Aortic endothelial cells synthesize a large chondroitin sulphate proteoglycan capable of binding to hyaluronate

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Confluent cultures of mouse aortic endothelial cells (END-D) were incubated with either [³⁵S]methionine or ³⁵SO₄²⁻, and the radiolabelled proteoglycans in media and cell layers were analysed for their hyaluronate-binding activity. The proteoglycan subfraction which bound to hyaluronate accounted for about 18% (media) and 10% (cell layers) of the total ³⁵S radioactivity of each proteoglycan fraction. The bound proteoglycan molecules could be dissociated from the aggregates either by digestion with hyaluronate lyase or by treatment with hyaluronate deca-saccharides. Digestion of [*methionine*-³⁵S]proteoglycans with chondroitinase and/or heparitinase, followed by SDS/polyacrylamide-gel electrophoresis, indicated that the medium and cell layer contain at least three chondroitin sulphate proteoglycans, one dermatan sulphate proteoglycan, and two heparan sulphate proteoglycans which differ from one another in the size of core molecules. Among these, only the hydrodynamically large chondroitin sulphate species with an *M_r* 550 000 core molecule was shown to bind to hyaluronate. A very similar chondroitin sulphate proteoglycan capable of binding to hyaluronate was also found in cultures of calf pulmonary arterial endothelial cells (A.T.C.C. CCL 209). These observations, together with the known effects of hyaluronate on various cellular activities, suggest the existence of possible specialized functions of this proteoglycan subspecies in cellular processes characteristic of vascular development and diseases.

INTRODUCTION

The endothelium forms the luminal surface of the vascular system and plays diverse physiological roles in blood–tissue homeostasis and in tissue remodelling during repair and differentiation. Correspondingly, morphological and functional changes of the endothelium are affected in such pathological conditions as atherosclerosis, hypersensitivity, inflammation and tumour metastasis (for reviews, see Levine *et al.*, 1983; Wight *et al.*, 1988).

The endothelium exists as a monolayer of highly flattened cells which are in intimate contact with the surrounding matrix and are thought to interact with matrix molecules in a dynamic fashion. One characteristic group of molecules, the proteoglycans, appear to be present in the matrix as well as on the endothelial cell surface. Thus electron microscopic studies (Simionescu *et al.*, 1981) have demonstrated that a significant number of anionic sites present on the surface of capillary endothelium could be removed by perfusion with chondroitinase ABC (EC 4.2.2.4; chondroitin ABC lyase) and/or heparitinase (EC 4.2.2.8; heparan sulphate lyase). Biochemical analysis (Oohira *et al.*, 1983; Hiss *et al.*, 1987) has shown that bovine aortic endothelial cells in culture synthesize and secrete heparan sulphate proteoglycans of different molecular sizes plus a small (*M_r* 130 000) chondroitin sulphate proteoglycan. We do

not know, however, how many subspecies or subpopulations are involved in the major endothelial proteoglycans and whether they have specialized functions. Previously, Marcum *et al.* (1986) reported that endothelial heparan sulphate proteoglycans contain a small subpopulation capable of binding to anti-thrombin, which may be responsible for the non-thrombogenic properties of the vascular endothelium. Kinsella & Wight (1986), on the other hand, demonstrated that the synthesis of chondroitin sulphate/dermatan sulphate proteoglycans is greatly increased when confluent endothelial cells are subjected to multiscratch wounding to induce migration, suggesting that a modification of the proteoglycan composition may be important in the proliferation and migration of endothelial cells after injury *in vivo*. Together, these results are suggestive of a diversity of endothelial proteoglycan subspecies that may be related to the diverse physiological roles of the endothelium.

Our interest in vascular endothelial cells and the surrounding matrix stems in part from our earlier observations concerning the strikingly increased synthesis of hyaluronate by mouse mammary carcinoma cell variants that had been selected for their increased efficiency in producing pulmonary metastasis after intravenous injection (Kimata *et al.*, 1983). Since the injected carcinoma cells must adhere to vascular endothelium before they can invade the endothelium and underlying basal lamina, it is a reasonable assumption that some hyaluronate-

Abbreviation used: PG/HA/LP-aggregate; proteoglycan–hyaluronate–link protein aggregate.

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binding substances are present in the matrix or on the surface of endothelial cells, which may be of key importance in the adhesion of the hyaluronate-coated carcinoma cells.

In this report, we show that aortic endothelial cells synthesize and secrete a large chondroitin sulphate proteoglycan which, unlike the other proteoglycan subpopulations synthesized, can interact with hyaluronate.

EXPERIMENTAL

Materials

Materials used in this study and their sources were as follows. L-[^{35}S]Methionine (1151 $\mu\text{Ci}/\text{mmol}$) was obtained from New England Nuclear (Boston, MA, U.S.A.); $^{35}\text{SO}_4^{2-}$ (carrier-free) was from the Japan Radioisotope Association (Tokyo, Japan); calf pulmonary arterial endothelial cells A.T.C.C. CCL 209 were from the American Type Culture Collection (Rockville, MD, U.S.A.); foetal calf serum was from Boehringer-Mannheim G.m.b.H. (Mannheim, Germany); tissue culture materials were from Becton Dickinson Overseas Inc. (Tokyo, Japan); DEAE-Sephacel, Sepharose CL-2B, Sepharose CL-6B and electrophoresis calibration kit (M_r markers) were from Pharmacia Japan (Tokyo, Japan); rooster comb hyaluronate (M_r 1 800 000), chondroitin 4-sulphate (M_r 40 000), chondroitin 6-sulphate (M_r 12 000), decasaccharide prepared from hyaluronate by digestion with testicular hyaluronidase, chondroitinase AC-II (EC 4.2.2.5), chondroitinase ABC, heparitinase and hyaluronate lyase (EC 4.2.2.1 from *Streptomyces*) were from Seikagaku Kogyo Co. (Tokyo, Japan; gifts from Dr. K. Yoshida). Pronase was from Kaken Kagaku Kogyo Co. (Tokyo, Japan); pepstatin was from the Institute of Microbiological Chemistry (Tokyo, Japan; a gift from Dr. T. Aoyagi). The monoclonal antibodies HK-84 and HK-102, prepared against the core protein of Engelbreth-Holm-Swarm mouse tumour heparan sulphate proteoglycan (Kato *et al.*, 1988) were gifts from Dr. M. Kato, Institute for Molecular Science of Medicine, Aichi Medical University, Japan.

The fraction containing hyaluronate/proteoglycan/link protein (HA/PG/LP) aggregates ('aA1 fraction') was prepared from Swarm rat chondrosarcoma as described by Faltz *et al.* (1979). The preparation was then subjected to associative Cs_2SO_4 gradient zonal rate sedimentation in the presence of hyaluronate decasaccharide (10 mol of hexuronate/mol of hyaluronate in aA1 fraction) to release link-protein-free proteoglycan molecules from hyaluronate chains (Kimata *et al.*, 1982). The bottom $\frac{1}{15}$ of the tube was collected and employed as a HA/PG/LP aggregate carrier.

END-D cell line

An endothelial cell line designated END-D was established by clonal selection on culture of C57BL/6 mouse aorta endothelial cells. END-D cells showed contact inhibition of growth. At confluency, they showed a morphology typical for endothelial cells. The endothelial cell phenotype of END-D was confirmed by immunohistochemical examination using human anti-(Factor VIII) antibody, which can recognize the Factor VIII (marker) of endothelial cells of C57BL/6 mouse aorta (Folkman *et al.*, 1979). In addition, on a per protein basis, angiotensin-converting enzyme activity was 100 times higher in END-D cell homogenates than in

baby hamster kidney (BHK) cell homogenates, a characteristic known to be specific for endothelial cells (Hayes *et al.*, 1978). Details of the procedures for establishment of END-D cells are described elsewhere (Takeuchi *et al.*, 1985) and can be obtained on application from the authors.

For metabolic labelling, 1×10^7 endothelial cells were plated in 100 mm-diam. plastic tissue culture dishes (Falcon) and cultured in 10 ml of Eagle's minimal essential medium, containing 10% (v/v) foetal calf serum, 0.05% (w/v) ascorbic acid and 0.029% (w/v) L-glutamine (medium A) in a humidified atmosphere containing 5% CO_2 until confluency (1×10^8 cells/plate; 3–4 days). The medium was then replaced with either 5 ml (per plate) of methionine-depleted medium A containing 36 μCi of [^{35}S]methionine or 5 ml of SO_4^{2-} -depleted medium A containing 77 μCi of $^{35}\text{SO}_4^{2-}$, and cultures were incubated as above for 24 h. At the end of incubation, the radioactive medium was collected from each culture. The cell layer on the dish was then collected by scraping with a rubber policeman.

Preparation of proteoglycans

Proteoglycans were extracted from cell layers with 4 M-guanidine hydrochloride, pH 8, containing a mixture of four proteinase inhibitors (10 mM-EDTA, 10 mM-N-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride and 0.36 mM-pepstatin) according to the method of Kimata *et al.* (1978) as outlined in Scheme 1(a). After the samples were dialysed against medium A containing 7 M-urea, the proteoglycans in both media and cell layer extracts were then separated from the bulk of non-proteoglycan proteins by ion-exchange chromatography procedures as outlined in Scheme 1(b). When the proteoglycans were prepared from $^{35}\text{SO}_4^{2-}$ -labelled cell layers and media, about 82% and 90% respectively of the total macromolecular ^{35}S radioactivity in each proteoglycan fraction was recovered.

Gel chromatography of proteoglycans and glycosaminoglycans

Proteoglycan samples were dialysed against 4 M-guanidine hydrochloride/0.1 M-Tris/HCl (pH 8)/1% (w/v) CHAPS/proteinase inhibitors (as above) and then chromatographed on a Sepharose CL-2B column (0.9 cm $\times$ 40 cm) equilibrated and eluted with the same buffer.

Glycosaminoglycan samples were prepared from the proteoglycan samples by alkaline β -elimination as described previously (Kimata *et al.*, 1983). The samples were chromatographed on a Sepharose CL-6B column (0.8 cm $\times$ 50 cm) equilibrated and eluted with the same buffer. The M_r of the radioactive peak was estimated from its K_{av} using the standard glycomaninoglycans (chondroitin 4-sulphate, M_r 40 000; chondroitin 6-sulphate, M_r 12 000).

Analysis by SDS/polyacrylamide-gel electrophoresis

Digestion of proteoglycan samples with chondroitinase AC-II, chondroitinase ABC and/or heparitinase and subsequent analysis by SDS/polyacrylamide-gel electrophoresis in 5–15% (w/v) polyacrylamide running gel/3.75% (w/v) polyacrylamide stacking gel was carried out as described previously (Oike *et al.*, 1980; Kato *et al.*, 1987). Radioactive bands were revealed by fluorography. M_r standards used were: laminin A chain, M_r 370 000; laminin B chain, M_r 200 000; phosphorylase b, M_r 94 000;

albumin, M_r 67000; ovalbumin, M_r 43000; carbonic anhydrase, M_r 30000.

Hyaluronate-binding experiments

The hyaluronate-binding activity of proteoglycans was analysed by the hyaluronate-dependent shift from the retarded proteoglycan peak to the excluded one on Sepharose CL-2B column chromatography, taking advantage of the property of the proteoglycan-hyaluronate aggregate of being excluded from the column (Hardingham & Muir, 1972; Hascall & Heinegard, 1974). In a typical experiment, 330000 c.p.m. ($3.4 \mu\text{g}$ as hexuronate) of $^{35}\text{SO}_4^{2-}$ -labelled proteoglycan fraction in 4 M-guanidine hydrochloride buffer A was mixed with high- M_r hyaluronate, and then dialysed against 0.1 M-Tris/HCl (pH 9.0)/proteinase inhibitors. In control experiments, hyaluronate was omitted from the reaction mixture. After the dialysis, the mixtures were applied to a Sepharose CL-2B column (0.9 cm $\times$ 40 cm) equilibrated with 0.1 M-Tris/HCl (pH 7.0)/proteinase inhibitors. When the effects of hyaluronate decasaccharide or hyaluronate lyase were examined, the dialysed fractions containing proteoglycans bound to hyaluronate were treated with 5 units of hyaluronate lyase (37 °C, 4 h) or 2.5 mg of the decasaccharide (room temperature, 2 h) before application to the column.

Affinity fractionation of hyaluronate-binding proteoglycans

Affinity fractionation utilizing HA/PG/LP-aggregate carrier was used for the separation of the proteoglycans from high- M_r non-proteoglycan proteins and from non-hyaluronate-bound proteoglycans. The experiments were carried out essentially as described by Kimata *et al.* (1986). Briefly, $^{35}\text{SO}_4^{2-}$ - or ^{35}S -methionine-labelled proteoglycan fractions ($3.4 \mu\text{g}$ as hexuronate) in 4 M-guanidine hydrochloride/buffer A were dialysed against 0.1 M-Tris/HCl (pH 9.0)/proteinase inhibitors and then mixed with HA/PG/LP-aggregate carrier ($25 \mu\text{g}$ as hexuronate). After incubation at 10 °C for 4 h, the mixture was subjected to associative zonal rate sedimentation on a linear Cs_2SO_4 gradient. In some experiments to confirm binding specificity, hyaluronate decasaccharide or hyaluronate lyase was added to the mixture of the dialysed fraction and the HA/PG/LP-aggregate carrier, and incubated as described above before application to Cs_2SO_4 zonal rate sedimentation.

Other methods

Enzymic analysis for glycosaminoglycan compositions was performed as described previously (Kimata *et al.*, 1983). Hexuronate was determined by the procedure of Bitter & Muir (1962) with glucuronolactone as a standard.

RESULTS

Analysis on SDS/polyacrylamide gels of proteoglycans synthesized by mouse endothelial cells

Confluent cultures of mouse endothelial cells (END-D) were metabolically labelled with ^{35}S -methionine as described in the Experimental section. Radioactive macromolecules secreted into media and those extracted from cell layers with 4 M-guanidine hydrochloride in the presence of proteinase inhibitors were equilibrated in 7 M-urea buffer with 0.25 M-NaCl and subjected to

Scheme 1. Extraction (a) and partial purification (b) of radio-labelled proteoglycans from media and cell layers of endothelial cell cultures

Buffer A: 20 mM-Tris/HCl (pH 8)/1 % (w/v) CHAPS/10mM-EDTA/10mM-N-ethylmaleimide/1mM-phenylmethanesulphonyl fluoride/0.36 mM-pepstatin.

DEAE-Sephacel chromatography to separate proteoglycans from the bulk of non-proteoglycan proteins, as outlined in Scheme 1. About 1.3 % (media) and 0.89 % (cell layers) of the total macromolecular ^{35}S radioactivity (media + cell layers) was recovered in the proteoglycan fractions.

The glycosaminoglycan side chains of the ^{35}S -methionine-labelled proteoglycan samples were removed by digestion with appropriate enzymes (chondroitinase AC-II, chondroitinase ABC or heparitinase) to yield labelled-protein-enriched core fractions. The resultant core molecules were examined by SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by fluorography (Fig. 1). Before enzyme treatment, a majority of the label in the proteoglycan

Fig. 1. SDS/polyacrylamide-gel electrophoresis of [^{35}S]-methionine-labelled proteoglycan core preparation under reducing conditions

[^{35}S]Methionine-labelled proteoglycan fractions, prepared from media (a) or cell layers (b) as in Scheme 1, were incubated without enzyme (lanes 1 and 5) or with either chondroitinase AC-II (lanes 2 and 6), chondroitinase ABC (lanes 3 and 7) or chondroitinase ABC plus heparitinase (lanes 4 and 8). The samples were separated by electrophoresis in a 5–15% gradient polyacrylamide gel in the presence of 2-mercaptoethanol. Radioactive bands were revealed by fluorigraphy. M_r values of core molecules, indicated by the arrows, were estimated from the positions of standard proteins (indicated by the arrowheads). The radioactive bands of M_r 90 000–140 000 in lanes 1 and 2 probably correspond to the bands of M_r 120 000–160 000 in lanes 3 and 4. The change in mobility has probably been caused by a large amount of protein contaminants (migrating around M_r 95 000) in the chondroitinase ABC preparation.

fraction from media was found in a broad band at or near the origin (Fig. 1a, lane 1). When the proteoglycan fraction was digested with chondroitinase AC-II, radioactive proteins with M_r values of 550 000, 470 000 and 430 000 were revealed (lane 2). When digested with chondroitinase ABC, the profile of the resultant core molecules (lane 3) was very similar to that of the chondroitinase AC-II-treated core molecules, except for the appearance of a new protein of M_r 41 000, indicating that a dermatan sulphate proteoglycan with a core molecule of M_r 41 000 is contained in the proteoglycan fraction. Further treatment with heparitinase resulted in the appearance of one additional protein with M_r 250 000 (lane 4). The fluorigraphs (Fig. 1a, lanes 1–4) show the presence of several other bands of low radioactivity. The formation of these bands, however, was not dependent upon the digestion with glycosaminoglycan lyases.

The proteoglycan fraction from media was thus shown to contain three chondroitin sulphate proteoglycans with different sizes of core molecules (M_r 550 000, 470 000 and 430 000), one dermatan sulphate proteoglycan with a core molecule of M_r 41 000 and one heparan sulphate proteoglycan with a core molecule of M_r 250 000.

The proteoglycan fraction prepared from cell layers was analysed in a similar way (Fig. 1b). The profiles of intact and enzyme-treated samples were qualitatively identical with those of the medium samples, except that

Fig. 2. Interaction of proteoglycans with hyaluronate and its inhibition by hyaluronate decasaccharide

$^{35}\text{SO}_4^{2-}$ -labelled proteoglycan fractions were mixed with (●) or without (○) hyaluronate, and then dialysed to associative conditions. The dialysed samples were applied to a Sepharose CL-2B column as described in the Experimental section. To aliquots of the dialysed samples was added hyaluronate decasaccharide. The mixtures were incubated at room temperature for 2 h and then applied to the column as described in the Experimental section (△). The overbars indicate the excluded fractions which were pooled for further analysis. The arrows indicate the void volume (V_0) and the total volume (V_1). (a) Sample from medium; (b) sample from cell layer. Fraction vol. was 0.4 ml.

a protein of M_r 410 000 appeared in place of the M_r 250 000 protein in the chondroitinase-plus-heparitinase digests (Fig. 1b, lanes 6, 7 and 8). Although the heparan sulphate proteoglycans were not studied in detail, it is of note that the subspecies of M_r 410 000 core exhibits a strong cross-reactivity with monoclonal antibodies HK-84 and HK-102 prepared against the core protein of EHS-tumour heparan sulphate proteoglycan low density form (Kato *et al.*, 1988).

Demonstration of hyaluronate-binding proteoglycan

Confluent cultures of mouse endothelial cells were metabolically labelled with $^{35}\text{SO}_4^{2-}$ and radiolabelled proteoglycan fractions were prepared from the media and cell layers as described in Scheme 1. Portions were dialysed to associative conditions with or without hyaluronate. The fractions were then subjected to Sepharose CL-2B column chromatography as described in the Experimental section: As Fig. 2 shows, the addition of hyaluronate caused a distinct shift of about 18% (medium) or 10% (cell layer) of the label from the retarded peak to the excluded one. It was of note that the marked decrease in label in the retarded peak took place around the fraction of K_{av} 0.38. The results suggest the presence in both medium and cell layer of a population of proteoglycan molecules which have the capacity to

interact with hyaluronate to form hyaluronate-proteoglycan aggregates. When aliquots of the proteoglycan fractions mixed with hyaluronate were treated with hyaluronate decaaccharide, there was a marked decrease of 90 % (medium) or 83 % (cell layer) in radioactivity in the excluded fraction, with a concomitant increase in radioactivity in the retarded fractions where the hyaluronate-dependent decrease had taken place (Fig. 2). Furthermore, treatment with hyaluronate lyase also gave essentially the same effect (results not shown). This behaviour of the labelled proteoglycans can be taken as confirming the presence of proteoglycan-hyaluronate aggregates. However, there is another possibility, i.e. that radioactive proteoglycans bind indirectly to hyaluronate via the contaminating non-proteoglycan protein(s) which were revealed in Fig. 1. As will be shown (Fig. 4), the presence of such radioactive molecules was not observed in the aggregates prepared from [^{35}S]methionine-labelled proteoglycan fractions, suggesting that the possibility is unlikely.

Characterization of the hyaluronate-binding proteoglycan

The Sepharose CL-2B column-excluded fractions were prepared from the mixtures of hyaluronate with [^{35}S]methionine- or $^{35}\text{SO}_4^{2-}$ -labelled medium and cell layer samples (see Fig. 2). After being subjected to digestion with hyaluronate lyase as described above, the fractions were again applied to the same Sepharose CL-2B column. More than 25 % (both medium and cell layer) of the [^{35}S]methionine label and about 10 % (medium) or 12 % (cell layer) of $^{35}\text{SO}_4^{2-}$ label were retained in the excluded fractions. This was also the case when the samples were digested with chondroitinase AC-II, although a slight decrease was observed in the retained radioactivity of the excluded fraction. The results suggest that the fractions contained mostly chondroitin sulphate proteoglycan(s) bound to hyaluronate, but also contained considerable amounts of high- M_r non-proteoglycan proteins and of other proteoglycans which are recovered in this fraction due to binding to such proteins.

To separate hyaluronate-binding proteoglycans from both non-proteoglycan proteins and other proteoglycans, affinity fractionation by associative zonal rate sedimentation on a linear Cs_2SO_4 gradient utilizing unlabelled HA/PG/LP-aggregate carrier was carried out as described in the Experimental section. As Fig. 3 shows, the addition of HA/PG/LP-aggregate carrier to $^{35}\text{SO}_4^{2-}$ -labelled proteoglycan fractions caused a marked shift of the label (about 20 % and 8 % of the label for medium and cell layer respectively) from the slowly sedimenting to the rapidly sedimenting band. When aliquots of the samples containing radioactive proteoglycans bound to HA/PG/LP-aggregate carrier were treated with hyaluronate decaaccharide or hyaluronate lyase, very little radioactivity was recovered as rapidly sedimenting aggregates (results not shown), suggesting that radioactive proteoglycans interacted with hyaluronate in the aggregate carrier. When the rapidly and slowly sedimenting $^{35}\text{SO}_4^{2-}$ -labelled fractions were subjected to repeated zonal rate sedimentation after a second attempt to interact with hyaluronate by another addition of HA/PG/LP-aggregate carrier, more than 85 % of the radioactivity was reproducibly recovered in both the rapidly and the slowly sedimenting fractions. Therefore, these fractions seemed to represent hyaluronate-binding

Fig. 3. Binding of radioactive proteoglycans to HA/PG/LP-aggregate carrier

Zonal rate sedimentation profiles on a Cs_2SO_4 gradient of $^{35}\text{SO}_4^{2-}$ -labelled proteoglycan fractions incubated under associative conditions with (●) or without (○) HA/PG/LP-aggregate carrier are shown. The overbars indicate the subfractions which were subsequently pooled for further analysis. The samples from the media and the cell layers gave very similar results; hence only the results with the former are shown here. Fraction vol. was 0.72 ml.

(aggregating) and non-aggregating proteoglycans respectively.

The rapidly and slowly sedimenting $^{35}\text{SO}_4^{2-}$ -labelled fractions were subjected to Sepharose CL-2B column chromatography as described in the Experimental section. The label derived from the rapidly sedimenting aggregate fraction was found in a broad peak, which had an earlier elution position (K_{av} 0.38 for samples from both media and cell layers; see Table 1) than the peak of the label from the slowly sedimenting fraction (K_{av} 0.62 and 0.72 for samples from media and cell layers respectively, results not shown). Thus the hyaluronate-binding proteoglycan molecules were larger in average hydrodynamic size than the population of non-aggregating proteoglycan molecules.

When $^{35}\text{SO}_4^{2-}$ -labelled glycosaminoglycan chains were prepared from the aggregating and non-aggregating proteoglycan samples and chromatographed on Sepharose CL-6B, the chains were eluted, in each case, as a broad peak of K_{av} 0.38, with an estimated M_r of 38,000 (Table 1).

Table 1. Hyaluronate-binding chondroitin sulphate proteoglycans from mouse and calf arterial endothelial cell cultures

Details of the analytical procedures and values for the physical properties of mouse proteoglycan shown here are indicated in the text.

Property	Fraction...	Hyaluronate-binding proteoglycan from cultures of:			
		Mouse endothelial cells		Calf endothelial cells	
		Medium	Cell layer	Medium	Cell layer
Intact molecule: K_{av} on Sepharose CL-2B		0.32	0.32	0.33	0.33
Core molecule: $10^{-3} \times M_r$		550	550	550	550
Chondroitin sulphate side chain: Average $10^{-3} \times M_r$		38	38	22	22
6-Sulphate/4-sulphate ratio		3.8	5.3	1.5	3.7

Further characterization of the labelled glycosaminoglycan chains was carried out using chondroitinase and heparitinase as described in the Experimental section. The chains derived from hyaluronate-binding proteoglycans (the samples from both media and cell layers) were almost totally digested with chondroitinase AC-II or chondroitinase ABC, indicating that they consist largely of chondroitin sulphate. In contrast, the glycosaminoglycans from non-aggregating proteoglycans appear to contain at least two distinct species, heparan sulphate and chondroitin sulphate, in $^{35}\text{SO}_4^{2-}$ -radioactivity ratios of 1:4 (media) and 1:2 (cell layers). It is also clear that the chondroitin sulphate chains from hyaluronate-binding and non-aggregating proteoglycans differ with respect to the 6-sulphate/4-sulphate ratio; this was 3.8 and 5.3 for the hyaluronate-binding proteoglycans in media and cell layers respectively, compared with 1.1 and 2.2 for the non-aggregating proteoglycans in media and cell layers respectively.

[^{35}S]Methionine-labelled proteoglycan fractions were subjected to interaction with HA/PG/LP-aggregate carrier under associative conditions, and the resultant radioactive aggregates (rapidly sedimenting fraction in Cs_2SO_4 gradient) were analysed for labelled proteoglycan core molecules. As Fig. 4(a) shows, the hyaluronate-binding proteoglycan fractions from both media and cell layers yielded a single radioactive band of M_r 550 000 after digestion with chondroitinase ABC. The hyaluronate-binding proteoglycan thus appears to be a single chondroitin sulphate proteoglycan species with a large core protein. A similar band also appeared in the gels containing the non-aggregating proteoglycan fraction (Fig. 4b, lanes 6 and 8). The nature of this component is not known at present, but it probably represents an immature form of the proteoglycan with the M_r 550 000 core, or a denatured form of M_r 550 000-core-containing proteoglycan molecules having conformations where the hyaluronate-binding sites have been altered, as has been demonstrated with disulphide bond-reduced cartilage proteoglycan (Hascall & Sajdera, 1969). Alternatively, the M_r 550 000 bands from the aggregating and non-aggregating proteoglycan fractions might correspond to different proteoglycan species, which coincidentally

Fig. 4. SDS/polyacrylamide-gel electrophoresis of hyaluronate-binding and non-binding [^{35}S]proteoglycans before and after digestion with chondroitinase ABC plus heparitinase

[^{35}S]Methionine-labelled proteoglycan fractions from media and cell layers were separated into hyaluronate-binding proteoglycan subfractions and non-aggregating subfractions as described in the text (cf. Fig. 2). The resulting subfractions were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions before or after digestion with chondroitinase ABC plus heparitinase. Radioactive bands were revealed by fluorography. M_r values of core molecules, indicated by the arrows, were estimated from the positions of standard proteins, indicated by the arrowheads. (a) Lane 1, hyaluronate-binding proteoglycan subfraction from media; 2, as in 1 but digested with the enzymes; 3, hyaluronate-binding proteoglycan subfraction from cell layers; 4, as in 3 but digested with the enzymes. (b) Lane 5, non-aggregating proteoglycan subfraction from media; 6, as in 5, but digested with the enzymes; 7, non-aggregating proteoglycan subfraction from cell layers; 8, as in 7 but digested with the enzymes. Radioactive bands at the origin after enzyme treatment (see lane 2 for example) were not usually seen. These were possibly due to the self-aggregation of the core molecule.

possess identical mobilities in the gels. The basis of this heterogeneity remains to be investigated.

Comparative studies with bovine pulmonary arterial endothelial cells

To see whether the synthesis of hyaluronate-binding large chondroitin sulphate proteoglycan is restricted to the mouse endothelial cell, we performed a series of comparative studies with a well-characterized endothelial cell line A.T.C.C. CCL 209 established from calf pulmonary arterial endothelium.

Approx. 3% (media) and 10% (cell layers) of the $^{35}\text{SO}_4^{2-}$ label was bound to the hyaluronate in HA/PG/LP-aggregate carrier, as judged by the same criteria employed for the examination of mouse endothelial cell proteoglycan (cf. Fig. 3).

As summarized in Table 1, the hyaluronate-binding proteoglycans from calf and mouse endothelial cell culture are very similar in both the mobility of the core molecule on SDS/polyacrylamide gels and the hydrodynamic size of the intact proteoglycan monomer, although there were some differences in the chain length and sulphation position of chondroitin sulphate chains.

DISCUSSION

In the present study, we have utilized mouse and calf aortic endothelial cell cultures to demonstrate the synthesis of hyaluronate-binding proteoglycan as well as to define the structure of this particular proteoglycan subpopulation. The hyaluronate-binding proteoglycan is hydrodynamically large and contains chondroitin sulphate chains of an average M_r of 39000 attached to a core molecule of M_r 550000. It is noteworthy that this proteoglycan is very similar to the hyaluronate-binding chondroitin sulphate proteoglycan (PG-M) synthesized by the chick limb bud (Kimata *et al.*, 1986) with respect to the size of the core molecule. Hyaluronate-binding proteoglycans of large size have also been found in tendon, aorta, bone mesenchyme, smooth muscle cell cultures and glial cell cultures (for a review, see Hassell *et al.*, 1986). At this time, however, little is known about the structural and functional relationship among these proteoglycans.

A fundamental body of knowledge on the proteoglycan-hyaluronate interaction has been obtained by studies of the large chondroitin sulphate proteoglycans in cartilage. In these molecules, there is a hyaluronate-binding site on the protein core (Hascall & Heinegard, 1974). A link protein is required to stabilize the proteoglycan-hyaluronate aggregates (Oegema *et al.*, 1977). Evidence indicates that the PG/HA/LP complex exists in intact cartilage (for review, see Hascall & Hascall, 1981). In a preliminary experiment, the rapidly sedimenting aggregate fractions were prepared in another way; unlabelled HA/PG/LP-aggregate carrier was first added to the $^{35}\text{SO}_4^{2-}$ -labelled proteoglycan fraction in 4 M-guanidine hydrochloride. The mixture was then dialysed to associative conditions and subjected to Cs_2SO_4 gradient zonal rate sedimentation. The simple addition of hyaluronate decaaccharide to the labelled aggregate thus obtained caused the complete dissociation of the label from the aggregate. The results suggest that the hyaluronate-binding proteoglycan of endothelial cells is somewhat different from the cartilage proteoglycan in the interaction with link protein, i.e. there is no

indication that the aggregate is stabilized by the link protein of cartilage origin, since the addition of hyaluronate decaaccharide is known to be unable to dissociate the proteoglycans from the cartilage link-protein-stabilized proteoglycan-hyaluronate aggregate (Kimura *et al.*, 1979, 1980). The results can be interpreted as reflecting a difference between the cartilage- and endothelium-type proteoglycans in their core protein structures, and hence in their manner of anchoring in the respective extracellular matrix. What appears to be a link protein has been described in bovine aorta (Gardell *et al.*, 1980), but whether it represents a link protein specific for the endothelial cell-type proteoglycan remains to be studied.

We previously characterized and compared the glycosaminoglycans of FM3A mouse mammary carcinoma cell line with differing capacities to form experimental pulmonary metastasis following intravenous injection (Kimata *et al.*, 1983). The studies showed a correlation between increased levels of hyaluronate, apparently occurring in the cell coat, and the enhanced abilities of injected FM3A variants to metastasize. Since one critical stage during blood-borne metastatic spread is circulating tumour cell arrest or attachment to the vascular endothelium, it is possible that some endothelial cell surface factors, such as the hyaluronate-binding proteoglycan, are responsible for the attachment of highly metastatic, hyaluronate-coated FM3A cells by virtue of their ability to interact with hyaluronate.

Chondroitin sulphate proteoglycans within the blood vessel wall have been implicated in many fundamental events associated with atherosclerosis (for reviews, see Levine *et al.*, 1983; Wight *et al.*, 1986). The demonstration of hyaluronate-binding proteoglycan in aortic endothelial cell cultures has raised an additional question concerning whether this particular proteoglycan sub-species is responsible for the development of atherosclerotic lesions.

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