Expression of reduced amounts of structurally altered aggrecan in articular cartilage chondrocytes exposed to high hydrostatic pressure

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The effect of hydrostatic pressure on proteoglycan (PG) metabolism of chondrocyte cultures was examined using a specially designed test chamber. Primary cultures of bovine articular chondrocytes at confluence were exposed for 20 h to 5 and 30 MPa continuous hydrostatic pressures and 5 MPa hydrostatic pulses (0.017, 0.25 and 0.5 Hz) in the presence of [^{35}S]sulphate. Northern blot analyses showed that chondrocyte cultures used in this study expressed abundant mRNA transcripts of aggrecan, typical of chondrocytes, but not versican. The cultures also expressed biglycan and decorin. Enzymic digestions with keratanase and chondroitinases AC, ABC and B and subsequent SDS/agarose gel electrophoresis confirmed the synthesis of aggrecans and small dermatan sulphate PGs. The continuous 30 MPa pressure reduced total PG synthesis by 37% as measured by [^{35}S]sulphate incorporation, in contrast to the 5 MPa continuous pressure which had no effect. The high static pressure also reduced total [^{3}H]glucosamine incorporation by 63% and total [^{14}C]leucine incorporation by 57%. The cyclic pressures showed a frequency-dependent stimulation (0.5 Hz, 11%) or inhibition (0.017 Hz, −17%) of [^{35}S]sulphate incorporation. Aggrecans secreted under continuous 30 MPa pressure showed a retarded migration in 0.75% SDS/agarose gel electrophoresis and they also eluted earlier on Sephacryl S-1000 gel filtration, indicative of a larger molecular size. The increased size was consistent with an increase of average glycosaminoglycan chain length as determined by Sephacryl S-300 gel filtration. No change in aggrecan size was observed with the lower (5 MPa) static or cyclic pressures. Continuous 30 MPa hydrostatic pressure slightly reduced the steady-state mRNA level of aggrecan, in parallel with the decline in PG synthesis measured by [^{35}S]sulphate incorporation. The results demonstrated that high hydrostatic pressure could influence the synthesis of PGs, especially of aggrecans, in chondrocytes both at the transcriptional and translational/post-translational levels.

INTRODUCTION

Chondrocytes of articular cartilage synthesize a number of different extracellular matrix components including proteoglycans (PGs) to produce a resilient tissue which resists compression during the loading of the joint. In cartilage, PGs are present mainly as large aggregates containing several aggrecan molecules attached to a single hyaluronan chain [1–3], the binding being stabilized by a link protein [4,5]. The aggrecan molecule has a core protein of 220 kDa as deduced from the human cDNA sequence [6], and contains a large number of covalently attached chondroitin sulphate (CS) and keratan sulphate (KS) glycosaminoglycan (GAG) chains, and *O*-linked and *N*-linked oligosaccharides [3,7]. In addition to aggrecan, chondrocytes are known to synthesize two species of small dermatan sulphate (DS)-containing PGs, named decorin and biglycan [8], and fibromodulin, a related PG containing KS [9,10].

Cartilage chondrocytes and their extracellular matrix are subjected to repeated alterations in their physical environment. Considerable interest has been focused on the different physical factors involved in the regulation of cartilage matrix production, as indicated by an increasing repertoire of loading systems *in vitro* [11–27]. Most of the models mimic conditions *in vivo* by compressing cartilage explants in tissue culture. With respect to PG synthesis by cartilage explants, continuous mechanical compression has been shown to decrease [^{35}S]sulphate incorporation into PGs [11,13,16–18,20]. However, the response to cyclic mechanical loading seems to depend on the amplitude and the frequency of the force. A stimulation is frequently found when relatively rapid cycles are applied [13,17,18,20,22,23].

Besides causing deformation and fluid flow, mechanical compression of cartilage raises the hydrostatic pressure in the tissue. Relatively little is known about the effects of hydrostatic pressure on cartilage and chondrocytes [14,15,25,26], especially at the physiological range of pressures [19,24,27,28]. The effect of the hydrostatic component of loading can be studied independently, since cell and tissue cultures can be readily compressed through the growth medium. Virtually no deformation then occurs since water is almost incompressible. Models utilizing hydrostatic pressure [15,19,24,28] possess the advantage of examining a single physical factor, unlike models based on mechanical tissue compression. An independent and potentially important role for the hydrostatic pressure in PG synthesis has been suggested in our earlier experiments on full-depth bovine articular cartilage explants [23]. Using short (50 ms) mechanical loadings which produce only 1–2% reduction in tissue thickness and very little fluid flow, but considerable hydrostatic pressure pulses, we observed a significant stimulation of [^{35}S]sulphate incorporation [23]. Indeed, it was subsequently demonstrated that certain frequencies of pure cyclic hydrostatic pressure on their own could increase [^{35}S]sulphate incorporation in chondrocytes [24].

In this study we report that a 20 h continuous loading *in vitro* with high (30 MPa) hydrostatic pressure inhibited GAG synthesis by 37% and 63% when measured by [^{35}S]sulphate and [^{3}H]glucosamine incorporations, respectively. Protein synthesis was reduced by 57% when determined by total [^{14}C]leucine

Abbreviations used: PG, proteoglycan; CS, chondroitin sulphate; KS, keratan sulphate; GAG, glycosaminoglycan; DS, dermatan sulphate; HA, hyaluronan; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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incorporation while only a slight decrease in aggrecan steady-state mRNA level was noticed. Furthermore, the size of aggrecan increased, while such effects were not found with lower continuous or cyclic pressures.

EXPERIMENTAL

Cell culture

Primary bovine articular chondrocyte cultures were obtained from 1 to 2 year old animals. Articular cartilage was prepared aseptically from the patellar surface of femurs, and chondrocytes were released from matrix by trypsin and collagenase and cultured to confluency as described earlier [24]. Morphologically, the cells had the polygonal appearance typical of chondrocytes in monolayers. Human foetal smooth muscle cells that were used as a control cell line in aggrecan/versican expression experiments were obtained from the intima-media of the aorta by digestion with trypsin and collagenase [29].

Loading *in vitro* with hydrostatic pressure

Technical data of the loading apparatus and the protocol used for pressurization experiments have been published previously [24]. Briefly, confluent cell culture dishes were filled with medium [Hepes-buffered Basal Medium Eagle (Flow Laboratories)] and sealed by a special membrane after excluding all air from the culture dish. Carrier-free [^{35}S]sulphate (Amersham Int.) was injected by a sterile needle through the membrane coating of the culture dishes to a final radioactivity of 20 μCi/ml, the needle hole was patched up with tape and the labelled dishes were pressurized for 20 h. Effects of the high hydrostatic pressure (30 MPa) on glycosylation and protein synthesis were also studied by total [^{3}H]glucosamine (50 μCi/dish, Amersham Int.) and total [^{14}C]leucine (50 μCi/dish in leucine-depleted medium, Amersham Int.) incorporations (20 h), respectively. Free radioactivity was removed by PD-10 desalting columns (Pharmacia), and the macromolecular fraction was measured by liquid scintillation.

The loading apparatus consisted of a water-filled, cylindrical loading chamber and a reference chamber of the same size, both situated in a room maintained at 37 °C. Continuous (5 and 30 MPa) or cyclic hydrostatic pressures (5 MPa) at frequencies 0.017, 0.25 and 0.5 Hz were applied to the dishes in the pressure chamber by a hydraulic control system. Sealed control dishes were immersed in a reference chamber for the same time period.

[^{35}S]Sulphate incorporation into proteoglycans

Autoradiographic Safranin O assay [30,31] was used to quantitate the radioactivity of PGs and GAGs in chromatographic fractions. The same method was modified to quantify the incorporation of [^{35}S]sulphate into secreted PGs. After loading experiments, culture medium was diluted 1:10 with 0.1% CHAPS (Sigma) in 0.15 M NaCl, and the diluted aliquots were pipetted into the wells of a 96-sample filtration device prefilled with 400 μl of 0.02% Safranin O (Fisher Scientific) in 50 mM sodium acetate buffer, pH 4.75. Image analysis of an autoradiography film exposed to the membrane sheet for 2 days was used as described previously [31] to quantify the incorporation rates of the samples. Alternatively, the membrane sheet was placed on a MeltiLex melt-on liquid scintillator (Wallac), heated at 90 °C for 5 min and the scintillator was allowed to solidify at room temperature. Radioactivity dissolved from the precipitation dots onto MeltiLex was measured by a MicroBeta liquid scintillation counter (Wallac). The yield of radioactivity obtained in the MeltiLex was directly related to the known radioactivities [32].

The amount of cell-associated, incorporated [^{35}S]sulphate was measured after 30 MPa loading from the control and pressurized cultures by overnight incubation of the cell layer in 0.1% Triton X-100 in 4 M guanidine/HCl containing 5 mM disodium EDTA, 100 mM 6-aminohexanoic acid, 0.03% NaN_3 and 10 mM benzamidine, pH 5.8, after washing of the cell cultures with PBS. The incorporated [^{35}S]sulphate in the medium and cell-associated fractions was measured by liquid scintillation after separation of macromolecules from the free precursor by gel filtration on PD-10 columns (Pharmacia).

Isolation of proteoglycans

After loading, the culture media were collected and immediately centrifuged (500 *g* for 10 min) to exclude the cells which had detached from the culture dish. A constant amount of the medium was transferred into tubes containing pre-weighed solid guanidine/HCl (Sigma) to give a final concentration of 4 M. The samples were eluted on PD-10 columns equilibrated with solution A (10 M formamide in 0.2 M NaCl/0.1% CHAPS/50 mM Na_2SO_4/5 mM disodium EDTA/100 mM 6-aminohexanoic acid/0.03% NaN_3/10 mM benzamidine, pH 6.5) to exchange guanidine/HCl into formamide and to separate most of the unincorporated precursor from [^{35}S]sulphate-labelled macromolecules. The macromolecular fraction was injected into a ProPak PA1 (5 mm × 40 mm, Dionex) pellicular anion-exchange h.p.l.c. precolumn at 1 ml/min by a Superloop (Pharmacia), and washed with solution A for 15 min and eluted with solution B (3 M NaCl in solution A). More than 90% of the total incorporated radioactivity was recovered in fractions 4–13 (each 1 ml) eluted by solution B.

Enzymic digestions

Proteinase K digestions [0.1% (w/v) Boehringer Mannheim] were performed in 0.1 M Tris/HCl buffer, pH 8.0, at 60 °C overnight. The enzyme digestion was terminated in a boiling-water bath for 5 min.

A variety of GAG-degrading enzymes (Seikagaku) were applied to PG samples. Overnight digestions with chondroitinase AC (40 munits/50 μl), ABC (40 munits/50 μl), and keratanase (5 munits/50 μl) were carried out in 0.1 M Tris/HCl buffer, pH 8.0, at 37 °C, while for chondroitinase B (2 munits/50 μl in the same buffer) the optimum temperature was 30 °C (manufacturer's instructions).

SDS/agarose gel electrophoresis of secreted proteoglycans

Six aliquots containing 20000 d.p.m. of incorporated [^{35}S]-sulphate from each culture medium were precipitated overnight at −20 °C with 4 vol. of 0.5% (w/v) sodium acetate in 94% (v/v) ethanol and washed three times with 0.5% (w/v) sodium acetate in 80% (v/v) ethanol. One sample was undigested, while others were digested with keratanase, chondroitinase AC, ABC or B and electrophoresed on 0.75% agarose gel as previously described [33]. Proteinase K-digested PGs were used as a reference sample for free GAG chains. Nasal cartilage PGs (2 μg) were included as a carrier. Autoradiograpahy combined with image analysis [34] was used to analyse the [^{35}S]sulphate-labelled PGs on the gels.

The presence of aggregating proteoglycans

The relative proportion of native aggregates in the newly synthesized PGs was studied using Sephacryl S-1000 gel permeation chromatography of plain medium samples. Aliquots

from each medium sample were diluted with elution buffer [0.1% CHAPS/0.15 M NaCl/50 mM Na_2SO_4/5 mM disodium EDTA/10 mM benzamidine/0.03% (w/v) NaN_3] to contain 50000 d.p.m. of incorporated [^{35}S]sulphate/ml and aliquots of 500 μl were injected onto a column (1 cm × 30 cm; Pharmacia) and eluted at flow rate of 20 ml/h.

The ability of PGs to aggregate was studied after incubation of medium samples with high molecular mass hyaluronan (HA; Healon, Pharmacia). Nasal cartilage PGs (100 μg) were pipetted into Eppendorf tubes and medium samples containing 50000 d.p.m. of incorporated [^{35}S]sulphate/ml were prepared. Aggregation was accomplished overnight at 4 °C after the addition of 4 μg of exogenous HA. The samples were then frozen and chromatographed immediately after thawing.

Comparison of glycosaminoglycan chain lengths

Portions of the ProPac PA1 anion-exchange-purified PGs were desalted, precipitated overnight at −20 °C with 4 vol. of 0.5% (w/v) sodium acetate in 94% (v/v) ethanol and washed twice with 0.5% (w/v) sodium acetate in 80% (v/v) ethanol. To enhance the susceptibility of the GAGs towards the degrading enzymes, the protein core of the PGs was first cleaved by proteinase K. Chondroitinase ABC and keratanase (10 m-units/250 μl) digestions were carried out separately after heat-denaturation of proteinase K. The carbohydrate chains were finally released from the remaining peptides by alkaline borohydride treatment [35]. Excess borohydride was destroyed with small aliquots of 5 M acetic acid on ice to approx. pH 5. The samples were diluted to a final volume of 1.5 ml and 500 μl aliquots were eluted on Sephacryl S-300 (Pharmacia) with 0.1% CHAPS (w/v)/0.15 M NaCl/50 mM Na_2SO_4/0.03% NaN_3 (w/v)/0.1 M sodium acetate, pH 6.5.

Sulphated disaccharides of CS

Carrier CS (10 μg, whale cartilage; Sigma) was added to the samples pooled from each treatment and GAGs were precipitated by 3 vol. of 0.5% sodium acetate/94% ethanol. After removal of ethanol by evaporation, the precipitates were digested with chondroitinase AC and separated by t.l.c. [36]. The 4- and 6-[^{35}S]sulphated CS isomers were detected and quantified by autoradiography and image analysis.

Extraction of RNAs

Total RNA was isolated from cell cultures using a single-step method [37]. The ratio of optical density at 260 versus 280 nm of all RNA samples was above 1.7. The average yield was 20 μg of total RNA/culture dish.

Northern blot analysis

Ribonucleic acids (5 μg/sample) were electrophoresed overnight on 1% agarose gels containing 16.7% (v/v) of 37% (w/v) formaldehyde [38] and transferred from agarose gels onto Genescreen Plus membranes (New England Nuclear). The membranes were hybridized as previously described [39], with minor modifications, using ^{32}P-labelled cDNA probes. Random priming (Multiprime DNA Labelling System, Amersham) was used for labelling the cDNAs with 5′[α-^{32}P]dCTP (Amersham) to a high specific radioactivity. For quantification of the hybridization signals, the membranes were probed with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [40].

cDNA probes

The following cDNA probes were used in the hybridizations: a decorin cDNA [41] for the full-length human decorin core protein; a biglycan cDNA [42] for the full-length human biglycan core protein; a human aggrecan cDNA, nucleotides 1107–1553 [6]; and a versican cDNA [43]. The probes were generous gifts from Drs. Krusius (decorin and versican probes), Fisher (biglycan probe), and Glumoff and Vuorio (aggrecan probe).

RESULTS

Characterization of [^{35}S]sulphate-labelled PGs

The size classes of PGs secreted into the medium by chondrocytes were characterized by SDS/agarose gel electrophoresis. Two main bands of [^{35}S]sulphate-labelled macromolecules (bands I and II, Figure 1a) were present in all samples. Macromolecules in the slower migrating band I had an M_r of about (2.5–3.0) × 10^6 [7] and represented large, aggregating PGs. The band II macromolecules have M_r of about (1.0–3.0) × 10^5 and represented small interstitial PGs. The band I PGs synthesized during the static 30 MPa loading showed a retarded migration in comparison with the corresponding PGs from control cultures (Figure 1a, lane 8). By densitometry, band I PGs constituted on average 85% of the [^{35}S]sulphate-labelled material in control cultures (Figure 1). None of the different types of pressures causes a clear change in this percentage (less than 2% difference).

Specific enzyme digestions were performed to further characterize the identity of the [^{35}S]sulphate-labelled PG bands. In

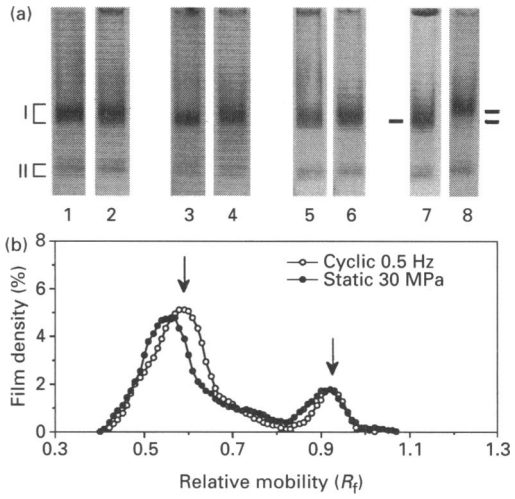

Figure 1 SDS/agarose gel electrophoresis of [^{35}S]sulphate-labelled PGs synthesized by chondrocyte cultures under high hydrostatic pressure

(**a**) Chondrocyte cultures were subjected to hydrostatic pressure for 20 h and simultaneously labelled with [^{35}S]sulphate. PGs secreted into medium were ethanol-precipitated and electrophoresed on 0.75% agarose gels as described previously [33]. Band I represents the large aggregating PGs, aggrecans, and band II the small interstitial PGs. Lanes 1, 3, 5 and 7 represent PGs from control cultures. Migration of PGs after cyclic pressurizations at 5 MPa with a frequency of 0.5 Hz (lane 2), 0.25 Hz (lane 4) and 0.017 Hz (lane 6), and after 30 MPa continuous pressurization (lane 8) are shown. The horizontal bars show the relative mobilities of aggrecans from control and statically loaded cultures (30 MPa). (**b**) Densitometric analysis of the PGs from cultures subjected to 5 MPa cyclic (○) and 30 MPa continuous (●) loading. The arrows show the maximum peaks of aggrecans and small interstitial PGs from control cultures.

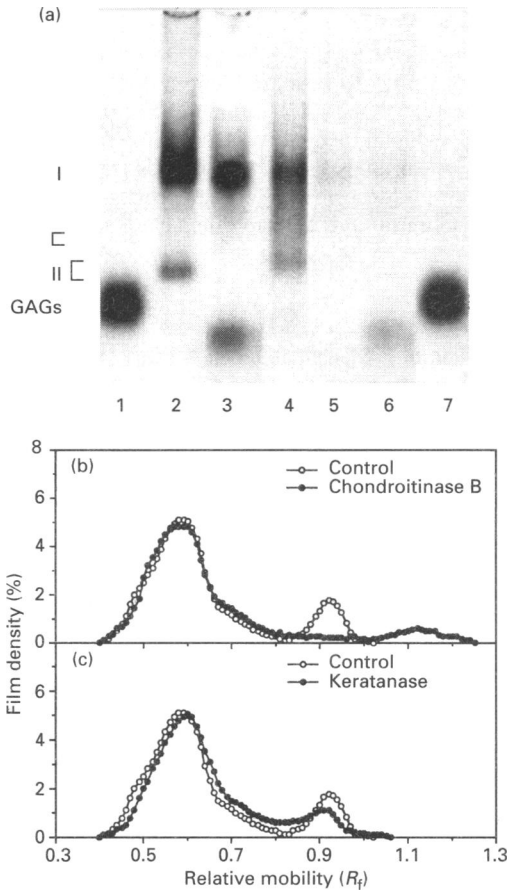

Figure 2 Effects of GAG-degrading enzymes on PGs isolated from statically pressurized and control chondrocyte cultures

Results from one control culture, representative of the PGs from control and pressurized cultures, are shown. (**a**) The following enzyme digestions were performed on ethanol-precipitated PGs: chondroitinase B (lane 3); keratanase (lane 4); chondroitinase AC (lane 5); and chondroitinase ABC (lane 6). Lane 2 was undigested and lanes 1 and 7 were digested with proteinase K to show the migration position of free GAG chains. The effects of (**b**) chondroitinase B and (**c**) keratanase on migration of PGs is shown in densitometric scans of the corresponding lanes.

Figure 2, data from a representative experiment are shown. Digestion of the samples with proteinase K was used to indicate the migration position of free GAG chains (Figure 2a, lanes 1 and 7). Proteoglycans in band II could almost completely be digested with chondroitinase B (a specific lyase enzyme for iduronic acid in CS/DS chains), indicating that band II PGs represent DS-PGs (decorin and biglycan). A part of the degradation products, moving faster than the band of proteinase K-released GAG chains, remained relatively large which showed that the DS chains contained blocks of iduronate-free sequences (Figure 2a, lane 3, Figure 2b). Chondroitinase B had no proteinase activity on gel electrophoresis molecular mass markers, digested with a 10-fold enzyme concentration of that used for PGs. Keratanase treatment slightly enhanced the mobility of band I, and produced a heterogeneous population of faster-migrating larger PGs (Figure 2a, lane 4). The possibility that fibromodulin accounted for a small reduction in band II after keratanase digestion was not further substantiated. Chondroitinase AC and ABC almost completely removed [^{35}S]sulphate-labelled PGs (Figure 1a, lanes 5 and 6, respectively).

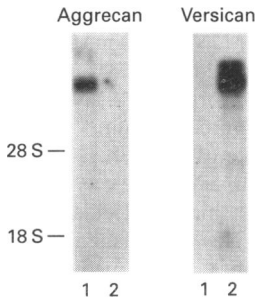

Figure 3 Expression of aggrecan but not versican mRNA transcripts by primary cultures of chondrocytes

Aliquots (5 μg) of total cellular RNA from primary chondrocytes (lane 1) and subcultured smooth muscle cells (lane 2) were analysed by Northern hybridizations. RNAs were electrophoresed overnight in 1% agarose gels containing 16.7% (v/v) of 37% (w/w) formaldehyde and transferred onto Genescreen Plus membranes. The membranes were sequentially hybridized using ^{32}P-labelled cDNA probes for aggrecan and versican.

A weak signal in a high M_r position of the gel after chondroitinase AC or ABC digestion was noticed on autoradiography, representing PG core proteins or other sulphated proteins (not determined). The nature of the fast migrating band in the GAG oligosaccharide region (Figure 2a, lane 6), that reproducibly appeared after chondroitinase ABC but not after chondroitinase AC treatment, remained obscure.

To distinguish between the synthesis of aggrecan and versican (both large, aggregating and CS-containing PGs), the presence of mRNA transcripts for these two PGs in chondrocytes was examined by Northern analysis. The results showed that the chondrocyte cultures used in this study expressed detectable amounts of only aggrecan, and not versican, whereas the opposite was true for the cultures of human foetal aortic smooth muscle cells (Figure 3). Versican mRNA could not be detected in the pressurized cultures either. Chondrocytes of this study also expressed abundant mRNA transcripts of decorin and biglycan (see below). Fibromodulin expression of chondrocyte cultures was not examined.

In conclusion, the chondrocytes used in this study produced PGs typical for cartilage extracellular matrix. Based on the results from Northern analyses and the susceptibility of the [^{35}S]sulphate-labelled PGs to keratanase and chondroitinases AC and ABC, the band I PGs contained predominantly aggrecans. The band II PGs consisted mainly of DS-PGs as shown by an almost complete enzymic degradation of the band with chondroitinase B.

Effect of continuous hydrostatic pressure on aggrecans

High continuous hydrostatic pressure caused a shift in the migration of band I PGs (aggrecans) in SDS/agarose gel electrophoresis (Figure 1, lane 8). Whether a lower continuous pressure could induce the same effect was tested on cultures exposed to 5 and 30 MPa continuous hydrostatic pressure. To avoid possible interference from serum proteins the PGs were partially purified by anion-exchange chromatography and an equal amount of carrier nasal PGs was included in the specimen before electrophoresis. The lower pressure did not affect the mobility of the two bands, whereas loading with the higher static 30 MPa pressure decreased the mobility of the aggrecan molecules (results not shown).

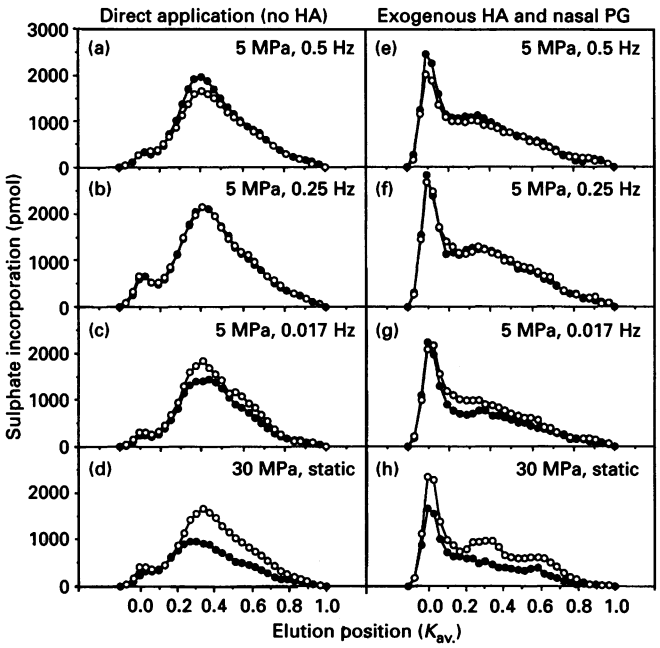

Figure 4 Aggregation of PGs from cyclically or statically pressurized chondrocyte cultures

Medium samples from control (○) and hydrostatically pressurized (●) culture dishes were chromatographed on Sephacryl S-1000 gel either directly (**a–d**) or after incubation overnight at 4 °C with exogenous HA (4 μg) and nasal PGs (100 μg) (**e–h**). Fractions were analysed for ^{35}S-labelled macromolecules by using the Safranin O precipitation method [31].

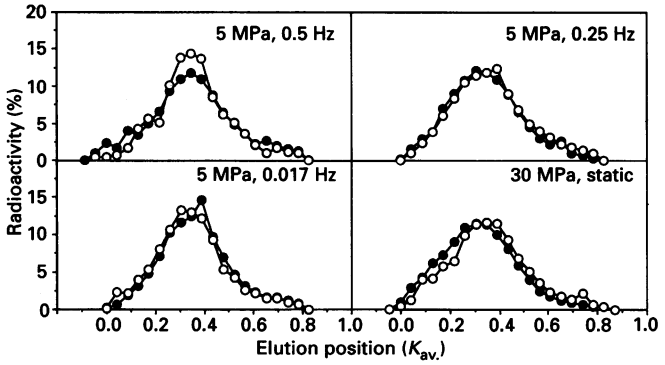

Figure 5 Relative chain length of the GAGs after cyclic or continuous hydrostatic pressure

Ion-exchange-purified PGs from control (○) and hydrostatically loaded (●) chondrocyte cultures were ethanol-precipitated, cleaved into peptides by proteinase K and treated with alkaline borohydride to release GAGs from the peptides. After neutralization, the GAGs were chromatographed on Sephacryl S-300 and fractions were analysed by using the Safranin O precipitation method [31].

Aggregation properties of the secreted proteoglycans

To estimate the amount of PGs in aggregates shortly after secretion, aliquots of the medium samples were directly chromatographed on Sephacryl S-1000. The amount of PGs that eluted in the void volume and represented aggregates was small in all samples (6–15% of the total) and no changes in the elution profiles were noticed after cyclic loadings (Figures 4a–4c). Unsealed control cultures also had a small percentage of aggregates, indicating that the sealing was not the reason for the relatively low proportion of the spontaneously formed aggregates. After static pressurization of 30 MPa, a pool of larger PG molecules was apparent in the non-aggregated aggrecans. The maximum radioactivity of the monomeric peak of aggrecan shifted from $K_{av.}$ 0.33 to 0.29 (Figure 4d).

Incubation with exogenous HA proved that 45–50% of the aggrecans synthesized during pressurization were aggregated with HA. There were no marked differences in the relative aggregation between the pressurized and control samples (Figures 4e–4h). The effect of exogenous HA suggested that the low degree of aggregation in the medium may have been due to a deficiency of HA or that the aggregation of PGs in these culture conditions was slow, requiring long-term incubations to be completed.

Structure of glycosaminoglycan chains

Whether the increased aggrecan size was due to larger GAG chains was studied by gel filtration of the GAG chains cleaved from the core protein by alkaline borohydride. The chain lengths in PGs isolated from the pressurized cultures were similar to those from the corresponding control cultures in cyclic loadings (Figures 5a–5c). Static 30 MPa loading resulted in a slightly larger average GAG chain length (Figure 5d). Keratanase digestions did not remarkably affect the elution profiles or positions shown. Chondroitinase ABC-resistant GAGs, representing KS, eluted a little later than those resistant to keratanase, but no changes appeared in their profiles after pressurization.

The ratio of monosulphated disaccharides produced after chondroitinase AC digestion was determined by t.l.c. Chondroitin 6-[^{35}S]sulphate represented 73–77% of the monosulphated isomers and its proportion remained unchanged in all experimental pressurizations.

Total proteoglycan synthesis in pressurized chondrocyte cultures

Total PG synthesis by chondrocyte cultures during cyclic (5 MPa at frequencies 0.017, 0.25 and 0.05 Hz) and continuous (5 and 30 MPa) hydrostatic pressurization was determined by [^{35}S]sulphate incorporation, and subsequent autoradiographic Safranin O assay. Cyclic modes of pressurization resulted in essentially similar findings to those previously described [24]. Fast cyclic pressure (0.5 Hz, 5 MPa) slightly increased (11%) total [^{35}S]sulphate incorporation into PGs, while pressurization at a low frequency (0.017 Hz, 5 MPa) decreased it by 17% (Table 1). In comparison with cyclic pressurizations, continuous loading with low static pressure (5 MPa) had no effect on [^{35}S]sulphate incorporation into PGs, whereas that with high static pressure (30 MPa) decreased it by 37% (Table 1). The amount of cell-associated (peri- and intra-cellular) PGs was only 20% of the total incorporated label in the control cultures. Therefore, changes in the retention of PGs in the pericellular pool hardly explains the alterations observed following cyclic pressures. During 30 MPa pressurization the cell layer-associated pool of [^{35}S]PGs was determined, and found to be 25%, indicating that an increase in this pool did not account for the reduction in secreted PGs. The marked decrease in [^{35}S]sulphate incorporation into PGs in response to high static pressure was further studied by [^{3}H]glucosamine and [^{14}C]leucine incorporation experiments. In accordance with the results obtained with [^{35}S]sulphate incorporation, continuous high pressure (30 MPa) decreased total [^{3}H]glucosamine and [^{14}C]leucine incorporations to about half of the control values (63% and 57%

Table 1 Total [^{35}S]sulphate incorporation into secreted proteoglycans during hydrostatic *in vitro* loading

Chondrocyte monolayers were exposed to hydrostatic pressure for 20 h and metabolically labelled with [^{35}S]sulphate during loading as described in the Experimental section. Incorporated label was separated from free precursor by gel filtration and radioactivity was quantified by liquid scintillation counting. Incorporation rates are expressed as pmol/h per dish mean ± S.D. ($n = 4$, except for 5 MPa continuous loading $n = 8$).

Loading regime	Control	Pressurized	Pressurized/ control
Cyclic loading (5 MPa)			
0.5 Hz	1076 ± 182	1196 ± 124	1.11
0.25 Hz	1411 ± 63	1385 ± 44	0.98
0.017 Hz	1112 ± 117	919 ± 254	0.82
Continuous loading			
5 MPa	1751 ± 91	1807 ± 121	1.03
30 MPa	1069 ± 144	677 ± 94	0.63

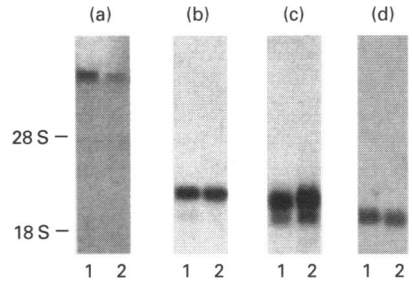

Figure 6 Expression of aggrecan, biglycan and decorin mRNA transcripts after continuous high hydrostatic pressurization

Northern transfer analysis was performed as described in Figure 3. The membranes were sequentially hybridized using ^{32}P-labelled cDNA probes for (**a**) aggrecan, (**b**) biglycan, (**c**) decorin and (**d**) GAPDH. Lane 1 presents Northern hybridizations from control cultures and lane 2 from loaded cultures.

decreases, respectively). This suggests a simultaneous reduction in glycosylation in general and an inhibition of protein synthesis.

Quantification of proteoglycan mRNAs by Northern blot analysis

Aliquots of total RNA isolated from pressurized and control chondrocyte cultures were electrophoresed on agarose gels and transferred onto nitrocellulose membranes, that were sequentially hybridized with specific cDNA probes for aggrecan, decorin and biglycan, and normalized to GAPDH mRNA. The hybridization results (Table 2) showed that continuous 30 MPa hydrostatic pressure slightly (although not significantly) reduced the steady-state mRNA level of aggrecan (Figure 6a, and Table 2). However, decorin and biglycan mRNA levels were increased in response to continuous 30 MPa hydrostatic pressure (Table 2), suggesting that the regulation of PG mRNA transcript levels was specific and not a part of a general influence of high hydrostatic pressure. Another explanation for the observed changes could be the presence of more than one population of cells that show a different response to the loading. The changes in decorin and biglycan mRNA transcripts were not reflected in the relative proportions of the large and small ^{35}S-sulphated PGs. In the cyclic pressurization experiments only minor changes were noticed, and no distinguishable pattern was obvious in any of the mRNAs analysed.

Table 2 Levels of proteoglycan mRNAs relative to GAPDH after hydrostatic loading of bovine articular chondrocytes

Chondrocyte monolayers were exposed to hydrostatic pressure for 20 h and mRNA was isolated and processed for Northern analysis after the loading as described in the Experimental section. The membrane was sequentially hybridized with cDNAs for aggrecan, biglycan, decorin and GAPDH. After densitometry of the autoradiographic films, the ratios of PG mRNA levels relative to GAPDH in the pressurized cultures were compared with those in control cultures. Data from two separate experiments (three experiments for continuous loading) are shown.

	PG mRNA levels relative to GAPDH (pressurized/control)		
Loading regime	Aggrecan	Biglycan	Decorin
Cyclic loading (5 MPa)			
0.5 Hz	0.93	1.01	1.23
0.25 Hz	1.55	1.05	1.46
0.017 Hz	1.07	1.00	1.18
Continuous loading (30 MPa)	0.75	2.66*	5.64*

* Changes are regarded as significant.

DISCUSSION

Numerous studies have demonstrated the complexity of the factors involved in the regulation of matrix production in articular cartilage during joint loading. A variety of different factors associated with loading are known to affect the synthesis of macromolecules in chondrocytes. These include, for example, osmotic pressure, extracellular pH and streaming potentials [16,17,44]. In addition, tensile forces stimulate sulphate incorporation in stretched chondrocyte cultures [12,45,46], and apparently also in the superficial zone of cartilage explants exposed to compressive mechanical loading [23]. Recently, relatively short hydrostatic impulses were reported to increase sulphate and proline incorporation in cartilage explants, while static pressure at high amplitudes decreased them [19]. In long-term cultures of anatomically intact articular cartilage, sulphate incorporation into small PGs was reduced after 1 week unless cyclic mechanical loading was applied to the cultures [22]. After 1 week's unloaded period, the reduced synthesis of the small PGs of cartilage cultures could be induced back to the original level by cyclic loading [22]. In the compressed part of the bovine deep flexor tendon fibrocartilage, 2 weeks of cyclic, unconfined compression for 20 min/day maintained a high level synthesis of large, cartilage-like PGs [21]. These reports have suggested that compression is an important factor in the regulation of tissue phenotype and PG synthesis.

De-differentiation of chondrocytes into fibroblastic phenotype has been known to occur with longer culture periods. Therefore, we first wanted to verify that the primary cell cultures used in these experiments expressed the chondrocyte phenotype and synthesized the PGs characteristic for articular cartilage chondrocytes. In SDS/agarose gel electrophoresis, specific enzymes degrading GAGs were utilized to show the presence of aggrecans and small PGs, representing decorin and biglycan. The mRNA transcripts of aggrecan, biglycan and decorin were abundantly expressed by the primary chondrocytes. In contrast, no detectable expression of versican mRNA could be demonstrated in control or pressurized cultures, further confirming the chondrocytic phenotype of our cultures.

In SDS/agarose gel electrophoresis, the relative proportions of aggrecans and DS-PGs in pressurized cultures were similar to those isolated from the control ones. No marked differences were observed in the aggregation properties, GAG chain lengths and ratios of chondroitin 6-[^{35}S]sulphate to chondroitin 4-[^{35}S]sulphate of PGs after cyclic loading. However, specific alterations were induced in aggrecans by the high continuous hydrostatic pressure. The high static pressure decreased total [^{35}S]sulphate incorporation and retarded the migration of aggrecans in SDS/agarose gel electrophoresis. This finding suggested an enlargement of aggrecans and was supported by the Sephacryl S-1000 gel filtration profile of the PGs. The increase in molecular size could, at least partially, be explained by longer GAG chains, as CS chains released by β-elimination from PGs collected after 30 MPa pressurization eluted earlier on Sephacryl-S-300. In rat chondrosarcoma, only 55 % of the serine residues in a completed aggrecan core protein are substituted with carbohydrate [47], so an increased substitution of the potential carbohydrate chain initiation sites on the core protein could increase the number of GAG chains on a single aggrecan molecule. Undersulphation of the GAG chains might also reduce the mobility of PGs in electrophoresis. However, undersulphation is not in accordance with the observed change in Sephacryl S-1000 gel permeation chromatography. An increase in the length of the core protein by alternative splicing is unlikely, as aggrecan mRNAs from control and loaded chondrocyte cultures had the same mobilities in agarose gels.

Pulse-chase experiments on bovine articular chondrocytes have shown that the average time from PG core protein synthesis to secretion is 32 min [48]. There are a number of examples of how chemically caused blocks in the biosynthetic machinery of chondrocytes change the structure of the secreted PGs. Disruption of microtubules by colchicine reduced the average size of PGs [49], while monensin inhibited sulphate incorporation and accumulated PGs in the medial/trans Golgi, leading to undersulphated GAG chains [50,51]. A possible retardation in the intracellular protein trafficking and secretion may, through a currently unknown mechanism, lead to the observed changes in PG structure during high hydrostatic pressure. Restrained core protein synthesis in cycloheximide-treated chondrosarcoma cell cultures decreased the [^{35}S]sulphate incorporation, while simultaneously the PG size and CS chain length were increased [52]. A limited core protein supply could also produce the reduced aggrecan synthesis and increased GAG chain size in the present study.

There is evidence that microtubules facilitate the secretion of macromolecules by directing them into the right compartments of the cell [53]. High hydrostatic pressure has multiple effects on the cytoskeletal elements [38,54–57]. Cytoskeletal disruption therefore may explain the reduced [^{35}S]sulphate incorporation in chondrocyte cultures under high pressure. Furthermore, it has been demonstrated that histone [58] and polysomal mRNAs [59] are associated with the cytoskeleton, and disruption of this association has been suggested to affect the stability of mRNAs [60].

The regulatory mechanism involved in the synthesis of aggrecan is complex and may be modulated at the level of both transcription and translation. The recent studies of Curtis et al. [61] have suggested that aggrecan synthesis can be regulated both by the steady-state level of aggrecan mRNA and the efficiency of the utilization of the mRNA. In cartilage explant cultures, serum increased the pool of mRNA coding for aggrecan, whereas insulin-like growth factor-1 caused no difference in aggrecan mRNA levels despite a 4-fold increase in aggrecan synthesis [61]. The complexity of PG synthesis has also been demonstrated by the regulation of decorin mRNA levels. Serum enhanced the amount of mRNA for the core protein of decorin in cartilage explants [61], although no stimulation of decorin or biglycan synthesis was noticed [62].

In summary, this is the first report on the influence of high, continuous hydrostatic pressure on the synthesis rate and structure of aggrecans in articular chondrocyte cultures and on the steady-state mRNA levels of PGs. The results indicate that hydrostatic pressure exhibits an influence on chondrocyte biosynthesis, independent of other effects induced by mechanical loading of cartilage. Under high pressure, PG and protein synthesis are inhibited while the structure of aggrecan synthesized under these conditions is specifically altered.

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