Proteoglycans from pig aorta

Comparative study of their interactions with lipoproteins

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Different proteoglycans (PGs) were isolated from pig aorta for aggregation studies with hyaluronic acid and human low-density lipoproteins (LDL). Extraction of the intima-media with 4M-guanidinium chloride and digestion of the residue with collagenase solubilized 91% of aortic hexuronic acid content. From the guanidinium chloride extract two PGs were isolated by ion-exchange and gel-permeation chromatography: proteochondroitin sulphate (PGI) with a protein-core apparent M_r of 250000 and proteodermatan-chondroitin sulphate (PGII) with a protein-core apparent M_r of 55000. Only PGI forms high- M_r aggregates with hyaluronic acid. From the collagenase digest two other PGs were isolated: proteoheparan sulphate and proteochondroitin sulphate (PGIII and PGIV respectively). PGIV had a smaller hydrodynamic size than PGI. PGI and PGII formed insoluble complexes with human LDL in the presence of Ca^{2+} . PGIII or PGIV did not form precipitates with the LDL. PGI and PGII, but neither PGIII nor PGIV, were bound to LDL-Sepharose. The main peaks of PGI and PGII were eluted from LDL-Sepharose with 60 mM- and 90 mM-NaCl respectively. The results indicate that aortic PGs have different interacting potentials with lipoproteins, depending on their M_r and their glycosaminoglycan composition.

INTRODUCTION

Arterial extracellular matrix contains elastin and collagen fibres, structural glycoproteins and PGs. PGs play an important role in the physiology of the vascular wall, in maintaining its structural integrity and regulating the permeability of the tissue. The arterial PGs isolated from different species are heterogeneous by size (Salisbury & Wagner, 1981), composition (Horn *et al.*, 1983) and turnover (Schmidt *et al.*, 1982).

The extraction of the intima media with dissociating agents solubilized mainly the proteogalactosaminoglycans and small amounts of proteoheparan sulphate (Oegema *et al.*, 1979). This proteoheparan sulphate was extracted in a higher yield after controlled collagenase/elastase digestion (Vijayagopal *et al.*, 1983). The major PGs containing chondroitin sulphate and dermatan sulphate were extracted from the intima-media of bovine (Oegema *et al.*, 1979; Kapoor *et al.*, 1981), human (Salisbury & Wagner, 1981) and pigeon (Wagner *et al.*, 1982) aortas with 4 M-GdnHCl in the presence of proteinase inhibitors. These PGs could be separated into different fractions: the high- M_r proteochondroitin sulphate and the proteodermatan sulphate (Salisbury & Wagner, 1981). The proteochondroitin sulphate aggregated with hyaluronic acid *in vitro* to form high- M_r complexes (Oegema *et al.*, 1979; Wagner *et al.*, 1983). The proteodermatan sulphate isolated from pigeon aorta did not aggregate with hyaluronic acid (Wagner *et al.*, 1983).

Certain GAGs and PGs are able to interact with lipoproteins (for review see Camejo, 1982). The reaction mechanism between LDL and GAGs can be conceived of as an ionic binding (Radhakrishnamurthy *et al.*, 1982). In comparative studies of GAG-lipoprotein interactions Iverius (1972) showed that the affinity of GAG-substituted

agarose for LDL was dependent on the structure and charge of glycan chains. Within the same type of GAG, the M_r , the charge density and the primary sequence of the chain (e.g. iduronic acid-rich clusters in the dermatan sulphate molecule) account for the differences in the affinity of GAGs for LDL (Fransson & Havsmark, 1981, 1982; Mourao & Bracamonte, 1984).

The interactions of aortic PGs with LDL are less well understood. In the presence of bivalent ions and at low ionic strength bovine aortic chondroitin sulphate-dermatan sulphate proteoglycans formed insoluble complexes with human LDL. In the absence of bivalent ions and at physiological salt concentration, the soluble complexes predominate (Vijayagopal *et al.*, 1981). The bovine aortic proteoheparan sulphate was shown to form soluble complexes with LDL (Vijayagopal *et al.*, 1983). It was reported also that the native PG structure favours the precipitation of lipoproteins (Vijayagopal *et al.*, 1981).

It is well established that GAGs are involved in the deposition of the lipids in the blood-vessel wall (for review see Radhakrishnamurthy *et al.*, 1982). Pig aorta develops atherosclerotic lesions similar to those observed in humans (Vesselinovitch, 1979). However, little information is available on the composition and macromolecular interactions of PGs from pig aortic wall. The aim of our experiments was to investigate the interactions of the PGs of pig aorta with lipoproteins of the LDL type.

MATERIALS AND METHODS

Materials

GdnHCl, urea, benzamidine hydrochloride and 6-aminoheptanoic acid were purchased from Merck

Abbreviations used: PG, proteoglycan; GAG, glycosaminoglycan; LDL, low-density lipoproteins; GdnHCl, guanidinium chloride.

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(Darmstadt, Germany). Collagenase (EC 3.4.24.3), CLSPA chromatographically purified from *Clostridium histolyticum* (425 units/mg) was purchased from Worthington Biomedical Corp. (Freehold, NJ, U.S.A.). Pronase (*Streptomyces griseus*) type IV, chondroitinase ABC (EC 4.2.2.4, *Proteus vulgaris*), chondroitinase AC (EC 4.2.2.5, *Arthrobacter aureus*) and hyaluronic acid from human umbilical cord were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hyaluronidase (EC 4.2.2.1, *Streptomyces hyaluroniticus*; 100 TRU/mg) was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). DEAE-Trisacryl was purchased from I.B.F. (Paris, France). Sepharose CL-2B, Sepharose CL-4B, Sepharose CL-6B, Sephadex G-25 (PD-10 column), CNBr-activated Sepharose 4B and protein M_r standards for polyacrylamide-gel electrophoresis were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Samples of GAG standards were prepared by Dr. M. B. Mathews (University of Chicago, Chicago, IL, U.S.A.). LDL isolated from human serum by sequential ultracentrifugation (Havel *et al.*, 1955) were prepared by Dr. M. Ayrault-Jarrier and Mrs. J. Burdin (U.E.R., St. Antoine, Paris, France). The purity of LDL was checked by immunoelectrophoresis with rabbit antiserum to human serum proteins.

All other chemicals used were of analytical grade or best grade commercially available.

Extraction of PGs

All the extracting buffer solutions were supplemented with the proteinase inhibitors 10 mM-disodium EDTA, 0.1 M-6-aminohexanoic acid and 5 mM-benzamidine hydrochloride. The extractions were carried out at 4 °C. The procedure used is outlined in Scheme 1.

Pig thoracic aortas were obtained from the local slaughterhouse and packed in ice for transport to the laboratory. The intimal and medial layers (50 g) were freed of adventitia and adjacent fat tissue, finely minced and rinsed with 0.14 M-NaCl. The tissue was extracted twice with 10 vol. of 4.0 M-GdnHCl in 50 mM-sodium acetate buffer, pH 5.8, for 24 h with gentle mechanical shaking. Extracts were centrifuged at 10000 *g* in a Beckman JA-21 centrifuge for 1 h.

The supernatant was removed (GdnHCl extract), and the remaining pellet was washed with distilled water until free of Cl⁻ and hydrolysed with bacterial collagenase (1 mg of enzyme/g of wet tissue) in 10 ml of 10 mM-CaCl₂/50 mM-Tris/HCl buffer, pH 7.6, for 48 h at 37 °C. The proteinase inhibitors 2 mM-phenylmethanesulphonyl fluoride and 10 mM-*N*-ethylmaleimide were added to the samples to inhibit non-specific proteolysis. The hydrolysis was stopped by adding urea to 6 M final concentration. The hydrolysate was centrifuged at 4000 *g* and the supernatant (collagenase digest) was removed and the pellet discarded.

Purification of PGs

The GdnHCl extract was concentrated 5-fold by ultrafiltration in an Amicon cell with PM 10 filter and dialysed against six 1-litre changes of 6 M-urea/50 mM-Tris/HCl buffer, pH 7.6. The extract was centrifuged at 10000 *g* for 10 min, then applied to a DEAE-Trisacryl column (26 mm × 260 mm) equilibrated with the same buffer. The column was eluted stepwise with 0.14 M-NaCl and 1 M-NaCl. The crude PGs, eluted with 1 M-NaCl, were dialysed against distilled water, freeze-dried,

dissolved in 4 M-GdnHCl/50 mM-sodium acetate buffer, pH 5.8, and chromatographed on Sepharose CL-4B. The fractions were analysed for hexuronic acid. The peaks containing PGI and PGII were dialysed against distilled water, freeze-dried and stored at -20 °C.

The collagenase digest was applied to a DEAE-Trisacryl column in urea/Tris buffer and eluted with stepwise NaCl concentrations as above. The fraction containing uronic acid was concentrated to one-fifth of the original volume and dialysed against 6 M-urea/0.14 M-NaCl/50 mM-sodium acetate buffer, pH 5.6. The dialysis residue was then applied to the same DEAE-Trisacryl column, equilibrated with dialysis buffer. The column was eluted with a linear gradient of 0.14–1.2 M-NaCl in 6 M-urea/50 mM-sodium acetate buffer, pH 5.6. The hexuronic acid-positive peaks were collected, dialysed against distilled water, freeze-dried, solubilized in 1 ml of 4 M-GdnHCl/50 mM-sodium acetate buffer, pH 5.6 and chromatographed on Sepharose CL-4B as above.

Characterization of PGs

The PGs (10 µg) were labelled with 0.5 mCi of ¹²⁵I by the chloramine-T method (Parker, 1976) in 0.05 ml of 0.5 M-phosphate buffer, pH 7.6. The labelled PGs were separated from free ¹²⁵I⁻ by gel filtration on Sephadex G-25 with haemoglobin as a carrier and dialysed against phosphate-buffered saline (0.15 M-NaCl/20 mM-sodium phosphate buffer, pH 7.5).

The digestion of labelled PGs with chondroitinase AC was performed by the method of Oike *et al.* (1980). Samples (0.12 ml) containing 2 µg of uronic acid were hydrolysed with 0.2 unit of enzyme in the presence of proteinase inhibitors at 37 °C for 3 h and 6 h. After incubation, the reaction was stopped by adding an equal volume of 8 M-urea/0.4% SDS. The samples were reduced with dithiothreitol (40 mM) at 100 °C for 3 min and separated by polyacrylamide-gel electrophoresis by the procedure of Laemmli (1970). The gels were stained with 0.25% Coomassie Blue, dried and autoradiographed on X-Omat Ortho G film (Kodak) in a micro chamber with an intensifying screen (Bonner & Laskey, 1974).

GAG chains were released from the core protein of the PGs by β-elimination in 50 mM-NaOH/1 M-NaBH₄ for 48 h at 48 °C, and were chromatographed on Sepharose CL-6B.

Aggregation of hyaluronic acid with PGs

Hyaluronic acid from human umbilical cord was fractionated on Sepharose CL-4B. The fraction recovered at the void volume (*V*₀) was used for aggregation assays. Labelled PG samples containing 5 µg of uronic acid were mixed with 0.1 µg of hyaluronic acid, incubated for 30 min at room temperature and applied to Sepharose CL-2B. The column was eluted with phosphate-buffered saline and the radioactivity of the fractions was measured in a Packard γ-radiation counter. Reduction and alkylation of PGs were carried out as described by Hascall & Sajdera (1970).

Interaction of LDL with PGs

Samples of human LDL corresponding to 5 µg of protein in 0.3 ml of 20 mM-CaCl₂/10 mM-Tris/HCl buffer, pH 7.4, were exposed to increasing concentrations of isolated PGs, containing up to 5 µg uronic acid content. The mixtures were kept at 37 °C for 30 min, and the turbidity was measured in a laser nephelometer

(Behring Institute, Marburg, Germany). The values were corrected by the individual precipitation of LDL and PG in separate blank assays.

Also, human LDL (2.5 mg of protein) was coupled to CNBr-activated Sepharose 4B by a standard procedure, as follows. CNBr-activated Sepharose was swollen in 50 ml of 0.1 M-HCl and washed with 0.5 M-NaCl/0.1 M-NaHCO₃. LDL was incubated for 30 min with the gel under gentle shaking. The gel was then washed with (1) 0.1% glycine/0.1 M-NaHCO₃, (2) 1 M-NaCl/0.1 M-sodium acetate buffer, pH 4.0, and (3) 1 M-NaCl/0.1 M-sodium borate buffer, pH 8.0. Some 80% of the applied LDL was bound to the gel, as calculated from the cholesterol content. The LDL-Sepharose column (9 mm × 40 mm) was equilibrated with 20 mM-CaCl₂/10 mM-Tris/HCl buffer, pH 7.6.

Identification of GAGs

The PG samples were hydrolysed with Pronase in 10 mM-CaCl₂/50 mM-Tris/HCl buffer, pH 7.8, for 48 h at 48 °C. Trichloroacetic acid was added to the hydrolysates to 10% final concentration. The samples were centrifuged and dialysed exhaustively against running tap water and distilled water. The dialysis residues were freeze-dried.

The GAG samples were hydrolysed with hyaluronidase in 50 µl of 1 M-sodium acetate buffer, pH 5.4, for 60 min at 60 °C. The pH was adjusted to 7.6 with 1% Na₂CO₃, and the samples were treated successively with 0.3 unit of chondroitinase AC and 0.3 unit of chondroitinase ABC for 5 h at 37 °C (Breen *et al.*, 1976). Samples (40 µl) containing about 10 µg of uronic acid were exposed to 10 µl of 10% (w/v) NaNO₂ in 50% (v/v) acetic acid during 90 min at room temperature to degrade heparan sulphate (Lagunoff & Warren, 1962).

The electrophoretic separation of GAGs was carried out on cellulose acetate gel (Cellogel; Chemetron, Milan, Italy) in 0.1 M-calcium acetate, pH 5.0, and in 0.1 M-HCl. The GAGs were stained with 0.2% Alcian Blue as described by Bartold *et al.* (1982).

The distribution of GAGs was estimated by photo-densitometric scanning of the electrophoresed GAGs on Cellogel at 560 nm with the Sebia gel-scanning system.

Analytical methods

Proteins were determined either colorimetrically (Lowry *et al.*, 1951), with bovine serum albumin as a standard, or by measuring the absorption at 280 nm. Uronic acid and hexosamines were determined by the methods of Bitter & Muir (1962) and Blix (1948) respectively. The glucosamine/galactosamine ratios were determined after the hydrolysis of GAGs in 4 M-HCl for 4 h at 100 °C followed by t.l.c. (Moczar & Moczar, 1970). The glucuronic acid/iduronic acid ratios were determined, after the hydrolysis of GAG chains by chondroitinase AC and chondroitinase ABC (Saito *et al.*, 1968), by the periodate/barbiturate method (Hascall *et al.*, 1972).

RESULTS

Extraction of PGs

The extraction procedure is outlined in Scheme 1. After 48 h of extraction with 4 M-GdnHCl, 55% of the total hexuronic acid content was solubilized. Two additional extractions solubilized 10% and 5% of the hexuronic acid content respectively. Further extractions did not liberate

Scheme 1. Experimental scheme for the purification of PGs from pig aorta

For full details see the text.

any appreciable amounts of the hexuronic acid content. Hydrolysis of the extraction residue with collagenase for 48 h released 21% of the total hexuronic acid content from the aorta. The centrifuged pellet from the collagenase digest contained less than 9% of the total intima-media hexuronic acid content.

The GAG composition of the extracts was investigated by specific enzymic assays and cellulose acetate electrophoresis. In the GdnHCl extract chondroitin sulphate and dermatan sulphate accounted for 58% and 32% of the total GAGs respectively. The remaining 10% appeared to be hyaluronic acid and/or heparan sulphate. In the collagenase digest the GAGs were composed of chondroitin sulphate (65.5%) and heparan sulphate (34.5%).

Purification of PGs

The GdnHCl extract and collagenase digest were each chromatographed on a DEAE-Trisacryl column stepwise with 0.14 M-NaCl and 1 M-NaCl (Kapoor *et al.*, 1981). The main uronic acid-positive peaks were eluted with 1 M-NaCl. The PG-containing peak obtained from the GdnHCl extract was chromatographed on Sepharose CL-4B in the presence of 4 M-GdnHCl. The elution profile is shown in Fig. 2(a). The PGs were separated into two fractions. The hexuronic acid-positive peaks were eluted near the void volume (K_{av} 0.05, for PGI) and with K_{av} 0.45 (for PGII). The materials absorbing at 280 nm were eluted at the void volume and at K_{av} 0.75. These materials were not further analysed.

The PG-containing peak of the collagenase digest eluted from DEAE-Trisacryl by 1 M-NaCl contained

Fig. 1. DEAE-Trisacryl chromatography of the collagenase-digested PGs (1 M-NaCl fraction)

The hexuronic acid-positive-peak material of the collagenase digest eluted by 1 M-NaCl from the DEAE-Trisacryl column at pH 7.6 was dialysed against 50 mM-sodium acetate buffer, pH 5.6, containing 6 M-urea and chromatographed on a column (16 mm × 130 mm) of DEAE-Trisacryl equilibrated with the starting buffer. Elution was performed with a linear gradient of 0.14–1.2 M-NaCl. The total elution volume was 120 ml and the flow rate was 12 ml/h, 3 ml fractions being collected. The effluent was analysed for protein (A_{280} , ----), uronic acid (●—●) and NaCl content (—). Fractions A and B were pooled as indicated by the horizontal bars.

heparan sulphate and chondroitin sulphate in the same proportion as the crude collagenase extract. When this fraction was chromatographed on Sepharose CL-4B, the chondroitin sulphate-containing and heparan sulphate-containing PGs could not be separated (result not shown), but when the material was rechromatographed on the same DEAE-Trisacryl column at pH 5.6 with a linear NaCl gradient (Fig. 1) two uronic acid-positive fractions were eluted, with 0.6 M-NaCl (fraction A) and with 0.7 M-NaCl (fraction B). For final purification the fractions were chromatographed on the Sepharose CL-4B in dissociative conditions (Figs. 2b and 2c). Fraction A was eluted as a single uronic acid-positive peak at K_{av} 0.35 (PGIII), and fraction B as a peak at K_{av} 0.25 (PGIV).

Characterization of PGs

The protein, hexuronic acid and hexosamine contents are given in Table 1. PGI and PGII contained equivalent amounts of proteins and hexuronic acid. PGIII and PGIV contained less protein than hexuronate. The ratio hexuronic acid/hexosamine was higher than 1:1 in all fractions except PGII.

For further characterization of the PGs, reductive alkaline hydrolysis was performed. Before hydrolysis the four PGs were eluted from the Sepharose CL-6B in dissociative conditions near the void volume of the column (Fig. 3). After treatment with alkaline NaBH_4 , the elution positions of the hexuronic acid-positive peaks liberated from PGs were more retarded. The results indicate that the PGs underwent β -elimination in the presence of alkaline borohydride. Similar displacement of

Fig. 2. Gel chromatography on Sepharose CL-4B of (a) PGs of GdnHCl extract eluted from DEAE-Trisacryl by 1 M-NaCl, (b) fraction A of the collagenase digest, and (c) fraction B of the collagenase digest

(a) The PGs (1.6 mg of uronic acid) were dissolved in 50 mM-sodium acetate buffer, pH 5.8, containing 4 M-GdnHCl and applied on the Sepharose CL-4B column (20 mm × 950 mm), equilibrated with the same buffer. The column was eluted at a rate of 18 ml/h, and 6 ml fractions were collected. (b) and (c) In each case the sample (0.3 mg as uronic acid) was dialysed against distilled water, freeze-dried and solubilized in GdnHCl/acetate buffer (as above). The column size was 10 mm × 550 mm, the elution rate was 6 ml/h and 1.5 ml fractions were collected. The fractions were analysed for uronic acid (●—●) and proteins (A_{280} , ----). PGs were pooled, dialysed against distilled water and freeze-dried. V_0 , elution volume of Blue Dextran; V_t , elution volume of Methyl Red.

the uronic acid-positive peaks was obtained when the GAG chains were liberated by Pronase digestion of the above PGs.

Table 1. Analysis of PGs isolated from pig intima-media

The PG preparations were obtained as described in the Materials and methods section. The GAGs were obtained from PGs by Pronase digestion. The uronic acid composition (expressed as percentage of total uronic acid) was determined after chondroitinase AC (D-glucuronic acid) and chondroitinase ABC digestion (D-glucuronic acid + L-iduronic acid), followed by quantification of unsaturated glucuronosyl residues. The hexosamine composition is expressed as the percentage of total hexosamines. Abbreviation: N.D., not determined.

	Composition (%)			
	PGI	PGII	PGIII	PGIV
Protein (Folin reaction)	35	37	18	16
Uronic acid (carbazole reaction)	36	30	42	45
L-Iduronic acid	3	43.6	N.D.	0
D-Glucuronic acid	97	56.4	N.D.	100
Hexosamines (Elson-Morgan reaction)	29	33	40	39
Glucosamine	3	8	84	7
Galactosamine	97	92	16	93

Fig. 3. Gel chromatography on Sepharose CL-6B of the pig aortic PGs before and after alkali treatment in the presence of NaBH₄. The PGs (●—●) and the GAGs (○---○) were obtained after β -elimination from PGI (a), PGII (b), PGIII (c) and PGIV (d). They were chromatographed on the column (8 mm $\times$ 550 mm), equilibrated with 50 mM-sodium acetate buffer containing 4 M-GdnHCl, at a flow rate of 6 ml/h. Uronic acid content (A_{535} , carbazole method) was measured in the 1.0 ml fractions. V_0 and V_t are the elution volumes of Blue Dextran and Methyl Red respectively. The arrow indicates the elution position of PGI (V_0).

For protein-core studies PGI and PGII were labelled with ^{125}I , digested by chondroitinase ABC, reduced with dithiothreitol and subjected to polyacrylamide-slab-gel electrophoresis. The result of autoradiography of the gel is shown on Fig. 4. The native PGs did not penetrate the gel. After chondroitinase digestion, a single protein band was detected on the gel from each PG of the GdnHCl extract. PGI protein core migrated as a diffuse band in the M_r range 200 000–300 000 (Fig. 4, lane b). PGII protein core was identified as a band of M_r 50 000–55 000 (Fig. 4, lane e). The different electrophoretic migrations of the labelled polypeptides indicate different sizes of the protein cores of PGI and PGII. Prolongation of the digestion with chondroitinase ABC from 3 to 6 h did not change the protein-core M_r values of PGI and PGII (Fig. 4, lanes e and f).

GAG composition

The GAGs liberated by β -elimination or Pronase treatment from PGI migrated on cellulose acetate strips coincidentally with the chondroitin sulphate standards. The GAG moieties were resistant to *Streptomyces* hyaluronidase and to HNO_2 treatment. They were hydrolysed by chondroitinase AC and chondroitinase

ABC. PGI contained over 97% of galactosamine and over 97% of glucuronic acid (Table 1). These results show that the glycan chains of PGI are composed of chondroitin sulphates. On the basis of its glucosamine/galactosamine ratio the amount of glucosaminoglycans, i.e. hyaluronate or heparan sulphate, was less than 3% of total GAGs in PGI.

Only PGII contained iduronic acid. The GAGs liberated from PGII were resolved in two components on cellulose acetate electrophoresis, co-migrating with chondroitin sulphate and dermatan sulphate standards. By digestion with chondroitinase AC and chondroitinase ABC, the bands were identified as chondroitin sulphate (28%) and dermatan sulphate (64%). The minor component was identified as heparan sulphate (about 8% of total GAGs). No hyaluronic acid was detected in PGII.

The carbohydrate portion of the PGIII contained 84% of glucosamine (Table 1). This glucosamine-containing material was resistant to hyaluronidase and chondroitinases and was degraded by HNO_2 . The assays indicated that PGIII contained heparan sulphate and that PGIV contained chondroitin sulphate as main GAG components.

Interaction of PGs with hyaluronic acid

The formation of the high- M_r aggregates between hyaluronic acid and PGI or PGII was studied by Sepharose CL-2B chromatography under associative conditions. The results are shown in Fig. 5. PGI and PGII were eluted as a broad peak with K_{av} 0.45 and 0.55. Reduction and carboxymethylation of the PGs did not change either their profiles or their elution positions. When non-reduced PGI was mixed with 1% of high- M_r hyaluronic acid, about 20% of the PG was excluded from Sepharose CL-4B. An increase in hyaluronic acid content did not change the amounts of PGI eluted in the void volume. The elution position of PGII was not modified by exogenous hyaluronic acid (Fig. 5b).

Interaction of PGs with LDL

The formation of the insoluble complexes between PGs and human LDL was investigated by laser nephelometry (Vijayagopal *et al.*, 1981). Fig. 6 shows the turbidity curves as a function of PG concentrations at constant concentration of LDL. Both PGI and PGII formed co-precipitates with LDL. No precipitation was obtained when PGIII or PGIV was incubated with LDL. At the lipoprotein concentration of $5\text{ }\mu\text{g}$ (as protein) in the reaction mixture, maximum turbidity was observed with $2\text{ }\mu\text{g}$ of PGI, whereas PGII formed a plateau of turbidity at concentrations between 3 and $12\text{ }\mu\text{g}$ of uronic acid. An excess of PGI inhibited complex-formation. When $15\text{ }\mu\text{g}$ of PGII was incubated with $5\text{ }\mu\text{g}$ of LDL, the turbidity decreased to one-third of the maximal value (result not shown). In agreement with previous experiments (Vijayagopal *et al.*, 1981), insoluble complexes formed only in the presence of bivalent cations such as Ca^{2+} or Mg^{2+} .

The dissolution of the precipitates at higher salt concentration (0.14 M-NaCl) suggests a role for the ionic strength in complex-formation (Radhakrishnamurthy *et al.*, 1982). To demonstrate ionic interactions between PGs and lipoproteins, the PGs were chromatographed on LDL-Sepharose. The results are given in Fig. 7. PGIII and PGIV were not retained on LDL-Sepharose (Fig. 7a). Hyaluronic acid and chondroitin sulphate standards

Fig. 4. SDS/polyacrylamide-gel electrophoresis and autoradiography of the GdnHCl extract before and after treatment by chondroitinase ABC

The labelled PGs were reduced with dithiothreitol and subjected to electrophoresis on a 5–20% acrylamide linear-gradient polyacrylamide gel. Lanes indicate the autoradiographs of: lane a, PGI; lane b, PGI hydrolysed by 0.2 unit of enzyme; lane c, PGII; lane d, PGII incubated for 3 h with the buffer without enzyme; lane e, PGII incubated for 3 h with 0.2 unit of enzyme; lane f, PGII incubated for 6 h with 0.2 unit of enzyme. Positions of the standard proteins and human reduced fibronectin are shown by the bars, with the M_r values shown. Details of hydrolysis by chondroitinase ABC are given in the Materials and methods section.

Fig. 5. Gel-permeation chromatography on Sepharose CL-2B of PGI (a) and PGII (b) in phosphate-buffered saline

The samples of ^{125}I -labelled PGs were either reduced and carboxymethylated ($\bigcirc$ — $\bigcirc$) before chromatography or incubated with hyaluronic acid ($\bullet$ — $\bullet$) as described in the Materials and methods section. The PGs were applied on the column (6 mm $\times$ 500 mm) and eluted with phosphate-buffered saline at a flow rate of 4.8 ml/h. The radioactivity was measured in the 0.5 ml fractions. V_0 and V_t are the elution volumes of *Escherichia coli* and Methyl Red respectively.

Fig. 6. Precipitation of human LDL by PGs from pig aorta

The different quantities of PGs (up to 5 μg of uronic acid) were incubated in 0.3 ml of 10 mM-Tris/HCl buffer, pH 7.4, containing 20 mM- CaCl_2 with 5 μg (as protein) of human LDL during 30 min. The turbidity measured by laser nephelometer represents the difference between the LDL-PG sample and the two control samples: without LDL and without PGs. Each point is the mean for three independent determinations.

were eluted in the void volume. PGI and PGII were retarded on the column. The PGs bound to LDL-Sepharose could be eluted with an NaCl gradient. The maximum of PGI was eluted at 0.06 M-NaCl, whereas the maximum of PGII was eluted at 0.09 M-NaCl. The results of affinity chromatography are in agreement with those of the turbidity measurements. They show that, according to the methods used in these investigations, only some aortic PGs are interacting with LDL.

Fig. 7. Affinity chromatography of PGIII and PGIV (a), PGI (b) and PGII (c) on LDL-Sepharose

The PG samples (30 μg of uronic acid) were solubilized in 0.5 ml of 10 mM-Tris/HCl buffer, pH 7.6, containing 20 mM- CaCl_2 and applied on the column (9 mm $\times$ 40 mm) equilibrated with the same buffer. The column was eluted with 5 ml of application buffer followed by a linear gradient of 0–0.2 M-NaCl (—) (7 ml). Uronic acid (histogram) was measured in 0.5 ml fractions. The arrow indicates the elution position of chondroitin sulphate standard.

DISCUSSION

Extraction of aortic intima-media with 4 M-GdnHCl or 8 M-urea results in a partial solubilization of PGs, and at least 30% of PGs (expressed as uronic acid) remained

associated with the collagen-elastin matrix (Oegema *et al.*, 1979; Horn *et al.*, 1983). In the present study, 90% of aortic hexuronic acid content was solubilized by 4 M-GdnHCl extraction followed by the digestion of the residue with collagenase. PGs were isolated from the extracts by ion-exchange and molecular-sieve chromatography (Kapoor *et al.*, 1981). Two proteogalactosaminoglycans (PGI and PGII) were separated from the 4 M-GdnHCl extract, and two other PGs, proteoheparan sulphate (PGIII) and proteochondroitin sulphate (PGIV), from the collagenase digest.

The results of our experiments confirm the observation made by other authors (Oegema *et al.*, 1979) that dissociative agents solubilize mainly chondroitin sulphate and dermatan sulphate PGs. The pig aortic PGs solubilized by GdnHCl are of similar size to the PGs from pigeon aorta (Wagner *et al.*, 1983) and human aorta (Salisbury & Wagner, 1981). PGI and PGII were shown to be heterogeneous by hydrodynamic size; however, they were completely separated by gel-permeation chromatography on Sepharose CL-4B. These two PGs differ by the length of their glycan chains (Fig. 3) and by the size of their protein cores (Fig. 4). Moreover, up to 20% of high- M_r proteochondroitin sulphate (PGI) interacts with hyaluronic acid to form aggregates. This PG behaves in this respect similarly to the cartilage proteoglycans (Hardingham & Muir, 1972). Unlike cartilage proteoglycans, however, PGI aggregated only partially with hyaluronic acid. The lesser interaction of PGI with hyaluronic acid may arise from: (a) competitive inhibition of complex-formation by endogenous hyaluronic acid; (b) partial degradation of hyaluronic acid-binding region during the preparation procedure; (c) partial dissociation of the complex during the chromatography on Sepharose CL-4B. Wagner *et al.* (1983) has shown that well-defined fractions of proteochondroitin sulphate from pigeon aorta underwent quantitative aggregation with hyaluronic acid when the residual hyaluronic acid present in these PGs was hydrolysed by hyaluronidase. PGII, similarly to the proteodermatan sulphate from pigeon aorta (Wagner *et al.*, 1983) and monkey aorta (Wight & Hascall, 1983), did not interact with hyaluronic acid.

When the tissue was pre-extracted with 4 M-GdnHCl, the major portion of the aortic proteoheparan sulphate was solubilized from the residue during a digestion with collagenase without elastase. The proteoheparan sulphate was partially separated from proteochondroitin sulphate by additional ion-exchange chromatography at pH 5.6 as proposed previously by Kapoor *et al.* (1981). PGIII and PGIV contained less protein than did PGI and PGII. Although collagenase digestion was carried out in the presence of the proteinase inhibitors, a partial degradation of protein core during the collagenase digestion cannot be excluded. The strong association of PGIII and PGIV with collagen, their GAG composition and their size suggest a probable basement-membrane nature of these PGs. The basement-membrane material isolated from different tissues or aortic myomedial cells contained different amounts of heparan sulphate and chondroitin sulphate. Moreover, only a portion of these PGs can be extracted by dissociative agents (Hassel *et al.*, 1980; Kanwar *et al.*, 1981; Heickendorff & Ledet, 1984; Parthasarathy & Spiro, 1984).

The complexity of vascular PGs suggests that they can play different physiological roles in normal and patho-

logically modified vessels. Since the early experiments by Gero *et al.* (1960), a great deal of data were published suggesting that aortic GAGs contribute to the deposition of lipids in the atherosclerotic lesions by insolubilizing lipoproteins (Bihari-Varga *et al.*, 1981; Camejo, 1982).

Our study has demonstrated the formation of insoluble complexes between human LDL and two distinct PG populations isolated from pig aorta. The differences in the formation of PG-LDL complexes are in agreement with the results of affinity chromatography of PGs on LDL-Sepharose. The precipitation of LDL depends on the nature of PGs and the composition of glycan chains (Vijayagopal *et al.*, 1981). Two proteochondroitin sulphates (PGI and PGIV) with similar GAG size but differing with respect to PG size exhibit different interacting potentials with LDL *in vitro*. Only the high- M_r species (PGI) formed a co-precipitate with LDL and was bound to LDL-Sepharose. As PGI and PGIV contained chondroitin sulphate chains of similar size, we may assume that the structural integrity of the PG molecule is required for the formation of the insoluble complex. However, the importance of GAG chain composition was shown by the differences in complex-formation and affinity for LDL-Sepharose between PGI and PGII.

Proteoheparan sulphate (PGIII) did not precipitate LDL. This observation is in agreement with the results obtained by Vijayagopal *et al.* (1983), suggesting that proteoheparan sulphate from bovine aorta is able to form only soluble complexes with human LDL. Fransson & Havsmark (1981) showed that the ratio of completely *N*-sulphated to *O*-sulphated blocks in the heparan sulphate chain limits its affinity for lipoproteins. The structure of aortic proteoheparan sulphate glycan chains may therefore influence complex-formation with lipoproteins.

An increase in dermatan sulphate content in the arterial wall was observed in the early stages of the development of atherosclerotic plaques (Stevens *et al.*, 1976; Radhakrishnamurthy *et al.*, 1982). PGII formed the most stable complexes with LDL. This PG may contribute to the retention of lipoproteins in pathologically modified arteries.

We are grateful to Dr. M. Ayrault-Jarrier and Mrs. J. Burdin for the gift of LDL. The support of C.N.R.S. (U.A. 580), I.N.S.E.R.M. (a fellowship to J.W.) and the Conseil Scientifique de l'Université Paris Val de Marne is gratefully acknowledged.

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Received 5 August 1985/2 December 1985; accepted 30 December 1985