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Immunolocalization of matrix proteins in different human cartilage subtypes

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Summary. Cartilage exerts many functions in different tissues and parts of the body. Specific requirements presumably also account for a specific biochemical composition. In this study, we investigated the presence and distribution pattern of matrix components, in particular collagen types in the major human cartilages (hyaline, fibrous, and elastic cartilage) by histochemical and immunohistochemical means.

Macroscopically normal articular cartilages, menisci, disci (lumbar spine), epiglottal, and tracheal tissues were obtained from donors at autopsy. Aurical and nasal cartilages were part of routine biopsy samples from tumor resection specimens. Conventional histology and immunohistochemical stainings with collagen types I, II, III, IV, V, VI, and X and S-100 protein antibodies were performed on paraformaldehyde-fixed and paraffin-embedded specimens.

The extracellular matrix is the functional component of all cartilages as indicated by the low cell densities. In particular major scaffold forming collagen types I (in fibrous cartilage) and II (in hyaline and elastic cartilages) as well as collagen type X (in the calcified layer of articular cartilages, the inner part of tracheal clips, and epiglottis cartilage) showed a specific distribution. In contrast, the "minor" collagen types III, V, and VI were found in all, collagen type IV in none of the cartilage subtypes.

In this study, we present a biochemical profile of the major cartilage types of the human body which is important for understanding the physiology and the pathophysiology of cartilages.

Key words: Immunohistochemistry, Human cartilage, Collagen, Matrix proteins

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Introduction

Cartilage exerts many functions in different tissues and parts of the body. In the joints the articular cartilage is mostly responsible for withstanding the mechanical load, whereas cartilages in the nose and the ear are mainly responsible for maintaining the form, and tracheal cartilage for preventing the organ from collapse during inspiration. These multiple functions require different biomechanical properties of their extracellular matrix and presumably also a different biochemical composition.

Cartilages principally consist of water containing dissolved solutes and the structural macromolecules, collagens and proteoglycans (Meachim and Stockwell 1979). These together make up more than 95% of the (dry) tissue mass. In addition to collagens and proteoglycans, other (glyco)proteins, enzymes, and their inhibitors are present in the extracellular matrix (ECM). Immunohistochemical studies on human tissues indicate that proportions and spatial distribution of cartilage constituents differ within cartilage subtypes. However, these studies typically investigated a certain function or pathologic state. The selection of matrix proteins and tissue subtypes was restricted to the specific scientific problem. Recently, a comprehensive immunochemical profile was presented for different cartilage subtypes in rabbits (Naumann et al., 2002). Studies like this are of interest not only with respect to understanding the physiology of the tissues and specific pathological conditions, but also the increasing need for evaluating engineered replacement tissues. A comparable profile is not available for human tissue. In the present study, we investigated the presence and distribution pattern of various collagen types and S-100 protein in human cartilages by immunohistochemical means. S-100 protein was included, as it was reported to be specifically expressed in human chondrocytes indicating the vitality and stability of the cartilaginous phenotype (Wolff et al., 1992). We selected six hyaline cartilages

(articular cartilage from knee, hip, and ankle, tracheal cartilage, nasal cartilage, and nucleus pulposus, two fibrous cartilages (meniscus and annulus fibrosus), and elastic cartilage (epiglottis and auricle). We investigated to what extent hyaline cartilage stands out from other cartilaginous tissues and discuss the impact of possible determinants to forming unique tissue characteristics.

Materials and methods

Tissue asservation and processing

Macroscopically normal articular cartilage from knee, hip and ankle, menisci, and disci (lumbar spine), epiglottal, and tracheal tissues (n=6, each) were obtained from donors at autopsy, within 48 hours of death. Donors were selected within an age range of 40–70 years with no evidence for musculoskeletal disorders or arthritic conditions. Auricle and nasal cartilages were part of routine biopsy samples from tumor resection specimens. Conventional histology and immunohistochemical stainings were performed on paraformaldehyde-fixed and paraffin-embedded specimens.

Histomorphological and histochemical demonstration of collagens and proteoglycans

From all samples Haematoxylin Eosin (HE) stainings were performed in order to evaluate cellularity and matrix integrity. Propidium iodide staining was used in order to assess cellularity and to identify apoptotic cell death (nuclear fragmentation).

Picrosirius Red and Elastica van Gieson's stain (EVG) for demonstrating collagens were performed according to standard histochemistry protocols (Romeis, 1989). The orientation of the collagen fibers was evaluated using polarized light microscopy. The presence of elastic fibers (elastins) was also demonstrated by the EVG stain.

Glycosaminoglycans were visualized by Safranin O staining according to the protocol from Rosenberg (Rosenberg, 1971). Occurrence and distribution of matrix constituents were assessed on a semiquantitative basis.

Immunohistochemical demonstration of S-100 cytoprotein and matrix components

The streptavidin-biotin-complex technique (Biogenex, Mainz, Germany) with alkaline phosphatase as detection enzyme was used for immunohistochemistry as described previously (Aigner et al., 1992). Various enzymatic pretreatments including hyaluronidase (Boehringer, Mannheim, Germany; 2 mg/ml in phosphate buffered saline (PBS), pH 5, for 60 minutes at 37°C), pronase (Sigma, Deisenhofen, Germany; 2 mg/ml in PBS, pH 7.3, for 60 minutes at 37°C), or bacterial protease XXIV (Sigma; 0.02 mg/ml; PBS, pH 7.3, for 60 minutes at 37°C) were tested. Table 1 lists antibodies and pretreatment and staining protocols for all antibodies, respectively. Fetal tissue was used to control for tissue specific reaction pattern of the antibodies: fetal bone showed strong immunopositive staining for collagen type I within the bone matrix. Cartilage was positive for collagen type II and collagen types I, III, V, and VI were found in the periosteal layer immediately next to osteocytes. Collagen type IV was selectively detected in the basal membranes e.g. of vessel walls. Collagen types V and VI were found pericellularly in the cartilage and collagen type X selectively in the hypertrophic zone of growth plate cartilage. References of previous published immunohistochemical studies provide additional evidence for the longstanding experience with the antibodies used here (Aigner et al., 1998, 1999, 2000; Dertinger et al., 2005). In addition to antibodies for the various collagen types we included that for S-100 protein. Observations for all immunostaining patterns were categorized for their intensity and prevalence (- = negative, + = weak and isolated, ++ = strong and widespread). For articular cartilages immunostaining was separately evaluated in the superficial, transitional, upper and deep radial and calcified layer. Tracheal cartilage was subdivided into the marginal and the inner area, intervertebral disci were subdivided in the annulus fibrosus and the nucleus

Table 1. Primary antibodies and enzymatic pretreatments used for immunohistochemical analysis.

ANTIGEN	SPECIES	SOURCE	DILUTION	OPTIMAL PRETREATMENT
collagen I	r	Biomex, Mannheim, Germany	1/1000	no pretreatment
collagen II	m	Oncogene, Boston, MA, USA	1/100	hyaluronidase/ 5x pronase
collagen III	r	Hoechst, Frankfurt, Germany	1/1000	hyaluronidase/ pronase
collagen IV	m	Dako-Cytomation, Hamburg, Germany	1/50	hyaluronidase/ pronase
collagen V	r	Biomex, Mannheim, Germany	1/5000	hyaluronidase/ pronase
collagen VI	r	Ruppert Timpl, München, Germany	1/10000	hyaluronidase/ protease
collagen X	m	Klaus van der Mark, Erlangen, Germany	1: 1/50; 2: 1/500	hyaluronidase/ protease
S-100 protein	r	Dako-Cytomation, Hamburg, Germany	1/20000	hyaluronidase/ protease

m: mouse monoclonal; r: rabbit polyclonal. Pretreatments: hyaluronidase (ovine testis, 2 mg/ml in phosphate buffered saline (PBS), pH 5, 60 min at 37°C); pronase (2 mg/ml in PBS, pH 7.3, 60 min at 37°C); protease XXIV (0.02 mg/ml in PBS, pH 7.3, 60 min at room temperature).

pulposus.

Results

Histomorphological analysis - Evaluation of tissue cellularity and cellular appearance

The cellularity (proportion of cells compared to the extracellular matrix) differed between the different cartilages evaluated. Cell density was low in articular cartilages (Fig. 1A,D) and fibrous cartilages (Fig. 1B). In contrast, non-articular hyaline cartilages i.e. nucleus pulposus (Fig. 1C) and trachea (Fig. 1E), as well as elastic cartilage (Fig. 1F) exhibited a slightly higher cellularity.

Generally, all cells of all cartilages stained positively for S-100 protein. The only exceptions were the surface (Fig. 1G) and calcified zones of the articular cartilage and the inner part of the tracheal clips (Fig. 1E). Of note, crosschecking of cell viability in these areas using nuclear staining (propidium iodide) demonstrated positive staining in the upper surface zone of the articular cartilage, but a very much reduced presence of viable cells in the calcified zone of the articular cartilage and the inner portions of the tracheal cartilage clips (Fig. 1E). In the inner portion of the tracheal clips, also "apoptotic" bodies (white arrows, figure 1I) were observed, whereas none were observed in any other cartilage (area).

Histochemical demonstration of matrix components

Proteoglycan-specific Safranin O staining differed between tissues. Uniquely, articular cartilages exhibited zonal variations in staining pattern with the Safranin O intensity being highest in the radial zone (Fig. 2A). The

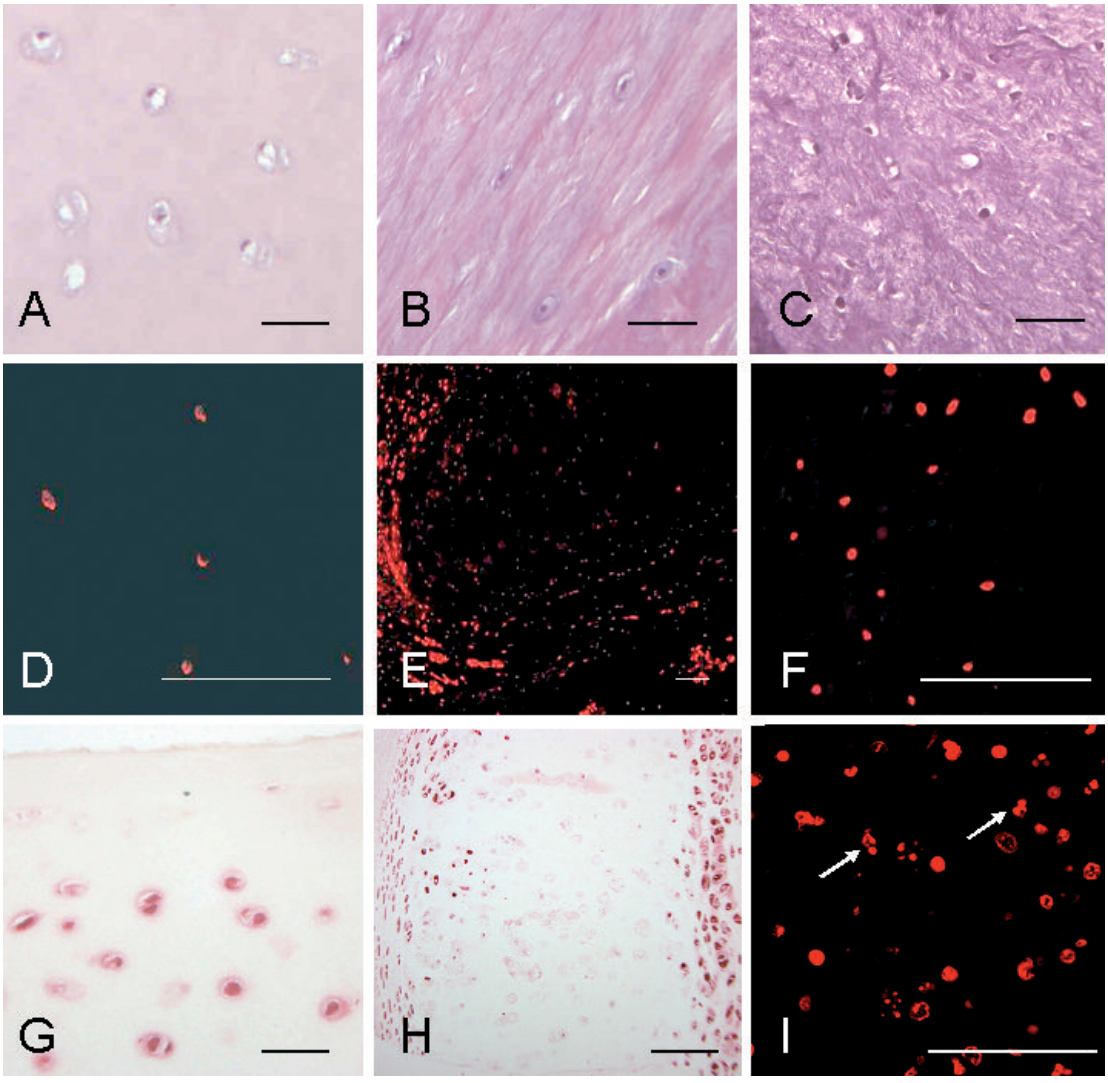

Fig. 1. Examples for HE staining **(A-C)** and nuclear staining with propidium iodide **(D-F, I)** show low cellularity in articular cartilages **(A, D)** and fibrous cartilages **(B)** in contrast to relatively high cellularity in non-articular hyaline cartilages, i.e. nucleus pulposus **(C)** and trachea **(E)**, and epiglottis cartilage **(F)**. Immunopositivity for S-100 protein was found in all but the surface zone of articular cartilages **(G)** and the inner part of the tracheal rings **(H)**. Nuclear staining demonstrated a reduced presence of viable cells in the inner part (asterisk) of the trachea rings **(E)**. Here **(I))**, nuclear fragmentation was observed (arrows). Magnification bars: A-C, G, H, 100 µm; D, F, I, 10 µm; E, 250 µm.

surface zone showed none, the transitional zone only moderate Safranin O staining. Hyaline non-articular cartilages, i.e. trachea (Fig. 2b) and elastic cartilages exhibited strongly positive Safranin O staining throughout the matrix. Fibrous cartilages showed weak to almost absent staining (Fig. 2C).

Fibril architecture, as indicated by cell arrangement and more obvious by polarization light microscopy (Fig. 2D, E) was anisotropic in (upper and deep zones of) articular cartilages, menisci, and annulus fibrous. In contrast, a rather isotropic collagen orientation was found in nose, ear, and epiglottis cartilage, and nucleus pulposus. Elastic fibers were only detected in the epiglottis (Fig. 2F) and ear cartilages.

Distribution of collagens within the cartilage matrices

A summary of immunostaining pattern of different cartilage subtypes for various collagen antibodies is given in Table 2. Example microphotographs are shown as well.

Immunolocalization showed collagen type I almost exclusively in fibrous cartilages of menisci (Fig. 3A) and part of the annulus fibrosus (Fig. 3B) of the intervertebral disci apart from some faint staining of a very thin layer at the surface of articular cartilages.

Collagen type II occurred in the ECM of basically all cartilage types investigated. However, the staining intensity was minor in fibrous cartilages e.g. meniscus (Fig. 3D) and strong in articular cartilage and the nucleus pulposus (Fig. 3E). In articular cartilages in some cases the uppermost surface layer was largely negative (Fig. 3F).

Collagen type III was found in all cartilage subtypes partly co-distributed with collagen types I and II. In articular cartilages (Fig. 3G) staining intensity was strongest in the transitional and the upper radial zones. In the deep radial zone weak interterritorial staining co-occurred with a moderate pericellular/territorial staining. Whereas epiglottis cartilage (Fig. 3H) exhibited patchy interterritorial matrix staining for collagen type III, it was restricted to the cell-associated matrix in ear cartilage (Fig. 3I).

No immunostaining was found for collagen type IV

Table 2. Summary of immunohistochemical staining pattern of different cartilage subtypes for various collagen antibodies.

cartilage source		collagen I	collagen II	collagen III		collagen IV	colllagen V		collagen VI		collagen X	
		it/t	it/t	it	t	it/t	it	pc/t	it	pc/t	it	t
knee	s	+/-	+/-	+/-	-	-	+/-	-	-	+/-	-	-
	t	-	++	+/++	++	-	+/++	++	+/-	++	-	-
	ur	-	++	+/++	++	-	+/++	++	+	++	-	-
	dr	-	++	+	++	-	+	++	+/-	++	-	+/-
	c	-	+	+	+	-	+	+	-	+	-	+
hip	s	+/-	+/-	+/-	-	-	+/-	-	-	+/-	-	-
	tr	-	++	+/++	++	-	+/++	+	+/-	++	-	-
	ur	-	++	+	++	-	+/++	++	+	++	-	-
	dr	-	++	+	++	-	+	++	+/-	++	-	+/-
	c	-	+	+	+	-	+	+	-	+	-	+
ankle	s	+/-	+/-	+/-	-	-	+/-	-	-	+/-	-	-
	t	-	++	+/++	++	-	+/++	++	+/-	++	-	-
	ur	-	++	+/++	++	-	+/++	++	+	++	-	-
	dr	-	++	+	++	-	-	++	+/-	++	-	-
	c	-	+	+	++	-	-	+	-	+	-	-
trachea	margin	-	++	+	+	-	++	++	-	++	-	+
	center	-	++	-	-/+	-	+	+	-	+	-	-/+
nose		-	++	-	+	-	+	++	+/-	++	-	-
epiglottis		-/+	++	+	+	-	+/++	++	-	++	-	+
ear		-	++	-	+	-	-/+	++	-/+	++	-	-
meniscus		+	+	+	+	-	+	++	+	++	-	-
disci	AF	+	+	-	+	-	+	++	+	++	-	-
	NP	-	++	+	+	-	+	++	-	++	-	-

-: negative; +: weak and/or isolated; ++: strong and widespread. it: interterritorial matrix; t: territorial matrix; pc: pericellular; s: superficial zone; t: transitional zone; ur: upper radial zone; dr: deep radial zone; c: calcified cartilage; AF: annulus fibrosus; NP: nucleus pulposus.

in any of the cartilage subtypes.

Immunolocalization of collagen type V was most prominent in the transitional and upper radial zone of articular cartilages (Fig. 4A). In areas with strong interterritorial staining negative halos around chondrocytes were observed. In contrast, areas where the interterritorial staining was less intense, typically exhibited a pericellular and territorial staining pattern. Like collagen type III, collagen type V appeared also in non-articular hyaline cartilages (such as nose (Fig. 4B) as well as in fibrous cartilages (such as menisci [Fig. 4C]). Type V collagen was largely positive throughout the matrix in epiglottis, but restricted to the territorial matrix in ear cartilage (data not shown).

Collagen type VI was detected in the pericellular matrix of all cartilage subtypes. Articular cartilage exhibited also a weak to moderate territorial or interterritorial collagen type VI positive staining (Fig. 4D). Interterritorial matrix staining appeared additionally in the upper radial zones. In some cases negative halos around chondrocytes were observed in this zone. In fibrous cartilages (menisci [figure 4F]) immunopositivity was also found concentrated round the cells, in between thick collagen bands being positive for collagen types I and III.

Immunolocalization of collagen type X was restricted to some moderate pericellular staining close to the tidemark of knee and hip samples, the inner part of the tracheal clips, and epiglottis cartilage.

Discussion

Our study provides a profile for matrix proteins in human cartilaginous tissues. Basic histomorphology and histochemistry already revealed important features shared by all cartilages: scarce cells (Stockwell, 1971) are surrounded by an abundant extracellular matrix, in which quantitatively the most important constituents are collagens, proteoglycans, and water. The abundance of the ECM corresponds to the fact that the matrix and not the cells (as in parenchymal organs) is the functional component of the cartilage tissues. In line with this, articular and fibrous cartilage prone to high biomechanical loading as compared to non-articular cartilages were less cellular, whereas nose, tracheal, ear and epiglottis cartilages with less or no biomechanical loading exhibited a relatively higher cellularity. The few areas (calcified layer of articular cartilages, inner part of the trachea clips) which showed a significant number of empty lacunae are most likely areas with cells which at least in part underwent terminal differentiation. This is reflected by the expression of collagen type X and

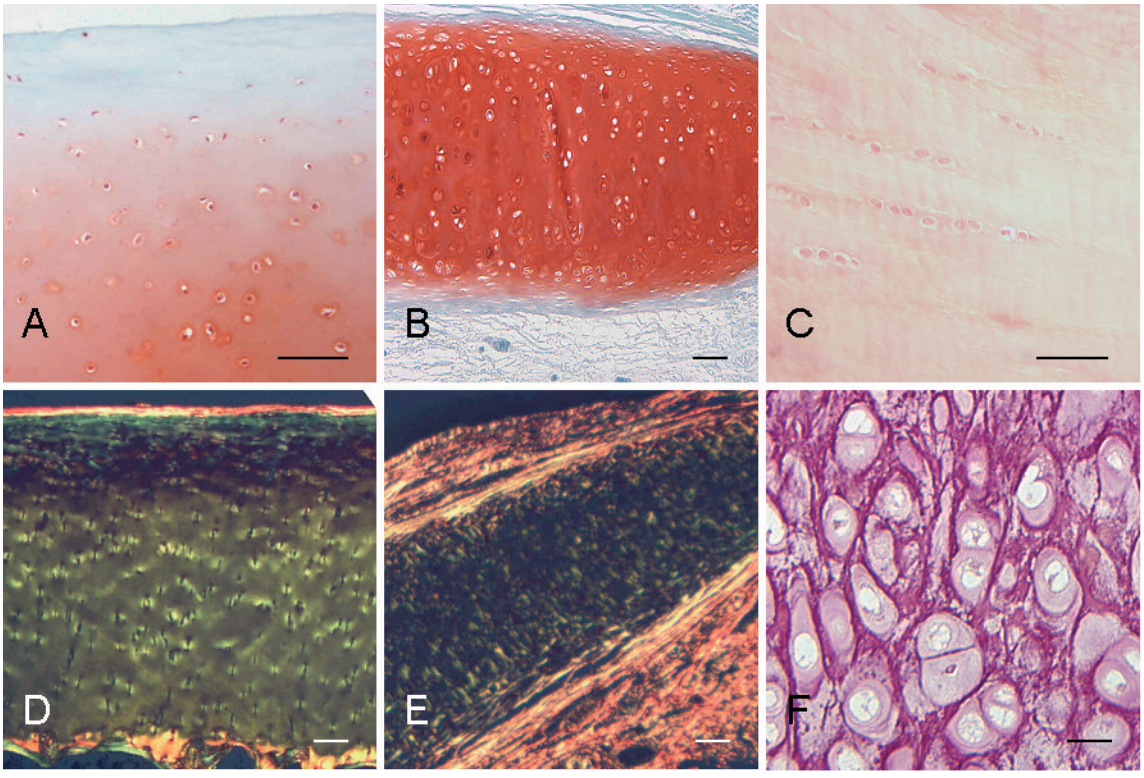

Fig. 2. Examples for histochemical demonstration of matrix components: Articular cartilages exhibited zonal variations in staining pattern with the Safranin O intensity being highest in the radial zone **(A)**. The surface zone showed none, while the transitional zone only moderate Safranin O staining. Hyaline non-articular cartilages, i.e. trachea **(B)** exhibited strongly positive Safranin O staining. Fibrous cartilages showed weak homogeneous to almost absent staining **(C)**. Fibril architecture, as indicated by cell arrangement and more obvious by polarization light microscopy was anisotropic in articular cartilages (**D**: upper and deep zones). In contrast, a rather isotropic collagen orientation was found in epiglottis cartilage **(E)**, and nucleus pulposus. Elastic fibers were detected in the epiglottis **(F)** cartilages. Magnification bars: A, C, 100 µm; B, D, E, 250 µm; F, 10 µm.

previous and ongoing cell death in these areas. Most likely, the latter is of apoptotic nature as in the fetal growth plate (Aigner et al., 2001), because we were able to show the presence of (rare) apoptotic bodies in these areas (see figure 1).

Picrosirius Red and EVG staining revealed collagens as the principle scaffold of the ECM. In line with previous ultrastructural studies (Meachim and Stockwell 1979), fibril architecture was anisotropic in (upper and deep zones of) articular cartilages, menisci, and annulus fibrous, i.e. tissues bearing weight and being exposed to tensile and shear stresses. Uniquely, articular cartilages exhibit a zonal structure based on an arcade-like architecture of the collagen fibrils (Benninghoff, 1920). The distribution of the remaining matrix constituents differs zone-dependently as well. In contrast, a rather isotropic collagen orientation is found in nose, ear, and epiglottis cartilage, and nucleus pulposus. Non-articular hyaline and elastic cartilages are in between articular and fibrous cartilages in terms of their structural rigidity. Their principle function is structural support for maintaining the shape of an organ. Apart from the nucleus pulposus they are only modestly involved in weight bearing. Solely elastic cartilages show elastic fibers providing the bending capacity needed in the epiglottis during breathing and swallowing and (at least in some animals) needed for ear orientation during listening.

The second basic constituent of all cartilages entrapped within the collagenous scaffold are the proteoglycans. Their heavily negatively loaded glycosaminoglycan side chains have a high water binding capacity and are responsible for the high swelling pressure typical for hyaline cartilages. Whereas this is essential for tissues mostly involved in impulse resorption such as articular cartilages and nucleus

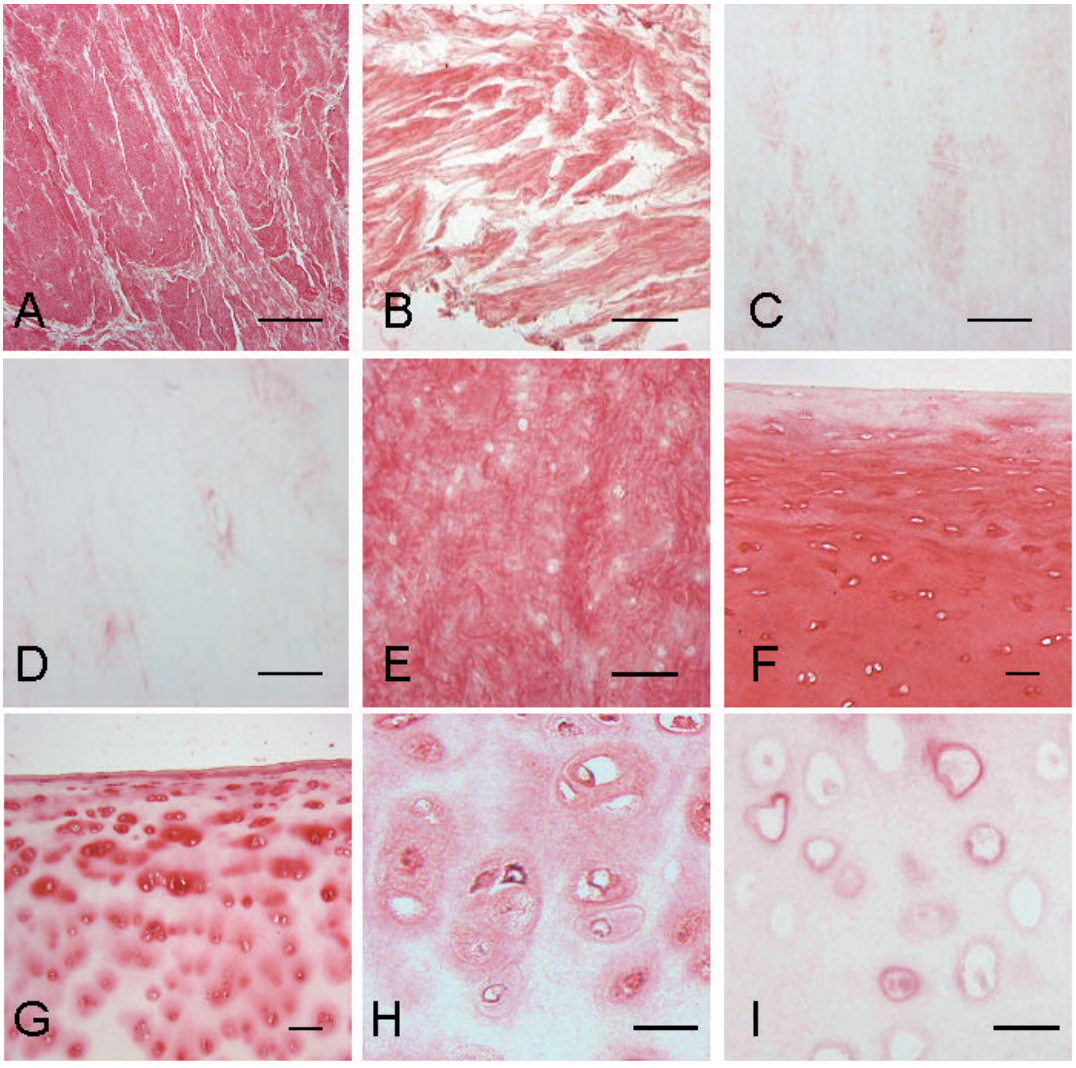

Fig. 3. Examples for immunostaining for collagen types I (first row), II (second row), and III (third row). Menisci **(A, D)** and annulus fibrosus **(B)** were strongly positive for collagen type I. In contrast, nucleus pulposus **(C, E)** exhibited strong collagen type II positive staining. In articular cartilages **(F)** in some cases the uppermost surface layer was negative for collagen type II antibody. Collagen type III, i.e. in articular cartilage **(G)** was largely co-distributed with collagen types I and II. Epiglottis cartilage **(H)** exhibited homogeneous interterritorial matrix staining for collagen type III. Immunopositivity was restricted to the territorial matrix in ear cartilage **(I)**. Magnification bars: A-E, 200 µm; F, G, 250 µm; H, I, 10 µm.

pulposus this is much less important in fibrous cartilages like menisci and the annulus fibrous which have primarily to withstand shear stresses and correspondingly have less proteoglycans.

Immunohistochemistry allows for a distinct analysis of matrix composition: A constitutive component of all cartilages is collagen type II: the collagen type II network, in which type II collagen forms an alloy with other collagens as well as non-collagenous proteins (Bruckner and van der Rest, 1994), appears to be the central structure responsible for the structural integrity of the cartilage tissues. Close interactions between collagen type II and highly concentrated proteoglycans in all but fibrous cartilages lend to these tissues their characteristic elasticity. The fact that type II collagen was not uniformly stained within tissues is most likely due to epitope masking of the densely packed collagen network. Potentially, it might also be due to partial degradation of the molecules within normal aged tissues (Squires et al., 2003). Similar caveats obviously apply to the staining pattern obtained with the other collagen antibodies as well, in particular as cartilage has got a very densely packed and heavily cross-linked matrix.

Fibrous cartilages, which need to withstand higher tensile strength, have additionally a strong signal for type I collagen associated with only a small amount of proteoglycans. Collagen type I forms the dominant network in these tissues (maybe intermixed with the still present collagen type II fibers).

The co-occurrence of collagen types I and III is widely accepted (McDevitt and Webber, 1990; Eyre, 2002; Eyre et al., 2002). Most likely in fibrous cartilages type III collagen intermixes with collagen type I fibers. Our results also confirm previous results showing type III collagen as an additional component of the collagen type II matrix in hyaline cartilages (Young et al., 2000). The co-localization of collagen types I and III in the absence of collagen type II at the surfaces of at least some articular cartilages most likely represents a remnant, in many samples already torn off, of the epichondral layer of fetal growth plate cartilage showing the same composition (Vornehm et al., 1996). The missing full chondrocytic differentiation of the cells in this very surface layer is also documented by the absence of staining for S-100 protein within the cells, which is a good marker of chondrocytic differentiation (Wolff et al., 1992).

As expected, collagen type IV, a major constituent of basement membranes, was not found in any of the cartilage subtypes investigated.

Another fibrillar collagen, type V collagen, belongs to the principal components of all cartilage subtypes. This minor collagen is thought to play a major regulatory role in determining fibril diameter and architecture of major collagen type I (Andrikopoulos et al., 1995). However, its function within collagen type II matrices is unclear.

In agreement with previous studies type VI collagen

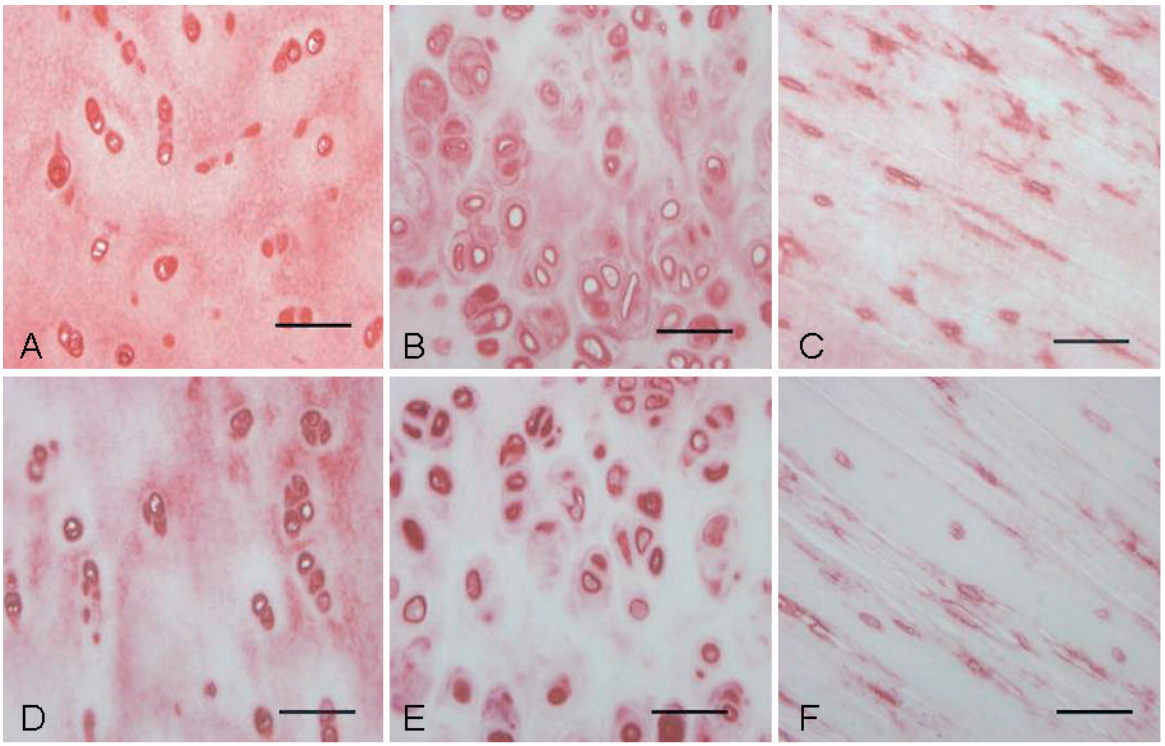

Fig. 4. All cartilage subtypes showed immunopositivity for the minor collagen types V (upper row) and VI (lower row): knee **(A, D)**, nose **(B, E)**, and menisci **(C, F)**. Magnification bars: 100 µm.

was in most cartilages concentrated pericellularly (Poole et al., 1987, 1988; Hambach et al., 1998; Söder et al., 2002). Interterritorial matrix staining for collagen type VI most likely reflects degradation products (Hambach et al., 1998). This is in contrast to Roberts et al. (2003), who observed collagen types VI and III staining solely cell-associated in hyaline-like repair cartilage in contrast to a widespread staining throughout the matrix in fibrous-like repair cartilage. However, epitope masking within the densely packed collagen fibrils might well explain the differences.

Immunopositive staining for collagen type X was restricted to the deep zones of articular cartilage adjacent to the tidemark, the inner parts but not the margins of the tracheal clips, and epiglottal cartilage. Collagen type X is a distinct marker of hypertrophic chondrocytes (Kirsch and von der Mark, 1990; Schmid and Linsenmayer, 1990). These results support the notion that the cells in these areas represent remnants of the hypertrophic zones of fetal growth cartilage.

The main difference between the human and the rabbit protein profiles (Naumann et al., 2002) is the more ubiquitous occurrence of collagen type I in the rabbit. In human cartilages collagen type I is restricted to fibrous cartilages and traces in the articular cartilage surface. In contrast, in the rabbit, collagen type I was detected in all cartilage tissues except articular cartilage. Collagen type X was detected in rabbit, but not in human nose and ear cartilages. In contrast, we found immunopositive staining for collagen type X in articular and epiglottal tissue in the human, which was not reported in the rabbit. Whether the subtle differences of the articular cartilages relates to changes in functional properties between both species, for example due to bipedal in contrast to quadruped locomotion, is unclear.

Articular cartilage is probably the best characterized of the cartilaginous tissues. The protein patterns we found are in good agreement with the literature reporting the occurrence of collagen types I, II, III, V, VI, and X (for review see Eyre 2002 (Young et al., 2000)). Staining patterns for different collagens are qualitatively almost similar for the different articular hyaline cartilages (i.e. hip, knee, and ankle). Also, immunostaining patterns of meniscus are in accordance with previously published studies (Bluteau et al., 1999), except that we could not detect collagen type X.

Overall, our investigations presents an analysis of the distribution of matrix collagens in the major cartilage subtypes in the human: clearly, type II collagen fibres appear to be the common biochemical backbone of all cartilage matrices with type I collagen being associated in fibrocartilages. The minor collagen types V and VI appeared to be either concentrated in the cell-associated matrix (hyaline cartilages) or dispersed throughout the matrix (fibrocartilage). The fact that almost all collagen types investigated (except type IV) could be demonstrated in all cartilage subtypes suggests that it is not so much the presence of a structural protein, but the concentration, the distribution, and the supramolecular assembly in the ECM that is required to meet specific biomechanical demands.

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