Phenotypic modulation of newly synthesized proteoglycans in human cartilage and chondrocytes

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Summary

The proteoglycans synthesized by human osteoarthritic femoral head cartilage and nonarthritic articular cartilage age-matched to the osteoarthritic cartilage specimens was studied in explant cultures and in chondrocytes generated by explant outgrowth from the cartilages. Twenty-four hours after explanation, both nonarthritic articular cartilage and osteoarthritic cartilage synthesized principally one large proteoglycan core protein that migrated on 3-5% acrylamide gels with an apparent molecular mass (M_r) of ~520 kDa after enzymatic digestion with chondroitinase ABC and keratanase. The proteoglycan was found in both the explant itself and in the medium compartment of the culture as well. This proteoglycan contained chondroitin-6-sulfate, keratan sulfate and the hyaluronan binding region as evidenced by immunoblotting with murine anti-proteoglycan monoclonal antibodies indicating that the proteoglycan was aggrecan. To a much lesser extent two additional proteoglycan core proteins were also found in the explant but were not seen in the culture medium compartment. These proteoglycans possessed apparent M_r's of ~480 kDa and ~390 kDa on 3-5% acrylamide gels after chondroitinase ABC and keratanase digestion. The medium compartment contained principally the ~520 kDa proteoglycan core protein. In osteoarthritic cartilage explants, the pattern of newly synthesized proteoglycans recovered from the tissue as assessed on 5-16% polyacrylamide gradient gels remained relatively the same from day 1 after explantation up to 36 days of culture. By contrast, the proteoglycans recovered from the culture medium contained chondroitin sulfate and keratan sulfate after 1, 7 and 21 days in culture but by 36 days appeared to contain only chondroitin sulfate. Chondrocytes generated from osteoarthritic cartilage and age-matched nonarthritic articular cartilage synthesized different patterns of large (greater than 200 kDa) proteoglycan. Whereas chondrocytes derived from osteoarthritic cartilage continued to synthesize principally the ~520 kDa proteoglycan core protein, the chondrocytes derived from nonarthritic cartilage synthesized in addition to this proteoglycan, abundant amounts of the other two proteoglycan core proteins as well.

Key words: Cartilage, Proteoglycan, Osteoarthritis, Human.

Introduction

HUMAN OSTEOARTHRITIS of the peripheral hip and knee joints is a severely debilitating disease found almost universally among the elderly which results in significant and irreversible destruction of synovial joint articular cartilage [1, 2]. Articular cartilage proteoglycan synthesis in response to an initial cartilage insult may be required to facilitate cartilage repair and perturbations in proteoglycan compensatory synthesis could be responsible for osteoarthritis disease progression [2]. In addition, the types of proteoglycans synthesized by osteoarthritic cartilage, as distinct from the specific amounts of proteoglycan, may play an important role in determining the functional characteristics of the cartilage extracellular matrix.

One hypothesis has stated that chondrocyte compensatory biosynthetic processes were compromised by a substantial increase in the transcription, biosynthesis and activation of matrix metalloproteinases (principally stromelysin-1 and collagenase) which degraded several matrix proteins required for normal cartilage function, including proteoglycans, collagen, link protein and fibronectin [2, 3-5]. This would be coupled to an imbalance between matrix metalloproteinase syn-
thesis and transcription and biosynthesis of the tissue inhibitor of metalloproteinase (TIMP-1) whose expression would not upregulated to the level seen with metalloproteinases [4].

Recent evidence, however, has established that significant modulation of chondrocyte biosynthetic pathways also occurs in human osteoarthritic cartilage. Thus, type X collagen synthesis, normally confined to hypertrophic chondrocytes of the zone of calcifying cartilage and growth plate cartilage was also detected in osteoarthritic cartilage [6, 7] and was indeed the major collagenous isotype synthesized by freshly isolated chondrocytes from osteoarthritic cartilage in vitro [8]. Substantial increases in the deposition of leucine-rich small proteoglycans, namely, decorin [9] and in the cartilage form of fibronectin [10] have also been reported in osteoarthritic cartilage.

In the present study, we sought to establish whether nonarthritic articular cartilage specimens age-matched to cartilage specimens obtained from osteoarthritic femoral heads acquired at the time of joint replacement synthesized proteoglycans that were substantially different from one another. In addition, modulation of the proteoglycan phenotype of human cartilage and chondrocytes generated by explant-outgrowth as a function of time-in-culture was studied.

**Materials and methods**

Chondroitinase ABC, chondroitinase-AC-II and endo-β-galactosidase (keratanase) were obtained from ICN Immunologicals (Irvine, CA, U.S.A.). Agarose and acrylamide for gel electrophoresis were obtained from FMC Bioproducts (Rockland, ME, U.S.A.) and ICN Biomedicals (Irvine, CA, U.S.A.), respectively. Sodium borohydride was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). All other chemicals were ACS grade or their equivalent. Anti-proteoglycan monoclonal antibody 1C6 (anti-hyaluronic acid binding region; HABR) was purchased from the Developmental Studies Hybridoma Bank (Baltimore, MD, U.S.A.). Monoclonal antibodies 3B3 (anti-chondroitin-6-sulfate), 2B6 (anti-chondroitin-4-sulfate) and 5D4 (anti-keratan sulfate) were generous gifts from Dr Bruce Caterson (University of North Carolina, Chapel Hill, NC, U.S.A.). Na\textsubscript{a} \textsuperscript{35}SO\textsubscript{4} (carrier free, specific activity, 43 Ci/mg at 100% isotope enrichment) was purchased from ICN. \textsuperscript{35}S-methionine (specific activity, 1333 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, U.S.A.). Eight-day-old chick embryo limb bud aggrecan (fraction AID1) was kindly provided by Dr David Carrino (Case Western Reserve University).

**HEALTH STATUS OF CARTILAGE DONORS**

All osteoarthritic cartilage specimens were obtained from osteoarthritic femoral heads of patients undergoing joint replacement for disabling osteoarthritis (Table I). Non-arthritic cartilage specimens were obtained from patients undergoing joint replacement as a consequence of hip fracture (Table I). A representative autoradiograph (see below) is shown for reporting results of proteoglycan synthesis studies.

**EXPLANT CULTURE**

Cartilage was diced into small pieces (1–2 mm\textsuperscript{3}) and treated briefly with testicular hyaluronidase (0.05% w/v) for 3 min at room temperature to remove adherent erythrocytes and synovial cells from the cartilage surface [11]. For explant-outgrowth, the cartilage was divided into groups of 10–15 minced pieces, distributed into 60 mm\textsuperscript{2} culture dishes (Falcon; Oxnard, CA, U.S.A.) and maintained in Dulbecco’s modification of Eagle’s medium (DMEM) which contained fetal bovine serum (10% v/v), penicillin-streptomycin (0.1% v/v) [11], mycostatin (0.1% v/v) and fungizone (1% v/v). The cultures were maintained in a humidified atmosphere of 10% CO\textsubscript{2}/90% air at 37°C. The minced tissue was not disturbed for 7 days and then the culture medium was replaced with fresh medium every 2–3 days thereafter until chondrocyte confluency was attained (usually by 30–40 days) [11, 12].

**RADIOISOTOPE INCORPORATION**

Cartilage specimens were radiolabeled with \textsuperscript{35}SO\textsubscript{4} (100 μCi/ml) in sulfate-free DMEM containing fetal bovine serum, antibiotic and antimycotics for 24 h on the day of explantation, while other cartilage specimens were treated in this manner on days 0, 6, 20 and 35 of explantation to determine the quality of the newly synthesized proteoglycans. All cartilage specimens were radiolabeled in parallel

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Diagnosis</th>
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<tbody>
<tr>
<td>1</td>
<td>79</td>
<td>Hip fracture (NA)</td>
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<tr>
<td>2</td>
<td>80</td>
<td>Osteoarthritis</td>
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<tr>
<td>3</td>
<td>84</td>
<td>Hip fracture (NA)</td>
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<td>4</td>
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<td>5</td>
<td>60</td>
<td>Hip fracture (NA)</td>
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<tr>
<td>6</td>
<td>65</td>
<td>Osteoarthritis</td>
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NA, nonarthritic.
with $[^{35}S]$-methionine (100 $\mu$Ci/ml) in methionine-free Eagle's medium supplemented with fetal bovine serum, antibiotics and antimycotics for the same time period. Chondrocytes derived by explant-outgrowth were briefly treated with pancreatic trypsin at 37°C [11, 12], the cells pelleted by low-speed centrifugation and replated at high initial cell density (2–3 x 10^5/ml) in 1.2 cm² surface area multiwell dishes (Falcon). After 2 days, the chondrocytes were radiolabeled with $^{35}$SO$_4$ or with $[^{35}S]$-methionine for 24 h.

PROTEOGLYCAN EXTRACTION

The cartilage explant or chondrocyte cell layer (cell-associated fraction; CAF) was extracted with 4 M guanidine hydrochloride (GuHCl)/0.2 M sodium acetate, pH 5.8 containing proteinase inhibitors [13] for 24 h at 4°C. The culture medium compartment was mixed 1:1 with 8 M GuHCl containing proteinase inhibitors and kept at 4°C overnight. The explant or CAF 4 M GuHCl extract and 4 M GuHCl extract of the culture medium compartment were separately exhaustively dialyzed using 6-8 kDa cutoff membranes against double-distilled de-ionized water at 4°C. The retained material was freeze-dried and stored at -80°C.

DENSITY GRADIENT ULTRACENTRIFUGATION

The newly synthesized proteoglycans were resolved from the bulk of the cellular proteins by density gradient ultracentrifugation under dissociative conditions [13]. The gradient fractions (D4 (top) to D1 (bottom)) were exhaustively dialyzed and freeze-dried. Final densities of these fractions were as previously reported [13].

COMPOSITE AGAROSE-POLYACRYLAMIDE GEL ELECTROPHORESIS (CA-PAGE)

D1, D2, D3 and D4 fractions radiolabeled with $^{35}$SO$_4$ were electrophoresed as previously described [14] and autoradiographs produced using Kodak X-Omat film and D-19 developer.

POLYACRYLAMIDE OR ACRYLAMIDE GEL ELECTROPHORESIS

CsCl density gradient fractions radiolabeled with either $^{35}$SO$_4$ or $[^{35}S]$-methionine were electrophoresed on 3–16% polyacrylamide slab gels containing 0.1% sodium dodecyl sulphate [14]. CsCl density gradient fractions were digested with either chondroitinase ABC, chondroitinase ABC plus keratanase or chondroitinase AC-II as previously described [15, 16] prior to electrophoresis. Autoradiographs were produced. In several cases, D1 fractions radiolabeled with $^{35}$SO$_4$ were digested with chondroitinase ABC and keratanase and electrophoresed on 3–5% acrylamide as previously described [13] and autoradiographs produced.

WESTERN BLOT ANALYSIS

D1 fractions radiolabeled with $[^{35}S]$-methionine were digested with chondroitinase ABC and keratanase prior to electrophoresis on 3–16% gels. The proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) by electroelution. The immobilon membrane was blocked with gelatin and then incubated with one of several anti-proteoglycan monoclonal antibodies (mAb). In the case of monoclonal antibody 1C6, the D1 fractions were additionally reduced and alkylated to reveal the hyaluronic acid binding region epitope [17, 18]. Immunoreactivity of proteoglycan core proteins with mAbs 1C6, 3B3, 5D4 and 2B6 were developed with rabbit or goat anti-mouse IgG or IgM (μ-chain specific) which was conjugated to alkaline phosphatase [16].

Results

PROTEOGLYCANS SYNTHESIZED BY NONARTHRITIC CARTILAGE AND OSTEARTHRITIC CARTILAGE EXPLANTS

D1 fractions were prepared from 4 M GuHCl extracts of osteoarthritic cartilage explants and from 4 M GuHCl extracts of the culture medium compartment. Qualitative alterations in the large (greater than 200 kDa) proteoglycans recovered from the D1 fractions were studied as a function of time in culture. D1 fractions radiolabeled with either $^{35}$SO$_4$, or $[^{35}S]$-methionine on the day of sample acquisition (day 0) or after 6, 20 and 35 days in culture were electrophoresed on 3–16% SDS-PAGE. The results of this analysis was compared to results obtained from chondrocytes derived from the osteoarthritic cartilage explants. This analysis showed that substantial modulation of newly synthesized proteoglycans occurred as a function of time-in-culture (Fig. 1). In the cartilage explant, after 24 h [Fig. 1(a), day 0], one major proteoglycan core protein (L$_1$) was detected and only after combined chondroitinase ABC and keratanase digestion. Significantly lesser amounts of two other proteoglycan core proteins (L$_2$ and L$_3$) were seen [Fig. 1(a), lane 3]. The requirement for both chondroitinase ABC and keratanase digestion to
Fig. 1. Proteoglycan synthesis by human osteoarthritic cartilage explants and chondrocytes. Human osteoarthritic (OA) femoral head cartilage was radiolabeled with $^{35}$SO$_4$ for 24 h on the day of explantation (day 0) and after 6, 20 and 35 days in culture. Chondrocytes (OA chondrocyte) derived from explant-outgrowths after 36 days were separated from the explant and radiolabeled for 24 h with $^{35}$SO$_4$. D1 fractions were prepared from the explant and the culture medium of the explanted cartilage and from the cell-associated fraction (CAF) and medium compartment of the chondrocyte cultures and digested with either chondroitinase ABC (ABC), chondroitinase ABC and keratanase (ABC/KSase) or not digested at all (none). D1 samples were then subjected to 3-16% SDS-PAGE and autoradiographs produced. $^{14}$C-methylated marker proteins are myosin, 200 kDa; phosphorylase b, 97.4 kDa; albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14.3 kDa. The percentage of $^{35}$SO$_4$ reaching equilibrium in the D1 fractions prepared from the explants after 24 h (day 0) was 70.6%, on day 7, 77.1%, on day 21, 71.3% and on day 36, 46.3%. The percentage of $^{35}$SO$_4$, reaching equilibrium in the D1 fractions prepared from the culture medium compartment after 24 h (day 0) was 45.8%, on day 7, 42.1%, on day 21, 54.2% and on day 36, 39.1%. The values for day 36 compare favorably with the results of other analyses of D1 fractions conducted on day 36 or somewhat older cultures; explant, 52.1 ± 6.2 (mean ± s.d., N = 3); medium compartment, 55.7 ± 15.8. PG, aggregating proteoglycan; PG-S, small proteoglycans.
visualize these proteoglycans persisted for the entire 36 days of the study.

In the culture medium compartment of the explants [Fig. 1(b)], up until day 21, chondroitinase ABC and keratanase digestion was required to visualize large proteoglycans and principally the L1 proteoglycan core protein was detected [Fig. 1(b), lanes 3, 6, 9]. By contrast, the L1 proteoglycan core protein seen after 36 days was produced after chondroitinase ABC digestion alone [Fig. 1(b), lane 11]. The migration of the L1 proteoglycan core protein did not change after the combined enzyme digestion [Fig. 1(b), lane 12].

In addition to the synthesis of large proteoglycan core proteins, osteoarthritic cartilage also synthesized proteoglycans that migrated on 3–16% polyacrylamide gels in the manner characteristic of small proteoglycans. These small proteoglycans (PG-S1, PG-S2) were not readily apparent until late (day 36) in the time-in-culture experiments [Figs 1(a) & 1(b), lane 10]. A broad band of radioactivity migrating between the 97.4 kDa and 30 kDa markers was visualized in D1 fractions without enzyme digestion [Fig. 1(a), lane 10]. This band was completely abolished after chondroitinase ABC digestion [Figs 1(a) and 1(b), lane 11]. This band was also detected in chondrocytes generated from the explant-outgrowth [Figure 1(c), lanes 1 and 4]. Chondrocytes generated from these osteoarthritic cartilage explants showed persistent synthesis of primarily the L1 proteoglycan core protein in both the cell-associated fraction (CAF) and in the culture medium compartment [Fig. 1(c)]. D1 fractions radiolabeled with 35S-methionine confirmed the results of these analyses on 35SO4-labeled D1 fractions (see below). The L1 proteoglycan core protein was immunoreactive with mAb 3B3 after chondroitinase ABC/keratanase digestion indicative of the presence of chondroitin-6-sulfate. Chondroitinase ABC digested D1 also was immunoreactive with mAb 5D4, indicative of the presence of keratan sulfate. Chondroitinase ABC/keratanase digested D1 also was immunoreactive with mAb 1C6 after reduction and alkylation, indicative of the presence of the hyaluronan binding domain. Only weak reactivity of D1 was obtained with mAb 2B6 after chondroitinase ABC/keratanase digestion. The immunoreactivity of the D1 fraction proteoglycan core proteins with mAb 3B3 after chondroitinase ABC/keratanase digestion was identical to the immunoreactivity of mAb 3B3 obtained with authentic chick limb bud aggregan. The presence of keratan sulfate, the hyaluronan binding domain and chondroitin-6-sulfate strongly indicated that the L1 core protein was aggregan.

In order to estimate the apparent size (Mr) of the principal L1 proteoglycan core protein synthesized by human cartilage in short-term culture, a D1 fraction radiolabeled with 35SO4 was digested with chondroitinase ABC and keratanase and electrophoresed on a 3–5% acrylamide slab gel with dithiothreitol and SDS in the running buffer. An autoradiograph was produced from the dried gel. Lane 1, 3H-methylated protein markers and the migration position of the α,β chains of dynein possessing Mr's of 480 kDa and 440 kDa, respectively [see 13]. Lane 2, 35SO4-labeled D1 fraction.

Fig. 2. Three per cent to 5% acrylamide gel electrophoresis of a human osteoarthritic femoral head cartilage D1 fraction. On the day of explantation, a sample of human osteoarthritic femoral head cartilage was radiolabeled with 35S04 for 24 h and a D1 fraction produced from the culture medium compartment. The D1 fraction was digested with chondroitinase/ABC and keratanase and electrophoresed on a 3–5% acrylamide slab gel with dithiothreitol and SDS in the running buffer. An autoradiograph was produced from the dried gel. Lane 1, 3H-methylated protein markers and the migration position of the α,β chains of dynein possessing Mr's of 480 kDa and 440 kDa, respectively [see 13]. Lane 2, 35SO4-labeled D1 fraction.
apparent $M_r$s were $\sim 390$ kDa and $\sim 480$ kDa, respectively.

For comparison purposes proteoglycan synthesis was studied on nonarthritic femoral head cartilage age-matched to the osteoarthritic femoral head cartilage samples (Table I). The percentage $^{35}$SO$_4$-labeled proteoglycan in the D1 fraction from two of these nonarthritic cartilage specimens was 72.3% and 71.8% which was similar to those average values obtained for osteoarthritic femoral head cartilage (Fig. 1).

Three percent to 16% SDS-PAGE showed that nonarthritic cartilage explants predominantly synthesized the $L_1$ proteoglycan core protein after 24 h in culture [Figs 3(a) and 3(b)]. A proteoglycan core protein migrating with the 200 kDa marker was detected in the medium compartment [Figs 3(a), lane 2, 3(b), lane 2] which was not seen in the 24 h osteoarthritic cartilage explants [Fig. 1(a), lane 3].

**COMPARISON OF PROTEOGLYCANS SYNTHESIZED BY CHONDROCYTES FROM OSTEOARTHRITIC CARTILAGE WITH CHONDROCYTES FROM AGE-MATCHED NONARTHRITIC CARTILAGE**

The proteoglycan profile produced by human chondrocytes generated by explant-outgrowth of osteoarthritic cartilage and nonarthritic cartilage was analyzed on $^{35}$SO$_4$-labeled CsCl density gradient fractions by autoradiography after CA-PAGE (Fig. 4). The proteoglycan profile produced by chondrocytes from nonarthritic cartilage and chondrocytes from osteoarthritic cartilage was virtually identical. Several proteoglycan subpopulations were visualized. The D1 fraction contained two proteoglycan populations ($P_{G_1}$ and $P_{G_2}$). We had previously shown that $P_{G_1}$ formed proteoglycan aggregates with hyaluronan whereas $P_{G_2}$ did not [17]. The fast migrating bands in the middle and low density D2/D3/D4 fractions (Fig. 4).

![Fig. 3. Proteoglycan synthesis by nonarthritic cartilage. Nonarthritic cartilage was radiolabeled with either (a) $^{35}$SO$_4$ or (b) $[^3]$S-methionine ($^{35}$S-Met) on the day of sample acquisition for 24 h. D1 fractions were either digested or not digested with chondroitinase ABC/keratanase (ABC/KSase) prior to electrophoresis on 3-16% gradient slab gels. The arrow shows the migration position of the $L_1$ proteoglycan core protein. The arrowhead denotes a proteoglycan core protein which is present in the medium compartment but not in the explant and is migrating slower than the 200 kDa marker protein. In (b), the arrow and the arrowhead mark the $L_1$ proteoglycan core protein and this additional proteoglycan core protein, respectively. The migration of the $S_1$ and $S_2$ core proteins is also indicated. Markers are $^{14}$C-methylated proteins as noted in Fig. 1.](image-url)
FIG. 4. Composite agarose-polyacrylamide gel electrophoresis of chondrocyte proteoglycans. Chondrocytes from nonarthritic or osteoarthritic cartilage were radiolabeled with $^{35}$SO$_4$ for 24 h. The newly synthesized proteoglycans in the medium compartment were subjected to CsCl density gradient centrifugation under dissociative conditions. Gradient fractions D4 (top), D3, D2 (middle) and D1 (bottom) [see 17] were electrophoresed and autoradiographs produced. PGL, aggregating proteoglycan [see 17]; PGn, nonaggregating proteoglycan [see 17]. NA, nonarthritic; OA, osteoarthritic.

lanes 1–3, 5–7) and the slow migrating band in the D4 fraction (Fig. 4, lanes 1 and 5) have not been as yet characterized.

The proteoglycans and other proteins transported into the medium compartment were further analyzed by 3–16% SDS-PAGE of [$^{35}$S]-methionine-labeled CsCl density gradient fractions (Fig. 5). The profiles of the chondrocytes from aged-matched nonarthritic cartilage (Fig. 5, lanes 1–4) and chondrocytes from osteoarthritic cartilage (Fig. 5, lanes 5–8) were virtually identical except for the almost complete absence of the L$_4$ and L$_m$ proteoglycan core proteins in the D1 fraction of the chondrocytes derived from osteoarthritic cartilage (Fig. 5, lane 8) and marked reduction in the intensity of the radio signal in the L$_1$ proteoglycan core protein relative to the S$_1$, S$_2$ core proteins (Fig. 5, lane 8). The L$_4$ proteoglycan core protein was also seen in the D2 fraction (Fig. 5, lanes 3 and 7). The percentage of $^{35}$S-labeled protein in the D1 + D2 fraction of the medium compartment in three nonarthritic aged-matched cartilage samples (Table I, patients 1, 3 and 5) was 47.4%, 47.1% and 46.6% compared to 38.8%, 60.7% and 61.8% for osteoarthritic femoral head cartilage samples (Table I, patients 2, 4, and 6). The S$_1$ and S$_2$ core proteins were seen primarily in the D1 and D2 fractions.

The proteoglycans in the D1 fraction were analyzed for susceptibility to chondroitinase ABC, chondroitinase AC-II digestion or combined chondroitinase ABC/keratanase digestion (Fig. 6). Under these conditions, most of the newly synthesized proteoglycans produced by chondrocytes from non-arthritic and from osteoarthritic cartilage were transported into the medium [Fig. 6(b)]. In the CAF [Fig. 6(a)], only the L$_4$ proteoglycan core protein was detected. The L$_4$ proteoglycan core protein migrated into the gel after chondroitinase ABC digestion [Fig. 6(a), lanes 2 and 6] or after chondroitinase AC-II digestion [Fig. 6(a), lanes 4 and 8]. The migration position of the L$_4$ proteoglycan core protein was not altered after additional keratanase digestion [Fig. 6(a), lanes 3 and 7]. By contrast, the medium compartment contained several proteoglycan subpopulations [Fig. 6(b)]. In the culture medium compartment of chondrocytes from nonarthritic cartilage, nonenzyme-digested D1 showed the presence of a proteoglycan that failed to enter the gel, together with two smaller proteoglycans [Fig. 6(b), lane 1]. After chondroitinase ABC digestion, proteoglycan core proteins, L$_4$ and L$_m$ could be visualized [Fig. 6(b), lane 2] together with radioactive bands S$_1$, S$_2$. Chondroitinase AC-II digestion [Fig. 6(b), lane 4] produced similar results with a modest increase in migration of these bands. When keratanase was added to chondroitinase ABC-digested D1, an additional core protein, L$_m$ could be seen [Fig. 6(b), lane 3]. In contrast to the above, in chondrocytes from osteoarthritic cartilage the L$_4$ proteoglycan core protein predominated in D1 fractions digested with either chondroitinase ABC or chondroitinase AC-II, respectively [Fig. 6(b), lanes 6 and 8, respectively]. Addition of keratanase to chondroitinase ABC-digested D1 produced only a faint band corresponding to the L$_m$ proteoglycan core protein; the L$_m$ proteoglycan core protein was never detected in any abundance [Figure 6(b), lane 7]. Radioactive bands corresponding to S$_1$, S$_2$ were also visualized after chondroitinase ABC, combined chondroitinase ABC/keratanase or chondroitinase AC-II digestion [Figure 6(b), lanes 6–8, respectively].
Discussion

The principle objective of this study was to systematically analyze the influence of explant and cell culture of human chondrocytes on the stability of the proteoglycan phenotype. Aberrant compensatory extracellular matrix biosynthesis has been suggested to account, in part, for the failure of articular cartilage to undergo repair as a consequence of osteoarthritic pathology [2]. Owing to the high prevalence of osteoarthritis among the elderly population, determinations of the fidelity of the proteoglycan repertoire necessitated that control cartilage for this study (i.e. nonarthritic) be age-matched to the osteoarthritic cartilage specimens.

The most abundant newly synthesized large proteoglycan core protein of human nonarthritic cartilage and osteoarthritic cartilage produced after 1 day in culture had identical apparent $M_r$ of ~520 kDa after chondroitinase ABC/keratanase digestion as estimated by its migration on 3–5% acrylamide. Immunoreactivity with a murine mAb reactive with keratan sulfate indicated that this core protein was aggrecan and not versican. Versican and aggrecan are closely related to each other [19]. Both core proteins possess a domain at the amino-terminal end which binds hyaluronan and a lectin domain at the carboxy-terminus whose naturally occurring ligand is unknown. Both proteoglycans contain chondroitin-sulfate. However, versican is devoid of keratan sulfate, whereas aggrecan possesses several keratan sulfate domains. Immunoblots showed that proteoglycan core protein L contained both the hyaluronan binding region and chondroitin-6-sulfate. Chondroitinase ABC/keratanase digestion of D1 fractions, however, raised the possibility that mAb 1C6 reactivity with the proteoglycan core protein L was to the G2 globular domain as well as to the hyaluronan binding region [20].

![Figure 5](image_url)

**Fig. 5.** Three per cent to 16% gradient gel electrophoresis of proteoglycans synthesized by human chondrocytes. Chondrocytes were radiolabeled with [35S]-methionine for 24 h. The culture medium compartment was subjected to CsCl density gradient centrifugation under dissociative conditions. Gradient fractions were digested with chondroitinase ABC/keratanase, electrophoresed on 3–16% gradient slab gels and autoradiographs produced. L, L1, L3 marks the position of three proteoglycan core proteins. S, S2 marks the position of the small proteoglycan core proteins. Rainbow™ marker proteins are myosin, 200 kDa; phosphorylase b, 92.5 kDa; bovine serum albumin, 66 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 21.5 kDa. NA, nonarthritic; OA, osteoarthritic.
Fig. 6. Three per cent to 16% gradient slab gel electrophoresis of D1 proteoglycans. Chondrocytes from nonarthritic cartilage or osteoarthritic cartilage were radiolabeled with [35S]-methionine for 24 h. The high-density D1 fraction from (a) the cell-associated fraction (CAF) and (b) the medium compartment produced by CsCl density gradient centrifugation under dissociative conditions was electrophoresed on 3–16% gradient polyacrylamide slab gels and autoradiographs produced. Some D1 fractions were digested with either chondroitinase ABC (ABC), ABC and keratanase (ABC/KSase) or chondroitinase AC-II (AC-II) prior to electrophoresis. Rainbow® markers are shown. The large proteoglycan core proteins, L₁, L₁₃, L₁₄ and the small core proteins, S₁, S₂ are indicated. Several other noncharacterized bands are shown in lanes 2, 4, 6 and 8 in (b) by +. Additional fast migrating bands present in D1 fractions electrophoresed without enzyme digestion are indicated in (b) by *. These bands appear to have a greater radiolabeling intensity in lanes 5-8 when compared to lanes 1-4.

The chondroitinase ABC/keratanase digestion of nonarthritic cartilage and osteoarthritic cartilage D1 fractions which was required to visualize migration of the newly synthesized proteoglycans into 3–16% gradient slab gels strongly indicated that proteoglycan core protein L₁ was aggrecan [Figs 1(a) & 1(b)]. In contrast, the results of protracted explant culture (36 days) suggested a modulation of the proteoglycan phenotype. This was especially true of the large proteoglycans transported into the medium compartment [Fig. 1(b), lane 12]. Thus, the susceptibility of the large proteoglycans to chondroitin ABC digestion in day 36 explant medium compartment and by chondrocytes derived from osteoarthritic cartilage was virtually identical [compare Fig. 1(b), lanes 11 and 12 with Fig. 1(c), lanes 5 and 6]. Either these large proteoglycans have a markedly reduced keratan sulfate content which was consistent with the fact that only chondroitinase ABC digestion was required to visualize the L₁ core protein, or the proteoglycan has a different sulfation pattern which could make it resistant to keratanase (A.R. Poole, personal communication). Alternatively, the medium compartment could be the repository of a versican-like proteoglycan while the explant itself retains aggrecan. A recent study indicated that osteoarthritic cartilage contained a large chondroitin sulfate proteoglycan which belonged to the versican family [21]. The versican core protein was estimated to be 550 kDa on polyacrylamide slab gels, which was similar in apparent size to the L₁ proteoglycan core protein found in the culture medium of human osteoarthritic cartilage explants after 36 days in culture.

The mechanism resulting in the synthesis of different forms of large proteoglycan core proteins by human chondrocytes from aged nonarthritic
articular cartilage in vitro remains to be elucidated. The gel patterns shown in Fig. 6(b) do not necessarily imply the existence of distinct and separate aggrecan core proteins. Certainly post-synthesis proteolytic cleavage of a single proteoglycan core protein could give rise to proteoglycan core proteins with characteristics similar to the migration pattern of D1 fractions from these chondrocytes as seen on 3–16% polyacrylamide gels after chondroitinase ABC/keratanase digestion. Alternatively, differences in amounts of N- or O-linked oligosaccharides or in glycosaminoglycan substitution among the three core proteins could explain the differences in migration. Previous studies have also stressed that alterations in proteoglycan biosynthesis by chondrocytes derived from osteoarthritic cartilage in vitro could imply dysfunction of proteoglycan biosynthesis in vivo [2, 22–25].

CA-PAGE showed marked similarities between the proteoglycan pattern of chondrocytes from nonarthritic cartilage and chondrocytes from osteoarthritic cartilage (Fig. 4). In the D1 fraction, two proteoglycan subpopulations were identified (i.e. PG\textsubscript{1} and PG\textsubscript{2}). The pattern demonstrated by these chondrocytes for newly synthesized proteoglycans was grossly similar to previously reported results of endogenous nonarthritic (aged 29 and 82 years) cartilage proteoglycans analyzed by CA-PAGE [26]. In that study, PG\textsubscript{1} reacted with antibody specific for the HABR and PG\textsubscript{2} with anti-decorin (small PG II) antibody [26].

We also found substantial synthesis of \[^{35}\text{SO}_4\] labeled small proteoglycans [Figure 1(a), lane 10] which resembled the migration profile of the leucine-rich proteoglycans, decorin and biglycan reported by others [27, 28]. Digestion of D1 fractions radiolabeled with \[^{35}\text{S}\]-methionine (Fig. 3) produced two core proteins that migrated somewhat slower than the 46 kDa marker protein. These bands were likely to be the glycosylated forms of biglycan and decorin, respectively. We had previously shown that these bands reacted with mAb 2B6 but not mAb 3B3 after chondroitinase ABC digestion, and did not react with mAb 2B6 after chondroitinase AC-II digestion [16], suggesting that these proteoglycans contained significant amounts of iduronate sequences, characteristic of dermatan sulfate proteoglycans. We have also shown by gel filtration chromatography that human osteoarthritic chondrocyte strains synthesized a greater amount of small proteoglycans than their nonarthritic cartilage counterparts and that this small proteoglycan was not derived from the large proteoglycans also synthesized by these cells [25]. A previous analysis of chondrocytes from osteoarthritic cartilage suggested that synthesis of a large proteoglycan containing predominantly dermatan sulfate and devoid of keratan sulfate indicated significant modulation from the proteoglycan phenotype of the parent tissue [22]. However, more recent analyses strongly support the view that dermatan sulfate proteoglycan synthesis is a normal product of articular cartilage [9, 27] and of chondrocytes in culture [28]. In the present studies, the proteoglycan core protein L\textsubscript{4} was strongly reactive with mAb 3B3 and only marginally reactive with mAb 2B6 after chondroitinase ABC/keratanase digestion indicating that L\textsubscript{4} probably did not contain any significant amounts of chondroitin-4-sulfate.

None of the analyses performed indicated the synthesis of a 59 kDa core protein known as fibromodulin [29] in the D1 fraction which also contained other small proteoglycans. The \[^{35}\text{SO}_4\] seen in the CAF or culture medium compartment migrating as a broad smear in nonenzyme digested D1 fractions was completely abolished by digestion with chondroitinase ABC indicating that this smear was in all likelihood proteoglycan fragments containing chondroitin sulfate. Keratanase digestion after initial digestion with chondroitinase ABC did not alter the migration profile of any \[^{35}\text{S}\]-methionine labeled D1 fraction bands migrating faster than the 69 kDa marker protein [Fig. 3(b), lane 2]. Characteristic mAb 5D4 immunoreactivity with fibromodulin [30] was also not detected in D1 fractions in this zone of the gel. We have, however, previously reported an mAb 2D3 (anti-keratan sulfate mAb with reactivity similar to mAb 5D4) reactive product in nonenzyme digested D1 fractions of rabbit chondrocytes [16] so that if a keratan sulfate proteoglycan was present in these D1 fractions, our method would have detected it.

Recent studies have refocused attention on phenotypic differences between human aged nonarthritic (often termed as 'normal cartilage') and osteoarthritic cartilage [2]. Specific elimination of osteophytic cartilage in these analyses, however, was an important requirement since the osteophytic cartilage 'cap' represented tissue arising during the course of the osteoarthritic process and possessed several characteristics of hyaline cartilage [31, 32]. The cartilage obtained for the present analyses was typical of the samples we have used previously [33]. Although there was significant destruction of the articular surface of the femoral head at the time of joint replacement surgery, there were also areas ('resident cartilage') retained on the femoral surface which were mildly discoloured or, at best, minimally fibrillated. This cartilage was generally obtained from the posterior or inferior aspect of the femoral head and was likely spared complete destruction.
probably because it resided in a reduced-weight bearing site. Previous histochemical analysis, however, showed that this cartilage was characterized by distinct signs of extracellular matrix degradation [33]. Some studies have attempted to compare 'histologically mild' osteoarthritic knee cartilage with 'normal' cartilage of the same joint [34]. These analyses revealed little difference between the proteoglycan synthesis capacity of the different topographical sites. We had previously shown that discoloured and fibrillated osteoarthritic femoral head cartilage synthesized a similar and overlapping profile of newly synthesized proteoglycans [35, 36]. Differences in cartilage thickness between nonarthritic and osteoarthritic cartilages may also confound the analysis of newly synthesized proteoglycans in explant culture [37], but intrinsic differences in proteoglycan synthesizing capacity of osteoarthritic and normal cartilage in vitro [38] would also contribute to the results obtained in the present study. Maintenance of human chondrocytes in high-density monolayer culture or in agarose [39] apparently regulated and maintained a stable chondrocyte phenotype. Thus, human chondrocytes from osteoarthritic cartilage maintained at high density, as did human osteoarthritic cartilage, actively transcribed the COL2A1 [40], COL9A1 and COL11A1 genes (unpublished observations).

Although the present study did not attempt to quantify the S1 and S2 core protein synthesis by human chondrocytes (Fig. 6), visual inspection of the autoradiographs suggested increased small proteoglycan synthesis by chondrocytes from osteoarthritic cartilage. Thus, the increased content of these small proteoglycans in osteoarthritic cartilage [9] and the association of dermatan-sulfate proteoglycans with anti-adhesive properties of extracellular matrix [41, 42] may also contribute to abortive attempts at repair of osteoarthritic cartilage lesions.

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