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Lectin-binding in normal and fibrillated articular cartilage of human patellae

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Summary. Fluorescein-isothiocyanate (FITC) labeled lectins were used to study the distribution of specific binding-sites in histological sections of normal and fibrillated articular cartilage of human patellae.

It has been shown that normal articular cartilage reveals lectin binding-sites for Concanavalin A (Con A) and wheat germ agglutinin (WGA), but not for soybean agglutinin (SBA), peanut agglutinin (PNA) and Ulex europaeus agglutinin (UEA).

In fibrillated cartilage the distribution pattern of Con A and WGA is completely changed. SBA, PNA and UEA show a distinct staining pattern in particular in the fibrillated areas of degenerated cartilage. Lectin-staining of the extracellular matrix and the chondrocytes in both normal and fibrillated cartilage did not show any correlation with material that was either PAS- or Alcian blue-positive. In comparison with the conventional PAS- and Alcian blue reaction lectin-staining proved to be superior.

Visualization of intra- and extracellular glycoconjugate-changes in normal and fibrillated cartilage in areas with no PAS and/or Alcian blue staining indicates that all layers of the cartilage are involved in the pathological process.

It is evident that lectins can demonstrate minute differences between normal and arthrotic cartilage and we therefore conclude that lectins are sensitive and specific tools for the study of degenerative joint diseases.

Key words: Articular cartilage – Glycosaminoglycans – Lectin-binding – Osteoarthrotic – Patella

Introduction

Lectins are used as histochemical markers to demonstrate glycoconjugate distribution in tissues in order to get more specific information about the localisation of carbohydrate moieties of macromolecules. A remarkable de-

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gree of heterogenity in the structure of tissue glycoconjugates has been demonstrated by the use of lectins (Spicer et al. 1983), which make this sensitive and specific method superior to the conventional PAS/Alcian blue-reactions. However, care has to be taken in the interpretation of lectin histochemical results, since fixation and decalcification seem to have an influence on the preservation of carbohydrate residues of macromolecules (Schünke et al. 1984).

Studies on lectin-binding to hyalin cartilage have been limited to non-human material of tracheal (Yamada and Shimizu 1977; Yamada 1978), costal (Yamada and Shimizu 1977) and non-decalcified growth-plate cartilage (Farnum and Wilsman 1984), and to elastic ear cartilage (Yamada 1978; Stoddart and Kiernan 1973). Lectin histochemistry has recently proved to be a useful tool to study glycoprotein-changes during chondrogenesis of mouse limb buds (Zimmermann and Thies 1984). In the present study the distribution-pattern of lectin-bindings is investigated in normal and fibrillated cartilage of human patellae.

A particular thick cartilage and a low fixed charge density, as compared to other joint surface, are pecularities of the femoropatellar joint (Ficat and Maroudas 1975). During load bearing, this joint is characterized by a high pressure together with small contact areas during movements in all joint positions (Wiberg 1941, Maquet 1976; Goodfellow et al. 1976). A high incidence of cartilage fibrillation even in young adults has been observed to occur more frequently than in other joints. Cartilage fibrillation is thought to be the primary lesion of osteoarthrotic joints. Deeply fibrillated cartilage showed a loss of glycosaminoglycans (Ficat and Maroudas 1975), the depletion of glycosaminoglycans being limited to the area of fibrillation as a local phenomenon. Characteristic cellular features of progressive degenerative changes are clusters of chondrocytes next to the fissures in the tissue. These clusters seem to be a heterogeneous population, since some are intensively proliferating chondrocytes with an abnormal increase of metabolism, while others do not show any metabolic activity with respect to the uptake of radioactive SO_4^{2-} (Dustmann et al. 1974).

Materials and methods

Specimens of articular cartilage from human patellae were collected from three autopsy cases without visible cartilage lesions or a clinical history of joint disease (20 years female, 23 years male and 24 years female). Other samples were collected from macroscopically fibrillated articular cartilage of humans without a clinical history of rheumatoid arthritis (72 years female, 75 years female and 76 years male).

Pathological grading of articular cartilage changes according to Collins (1949) was based on macroscopic inspection of the patellar surface, supplemented by histological examination of cartilage sections. Patellae were classified as normal (Grade 0) when the articular cartilage showed a smooth surface without macroscopically visible degenerative changes. Specimens of cartilage with a visibly fibrillated surface were taken from areas (mostly lateral facet) where fibrillation extends down to the middle layer (Grade II). For grading, surfaces were also inspected with a stereomicroscop at $\times 2$ magnification. Until cryostat sectioning the samples were frozen in 0.9% saline at $-20°$ C.

FITC-labeled lectins from Triticum vulgaris (wheat germ, WGA), Arachis hypogaea (peanut, PNA), Glycine max (soybean, SBA), Ulex europaeus (UEA) and Canavalia ensiformis (Con A) as well as their inhibitoring sugars were purchased from MEDAC, Hamburg/FRG

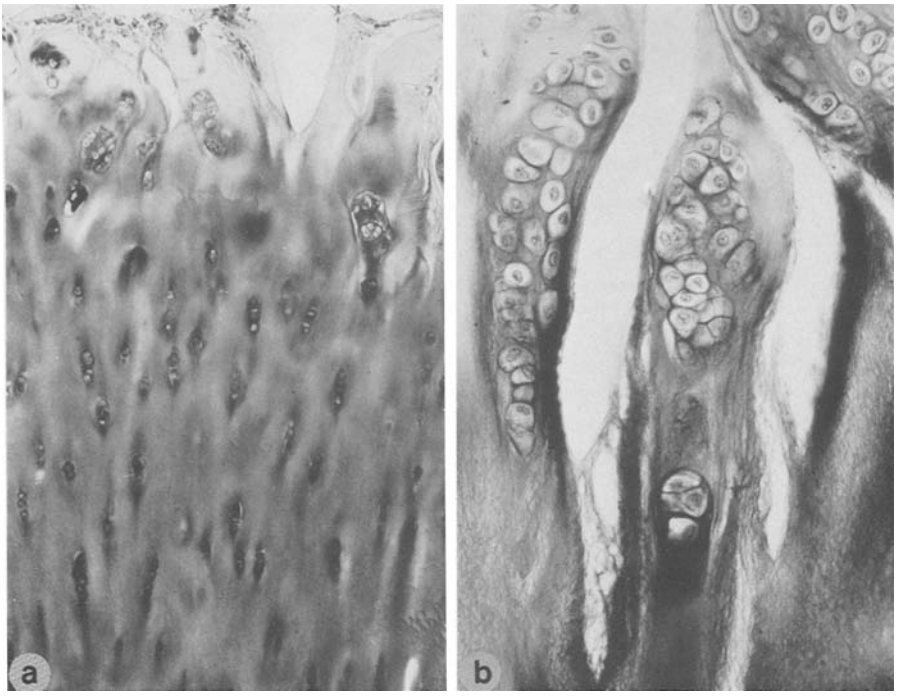

Fig. 1 a, b. Giemsa-stained sections of fibrillated articular cartilage. **a**, Deep fissures with adjacent clusters in the upper part of osteoarthrotic cartilage, × 50. **b** typical clusters of chondrocytes adjacent to deep fissures. × 250. Note: All figures show the joint cavity upward and the subchondral bone downward

(Product of E.Y. Lab., Santa Mateo, Calif., USA). Prior to fixation, cartilage slices of approximately 2 mm thickness from the entire transverse diameter of the patella with its subjacent bone were cut always keeping the tissue moist with cold 0.9% saline. Fixation of the cartilage in Bouin's fluid was carried out for 24 h at 4° C. After thorough washing of the specimens in 80% ethanol (6 h with 5 changes), decalcification was performed in 20% EDTA (pH 7.2) at 37° C for 16 to 25 days. Termination of decalcification was determined by X-rays, with routine checks every third day.

After washing in tab water (4 h) and dehydration the tissue was embedded in paraplast, sectioned and mounted on gelatine coated slides.

For lectin-binding experiments 7 μm thick tissue sections were deparaffinated in xylene, rehydrated with a graded series of ethanol and finally passed into phosphate-buffered saline (0.01 M phosphate-buffer, pH 7.4, plus 0.15 M NaCl, PBS), supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1 mM MnCl (Spicer et al. 1983). Incubation of the sections with various lectin solutions (1 mg/ml) was performed in a moist chamber at room temperature for 30 min. After washing of the slides with PBS (3 × 15 min), sections were mounted in 10% glycerol in PBS.

Slides were examined using a Zeiss-microscope equipped with a fluorescence illuminator according to PLOEM. Micrographs were taken by hand (maximum exposure time 60 s) using Ilford HP5-135 films.

Specifity of lectin binding was checked in control experiments where the lectin binding was completely inhibited, when the lectin was mixed prior to incubation with the corresponding sugars at a concentration of 0.2 M: N-acetyl-D-galactosamin for SBA, N-acetyl-D-glucosamin for WGA, L-fucose for UEA, D-galactose for PNA and D-mannose (D-glucose) for Con A (Goldstein and Hayes 1978).

To estimate the autofluorescence of the tissue, PBS-mounted sections were used. Unfixed cryostat-sections were taken to exclude artefacts due to fixation and decalcification.

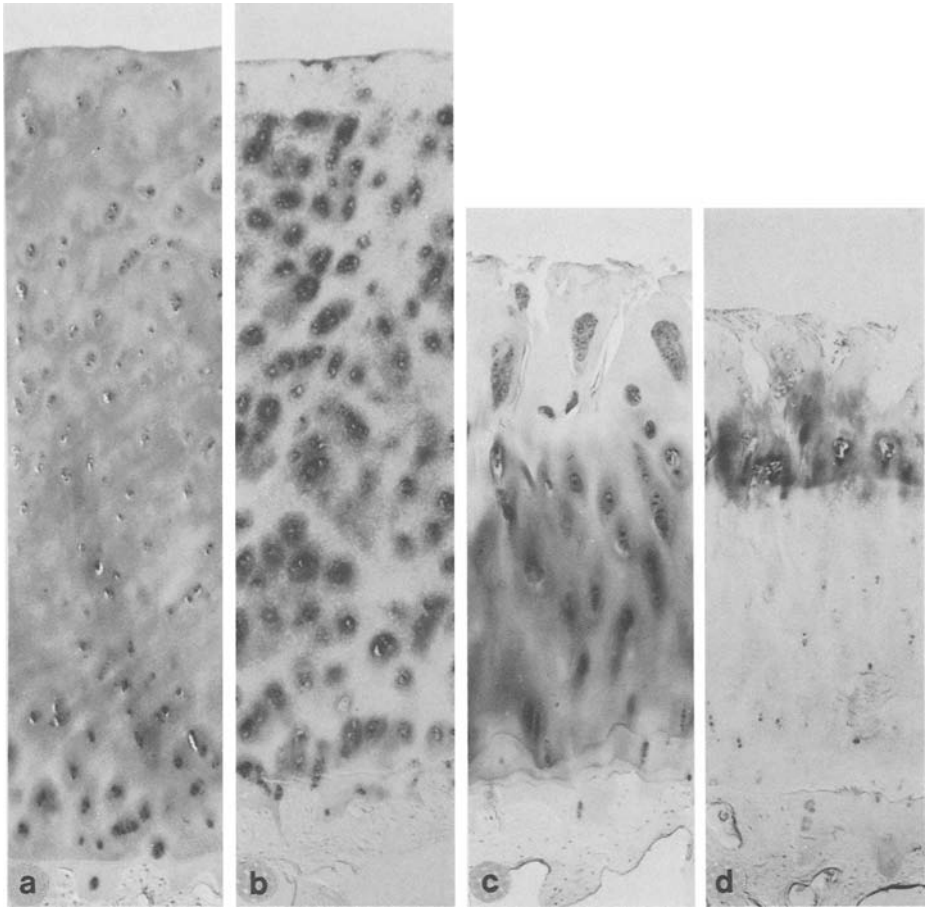

Fig. 2a–d. PAS- and AB-stained sections of normal and fibrillated articular cartilage. **a**, normal articular cartilage, PAS. **b**, normal articular cartilage, AB (pH 1.0). **c**, fibrillated articular cartilage, PAS. **d**, fibrillated articular cartilage AB (pH 1,0). **a–d** $\times 40$

For conventional histology, the sections were stained with HE, Giemsa, PAS and Alcian blue 8GX (AB) at pH 1.0 and 2.5 as described by Pearse (1968).

Results

1. Conventional histochemistry

Fibrillated articular cartilage of Giemsa-stained tissue sections showed numerous fissures especially in the superficial and in the upper part of the middle layer of the cartilage. Adjacent to the fissures there are several clusters (Fig. 1a, b). Normal articular cartilage contained PAS-positive material throughout the entire transverse diameter of the tissue (Fig. 2a). In the upper two thirds, the interterritorial matrix was intensely stained, while staining of the territorial matrix around the individual chondrons was only

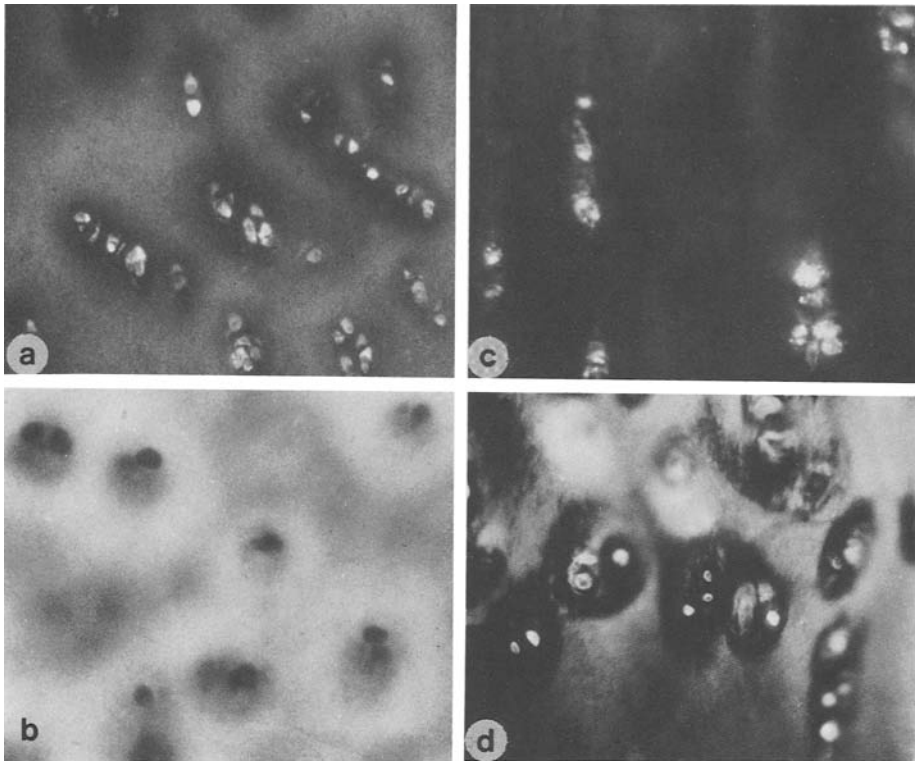

Fig. 3a–d. Binding-patterns of FITC-labeled Con A and WGA in deep and middle layers of normal and fibrillated articular of human patellae. **a**, normal articular cartilage, Con A. **b**, normal articular cartilage, WGA. **c**, fibrillated articular cartilage, Con A, **d**, fibrillated articular cartilage, WGA. **a–d** × 200

moderate. In the deep zone adjacent to the calcified layer there is a strong pericellular staining, while interterritorial PAS-staining in this area is less intensive. Chondrocytes were evenly positive throughout the full depth of cartilage. With the Alcian blue technique (pH 1.0) strong and intensive staining of chondrocytes and their territorial matrix was found throughout the entire depth of the cartilage, whereas only moderate staining was seen in the interterritorial regions (Fig. 2b).

In fibrillated articular cartilage PAS-positive material with a strong territorial and moderate interterritorial staining reaction is localized predominantly in the deep and middle layers of the cartilage (Fig. 2c). In contrast the extracellular matrix in the fibrillated areas is completely PAS-negative, whereas most of the clusters are heavily stained.

Intensive AB-staining (pH 1.0) in fibrillated cartilage was restricted to a narrow band of extracellular matrix adjacent to the upper end of PAS-positive regions. Clusters of chondrocytes in this area and their surrounding extracellular matrix are heavily stained. In the deep and middle layers Alcian blue staining appears to be absent or much reduced (Fig. 2d). Clusters in fibrillated areas were stained positive with AB.

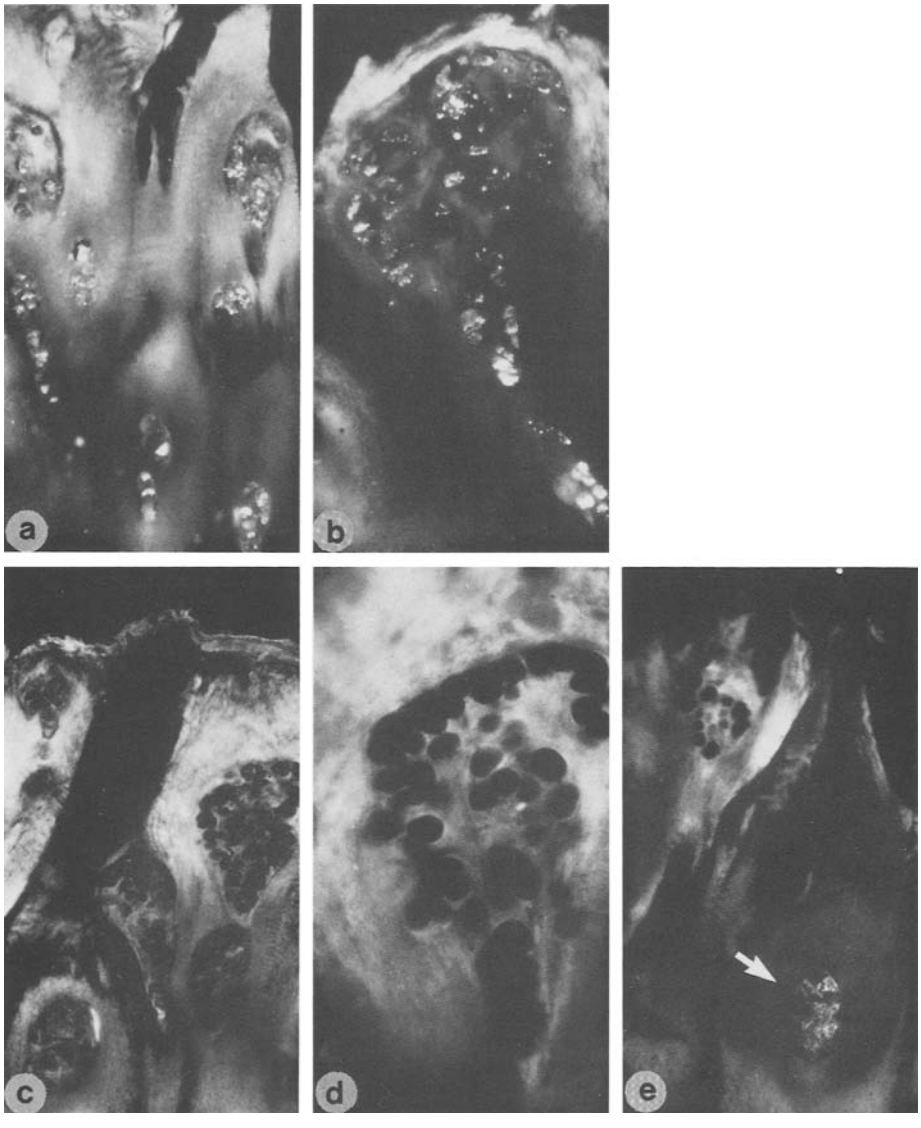

Fig. 4a–e. Binding-patterns of FITC-labeled Con A and WGA in fibrillated regions of osteoarthrotic cartilage. **a** , Con A ×150, **b**, cluster with Con A-positive material in the chondrocytes ×390. **c**, WGA ×150. **d**, cluster with WGA-positive extracellular matrix ×390. **e**, WGA.Clusters in different distances from the joint cavity. Cluster in the deeper part of the fibrillated area (*arrow*) with WGA-positive chondrocytes ×150

2. Lectin-binding in normal articular cartilage

In normal articular cartilage Con A binding was intensely positive in chondrocytes (Fig. 3a), negative in the territorial extracellular matrix and moderately positive within the interterritorial regions throughout the full thickness of cartilage. With WGA there was a highly specific territorial staining sur-

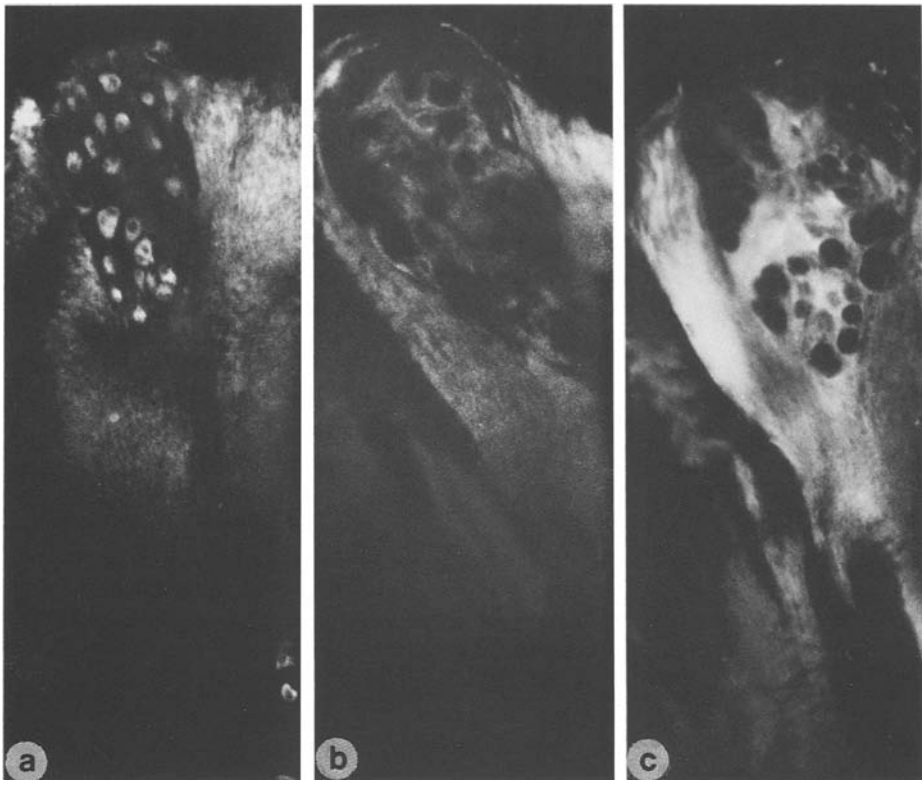

Fig. 5a–c. Binding-patterns of FITC-labeled UEA, SBA and PNA in clusters and surrounding extracellular matrix of fibrillated articular cartilage. **a**, UEA. **b**, SBA, **c**, PNA. **a–c** × 215

rounding individual chondrocytes (Fig. 3b), while staining of the extracellular matrix in the interterritorial regions was less intensive. Chondrocytes were completely negative in all layers of normal articular cartilage. No staining was obtained with SBA, PNA and UEA.

3. Lectin-binding in fibrillated articular cartilage

In fibrillated cartilage, Con A binding-sites were found in chondrocytes, localized either in the deeper parts as groups of two to four cells (Fig. 3c) or as typical cell clusters within the upper part of the cartilage adjacent to the fissures (Fig. 4a, b). In deep and middle layers Con A binding of the extracellular matrix was completely negative (Fig. 3c). In contrast, the extracellular matrix of the fibrillated area was heavily stained with Con A, particulary around the individual clusters (Fig. 4a, b).

With WGA there was an intensive cytoplasmic staining of chondrocytes belonging to the deep and middle layers of fibrillated cartilage (Fig. 3d). The territorial matrix in these regions was unstained, whereas the interterritorial extracellular matrix revealed strong WGA-binding. In fibrillated areas (Fig. 4c, d) most of the clusters were surrounded by heavily stained

extracellular matrix, while the condrocytes within these clusters were completely negative. In contrast, clusters in the deeper parts of the fibrillated area showed WGA-positive chondrocytes (Fig. 4e), whereas their surrounding extracellular matrix remained negative.

With SBA, PNA and UEA intensive staining of extracellular matrix was obtained only in the fibrillated area of the cartilage (Fig. 5a–c). In addition, individual clusters were positively stained with UEA (Fig. 5a). Chondrocytes as well as extracellular matrix of the deep and middle layers showed no specific binding-sites for these lectins.

Discussion

Binding of FITC-conjugated lectins to tissue sections of articular cartilage has been shown to be influenced by fixation and decalcification. A good correlation of lectin-staining in Bouin-fixed cartilage of rat femoral head has been found when compared with cryostat-sections of unfixed material (Schünke et al. 1984). In contrast, formalin fixation heavily diminishes Con A binding-sites especially within the cytoplasm of the chondrocytes. Similar observations concerning the influence of fixation on rat spermatic cells in tissue sections have been made by Söderstrom et al. (1983), who found a significant enhancement of lectin fluorescence obtained by Bouin fixation as compared to formalin fixation.

In addition, decalcification is also considered to be a critical point during tissue processing. Decalcification in 20% EDTA (pH 7.2) in contrast to 5% nitric acid (Ippolito et al. 1981) retained most of the sensitive glycoconjugates, responsible for the lectin-binding.

It has been shown in the present study that normal articular cartilage from human patellae possesses lectin binding-sites for Con A and WGA. Con A binding is intensively positive in chondrocytes, negative in territorial and moderately positive in interterritorial matrix throughout the full thickness of cartilage. In fibrillated cartilage the labeling pattern of Con A has changed. Whereas Con A binding-sites are still present in chondrocytes, they are absent throughout the whole extracellular matrix of the deep and middle layers. However, there is a strong specific staining of the extracellular matrix throughout the whole fibrillated area, particularly around the individual clusters. Most of the chondrocytes in these clusters show positive Con A-staining within the cytoplasma.

Cytoplasmic staining with Con A has been observed in chondrocytes from different species (Yamada 1978; Stoddart and Kiernan 1973). It has been considered to represent the cellular compartement of rough endoplasmic reticulum (RER) (Farnum and Wilsman 1984). In RER high mannose branched-chains are added on to asparagin-linked oligosaccharides (Corfield and Schauer 1979), for which Con A has its binding specifity. Glycosylated type II collagen of the extracellular matrix contains glucose and may therefore represent the Con A binding-sites. The precise pattern of Con A binding-sites in the matrix suggests that either the availability or the quantity of glucose residues on collagen varies significantly in the different regions.

With WGA, intensive specific territorial staining surrounds individual chondrocytes in normal articular cartilage, while staining of the extracellular matrix in interterritorial regions is less intensive. Chondrocytes are completely negative throughout the cartilage matrix. In fibrillated cartilage WGA-staining pattern has completely changed. Although the extracellular matrix is still heavily stained, territorial regions are completely negative in deep and middle layers. Chondrocytes, in contrast, are particularly WGA positive in these areas. This suggests a possible restriction of secretion of WGA-. positive material as judged also from a complete negative halo surrounding the chondrocytes. Throughout the fibrillated area there is an especially strong WGA-staining of the extracellular matrix. However, there is no homogeneous staining of clusters in the fibrillated regions. Chondrocytic labeling with WGA is restricted to clusters which are localized in deeper areas of the fibrillated zone. These clusters have a WGA-negative territorial matrix. In contrast, clusters adjacent to the surface of the joint cavity exceptionally may reveal no WGA affinity, but show intense territorial WGA-staining.

WGA and Con A represent lectins whose binding-sites are relatively frequent throughout tissues, however, their binding-patterns are considered to be non-overlapping. It has been assumed that cytoplasmic labeling of Con A represent affinity for the RER, while juxtanuclear labeling of WGA possibly corresponds to the Golgi apparatus (Farnum and Wilsman 1984; Ratcliffe et al. 1984). In vitro WGA-binding has been demonstrated to have a specific affinity for both N-acetyl-D-glucosamin and neuraminic acid residues. Binding affinities of this lectin in the extracellular matrix therefore could demonstrate N- and O-linked oligosaccharides (neuraminic acid), hyaluronic acid and keratan sulfate (N-acetyl-D-glucosamine). According to Toda et al. (1981) sulfated glycosaminoglycans such as keratan sulfate specifically react with WGA.

In contrast, terminal sugar residues labelled by SBA (N-acetyl-galactosamine), PNA (D-galactose) and UEA (fucose) are only infrequently found in tissues. Application of these three lectins to tissue sections of healthy articular cartilage results in a completely negative staining pattern. In fibrillated areas, however, SBA, PNA and UEA have distinct staining pattern. Each of these three lectins reveals a specific affinity for the extracellular matrix adjacent to the fissures and surrounding some of the clusters. In addition, UEA, a fucose specific lectin, stains chondrocytes of the individual clusters.

This is even more remarkable since the extracellular matrix of this very region is completely negative with the PAS- and Alcian blue reaction. Generally, sequences of disaccharides, representing the glycosaminoglycans of the extracellular matrix in healthy articular cartilage are the bridging agent between collagen and proteoglycans (Hascall and Hascall 1983). Therefore they are masked for the detection by terminal sugar detecting lectins. The bondage breakdown in fibrillated cartilage make these residues accessible to detection by lectins. This is particular due to SBA and PNA, which are binding to N-acetyl-D-galactosamin and D-galactose, respectively, parts

of repeating disaccharides units representing chondroitinsulfate and keratan sulfate. Therefore fucose as a part of the linkage region also becomes accessible to UEA. It is known that biochemical changes are correlated with the degree of fibrillation. Deeply fibrillated cartilage, for example, shows a decrease in both rate of synthesis and the content of glycosaminoglycan (Brocklehurst et al. 1984). However, the possibility that pathological glycoproteins are produced instead of bondage breakdown cannot be excluded.

In accordance with previous observations (Stockwell 1970) PAS- and Alcian blue-positive material of healthy articular cartilage is uniformly distributed throughout the whole depth of cartilage. Generally, the PAS-reaction is absent or slightly moderate in areas, where Alcian blue staining is strong and vice versa. As already observed by Van der Valk and Hageman (1982) and Watanabe et al. (1981) there is no clear correlation between the binding of any lectin and the distribution pattern of material that was either PAS- or Alcian blue-positive.

In fibrillated articular cartilage of human patellae, however, staining patterns of PAS- and AB- positive material have completely changed. In histological sections, the distribution of PAS-positive material corresponds mainly to the deep and middle layers. In fibrillated areas, however, the extracellular matrix is always PAS-negative, whereas most of the clusters are heavily stained. According to the AB-staining pattern (pH 1.0), sulfated glycosaminoglycans have been reduced predominantly to a narrow band of extracellular matrix adjacent to the upper end of the PAS-positive regions. Clusters of chondrocytes in this area and their surrounding extracellular matrix are intensely stained. In the extracellular matrix of deep and middle layers, however, AB-staining appears to be absent or much reduced.

With UEA, PNA and SBA changes of sugar residues in areas next to the luminal surface have been demonstrated. This layer has not been stained either by PAS nor by Alcian blue which indicates the superiority of lectin staining. Additionally, the deep layers which have not been stained with Alcian blue show particular staining pattern with Con A and WGA. This indicates that all layers of the cartilage are involved in the pathological process.

It is evident that lectins can demonstrate minute differences between normal and arthrotic cartilage which could not be observed by PAS and Alcian blue. We therefore conclude, that lectins are sensitive tools for the study of the arthrotic process, since we were able to demonstrate the involvement of all layers of the cartilage in this process.

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