Structural glycoproteins from rabbit aortic media

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(Received 25 October 1982/Accepted 10 January 1983)

Rabbit aortic intima-media fragments were incubated with [¹⁴C]mannose and [³H]fucose for 6 h to detect glycoproteins synthesized *in situ*. The radioactively labelled and the non-labelled samples were extracted with 0.2 mM-CaCl₂/0.5 mM-dithiothreitol/0.5 mM-ATP and chloroform/methanol/water (4:4:1, by vol.). The delipidated residue was extracted with 5 M-guanidinium chloride/0.05 M-dithiothreitol/0.1 M-Tris/0.4% Na₂EDTA, pH 7.5, before (extract 1) and after hydrolysis with collagenase (extract 2). The proteins in extracts 1 and 2 were S-carboxamidomethylated and separated by molecular-sieve chromatography, polyacrylamide-gel electrophoresis and isoelectric focusing in sucrose gradients in urea. The apparent molecular weights of glycoproteins were 36 000 (glycoprotein I) from extract 1, 50 000 (glycoprotein II) and 130 000 (glycoprotein III) from extract 2. The molecular weights of the non-labelled and radioactively labelled glycoproteins were identical. Glycoproteins I, II and III contain large amounts of polar amino acids and methionine. They contain neither hydroxyproline nor 3-methylhistidine. A hydroxyproline-containing component of 160 000-apparent-mol.wt. relatively rich in polar amino acids and labelled with incorporated sugars was isolated from extract 1. The incorporation *in vitro* of radioactive sugars into glycoproteins I, II, III and collagenous glycoproteins indicates that they are synthesized in the surviving aorta by the smooth-muscle cells.

The glycoproteins isolated from aortic media have been characterized according to arbitrarily chosen criteria such as solubility (Radhakrishnamurthy *et al.*, 1964; Barnes & Partridge, 1968; Moschetto & Havez, 1969; Klemer & Nager, 1967; Moczar & Robert, 1970; Maier & Buddecke, 1971; McCullagh *et al.*, 1973; Moczar *et al.*, 1977), association with elastin (Robert *et al.*, 1971; John & Thomas, 1972) or localization in the arterial intima and media (Wagh & Robert, 1972).

Previously described techniques for separating aortic structural glycoproteins involved extractions at high pH (Barnes & Partridge, 1968) and/or hydrolytic cleavage of collagen with trichloroacetic acid (Moczar & Robert, 1970), treatments that may result in hydrolysis of the glycoproteins (Moczar *et al.*, 1975). Thus it was not yet clear whether the apparent heterogeneity of the aortic glycoproteins obtained by the above procedures was representative of a real molecular heterogeneity or whether it was due to artefacts of the extraction procedure.

A 2.5×10^5 -mol.wt. glycoprotein isolated from monkey arterial smooth-muscle-cell cultures was tentatively identified as a microfibrillar component of elastin (Muir *et al.*, 1976). A glycoprotein of 200 000 mol.wt. was partially purified from calf aortic media and from confluent cultures of smooth-muscle cells obtained from this tissue; it reacted with fibronectin antibodies (Schwartz *et al.*, 1980).

There is little information available on the synthesis of glycoproteins *in situ* in the arterial wall (Maier & Buddecke, 1971; Robert *et al.*, 1973), thus it is difficult to correlate the data obtained in cell cultures with the glycoprotein composition of aorta characterized by preparative techniques. We wanted to find out whether the glycoproteins extracted at high ionic strength from the aorta, designated 'structural glycoproteins' (Moczar & Robert, 1970; Moczar *et al.*, 1977; Robert & Moczar, 1982) are synthesized by arterial cells or if they are derived from the plasma. We studied therefore the incorporation *in vitro* of radioactive sugars in the surviving rabbit aortic intima-media (Moczar *et al.*, 1979).

Abbreviation used: SDS, sodium dodecyl sulphate.

Experimental

Materials

Eagle's minimum essential medium (MEM 211), glutamine (Gln 3100), penicillin and streptomycin were obtained from Eurobio (France), mycostatin from Gibco Biocult, Glasgow, Scotland, U.K. D-[U-¹⁴C]Mannose (300 μ Ci/mmol) was purchased from CEA, Saclay, France, and L-[1-³H]fucose (6 Ci/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K. Collagenase (600 units/mg) was obtained from Worthington Biochemical Co., Freehold, NJ, U.S.A. Bovine serum albumin, myoglobin, hen's-egg albumin and bovine γ -globulin were obtained from Sigma, St. Louis, MO, U.S.A. Ferritin, catalase, lactate dehydrogenase and bovine serum albumin (Electrophoresis Calibration Kit) was obtained from Pharmacia, Uppsala, Sweden. *N*-Ethylmaleimide, phenylmethanesulphonyl fluoride, guanidinium chloride, sucrose and dithiothreitol were purchased from Merck, Darmstadt, Germany.

Acrylamide and *NN'*-methylenebisacrylamide were purchased from Koch-Light, Colnbrook, Bucks., U.K., and Ready-Solv GP scintillation liquid from Beckman (Irvin, CA, U.S.A.). Ampholyte (Servalyt) was obtained from Serva, Heidelberg, Germany. Ultrafiltration membrane type PM10 was obtained from Amicon, High Wycombe, Bucks., U.K.

Aorta

Rabbits were killed by cervical dislocation and allowed to bleed through the carotid artery. Aortas were excised from thirty 3-month-old inbred rabbits (Ecole Vétérinaire, Maisons-Alfort, France). For incubation in the presence of radioactive precursors, the rabbit aortic intima-media was cleaned from adventitia and endothelial cells and dissected to 1 mm² fragments in Eagle minimum essential medium. For the preparative separation of glycoproteins the aortic intima-media was cleaned in 0.9% NaCl supplemented with 2 mM-phenylmethanesulphonyl fluoride, 10 mM-*N*-ethylmaleimide and 0.1% EDTA as proteinase inhibitors (Sear *et al.*, 1977).

Incorporation of radioactive precursors into macromolecules

The macromolecular-incorporation kinetics of D-[U-¹⁴C]mannose and L-[1-³H]fucose were investigated in rabbit aortic intima-media fragments (50 mg fresh wt.) incubated in 5 ml of MEM medium 2011 containing 100 units of penicillin/ml, 100 μ g of streptomycin/ml and 100 units of mycostatin/ml in the presence of 25 μ Ci of D-[U-¹⁴C]mannose or 25 μ Ci of L-[1-³H]fucose respectively. The samples were shaken at 37°C for up to 18 h.

The incubated samples were washed with 10% trichloroacetic acid containing 0.5% mannose and

fucose respectively until no radioactivity was detected in the supernatant, and the radioactivity in chromatographically isolated monosaccharides was determined (Table 2 below). To investigate the radioactivity incorporation pattern of glycoproteins, aortic-media samples of about 200 mg fresh weight were incubated in triplicate in the presence of 25 μ Ci of D-[U-¹⁴C]mannose and 25 μ Ci of L-[1-³H]fucose respectively for 6 h. The incubated samples were chilled on an ice bath, washed with 0.5% mannose or 0.5% fucose respectively, containing 2 mM-phenylmethanesulphonyl fluoride, 10 mM-*N*-ethylmaleimide and 0.1% EDTA as proteinase inhibitors, and extracted as indicated below.

Extraction of glycoproteins

The flow sheet of the extraction is given in Scheme 1. The radioactively labelled aortic media was homogenized in a tissue grinder (Kontes, NJ, U.S.A.) and the non-incubated samples for 3 $\times$ 20 s in a Waring (New Hartford, CN, U.S.A.) Blender.

Scheme 1. Extraction of the intima-media of rabbit aorta

Details are given in the Experimental section. Non-labelled aortas were homogenized in 0.9% NaCl/0.2 M-6-amino-hexanoic acid to remove the bulk of serum contaminants. The samples incubated with the radioactive sugars were washed to remove the incubation medium before the extractions. Guanidinium chloride extracts 1 and 2 were obtained before and after hydrolysis of collagen respectively.

The following extracting agents were used with a tissue/buffer ratio of 1:10 (w/v) in the presence of the proteinase inhibitors indicated below. The non-radioactive samples were homogenized in 0.9% NaCl/0.2 M-6-aminohexanoic acid solution to remove the bulk of serum contaminants before the extractions. The homogenate was centrifuged as indicated below, and the supernatant discarded. Two extractions for 6 and 16 h at 4°C were carried out with 0.2 mM-CaCl₂/0.5 mM-ATP/0.5 mM-dithiothreitol (Sender, 1971). The suspensions were centrifuged at 35000g in a Beckman L3-50 rotor at 4°C for 40 min. The centrifuged pellet was delipidated with 5 vol. of chloroform/methanol/water (4:4:1, by vol.) as described by Kang *et al.* (1978) and the chloroform was washed out with 2 vol. of acetone/methanol (1:1, v/v). The organic phase was centrifuged at 4000g at 4°C in a Minifuge 2 (Heraeus, Osterode, Germany). The delipidated residue was extracted at room temperature with 5 M-guanidinium chloride/0.1 M-Tris/0.1% EDTA/0.05 M-dithiothreitol, pH 7.5, successively for 8 and 16 h (Ross & Bornstein, 1969; Serafini-Fracassini *et al.*, 1975). The supernatants were centrifuged at 35000g for 30 min at 4°C in a Beckman L3-50 rotor. The sediment was washed with Cl-free distilled water by centrifuging at 4°C and hydrolysed with bacterial collagenase at pH 7.4 in 0.05 M-Tris/HCl-0.01 M-CaCl₂ buffer; 10 mM-N-ethylmaleimide and 2 mM-phenylmethanesulphonyl fluoride were added as proteinase inhibitors. The digestion was carried out in 50 µl of buffer solution with 10 µg of collagenase per mg of tissue dry weight submitted to extraction. Two consecutive hydrolyses for 24 h were carried out, and the suspension was centrifuged at 35000g for 50 min at 4°C. The pellet was extracted twice with the 5 M-guanidinium chloride buffer as indicated above. To detect the non-specific proteolytic activity of the collagenase in the pres-

ence of proteinase inhibitors, the enzyme was incubated with hen's-egg albumin. The electrophoretic mobility of hen's-egg albumin was not modified under the above conditions of incubation.

Measurement of radioactivity

This was done as described previously (Lafuma *et al.*, 1982).

Gel chromatography

The preparative separation techniques are outlined in Scheme 2. Gel chromatography was performed at room temperature on a column (5 cm × 75 cm) of Sepharose 4B (about 40 mg of protein was charged on the column). The excluded peak of the Sepharose 4B column from extract 1 (A1) (Fig. 3 below) was chromatographed on Sephacryl S-300 (1 cm × 100 cm). Elution was carried out with 6 M-urea/0.02 M-Na₂EDTA/0.2% SDS at pH 7.4, and 1 ml fractions were collected at an elution rate of 1.9 cm/h and 7.6 cm/h for Sepharose 4B and Sephacryl S-300 respectively. The elution was monitored at 280 nm with ISCO UA5 absorbance monitor. Columns were calibrated for molecular-weight determination with Blue Dextran 2000 (void volume), bovine liver catalase (mol.wt. 232 000), bovine serum albumin (mol.wt. 67 000) and myoglobin (mol.wt. 17 200). The total volume of the column was determined with dinitrophenol.

Electrophoresis

Polyacrylamide-gel electrophoresis was carried out as described by Weber & Osborn (1969) under reducing conditions in 6 M-urea (Fairbanks *et al.*, 1971; Moczar *et al.*, 1977) in 4.5% gels in glass tubes of 0.6 cm × 12 cm internal dimensions and in slab gels 12.5 cm × 26 cm. The proteins were stained with 0.005% Coomassie Blue (w/v) in 30% methanol/acetic acid (91:9, v/v) for 3 h. The

Scheme 2. Fractionation of guanidinium chloride extracts 1 and 2 from intima-media of rabbit aorta. The extracts were obtained as outlined in Scheme 1. Details are given in the Experimental section.

glycoproteins were detected with periodic acid/Schiff reagent. The photodensitometric evaluation of the proteinograms was done at 560nm in an ISCO UA5 gel scanner. Ferritin (mol.wt. 440000), catalase (232000), bovine serum albumin (67000), bovine γ -globulin heavy chain (50000) and light chain (23500) were used as standards for molecular-weight estimations. To detect the radioactive proteins, the gels were sliced into 1.5 mm segments and treated with 1 ml of 30% H_2O_2 containing 0.3% NH_3 at 70°C for 3 h and at room temperature overnight (Rauterberg *et al.*, 1966). The samples were supplemented with 10 ml of Ready-Solv GP scintillation liquid and the radioactivity was measured after an incubation for 4 days (Sear *et al.*, 1977).

Isoelectric focusing

Preparative isoelectric focusing of proteins separated by gel chromatography was carried out in sucrose gradients in urea (Moczar *et al.*, 1977; Lafuma *et al.*, 1982) in an ISCO (Lincoln, NE, U.S.A.) model 212 column; about 5 mg preparation was used. Servalyt carrier ampholytes covering the ranges pH2–11 and 4–6 (Serva, Heidelberg, Germany) were used.

Analytical methods

Freeze-dried samples (about 400 μg of protein) were hydrolysed in 6M-HCl for 24 h at 105°C in Pyrex tubes sealed *in vacuo*. The HCl was evaporated under reduced pressure in a desiccator in the presence of KOH pellets. Amino acid analysis was performed on an LKB (Bromma, Sweden) model 3201 automatic amino acid analyser. To separate 3-methylhistidine, characteristic of actin, the amino acid analyser was run with 0.38M-sodium citrate buffer, pH5.28.

The monosaccharides in glycoprotein I were identified by g.l.c. The glycoproteins were submitted to methanolysis and trifluoroacetylation (Zanetta *et al.*, 1972). The trifluoroacetyl-sugar derivatives were analysed by g.l.c. with a Pye (Cambridge, U.K.) 104 chromatograph using Chromosorb W 80/100 and 5% (w/w) OV 210 liquid phase (Varian, Palo Alto, CA, U.S.A.).

[^3H]Sugar labelling of non-radioactive glycoproteins

Focused proteins (100 μg) were dialysed against 0.05M-potassium phosphate/0.15M-NaCl, pH8.2, to avoid precipitation of sparingly soluble proteins. Portions (1 ml) were incubated at 37°C and at pH7.4 with 25 μl (2.5 mg/ml) of galactose oxidase (type IV; 50 units/mg; EC 1.1.3.9; Sigma, St. Louis, MO, U.S.A.) solution for 3 h. The oxidized products were reduced with 100 μCi of NaB^3H_4 (4.5 Ci/mmol; CEA) for 1 h at room temperature (Gahmberg &

Hakamori, 1973). The reaction mixture was acidified with acetic acid and dialysed against 0.05M-potassium phosphate buffer, pH8.2. The samples were separated by SDS/polyacrylamide-gel electrophoresis and the radioactivity profiles were determined.

Results

Biosynthetic labelling and extraction of glycoproteins

The incorporation of [^{14}C]mannose and [^3H]fucose was linear with time between 2 and 6 h of incubation. The separation of the biosynthetically labelled proteins was carried out from the fragments of aortic media-intima incubated with ^{14}C - and ^3H -labelled sugars for 6 h. The flow sheet of the extraction and the designation of the extracts are shown in Scheme 1. The extraction with 0.2mM- CaCl_2 /0.5mM-dithiothreitol/0.5mM-ATP was carried out to remove the bulk of the actin (Sender, 1971). This extract from aorta showed several protein bands on polyacrylamide-gel electrophoresis. One of the components co-migrated with actin prepared from rabbit skeletal muscle. The extraction with CaCl_2 /dithiothreitol/ATP buffer was followed by a delipidation with chloroform/methanol/water.

The distribution of the radioactivity in the extracts is given in Table 1. The macromolecular incorporation pattern in the extracts was different for [^{14}C]mannose and [^3H]fucose. The highest ^{14}C label was recovered in the macromolecules extracted with the guanidinium chloride buffer before hydrolysis with collagenase (extract 1). When the aortic intima-media fragments were incubated with [^3H]fucose, the collagenase digest accounted for the

Table 1. Incubation of rabbit aortic intima-media with D-[^{14}C]mannose and L-[^3H]fucose for 6 h

The distribution of the radioactive macromolecular sugar label in the extracts obtained from the incubated aorta (Scheme 1) is shown. Results are expressed as percentages of the total incorporated radioactivity recovered in the extracts. Individual values varied by $\pm 20\%$ around the average values shown in the Table.

Extract	Radioactivity incorporated (%)	
	^{14}C	^3H
0.2 mM- CaCl_2 /0.5 mM-dithiothreitol/ 0.5 mM-ATP	1.0	2.0
Chloroform/methanol/water (4:4:1, by vol.)	22.4	Trace
Extract 1	40.6	35.0
Collagenase digest	28.7	55.0
Extract 2	7.3	8.0

Table 2. Incorporation of D-[U-¹⁴C]mannose into macromolecules in the rabbit aortic intima-media

Aortic fragments were incubated with D-[U-¹⁴C]-mannose for 6 h as described in the Experimental section. The extraction of the incubated samples was done as shown in Scheme 1 and described in the Experimental section. The samples were hydrolysed with 1.5 M-HCl for 2 h. The hydrolysates were neutralized with Amberlite 45 (OH form) resin and purified from the peptides on mixed Amberlite G120 (200/400 mesh) and Amberlite IR 45 (OH form) (6 mm × 25 mm) columns (Moczar & Moczar, 1970). The sugars were separated by descending paper chromatography on Schleicher and Schüll (Dassel, Germany) 2043b paper with the solvent system butan-1-ol/pyridine/0.1 M-HCl (5:3:2, by vol.). Non-radioactive monosaccharides were used as migration markers and the hexoses were eluted with 0.1 M-HCl/90% ethanol and detected with aniline/phthalate reagent (Hughes & Jeanloz, 1964). The radioactivity of the eluates was determined. Results are expressed as percentage of total radioactivity of monosaccharide residues in the extracts.

Monosaccharides	¹⁴ C radioactivity (%)	
	Extract 1	Collagenase digest
Mannose	52	18
Fucose	18	18
Glucose	16	24
Galactose	6	32
Glucosamine	8	8

highest percentage of recovered radioactivity. When the delipidation was omitted, the radioactive yield increased in extract 1, especially with [¹⁴C]mannose tracer. To obtain reproducible results, the extraction with chloroform/methanol/water was carried out before the solubilization of proteins with the guanidinium chloride buffer. As the ³H and ¹⁴C label in the CaCl₂/ATP/dithiothreitol extracts was less than 2% of the total radioactive-sugar incorporation, the glycoprotein composition of these extracts was not studied.

To investigate the macromolecular incorporation of [³H]fucose and [¹⁴C]mannose, portions of the two major ³H- and ¹⁴C-labelled extracts (guanidinium chloride extract 1 and collagenase digest respectively) were submitted to partial hydrolysis and the sugars were separated by paper chromatography. After incubation of aortic fragments with [¹⁴C]-mannose for 6 h, 50% of the macromolecular ¹⁴C label in extract 1 co-chromatographed with D-mannose (Table 2). The residual ¹⁴C label accounted for the incorporation of L-fucose, D-galactose, D-glucose and D-glucosamine. The radioactivity recovered in the monosaccharide residues accounted for about 80% of the radioactivity in the guanidinium chloride extract 1 and the collagenase digest.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of proteins in guanidinium chloride extract 1 from intima-media of rabbit aorta

Intima-media fragments were incubated with D-[U-¹⁴C]mannose and L-[³H]fucose for 6 h as described in the Experimental section and extracted as outlined in Scheme 1 and described in the Experimental section. The gels were stained with periodate/Schiff reagent (----) and Coomassie Blue (—) and then sliced and assayed for ³H radioactivity (●). The arrows indicate the electrophoretic mobilities of components with (a) 200 000, (b) 160 000 (collagenous glycoprotein component), (c) 130 000 (glycoprotein III), (d) 68 000, (e) 36 000 (glycoprotein I) apparent molecular weight.

With [³H]fucose, about 70% of the incorporated radioactivity were identified as fucose. Thus the recorded incorporation of ³H and ¹⁴C-labelled monosaccharides can be considered as a reliable measure of the biosynthesis of oligosaccharides of glycoproteins.

Separation of glycoproteins

The separation of proteins by SDS/polyacrylamide-gel electrophoresis indicated the presence of periodate/Schiff-stained and radioactive-sugar-labelled bands of 36 000, 68 000, 130 000, 160 000 and 220 000 apparent mol.wt. in extract 1 (Fig. 1). The 36 000-mol.wt. component had the highest intensity with periodate/Schiff stain, as shown by gel scanning. From extract 2, the major periodate/Schiff-positive and radioactively labelled component migrated with 50 000 apparent mol.wt. Glycoprotein components of 130 000 and 160 000 mol.wt. were detected by their ³H and ¹⁴C labels (Fig. 2). The macromolecular-incorporation patterns with [³H]-fucose and [¹⁴C]mannose tracers in extracts 1 and 2 were similar.

The electrophoretic mobilities of the Coomassie Blue- and periodate/Schiff-stained bands from the extracts obtained from radioactive labelled and non-radioactive aortic intima-media were identical.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of proteins in guanidinium chloride extract 2 from intima-media of rabbit aorta

Intima-media fragments were incubated with D-[U- ^{14}C]mannose and L-[1- ^3H]fucose for 6 h as described in the Experimental section and extracted as outlined in Scheme 1 and described in the Experimental section. The gels were stained with periodate/Schiff reagent (—) and Coomassie Blue (—) and then sliced and assayed for ^3H radioactivity (●). The arrows indicate the mobilities of components with (a) 200000, (b) 160000, (c) 130000 (glycoprotein III) and (d) 50000 (glycoprotein II) apparent molecular weight.

Gel filtration

The preparative separation techniques and the designation of the glycoprotein fractions are outlined in Scheme 2.

Extract 1. The *S*-carboxamidomethyl-proteins from extract 1 were resolved into an excluded (A1) and a retarded (B1) fraction by Sepharose 4B chromatography (Fig. 3). Fraction B1 was eluted over the molecular-weight range 30000–40000. It was resolved by SDS/polyacrylamide-gel electrophoresis into one major periodate/Schiff-positive ^3H - and ^{14}C -labelled component of 36000 apparent mol.wt. and two Coomassie Blue-stained bands of 36000 and 45000 apparent mol.wts. When fraction B1 was hydrolysed with collagenase, the electrophoretic mobility of the glycoprotein did not change, but the protein of 45000 mol.wt. was degraded. Fraction 1 contained protein bands of 300000, 220000, 160000 and 130000 apparent mol.wt. on polyacrylamide-gel electrophoresis. The periodate/Schiff-positive and radioactive-sugar-labelled components migrated with 220000, 160000 and 130000 mol.wts. Fraction A1 contained about 20 hydroxyproline residues per 1000 amino acid residues. When submitted to bacterial collagenase digestion followed by SDS/polyacrylamide-gel electrophoresis, the 160000-mol.wt. band disappeared, and the protein bands corresponding to 220000 and

Fig. 3. Gel filtration on Sepharose 4B of proteins extracted with 5M-guanidinium chloride/0.05M-dithiothreitol/0.1M-Tris/0.4% Na_2EDTA , pH 7.5, from rabbit aortic intima-media

The proteins were extracted with the 5M-guanidinium chloride buffer from the rabbit aortic intima-media before (extract 1) and after (extract 2) hydrolysis of collagen with collagenase. The extraction procedure and the designation of the extracts are illustrated in Scheme 1 and the separation techniques in Scheme 2 respectively. The guanidinium chloride extracts 1 and 2 were concentrated by ultrafiltration on Amicon PM10 membrane to one-tenth of the original volume. The proteins were *S*-carboxamidomethylated in guanidinium chloride with iodoacetamide as described by Haeberli *et al.* (1975). The samples were dialysed against 6M-urea/0.05M- Na_2HPO_4 , pH 7.2. The elution position of the standards are given as follows: V_0 , Blue Dextran 200; 1, bovine serum albumin (mol.wt. 68000); 2, myoglobin (17200). Fractions were pooled as indicated in (a) and (b), concentrated on an Amicon PM10 membrane, and dialysed against 6M-urea containing Dowex XR resin (acetate form) to remove SDS before isoelectric focusing.

130000 mol.wt. remained unchanged. Besides the 220000- and 130000-mol.wt. components, several new polypeptide bands were also detected in the molecular-weight range 30000 and 120000. It appears, therefore, that the 160000-mol.wt. fraction contains collagenase-sensitive sequences.

When extract A1 from labelled or non-labelled samples was fractionated on a Sepharose S-300 column, a fraction emerged in the void volume and the [^3H]fucose labelled peak was eluted at a position corresponding to 160000 apparent mol.wt. (A11) (result not shown).

Extract 2. From the excluded A2 peak the ^3H - and ^{14}C -labelled proteins were of 400000, 200000, 160000 and 130000 molecular weight when subjected to SDS/polyacrylamide-gel electro-

Fig. 4. Isoelectric focusing of proteins extracted with 5M-guanidinium chloride/0.05M-dithiothreitol/0.1M-Tris/0.4% Na₂EDTA, pH7.5, from intima-media of rabbit aorta

The extraction procedure and the designation of the extracts are illustrated in Scheme 1, and the separation of glycoproteins in Scheme 2. The molecular-sieve chromatography of extract 1 and 2 is shown in Fig. 3. Separation was monitored by continuous recording of the A_{280} (—) of the collected fractions. Fractions were assayed for pH (■) and radioactivity (the histogram bars indicate the radioactivities measured in the fractions). The arrows indicate the isoelectric points of: (a) glycoprotein I from peak B1; (b) collagenous glycoprotein component from peak A11; (c) glycoproteins II and III from peak B2.

phoresis. Peak A2 was recovered in small amounts and its protein components were not further investigated. The retarded peak B2 was eluted in molecular-weight range 50 000–60 000. It contained a major periodate/Schiff-positive component, from extract 2, of 50 000 apparent mol.wt. on SDS/polyacrylamide-gel electrophoresis. The fractions separated by gel chromatography were purified by isoelectric focusing.

Isoelectric focusing

The separation pattern of proteins from peak B1 (Fig. 3) is shown in Fig. 4(a). The major protein peak focused at pI5.7 was labelled with [¹⁴C]-mannose. Electrophoretic analysis in SDS/polyacrylamide gels indicates that the pI5.7 product

accounted for the recovery of the glycoprotein of 3600 mol.wt. (Glycoprotein I). Fraction A11 was resolved into a major protein peak of pI7.0 (collagenous glycoprotein component) and a minor component of pI5.1. The pI5.1 peak had a high [³H]- and [¹⁴C]-sugar label (Fig. 4b). The [³H]-fucose- and [¹⁴C]-mannose-labelled proteins from peak B2 were focused at pI5.3 and 5.1 (Fig. 4c).

Labelling of glycoproteins with NaB³H₄

To obtain further evidence for the presence of covalently linked oligosaccharides in the pI5.3 and pI5.1 polypeptides, and information on their molecular-weight distribution, both components were also prepared from non-labelled aortas. The non-radioactive focused peak materials were treated successively with galactose oxidase and NaB³H₄. The ³H-labelled reaction products were investigated by SDS/polyacrylamide-gel electrophoresis. The galactose oxidase- and NaB³H₄-treated pI5.3 and pI5.1 components migrated with apparent mol.wts. 50 000 and 130 000 respectively. Electrophoretic analysis and isoelectric focusing indicated that the major periodate/Schiff-positive and [³H]- and [¹⁴C]-sugar-labelled component from extract 2 (glycoprotein II) was recovered at pI5.3. The presence of incorporated [³H]fucose and [¹⁴C]mannose in the pI5.1 protein and the radioactive derivative obtained from it on galactose oxidase and NaB³H₄ treatment, indicates that the pI5.1 component is a glycoprotein of 130 000 mol.wt. (glycoprotein III).

Composition of glycoproteins

Amino acid analysis (results not shown) indicated that glycoproteins I, II, III and collagenous glycoprotein are rich in polar amino acids and methionine. The absence of *N*-methylhistidine indicates that glycoproteins I, II and III are not contaminated with actin. Molecular-sieve chromatography followed by isoelectric focusing of the *S*-carboxamidomethylated proteins proved to be an adequate technique to separate aortic glycoproteins from actin. When fraction B1 was focused over the pH range 2–11, a small amount of hydroxyproline was present in glycoprotein I. The hydroxyproline content of the preparation could be eliminated by a second focusing over the pH range 4–6.

Unlike glycoproteins I, II and III, collagenous glycoprotein contains 30 residues of hydroxyproline and 148 residues of glycine per 1000 amino acid residues. The amount of polar amino acids in collagenous glycoprotein is similar to that found in glycoproteins I, II and III.

The oligosaccharide moiety of glycoprotein I from rabbit aorta contains *N*-acetylglucosamine galactose and mannose residues in molar proportions 1 : 1 : 1.4 and traces of sialic acid and fucose as shown by g.l.c. The incorporation of radioactive sugars into

the four glycoproteins indicate that they are synthesized by the arterial smooth-muscle cells and do not originate from the blood plasma.

Discussion

Our previous experiments on structural glycoproteins (Moczar *et al.*, 1977) and glycopeptides (Moczar *et al.*, 1970) from porcine aorta indicated that their oligosaccharide chains are composed of *N*-acetylglucosamine, galactose, mannose, sialic acid and fucose. Thus radioactive mannose and fucose were used to monitor the glycoprotein label in the surviving aortic intima-media. The simultaneous occurrence of biosynthetic and catabolic processes may lead, however, to a heterogeneous labelling pattern. In order to ascertain that we were not dealing with artefacts, the extraction and separation of proteins from the aortic intima-media incubated *in vitro* with radioactive precursors and from native, non-incubated aorta samples were carried out separately, and the electrophoretic mobilities of the extracted proteins were compared.

The biosynthetic pathway of mannose-containing oligosaccharides in glycoproteins proceeds via polyphenyl phosphate mannose intermediates (Spira *et al.*, 1976; Hemming, 1979). To remove contaminating glycolipids from glycoproteins, the homogenized tissue pellet remaining after the extraction with $\text{CaCl}_2/\text{ATP}/\text{dithiothreitol}$ was extracted with chloroform/methanol/water mixture as described for dolichol derivatives (Kang *et al.*, 1978).

In common with other connective-tissue glycoproteins (Anderson, 1976), aortic glycoproteins are sparingly soluble at low ionic strength (Barnes & Partridge, 1968; Moczar & Robert, 1970; McCullagh *et al.*, 1973; Moczar *et al.*, 1977). To separate glycoproteins associated with elastin from those readily extractable from the collagen-elastin matrix, the extractions of the delipidated residue with guanidinium chloride/dithiothreitol buffer were carried out before and after hydrolysis of collagen (Robert & Moczar, 1982). Aggregation of extracted proteins during molecular-sieve chromatography and isoelectric focusing was avoided by *S*-carboxamidomethylation (Lafuma *et al.*, 1982). This extraction procedure resulted in a rather selective solubilization of glycoprotein I in extract 1 and of glycoprotein II in extract 2. A glycoprotein with a molecular weight (36000) similar to that of glycoprotein I was isolated previously from the porcine (Moczar *et al.*, 1977) and calf aortic media (M. Moczar, unpublished work). Accordingly, this type of glycoprotein appears to be a component of the aortic wall in different species.

As glycoprotein II was not detected in guanidinium chloride extract 1 and could be isolated only after collagenolytic hydrolysis of the aortic tissue,

this molecule could be a cleavage product from a collagenous protein. The first guanidinium chloride extract did also contain, however, a collagenase-sensitive protein in fraction A1. It is tempting to correlate these findings with the data of Sear *et al.* (1981) on the occurrence of fucosylated non-collagenous polypeptides of 50000 and 30000 mol.wt. in the collagenous glycoprotein isolated from elastic fibres. The composition of the collagenous glycoprotein is different from that of type IV collagen (Timpl *et al.*, 1978) and of non-collagenous structural glycoproteins such as laminin (Timpl *et al.*, 1979) and fibronectin (Yamada *et al.*, 1978). The simultaneous presence of hydroxyproline and large amounts of polar amino acids in glycoprotein II is similar to that of the baboon lung glycoprotein of 140000 mol.wt. (Lafuma *et al.*, 1982) and of the collagenous glycoprotein isolated from calf aorta (Gibson & Cleary, 1982). The nature of the [^3H]fucose label in the collagenous digest is not known. It may be due to the presence of fucosylated non-collagenous polypeptides cleaved from basement-membrane-type collagens with collagenase (Sear *et al.*, 1981).

The above results can be compared with those obtained previously on pulmonary-tissue glycoproteins (Lafuma *et al.*, 1982). It appears that both tissues elaborate several glycoproteins. The spectrum of these glycoproteins (gel-electrophoretic profile and specific radioactivity) is different for both tissues and may well be a marker of tissue-specific differentiation of mesenchymal cells.

The work was supported by CNRS (Groupe de Recherche No. 40), INSERM and by the Délégation Générale à la Recherche Scientifique et Technique (grant no. 787260) and the Conseil Scientifique de l'Université Paris-Val de Marne.

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