Distribution of chondroitin 4-sulfate epitopes (2/B/6) in various zones and compartments of articular cartilage in guinea pig osteoarthrosis

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ABSTRACT – We studied changes in the morphology of tibial articular cartilage in early guinea pig osteoarthrosis (OA) at 6 and 12 months of age with quantitative light microscopy, and the distribution of chondroitin-4-sulfate with quantitative ultrastructural immunolabeling, using the 2/B/6 epitope. Labeling was correlated to previous chromatography findings concerning proteoglycan (PG) concentration in animals of the same age. The cell volume fraction had decreased at 12 months in the superficial zone of cartilage with OA (medial condyle) as well as in cartilage without OA (lateral condyle), being lower medially than laterally. The PG concentration differed between the zones and matrix compartments. Medially, a reduction in PG concentration occurred between 6 and 12 months in the interterritorial compartment of the two uppermost zones. Laterally, the concentrations increased. In general, the pericellular PG concentration was higher than the interterritorial in the two uppermost zones. The striking variation in structural and labeling responses in the various zones and compartments indicate a heterogeneous tissue response in guinea pig OA that will probably affect sampling in biochemical analyses of cartilage homogenates and synovial fluid.

Most animal osteoarthrosis (OA) models—e.g., transection of the anterior cruciate ligament (Hulth et al. 1970, Pond and Nuki 1973, Brandt et al. 1991) or meniscectomy (Colombo et al. 1983, Little et al. 1997)—involve a joint injury inducing secondary OA. However, there are also naturally occurring arthropathies in animals—e.g., Hartley guinea pigs that spontaneously develop a condition, which is morphologically similar to human OA (Bendele and Hulman 1988). Guinea pig OA of the knee develops in a reproducible manner, first affecting the central portion of the medial condyle—the part of the joint that is not covered by a meniscus and which is presumably subjected to the highest load—where gross lesions are invariably present at 12 months (de Bri et al. 1995). An increase in the severity of guinea pig OA is paralleled by a decrease in proteoglycan (PG) and collagen concentrations (Wei et al. 1997). The lower PG concentration is probably largely due to a reduction in synthesis (Wei et al. 1998). The mechanical properties of cartilage are determined by its macromolecular matrix constituents—i.e., collagens and PGs—and its large content of water. Aggrecan is the most abundant PG, which provides elasticity by means of a high concentration of fixed charges of its chondroitin sulfate side-chains, that mainly have sulfate at the 4th or 6th position of the hexosamine moieties.

The composition of matrix can be analyzed at different anatomical sites biochemically, but it is more difficult to clarify the more delicate patterns of distribution in zones and matrix compartments. Immunohistochemical techniques give a higher resolution in the study of specific chemical structures. Antibodies against PG-related epitopes have been used to study the distribution of these sub-

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stances in cartilage (Caterson et al. 1985). In the present study, we used the 2/B/6 preparation which reacts with an unsaturated, terminal 4-sulfated chondroitin disaccharide, obtained after partial chondroitinase digestion of the chondroitin sulfate chain (Couchman et al. 1984).

We examined changes in PG distribution in relation to zones and matrix compartments during the development of guinea pig OA.

Animals and methods

Ten 6- and 12-month-old male Hartley guinea pigs (n = 5) (Møllegård, Copenhagen, Denmark) were used for light microscopy and ultrastructural studies. The 2/B/6 antibody was a generous gift from Prof. Bruce Caterson (Cardiff, Wales). Goat-anti-mouse IgG antibodies conjugated with 10 nm gold particles were obtained from Amersham (Buckinghamshire, UK), nonspecific goat serum and chondroitinase ABC from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

The animals were heparinized, anesthetized and fixed by vascular perfusion, with an equal mixture of 0.3% phosphate-buffered glutaraldehyde and 0.3% paraformaldehyde (Svensson et al. 1987). The proximal tibiae were dissected and cut sagitally into medial and lateral portions. From the nonmeniscus-covered parts, half a mm thick vertical slices, including cartilage and part of the subchondral bone, were cut with a sharp chisel and then immersed in the same fixative for 2 h. Specimens were dehydrated at –45 °C and embedded in a polar resin (Hultenby et al. 1991).

1 μm thick sections from 2–4 blocks of each condyle were used for light microcopy. One micrograph covering the 4 zones was taken from each section. Volume densities (Vv) of matrix and chondrocytes in the various zones were estimated by point counting (Gundersen et al. 1988), including the entire lacunae in the estimate of the cell Vv.

The superficial zone was regarded as the top layer of cartilage with flattened and relatively small cells with their long axes parallel to the articular surface. The intermediate zone was the area beneath the superficial zone, characterized by its larger oval or round cells. The next, deep zone was the underlying area down to the calcified zone, as defined by the position of the tidemark. In the deep zone, the chondrocytes increase in size and tend to be disposed in columns with their axes more or less perpendicular to the articular surface. The calcified zone is the remaining cartilage beneath the tidemark and down to the subchondral bone (Meachim and Stockwell 1979).

In the electron microscopy studies, 2 central ultrathin sections (35–40 nm) from 2 blocks of each condyle were cut and placed on formvar-coated nickel grids. Sections were digested with chondroitinase ABC, 0.2 units/mL in 0.1 M Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin for 15 min at room temperature. To block nonspecific binding, sections were placed on 10% normal goat serum, and then incubated in a humidified chamber overnight, with the specific antibody diluted 1:500 in 0.1 M phosphate buffer and containing 0.1% normal goat serum (PBG). The sections were then thoroughly rinsed in 0.1 M PBG, and bound antibodies were detected with secondary gold-conjugated antibodies. All immunological incubations were done simultaneously, using the same stock solutions. Subsequently, the sections were contrasted with 4% uranyl acetate and lead citrate and examined with a Philips 400T electron microscope at 80 kV, using undigested sections as controls.

Electron micrographs, final magnification ×66,000, were used to detect the chondroitin-4-sulfate distribution along the superficial, intermediate, deep and calcified zones. In each zone, 2 matrix compartments (pericellular and interterritorial) were defined on printed copies, apart from the calcified zone, where it is difficult to distinguish the pericellular compartment. This compartment was defined as the area within 1 μm of the cell surface. The interterritorial compartment, which has thicker collagen fibrils, was sampled at low magnification on the microscope screen, midway between two cells, and high magnification pictures were taken, using a stratified random sampling technique.

From each section 21 micrographs were prepared from the 4 zones: 3 micrographs from the interterritorial and 3 from the pericellular matrix of each unmineralized zone and 3 from the calcified zone. The grain counts were used as relative values comparing various parts of the same section and

then correlated to concentration values previously obtained by high-performance liquid chromatography from a material with the same age distribution of animals (Wei et al. 1997). Therefore, the epitope concentration, measured as density of gold grains, was recalculated to give the amounts of 4-sulfated chondroitin sulfate disaccharides in chemical terms, considering the volume densities of the matrix, and allowing the estimation of concentrations in the various tissue compartments. The amount of label was estimated in each zone as the product of labeling density (a_i) and the corresponding zonal volume density (matrix in a zone as a proportion of total tissue volume, Vv_{zone}), the sum of these products $\{A=\Sigma(a_i \times Vv_{zone})\}$ reflecting the concentration of epitope in the total tissue volume. The value obtained (A) and the corresponding chemically determined concentration of chondroitin-4-sulfate disaccharides ([CS4]) were then used to calculate a constant ($k=[CS4]/A$) that permitted the translation of any labeling density into estimates of concentration ($[CS4]_i=k \times a_i$).

Statistics

Results were analyzed using a two-way ANOVA with repeated measures on one factor. The within factor was Condyle (levels: medial and lateral side) and the between factor was Age (levels: 6 and 12 months). In case of significant interaction, simple effects were examined—i.e., effects of one factor holding the other factor fixed. The p-values were then corrected, using the Bonferroni procedure. P-values less than 0.05 were considered statistically significant. Due to technical problems in processing the tissue, one 12-month lateral condyle could not be analyzed. An additional lateral condyle of an animal from the same litter was therefore processed and used as a matched pair.

Results

At 6 months, no gross changes were detected on either tibial condyle. However, at 12 months, we found macroscopic roughening and fibrillation of the articular surface, and cartilage destruction. These were consistent findings on the medial but not the lateral condyle. With the light microscope, the superficial layer seemed slightly less cellular on the medial side as early as at 6 months. At 12 months, cell Vv on the medial side was considerably lower in the superficial zone (Table 1). In the deep zone, cell clustering and hypertrophy were salient features medially (Figure 1).

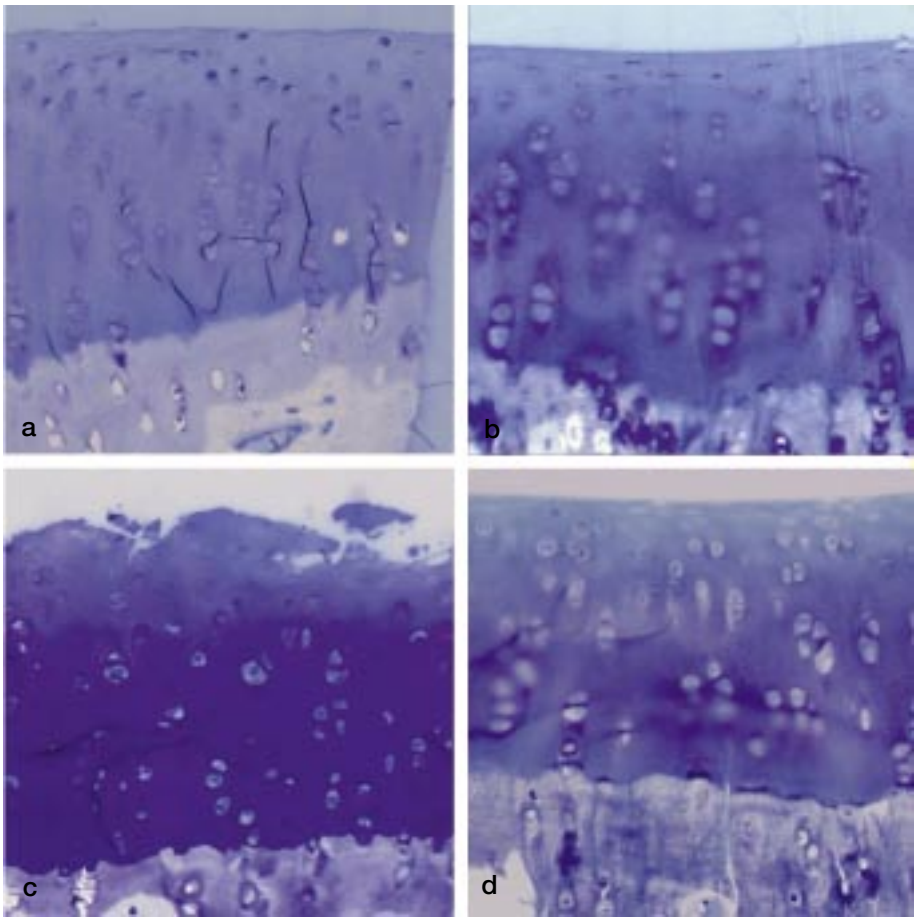
Table 1. Cell volume density (proportion of cells to the total volume of the zone) in articular cartilage of medial (OA) and lateral (without OA) tibial condyles of 6- and 12-month-old guinea pigs. Mean (SD)

	Cell volume density			
	Medial condyle		Lateral condyle	
Cartilage zone	6 months	12 months	6 months	12 months
Superficial	6.4 (3.6)	0.8 (1.6) [a]	9.3 (4.8) [b]	6.3 (2.9) [ab]
Intermediate	7.7 (2.1)	5.4 (1.6)	8.3 (2.8)	6.8 (4.5)
Deep	8.4 (1.5)	7.5 (1.7)	9.2 (1.3)	8.2 (1.7)

[a] p = 0.02, as compared to the corresponding value at 6 months.
[b] p = 0.03, as compared to the medial condyle at 12 months.

The PG concentration in the superficial and intermediate zones decreased between 6 and 12 months medially, while it increased laterally. These changes were seen in both the pericellular and territorial compartments. At 6 months, the PG concentration was the same or slightly higher medially, while at 12 months, it was almost 100% higher laterally than medially, except in the deep and calcified zone. The levels in the deep zone increased between 6 and 12 months at both sides, except in the interterritorial compartment medially where it remained unchanged. The levels in this zone were higher medially than laterally (Table 2).

One striking finding was that the tissue responses varied, not only between the various zones, but also that the amounts of PG available for incorporation in the interterritorial matrix were also affected. Medially, in the superficial zone, the pericellular concentration decreased by 61% between 6 and 12 months; the corresponding figure in the intermediate zone was 52%, while the concentration in the deep zone increased by 37%. The pericellular PG concentration was usually higher than the interterritorial in the two uppermost zones.



Histological sections from the medial (a) and lateral (b) tibial condyles of a 6-month-old guinea pig without osteoarthrosis. At 12 months, the medial condyle (c) shows osteoarthrosis, with fibrillation and a few chondrocytes remaining in the superficial and intermediate zones. The corresponding joint surface of the lateral condyle (d) is intact, but there are a few cells in the superficial zones. (toluidine blue).

Table 2. Chondroitin-4-sulfate concentrations in the pericellular (pc) and interterritorial (it) compartments in various zones of the medial and lateral condyles of the tibial articular cartilage in 6- and 12-month-old animals (µg UA/mg dry tissue weight). Mean (SD). Calculations based on labeling density, matrix volume density and the total concentration of chondroitin-4-sulfate disaccharides determined chromatographically (5). (For calculations, see methods)

		6 months		12 months		P-values			
Zone	Compartment	Medial condyle	Lateral condyle	Medial condyle	Lateral condyle	Med vs Lat 6 months	Med vs Lat 12 months	Med 6 vs 12 months	Lat 6 vs 12 months
Superficial	pc	8.2 (5.3)	4.8 (1.8)	3.2 (2.4)	11.1 (4.0)	0.1	0.03	0.2	0.02
	it	4.5 (0.9)	3.5 (1.0)	1.8 (1.3)	7.9 (2.8)	0.7	<0.001	0.01	0.02
Intermediate	pc	6.7 (2.1)	5.0 (1.5)	3.2 (2.2)	10.0 (3.9)	0.08	<0.001	0.07	0.05
	it	5.3 (0.8)	4.5 (1.0)	3.5 (1.0)	7.4 (1.0)	0.3	<0.001	0.03	0.003
Deep	pc	8.4 (2.1)	4.3 (1.1)	11.4 (1.4)	9.7 (3.5)	0.02	0.02	0.002	0.002
	it	10.2 (0.8)	6.7 (0.5)	9.6 (0.9)	8.2 (0.7)	0.004	0.05	0.8	0.009
Calcified	it	4.1 (0.6)	3.1 (0.6)	1.5 (0.5)	2.0 (0.4)	0.002	0.07	<0.001	0.01

Discussion

The development of OA in the guinea pig knee follows a distinct pattern, starting in the nonmeniscus-covered part on the medial condyle and later occurring laterally. The two condyles may thus represent two phases of the natural history of this disorder. Although clinical samples suggest that there is no normal cartilage in a joint with OA (Heinegård et al. 1998), the characteristic morphological patterns of guinea pig OA show that, at least initially, there are focal structural changes and probably biochemical ones as well. Presumably, molecular changes take place long before changes can be detected with the microscope.

We estimated the volume density of cells. The estimate of the cell number of cells requires the use of numerous parallel sections, and identification of individual cells on separate sections, which is difficult in cartilage. However, the many empty lacunae in the superficial zone (Figure) supports the view of a decrease in the number of cells, which is in line with previous observations of regional chondrocyte depletion in guinea pig OA cartilage (Bendele and Hulman 1988) as well as in the late stage of human OA (Blanco et al. 1998, Aigner et al. 2001). Our finding of cell depletion even at the lateral side at 12 months, without light microscopic OA (the lateral condyle also regularly develops OA at 24 months) (de Bri et al. 1995), indicates that cellular changes are early events in guinea pig OA.

The sections in the present study were treated with a limited chondroitinase digestion that cleaves the chondroitin sulfate chains at random, and all but the nonreducing terminal uronic acid may develop into the 4.5-unsaturated epitope. With the tissue embedded in the plastic resin, the reaction will take place only on the section surface with no dependence on penetration of enzyme or antibodies, nor will there be any rearrangement of protein cores in the tissue. The epitopes that can be developed in this way are located anywhere in GAG chains and extend from the section surface, depending on its relation to the cut surface. Therefore, the distribution of gold grains in a section reflects that of the total 4-sulfated chondroitin disaccharides in the tissue, regardless of possible variations in sulfation along the chain. Furthermore, the fairly constant sulfation pattern seen previously (Wei et al. 1997) suggests that the 2/B/6 labeling would also mirror the overall distribution of chondroitin sulfate. The principal reduction in PG concentration in the OA-affected medial condyle between 9 and 12 months, coinciding with more advanced development of OA (Wei et al. 1997) seems to be confined to the superficial and intermediate zones. The varying responses of the compartments in various zones indicate a process starting in the cell, first affecting the pericellular matrix and then the territorial matrix. In addition, in situ hybridization experiments on OA cartilage have shown suppression of anabolic activity of chondrocytes in the superficial zone (Aigner et al. 1997).

A comparison of cultured chondrocytes from cartilage slices of different zones have shown that chondrocytes derived from the superficial zone synthesize less PG than cells from deeper zones (Aydelotte et al. 1988). This is in line with physiochemical (Venn and Maroudas 1977) and biochemical studies (Franzén et al. 1981) on intact cartilage showing the lowest PG content in the superficial zone, the highest in the intermediate zone, and slightly lower in the deep zone. We found the highest concentration in the deep zone. However, this difference may be explained by species, sampling, the technique used and, of course, by the OA stage.

In the two upper zones, PG concentration was higher in the pericellular than in the interterritorial compartment. This pericellular concentration depends on the rate of synthesis by the cell and the pericellular turnover. The pericellular and interterritorial degradation occur in separate metabolic pools (Sandy 1992), a smaller proportion of the PGs being rapidly degraded pericellularly soon after secretion. The remaining fraction is available for transfer into the interterritorial matrix, where the turnover occurs at a considerably slower pace. This transfer depends on the pericellular concentration, which therefore reflects the rate of PG incorporation into the interterritorial matrix.

In a previous paper, we found a decrease in synthesis rather than an increase in degradation as an early event in guinea pig OA (Wei et al.1998). The labeling pattern in the present study indicates that this decrease in deposition of PGs is mainly confined to the superficial and intermediate zones,

while the concentration in the deep zone is almost unchanged or increased. Further down, in the supposedly metabolically less active calcified zones, the concentration of PG was lower. Since it was the only zone that showed a reduction on both sides, this may not have been caused by OA.

A comparison between the medial and lateral condyles, suggests that the process is slow, because despite depletion of the superficial cells, labeling increased laterally during the 6-month period. Thus the responses of the various parts of the cartilage and of the different zones seem to differ. Analyses of pooled samples from entire cartilages and synovial fluid may therefore mirror a wide variety of reactive processes as well as various stages of OA development.

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