Arterial basement-membrane-like material isolated and characterized from rabbit aortic myomedial cells in culture

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(Received 25 October 1982/Accepted 1 February 1983)

Arterial basement-membrane-like material was isolated from rabbit aortic myomedial cell cultures by sonication and differential centrifugation. Isolated basement-membrane-like material was shown to be free of both cellular and matrix contaminants, on the basis of determinations of DNA, RNA, cholesterol, phosphorus and ($\text{Na}^+ + \text{K}^+$)-activated ATPase, combined with electron microscopy. Amino acid analyses showed that arterial basement-membrane-like material was composed of predominantly non-collagenous amino acids. Evaluated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, reduced basement-membrane-like material comprised six major and about 30 minor components in the M_r range 10 000–600 000. One of the major peptides (M_r 225 000) was disulphide-linked. Periodic acid–Schiff staining of gels indicated that most high-molecular-weight components were glycoproteins. Two-dimensional gel electrophoresis resolved reduced basement-membrane-like material into more than 100 components, with pI from 5 to 7. The disulphide-linked M_r 225 000 peptide appeared heterogeneous, with pI of 5.6–6.0, and was considered to represent fibronectin. All major peptides were of non-collagenous nature, on the basis of their susceptibility to pepsin and resistance to collagenase. Purified myomedial basement-membrane-like material contained collagenous peptides, as indicated by the presence of hydroxyproline and hydroxylysine. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of pepsin-treated and reduced basement-membrane-like material revealed five high-molecular-weight collagenous components appearing in the M_r range 105 000–375 000 relative to type I collagen standards.

Previous characterization of basement membranes was based on morphological methods such as periodic acid–Schiff stain and electron microscopy. The myomedial BM-like material in tunica media is an ill-defined but abundant component of the smooth-muscle cell, coating the entire surface of the cells (Ross, 1971; Campeley-Campbell *et al.*, 1979). However, in electron micrographs myomedial BM-like material does not appear to contain the various layers described for glomerular basement membranes, but shows large variation in both width and electron density. Our knowledge of the chemical composition of myomedial BM-like material is rather small. Previous chemical studies have been concerned solely with the collagenous part of myomedial BM-like material, involving proteolytic digestion of whole arterial tissue (Trelstad, 1974;

Chung *et al.*, 1976). The introduction of tissue cultures of arterial myomedial cells offers the possibility of obtaining a pure preparation of myomedial BM-like material.

It is the purpose of the present study to describe the isolation procedure and to present the results of compositional studies performed on arterial BM-like material isolated from myomedial cells in culture. The results indicate that arterial BM-like material obtained by a sonication technique consists of non-collagenous glycoproteins with a heterogeneous polypeptide distribution and several high-molecular-weight collagenous peptides.

Materials and methods

Cell cultures

Arterial myomedial cells were obtained from thoracic aortae of male white Danish rabbits

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

weighing 1½–2 kg. Tunica media was made into explants (diameter 1–2 mm), which were then incubated in batches of 13–15 pieces per tissue-culture flask (264 ml; Nunc, Roskilde, Denmark) (Ledet *et al.*, 1976; Ledet & Vuust, 1980). The incubation medium was composed of Eagle's basal medium, Hanks balanced salt solution, neomycin sulphate (50 µg/ml), ascorbic acid (50 µg/ml) and 10% rabbit serum (Gibco, U.K.). Non-trypsin-treated cultures of arterial myomedial cells contained 5–7 mg of protein per flask after a growth period of about 5–6 weeks.

Preparation of BM-like material

The isolation procedure was mainly based on the method of Krakower & Greenspon (1951) and Spiro (1967). The medium was decanted and cell layer washed three times with cold 0.15 M-NaCl, followed by incubation for 15 min at 4°C with 1 M-NaCl. All NaCl solutions were buffered with 10 mM-Tris/HCl, pH 7.4, and added with the proteinase inhibitors 0.5 mM-*N*-ethylmaleimide, 0.5 mM-phenylmethanesulphonyl fluoride and 1 mM-benzamidine hydrochloride (Sigma). The cells were removed, and sonicated (MSE sonicator, model Soniprep) in the 1 M-NaCl buffer, at a wavelength of 16 µm for three intervals of 30 s with cooling in ice/water between bursts. The sonicated cell suspension was evaluated by phase-contrast microscopy. The disrupted cell suspension was then passed through a 300 µm sieve to remove explants. BM-like material was sedimented by centrifugation at 2100g for 10 min at 4°C and washed sequentially in 1 M-NaCl (three times), 0.15 M-NaCl (twice) and finally in sterile distilled water. The membrane pellet was freeze-dried and stored at –18°C until use.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed on 7½% polyacrylamide column gels (0.5 cm × 10 cm) containing 0.1% SDS in the sodium phosphate/urea buffer system of Goldberg *et al.* (1972). However, radio-labelled BM-like material was examined on 0.75 mm-thick slab gels. The procedure was that described by Laemmli (1970) with a 5–15% exponential-gradient separating gel, a 4% stacking gel and Tris/glycine buffers. Each sample contained approx. 100 µg of protein, except for the periodic acid–Schiff-stained gels, which were loaded with 200–300 µg of protein. The gels were stained for protein with Coomassie Brilliant Blue and for glycoprotein by the periodic acid–Schiff-staining method of Fairbanks *et al.* (1971). The following standards for M_r determinations were used: pepsin (35 000), bovine serum albumin (67 000), phosphorylase *a* (94 000), β -galactosidase (130 000) and ferritin (220 000, 440 000).

Two-dimensional polyacrylamide-gel electrophoresis

Labelled BM-like material was further examined by two-dimensional gel electrophoresis mainly as described by O'Farrell (1975). The second dimension comprised a 7½% polyacrylamide slab gel made in a urea/phosphate buffer (Goldberg *et al.*, 1972).

Autoradiography and metabolic labelling

The membrane material was labelled for autoradiography by incubating the cells with 10 µCi of [³H]amino acid mixture/ml (Amersham International) for 24 h. Fluorography of the gels was done as described by Bonner & Laskey (1974), with Kodak NS-2T X-ray films.

The content of RNA in BM-like material was evaluated after growing the cells in [U-¹⁴C]uridine (0.5 µCi/ml, 450–540 Ci/mol; Amersham International) for 24 h. Its content of DNA was evaluated after incubating cell cultures for 24 h with [methyl-³H]thymidine (0.5 µCi/ml, 2 Ci/mol; Amersham International).

Chemical determinations

The contents of cholesterol and phosphorus in isolated BM-like material were evaluated by the procedures of Hanel & Dam (1955) and Bartlett (1959) respectively. Protein was measured by the method of Lowry *et al.* (1951), with bovine albumin as standard. Contamination with plasma membranes was evaluated by measuring (Na⁺ + K⁺)-activated ATPase by the procedure of Jørgensen (1974). The DNA content of BM-like material was measured by a diphenylamine reaction (Burton, 1956). DNA determinations were performed on pooled membrane pellets (about 1 mg of protein).

Amino acid analyses

Amino acids were determined with a Beckman 121 MB amino acid analyser after hydrolysis of the samples in 6 M-HCl for 20 h at 110°C in sealed tubes. A more accurate analysis of the ratios proline/hydroxyproline and lysine/hydroxylysine was evaluated by incubating the cell cultures with [U-¹⁴C]proline (1 µCi/ml, 250 Ci/mol; Amersham International) or [U-¹⁴C]lysine (3 µCi/ml, 300 Ci/mol; Amersham International) for 24 h respectively. Proline and hydroxyproline liberated by acid hydrolysis were estimated by ion-exchange chromatography (Dowex 50-X8) (Firshein & Schill, 1966), and lysine and hydroxylysine were measured by t.l.c. (Cohen & Vogt, 1975).

Proteolytic cleavage of BM-like material

For digestion with pepsin (Calbiochem), BM-like material was incubated for 2 h at 30°C (Crouch & Bornstein, 1979). The reaction was terminated by

addition of 0.5M-NaOH and the digested material was dialysed against distilled water and freeze-dried before SDS/polyacrylamide-gel electrophoresis. For digestion with collagenase (Sigma, grade IV), samples were incubated with bacterial collagenase (5 μ g/ml), 0.5mM-CaCl₂ and 0.5mM-*N*-ethylmaleimide for 6h at 37°C (Crouch & Bornstein, 1979). For controls, BM-like material was incubated under the same conditions, except for the addition of pepsin or collagenase.

Electron microscopy

Electron-microscopic examinations were performed on both cell layer and isolated BM-like material. The cells were fixed and embedded *in situ* in the culture flasks. As the primary fixative glutaraldehyde was used, and all material was post-fixed in osmium before embedding in Epon. This sections were prepared with an LKB Ultratome and then stained with aqueous uranyl acetate. The stained sections were viewed with a Jeol 100B or a Philips electron microscope.

Results

Cell cultures

Observations of the cell cultures by phase-contrast microscopy revealed a multilayer and a growth pattern typical for myomedial cells (Fig. 1) (Ross, 1980). Electron microscopy confirmed that the cells were arranged in a multilayer and moreover contained characteristic myofilaments and dense bodies (Fig. 2). BM-like material appeared finely granular and covered the cell surface in abundance, although some areas devoid of BM-like material were occasionally observed.

Isolation of myomedial BM-like material

The effect of various sonication times was evaluated by a combination of phase-contrast microscopy and SDS/polyacrylamide-gel electrophoresis. It became clear that sufficient disruption of cells was only obtained by sonication times of 3 $\times$ 30s or 6 $\times$ 30s. The SDS/polyacrylamide-gel electrophoresis disclosed no obvious differences in number and position of bands (Figs. 3a and 3b). To minimize the sonication time, the 3 $\times$ 30s procedure was selected.

In subsequent studies we analysed the influence of three different centrifugal forces (900g, 1500g, 2100g) on BM-like material. The position and number of bands after SDS/polyacrylamide-gel electrophoresis revealed no difference in band pattern (Figs. 3c and 3d), and centrifugation at 2100g was then chosen. Isolated BM-like material constituted up to 10–15% of total cell protein. From each culture flask 200–700 μ g of BM-like material could be obtained.

Fig. 1. Phase-contrast micrograph of a 4-week-old primary culture of arterial myomedial cells

Myomedial cells were grown out from tunica media explants obtained from rabbit aorta and cultured until stationary growth phase without trypsin treatment. The micrograph demonstrates the characteristic growth pattern of myomedial cells, which form 'hills' (arrow) and 'valleys' as described by Ross (1980). Magnification $\times$ 400.

Fig. 2. Electron micrograph of a 5-week-old primary culture of rabbit aortic myomedial cells

The cells were fixed and embedded in the culture flask. The electron micrograph shows that the cells are arranged in a multilayer, with abundant amounts of BM-like material (arrows) covering cell surfaces. Magnification $\times$ 26 000.

Purity of BM-like preparation

The purity was assessed by electron-microscopic examination, chemical analyses and metabolic labelling. Electron microscopy showed that pellets consisted of sheets of finely granular membranes which

at low (Fig. 4a) and high magnification (Fig. 4b) showed no contamination with nuclei, cell membranes, elastin, collagen fibres or cytoplasmatic organelles.

Incubation studies with [methyl- ^3H]thymidine revealed that there was very little DNA in the membrane preparations (Table 1). Chemical determination of DNA showed that the content in three different pooled preparations (each containing about 2 mg of protein) was below the limit of detection. When the content of RNA was evaluated by [U- ^{14}C]uridine incorporation, only 0.7% of radioactivity incorporated into myomedial cells was found in isolated BM-like material (Table 1).

In three different preparations there was no trace of ($\text{Na}^+ + \text{K}^+$)-activated ATPase (300–500 μg of protein each). Analyses of total phosphorus showed that the present preparation contained 1.22 $\mu\text{g}/\text{mg}$ of protein. The amount of cholesterol was correspondingly low (Table 1).

Fig. 3. Influence of sonication time and centrifugal force on the peptide pattern of isolated BM-like material. Arterial myomedial cells were sonicated for $3 \times 30\text{ s}$ (a) or $6 \times 30\text{ s}$ (b). In both cases BM-like material was sedimented by centrifugation at 2100g and purified by extensive washing as described in the Materials and methods section. Gels (c) and (d) show BM-like material obtained by sonication for $3 \times 30\text{ s}$ and centrifugation for 10 min at 900g (c) or 2100g (d). Electrophoresis was performed under reducing conditions in 7.5% polyacrylamide column gels in the presence of 0.1% SDS. Proteins were stained with Coomassie Blue.

Fig. 4. Electron micrographs of purified BM-like material isolated from arterial myomedial cells in culture.

After disruption of myomedial cells by sonication for $3 \times 30\text{ s}$, BM-like material was sedimented by centrifugation at 2100g for 10 min and washed in buffered 1M-NaCl and 0.15M-NaCl several times and finally in sterile distilled water. The final pellet containing purified BM-like material was fixed in glutaraldehyde and embedded in Epon. Isolated BM-like material at low magnification (a, $\times 6000$) and at high magnification (b, $\times 60000$). Note absence of nuclei, cytoplasmic organelles, cell membranes, elastin and collagen fibres.

Table 1. Arterial myomedial BM-like material

Three to five different preparations of BM-like material were analysed for possible contaminants as described in the Materials and methods section. Means $\pm$ s.e.m. are given for the numbers of analyses indicated in parentheses.

Cholesterol ($\mu\text{g}/\text{mg}$)*	0.17 ± 0.02 (3)
Phosphorus ($\mu\text{g}/\text{mg}$)*	1.22 ± 0.73 (4)
DNA ($\mu\text{g}/\text{mg}$)*	<0.01 (5)
[^{14}C]Uridine (%)†	0.71 ± 0.11 (4)
[^3H]Thymidine (%)†	0.19 ± 0.04 (3)

* $\mu\text{g}/\text{mg}$ of BM-like protein.

† Radioactivity in isolated BM-like material as % of radioactivity incorporated into cells. In these experiments BM-like material constituted $14.2 \pm 2.33\%$ (s.e.m.) of total cell protein.

Amino acid composition

Amino acid analyses indicated that glutamic acid, leucine and aspartic acid were the most abundant constituents of isolated BM-like material (Table 2). The contents of glycine, hydroxyproline and hydroxylysine were remarkably low. The concentrations of hydroxyproline and hydroxylysine were evaluated also by radioactive labelling. Hydroxyproline amounted to $8.89 \pm 0.58\%$ (S.E.M.; $n=4$) relative to proline, and hydroxylysine amounted to $1.75 \pm 0.05\%$ (S.E.M.; $n=3$) relative to lysine.

SDS/polyacrylamide-gel electrophoresis

Initially we evaluated the solubility of BM-like material labelled with [^3H]amino acids by using 2% SDS supplemented with mercaptoethanol; 90–95% of BM-like material was solubilized. BM-like material subjected to SDS/polyacrylamide-gel electrophoresis under reduced conditions gave a pattern of approx. 35–37 components, ranging in apparent M_r from 10 000 to 600 000 (Fig. 5a). Six major components were found, with apparent M_r values 265 000, 225 000, 205 000, 200 000, 55 000 and 45 000. It is noteworthy that no bands with positions corresponding to collagen $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ chains were observed on the gels.

The major changes caused by non-reduction were almost confined to high-molecular-weight peptides.

The M_r -225 000 peptide migrated to a position at M_r 450 000 under non-reducing conditions (Fig. 5b).

Myomedial BM-like material treated with bacterial collagenase exhibited minor changes in band pattern on an exponential SDS/polyacrylamide gel under reducing conditions. A component of M_r approx. 300 000 was susceptible to bacterial collagenase (Fig. 6a).

When column gels were stained for carbohydrate by the periodic acid–Schiff technique, nearly all major bands were found to be positive. It was characteristic that the colour reaction developed most strongly in the group of high-molecular-weight components (results not shown).

Fig. 5. Fluorescence autoradiograms of BM-like material isolated from myomedial cells in culture labelled with $10 \mu\text{Ci}$ of [^3H]amino acids/ml

Electrophoreses were performed on 5–15% exponential-gradient polyacrylamide slab gels in the presence of 0.1% SDS. (a), Estimation of apparent M_r of polypeptides components of reduced myomedial BM-like material. Standards for M_r determination were ferritin (440 000), β -galactosidase (130 000), phosphorylase (94 000), bovine albumin (67 000) and pepsin (35 000). (b), Reduced (left) and non-reduced (right) BM-like material, showing the appearance of a M_r -450 000 peptide and the disappearance of a M_r -225 000 peptide under non-reducing conditions. Numbers to the left and right of the gels are M_r values ($\times 10^{-3}$).

Table 2. Amino acid composition of BM-like material
BM-like material was obtained from cultured aortic myomedial cells by a sonication/differential-centrifugation technique. Amino acids were determined on 24 h hydrolysates of isolated BM-like material. The numbers represent the mean values from two preparations.

Amino acid	Content (residues/1000 residues)
Aspartic acid	90
Threonine	56
Serine	63
Glutamic acid	122
Proline	65
Hydroxyproline	6
Glycine	78
Alanine	72
Valine	65
Methionine	19
Isoleucine	40
Leucine	90
Tyrosine	33
Phenylalanine	45
Lysine	60
Hydroxylysine	2
Histidine	23
Arginine	55
Half-cysteine	16

Fig. 6. Proteolytic cleavage of myomedial BM-like material

BM-like material was prepared from cultures of arterial myomedial cells, which were labelled with [^3H]amino acids. Isolated BM-like material was resolved by SDS/polyacrylamide-gel electrophoresis on a 5–15% exponential-gradient gel after reduction with mercaptoethanol, and the labelled peptides were detected by fluorescence autoradiography. (a), Right-hand gel, BM-like material digested with bacterial collagenase ($5\text{ }\mu\text{g/ml}$) for 6 h, 37°C . The control gel (minus collagenase) is shown to the left. The apparent M_r of a collagenase-susceptible peptide is indicated. (b), Left-hand gel, pepsin-resistant components of BM-like material. Incubation with pepsin was performed for 2 h, 30°C . The apparent M_r values ($\times 10^{-3}$) of pepsin-resistant peptides were estimated relative to calf skin collagen α_1 , β and γ chains and are indicated. (b), Right-hand gel demonstrates the collagenous nature of the pepsin-resistant components, since all peptides were degraded by bacterial collagenase.

Pepsin-resistant collagenous components

Since pepsin treatment of myomedial BM-like material resulted in extensive degradation, pooled preparations containing $700\text{--}1000\text{ }\mu\text{g}$ of protein (before pepsin treatment and dialysis) had to be used for SDS/polyacrylamide-gel electrophoresis. Fig. 6(b) shows an autoradiogram of pepsin-treated reduced BM-like material. BM-like material contained at least five pepsin-resistant bands, which

were all susceptible to bacterial collagenase (Fig. 6b), indicating that the peptides represented collagenous material. Estimation of apparent M_r values with type I collagen as standard showed that one major collagenous component had M_r about 105 000, corresponding to the M_r obtained for the B chain of type V collagen (Foidart *et al.*, 1981). Myomedial BM-like material contained also a pepsin-resistant component of M_r about 135 000. A collagenous peptide with approximately the same M_r has been isolated from other basement membranes (Kefalides & Winzler, 1966; Sage *et al.*, 1979; Foidart *et al.*, 1981). Two distinct components were discernible on our gels (Fig. 6), with apparent M_r values of about 200 000–210 000, in addition to a broad band with M_r of about 375 000.

Two-dimensional-gel electrophoresis

By two-dimensional-gel electrophoresis, reduced BM-like material comprised more than 100 components in pH range 5.0–7.0. Almost the same kind of data have been obtained for the glomerular basement membrane (Sato & Spiro, 1976). Our analysis showed that the M_r -225 000 peptide was heterogeneous, with pI 5.6–6.0.

Discussion

The present study has indicated that it is possible to isolate BM-like material from myomedial cells in culture. It was our purpose to provide a description of the chemical composition of whole myomedial BM-like material rather than to focus on single components, and it was therefore essential to avoid proteolytic degradative methods. The procedure used of sonication and differential centrifugation was found suitable as it was for isolation of other basement membranes (Spiro, 1967; Westberg & Michael, 1970; Butkowski *et al.*, 1979). The fact that extended sonication had no influence on the peptide pattern of BM-like material as evaluated by SDS/polyacrylamide-gel electrophoresis is in accordance with the results of Freytag *et al.* (1976). Moreover, Spiro (1970) and Westberg & Michael (1970) found no changes in hydroxyproline and phospholipid contents of glomerular basement membranes isolated after prolonged sonication.

Contamination with extracellular matrix components and intracellular proteins was evaluated by thorough chemical, enzymic and electron-microscopic examinations. The amino acid composition of isolated BM-like material was different from that obtained from microfibrils (Muir *et al.*, 1976) and elastic fibres (Ross & Bornstein, 1969). Moreover, the negligible quantities of phosphorus and cholesterol in the present preparation were in the same range as those obtained previously in other base-

ment-membrane preparations (Spiro, 1967; Westberg & Michael, 1970; Butkowski *et al.*, 1979). The fact that BM-like material obtained from myomedial cells in culture exhibited no ($\text{Na}^+ + \text{K}^+$)-activated ATPase activity, combined with electron-microscopic examinations, support the chemical indications of a highly purified preparation of BM-like material.

It is noteworthy that there were no bands with molecular weights corresponding to type I collagen α chains; thus the content of hydroxyproline in BM-like material could not represent contamination with interstitial collagens. However, immunoelectron-microscopic studies have demonstrated the presence of collagen types IV and V within basement membranes of the kidney (Roll *et al.*, 1980). Moreover, B chains of type V collagen, which in tunica media is located pericellularly in relation to the smooth-muscle cells (Madri *et al.*, 1980), have been extracted from the media of human aortic tissue (Chung *et al.*, 1976; Ooshima, 1981) and cultures of arterial myomedial cells (Mayne *et al.*, 1978). It is at present unclear if some of the collagenous peptides in our BM-like preparation correspond to collagen types IV and V. From radioactive-hydroxyproline measurements it is suggested that the collagenous peptides in the present study make up approx. 5–10% of arterial myomedial BM-like material.

The M_r -225 000 peptide of reduced myomedial BM-like material was considered to represent fibronectin, since this peptide was disulphide-bonded, pepsin-susceptible, collagenase-resistant and appeared heterogeneous in the same pH range as described for fibronectin (Vartio & Vaheri, 1981). Previous studies *in vitro* have demonstrated that arterial myomedial cells produce fibronectin (Burke *et al.*, 1977; Ledet & Vuust, 1980), and morphological observations suggest that some basement membranes contain fibronectin as a part of the structure (Courtoy *et al.*, 1980). A glycoprotein called laminin isolated from BM material obtained from the EHS sarcoma has been shown to contain two chains of M_r 200 000 and 440 000 after reduction (Timpl *et al.*, 1979). This peptide may well be a part of the present BM-like material, since bands were observed with the described M_r values.

Studies of basement membranes isolated from a large number of tissues, e.g. renal glomerulus (Kefalides & Winzler, 1966; Spiro, 1976), renal tubules (Butkowski *et al.*, 1979), lens capsule (Fukushi & Spiro, 1969), have indicated that the composition varies substantially with each basement membrane as reflected by amino acid analyses. Isolated myomedial BM-like material contains less collagenous components than do other characterized basement membranes, which also emphasizes that, although chemically related, there are

differences in the chemical structure of basement membranes.

The data provided in the present study showed that arterial BM-like material, which comprises rather small amounts of collagenous but large amounts of non-collagenous peptides, can easily be obtained from cultures of myomedial cells for dynamic as well as pathophysiological studies.

The excellent technical assistance of Kirsten Mikkelsen, is gratefully acknowledged. Electron microscopy was performed by Professor Steen Olsen, University Institute of Pathology and Dr. Jan Nørgaard, Department of Anatomy A, University of Aarhus. Dr. Torben Elbæk Petersen, Department of Molecular Biology and Plant Physiology, and Dr. Peter Leth Jørgensen, Department of Physiology, University of Aarhus, performed the amino acid analyses and ATPase activity measurements respectively. The study was supported by the Danish Medical Research Council, Chief Physician Garde and Mrs. Garde's Foundation, King Christian X Foundation, NOVO Foundation, Danish Heart Association and Nordic Insulin Foundation. L. H. is the recipient of a Research Fellowship at the University of Aarhus.

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