRG) were used for visualization of the antigens. Nuclear DNA was counterstained with propidium iodide.

CONTROL EXPERIMENTS

In control experiments, primary or secondary antibodies were replaced by PBS and the samples processed as described above. Additionally, in some test specimens similar dilutions of non-immune serum (BioGenex, San Ramon) replaced primary antibodies. All control samples were negative.

CONVENTIONAL ELECTRON MICROSCOPY

Conventional transmission electron microscopy was performed along the protocol published by Spurr[42] and modified by Schulz[43]. Briefly, small cartilage pieces were fixed with glutaraldehyd-formol overnight (1.25% glutaraldehyde, 2% formaline) and cleared in phosphate buffer (18.76 mg/ml NH_2PO_4, 4.28 mg/ml NaOH, pH 7.5). After post-fixation with 1% osmiumtetroxide for 2 h and re-washing in the phosphate-buffer the samples were dehydrated in an acetone series (30%, 60%, 70%, 80%, 90%, 100%, 100% acetone; 30′ each). Afterwards, samples were infiltrated with an epoxy resin-acetone series (50% 30 min, 75% overnight, two times 100% 2 h) and embedded in pure epoxy resin according to Spurr and Schulz in standard gelatin capsules[42,44]. The epoxy resin polymerized over night at 70°C. For light microscopical control, semi-thin sections (0.5–1 μm) were made and stained with methylen blue and azure II[45]. For electron microscopical analysis ultra-thin sectioning (50–100 nm) were placed on Formvar laminated copper grids and stained with uranylacetate and ‘leadcitrate’.

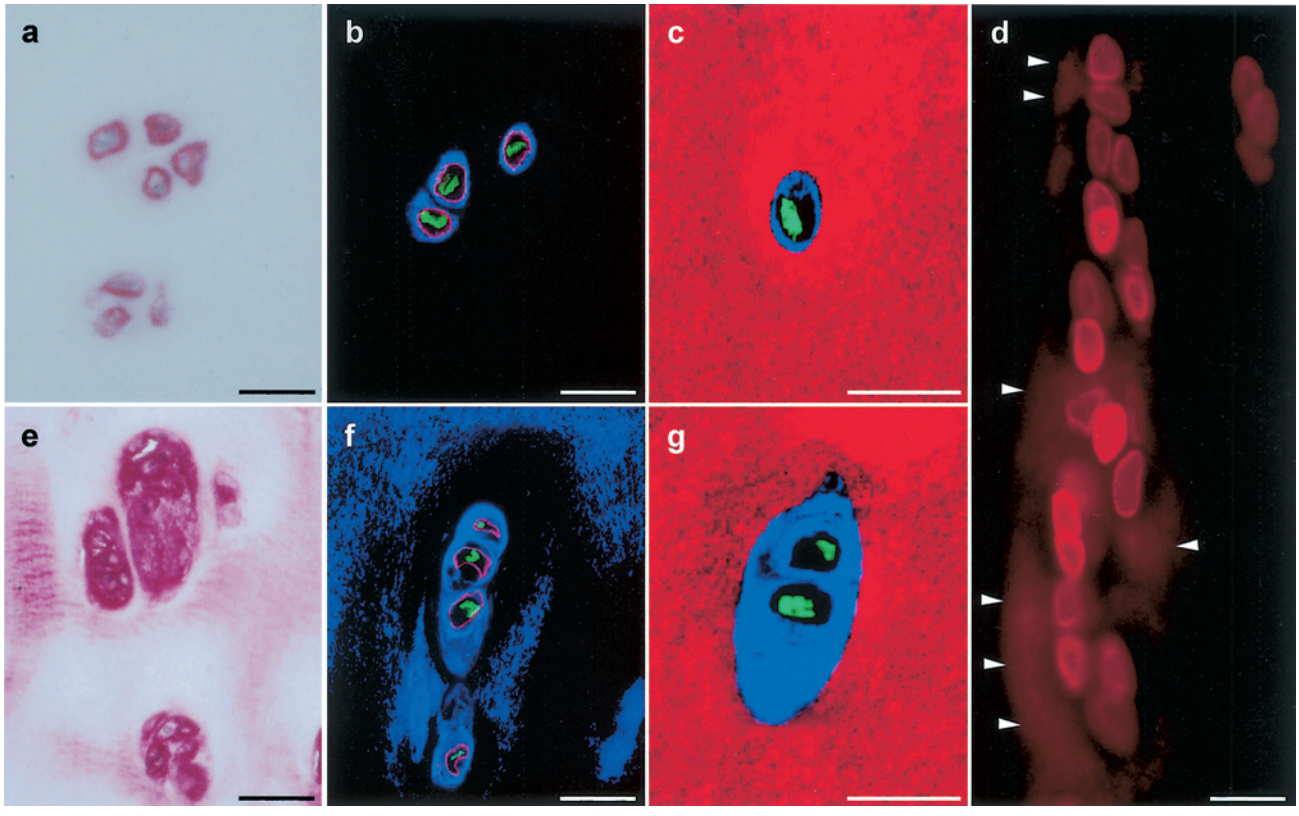

Fig. 1. Immunostaining for type VI collagen revealed a clear pericellular concentration of type VI collagen in normal (a) and osteoarthritic cartilage (e). This was confirmed by confocal laser scanning fluorescence microscopy [normal (b), (c); osteoarthritic (f), (g); blue (b), (c), (f), (g): type VI collagen; green (b), (c), (f), (g): nuclear staining; red (b), (f): β1-integrin; red (c), (g): type II collagen]. (d): Fluorescence immunostaining for type VI collagen in a semithick-section of normal articular cartilage (80 μm) visualized the columnar arrangement of chondrocytes in the deep cartilage zone of human articular cartilage (indicated by the red immunostaining of the type VI collagen positive pericellular matrix). It also clearly showed a faint staining for type VI collagen in the cell associated interterritorial matrix (arrowheads). (Magnification bars: 50 μm.)

IMMUNOELECTRON MICROSCOPY

For immunoelectron microscopy a pre-embedding protocol was used: semi-thick (50–100 μm) cartilage Vibratome® sections were stained immunohistochemically similar to Vibratome® sections stained for laser confocal microscopy. Sections were treated with hyaluronidase (2 mg/ml in PBS, pH 5) and protease XXIV (0.2 mg/ml, PBS pH 7.3) for 2 h. Subsequently, they were incubated with the primary antibodies overnight at 4°C at a ten times higher concentration than that used for conventional immunohistochemistry. After three washings with TBS, epitope-bound antibodies were detected using the same link antibodies as for conventional immunohistochemistry. The 'label' was either labeled with peroxidase or 1 nm gold particles. To all solutions (including antibodies) 0.05% saponin (Sigma) was added. If a peroxidase labeled antibody was used the sections were washed with TBS buffer three times and then incubated in DAB-solution (0.5 mg/ml DAB, 1 μl/ml of 30% H_2O_2, 50 mM Tris-HCl pH 7.6) for 1 h. The reaction was stopped with TBS buffer. For immunogold labeling, after washing three times with 'auro wash' buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 150 mM NaCl, 0.8% BSA, 0.1% IgG, and 0.02%NaN_3, pH 7.4) the sections were incubated with a gold labeled secondary antibody (Auro Probe, Amersham) diluted to 1:50 in 'auro wash' buffer for 2 h. After removal of the antibody, three washings with PBS (10 mM Na_2HPO_4, 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4), the sections were incubated with 2% glutaraldehyde (Fluka) in PBS for 10 min. After washing three times with PBS the sections were incubated with a silver enhancement kit (IntenSE, Amersham) for 2–10 min until sufficient staining was obtained. Subsequently, the reaction was stopped by washing with PBS and the stained sections were processed for electron microscopy as described above.

Results

NORMAL ARTICULAR CARTILAGE

Conventional immunohistochemistry [Fig. 1(a)] and confocal scanning microscopy [Fig. 1(b),(c)] revealed in normal articular cartilage type VI collagen staining concentrated in the pericellular matrix [Fig. 1(a)–(d)] with cells being less positive in the superficial zone. Some of the cells in the calcified zone as well as exceptional cells in the other zones were negative. Additionally, very faint immunostaining was detected in the cell-associated interterritorial matrix, but only after short fixation times [Fig. 1(d)]: arrow heads].

Double-labeling confocal scanning microscopy experiments using antibodies to type II and VI collagens showed a nearly exclusive distribution of both collagen types around the cells [Fig. 1(c)], though a slight overlap could

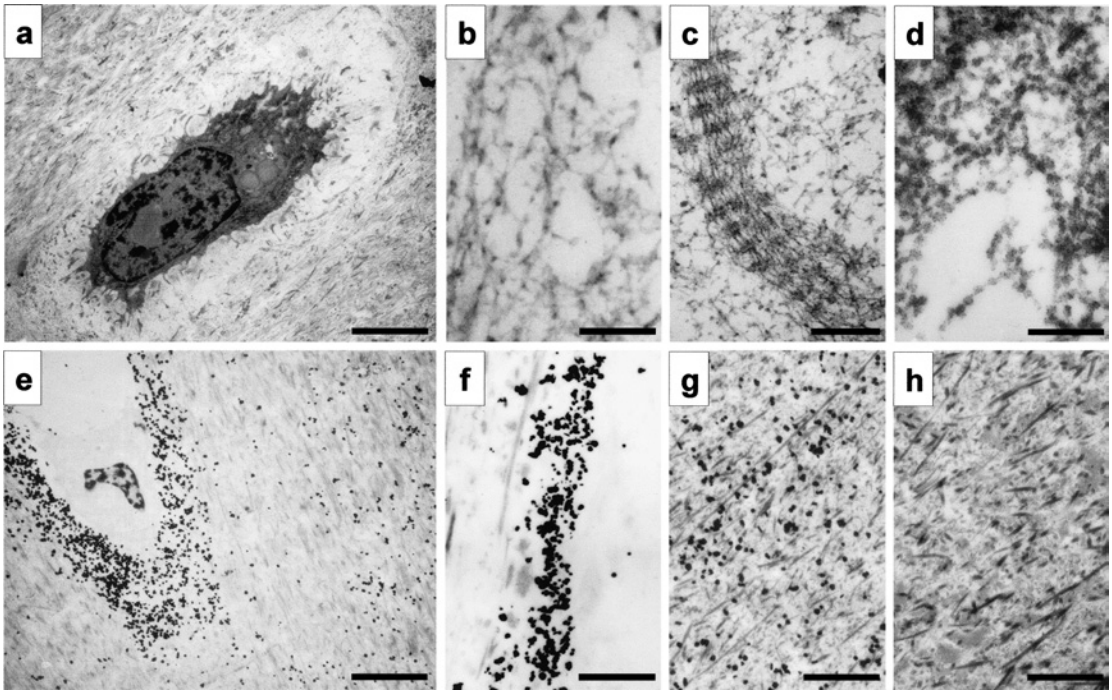

Fig. 2. Normal articular cartilage: conventional transmission electron microscopy showed a fine fibrillar matrix surrounding the chondrocytes [(a); (b): detail] and only exceptionally banded structures (c). The beaded appearance with the 100 nm periodicity was in particular apparent after peroxidase-labeled pre-embedding immunoelectron microscopy [(d): α3(VI)]. Immunogold-labeling showed the presence of [α3(VI)-]epitopes mainly in the pericellular matrix (e) and suggested some physical overlap with the type II collagen network (f). Labeling was also seen in the cell associated interterritorial matrix (e), (g) whereas the territorial cartilage matrix (e) showed hardly any and the interterritorial matrix far off the cells (h) showed virtually no labeling. [Magnification bars: (a), (e): 10 μm; (b): 2 μm; (c): 0.3 μm; (d): 0.5 μm; (f)–(h): 5 μm.]

not be excluded from this analysis. Double-labeling experiments using type VI collagen and β1-integrin antibodies showed an immediate association of type VI collagen fibrils to the cell surface [Fig. 1(b)].

In normal articular cartilage, conventional electron microscopy showed the typical fine fibrillar material in the pericellular chondrocyte matrix [Fig. 2(a),(b)]. Exceptionally, single banded structures of typical 110 nm periodicity were observed even in one case of a very young individual (28 years) with macroscopically and histologically normal articular cartilage [Fig. 2(c)].

Immunoelectron microscopy using immunogold-labeling confirmed the presence of type VI collagen in the pericellular space [Fig. 2(e)] and immunolabeling using the peroxidase technique showed beaded filaments with typical 110 nm periodicity [Fig. 2(d)]. Immunolabeling showed some overlap of type VI collagen with the type II collagen network [Fig. 2(f)] and confirmed the presence of type VI collagen epitope staining in the cell-associated interterritorial cartilage matrix [Fig. 2(g)], whereas the interterritorial matrix far off the cells was virtually negative [Fig. 2(h)]. Neither conventional electron microscopy nor immunoelectron microscopy was able to show typical beaded filaments in the cell-associated interterritorial matrix. The immunolabeling appeared not to be associated with the type II collagen fibrils.

OSTEOARTHRITIC CARTILAGE

Conventional immunohistochemistry and confocal laser scanning microscopy clearly showed extended areas of strong pericellular type VI positivity, in particular in the middle and deep zones of osteoarthritic cartilage [Fig. 1(e)–(g)].

Double-labeling confocal scanning microscopy for collagen types II and VI showed similar to normal cartilage no evident overlap of both collagen types [Fig. 1(g)]. Again, a close association of type VI collagen with the cell membrane was established by double-labeling with β1-integrin antibodies [Fig. 1(f)]. In all samples, extensively enhanced staining was seen in the interterritorial cartilage matrix [Fig. 1(e),(f)].

Conventional transmission electron microscopy revealed in osteoarthritic cartilage in the enlarged pericellular area around many of the chondrocytes banded structures with the typical 110 nm periodicity as well as fine filaments [Fig. 3(a)–(c)].

Immunoelectron microscopical analysis confirmed the extended pericellular, type VI positive zone [Fig. 3(d)]. This was particularly true for the chondrocytes of the middle and deep zones. Many of the cells were surrounded by excessive banded structures [Fig. 3(a),(c)], which were hardly labeled by the type VI collagen antibody [Fig. 3(f)]. Immunogold-labeling also confirmed in the cell-associated interterritorial matrix the increased prevalence of type VI collagen staining [Fig. 3(e)], but again no association with the type II collagen fibers was evident.

Discussion

Our ultrastructural analysis of normal articular cartilage using conventional transmission and immunoelectron microscopy confirms and extends previous work[6,9]. Chondrocytes of normal, but also—at least in part—of

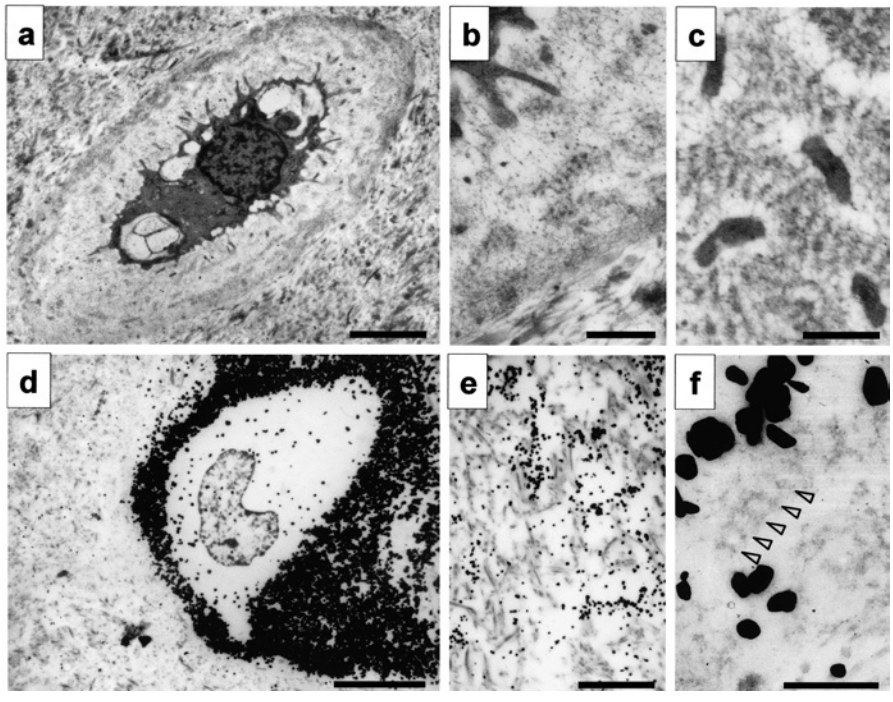

Fig. 3. Osteoarthritic articular cartilage: conventional transmission electron microscopy showed that the chondrocytes in osteoarthritic cartilage were surrounded frequently by the banded form of type VI collagen [(a): overview; (b), (c): details]. Immunogold-labeling confirmed the presence of type VI collagen in the extended pericellular zone (d) as well as in the interterritorial matrix (e). The banded structures were hardly labeled by the antibodies in the immunogold-labeling (f). [Magnification bars: (a), (e): 5 μm; (b): 1 μm; (c), (f): 0.5 μm; (d): 10 μm.]

osteoarthritic articular cartilage are surrounded by a fine network of type VI collagen filaments (besides other matrix components). Abundant cross-striated fibrous type VI collagen aggregates with the characteristic 110 nm periodicity[6,7] are found around osteoarthritic chondrocytes[10], but exceptionally also around normal chondrocytes[46], even in the young, as shown in this study. On the ultrastructural level, the presence of type VI collagen epitopes was confirmed also in the cell-associated interterritorial cartilage matrix[3,34] in particular in osteoarthritic cartilage. However, no association of the type VI collagen staining with the type II collagen fibrils was found in the areas positive for both collagen types II and VI. Thus, we found no evidence for a separate fibrillar type VI collagen network in the non-pericellular cartilage matrix as suggested by Keene and colleagues[3]. More likely, the interterritorial staining reflects an in osteoarthritic articular cartilage enhanced degradation of type VI collagen chains leading to increased type VI collagen degradation fragments diffusing from the pericellular matrix. This would fit previous biochemical data showing an increased degradation of type VI collagen in osteoarthritic cartilage[10,47] and support the assumption of a basic physiological turnover of type VI collagen also in normal articular cartilage[34]. Our data confirm a close linkage of the type VI collagen filaments to the cell surface, which is retained even after shrinkage of the cell membrane by fixation procedures. This linkage might be mediated by other molecules or by direct binding to cell-membrane integrins[9,23,24].

Our results suggest a physical overlap of the type VI collagen network with the type II collagen positive matrix in normal and osteoarthritic cartilage. Thus, besides physicochemical binding to other collagens, decorin, fibromodulin, hyaluronan, fibronectin[27–29], direct interwearing of both networks might play a role in anchoring the chondrocytes via type VI collagen to the territorial and via the territorial to the interterritorial cartilage matrix. This supports the notion that type VI collagen is one central molecular component forming a mechanical interface between the rigid type II collagen based territorial and interterritorial cartilage matrix and the cells, which are, as such, rather fluid.

In osteoarthritic cartilage, a very much enlarged ('swollen'[32]), type VI collagen positive pericellular area with still only a slight overlap with the type II collagen network is observed. It remains unclear whether this increase of the pericellular volume is due to distension of the territorial cartilage matrix or requires more or less extensive enzymatic degradation of the territorial cartilage matrix[48].

Own and other studies suggest a significant basal turnover of type VI collagen in normal and an enhanced turnover in osteoarthritic cartilage. So far, it is rather unclear how type VI collagen is degraded. Type VI collagen has been shown to be largely resistant to classical collagen degrading enzymes[6,49]. Also unclear is how the banded isoform of type VI collagen develops[7,30,50], although recent ultrastructural data further confirm their formation directly from the fine fibrillar subform[51]. Our data suggest two types of degradation of type VI collagen in articular cartilage and maybe other connective tissues: one 'physiological' form of degradation resulting in diffusion of type VI collagen fragments off the cells into the cell-associated interterritorial cartilage matrix as indicated by corresponding immunostaining. Secondly, transformation of type VI collagen fibrils leading to banded structures, which appear mostly in diseased cartilage. These banded structures might (largely) lack the N-globular epitopes of the α3(VI)-chain recognized by our antibody, whereas the triple-helical domain appears to be retained and can be visualized by electron microscopy[51]. Functionally, the banded isoform of type VI collagen might imply a significant functional loss of

type VI collagen at least in the pericellular matrix of chondrocytes (in osteoarthritic cartilage) as this isoform presumably implicates the reduced abundance of fine flexible filaments attached to the cell surface. Rather these banded structures represent large rigid conglomerates no longer providing the flexible mechanical interface to the type II collagen network.

Overall, we could show the enhanced prevalence of type VI collagen in the pericellular and in the cell associated interterritorial matrix of osteoarthritic cartilage and confirm the overall severe alteration of the pericellular matrix in osteoarthritic articular cartilage. Our results suggest two ways of degradation of type VI collagen, one 'physiological' and one leading to the banded structures. The latter are presumably rather rigid and functional insufficient. This transformational conformation of type VI collagen might be one primary reason for the significant functional derangement of the pericellular cartilage matrix rather than the a pure net loss of collagen type VI molecules in osteoarthritic articular cartilage.

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