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Comparison of small proteoglycans from skin fibroblasts and vascular smooth-muscle cells

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Physicochemical and chemical properties of small proteoglycans containing galactosaminoglycan chains from cultured human skin fibroblasts and human smooth-muscle cells were compared to determine the extent of structural similarity. The proteoglycan secreted by smooth-muscle cells was of larger molecular size and of higher buoyant density, due to longer glycosaminoglycan chains, than the secretion product of skin fibroblasts. Additionally, both proteoglycans differed in the ratio of iduronic acid and glucuronic acid residues. On the other hand, degradation of secreted [^{3}H]leucine-labelled proteoglycans with chondroitin ABC lyase followed by SDS/polyacrylamide-gel electrophoresis resulted in the appearance of core protein bands of identical size (M_r 48000 and 45000, depending on the number of asparagine-bound oligosaccharides). An M_r value of 40000 was determined for the core protein of cells pretreated with tunicamycin. An antibody against the core protein from fibroblast secretions was cross-reactive with the core protein from smooth-muscle cells. Core protein accumulating intracellularly after treatment with carbonyl cyanide *m*-chlorophenylhydrazone exhibited, on reduction and alkylation, an isoelectric point of 7.8 in both cell types. Limited proteolysis by staphylococcal V8 serine proteinase or endoproteinase Lys-C led in both instances to the formation of peptides of identical size. Peptides bearing asparagine-bound oligosaccharides were free of glycosaminoglycan chains. Similar peptide patterns were obtained when ^{125}I-labelled core proteins were digested with either trypsin or chymotrypsin. Thus small proteoglycans from fibroblasts and smooth-muscle cells can be differentiated by their glycosaminoglycan moieties but not by the nature of their core proteins.

INTRODUCTION

Proteoglycans and their constituent glycosaminoglycan chains are important elements of connective tissues, as they form structural links between the fibrous components of the extracellular matrix and the resident cells (for reviews see Hascall & Hascall, 1981; Muir, 1983). In recent years the existence of a ubiquitous family of galactosaminoglycan-chain-bearing proteoglycans, much smaller than typical cartilage proteoglycans, has been revealed. Such small proteoglycans have been isolated from skin (Damle *et al.*, 1982), aorta (Kapoor *et al.*, 1981; Schmidt *et al.*, 1982; Wagner *et al.*, 1983), cornea (Gregory *et al.*, 1982), sclera (Cöster & Fransson, 1981), cartilage (Heinegård *et al.*, 1981), bone (Franzén & Heinegård, 1984), foetal membranes (Brennan *et al.*, 1984), tendon (Anderson, 1975), periodontal ligament (Pearson & Gibson, 1982), uterine cervix (Uldbjerg *et al.*, 1983) and colon (Iozzo & Wight, 1982). The size of the core protein was reported to be between about M_r 40000 and M_r 50000. Few galactosaminoglycan chains or at the extreme only one chain (Chopra *et al.*, 1985) were found to be covalently linked to the core protein that contained in addition glycoprotein-type oligosaccharides.

The galactosaminoglycan chains of these small proteoglycans may exhibit great differences in chain length and chemical composition. In cartilage, the chains are exclusively (Heinegård *et al.*, 1981) or predominantly (Rosenberg *et al.*, 1985) of chondroitin sulphate type, but they contain a high proportion of iduronic acid in skin and sclera (Damle *et al.*, 1982; Cöster & Fransson, 1981) and were therefore named dermatan sulphate. Glycosaminoglycan chains of the small proteoglycan from aorta may consist of equal proportions of glucuronate-containing and iduronate-containing disaccharides (Fransson & Havsmark, 1970).

It is not yet known whether a single or several distinct genes encode for the core protein of the small proteoglycans. Heinegård *et al.* (1985) found that proteoglycans from bovine aorta and cartilage on one hand, and proteoglycans from cornea, sclera, tendon and bone on the other hand, were immunologically identical and gave similar peptide maps. Partial immunological cross-reactivity was found for proteoglycans from the two groups. On the basis of immunological studies Rosenberg *et al.* (1985) proposed that even within a single tissue, bovine articular cartilage, two different core proteins of similar size may be present.

Previous studies in this laboratory have shown that a small proteodermatan sulphate is a major secretory product of cultured human skin fibroblasts (Glössl *et al.*, 1984). Its core protein (M_r = 38000) bears two or three asparagine-linked oligosaccharides in addition to the galactosaminoglycan chains. *O*-Glycosidically linked oligosaccharides are absent. As cultured smooth-muscle cells retain at least some of their characteristics with respect to proteoglycan biosynthesis (Wight & Ross, 1975; Wight & Hascall, 1983), we have compared structural aspects of small proteoglycans secreted by human skin fibroblasts and human smooth-muscle cells.

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It is shown that the small proteoglycans from both sources differ in their macromolecular properties and in their glycosaminoglycan composition. Their core proteins, however, were indistinguishable by several criteria.

MATERIALS AND METHODS

Materials

The following materials were purchased from the suppliers indicated: $Na_2{}^{35}SO_4$ (25–40 Ci/mg of sulphate) and $Na^{125}I$ (13–17 Ci/mg of iodine) from Amersham–Buchler; L-[4,5-^{3}H]leucine (45 Ci/mmol) and [^{14}C]-methylated M_r standards from New England Nuclear; chondroitin ABC lyase (EC 4.2.2.4) and chondroitin AC lyase (EC 4.2.2.5) from Seikagaku Kogyo; chymotrypsin (EC 3.4.21.1) treated with L-tosyl-lysylchloromethane, trypsin (EC 3.4.21.4) treated with L-tosylphenylalanyl-chloromethane, tunicamycin, carbonyl cyanide *m*-chlorophenylhydrazone and Protein A–Sepharose from Sigma Chemical Co.; endoproteinase Lys-C (suggested classification EC 3.4.21.14) from Boehringer–Mannheim; staphylococcal serine proteinase (from *Staphylococcus aureus* V8; EC 3.4.21.19) from Miles Laboratories; DEAE-Trisacryl and Ampholines from LKB Instrument Co. Polyclonal rabbit antiserum against proteodermatan sulphate from human skin-fibroblast secretions was that used previously (Glössl *et al.*, 1984).

Cell culture

Human skin fibroblasts were maintained in culture as described previously (Cantz *et al.*, 1972). Tissue specimens from human aorta and vena saphena were obtained from the Department of Thorax Surgery of this university by the courtesy of Dr. H. Dittrich and Dr. P. Clajus. Cells were grown from the inner portion of the media and from the intima under analogous conditions. The cells formed monolayers characteristic for smooth-muscle cells, e.g. the cells tended to grow in 'hills' and 'valleys' (Ross *et al.*, 1974). Cells were used for experiments between the third and the eighth passage.

Isolation of small proteoglycans and of their core proteins

To obtain [^{35}S]sulphate-labelled or [^{3}H]leucine-labelled proteoglycans confluent cultures were kept for 6–48 h in modified Waymouth MAB 87/3 medium (Gorham & Waymouth, 1965; as formulated in the GIBCO catalogue) containing the respective radioactive precursors exactly as described previously (Glössl *et al.*, 1984). Unlabelled proteoglycans were prepared from the media of cells that had been incubated for 3–4 days with Waymouth MAB 87/3 medium containing additionally 30 nM-sodium selenite and transferrin (4 μg/ml). All purification steps were done in the presence of the proteinase inhibitors 6-aminohexanoic acid (0.1 M), EDTA (10 mM), *N*-ethylmaleimide (10 mM) and benzamidine hydrochloride (5 mM). [^{3}H]Leucine-labelled proteoglycans were immune-precipitated as described previously (Glössl *et al.*, 1984), with minor modifications (Glössl *et al.*, 1986). Portions of adsorbed proteoglycans were then treated with 20 munits of chondroitin ABC lyase or buffer alone (Saito *et al.*, 1968) in the presence of proteinase inhibitors and processed for SDS/polyacrylamide-gel electrophoresis as described previously (Glössl *et al.*, 1984). Alternatively, the material was solubilized by heating in 1% (w/v) SDS in water, and the supernatant was dried in a Speed Vac Concentrator (Bachofer, Reutlingen, W. Germany) by centrifugation under reduced pressure. SDS and salt were removed by washing with methanol. For the preparation of unlabelled core protein culture media were mixed with proteinase inhibitors and made 0.1% with respect to Triton X-100 and 20 mM with respect to Tris/HCl, pH 7.4, before chromatography on a DEAE-Trisacryl column (10 ml bed volume/100 ml of medium) equilibrated with 20 mM-Tris/HCl buffer, pH 7.4, containing proteinase inhibitors and 0.1% Triton X-100 (buffer A), and 0.15 M-NaCl. The column was eluted stepwise with 3 vol. each of 0.15 M-NaCl, 0.3 M-NaCl and 1.0 M-NaCl, all in buffer A. Appropriate fractions from the last step were pooled, dialysed against 0.1% Triton X-100 and freeze-dried. The detergent was removed by washing with methanol. Samples from smooth-muscle cells were dissolved in 50 mM-sodium acetate buffer, pH 5.8, containing 4 M-guanidinium chloride, 0.5% Triton X-100 and proteinase inhibitors before chromatography on a 1 cm × 151 cm column of Sepharose CL-4B equilibrated with the same buffer. Fractions corresponding to the elution volumes of labelled small proteoglycans were pooled, dialysed against 0.1% Triton X-100 and further processed as above. For the final preparation of core proteins samples from both fibroblasts and smooth-muscle cells were digested with 50 munits of chondroitin ABC lyase and then subjected to preparative SDS/polyacrylamide-gel electrophoresis. Gel areas corresponding to the size of the core proteins of the small proteoglycans were excised and subjected to electro-elution (Strålfors & Belfrage, 1983). The purity of the preparation was tested by SDS/polyacrylamide-gel electrophoresis and staining with $AgNO_3$ (Merril *et al.*, 1981).

Characterization of intact proteoglycans and glycosaminoglycan chains

[^{35}S]Sulphate-labelled proteoglycans from the culture medium were chromatographed on a Sepharose CL-4B column as described above. Appropriate fractions were pooled, dialysed against 0.1% Triton and freeze-dried. For density-gradient centrifugation the material was first dissolved in 2 ml of water containing 100 μg of chondroitin sulphate. After the addition of 8 ml of ethanol and 100 mg of potassium acetate, Triton X-100-free proteoglycan was obtained in the precipitate, which was dissolved in 50 mM-sodium acetate buffer, pH 5.8, containing 4 M-guanidinium chloride and proteinase inhibitors. CsCl was added to give a density of 1.40 g/ml, and the solution was centrifuged at 107000 $g_{av.}$ for 63 h at 10 °C. The centrifuge tubes were frozen in liquid N_2 and cut into 11 fractions for density and radioactivity determinations.

Fractions from the Sepharose CL-4B column were also subjected to a β-elimination reaction (0.15 M-NaOH, 4 h at 37 °C). After neutralization with acetic acid, drying and washing with methanol, the samples were dissolved in water. For determinations of molecular size a portion of the sample was chromatographed on a calibrated Sephacryl S-300 column (1 cm × 98 cm) equilibrated and eluted with 4 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8, containing 0.1% Triton. The composition of glycosaminoglycan chains was calculated after parallel digestion with chondroitin AC lyase (50 munits, 16 h at 37 °C) and chondroitin ABC lyase (50 munits, 30 min at 37 °C) followed by descending

paper chromatography in butan-1-ol/acetic acid/1 M-NH_3 (2:3:1, by vol.) as described by Saito *et al.* (1968). The paper was cut into 1 cm segments that were eluted with 2 ml of water before liquid-scintillation counting. The distribution of D-glucuronic acid residues along the glycosaminoglycan chains was analysed by degradation with chondroitin AC lyase and chromatography of the products on a Sephadex G-50 column (1 cm × 147 cm) equilibrated and eluted with 10 mM-Tris/HCl buffer, pH 7.2, containing 1% SDS and 1 mM-NaN_3.

Characterization of proteoglycan core proteins

For isoelectric focusing of glycosaminoglycan-free core protein the property of carbonyl cyanide *m*-chlorophenylhydrazone in blocking the transport of secretory macromolecules at the level of the endoplasmic reticulum was used (Tartakoff & Vassalli, 1977). Cells were preincubated for 1 h with the drug (50 μM) and then labelled with [^{3}H]leucine (35 μCi/ml) and [^{35}S]sulphate (20 μCi/ml) for 4 h in the continuous presence of the drug. Cell-associated core proteins and proteoglycans were extracted (Glössl *et al.*, 1984) and isolated by immune precipitation as described above. Antigenic material was solubilized with 8 M-urea in 10 mM-sodium phosphate buffer, pH 8.0 (2 h at 37 °C). After removal of Protein A–Sepharose the samples were reduced and treated for 30 min with 30 mM-iodoacetamide to avoid re-formation of immune complexes. After dialysis against 0.1% Triton X-100 samples of 1.5 ml containing 7 M-urea, 10% (v/v) glycerol, 1% (v/v) Ampholine, pH 5–8, and 1% (v/v) Ampholine, pH 7–9, were made 5% with respect to acrylamide (bisacrylamide = 3% of total acrylamide) and polymerized in 5 mm tubes. Focusing was performed for 5.5 h at 4 °C, 0.12 W being applied. At the end of the run the gel was frozen and cut in 1.6 mm slices, which were equilibrated with 2 mM-KCl for pH determination and then solubilized with 30% (v/v) H_2O_2/NH_3 (99:1, v/v) (6 h at 50 °C) for radioactivity measurements.

For iodination core proteins equivalent to 0.2 μmol of hexuronic acid with skin-fibroblast secretions and 0.1 μmol hexuronic acid with secretions from arterial smooth-muscle cells were prepared by preparative SDS/polyacrylamide-gel electrophoresis and electro-elution. Freeze-dried protein was dissolved in 100 μl 0.2 M-sodium phosphate buffer, pH 7.0, and incubated for 30 min at room temperature with 200 μCi of Na^{125}I (3 μl) and one Iodobead (Pierce Chemical Co.). The bead was removed, and the reaction was quenched by adding 50 μl of 10 mM-$NaHSO_3$ and 100 μl of 60 mM-KI in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and 500 μg of cytochrome *c*. After sequential dialysis against 24 mM-KI in 0.1% Triton X-100, 0.1% Triton X-100 and SDS sample buffer, the retained dialysis residue was treated with 1 mg of dithioerythritol for 30 min at 56 °C and subjected to SDS/polyacrylamide-gel electrophoresis. After auto-radiographic localization of core-protein bands in the fixed and dried gel, appropriate areas were cut out, pulverized with a spatula and subjected to proteolytic digestion. For trypsin treatment the samples were incubated with 1 ml of 32 mM-ammonium bicarbonate buffer, pH 8.5, containing 50 μg of trypsin and 200 μg of bovine serum albumin for 18 h at 37 °C. Chymotryptic degradation was performed analogously except that 100 μg of enzyme in a 50 mM buffer was used. After 6 h, the same amount of the respective proteinases was added. The samples were then centrifuged, and the supernatants

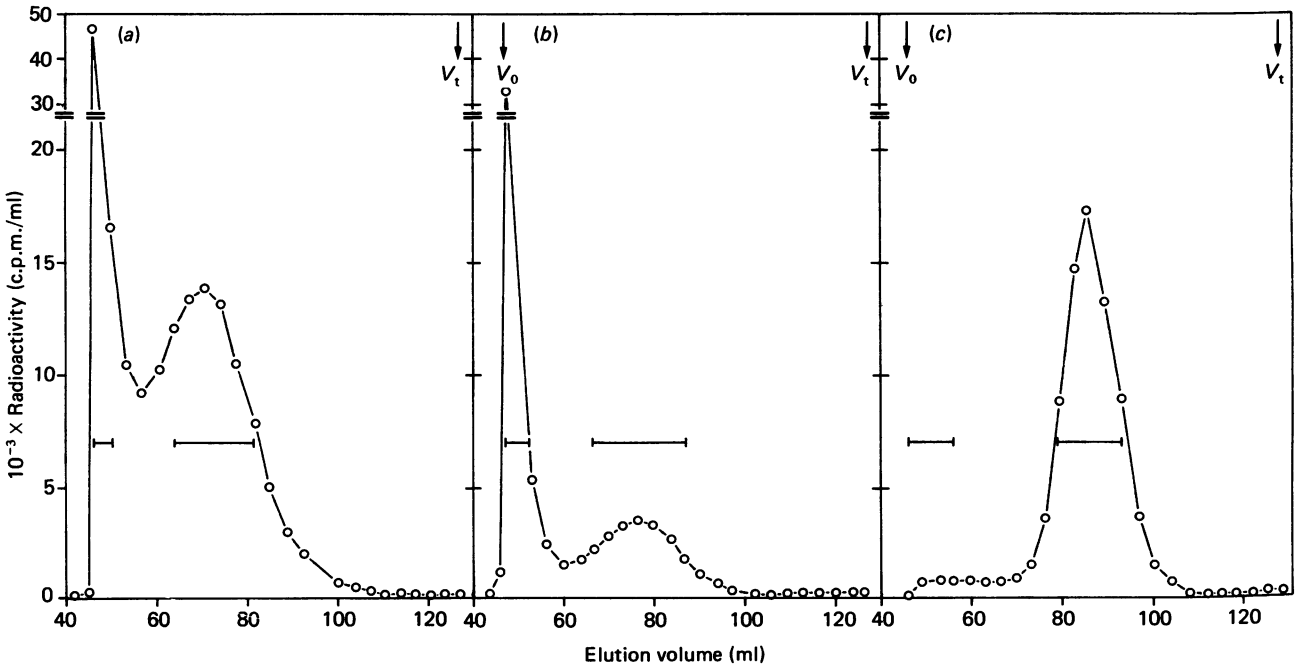

Fig. 1. Gel chromatography on Sepharose CL-4B of [^{35}S]sulphate-labelled proteoglycans from the secretions of venous smooth-muscle cells (*a*), arterial smooth-muscle cells (*b*) and skin fibroblasts (*c*)

Proteoglycans were obtained after 48 h of incubation with 10 μCi of [^{35}S]sulphate/ml. Pools used for further experiments are indicated by the bars. V_0, void volume; V_t, total volume.

Table 1. Relative compositions of glycosaminoglycan chains from fibroblast and smooth-muscle-cell secretions

Confluent cultures were labelled for 48 h in the presence of 10 μCi of [^{35}S]sulphate/ml. Proteoglycans were precipitated by $(NH_4)_2SO_4$ and chromatographed on a Sepharose CL-4B column. Glycosaminoglycan chains from appropriate fractions (see Fig. 1) were liberated by β-elimination and incubated with either chondroitin AC lyase or chondroitin ABC lyase before descending paper chromatography. Details are given in the Materials and methods section. Abbreviations: DS, dermatan sulphate; C-4-S, chondroitin 4-sulphate; C-6-S, chondroitin 6-sulphate; ΔDiS, unsaturated disaccharides.

	Composition (% of total radioactivity)				4-Sulphated ΔDiS*
	Chondroitin ABC lyase-resistant	DS	C-4-S	C-6-S	6-sulphated ΔDiS
Small proteoglycan					
Fibroblasts	4	87	5	3	4.24
Arterial smooth-muscle cells	6	62	18	15	3.25
Venous smooth-muscle cells	6	50	22	22	2.68
Large proteoglycan					
Fibroblasts	18	24	21	38	0.81
Arterial smooth-muscle cells	2	10	23	65	0.41
Venous smooth-muscle cells	3	12	29	55	0.65

* Determined after digestion with chondroitin ABC lyase.

were dried under N_2, dissolved in water and dried again. Peptides were dissolved in a small volume of acetic acid/formic acid/water (3:1:16, by vol), applied to a 20 cm × 20 cm cellulose F plastic sheet (Merck) and subjected to electrophoresis in the first dimension followed by t.l.c. in the second dimension as described by Elder *et al.* (1977). Fuchsin was used as an internal standard.

For identification of peptides bearing asparagine-bound oligosaccharides and/or glycosaminoglycan chains [^{3}H]leucine-labelled small proteoglycans from cultures incubated in the presence (1 μg/ml) or in the absence of tunicamycin were subjected to limited proteolysis. For degradation by staphylococcal serine proteinase, the incubation mixture contained, in a final volume of 80 μl, 20000–100000 c.p.m. of proteoglycan, 0.5 μg of enzyme, 62.5 mM-Tris/HCl buffer, pH 7.8, 0.25 mM-EDTA, and 0.1% SDS. After 3 h at 37 °C, the sample was made 15 mM with respect to benzamidine hydrochloride and loaded on a 0.2 ml DEAE-Trisacryl column prepared in a Pasteur pipette. The resin had been equilibrated with buffer A containing 0.15 M-NaCl except that the concentration of benzamidine hydrochloride had been increased to 15 mM. Unbound material (fraction A) was eluted with 800 μl of 0.2 M-NaCl in buffer A, whereas glycosaminoglycan-bearing peptides were desorbed with 1.2 ml of 1 M-NaCl in buffer A (fraction B). Bovine serum albumin (10 μg) was added to fraction A before it was dried in a Speed Vac concentrator, washed successively with 20% (w/v) trichloroacetic acid and ethanol and then subjected to SDS/polyacrylamide-gel electrophoresis. Fraction B was dialysed against 0.1% Triton X-100, concentrated, washed with methanol and subjected to digestion with chondroitin ABC lyase before electrophoresis.

The mixture for degradation by endoproteinase Lys-C contained proteoglycan (10000–50000 c.p.m.), 0.4 unit of enzyme, 50 mM-ammonium bicarbonate buffer, pH 8.6, 1 mM-EDTA, and 0.1% SDS in a final volume of 20 μl. After 3 h at 37 °C the sample was made 2.5 mM with respect to L-tosyl-lysylchloromethane and then processed as described for proteinase-V8-generated peptides.

Other methods

SDS/polyacrylamide-gel electrophoresis [Laemmli (1970), as modified by Hasilik & Neufeld (1980)] followed by fluorography (Bonner & Laskey, 1974) were

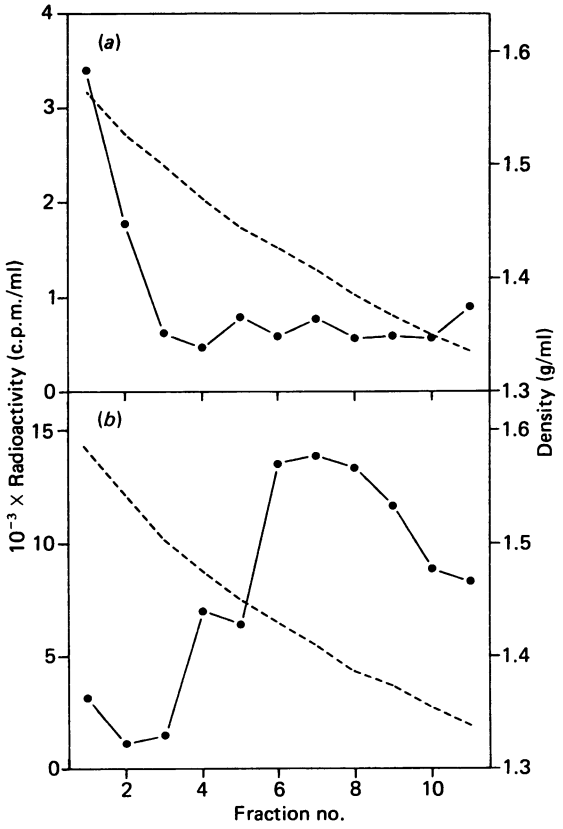

Fig. 2. Density-gradient centrifugation of [^{35}S]sulphate-labelled small proteoglycans from the secretions of venous smooth-muscle cells (*a*) and skin fibroblasts (*b*)

Small proteoglycans were obtained after gel chromatography on a Sepharose CL-4B column as described in the legend to Fig. 1. The broken line indicates the density gradient.

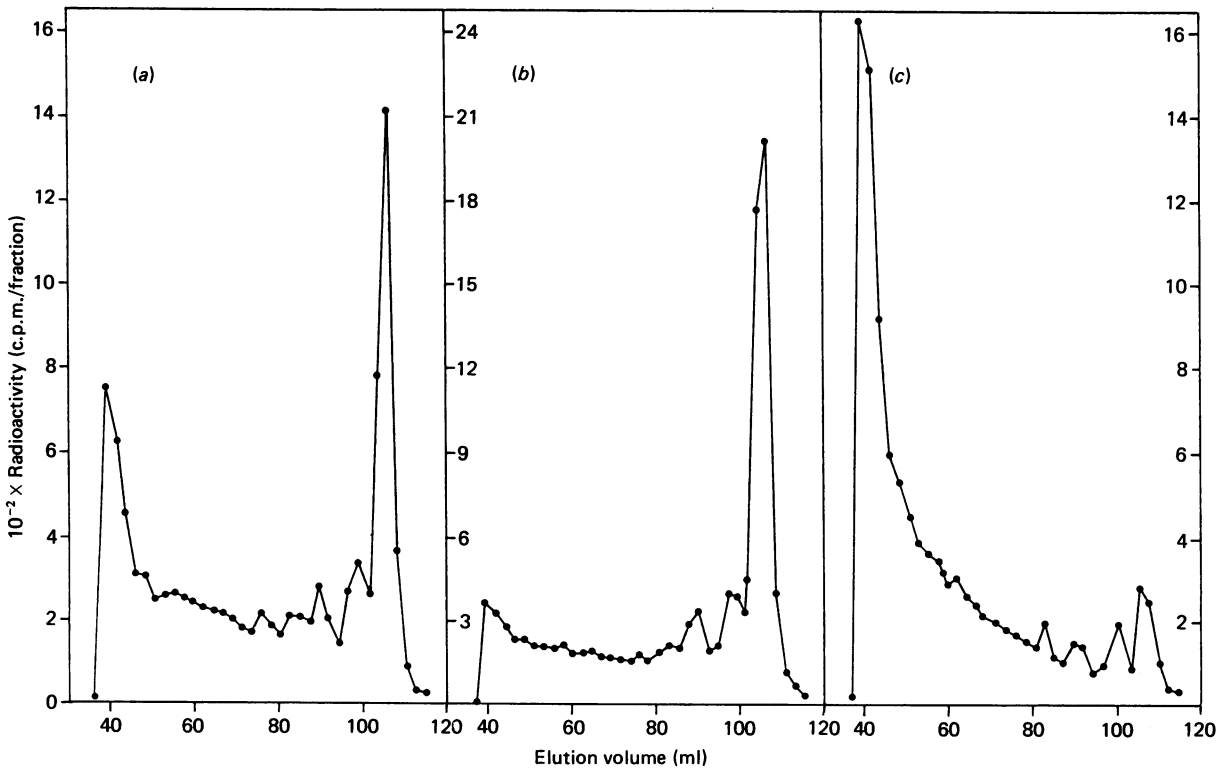

Fig. 3. Gel chromatography on Sephadex G-50 of [^{35}S]sulphate-labelled glycosaminoglycan fragments obtained from the secretions of arterial smooth-muscle cells (*a*), venous smooth-muscle cells (*b*) and skin fibroblasts (*c*)

Small proteoglycans were obtained as indicated in the legend to Fig. 1. Glycosaminoglycan chains were released by a β-elimination reaction and treated with chondroitin AC lyase.

performed as described previously. ^{125}I in gels and on thin-layer plates was detected by means of a du Pont Cronex Lightning-Plus intensifying screen. Hexuronic acids were quantified by the method of Bitter & Muir (1962).

RESULTS

Characterization of intact proteoglycans and glycosaminoglycan chains

Small [^{35}S]sulphate-labelled proteoglycans from secretions of skin fibroblasts and smooth-muscle cells differed remarkably in their hydrodynamic size as judged by chromatography on Sepharose CL-4B under dissociative conditions (Fig. 1). The retarded peak, which represents predominantly galactosaminoglycan-containing proteoglycans (see below), was eluted with mean $K_{av.}$ values of 0.28, 0.33 and 0.44 with venous smooth-muscle cells, arterial smooth-muscle cells and fibroblast secretions respectively. Additionally, marked differences in the proteoglycan distribution pattern were observed. In contrast with skin fibroblasts, the main secretion product of smooth-muscle cells eluted in the exclusion volume of Sepharose CL-4B. All further results, except that described in Table 1, were obtained by investigating the small-sized proteoglycans that would be retarded by Sepharose CL-4B.

On density-gradient centrifugation proteoglycan from venous smooth-muscle cells was found in the bottom fraction (density > 1.55 g/ml) whereas proteodermatan sulphate from fibroblasts exhibited a density of about 1.4 g/ml (Fig. 2). This result suggests that the product of smooth-muscle cells exhibits a higher carbohydrate/protein ratio than that of fibroblasts. The glycosaminoglycan chains of the small proteoglycans could indeed be distinguished by their molecular size. Chains liberated from the core protein by a β-elimination reaction were eluted from a Sephacryl S-300 column as symmetrical peaks with mean $K_{av.}$ values of 0.13, 0.14 and 0.23 for glycosaminoglycans from venous smooth-muscle cells, arterial smooth-muscle cells and fibroblasts respectively (results not shown). This would correspond to mean M_r values of 59000, 56000 and 40000 respectively. It should be noted, however, that only standards with M_r values of 12400, 19000, and 37000 were available for calibration.

Glycosaminoglycans from the different sources differed not only in chain length but also in the structure of the repetitive disaccharide units (Table 1). As shown previously (Hoppe *et al.*, 1985), the small proteoglycan from skin fibroblasts contained predominantly disaccharides of the type L-IdoA-1,3-GalNAc-4-sulphate. A much greater content of glucuronic acid was calculated to be present in the corresponding glycosaminoglycans from smooth-muscle cells. A greater proportion of *N*-acetylgalactosamine 6-sulphate residues was found additionally. Proteoglycans eluted in the exclusion volume of the Sepharose CL-4B column were rich in glucuronic acid. However, the material from skin fibroblasts contained twice as much iduronic acid as that from smooth-muscle cells. In all pools, the amount of material resistant to degradation by chondroitin ABC lyase was so small that the outcome of density-gradient

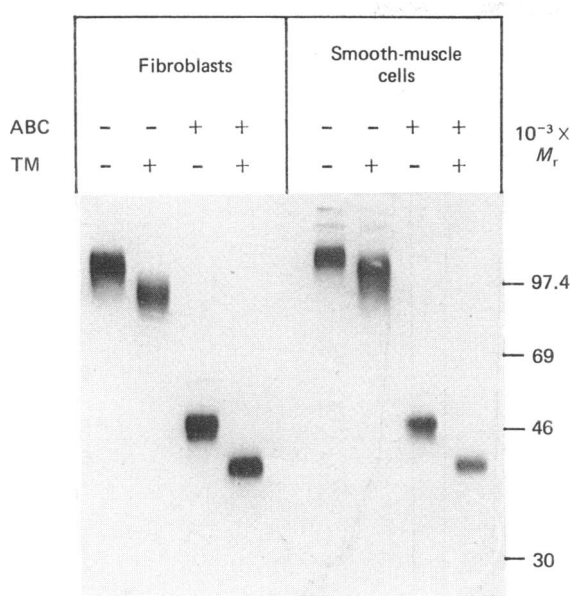

Fig. 4. Gel electrophoreses in the presence of SDS of [^{3}H]leucine-labelled small proteoglycan from arterial-smooth-muscle-cell and fibroblast secretions

Cultures were preincubated for 15 h in the presence of tunicamycin (TM, 1.0 μg/ml) or dimethyl sulphoxide (70 mM), the solvent of the drug. Labelling with [^{3}H]leucine (40 μCi/ml) was for 6 h in the continuous presence of tunicamycin. Small proteoglycans were isolated by immune precipitation and treated with chondroitin ABC lyase (ABC) or buffer alone before SDS/polyacrylamide-gel electrophoresis. The total acrylamide concentration of the separation gel was 15.2%. [^{14}C]Methylated M_r standards were phosphorylase *b* (M_r 97400), bovine serum albumin (M_r 69000), ovalbumin (M_r 46000), carbonic anhydrase (M_r 30000) and cytochrome *c* (M_r 12300).

centrifugation and chain-size determination should not have been significantly influenced by the presence of proteoheparan sulphate.

The distribution of glucuronic acid residues along the glycosaminoglycan chains of small proteoglycans was studied by digestion with chondroitin AC lyase followed by chromatography on Sephadex G-50. It can be deduced from Fig. 3 that the glycosaminoglycan chains of fibroblast proteoglycans contain only minor amounts of clusters of glucuronic acid-containing disaccharides. Disaccharides are released by the enzyme only if either glucuronic acid is the antepenultimate sugar at the non-reducing end of the chain or if blocks of chondroitin sulphate-type disaccharides are present. In contrast, small proteoglycans of smooth-muscle cells contain repetitive chondroitin sulphate-type disaccharides but also single disaccharides of this type along the chains.

Characterization of core proteins

Immunological cross-reactivity. Polyclonal antibodies against the core protein of the small proteoglycan from fibroblast secretions were well suited for immune precipitation of the small proteoglycan from secretions of smooth-muscle cells. On labelling with [^{3}H]leucine small proteoglycans could be immune-precipitated from the media of both cell types. They exhibited a slower mobility than phosphorylase *b* (M_r 97400) during SDS/polyacrylamide-gel electrophoresis (Fig. 4). Exhaustive digestion with chondroitin ABC lyase either before or after antibody treatment resulted in the appearance of core proteins with M_r values of 48000 and 45000 respectively. An M_r value of 40000 was found when the cells were treated with tunicamcyin to inhibit the formation of asparagine-linked oligosaccharides. In fibroblasts an M_r value of 38000 was determined previously for the intracellularly located core protein containing neither dermatan sulphate stubs nor *N*-linked

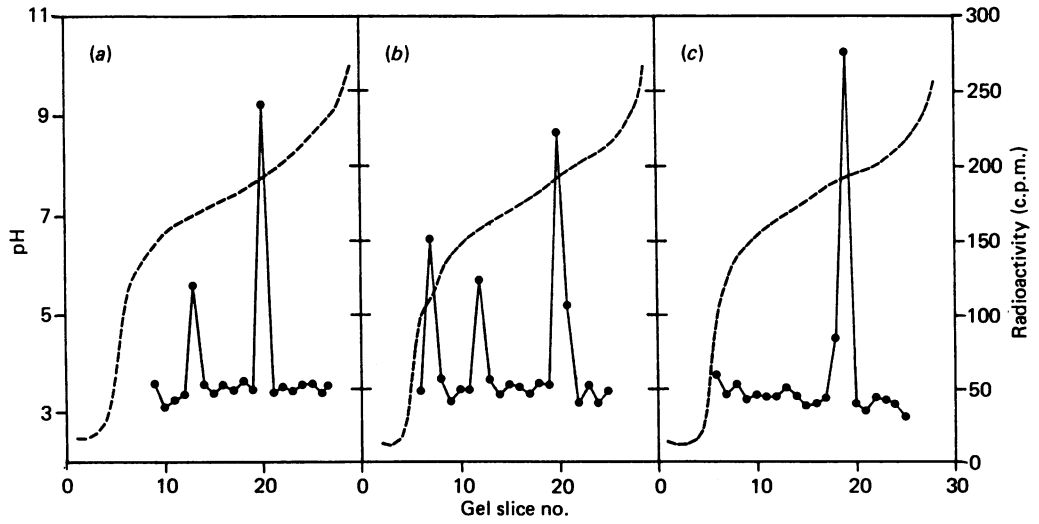

Fig. 5. Isoelectric focusing of [^{3}H]leucine-labelled core protein from arterial smooth-muscle cells (*a*), venous smooth-muscle cells (*b*) and skin fibroblasts (*c*)

Cultures were preincubated for 1 h in the presence of 50 μM-carbonyl cyanide *m*-chlorophenylhydrazone and then incubated for 4 h with 35 μCi of [^{3}H]leucine/ml, the drug being present continuously. [^{35}S]Sulphate (20 μCi/ml) was included for control purposes. The broken line indicates the pH gradient.

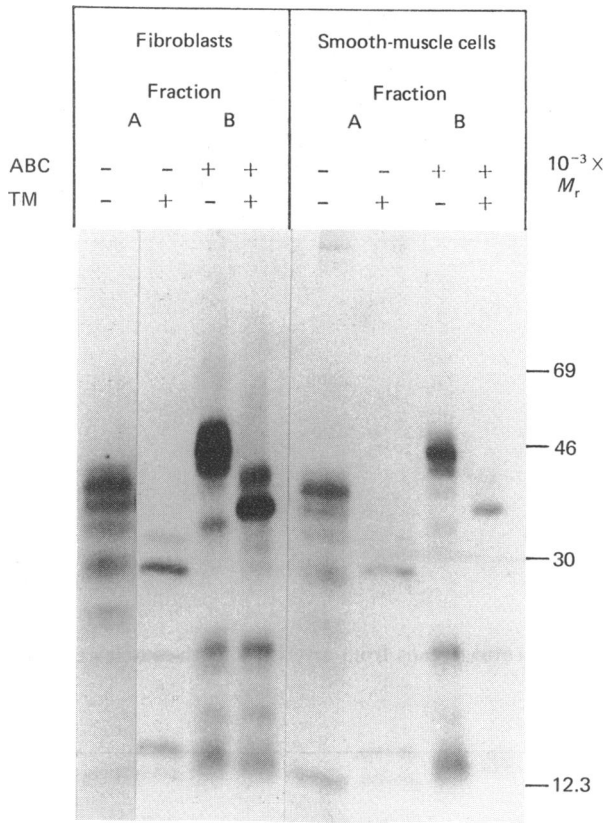

Fig. 6. Peptide patterns of core proteins of small proteoglycans from arterial-smooth-muscle-cell and fibroblast secretions after partial proteolysis by endoproteinase Lys-C

[^{3}H]Leucine-labelled small proteoglycans from the media of cultures that had been kept in the presence or in the absence of tunicamycin were isolated as described in the legend to Fig. 4. After proteolysis, the digest was separated on DEAE-Trisacryl into peptides without (fraction A) or with (fraction B) glycosaminoglycan chains. The latter fraction was treated with chondroitin ABC lyase before polyacrylamide-gel electrophoresis in the presence of SDS. The acrylamide concentration of the separation gel was 15.2%.

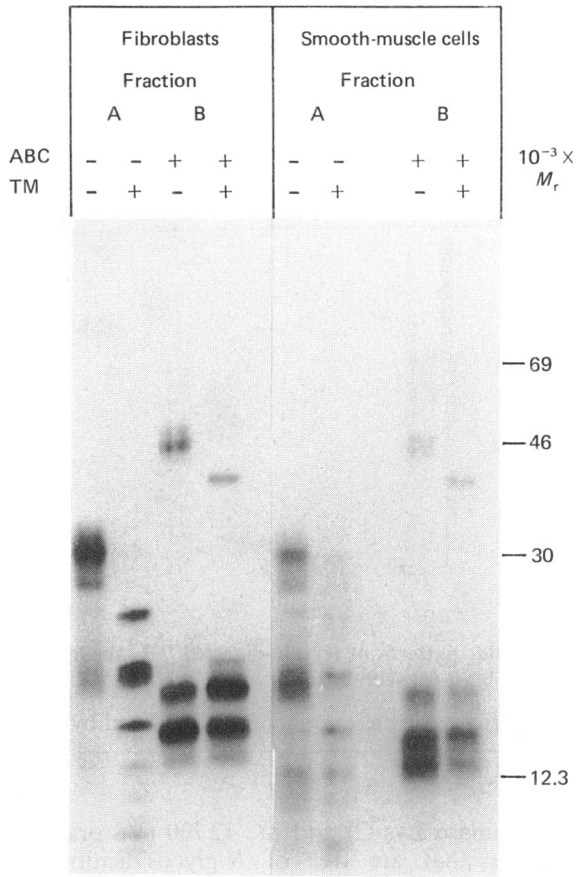

Fig. 7. Peptide patterns of core proteins of small proteoglycans from arterial-smooth-muscle-cell and fibroblast secretions after partial proteolysis by staphylococcal V8 proteinase

[^{3}H]Leucine-labelled proteoglycans were isolated as described in the legend to Fig. 4 and processed analogously to the description given in the legend to Fig. 6.

oligosaccharides (Glössl *et al.*, 1984). Immune precipitation resulted in the isolation of a representative species of small proteoglycans. When [^{35}S]sulphate-labelled proteoglycans were repeatedly treated with antibody-coated Protein A–Sepharose, the first precipitation step was at least 90% complete regardless of the source of proteoglycan. Chromatography of unbound material on a Sepharose CL-4B column did not reveal a peak of small proteoglycan (result not shown).

Isoelectric focusing. Core protein obtained by treatment of intact proteoglycan with chondroitin ABC lyase is unsuited for isoelectric focusing since glycosaminoglycan stubs remain attached to the core protein. We therefore took advantage of the fact that treatment of cells with carbonyl cyanide *m*-chlorophenylhydrazone leads to an intracellular accumulation of core protein (Glössl *et al.*, 1986). After incubation of cells in the presence of [^{3}H]leucine core protein from the cell layer was immune-precipitated and after reduction and treatment with iodoacetamide subjected to isoelectric focusing. Fig. 5 shows that most of the ^{3}H-labelled material from both cell types exhibited a pI of 7.8. This pI, however, is that of a reduced and alkylated protein, since the immune complexes had to be destroyed irreversibly. Cross-reactive material from smooth-muscle cells contained additional proteins of lower pI. Material with a pI of 5.3 was also labelled with [^{35}S]sulphate and may therefore represent an intermediate of the chain elongation process.

Limited proteolysis. [^{3}H]Leucine-labelled proteoglycans were isolated from the media of fibroblasts and smooth-muscle cells by immune precipitation and subjected to limited proteolysis by endoproteinase Lys-C and staphylococcal V8 serine proteinase. Peptides bearing glycosaminoglycan chains were separated from the other peptides by ion-exchange chromatography before the polysaccharide was removed by chondroitin ABC lyase. Though the extent of proteolysis differed somewhat in different preparations, the size of the peptides generated by both proteinases was indistinguishable for core proteins from fibroblasts and smooth-muscle cells (Figs. 6 and 7).

Comparing the peptide patterns of core proteins with and without asparagine-linked oligosaccharides, the following conclusions could be drawn. Glycosaminoglycan chains were located on peptides of M_r 12500

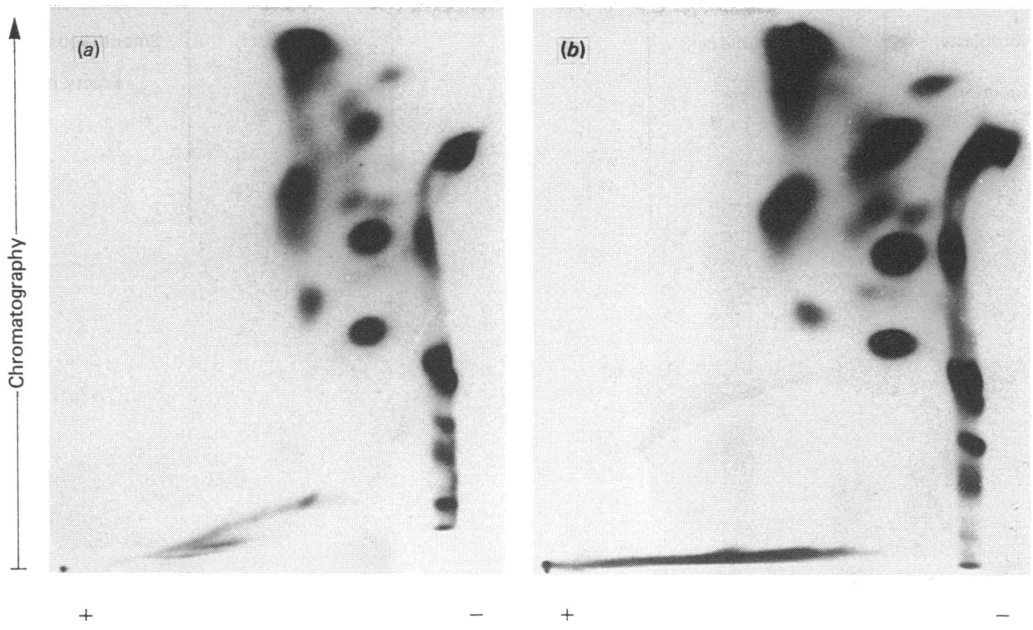

Fig. 8. Peptide patterns of trypsin-digested ^{125}I-labelled core proteins of small proteoglycans from arterial-smooth-muscle-cell (*a*) and fibroblast (*b*) secretions

The polarity during electrophoresis is indicated by the + and − signs.

(endoproteinase Lys-C) and M_r 12700 (V8 proteinase) respectively that are free of *N*-glycosidically linked oligosaccharides. Peptides of this size were found to be retained by DEAE-Trisacryl before chondroitin ABC lyase treatment. Conversely, the largest peptides not being adsorbed on the ion-exchange matrix must be free of glycosaminoglycan chains. In the case of degradation by endoproteinase Lys-C such peptides from control cultures exhibited an M_r value of 37000 and those from tunicamycin-treated cultures an M_r of 30500. On the other hand, peptides with M_r values of 19500 (endoproteinase Lys-C, fraction B), 16800 (V8 proteinase, fraction B), and most probably also of 26900 (endoproteinase Lys-C, fraction A), are free of asparagine-linked oligosaccharides, since they were generated from both tunicamycin-treated and untreated cultures. These results suggest that the core protein of the small proteoglycan from fibroblasts and smooth-muscle cells contains distinct domains bearing either glycosaminoglycan chains or asparagine-bound oligosaccharides.

Peptide mapping. Core protein was prepared without employing immunological procedures, iodinated and exhaustively digested with trypsin and with chymotrypsin. The peptides were separated by two-dimensional high-voltage electrophoresis/t.l.c. The patterns observed for the small proteoglycans from fibroblast and smooth-muscle cell secretions were remarkably similar to each other (Figs. 8 and 9).

DISCUSSION

Comparative studies on the core proteins of different proteoglycan species from mesenchymal tissues are hampered by their relatively long half-life, which makes possible a random attack by tissue proteinases (see, e.g., Werb & Dingle, 1976), and by their tight association with other macromolecules. In contrast, proteoglycans can easily be purified from tissue-culture media, and evidence for proteolysis of core proteins in conditioned culture medium has not yet been obtained. During culture, however, cells may de-differentiate and lose their ability to express a tissue-specific proteoglycan type. For example, chondrocytes produce type II collagen and cartilage-specific proteoglycans only under special tissue-culture conditions (Glaser & Conrad, 1984).

Physicochemical and chemical properties of the small proteoglycans from fibroblast and smooth-muscle-cell secretions were clearly different from each other. The product of smooth-muscle cells is of larger molecular size and bears longer glycosaminoglycan chains, which have a higher proportion of glucuronic acid and *N*-acetylgalactosamine 6-sulphate residues than the counterpart from skin fibroblasts. The small proteoglycan from bovine arterial tissue was similar to the small proteoglycan from human smooth-muscle cells with respect to total size, length of the glycosaminoglycan chains and the glucuronic acid/iduronic acid ratio (Schmidt *et al.*, 1982). In accordance with previous studies on glycosaminoglycans from primate arteries (Wight & Ross, 1975) we therefore conclude that smooth-muscle cells express distinct structural features of their proteoglycans when cultured *in vitro*. There are some ambiguities with regard to the characterization of glycosaminoglycan chains by enzymic degradation alone. Galactosaminoglycan sequences may resist degradation by chondroitin AC lyase in spite of a low iduronic acid content (Yanagishita *et al.*, 1979). However, repeated addition of this enzyme and prolonged incubation did not result in an increased production of unsaturated disaccharides.

In spite of the differences of the glycosaminoglycan moieties, the core proteins of small proteoglycans from fibroblasts and smooth-muscle cells could not be distinguished. Both were immunologically related, they

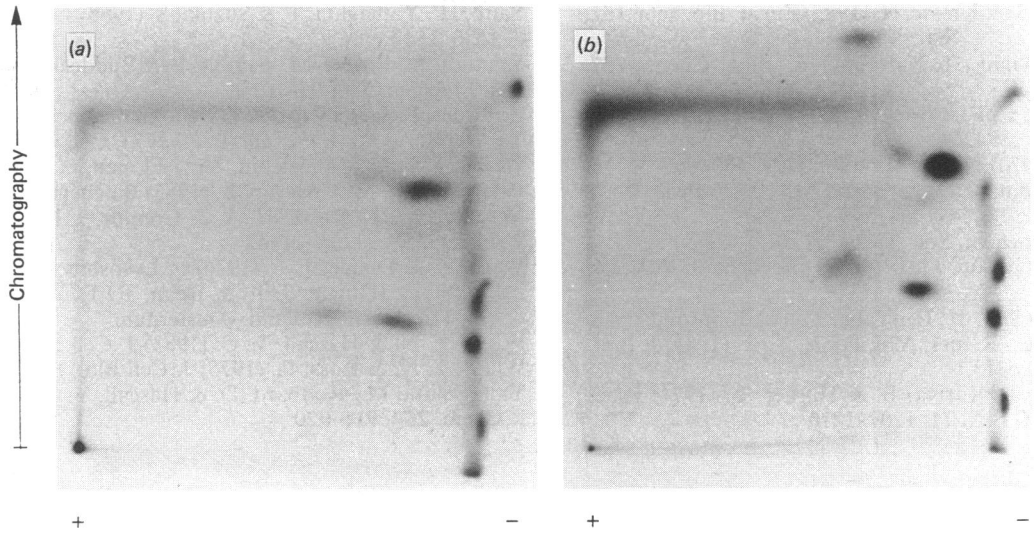

Fig. 9. Peptide patterns of chymotrypsin-digested ^{125}I-labelled core proteins of small proteoglycans from arterial-smooth-muscle-cell (*a*) and fibroblast (*b*) secretions

The polarity during electrophoresis is indicated by the + and − signs.

were of identical size and isoelectric point, and both contained the same number of asparagine-bound oligosaccharides. Limited proteolysis by either staphylococcal V8 serine proteinase or endoproteinase Lys-C of [^{3}H]leucine-labelled core proteins as well as exhaustive proteolysis by trypsin or chymotrypsin of ^{125}I-labelled proteins resulted in very similar patterns. These results, however, do not provide final proof of their identity, which must await amino acid sequencing.

Heinegård *et al.* (1985) proposed the existence of two groups of small proteoglycans in bovine tissues, arterial and cartilage proteoglycans belonging to a different group than those from tendon, sclera, cornea and bone. The homogeneity of core proteins, however, had not been rigorously proven, and the possible occurrence of the artifacts mentioned above not been excluded. Although the results described in the present paper favour the assumption of identical core proteins in skin and vessel walls, they do not exclude the possibility that different classes of small proteoglycans exist in other tissues.

Considering the core proteins in the two cell types as identical, the differences in the chemical composition of the glycosaminoglycan chains could not be explained by the assumption that the actual activity of polymerizing or modifying enzymes is affected by the nature of the core protein. Alternatively, different cell types may be characterized by different ratios of the various enzymes or by variations in the transit time of proteoglycans in certain subcellular compartments. Studies on the influence of monensin on the biosynthesis of the small proteoglycan in fibroblasts suggested that chain polymerization and 6-sulphation occur predominantly in a different subcompartment of the Golgi apparatus from that in which epimerization of glucuronic acid and 4-sulphation occur (Hoppe *et al.*, 1985). The increased chain length and the higher *N*-acetylgalactosamine 6-sulphate/*N*-acetylgalactosamine 4-sulphate and D-glucuronic acid/L-iduronic acid ratios in smooth-muscle cell proteoglycan could then be explained by the assumption that it resides for a longer period of time in the first subcompartment than does the corresponding product of fibroblasts.

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