Glomerular mesangial cells *in vitro* synthesize an aggregating proteoglycan immunologically related to versican

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Recent studies have shown that mesangial cells derived from human adult glomeruli synthesize a number of ^{35}S-labelled proteoglycans including a large chondroitin sulphate proteoglycan (CSPG), two dermatan sulphate proteoglycans (biglycan and decorin) and two heparan sulphate proteoglycans [Thomas, Mason and Davies (1991) Biochem. J. 277, 81–88]. In the present study we have examined the interaction of these proteoglycans with hyaluronan (HA) using associative gel chromatography. Only the large CSPG bound to HA, with 60% of those molecules in the medium and 80% of those in the cell layer being able to interact. Reduction and alkylation, or treatment of the monomer CSPG with proteinases, prevented the formation of aggregates, suggesting that the core protein was involved. The aggregates formed between purified CSPG and HA could be dissociated in the presence of HA–oligosaccharides of at least 10 monosaccharides in length. The inclusion of link protein with CSPG and HA promoted the formation of aggregates. Experiments with ^{3}H-labelled mesangial-cell proteoglycans confirmed that only the large CSPG, with core protein molecular masses of 400 kDa and 500 kDa, interacted with HA. After chondroitin ABC lyase treatment of CSPG isolated from conditioned culture medium, several bands similar to those observed with ^{3}H-labelled core proteins were identified using a polyclonal antiserum that recognizes versican. A monoclonal antibody recognizing the 1-C-6 epitope in the G1 and G2 globular regions of aggrecan did not recognize either mesangial-cell CSPG or bovine aortic versican. Northern-blot analysis confirmed that human mesangial cells express versican. Thus human mesangial large CSPG is a member of the versican family of proteoglycans. The interaction of CSPG and HA within the glomerulus may be important in glomerular cell migration and proliferation.

INTRODUCTION

The glomerular mesangial cell is surrounded by a pericellular matrix the composition of which helps to determine the physical, mechanical and functional properties of the glomerulus. This matrix is composed mainly of type-IV collagen together with varying amounts of fibronectin, laminin and proteoglycans [1,2]. Histochemical and immunohistochemical studies indicate that proteoglycans of both heparan sulphate and chondroitin sulphate/dermatan sulphate varieties are present [3–6]. Cultures of adult human mesangial cells synthesize a large chondroitin sulphate proteoglycan (CSPG), situated mainly in the cell layer, and small dermatan sulphate proteoglycans (DSPGs), which are found almost exclusively in the culture medium [7]. The DSPGs were identified as PG-I (biglycan) and PG-II (decorin), with the former molecule accounting for the majority of these species of proteoglycan. The mesangial-cell CSPG was not fully characterized but had some properties which were similar to the large CSPGs synthesized by vascular smooth-muscle cells, aortic endothelial cells and fibroblasts [8–12]. These molecules belong to a family of proteoglycans related functionally to the aggregating CSPG of cartilage (aggrecan), as they are all capable of interacting with hyaluronan (HA) to form proteoglycan–HA aggregates.

HA is a critical component of most extracellular matrices and there is evidence that its physical and chemical properties not only participate in the maintenance of organized structures but also regulate cell migration, adhesion and proliferation (for a review see ref. [13]). A role for HA in the function of the normal glomerulus has not been fully explored. It has been reported that mesangial cells derived from rat kidney synthesize HA [14] and a recent report indicated the presence of an HA-binding receptor in kidney tissue, which appears to be located on the plasma membrane and may provide a mechanism by which HA interacts with the surfaces of glomerular cells [15]. A second possible role for HA within the mesangium could involve the interaction with the large CSPGs known to be synthesized by mesangial cells [7,14]. The present investigation shows that the CSPGs do interact with HA and provides immunochemical evidence that they are related to the versican family of proteoglycans.

MATERIALS AND METHODS

Cell culture

Human mesangial cells were cultured and characterized by methods previously reported by us [7]. All experiments were performed with material extracted from cells radiolabelled between the third and fifth passages. For metabolic labelling cells were cultured on 35-mm-diam. plastic dishes (Falcon) and incubated with 50 μCi/ml [^{35}S]sulphate (specific radioactivity 950–1350 Ci/mmol; Amersham International, Amersham, Bucks., U.K.) for 24 h in fresh medium containing 15% (v/v) fetal-calf serum and 10% of the normal inorganic sulphate as described for human glomerular epithelial cells [16]. To label the

Abbreviations used: CSPG, chondroitin sulphate proteoglycan; DSPG, dermatan sulphate proteoglycan; HA, hyaluronan; mAb, monoclonal antibody; ECL, enhanced chemiluminescence.

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core protein, cells were incubated in leucine-free medium (Gibco) for 48 h with L-[4,5-^{3}H]leucine (20 μCi/ml; Amersham). In order to label HA, cells were incubated with D-[6-^{3}H]glucosamine (20 μCi/ml; Amersham) for 24 h.

Extraction and isolation of proteoglycans

After incubation with radiolabelled precursors the proteoglycans in the culture medium and cell layer were extracted in the presence of proteinase inhibitors, as described previously [16]. To recover the total proteoglycan population the culture medium and cell layer were buffer-exchanged on a Sephadex G-50 column (2 cm × 15 cm) equilibrated with 8 M urea, 20 mM Bis-Tris/HCl, pH 6.0, containing 0.15 M NaCl and 0.5 % CHAPS (urea buffer), and the labelled material eluting in the void volume was applied to a Mono Q column (Pharmacia) equilibrated in the same urea buffer. The column was washed with 5 volumes of urea buffer and the bound material of low affinity was eluted with a linear gradient of 0.15–0.75 M NaCl, followed by a second gradient of 0.75–1.2 M NaCl to obtain the proteoglycan fraction. Fractions (0.5 ml) were collected and assayed for radioactivity. Fractions containing labelled material, which were eluted between 0.4 and 0.6 M NaCl (designated the HA pool) and between 0.8 and 0.9 M NaCl (total proteoglycan pool), were pooled separately. To obtain samples of the different proteoglycans the total proteoglycan pool was either digested with chondroitin ABC lyase or treated with nitrous acid and further fractionated as detailed previously [7,16].

A 4 M guanidine hydrochloride extract was prepared from 20 μM sections of normal human articular cartilage from a 31-year-old patient [17]. Pig laryngeal cartilage aggrecan (A1D1) was prepared according to the method of Hardingham et al. [18]. An authentic sample of bovine aortic versican was kindly donated by Professor D. Heinegard (University of Lund, Lund, Sweden) [19].

Gel chromatography of proteoglycans

Proteoglycans were subjected to analytical gel-permeation chromatography at room temperature on a column (6 mm × 1.5 m) of Sepharose CL-2B or CL-4B equilibrated and eluted with either 4 M guanidine hydrochloride, 50 mM sodium acetate, pH 6.0, containing 0.5 % Triton X-100 (dissociative buffer), or 0.5 M guanidine hydrochloride, 0.1 M Tris/HCl, pH 7.0, 0.5 % CHAPS (associative buffer).

Characterization of newly synthesized HA

The [^{3}H]HA pool eluted from the Mono Q column described above was applied to a column (2 cm × 15 cm) of Sephadex G-50 equilibrated in 50 mM NH_4HCO_3. The material eluting in the void volume was freeze-dried, taken up in 0.1 M sodium acetate buffer, pH 6.0, containing papain (3 μg/ml) but no cysteine or EDTA [20] and digested at 60 °C for 16 h. After incubation the material was passed over a Sephacryl S-500 column equilibrated in dissociative buffer and analysed for radioactivity.

Proteoglycan–HA-binding experiments

The ability of mesangial-cell CSPG to form aggregates with HA was assessed by analysing the shift in the elution from the included volume to the excluded volume of an associative Sepharose CL-2B column after interaction with the glycosaminoglycan. Preparations of total ^{35}S-labelled proteoglycans from culture medium or the cell layer, or samples of individually purified ^{35}S-labelled proteoglycans were incubated with HA (1 mg/ml, human umbilical; Sigma) and then dialysed extensively against associative buffer. After dialysis the mixture was chromatographed on an associative Sepharose CL-2B column.

In other experiments the ability of mesangial-cell CSPG to form aggregates with HA was compared with that of pig laryngeal cartilage aggrecan. Aggrecan and link protein were isolated as described previously [21] and dissolved in 4 M guanidine hydrochloride, 50 mM sodium acetate, pH 5.8 (1 mg/ml). To this was added mesangial-cell [^{35}S]CSPG (40×10^3 d.p.m./ml) with or without link protein (25 μg/ml) and HA (200 μg/ml). The mixture was dialysed extensively against associative buffer and chromatographed on an associative Sepharose CL-2B column.

Analytical methods

Oligomers of HA were prepared by digesting high-molecular-mass HA (25 mg/ml) with 880 units/ml of testicular hyaluronidase (type IV; Sigma) at 37 °C for 6 h, and the released oligosaccharides separated on a column of Sephadex G-50 (superfine) eluted with 50 mM NH_4HCO_3 [22]. Fractions having a $K_{av.}$ between 0 and 0.3; 0.3 and 0.45; and > 0.45 were combined separately, freeze-dried, dissolved in water, freeze-dried a second time and finally dissolved again in water (1 ml). Uronic acid was determined by the procedure of Bitter and Muir [23] with glucuronolactone as a standard. Heparan sulphate proteoglycans were degraded chemically with nitrous acid at pH 1.5 for 10 min [24,25]. CSPGs were digested with chondroitin ABC lyase [26] in the presence of proteinase inhibitors, as described by Oike et al. [27]. Mesangial-cell CSPG was reduced with 5 mM dithiothreitol for 5 h at 45 °C and alkylated by incubation overnight at 4 °C in the dark with 35 mM iodoacetamide.

SDS/PAGE and Western-blot analysis

Electrophoresis was performed on either 3–12 % or 5–15 % (with a 4 % stacking gel) polyacrylamide gradient gels using the buffer system of Laemmli [28]. Samples for Western-blot analysis were purified from the conditioned culture medium of confluent human mesangial cells and stored with proteinase inhibitors [7]. Samples were digested with chondroitin ABC lyase (proteinase-free; ICN Biochemicals) (at 37 °C for 4 h) and in some experiments with a combination of chondroitin ABC lyase and keratanase (proteinase-free; ICN Biochemicals). After digestion the products were separated by electrophoresis and transferred to Immobolin-P (Millipore, U.K.) and probed with rabbit anti-(bovine versican) serum (kindly provided by Professor D. Heinegard, Lund, Sweden), monoclonal antibody (mAb) 1-C-6, mAb 2B6 and mAb 3B3. The versican antiserum was raised against a large CSPG isolated from bovine aorta and does not cross-react with aggrecan isolated from bovine cartilage and sclera [19]. mAb 1-C-6 recognizes an epitope which is present on both globular regions of aggrecan [29]. For the detection of this epitope Immobilin blots were reduced with 10 mM dithiothreitol. mAb 2B6 and mAb 3B3 recognize the unsaturated 4- and 6-sulphated disaccharides remaining after digestion of CSPGs with chondroitin ABC lyase [5]. Proteins were visualized using enhanced chemiluminescence (ECL) (Amersham International). For fluorography the fractions containing proteoglycans labelled with [^{3}H]leucine (see the Results section) were pooled, dialysed

against distilled water and freeze-dried. The labelled proteoglycans were then incubated with chondroitin ABC lyase and submitted to SDS/PAGE in a 3–12% (w/v) polyacrylamide gradient gel. The gels were impregnated with Amplify (Amersham), dried under vacuum and the labelled proteins were visualized by fluorography.

RNA isolation and Northern-blot analysis

Total RNA from fifth passage human mesangial cells was isolated by a rapid RNA extraction protocol [30]. After quantification total RNA (10 μg) was separated for 12 h in a formaldehyde/MOPS/1.2% agarose gel by standard techniques and transferred to a nylon membrane (NEN). Membranes were hybridized with a random-primed ^{32}P-labelled human versican cDNA (Bioquote Ltd., York, U.K.) [31,32] in standard formaldehyde conditions. This probe was derived from human IMR-90 lung fibroblasts and encodes for nucleic acids 1475–2802 plus the entire 3′-untranslated region. The membranes were also hybridized with a labelled human decorin cDNA (a kind gift from Dr. Larry Fisher, NIDR, NIH, Bethesda, MD, U.S.A.) [33] and a human aggrecan cDNA (a kind gift from Dr. J. Dudhia, Kennedy Institute, London, U.K.). After post-hybridization washing in 0.5 × SSC/0.1% SDS (SSC, 0.15 M NaCl/0.015 M sodium citrate), membranes were exposed to X-ray film for 4 days.

RESULTS

Interaction of radiolabelled mesangial-cell proteoglycans with HA

Mesangial cells in culture were incubated with [^{35}S]sulphate for 24 h and the labelled proteoglycans extracted from the culture medium were analysed by associative chromatography on a Sepharose CL-2B column as described in the Materials and methods section. The elution profile (Figure 1a) showed that the majority of the ^{35}S-labelled proteoglycans were well retarded by the gel ($K_{av.}$, 0.3–0.8) and only traces appeared in the void volume. The addition of HA (1 mg/ml) to the culture medium proteoglycans resulted in a significant increase in the amount of proteoglycan appearing in the void volume and this was accompanied by an equal reduction in labelled molecules eluting at $K_{av.}$, 0.3–0.5 (Figure 1a). The ^{35}S-labelled proteoglycan in the void volume was pooled and rechromatographed under dissociative conditions on the same CL-2B column (Figure 1b). Under these conditions the majority of the ^{35}S label was eluted at a $K_{av.}$ value of 0.45. Chondroitin ABC lyase digestion removed this peak with the appearance of labelled chondroitin sulphate disaccharides in the total volume of the column (results not shown). Similar results were obtained with the total ^{35}S-labelled proteoglycan extracted from the cell layer (results not shown). When the experiments were repeated with [^{3}H]leucine-labelled culture-medium proteoglycans, associative CL-2B analysis (Figure 1c) indicated that a similar proportion of the labelled material aggregated with HA. These results suggest that human mesangial cells synthesize and secrete a population of CSPGs which have the capacity to interact with HA to form aggregates.

To confirm these results, the large CSPG, decorin, biglycan, HSPG-I and HSPG-II synthesized by human mesangial cells *in vitro* were purified by ion-exchange and gel-filtration chromatography before and after selective degradation of the glycosaminoglycan side chains as described previously {see Figures 1(a), 2, 5, and Table 1 from [7]}. Each proteoglycan was incubated separately with HA and subjected to gel chromatography on CL-2B under associative conditions. Biglycan and decorin, as well as HSPG-I and HSPG-II, failed to undergo aggregation in the presence of HA. In contrast the results shown in Figure 2 indicate that about 60% of the ^{35}S-labelled large CSPGs formed aggregates with HA. When the CSPGs which were not interacting were separated and re-incubated with HA they still eluted in the included volume of the CL-2B column (results not shown). In comparable experiments with large CSPG isolated from the cell layer, 80% bound to HA. The addition of decasaccharides (Figure 3a) or higher-molecular-mass oligomers (results not

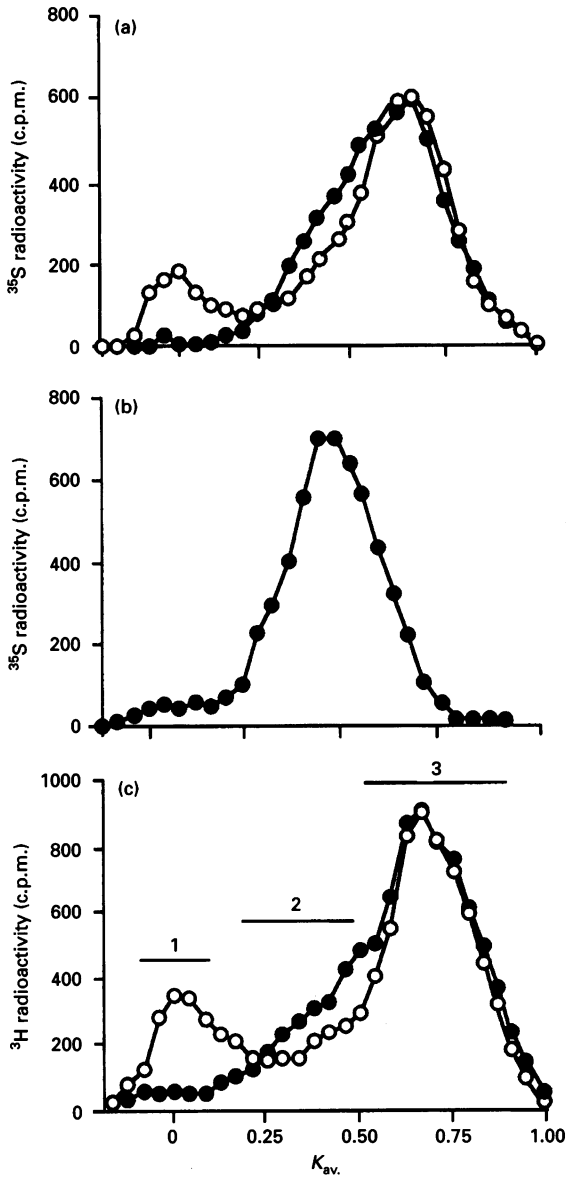

Figure 1 Interaction of HA with human adult mesangial-cell proteoglycans

Human mesangial cells were metabolically labelled with either [^{35}S]sulphate or [^{3}H]leucine for 24 h and the radiolabelled ^{35}S-proteoglycans extracted from the culture medium by Mono Q ion-exchange chromatography as described in the Materials and methods section [7]. (**a**) Aliquots of the labelled ^{35}S-proteoglycans were mixed with (○) or without (●) HA (1 mg/ml), dialysed against associative buffer and chromatographed on an analytical associative Sepharose CL-2B column; (**b**) the ^{35}S-labelled proteoglycans appearing in the V_0 [see (**a**)] were pooled and dialysed against dissociative buffer, and re-chromatographed on an analytical dissociative Sepharose CL-2B column; (**c**) ^{3}H-labelled proteoglycans were processed exactly as described in (**a**) above. The horizontal bars in (**c**) indicate the fractions pooled for further analysis.

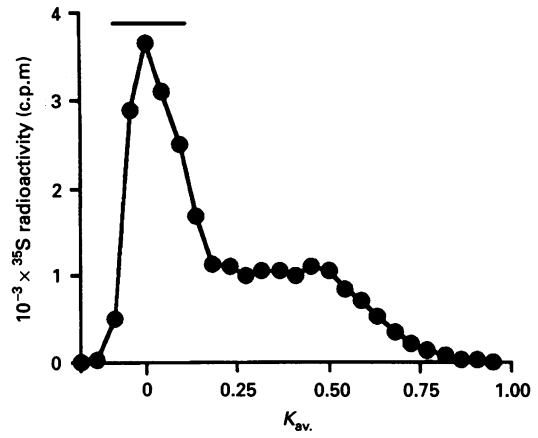

Figure 2 Interaction of purified human mesangial-cell proteoglycans with HA

Confluent cultures of human mesangial cells were labelled metabolically for 24 h with [^{35}S]sulphate, and the cell medium proteoglycans separated from proteins and labelled glycoproteins by Mono Q ion-exchange chromatography. The ^{35}S-labelled proteoglycans that were eluted with 1.2 M NaCl were digested with cold nitrous acid and re-chromatographed on an analytical dissociative Sepharose CL-4B column to separate the large CSPG from the small DSPGs. Fractions containing CSPG were pooled, dialysed against associative buffer, and incubated with HA (1 mg/ml). The mixture was then chromatographed on an associative CL-2B analytical column. Fractions indicated by the horizontal bar were pooled for further experiments.

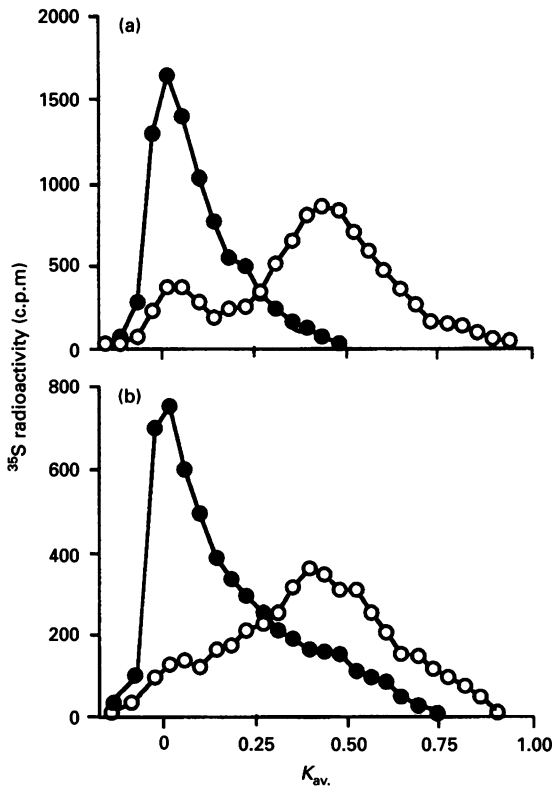

Figure 3 Effect of HA oligosaccharides and reduction and alkylation on CSPG–HA aggregates

Aggregating mesangial-cell CSPGs were prepared as indicated in Figure 2 and (**a**) incubated with HA (0.2 mg/ml) in the presence (○) and absence (●) of HA decasaccharide (2.5 mg/ml) and chromatographed on an associative CL-2B column; (**b**) CSPG–HA aggregates (see Figure 2) were pooled and passed over a dissociative CL-2B column to separate HA from monomer CSPG. Aliquots of the monomer in dissociative buffer were either incubated alone or reduced and alkylated, dialysed into associative buffer and then reincubated with HA (0.2 mg/ml). The mixture containing the non-reduced (●) and reduced (○) CSPG was chromatographed on an associative Sepharose CL-2B column.

shown) resulted in a significant reduction in the amount of ^{35}S-CSPG eluting in the void volume. In contrast the incubation of HA/proteoglycan aggregates with octasaccharides or smaller oligomers had no effect on the elution profile (results not shown).

To investigate the specificity of mesangial-cell proteoglycan–HA interaction we prepared aggregating CSPG as described in Figure 2. The aggregates were pooled and passed over a dissociative CL-2B column to separate the HA from the monomer CSPG. The interaction of this purified CSPG population with HA was abolished by prior digestion with papain or trypsin, indicating that the core protein was involved in some way. The importance of the core protein was emphasized further by experiments which demonstrated that reduction and alkylation of the purified aggregating CSPG before incubation with HA significantly reduced its ability to form aggregates (Figure 3b).

In further experiments the ability of CSPG to interact with HA was compared with the same interaction with pig cartilage aggrecan. Aggrecan and ^{35}S-labelled mesangial-cell CSPG together were dialysed to associative conditions and chromatographed on an associative Sepharose CL-2B column as described in the Materials and methods section. The CSPG has an overall smaller hydrodynamic size than aggrecan (Figure 4a). When limiting amounts (200 μg/ml) of HA were added before dialysis both proteoglycans showed some shift towards the excluded volume of the column in subsequent associative Sepharose CL-2B chromatography (Figure 4b). However, when both HA and link protein were added before dialysis, both proteoglycans elute predominantly in the excluded volume (Figure 4c). These results indicate that the CSPG can be incorporated into mixed proteoglycan–HA aggregates stabilized by link protein.

Characterization of the mesangial-cell CSPG

To investigate the nature of the core protein of the mesangial-cell CSPG, fractions were pooled as indicated in Figure 1(c), dialysed, digested with chondroitin ABC lyase and the products examined by SDS/PAGE under reducing conditions followed by fluorography. The results are shown in Figure 5. Pool 1 contained a large CSPG which yielded two major core proteins with apparent molecular masses of 500 and 400 kDa and a minor band at 350 kDa (lane 1). These bands were not present in control incubations without chondroitin ABC lyase present (results not shown). Thus the pool I proteoglycan corresponds to the mesangial-cell large CSPG previously described by us, which was shown to have similar core protein heterogeneity [7]. Similar treatment of the proteoglycans in pool 2 failed to yield the 500 kDa protein but did show a clear band at 400 kDa together with some minor bands of lower molecular mass (Figure 5, lane 2). Treatment of the third pool of proteoglycans with chondroitin ABC lyase resulted in the appearance of core proteins with molecular masses consistent with decorin and biglycan (Figure 5, lane 3).

Western-blot and Northern-blot analysis of mesangial-cell CSPG

Two large CSPGs have been identified in the tissue, which interact with HA. These are aggrecan, the major proteoglycan of hyaline cartilage [34,35], and versican [32], which was isolated from a number of tissues as described above. To investigate the

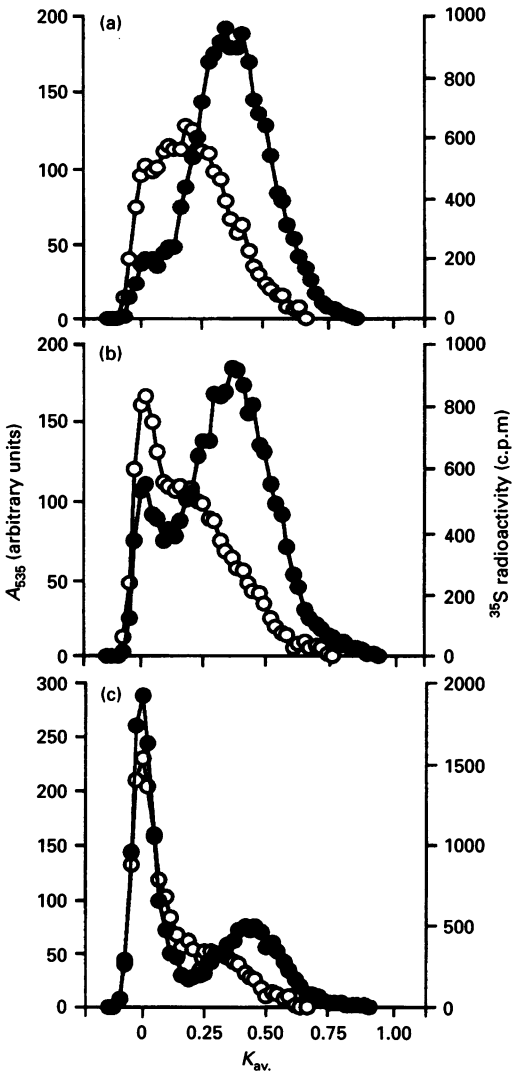

Figure 4 Comparison of the HA-binding properties of mesangial CSPG with pig aggrecan

Aggregating CSPG was obtained as shown in Figure 2 and pig aggrecan as described in the Materials and methods section. To the aggrecan (1 mg/ml) was added 40000 c.p.m./ml of ^{35}S-labelled CSPG and (**a**) no HA, (**b**) 200 μg/ml HA and (**c**) 200 μg/ml HA plus 25 μg/ml link protein. The mixtures were dialysed against associative buffer and then chromatographed on a Sepharose CL-2B column under associative conditions. In each fraction aggrecan was determined using the dimethyl Methylene Blue assay (○) and ^{35}S-labelled CSPG measured by β-counting (●).

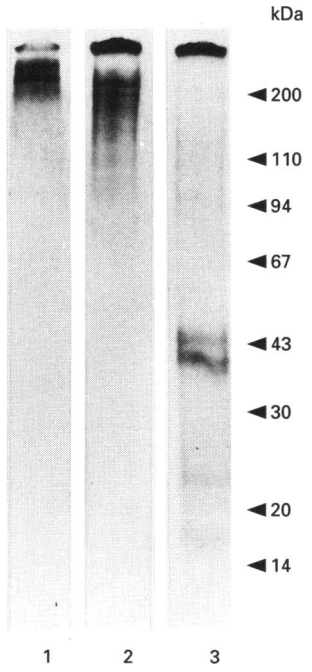

Figure 5 SDS/PAGE of [^{3}H]leucine-labelled mesangial-cell proteoglycans

The pooled fractions from Figure 1(c) were dialysed against water, freeze-dried, incubated with chondroitin ABC lyase and submitted to SDS/PAGE on a gradient gel (3–12%): the gels were dried and radioactive bands revealed by fluorography. Molecular mass values of the core-protein molecules were estimated from the positions of standard proteins. Proteins with molecular masses > 200 kDa were estimated by extrapolation.

nature of the large CSPG synthesized by the mesangial cells unlabelled proteoglycans were prepared from the conditioned medium. For these experiments the fetal-calf serum used for the cell culture had been previously passed over DEAE-Sepharose to remove bovine proteoglycans. The mesangial-cell proteoglycans were investigated by PAGE and Western-blot analysis together with a crude proteoglycan extract from human articular cartilage and an authentic sample of bovine aortic versican. The proteoglycans were digested with chondroitin ABC lyase and keratanase and electrophoresed under non-reducing conditions on 5–15% (w/v) polyacrylamide gradient gels. After transfer to Immobolin the separated proteins were probed with a polyclonal anti-versican serum and mAbs 3B3 and 2B6. A Western blot of the mesangial-cell proteoglycan reacted with the anti-versican serum to reveal three major bands (Figure 6, lane 1) in the same molecular mass range as detected by metabolic labelling with [^{3}H]leucine (Figure 5). After allowing time for all the ECL activity on the Immobolin to decay, it was reprobed with either mAb 2B6 or 3B3. These experiments showed that the bands which reacted with the versican antiserum contained both 4- and 6-sulphated linkage region disaccharides (Figure 6, lanes 3 and 4). These mAbs also detected lower-molecular-mass components corresponding to biglycan and probably decorin, which are present in the medium of human mesangial-cell cultures [7]. In a separate experiment the mesangial-cell CSPGs, human cartilage proteoglycans and bovine versican were probed with the anti-versican serum and mAb 1-C-6. All lanes were intentionally overloaded. All these proteoglycan preparations reacted with the anti-versican serum (Figure 6, lanes 5–7). This indicates the presence of versican-like proteoglycans in adult human articular cartilage. Neither the mesangial-cell CSPG or bovine versican reacted with mAb 1-C-6 (Figure 6, lanes 8–10).

We next confirmed, by carrying out Northern-blot analysis for mRNA, that human mesangial cells expressed versican. Hybridization of membranes with cDNA probes for versican and decorin revealed mRNA species of 8.2, 1.9 and 1.6 kb (Figure 7). The large species correspond to the mRNA for versican [32] and the smaller species to mRNA for decorin [33]. In parallel experiments we failed to detect mRNA for aggrecan core protein.

Synthesis of HA by mesangial cells

In view of the above interaction between CSPG and HA we investigated HA synthesis by human adult mesangial cells. Cells

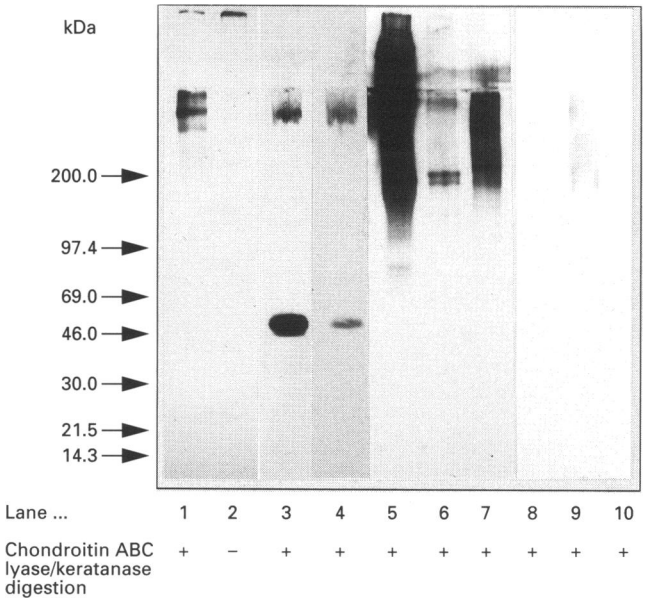

Figure 6 Identification of versican synthesized by human mesangial cells and extracts of human articular cartilage

Purified extracts of human mesangial-cell proteoglycans (lanes 1–5 and 8), human articular cartilage aggrecan (lanes 6 and 9) and bovine aortic versican (lanes 7 and 10) were subjected to gradient SDS/PAGE and electroblotted on to Immobolin. Analysis was performed with (+) or without (−) digestion with chondroitin ABC lyase and keratanase. The blots were probed with: lanes 1, 2, 5, 6 and 7, anti-versican serum; lane 3, mAb 2B6; lane 4, mAb 3B3; lanes 8–10, mAb 1-C-6. Arrows indicate migration positions of molecular-mass markers.

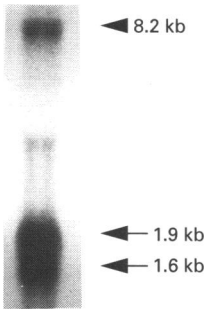

Figure 7 Northern-blot analysis of human mesangial-cell proteoglycans

Total RNA was purified from confluent human mesangial cells and analysed by Northern-blot analysis. Specific mRNAs were hybridized with cDNA probes for versican (arrowhead) and decorin (arrows) (see Materials and methods section).

were incubated with [^{3}H]glucosamine and the labelled material in the cell layer and the culture medium was buffer-exchanged and subjected to ion-exchange chromatography on a Mono Q column as described in the Materials and methods section. The HA pool was digested with papain and analysed by gel chromatography on Sephacryl S-500 (Figure 8). Papain treatment served to degrade any ^{3}H-glycoproteins present and the digestion products were eluted in the included volume of the column. The material eluting in the void volume was pooled and digested with hyaluronidase from *Streptomyces* sp. and the products analysed on the same S-500 column (Figure 8). These experiments show that the papain-resistant [^{3}H]glucosamine-labelled material was

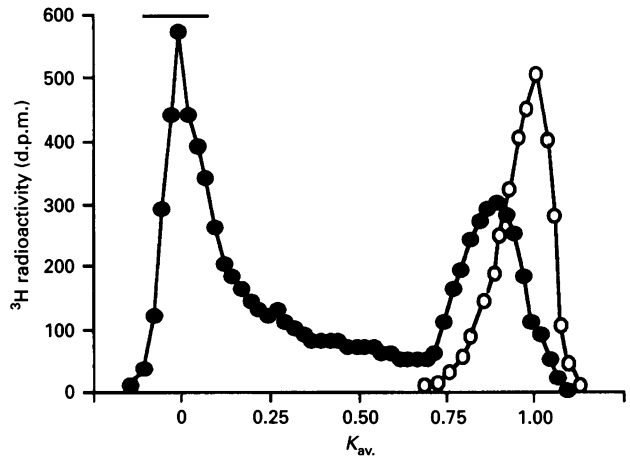

Figure 8 Separation of [^{3}H]HA synthesized by human adult mesangial cells

Cells were labelled with [^{3}H]glucosamine for 24 h and the labelled macromolecules extracted from the culture medium by ion-exchange chromatography on a Mono-Q column as detailed in the Materials and methods section. Fractions which were eluted from the anion-exchange column between 0.4 and 0.6 M NaCl were pooled and passed over a Sephadex G-50 column equilibrated with 50 mM NH_4HCO_3. The material eluting in the void volume was freeze-dried, and digested with papain. The digest was chromatographed over a Sephacryl S-500 column equilibrated in dissociative buffer (●). The fractions indicated by the horizontal bar were pooled and digested with *Streptomyces* hyaluronidase and rechromatographed on the same column (○).

completely degraded by the HA lyase, and thus indicate that human mesangial cells synthesize HA. The results further showed that > 80% of this macromolecule was located in the culture medium.

DISCUSSION

In a previous study we demonstrated that adult human mesangial cells in culture synthesize two DSPGs, biglycan and decorin, a large CSPG and two heparan sulphate proteoglycans. The CSPG was not positively identified but after chondroitin ABC lyase treatment of ^{125}I-labelled molecules protein cores of 500 kDa and 400 kDa were obtained [7]. In the present study this heterogeneity was confirmed after metabolically labelling cultures with [^{3}H]leucine. In the final purification step for these experiments the CSPG was separated from other proteoglycans in the medium on the basis of its ability to form aggregates with HA. Two major ^{3}H-labelled core proteins of molecular masses 400 and 500 kDa, together with a minor band at 350 kDa, were identified. Moreover when the partially purified proteoglycans from the culture medium were treated with the same lyase, electrophoresed, blotted and probed with a polyclonal antibody raised against bovine versican [19] similar bands were revealed. Thus the large CSPG synthesized by mesangial cells is identified as a member of the versican family of proteoglycans. The heterogeneity of the core protein may be due to differences in substitution by N- or O-linked oligosaccharides. There are 20 potential N-glycosylation sites and 22 sites with three or more consecutive serine-threonine clusters in the versican core-protein sequence [32]. It was also noticeable that several bands with apparent molecular masses of 400, 300 and 200 kDa were revealed on SDS/PAGE of chondroitin ABC lyase-treated non-aggregating proteoglycans. These multiple bands could also arise from proteolytic processing of the large forms. The 300 kDa form

failed to interact with HA, probably due to proteolytic processing of the HA-binding region so as to prevent interaction.

In our experiments final confirmation that human mesangial cells express versican was obtained by Northern-blot analysis of total mRNA preparations. A cDNA probe for the versican sequence hybridized with an 8.2 kb transcript, which is characteristic for this proteoglycan [32]. The appearance of the single band indicates that it is unlikely that major alternatively spliced messages for this molecule are processed. It is interesting, however, to note that cDNA analysis of PG-M, a large CSPG with high sequence similarity to versican revealed spliced multiforms [36]. PG-M also showed core-protein heterogeneity.

The large CSPGs were capable of interacting under associative conditions with HA to form large aggregates. The specificity of this interaction was emphasized by several experiments. First, the interaction was specific for the type of proteoglycan, as neither dermatan sulphate nor heparan sulphate proteoglycans formed complexes. Secondly, the interaction was mediated via a domain in the core protein, since trypsin treatment of monomer proteoglycan in the presence or absence of HA prevented the formation of aggregates. Thirdly, the CSPG monomer could be displaced readily from the aggregates by the addition of decasaccharides or larger oligomers of HA [35,37].

Reports in the literature have indicated the existence of structural heterogeneity among large CSPGs extracted from several different tissues and from cells *in vitro* [8,9,11,19,36,38]. This heterogeneity may reflect their biological properties, as those from bovine and pig aorta were subfractionated according to their binding capacity to low-density lipoproteins [39] and their ability to bind antithrombin III [40] respectively. Using associative CsCl centrifugation, Morita et al. [12] separated two populations of large CSPGs from the conditioned medium of cultured mouse aortic endothelial cells based on their ability to form aggregates with HA. Furthermore although the protein cores of proteoglycans secreted into the medium and those isolated from the cells appeared to be polydisperse, only those with an apparent molecular mass of 550 kDa appeared to bind to HA. In the present study we also demonstrated similar core-protein heterogeneity but in contrast with the endothelial-cell-derived molecules the mesangial-cell species that bind HA consist of proteoglycans with both 500 kDa and 400 kDa core proteins. Another possibility is that they are derived from other proteoglycans such as the large dermatan proteoglycan expressed in human fibroblasts [41]. This proteoglycan, which does not interact with HA to form aggregates, is composed of two unrelated core proteins of 460 and 300 kDa.

Two groups of aggregating CSPGs have been described [38,42]. The most studied is aggrecan, the principal proteoglycan of hyaline cartilage. This proteoglycan forms aggregates with HA through a highly specific, non-covalent interaction mediated via the G1 domain located in the N-terminal region. With aggrecan each interaction is stabilized by a single link protein, which shows sequence similarity with G1. The second form of aggregating CSPG has been named versican [32]. This molecule is synthesized by human fibroblasts and aortic smooth-muscle cells. In addition the large CSPG of mouse aortic endothelial cells closely resembles versican [12]. The cDNA of fibroblast versican has been sequenced and comparison with that of aggrecan clearly indicates that it is a different gene product [31,32]. An apparent difference is the presence in versican of two epidermal growth factor-like regions situated adjacent to the G3 region. These were not detected in chick or rat aggrecan cDNA, but the occurrence of a single epidermal growth factor-like domain has been reported in some cDNA clones for human aggrecan core protein [43].

While the experiments of LeBaron and co-workers [42] and Morita and co-workers [12] showed that versican interacts with HA, they did not investigate whether the aggregates which were formed could be stabilized with link protein in a manner analogous to aggrecan. Our data indicates that mesangial-cell CSPG does not contain the 1-C-6 epitope present in the G1 domain of aggrecan, the site of interaction with link protein. Nevertheless experiments in which cartilage link protein was added to mixtures of CSPG and HA suggest that it promotes the stabilization of aggregates (see Figure 4c).

Since the mesangial-cell-derived large CSPG is recognized by an antibody raised against bovine versican the immunological data strongly suggest that this large aggregating CSPG is related to versican rather than aggrecan. The presence, in our experiments, of mRNA for versican, but not aggrecan, in human mesangial cells supports this conclusion.

An unexpected finding in the present investigation was that the extracts of human adult articular cartilage contained protein that was recognized by anti-versican serum. This extends the recent observation of Grover and Roughley [44], who demonstrated the presence of mRNA for versican in human chrondrocytes using PCR, and represents a novel location for the distribution for this proteoglycan.

Preliminary data suggest that a large CSPG may play an important role in mesangial-cell proliferation [45]. This finding is consistent with reports that show that CSPGs are expressed at a higher level in proliferating vascular smooth-muscle cells when compared with quiescent cells [46]. Moreover, HA is involved in a diverse array of biological processes, including cell differentiation, cell adhesion and proliferation [13]. Since mesangial cells synthesize HA the interaction of this molecule with CSPGs may be involved in the proliferation of these cells. However, additional studies are required to clarify the role of HA and HA-binding proteoglycans within the glomerulus.

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