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In vitro production of proteoglycans in the articular-epiphyseal cartilage of growing pigs

S. EKMAN[1]*, E. UNGER[2] and L. KJELLÉN[2]

[1] *Department of Anatomy & Histology, and Department of Pathology, Swedish University of Agricultural Sciences, Box 7028, S-750 07 Uppsala, Sweden*

[2] *Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, The Biomedical Centre, Box, 575, S-751 23 Uppsala, Sweden*

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The failure of cartilage mineralization in osteochondrotic cartilage may be due to an impaired proteoglycan production. The *in vitro* production of proteoglycans was therefore studied in the joint cartilage of growing pigs, aged 9–18 weeks, after incubation of cartilage samples with ^{35}S-sulfate. Cartilage was obtained from different areas of the femoral condyles and samples from these areas were further divided into three layers, where the superficial layer contains articular cartilage and the basal layers consist of growth cartilage. There was no significant difference in the overall amount of ^{35}S-proteoglycans synthesized in different areas of the condyles. However, the total production of ^{35}S-proteoglycans per mg tissue was highest in the basal layer in all areas. This was not due to a larger number of cells; the superficial layer contained more DNA per mg tissue than the basal layer. Gel chromatography on Sepharose CL-2B of the cartilage extracts, which resulted in the separation of large proteoglycans ($K_{av} \sim 0.4$) from proteoglycans of small hydrodynamic size ($K_{av} \sim 0.8$), showed that the relative amount of large proteoglycans increased with the distance from the articular surface. Again, no difference in the relative amounts of large and small proteoglycans were found when cartilage from different areas were compared. Osteochondrotic cartilage was detected in the pigs aged 12–18 weeks. In areas where osteochondrotic cartilage were present, the total production of ^{35}S-proteoglycans was lowered and the relative amount of large proteoglycans was less than that found in the adjoining areas devoid of osteochondrotic lesions. The data available indicate that the higher relative amount of small proteoglycans in the osteochondrotic cartilage was partly caused by degradation of the large proteoglycans (aggrecan).

Keywords: Cartilage; osteochondrosis; proteoglycan; swine

Introduction

The joint cartilage in the growing pig is composed of articular cartilage and epiphyseal growth cartilage [1]. The matrix in the epiphyseal growth cartilage will calcify and is involved in the endochondral ossification.

Osteochondrosis is a generalized skeletal disease that affects the epiphyseal and metaphyseal growth cartilages and results in an impaired endochondral ossification [2]. In pigs the frequency of osteochondrosis is reported to be more than 90% in the Swedish Landrace and Yorkshire breeds [2]. Reiland [2] found microscopic lesions in 4-month-old pigs, without lesions in the articular cartilage. The epiphyseal growth cartilage of the medial femoral condyle and the growth plate (metaphyseal growth cartilage) of the distal end of ulna were two predilection sites.

Early osteochondrotic lesions of the epiphyseal growth cartilage are characterized by areas of chondronecrosis surrounded by clustered chondrocytes in the resting, proliferative and hypertrophic zones [3, 4]. In addition, calcification of the hypertrophic zone and endochondral ossification are impaired. Retained necrotic cartilage can also be found in the subchondral bone, due to continued ossification in the surrounding growth cartilage. Early osteochondrotic lesions in the growth plates (metaphyseal growth cartilage), preceding the chondronecrosis, are characterized by groups of chondrocytes not maturing into hypertrophic cells [5]. In agreement with this observation, we recently demonstrated, using immunocytochemistry,

* To whom correspondence should be addressed at: Department of Pathology, Swedish University of Agricultural Sciences, Box 7028, S-750 07 Uppsala, Sweden.

that the matrix in the chondronecrotic areas resembled that of the proliferative zone [6], which made us suggest that the chondrocytes had stopped during maturation. This would indicate that the pathogenesis of the lesions in the metaphyseal and epiphyseal growth cartilages is similar.

The hypertrophic chondrocytes produce a unique matrix, suitable for calcification (see [7] for review). A number of factors control calcification, among them proteoglycans, which are believed to influence significantly the process of mineralization (see [8, 9] for review).

The proteoglycans are large macromolecules consisting of a core-protein to which chains of glycosaminoglycans are attached. Their structural and functional diversity is great [10]. Different types of cartilage proteoglycans have been isolated: among them a large aggregating proteoglycan (aggrecan) and two types of small proteoglycans (decorin and biglycan) (see [11, 12] for review). Upon chromatography on Sepharose CL-2B of cartilage extracts, the proteoglycans are eluted in two peaks, where the first peak contains the large proteoglycans separated from the proteoglycans of smaller hydrodynamic size in the second peak. The second peak may also contain degradation products of the large proteoglycans.

By comparison of the proteoglycans produced by cells of the osteochondrotic area and those synthesized by hypertrophic chondrocytes in normal cartilage it may be possible to identify differences that would suggest the presence of non-maturing chondrocytes in the osteochondrotic cartilage. A difference in proteoglycan production could also explain the insufficient calcification and subsequent impaired endochondral ossification of the osteochondrotic cartilage. In the present investigation, the *in vitro* synthesis of proteoglycans in different areas as well as in different depths of the articular-epiphyseal cartilage complex of the growing pig, has been studied.

Materials and methods

Pigs aged 9–18 weeks were studied. The following animals were used; two Swedish Landrace pigs (16- and 18-week-old), four Swedish Landrace-Yorkshire crossbred pigs from the same litter (9-, 12-, 13- and 16-week-old) and one 16-week-old Swedish Landrace-Yorkshire crossbred pig. All animals were male and their feed ratios were according to Swedish standards [13]. Articular and epiphyseal cartilage from the medial and lateral femoral condyles were obtained within 20 min after stunning and bleeding. Tissue samples (3–5 mm thick) were taken from different areas (A, B, C) and depths (1, 2, 3) of the cartilage of each condyle (see Fig. 1). For studies of different depths, the cartilage in each area was divided into three approximately equal parts. Depth 1 contains articular cartilage and in basal parts, resting cells from the growth cartilage. Depth 2 is a mid layer and contains mostly resting cells from the growth cartilage. The third depth (3) is composed of the growth cartilage, with resting, proliferative, hypertrophic and calcifying regions. In femoral joints, where osteochondrotic cartilage was seen macroscopically, the retained cartilage (i.e. necrotic cartilage retained into the subchondral bone due to continuing adjacent ossification) was included in the third (depth 3) layer or analysed separately. The samples were weighed, rinsed in F 10 medium buffered with 20 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic

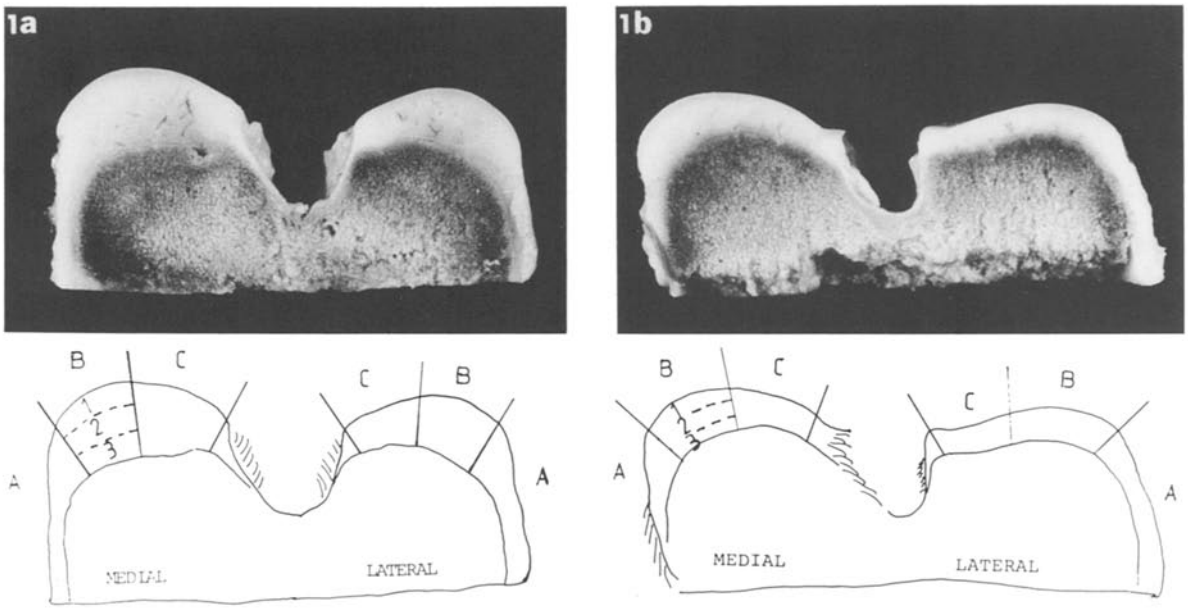

Figure 1. Transverse section of the femoral condyles from (a) a 9-week-old and (b) a 12-week-old Swedish Landrace-Yorkshire crossbred pig. Note the sample areas A, B, C, and 1, 2, 3.

acid) and subsequently cut into small pieces (thickness less than 0.2 mm).

For light microscopic examination, 3–5 mm thick slabs of the entire thickness and width of the articular and epiphyseal cartilage and a small area of subchondral bone were taken from each condyle adjacent to the tissue samples collected for proteoglycan analysis. The slabs were immersed in a 4% aqueous solution of buffered formaldehyde, embedded in paraffin, cut and stained with Haematoxylin and Eosin (HE).

The cartilage pieces of depths 1–3 from areas B and C of the lateral and medial condyles (approximately 20–80 mg wet weight, each) and whole thickness pieces of areas A–C in the medial and lateral condyles (approximately 100–200 mg wet weight, each) were cultured at 37 °C for 24 h in a rotating incubator in 1 ml HEPES buffered F 10 medium, containing, if not otherwise indicated, 0.1 mCi ml^{-1} of carrier-free ^{35}S-sulfate (New England Nuclear). Pilot experiments demonstrated that the incorporation of label was linear during this time. The medium was saved and the cartilage pieces were extracted with 4 M guanidine-HCl in 0.05 M sodium acetate buffer, pH 5.8 containing 1 mM NEM (*N*-ethylmaleimide), 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.2% Triton X-100, 10 mM EDTA (ethylene-diamine-tetra-acetic acid), 100 mM ε-aminocaproic acid for 48 h at 4 °C.

A fluorimetric procedure was used for DNA-analysis of cartilage extracts [14]. The guanidine HCl of the cartilage extracts was removed by dialysis prior to digestion with papain at 60 °C for 16 h. A mixture of 0.4 ml sample (papain digested cartilage), 0.4 ml ethidium bromide (EBr) and 1.2 ml phosphate-buffered saline (PBS: 8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 0.2 g l^{-1} KH_2PO_4, 1.15 g l^{-1} Na_2HPO_4; pH 7.4) was prepared. The EBr fluorescence was measured at 600 nm (excitation at 363 nm), using pure salmon DNA as standard.

The total amount of ^{35}S-macromolecular radioactivity in the extracts was determined after dialysis against extraction buffer. Some of the medium samples were also analysed for total macromolecular ^{35}S-radioactivity and were found to contain less than 10% of the ^{35}S-radioactivity present in the corresponding cartilage extracts. The residues were incubated in 2 ml 0.5 M NaOH at 4 °C for 24 h followed by dialysis against 50 mM TRIS-HCl, pH 8 and the amount of radioactivity was determined. The amount of ^{35}S-proteoglycans extracted from the cartilage with guanidine-HCl varied between 65–75% of the total macromolecular ^{35}S-radioactivity present in the cartilage. No difference in extractability between samples from normal and pathological tissue was seen.

Gel chromatography under dissociative conditions were performed on a Sepharose CL-2B column (0.5 × 85 cm) eluted in extraction buffer. Fractions of 0.2 ml were collected and analysed for ^{35}S-radioactivity. Dextran blue (Pharmacia Fine Chemicals, Uppsala, Sweden) and DNP-alanine (Sigma Chemical Co, St Louis, MO, USA) were used as markers for void (V_o) and total (V_t) volume, respectively. The relative proportion of large proteoglycans (peak I, $K_{av} \approx 0.4$) and small proteoglycans (peak II, $K_{av} \approx 0.8$) in each extract was calculated from the chromatograms; macromolecules eluting at K_{av}-values $\leqslant 0.6$ on Sepharose CL-2B were considered to represent large proteoglycans and those eluting later to represent small proteoglycans (see Fig. 4).

The ability of the proteoglycans to form aggregates with hyaluronic acid was determined by gel chromatography on a Sepharose CL-2B column under associative conditions. The ^{35}S-labelled proteoglycans alone or with the addition of hyaluronic acid (HA; final concentration 30 μg ml^{-1}) and bovine cartilage chondroitin sulfate proteoglycan (final concentration 3 mg ml^{-1}) were dialysed overnight at 4 °C against 0.5 M sodium acetate buffer, pH 5.8, containing a mixture of protease inhibitors (10 mM EDTA, 1 mM NEM, 1 mM PMSF, 1 μg ml^{-1} pepstatin). The bovine proteoglycan was obtained from Dr Bruce Caterson, University of North Carolina, Chapel Hill. Gel chromatography under associative conditions was performed on columns (0.5 × 85 cm) eluted in the acetate buffer used for dialysis. Fractions of 0.2 ml were collected and analysed for ^{35}S-radioactivity.

Results

Articular and epiphyseal growth cartilage was obtained from the medial and the lateral condyles of pigs aged 9–18 weeks. The joint cartilage consisted of a superficial avascular articular cartilage and a thick growth cartilage (Fig. 2a). The articular cartilage was intact in all the pigs examined. Areas of necrotic cartilage were not present in the pig aged 9 weeks. In the epiphyseal growth cartilage from the pigs aged 12, 13, 16 and 18 weeks, areas of chondronecrosis (Fig. 2b) were found in both the medial and lateral condyles. This necrotic cartilage could be found in all regions (resting, proliferative, hypertrophic) of the growth cartilage in the B and C areas (loaded cartilage areas; Fig. 1). The areas of necrosis were surrounded by chondrocytes in cluster formations (Fig. 2b). The Swedish Landrace pigs (16 and 18 weeks) and the Swedish Landrace-Yorkshire crossbred pigs (16 weeks) also had areas of necrotic cartilage extending into the subchondral bone in C-areas of both lateral and medial femoral condyles. Since the adjacent endochondral ossification had continued, these areas could be seen on macroscopic examination as small retentions (2–10 mm) of cartilage in the subchondral bone (CM-P).

The morphology, including the ultrastructure, of the cartilage from the pigs included in this study has been described by Ekman and co-workers [4].

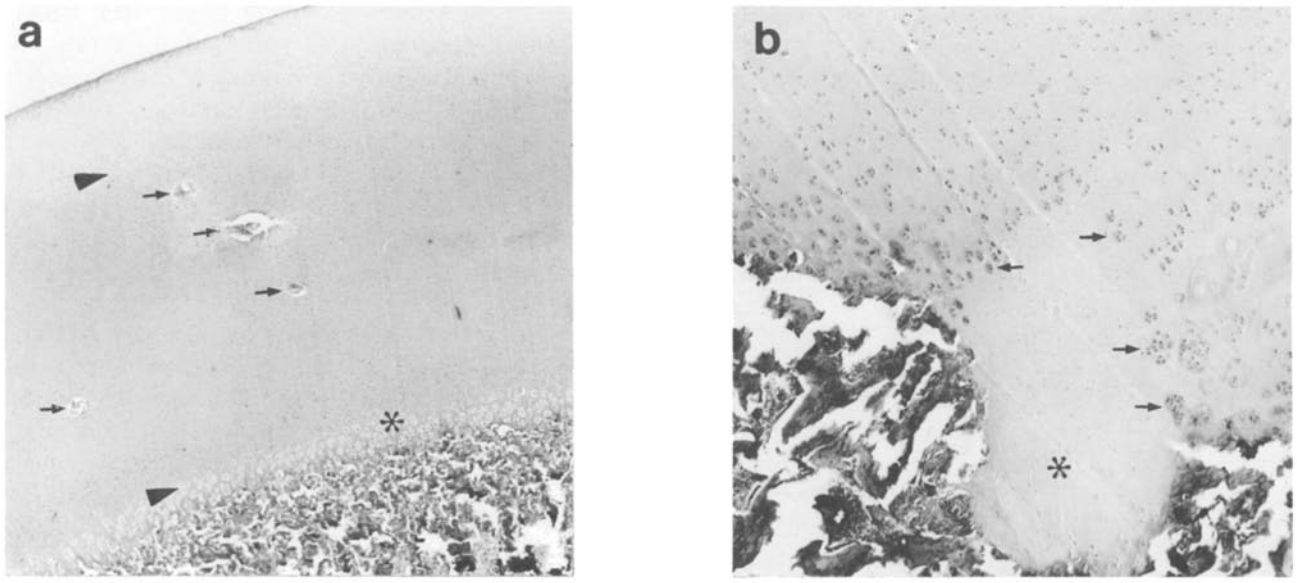

Figure 2. Micrographs of HE stained sections of the lateral femoral condyle from an 18-week-old Swedish Landrace pig. ×50. (a) Vascular channels (small arrows) are seen in the growth cartilage (between arrowheads) which occupies about 2/3 of the joint cartilage. Note the basal layers of the growth cartilage with proliferative and hypertrophic condrocytes (*). (b) A large area of chondronecrosis (*) is present in the basal growth cartilage and subchondral bone. The endochondral ossification has ceased focally. The necrotic cartilage is surrounded by chondrocytes in cluster formations (arrows).

Total production of ^{35}S-proteoglycans

Cartilage obtained from the A, B and C areas (Fig. 1) of the medial and lateral femoral condyles from the crossbred littermates (aged 9, 12, 13 or 16 weeks) and from the 16-week-old Swedish Landrace pig were divided into different layers (1, 2 and 3; Fig. 1) and the production of ^{35}S-proteoglycans was studied. The morphology of material adjacent to the cartilage used for proteoglycan production was examined and found to contain chondronecrotic areas in layer 3 (B and C areas) in all except the 9-week-old pig. Hence, the tissue samples is a combination of normal and pathological tissue. The total incorporation of ^{35}S-sulfate in the joint cartilage was found to be highest in the third layer (depth 3; which contains the growth cartilage) for all sample areas from the pigs aged 9, 12 and 13 weeks (Fig. 3). However, the high production of ^{35}S-proteoglycans in the basal layer was not due to a higher cell density since the DNA content of depth 3 cartilage was less or equal to that of the upper layers (Fig. 4). The AL and CL areas of the 16-week-old crossbred pig and the C-areas from the 16-week-old Swedish Landrace pig had a different ^{35}S-sulfate incorporation pattern, with no obvious high relative production of ^{35}S-proteoglycans in the third layers (Fig. 3). A pathological ^{35}S-incorporation pattern was also seen in macroscopically retained cartilage from the 16-week-old Swedish Landrace pig, which was separately analysed (CM-P, Fig. 3).

Small areas of macroscopical osteochondrotic cartilage (i.e. retained cartilage) were also dissected out from the Swedish Landrace-Yorkshire crossbred pigs (13 and 16 weeks) and two Swedish Landrace pigs (16 and 18 weeks). However, the total *in vitro* incorporation of ^{35}S-sulfate in these pieces was very low due to the presence of necrotic cartilage and some could not be further analysed. In the 18-week-old pig, the total incorporation of ^{35}S-sulfate was eight times higher in the normal growth cartilage (area B) compared with that of the osteochondrotic cartilage (area C).

Relative amounts of large and small proteoglycans

When the cartilage extracts, obtained from different areas of the medial and lateral femoral condyles of the five cross-bred littermates, were chromatographed on a Sepharose CL-2B column, the macromolecular ^{35}S-radioactivity was separated into two peaks representing large (I) and small (II) cartilage proteoglycans (Fig. 5a and b). No differences were found when comparing cartilage extracts from different non-pathological areas or from pigs of different ages. To study the normal distribution of large and small proteoglycans in the different layers, cartilage extracts from the 9-week-old pig, devoid of osteochondrotic cartilage, were analysed (Table 1). Most of the ^{35}S-proteoglycans of all three layers were present in peak I but the predominance of the large proteoglycans was more marked in the basal growth cartilage (depth 3). However, the basal layer of C-areas with osteochondrotic lesions generally contained higher relative amounts of 'small proteoglycans' than did the basal layer of the adjoining normal B-areas, as shown in Fig. 5a and b for cartilage extracts from a 16-week-old Swedish Landrace-Yorkshire crossbred pig. Similar results

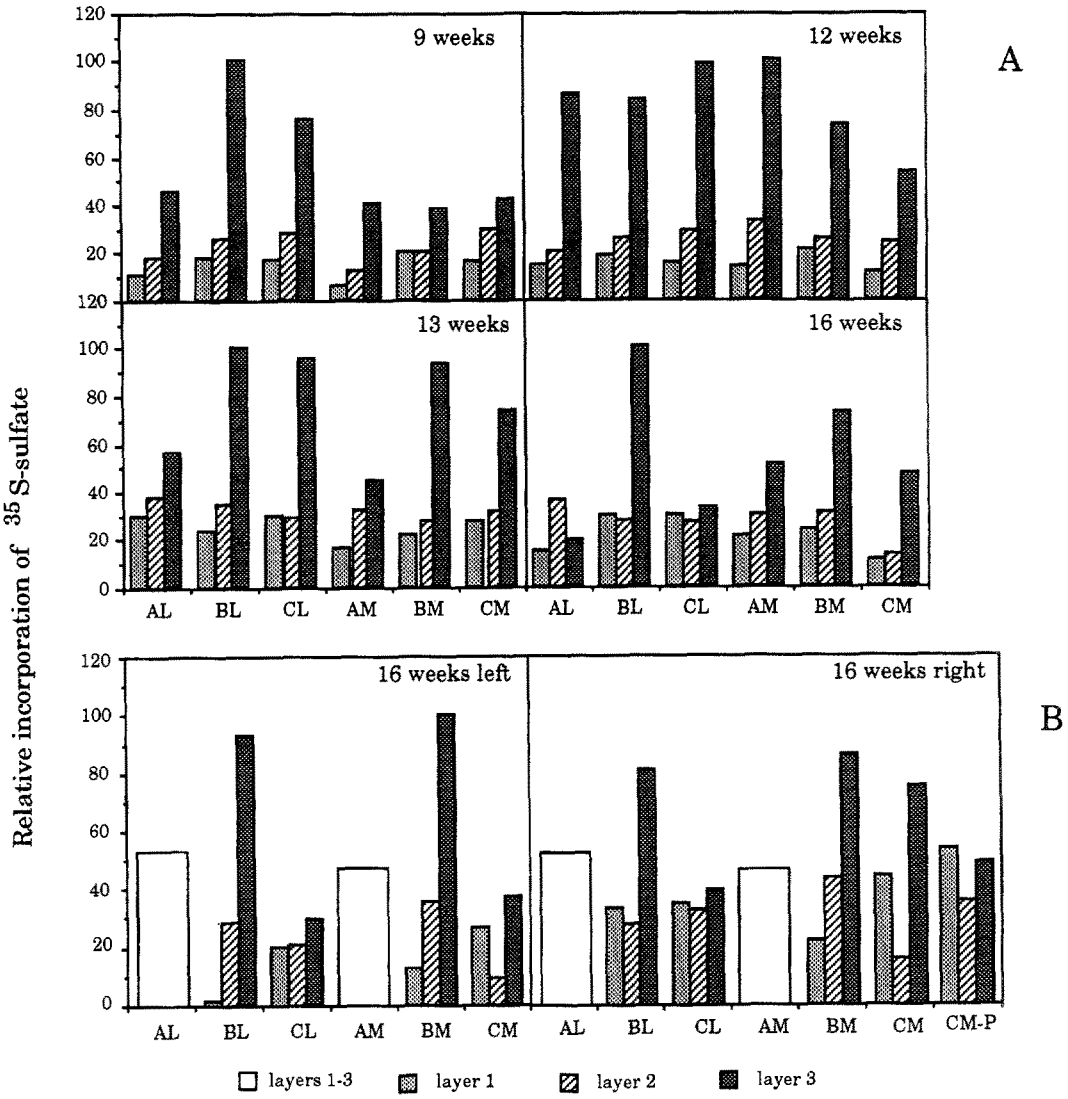

Figure 3. The relative *in vitro* incorporation of ^{35}S-sulfate per mg tissue in different areas (A, B, C) and different layers (1, 2, 3) of lateral (L) and medial (M) femoral articular-epiphyseal cartilage from (a) four (9, 12, 13, 16 weeks) Swedish Landrace-Yorkshire crossbred pigs (littermates) and (b) left and right femoral condyles from a 16-week-old Swedish Landrace pig. CM-P, layer 3 contains macroscopically retained cartilage in the subchondral bone. The highest value obtained for each pig was set at 100. Open bars = not divided into layers.

Table 1. Relative amounts of extracted ^{35}S-proteoglycans present in peak I after chromatography on Sepharose CL-2B, of cartilage from a 9-week-old Swedish Landrace-Yorkshire crossbred pig.

Cartilage area	*Layers*		
	Superficial (1) (%)	*Mid (2)* (%)	*Basal (3)* (%)
AM	72	75	86
BM	75	78	82
CM	71	74	82
AL	56	72	83
BL	75	80	76
CL	74	72	82

were obtained also for the 16-week-old Swedish Landrace pig and the 18-week-old pig (data not shown).

To investigate whether the large proteoglycans in the osteochondrotic cartilage retained the capacity to aggregate with hyaluronic acid, the cartilage extracts from the 16-week-old Swedish Landrace-Yorkshire crossbred pig (Fig. 5a and b) were dialysed to associative conditions in the presence of hyaluronic acid and carrier bovine cartilage proteoglycans, and again analysed by gel chromatography on Sepharose CL-2B. As shown in Fig. 5c and d, a portion of the proteoglycans of both the control (22%) and osteochondrotic cartilage (18%) formed aggregates with hyaluronic acid. However, in the osteochondrotic cartilage, low molecular weight proteoglycans appeared to be present in the aggregates since the peak of small proteoglycans

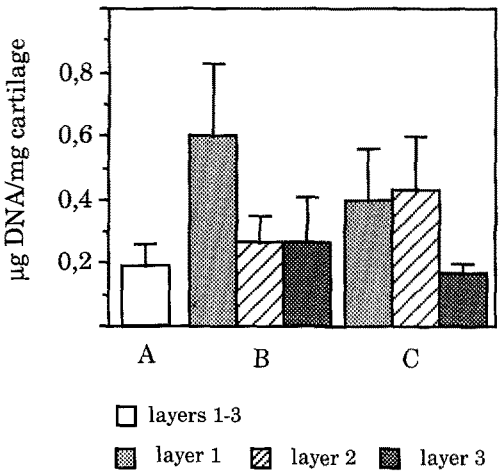

Figure 4. Content of DNA in cartilage samples in different areas (A, B, C) and different layers (1, 2, 3) obtained from the 16-week-old Swedish Landrace pig (Fig. 3b). Samples from both lateral and medial femoral articular-epiphyseal cartilage complex were analysed. Mean values and SD for three to five samples.

contained less ^{35}S-radioactivity after gel chromatography under associative (18%) than under dissociative (28%) conditions (compare Fig. 5b and d). This was not the case for the control cartilage, where the peak of low molecular weight proteoglycans represented 13% and 11% respectively, under dissociative and associative conditions. The aggregating 'small proteoglycans' in osteochondrotic cartilage are most likely degradation products of the large proteoglycans, containing the hyaluronic acid binding region, demonstrating that at least part of the increased relative amount of 'small proteoglycans' in osteochondrotic cartilage is due to degradation of the large proteoglycans. This is also reflected in the higher proportion of large non-aggregating proteoglycans in osteochondrotic than in control cartilage. The ^{35}S-macromolecules eluting in peak II under associative conditions (Fig. 5c and d) most likely represents small proteoglycans (biglycan/decorin type), possibly with the addition of non-aggregating fragments of aggrecan.

Discussion

One aim of the present investigation was to study the production of newly synthesized proteoglycans in different layers of normal articular-epiphyseal growth cartilage and osteochondrotic growth cartilage. We also wanted to see if the proteoglycan production was uniform in different areas of the condyles so that cartilage from an area close to an osteochondrotic lesion could be used as a control, when synthesis of proteoglycans in cartilage from the lesion was studied. The second aim was to test our hypothesis that the presence of non-maturing hypertrophic chondrocytes precede the chondronecrosis.

In the present study no consistent difference in ^{35}S-

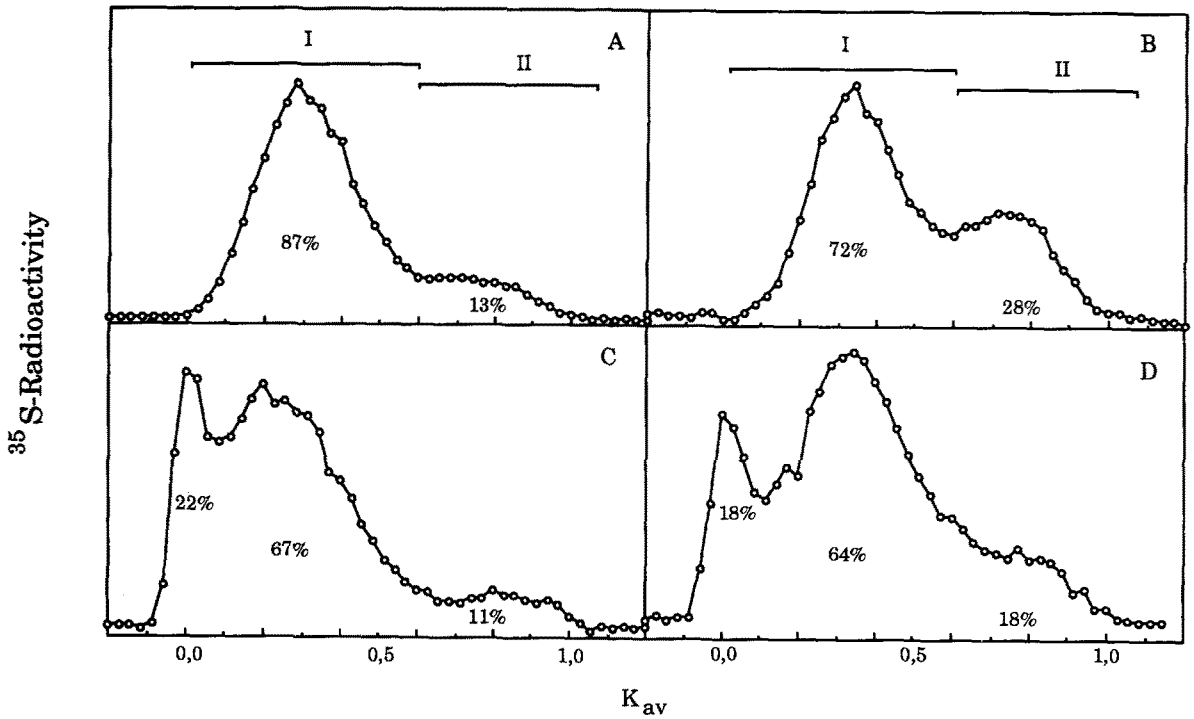

Figure 5. Gel chromatography on columns of Sepharose CL-2B, of cartilage extracts from (a), (c) normal (B-area, layer 3) and (b), (d) osteochondrotic cartilage (C-area, layer 3) of a 16-week-old Swedish Landrace-Yorkshire crossbred pig. The samples were analysed before (a), (b) and after (c), (d) addition of hyaluronic acid and carrier bovine cartilage chondroitin sulfate proteoglycans followed by dialysis to associative conditions.

incorporation into proteoglycans in the A, B and C areas of the condyles obtained from the four littermates could be seen. This is in accordance with Slowman and Brandt [15], who found no difference in the rate of net ^{35}S-glycosaminoglycan synthesis in organ culture of loaded and unloaded cartilage from dogs. The relative proportion of large and small proteoglycans was also similar in different areas.

When different layers of the cartilage were studied, the cartilage in the basal layer was found to synthesize larger amounts of ^{35}S-proteoglycans. This was not the case for layer 3 of the AL and CL areas from the 16-week-old crossbred pig and layer 3 of the C-areas from the 16-week-old Swedish Landrace pig (Fig. 3). While the absence of an active growth cartilage may be the reason for this reduction in the AL area, the lowered incorporation in layer 3 of the C-areas may reflect the presence of osteochondrotic cartilage.

As shown for the 9-week-old pig, layer 3 contained cartilage that synthesized a higher proportion of large proteoglycans compared to cartilage from the other layers (Table 1). DNA analyses indicated that this was not due to a higher cell density of this cartilage (Fig. 4). This is in accordance with several previous studies on proteoglycans extracted from serial sections through adult articular cartilage from cows [16], humans [17] and pigs [18], which indicate that even articular cartilage devoid of growth cartilage contains a deep layer of active chondrocytes synthesizing large amounts of high molecular weight proteoglycans. Aydelotte and co-workers [19] also showed that cultured chondrocytes from the deep zones of bovine articular cartilage synthesized more proteoglycans than did cells from the superficial zone, and that a higher proportion of the proteoglycans synthesized by deep zone chondrocytes were large, aggregating proteoglycans.

Microscopical areas of necrotic cartilage were found in both C and B areas in cartilage from pigs aged 12–18 weeks. Macroscopical osteochondrotic lesions were present in the C areas of the joint cartilage from the 16- and 18-week-old pigs. The osteochondrotic lesions develop over time and has microscopically been described [4] as areas of chondronecrosis in the growth cartilage that eventually form a retention of cartilage into the subchondral bone. At a later event an osteochondritis dissecans with secondary osteoarthrosis will progress. In studies of the aetiopathogenesis of osteochondrosis, the very early lesions prior to retention of a mostly necrotic cartilage are of interest. The deep layers of cartilage from C-areas with osteochondrotic lesions, showed a low incorporation of ^{35}S-sulfate and a reduced relative content of large proteoglycans compared with the B areas of the same condyles. This may be due to a reduced number of viable cells present in the cartilage area containing the lesion. It is also possible that the clustered chondrocytes surrounding the degenerated cartilage have a lowered capacity to synthesize proteoglycans. However, the apparent increase in the relative amount of small proteoglycans was at least partly due to an enhanced degradation of the newly synthesized large proteoglycans, as shown by the ability of 'small proteoglycans' in the osteochondrotic cartilage to aggregate with hyaluronic acid (Fig. 5).

Proteoglycan aggregates and high concentrations of proteoglycans have been shown to inhibit mineral growth *in vitro* [20]. It has been suggested that changes in the proteoglycan structure and organization [21, 22] without a net loss of proteoglycans [9] may be sufficient for calcification to occur. The proportion of non-aggregated monomers have been shown to increase and the proteoglycan aggregate size to decrease in the hypertrophic region (see [23] for review). Nakano and co-workers [24] studied proteoglycans in 7-month-old pigs with osteochondrosis and secondary osteoarthrosis and found that the proteoglycans from these areas were less aggregated. This is similar to the results obtained by Heinegård and co-workers [25], studying osteoarthrosis in dogs. Hence, the findings of Nakano and co-workers [24] may relate more to the secondary osteoarthrosis than to the osteochondrosis. Our results, which concern changes in proteoglycan production in early osteochondrosis, indicate that the reduced ability to form aggregates with hyaluronic acid may be explained by an enhanced degradation of aggrecan in the development of the disease. From the present results, we cannot distinguish between early cell-death and the presence of non-maturing cells, as the key to the enhanced degradation. Further studies of osteochondrosis should include *in situ* techniques, where the morphology of the lesion can be related to the presence of specific changes in the assembly of the macromolecular components within the extracellular matrix.

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