The carbohydrate components of arterial basement-membrane-like material

Studies on rabbit aortic myomedial cells in culture

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The carbohydrate composition of arterial basement-membrane-like material was investigated. Basement-membrane-like material was isolated from cultures of aortic myomedial cells by a sonication/differential-centrifugation technique. Purified basement-membrane-like material contained a total of 5% sugars, comprising glucose, galactose, mannose, fucose, sialic acid, glucosamine and galactosamine in the approximate molar proportions 3.2:3.5:3.4:3.2:1:5.5:3.1. In addition, small amounts of xylose were found. Analyses for uronic acid showed that glycosaminoglycans comprised about 1% of isolated basement-membrane-like material. The carbohydrate composition indicated the presence of complex-type oligosaccharides in addition to hydroxylysine-linked disaccharides. [^3H]Glucosamine-labelled glycopeptides obtained by proteinase digestion and gel filtration were resistant to endo- β -*N*-acetylglucosaminidase D, but more than 10% were susceptible to α -mannosidase, demonstrating the presence of high-mannose-type oligosaccharides. The distribution of carbohydrates among peptides of basement-membrane-like material on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was investigated after labelling with [^3H]mannose, [^3H]fucose, [^3H]galactose and [^3H]glucosamine. Among peptides that appeared to carry carbohydrates were a proteoglycan(s) and seven glycoproteins in the molecular-weight range 120 000–700 000.

Ultrastructurally, BM-like material is found as a continuous layer surrounding the myomedial cells in tunica media of aorta as well as in cultures of aortic myomedial cells (Pease & Molinari, 1960; Ross, 1971). The physiological function of this periodic acid–Schiff-positive structure is unknown, but the characteristic position suggests that BM-like material serves as a barrier between cells and environment, thus being of importance for the access of hormones, lipoproteins etc. to the cells.

More recently, myomedial BM-like material has been isolated from cultures of aortic myomedial cells. Analyses of the protein components showed that myomedial BM-like material contained several high-molecular-weight collagenous peptides and a larger number of non-collagenous components, some of which were glycoproteins, as indicated by their periodic acid–Schiff reactivity (Heickendorff & Ledet, 1983). It is the purpose of the present study to provide information about the carbohydrate com-

ponents of BM-like material isolated from cultures of arterial myomedial cells. The results indicate that myomedial BM-like material is a heterogeneous structure comprising several glycoproteins and a proteoglycan(s). The carbohydrate analyses suggest that complex carbohydrates, high-mannose-type oligosaccharides, hydroxylysine-linked disaccharides and small amounts of glycosaminoglycans are contained in myomedial BM-like material.

Materials and methods

Cell cultures

Arterial myomedial cell cultures were obtained from rabbit thoracic aortae of white Danish Country rabbits weighing 1½–2 kg as described previously (Ledet & Vuust, 1980). The incubation medium was composed of Eagle's basal medium, Hanks balanced salt solution, ascorbic acid (50 µg/ml), neomycin sulphate (50 µg/ml) and 5% newborn-calf serum plus 5% rabbit serum (Gibco, Paisley, Scotland, U.K.).

Abbreviations used: BM-like material, basement-membrane-like material; SDS, sodium dodecyl sulphate.

Preparation of BM-like material

BM-like material was isolated from 4–5-weeks-old primary non-trypsin-treated cultures of arterial myomedial cells by a procedure that has been described in detail by Heckendorff & Ledet (1983). Briefly, myomedial cells were disrupted in 1 M-NaCl at 4°C and passed through a 300 µm nylon sieve. BM-like material was sedimented by centrifugation at 2100 g for 10 min at 4°C and purified by washing in 1 M-NaCl (three times), 0.15 M-NaCl (twice) and finally in sterile distilled water. All solutions were buffered with 10 mM-Tris/HCl, pH 7.4, and contained the proteinase inhibitors 0.5 mM-*N*-ethylmaleimide, 0.5 mM-phenylmethanesulphonyl fluoride and 1 mM-benzamidinium hydrochloride. Isolated BM-like material was freeze-dried and stored at -18°C until use.

Carbohydrate analyses

All carbohydrate analyses were performed on two to five different preparations containing 1–4 mg (dry wt.) of BM-like material. Neutral sugars and amino sugars were liberated from myomedial BM-like material by hydrolysis in 1 M-HCl and 4 M-HCl, respectively, for 5 h at 100°C. The hydrolysate was separated into neutral sugar and hexosamine fractions by passage through coupled columns of Dowex 50-X4 (H⁺ form; 200–400 mesh) and Dowex 1-X8 (formate form; 200–400 mesh) (Spiro, 1967). The carbohydrates of the neutral-sugar fractions were identified by descending paper chromatography on Whatman no. 1 paper in butan-1-ol/ethanol/water (10:1:2, by vol.) (Spiro, 1967). Sugars were located by the alkaline AgNO₃ procedure (Wheat, 1966). For quantitative determination, the neutral sugars were separated as borate complexes by anion-exchange chromatography on DA-X4-11 resin (Dionex, Sunnyvale, CA, U.S.A.) and eluted as described by Spiro (1972). Neutral sugars were determined by an orcinol reaction (Weiber & Moshin, 1953). The average recovery was 77% when standard mixtures of hexoses were subjected to the whole procedure (hydrolysis included). Glucosamine and galactosamine were separated on cationic resins (DC-6A; Dionex) with citrate buffers of pH 5.0 (Spiro *et al.*, 1976). The hexosamine content was evaluated by a neocuproin/HCl procedure (Lee, 1972). When standards of hexosamines were analysed by this procedure the average recovery was 88%. Fucose was determined directly by Dische & Shettles' (1948) reaction including a sample blank treated with H₂SO₄ and omitting the cysteine reagent. Sialic acid was analysed by the thiobarbituric assay of Warren (1959) after hydrolysis in 0.05 M-H₂SO₄ at 80°C for 1 h. The content of uronic acid was determined by the modified carbazole reaction, with glucuronic acid as standard (Bitter & Muir, 1962). Coloration owing to protein and

neutral sugars was corrected for by including blanks containing bovine albumin and the appropriate mixture of hexoses. Protein was determined by the method of Lowry *et al.* (1951), with bovine albumin as standard.

Metabolic labelling

The distribution of the individual carbohydrates among the peptides of isolated BM-like material was appraised by incubating myomedial cell cultures for 24 h with the following sugars (20 µCi/ml): [2-³H]-mannose (10–20 Ci/mmol), [6-³H]-glucosamine (20–40 Ci/mmol), [1-³H]-fucose (0.5–2 Ci/mmol), [1-³H]-galactose (5–29 Ci/mmol), in addition to [³⁵S]-sulphate (20–40 Ci/mg) or 10 µCi of [4,5-³H]-leucine (40–60 Ci/mmol)/ml. All radiochemicals were obtained from Amersham International.

To estimate the degree of conversion of the radioactive sugars into other sugar compounds, cell cultures were labelled with [2-³H]-mannose, [6-³H]-glucosamine, [1-³H]-galactose or [1-¹⁴C]-galactosamine (2 µCi/ml) for 24 h. For each labelled sugar compound, BM-like material was analysed separately for incorporation into hexosamines and hexoses after hydrolysis in 4 M- and 1 M-HCl, respectively. The released sugars were separated by ion-exchange chromatography as described above, and the radioactivity present in the individual sugars was determined by liquid-scintillation counting, by using a toluene/Triton X-100/ethanol 'cocktail' (8:5:2, by vol.) containing 1.33 g of Permablend III/1 (Packard). The conversion of [³H]-glucosamine into sialic acid was determined by incubation of labelled BM-like material with a *Clostridium perfringens* neuraminidase (Boehringer Mannheim, W. Germany) as described by Spiro (1967). Sialic acid-free BM-like material was precipitated and washed with cold 5% trichloroacetic acid. For a control, BM-like material was treated under the same conditions except for the addition of enzyme.

SDS/polyacrylamide-gel electrophoresis

For periodic acid-Schiff staining (Fairbanks *et al.*, 1971), electrophoresis was performed on 7%-polyacrylamide column gels (Goldberg *et al.*, 1972). Labelled material was separated on 5–15%-acrylamide exponential-gradient slab gels (Laemmli, 1970). The slab gels were stained for protein with Coomassie Brilliant Blue and subjected to fluorography by using Kodak XAR-5 films (Bonner & Laskey, 1974). The following standards for molecular-weight determination were used: ferritin (440 000, 220 000), β-galactosidase (130 000), phosphorylase *a* (94 000), bovine serum albumin (67 000) and pepsin (35 000).

Glycopeptides

[³H]-Glucosamine-labelled BM-like material was incubated with bacterial proteinase (type XIV,

Sigma) (enzyme/substrate ratio, 1:10, w/w) in 0.05M-Tris/HCl buffer (pH 7.8)/0.5mM-CaCl₂ under toluene for 48 h at 37°C. Glycopeptides were obtained by Sephadex G-50 chromatography (fine grade; Pharmacia) (column size 0.9 cm × 70 cm) in 0.2M-ammonium acetate buffer, pH 5.0. Flow rate was 8 ml/h. The columns were calibrated with Blue Dextran (Pharmacia), maltoheptaose (mol.wt. 1153; Boehringer Mannheim), stachyose (mol.wt. 667; Sigma) and [³H]mannose. Labelled glycopeptides were treated with 1 unit of α -D-mannosidase from jack bean (Boehringer Mannheim; 1 unit will hydrolyse 1 μ mol of *p*-nitrophenyl α -D-mannoside/min) in 0.2 ml of 0.05M-sodium acetate buffer, pH 4.0, for 3 h at 37°C (Irimura *et al.*, 1981). Digestion with 0.01 unit of endo- β -N-acetylglucosaminidase D (activity was defined in the Miles Manual) from *Diplococcus pneumoniae* (Miles Scandinavia, Birkerød, Denmark) was performed in 0.2 ml of 0.15M-imidazole buffer, pH 6.5, at 37°C for 24 h. After heat inactivation the samples were analysed by gel filtration on Sephadex G-25 (superfine grade; Pharmacia) as described above.

Results

Carbohydrate analyses

Carbohydrate analyses showed that myomedial BM-like material contained a total of 5.0 mg of sugar residues per 100 mg of protein (assuming that the hexosamines are in the *N*-acetyl form), which explains the periodic acid-Schiff reactivity of this structure. In the neutral-sugar fraction, glucose, galactose, mannose and fucose could be identified according to their migration by descending paper chromatography. Quantitative determinations of hexoses and hexosamines showed that glucose, galactose, mannose, fucose, sialic acid, glucosamine and galactosamine were present in the approximate molar proportions 3.2:3.5:3.4:3.2:1:5.5:3.1, determined on two to five different preparations of BM-like material. In addition, xylose was present. The results of the carbohydrate analyses are shown in Table 1. Fucose was analysed after ion-exchange chromatography of the neutral-sugar fraction obtained by hydrolysis in 1M-HCl for 5 h as well as by the direct Dische-Shettles (1948) reaction. The values obtained by the direct reaction (listed in Table 1) were 20% higher than those by the former procedure [$4.5 \pm 0.43 \mu\text{g}$ (S.E.M., $n = 3$) of fucose/mg of BM-like material], indicating that some destruction of fucose takes place during the 5 h hydrolysis in 1M-HCl. Preparations of BM-like material were analysed for uronic acid by the modified carbazole reaction; $4.5 \mu\text{g}$ of uronic acid was found per mg of BM-like material, consequently approx. 1% of myomedial BM-like material is considered to represent glycosaminoglycans.

Table 1. Carbohydrate composition of isolated arterial BM-like material

BM-like material was obtained from cultures of aortic myomedial cells by a sonication/differential-centrifugation technique. After hydrolysis in 1M-HCl for 5 h at 100°C, neutral sugars were analysed by borate-complex anion-exchange chromatography by using an orcinol reaction. Amino sugars were released from purified BM-like material by hydrolysis in 4M-HCl for 5 h at 100°C and separated by cation-exchange chromatography. Amino sugars were determined by a neocuproin/HCl procedure. No correction for recovery was made. Fucose, sialic acid and uronic acid were each determined directly on preparations of BM-like material as described in the text. Means $\pm$ S.E.M. are given, for the numbers of preparations indicated in parentheses.

	Sugar content ($\mu\text{g}/\text{mg}$ dry wt. of BM-like material)
Glucose	5.6 ± 0.53 (3)
Galactose	6.2 ± 0.19 (3)
Mannose	5.9 ± 0.25 (3)
Fucose	5.6 ± 0.43 (5)
Sialic acid	3.0 ± 0.09 (3)
Xylose	1.8 ± 0.23 (3)
Glucosamine*	11.8 ± 0.27 (2)
Galactosamine*	5.2 ± 1.20 (2)
Uronic acid	4.5 ± 0.51 (3)

* Calculated as *N*-acetylhexosamines (mean $\pm$ range).

Interconversion of labelled sugars

It became clear from the incorporation studies that [³H]mannose was quite stable (Table 2). When myomedial BM-like material was labelled with [³H]galactose, some two-thirds of incorporated radioactivity could be identified as [³H]galactose, the rest being converted into other neutral sugars, predominantly glucose. About 26% of [³H]glucosamine was epimerized to [³H]galactosamine, but no neutral sugars were formed. These labelled sugars were considered useful for characterization of glycoproteins contained in arterial BM-like material. However, when [¹⁴C]galactosamine was used as substrate, most was epimerized to glucosamine. Treatment of [³H]glucosamine-labelled BM-like material with neuraminidase showed that only $8.8 \pm 0.4\%$ (S.E.M., $n = 3$) was converted into sialic acid.

SDS/polyacrylamide-gel electrophoresis

When BM-like material was separated by SDS/polyacrylamide-gel electrophoresis under reducing conditions followed by periodic acid-Schiff staining, most high-molecular-weight peptides appeared positive (results not shown). Fig. 1 shows the fluorescence autoradiograms of myomedial BM-like material labelled with [³H]leucine, [³H]mannose, [³H]galactose, [³H]fucose, [³H]glucosamine and

Table 2. *Interconversion of radioactive sugars released from labelled BM-like material*

Labelled BM-like material was obtained by a sonication/differential-centrifugation procedure from arterial myomedial cell cultures which were labelled with [³H]mannose, [³H]galactose, [³H]glucosamine or [¹⁴C]galactosamine (2 µCi/ml) in separate experiments. Sugars were released from purified BM-like material by hydrolysis and measured by ion-exchange chromatography as described in the Materials and methods section. For each labelled sugar the amount of radioactivity present in the various sugar fractions was quantified and expressed as a percentage of the total amount incorporated into BM-like material. All values represent the mean of three to five analyses. The S.D. was less than 10% in all determinations.

Substrate	Sugars released from BM-like material					
	Monosaccharide					Amino sugar
	Galactose	Mannose	Fucose	Glucose	Xylose	Glucosamine Galactosamine
[³ H]Mannose	0%	97%	3%	0%	0%	← Less than 1%† →
[³ H]Galactose	67%	6%	4%	24%	1%	← Less than 1%† →
[³ H]Glucosamine	← Less than 1%* →					74% 26%
[¹⁴ C]Galactosamine	← Less than 1%* →					73% 27%

* The combined monosaccharides comprised less than 1% measured as incorporated radioactivity eluted in the neutral sugar fraction of coupled Dowex columns after hydrolysis in 1 M-HCl for 5 h.

† Determined as radioactivity eluted in the hexosamine fraction from Dowex 50-X4 columns after hydrolysis of BM-like material in 4 M-HCl for 5 h.

Fig. 1. *Peptides and glycoproteins of purified arterial myomedial BM-like material detected by metabolic labelling*

Arterial myomedial cell cultures were incubated for 24 h with [³H]leucine (10 µCi/ml), [³H]mannose, [³H]galactose, [³H]fucose, [³H]glucosamine or [³⁵S]-sulphate (20 µCi/ml). Arterial BM-like material was isolated from labelled cells by a sonication/differential-centrifugation technique. Purified BM-like material was reduced and subjected to SDS/polyacrylamide-gel electrophoresis on a 5–15%-acrylamide exponential-gradient gel (Laemmli, 1970). Labelled components were detected by fluorescence autoradiography. BM-like material labelled with [³H]leucine (lane 1), [³H]mannose (lane 2), [³H]galactose (lane 3), [³H]fucose (lane 4), [³H]glucosamine (lane 5) or [³⁵S]sulphate (lane 6) is shown.

[³⁵S]sulphate, indicating that BM-like material contains a large number of peptides, of which several appear to be glycoproteins. These could be classified as follows. I, A component(s) that barely penetrated the gel contained sulphate, large amounts of glucosamine and galactose, but no mannose and only small amounts of protein. This component(s) is considered to represent a proteoglycan(s). II, This group comprised several peptides which contained the common constituents of glycoproteins, namely glucosamine, mannose, fucose, galactose and no sulphate. Two peptides which appeared with molecular weights of 700 000 and 280 000 seemed to be extremely heavily glycosylated. Another series of peptides showed a pattern of low carbohydrate/protein ratio. Particularly, five peptides were observed, with molecular weights of approx. 500 000, 400 000, 225 000, 210 000 and 120 000. III, In this group the peptides seemed to contain mannose as the predominant sugar. Here the molecular weights of the major components were estimated to be 100 000, 64 000 and 48 000. The labelling pattern thus points to considerable complexity of the carbohydrate components. In spite of the remarkable heterogeneity of BM-like material, emphasis should be made especially on three components, the proteoglycan(s) and the two glycoproteins appearing with mol.wts. of 700 000 and 225 000, since they seemed to contain most of the carbohydrates of BM-like material. Previously the 225 000-Da glycoprotein

Molecular weights of major peptides (left) and glycoproteins (right) of BM-like material are indicated.

Fig. 2. *Sephadex G-50 chromatography of proteinase-digested BM-like material*

Labelled BM-like material was digested with bacterial proteinase (0.2 mg/ml) for 48 h and analysed by gel filtration. The column (0.9 cm $\times$ 70 cm) was eluted with 0.2 M-ammonium acetate, pH 5.0, and 1.55 ml fractions were collected and assayed for radioactivity. The elution volumes of Blue Dextran (V_0) and [^3H]mannose (V_1) are indicated. ●, [^3H]Glucosamine-labelled BM-like material; ▲, [^3H]mannose-labelled BM-like material.

has been suggested to represent fibronectin (Heickendorff & Ledet, 1983), whereas the identity of the 700 000-Da carbohydrate-rich component remains to be established.

Glycopeptides

Sephadex G-50 chromatography of carbohydrate constituents obtained by proteinase digestion showed that [^3H]glucosamine-labelled components comprised glycosaminoglycans that were eluted with the void volume (Fig. 2). The identification of this high-molecular-weight material was based on HNO_2 treatment and enzymic degradation (L. Heickendorff & T. Ledet, unpublished work). Glycopeptides were retarded on the column and were eluted as a broad peak. All [^3H]mannose-labelled components were eluted in this peak (Fig. 2). The structure of the oligosaccharides was evaluated by digestion with specific glycosidases. [^3H]Glucosamine-labelled glycopeptides were resistant to endo- β -N-acetylglucosaminidase D, since all glycopeptides were eluted with the void volume of Sephadex G-25

Fig. 3. *Sephadex G-25 chromatography of [^3H]glucosamine-labelled glycopeptides (a) and α -D-mannosidase-treated glycopeptides (b)*

Labelled glycopeptides were obtained by Pronase digestion followed by Sephadex G-50 chromatography. Glycopeptides were incubated with 1 unit of jack-bean α -D-mannosidase in 0.05 M-sodium acetate buffer, pH 4.0, for 3 h at 37°C and analysed by gel filtration on Sephadex G-25 (superfine grade). Column size, 0.9 cm $\times$ 70 cm; eluent, 0.2 M-ammonium acetate, pH 5.0; rate 8 ml/h. Fractions (1 ml) were collected and assayed for radioactivity. The elution volumes of Blue Dextran (V_0) and [^3H]mannose (V_1) are indicated.

(results not shown). α -D-Mannosidase, which releases mannose residues from chains with unsubstituted terminal mannose residues, degraded $10.8 \pm 0.5\%$ (S.E.M., $n = 4$) of [^3H]glucosamine-labelled glycopeptides (Fig. 3).

Discussion

Previously we demonstrated that purified BM-like material comprises several high-molecular-weight collagenous peptides, but no type I or type III collagens (Heickendorff & Ledet, 1983). The carbohydrate composition of myomedial BM-like material is compatible with the presence of glucose-galactose units linked to the collagenous peptides. Such disaccharides have previously been found in basement membranes from several tissues (Spiro, 1976). The lower glucose content of myomedial BM-like material corresponds to previous data showing a predominance of non-collagenous components (Heickendorff & Ledet, 1983).

The presence of fucose, sialic acid, glucosamine and mannose indicate that myomedial BM-like material contains complex oligosaccharides. The oligosaccharides were resistant to endo- β -N-acetyl-

glucosaminidase D. This enzyme is active only towards complex carbohydrates containing a free mannose residue attached to the mannose-glucosamine-glucosamine core (Muramatsu, 1978). It is noteworthy that α -mannosidase treatment demonstrated that about 10% of [^3H]glucosamine-labelled oligosaccharides contain unsubstituted mannose residues at the terminal reducing end, indicating that the susceptible oligosaccharides may be of high-mannose type (Kornfeld & Kornfeld, 1976). This is also in agreement with the SDS/polyacrylamide gels showing that some glycoproteins of myomedial BM-like material contain mannose as the principal sugar. The number of high-mannose-type oligosaccharides is most probably underestimated, since the α -mannosidase-resistant oligosaccharides may contain [^3H]glucosamine residues not only in the core region but also in the outer chains. The terminal sugar in the more common α -mannosidase-resistant glycopeptides is probably mainly sialic acid, since this sugar occurs in large amounts in myomedial BM-like material. Moreover, we have demonstrated that about 80% of the oligosaccharides carry negative charges, consistent with extensive sialylation (L. Heckendorff & T. Ledet, unpublished work).

We have found that glycosaminoglycans constitute about 1% of myomedial BM-like material, and similar data have been presented for glomerular basement membranes (Kanwar & Farquhar, 1979). SDS/polyacrylamide-gel electrophoresis of myomedial BM-like material revealed the presence of a sulphated high-molecular-weight component(s), indicating that the glycosaminoglycans exist in the form of a proteoglycan(s). More recently, heparan sulphate has been isolated from purified myomedial BM-like material (L. Heckendorff & T. Ledet, unpublished work), which is also the predominant type in other basement membranes (Kanwar & Farquhar, 1979). The content of xylose relative to uronic acid was higher than expected if it was derived only from xylosyl-serine linkages in a proteoglycan (Rodén, 1980). Xylose, which is not considered as a normal constituent of animal glycoproteins, has been found in glycoproteins from pig plasma membranes (King & Tabiowo, 1982), but the significance of this finding is not clear.

In studies involving metabolic labelling of carbohydrates, the biosynthetic fate of the radioactive sugars used is a crucial point. [^3H]Mannose and [^3H]galactose have been found to be converted into other sugars, whereas [^3H]fucose is considered to be stable, on the basis of observations from several cell-culture systems (Yurchenco *et al.*, 1978). The observed stability of [^3H]mannose, [^3H]glucosamine and [^3H]galactose in the present study may be caused by the presence of 5.6 mM-glucose in the incubation medium during labelling, thus providing the cells with sufficient unlabelled precursors.

Our previous data have suggested that the 225 000-Da peptide contained in isolated BM-like material represents fibronectin (Heickendorff & Ledet, 1983). In the present investigation this peptide was found to be one of the most prominent glycoproteins of arterial BM-like material, and appeared to contain mannose, galactose, fucose and glucosamine, which is compatible with chemical analyses of fibronectin (Ruoslahti *et al.*, 1981). The presence of fibronectin in myomedial BM-like material is in accordance with immunohistochemical studies showing that tunica media of aorta contains fibronectin associated with myomedial BM-like material (Stenman & Vaheri, 1978).

The carbohydrate composition of BM-like material isolated from cell cultures of aortic myomedial cells showed that complex oligosaccharides, high-mannose-type oligosaccharides, glucose-galactose units and small amounts of glycosaminoglycans constitute the carbohydrate components. Moreover, there is a disparity in the distribution of the carbohydrate units among the various peptides. The physiological consequence of the composition and dispersion of the carbohydrates in the BM-like material is unknown, but may be important for the interactions of the cell and its environment.

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