Biochimica et Biophysica Acta 898 (1987) 37–52
Elsevier

BBA 73372

Separation of luminal and abluminal membrane enriched domains from cultured bovine aortic endothelial cells: monoclonal antibodies specific for endothelial cell plasma membranes

Stuart Jaffe [a], Peter D. Oliver [b], Shakeel M. Farooqui [a], Patricia L. Novak [c], Nino Sorgente [c] and Vijay K. Kalra [a]

[a] *Department of Biochemistry,* [c] *Departments of Ophthalmology and Pathology, School of Medicine, University of Southern California,* [c] *The Vascular Research Laboratory, Estelle Doheny Eye Foundation, Los Angeles, CA (U.S.A.) and* [b] *Department of Anatomy, McGill University, Montreal (Canada)*

(Received 23 July 1986)
(Revised manuscript received 6 November 1986)

Key words: Endothelial cell; Plasma membrane; Iodination; Monoclonal antibody; Western blot; Freeze-fracture; Electron microscopy

Two kinds of membrane (luminal and abluminal membrane domains) fractions have been isolated from bovine aortic endothelial cells by fractionation of whole cell homogenate on discontinuous sucrose density gradients. The luminal membrane domain was enriched 12–16-fold for angiotensin-converting enzyme activity and 8–10-fold in alkaline phosphatase activity. The abluminal membrane domain displayed an enrichment of 8-fold in $(Na^+ + K^+)$-ATPase activity. Both of the membrane domains were minimally contaminated with mitochondria, microsomes and Golgi bodies, as assessed by their corresponding marker enzyme activities. ^{125}I-labeling of endothelial cell monolayers by the Enzymo-Bead lactoperoxidase-catalyzed iodination procedure, followed by isolation of membranes, revealed that the radioactivity was predominantly associated with membranes enriched in angiotensin-converting enzyme activity, corresponding to the luminal membrane domain. However, when cells were radioiodinated in suspension culture, radioactivity was found equally associated in both the luminal and abluminal membrane fractions. Electron microscopy of freeze-fractured and sectioned material showed both luminal and abluminal membrane domains to be in the form of vesicles varying in size from 100 to 400 nm in diameter. To characterize the separation of endothelial cell membrane domains, we have attempted to prepare monoclonal antibodies specific for endothelial cells. Several clones were obtained, producing antibodies which bound to endothelial cells of arterial, venous and capillary origin. Two antibodies of these clones, $XIVC_6$ and XVD_2, were studied in more detail. In the ELISA assay, these antibodies reacted with bovine vascular endothelial cells, but not with human umbilical cord endothelial cells, nor with bovine corneal endothelial cells, smooth muscle cells or fibroblasts. Both of these antibodies are directed against an antigen of approximately 130 kDa, under reducing and non-reducing conditions, as assayed by the immunoprecipitation method. Western blot analysis of luminal and abluminal membrane fractions revealed that only MAb XVD_2 reacted with an antigen, indicating that the antibody $XIVC_6$ is directed against an epitope which is denatured by SDS. Moreover,

Abbreviations: BAEC, bovine aortic endothelial cells; GAM-FITC, goat antimouse Ig, fluorescein isothiocyanate (FITC) conjugated; ELISA, enzyme-linked immunosorbent assay.

Correspondence: V.K. Kalra, Department of Biochemistry, University of Southern California School of Medicine, 2025 Zonal Avenue, Los Angeles, CA 90033, U.S.A.

MAb XVD_2 preferentially reacted with the luminal membrane compared to the abluminal membrane domain of the endothelial cell. These monoclonal antibodies do not react with platelet membrane proteins, indicating that this 130 kDa membrane antigen is not common to both endothelial cells and platelets. These monoclonal antibodies will be useful for monitoring the separation of luminal and abluminal membrane domains and in the immunoaffinity purification of these domains.

Introduction

Endothelial cells form the lining of blood vessel walls, providing a physical barrier between the circulating blood and the surrounding tissues. The lining of vascular endothelium plays an active role in the adherence of stimulated platelets [1], polymorphonuclear leukocytes [2–6], monocytes [7], sickled red blood cells [8], and very likely in tumor cell adherence and migration during the process of metastasis [9–11].

Recently, it has been shown that the blood coagulation factors IX, IXa and X also bind to endothelial cells with high affinity [12,13], suggesting that endothelial cells may have a more direct role in the regulation of blood coagulation, in addition to secreting plasminogen activator [14] or inhibiting thrombin formation [15]. Alterations in endothelial cell structure and/or function very likely play a role in thrombosis and in the macro- and micro-angiopathies which are the hallmarks of diseases such as diabetes and atherosclerosis [16,17].

Endothelial cells, like epithelial cells, exhibit a polarized organization that reflects different functions carried out at the different cell surfaces. The luminal membrane domain is involved in the interaction with blood components, the exchange of ions, and transport of hydrophilic macromolecules. The lateral surface, in contact with neighboring endothelial cells, is specialized in cell adhesion and intracellular communication. The abluminal membrane domain is in contact with the basal lamina and is involved in the transport of small and large molecules which provide nutrients for underlying smooth muscle cells and other tissue cells. These domains have been characterized morphologically in capillary endothelium by studying the distribution of injected polycationized ferritin and alcian blue in mice [18]. Recently, Horvat et al. [19] demonstrated different domains in endothelial cells utilizing immunoelectron microscopy of podocalyscin. It has been shown that podocalyscin is predominantly localized to the luminal membrane domain. However, relatively little information is available on the biochemical composition and distribution of membrane constituents in the endothelial cell plasma membranes, and what role these play in the molecular events associated with the adherence of blood cells to endothelial cells. Likewise, it is not known which constituents of the endothelial cell membrane confer to the endothelium its non-thrombogenic property.

In order to define the role of membrane components in the luminal membrane domain that function in endothelial cell physiology and adherence, we have subfractionated bovine aortic endothelial cells and obtained two separate plasma membrane domains that are enriched in either alkaline phosphatase and angiotensin-converting enzyme activity, or $(Na^+ + K^+)$-ATPase activity. The labeling of cells in monolayer, utilizing the lactoperoxidase-catalyzed iodination procedure, showed that the fraction enriched in angiotensin-converting enzyme activity also contained most of the incorporated radioactivity, and thus corresponded to the luminal membrane domain. In an effort to obtain more markers specific to luminal and abluminal endothelial cell domains, we raised monoclonal antibodies to bovine aortic endothelial cell surface antigens. We describe the development of two monoclonal antibodies directed against a bovine endothelial cell membrane protein(s) having a molecular weight of 130 000. Using Western blot analysis, we demonstrate that one of these antibodies reacts with an antigen located primarily on the luminal membrane domain of the endothelial cells.

Materials and Methods

Chemicals

$Na^{125}I$ (15–20 Ci/mmol) was purchased from

ICN (Irvine, CA); Enzymo-Bead reagent was obtained from Bio-Rad (Richmond, CA). All other chemicals were of reagent grade.

Cell and cell cultures

Bovine aortic endothelial cells (BAEC) were obtained from 2- to 3-day-old calves, as previously described [20,21]. The cells were maintained in RPMI 1640 (Irvine Scientific, Santa Ana, CA) containing 10% heat inactivated fetal bovine serum, garamycin (50 μg/ml), and fungizone (5 μg/ml).

Purification of human and bovine platelets

Human and bovine platelets were prepared from whole blood following the procedures of Rudnick et al. [21]. Briefly, 50 ml of human and bovine whole blood was centrifuged at $160 \times g$ for 15 min at 4°C. The supernatant was collected and subjected to a second centrifugation at $4500 \times g$ for 20 min. The supernatant was discarded and the pellet was suspended in 1 ml of 10 mM phosphate-buffered saline (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF).

Isolation of plasma membranes

Monolayer cultures of endothelial cells grown to confluency were washed twice with 5 ml of phosphate-buffered saline (pH 7.4) (5 mM Na_2HPO_4/NaH_2PO_4 and 145 mM NaCl), and the cells from each flask were scraped with a rubber jacketed spatula into 5 ml of phosphate-buffered saline. The cells ($8 \cdot 10^6$/flask) from 40 flasks were scraped and collected by centrifugation at $1800 \times g$ for 10 min. The cell pellet was suspended in 25 ml of phosphate-buffered saline and homogenized in a Dounce homogenizer with a tight B pestle (Kontes Glass Co., Vineland, NJ). Twenty excursions were sufficient to effect cell breakage. The homogenate was centrifuged at $1800 \times g$ for 10 min and the supernatant solution was removed. The pelleted material was homogenized in a Dounce glass homogenizer with a tight B pestle (Kontes Glass Co., NJ) (15 strokes) and the suspension was further homogenized using a Polytron (Brinkman Instruments, Westbury, NY) equipped with a PT-10 probe at a power setting of 5 for 90 s, followed by centrifugation at $1800 \times g$ for 10 min. The combined supernatants were centrifuged at $30\,000 \times g$ for 45 min at 4°C, and the resulting pellet was resuspended in 1 ml of 250 mM sucrose solution buffered with 10 mM Tris (pH 7.4) and layered over a discontinuous sucrose density gradient of the following composition: 1.5 ml of 60% sucrose then 2 ml each of 40%, 32%, 27% and 20% sucrose. The centrifugation was carried out in an SW41 Ti rotor (Beckman Instruments, Irvine, CA) at 21 000 rpm ($60\,000 \times g$) for 90 min.

The bands of turbid material at the sucrose interfaces in the gradient were removed with a Pasteur pipet and designated as follows: 0–20%, I; 20–27%, II; 27–32%, III; 32–40%, IV; and 40–60%, V. Each fraction was assayed for specific marker enzymes: glucose-6-phosphatase [22] (microsomes); succinate dehydrogenase [23] (mitochondria); thiamin pyrophosphatase [24] (Golgi bodies); alkaline phosphatase [25]; and ouabain-sensitive ($Na^+ + K^+$)-ATPase [26,27] (plasma membranes). Orthophosphate was estimated by the method of Fiske and SubbaRow [28]. ($Na^+ + K^+$)-ATPase activity was also measured by the potassium stimulated *p*-nitrophenylphosphatase reaction [29].

Assay of angiotensin-converting enzyme activity

Angiotensin-converting enzyme was assayed following the method of Cushman and Cheung [30]. Briefly, each fraction was assayed in a total volume of 250 μl containing 100 mM potassium phosphate buffer, 300 mM sodium chloride, and 5 mM hippuryl-L-histidyl-L-leucine. After 30 min, the reaction was terminated by the addition of 250 μl 1 M HCl. The HCl was added before the enzyme in zero-time controls. The hippuric acid formed by the action of angiotensin-converting enzyme on hippuryl-L-histidyl-L-leucine was extracted from the incubation medium into 1.5 ml of ethyl acetate. The tubes were centrifuged and 1.0 ml aliquots of ethyl acetate were transferred into a clean tube and evaporated at 120°C. The contents were redissolved in 1 ml of water and the absorbance was read at 228 nm. The enzyme activity was calculated by the equation (A_{228} (exp.) $- A_{228}$ (control)) $\times 5.6 \times 10^{-3}$. One unit of enzyme is defined as the amount catalyzing the formation of 1 μmol hippuric acid in one minute at 37°C under standard assay conditions.

Lactoperoxidase-catalyzed radioiodination of cell surface proteins

Bovine aortic endothelial cells grown to confluency were washed twice with 10 ml of phosphate-buffered saline. The radioiodination [31] of cells was carried out using immobilized preparations of lactoperoxidase and glucose, i.e., Enzymo-Bead reagent (Bio-Rad Laboratories, Richmond, CA). To washed cell monolayers ($8 \cdot 10^6$ cells per flask) or cells in suspension were added 1.5 ml of phosphate-buffered saline, 100 μl of rehydrated Enzymo-Bead reagent, 100 μl of 1% β-D-glucose and 10 μl of $Na^{125}I$ (2.0 mCi). The iodination was allowed to proceed for 10 min at 4°C with gentle shaking. The supernatant was removed and the cells washed twice with 10 ml of ice cold phosphate-buffered saline. Radioiodinated cells ($1.6 \cdot 10^7$ cells) scraped from the flasks in phosphate-buffered saline were mixed with non-labeled cells ($3.6 \cdot 10^8$ cells) for isolation of plasma membranes, as described above. An aliquot of each fraction in the discontinuous gradient was precipitated with 10% trichloroacetic acid. The precipitate was washed and radioactivity was determined using a Beckman 4000 gamma counter.

Production of monoclonal antibodies

Bovine aortic endothelial cells (BAEC) were detached from the flasks with a rubber spatula, and $1 \cdot 10^7$ cells were injected intraperitoneally in BALB/c mice in 0.5 ml of phosphate-buffered saline at 10 day intervals. Four days after the third injection, the spleens were removed and the cells harvested for fusion.

Cell hybrids were prepared as described by Galfre et al. [32], except that spleen cells were fused with the myeloma cell line SP 2/0. Spleen cells were initially prepared in Dulbecco's phosphate-buffered saline containing 2% fetal bovine serum. The cells were then washed with Dulbecco's modified Eagle's medium (DMEM; Irvine Scientific, Santa Ana, CA) in the absence of serum, and $1 \cdot 10^8$ spleen cells were mixed with approx. $3 \cdot 10^7$ SP 2/0 myeloma cells. The mixture was centrifuged at $600 \times g$ for 7 min and the pellet collected. The pellet was then suspended in 0.8 ml of 45% poly(ethylene glycol) (PEG 1500; Aldrich Chemical Co., Milwaukee, WI) in DMEM at 37°C and the suspension incubated for 1 min after which 20 ml of DMEM lacking PEG was added slowly. The cells were centrifuged and resuspended in DMEM containing 10% fetal bovine serum. Selection for hybrid clones was accomplished through the use of HAT medium [33]. The resultant hybridomas were tested for production of antibodies against BAEC by means of an indirect enzyme-linked immunoabsorbent assay (ELISA).

ELISA

Endothelial cells, fibroblasts, smooth muscle cells, corneal endothelial cells from bovine, and human umbilical vein endothelial cells ($5 \cdot 10^4$) were plated into 96-well microtiter plates in RPMI 1640 containing 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA). After overnight culture, the cells were rinsed with phosphate-buffered saline, fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1–2 h, then rinsed extensively with phosphate-buffered saline and stored at 4°C in phosphate-buffered saline containing 0.1% bovine serum albumin (PBS-BSA) and 0.1% NaN_3. Prior to use, the plates were washed with PBS-BSA and 40 μl of hybridoma supernatant was added to each well. After one hour of incubation at room temperature, the wells were rinsed four times with PBS-BSA containing 0.2% Tween-20. To each well was then added 100 μl of peroxidase conjugated goat antimouse IgG diluted 1 : 100 with PBS-BSA. After 30 min, the wells were rinsed four times and incubated in 50 μl of 0.05 M citrate-0.1 M phosphate buffer (pH 5.0) containing 0.01% H_2O_2 and 0.04% *o*-phenylenediamine for 30 min. The reaction was stopped with 50 μl of 2 M H_2SO_4 and the absorbance in each well was measured on an ELISA reader at 492 nm. An irrelevant monoclonal antibody directed against a viral protein was used as a negative control; the original mouse serum was used as a control. Clones secreting antibody with an affinity to BAEC were subsequently subcloned twice by limited dilution.

Immunoflourescence

The positive supernatant was screened for specificity toward endothelial cells in tissue sections of calf thymus, aorta and kidney. The tissues were fixed, dehydrated in a graded series of

ethanol, infiltrated two times with PEG 1000 for 1 h at 42°C, and then placed in PEG 1500 in embedding molds. Sections were cut at 4–6 μm, and then rinsed for 1 h in phosphate-buffered saline, followed by a second rinse in phosphate-buffered saline and 1% BSA for 1 h. The sections were transferred to conical centrifuge tubes and incubated for 30 min with the supernatants; they were then rinsed in PBS-BSA, incubated in a GAM-FITC (goat-antimouse Ig, fluorescein isothiocyanate (FITC) conjugated) for 30 min, rinsed four times with PBS-BSA and mounted on gelatin coated slides with a 9:1 glycerol/phosphate-buffered saline solution containing 0.1% *o*-phenylenediamine, and adjusted to a final pH of 8.2 with 0.1 M KOH (pH 9.5). Sections were examined using immunofluorescence microscopy.

Purification of monoclonal antibodies

Purification of monoclonal antibodies was accomplished by adsorption of hybridoma supernatant onto a protein A-Sepharose column (Pharmacia). The column was washed with 10 mM phosphate-buffered saline (pH 7.4) and antibody was eluted with 0.1 M glycine [pH 2.8}. Fractions containing monoclonal antibody were then pooled, pH adjusted to 7.4, and dialyzed overnight against 5 mM phosphate-buffered saline (pH 7.4) at 4°C.

Immmunoprecipitation of antigen

Monolayers of endothelial cells ($40 \cdot 10^6$ cells) were labeled with 5 μCi/$8 \cdot 10^6$ cells of ^{35}S-methionine (spec. act. 800 Ci/mmol; ICN, Irvine, CA) for 24 h. The monolayers wer then washed three times with Tris-buffered saline (140 mM NaCl/10 mM Tris (pH 7.4)) and incubated with either 0.5% Nonidet P-40 or 0.5% Triton X-100 in Tris-buffered saline for 18 h at 4°C. The lysates were centrifuged at $300 \times g$ and the supernatant was removed. The supernatant was incubated with each monoclonal antibody (50 μg protein) for 1 h at room temperature. Protein A-Sepharose (100 mg/ml) was then added and incubated for an additional 2 h. After centrifugation, the precipitate was washed three times with Tris-buffered saline to remove non-specifically adsorbed protein. The pellet was solubilized in 2% SDS in 100 mM Tris (pH 8.2) in a boiling water bath for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis on a 5% gel in the presence or absence of 2-mercaptoethanol. The gel was then dried and subjected to autoradiography using Kodak X-OMAT film.

Immunoblots of membranes

Fractions II and IV corresponding tentatively to luminal and abluminal membrane domains of endothelial cells were subjected to SDS polyacrylamide gel electrophoresis on 5% gels in the presence or absence of 2-mercaptoethanol. The electrophoretically separated proteins were transferred to nitrocellulose membranes, as described by Towbin et al. [34].

Nitrocellulose membranes were incubated in phosphate-buffered saline (pH 7.4) containing 3% bovine serum albumin for 1 h at 37°C, followed by incubation overnight at 4°C with purified monoclonal antibodies diluted 1:100 with the PBS-BSA. After incubation with antibody, the transfers were rinsed three times with phosphate-buffered saline and incubated in peroxidase-conjugated antimouse IgG (Hyclone) diluted 1:1000 with phosphate-buffered saline in 1% bovine serum albumin [35]. The transfers were then rinsed three times with phosphate-buffered saline and developed with a solution of five parts 0.01% H_2O_2 in phosphate-buffered saline and one part of a solution of 3 mg/ml 4-chloro-1-naphthol in methanol.

Subtyping

The subtype of the immunoglobulin present in the hybridoma supernatant was determined using a monoclonal antibody subisotyping ELISA system (Hyclone Laboratories, Logan, Utah) consisting of rabbit antibody to mouse IgG_1, IgG_2, IgG_{2b}, IgG_3, IgM and IgA.

Electron microscopy

Pellets of membranes were fixed using 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C [36]. The pellets were then rinsed in 0.1 M cacodylate buffer and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer on ice. Following osmication, the pellets were washed two times in distilled water, rinsed three times in 0.1 M sodium acetate buffer, and stained en bloc with 1% uranyl acetate in 0.05 M acetate buffer for 2 h at room tempera-

ture. After staining, the pellets were washed two more times with acetate buffer and distilled water, respectively. Dehydration was in ethanol, after which the membranes were embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Thin sections were cut using a Porter-Blum MT II B ultramicrotome and stained sequentially with uranyl acetate and lead citrate. Sections were examined and photographed with a Zeiss EM 10 electron microscope.

Freeze-fracture specimens were prepared after fixation of the membrane pellet overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C [36]; the pellets were then rinsed in 0.1 M cacodylate buffer three times, 15 min each. The pellets were then processed through 10%, 20% and 30% glycerol, and prepared in 0.1 M sodium cacodylate buffer for 20 min each. The pellets were then removed from the 30% glycerol, placed on gold alloy discs, and frozen in Freon 22 cooled by liquid nitrogen [37]. Fracturing was accomplished with a Balzers 301 unit utilizing a double replica device. Platinum carbon shadowing was controlled with an electron gun and a quartz-crystal film thickness monitor. Replicas were cleaned in absolute methanol, sodium hypochlorite (clorox), and 50% H_2SO_4, rinsed in distilled water, and picked up on Parlodion-coated 100-mesh copper grids. Specimens were examined and photographed with a Zeiss EM 10 electron microscope.

Lipid analyses

Lipids were extracted from membranes using chloroform/methanol (2:1, v/v) by the method of Folch et al. [38]. Lipid extracts were dried under nitrogen and then redissolved in 100 μl of chloroform. Phospholipids were separated by two-dimensional thin-layer chromatography on Silica gel H plates (Brinkman Instruments) using a solvent system consisting of chloroform/methanol/acetic acid/water (50:25:8:4, v/v) in the first dimension, and chloroform/methanol/water (5:10:1, v/v) in the second dimension, as described by Jain [39]. Phospholipid-phosphorus was determined in the scraped spots after digestion with perchloric acid at 170°C for 90 min [40]. The identity of spots was established from the mobility of known standards of phospholipids. Protein was measured according to the method of Lowry et al. [41] using bovine serum albumin as standard.

Results

Isolation and enzymatic characterization of luminal membranes

To isolate the plasma membrane fraction, bovine aortic endothelial cells (BAEC) were homogenized in a Dounce homogenizer followed by low speed centrifugation to remove nuclei, debris and mitochondria. Table I summarizes the specific activities and enrichment of marker enzymes obtained during the membrane fractionation of BAEC. Fraction II, sedimenting at the 20–27% sucrose density interface, was enriched 8–10-fold in alkaline phosphatase activity, while fraction IV, sucrose density 32–40%, was enriched 6–8-fold in ouabain-sensitive $(Na^+ + K^+)$-ATPase activity, compared to the original homogenate. In fraction II, there was a minimal increase in $(Na^+ + K^+)$-ATPase activity, while in fraction IV the specific activity of alkaline phosphatase was 1.2-fold compared to the homogenate. The enrichment factors in fraction II for succinate dehydrogenase and glucose-6-phosphatase were 0.03- and 1.7-fold, respectively, suggesting little contamination by mitochondria, although some contamination by microsomes and Golgi complex were present. Similarly, fraction IV exhibited little contamination with mitochondria, although a slight enrichment (about 2-fold of the microsomal membrane marker glucose-6-phosphatase) suggests some microsomal contamination. The recovery of membrane fractions II and IV was relatively low corresponding to 8 and 4% of the total alkaline phosphatase and $(Na^+ + K^+)$-ATPase activity, respectively. These results indicate that fractions II and IV represent different regions of the membrane of endothelial cells, similar to the results obtained with intestinal epithelial cells which contain brush-border membranes enriched in alkaline phosphatase activity and baso-lateral membranes containing primarily $(Na^+ + K^+)$-ATPase activity [42].

Since angiotensin-converting enzyme has been shown to be exclusively located on the luminal side of vascular endothelium [43] utilizing immunocytochemical techniques, we also studied the distribution of angiotensin-converting enzyme and

TABLE I

DISTRIBUTION AND ACTIVITIES OF PROTEIN AND SUBCELLULAR MARKER ENZYMES IN THE FRACTIONATION OF BOVINE AORTIC ENDOTHELIAL CELLS

Bovine aortic endothelial cell cultures were prepared and fractionated as described in Materials and Methods. Fractions were collected at each interface as follows: II (20–27% sucrose); IV (32–40% sucrose). The data are representative of six independent membrane preparations. The values are the means ± S.E. Numbers in parenthesis are the percent recovery of protein and enzyme units compared to 100% in homogenate.

Fraction	Protein (mg)	$(Na^+ + K^+)$-ATPase [a]	Alkaline [a] phosphatase	Glucose-6-phosphatase [a]	Succinate dehydrogenase [b]	Thiamin pyrophosphatase [a]
Homogenate	76.82 (100%)	2.14 ± 0.61 (100%)	2.04 ± 0.86 (100%)	0.13 ± 0.42 (100%)	27.60 ± 0.80 (100%)	0.12 ± 0.08 (100%)
Membrane Pellet	2.12 (2.8%)	3.53 ± 0.72 (9.6%)	6.78 ± 2.53 9.2%)	0.12 ± 0.15 (2.81%)	1.00 ± 0.60 (1%)	0.09 ± 0.02 (2%)
Fraction II	0.75 (0.97%)	4.55 ± 1.04 (2%)	16.21 ± 5.10 (7.7%)	0.23 ± 0.03 1.7%)	0.80 ± 0.05 (0.3%)	0.02 ± 0.01 (0.18%)
Fraction IV	0.25 (0.33%)	15.85 ± 2.16 (3.9%)	2.82 ± 1.65 (0.8%)	0.25 ± 0.04 (1.20%)	0.50 ± 0.04 (0.11%)	0.02 ± 0.003 (0.12%)

[a] Enzyme specific activities, expressed as μmol/min per mg of protein.
[b] Enzyme specific activities, expressed as nmol/min per mg of protein.

$(Na^+ + K^+)$-ATPase in density gradient fractions of the membrane pellet. As show in Fig. 1, the angiotensin-converting enzyme is highly enriched in fraction II, while $(Na^+ + K^+)$-ATPase specific activity was high in fraction IV. Both angiotensin-converting enzyme and $(Na^+ + K^+)$-ATPase activities were of intermediary value in fraction III.

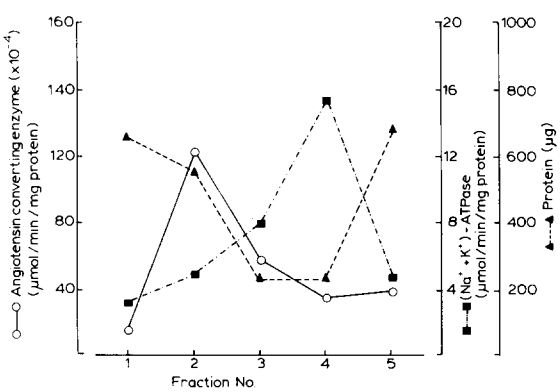


Fig. 1. Enzyme activity profile of sucrose density gradient fractions. Membranes isolated as described under Materials and Methods were loaded onto a discontinuous sucrose density gradient and centrifuged for 90 minutes at 60000 × *g*. Five fractions were collected and $(Na^+ + K^+)$-ATPase and angiotensin-converting enzyme activities were measured. Angiotensin-converting enzyme specific activity in homogenate was 8.25 ± 0.8 ($n = 6$).

Characterization of plasma membrane fractions

Since the endothelial cells used to prepare the membranes were grown in monolayer, and thus had part of the cell surface exposed to media and part in contact with the substratum, as is the case in vivo, we sought to determine whether the two membrane preparations obtained (fractions II and IV) represented separate domains. For this purpose, monolayers of BAEC were radioiodinated for 10 min utilizing the impermeable lactoperoxidase-catalyzed iodination procedure [44,45], followed by fractionation of plasma membrane fractions on a sucrose gradient. As shown in Fig. 2, most of the incorporation of ^{125}I was observed in fraction II, while fraction IV contained only 14% of the radioactivity found in fraction II. However, when endothelial cells in suspension were radioiodinated, both fractions II and IV were labeled, and the extent of label in fraction IV was higher when cells were labeled in suspension compared to cells labeled in monolayer. Since fraction II contains alkaline phosphatase activity and its surface domain is specifically labeled with impermeable ^{125}I, we assume that this fraction corresponds to

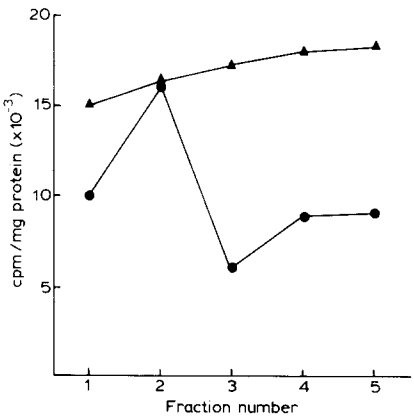


Fig. 2. Distribution of ^{125}I in BAEC membrane fractions separated on sucrose density gradient. Bovine aortic endothelial cells ($1.6 \cdot 10^7$ cells) were labeled with ^{125}I utilizing solid-phase lactoperoxidase, as described in Materials and Methods. The radiolabeled cells, mixed with non-iodinated cells ($3.6 \cdot 10^8$ cells), were homogenized and fractionated on a sucrose density gradient. The bands of turbid material at the interfaces in the gradient were removed and designated as fractions: I (0–20%); II (20–27%); III (27–32%); IV (32–40%); V (40–60%%). Radioactivity and protein were estimated in each fraction. The data show the incorporation of radioactivity after subtraction of the background radioactivity. ▲, cells labeled in suspension; ●, cells labeled in monolayer.

the luminal domain, whereas fraction IV presumably represents the abluminal cell surface domain. The radioactivity associated with fraction IV, when cells are labeled in monolayer, presumably is the result of slow permeation of substrate to the contraluminal surface of cells in contact with the substratum during the labeling procedure.

Electron microscopic examination of membrane fractions

Fig. 3 shows representative views of thin sections taken from pellets of the isolated fractions II and IV. The pellet of fraction II (Fig. 3a) consisted mainly of smooth membrane vesicles of various sizes (100–400 nm), including some Golgi bodies, though some membrane sheets were still present. The pellet of fraction IV (Fig. 3b) contained smooth membrane vesicles in a broad range of sizes (50–500 nm), mitochondria, and dense amorphous material. There appeared to be no contamination by mitochondria in fraction II. Freeze-fracture electron micrographs (Fig. 4) of luminal membranes revealed predominately higher particulate membrane with diameters of 100–400 nm. No differences in the particle density were observed between the convex and concave fracture faces of the cytoplasmic membrane leaflet.

Lipid composition of fraction II (luminal) and fraction IV (abluminal) plasma membrane of endothelial cells

Three major phospholipids (Table II, consisting primarily of phosphatidylcholine (PC) ($39 \pm 7\%$), phosphatidylethanolaminė (PE) ($19 \pm 2\%$), and sphingomyelin ($19 \pm 1.5\%$), were present in fraction II. The other components were phosphatidylserine (PS) and phosphatidylinositol (PI) (12%). Similarly, fraction IV was enriched in PC ($44 \pm 6\%$), sphingomyelin ($13 \pm 1.8\%$), and PE ($20 \pm 2\%$), while PI and PS were present in lesser amounts to the extent of $12 \pm 1.4\%$. In conclusion, there were not statistically significant differences in the phospholipid composition between fraction II and fraction IV membranes.

Tissue specificity of monoclonal antibodies $XIVC_6$ and XVD_2

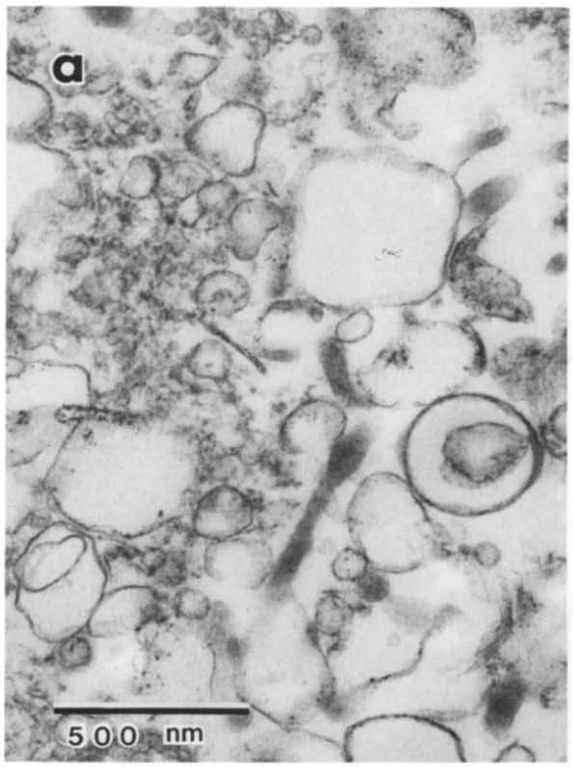
Of over 500 wells of primary hybridoma cultures tested, 63 hybridomas reacted with cultured bovine aortic endothelial cells (BAEC) using the ELISA assay. When these hybridomas were tested using an immunofluorescence assay on poly(ethylene glycol) (PEG) sections of bovine thymus, only

TABLE II

PHOSPHOLIPID COMPOSITION OF FRACTION II (LUMINAL) AND FRACTION IV (ABLUMINAL) MEMBRANES ISOLATED FROM BOVINE AORTIC ENDOTHELIAL CELLS

Phospholipids from bovine aortic endothelial cell membrane fractions were extracted, fractionated and quantitated as described under Materials and Methods. The values are the means ± S.E. of 6–8 membrane preparations.

Lipids	% of total phospholipids	
	Fraction II membranes	Fraction IV membranes
Phosphatidylethanolamine	19.38 ± 1.81	20.02 ± 2.05
Phosphatidylcholine	38.90 ± 7.29	44.55 ± 7.65
Sphingomyelin	19.3 ± 1.56	12.0 ± 1.82
Phosphatidylserine + phosphatidylinositol	12.76 ± 2.50	12.39 ± 1.43
Unidentified phospholipids	4.66 ± 0.55	6.62 ± 1.75



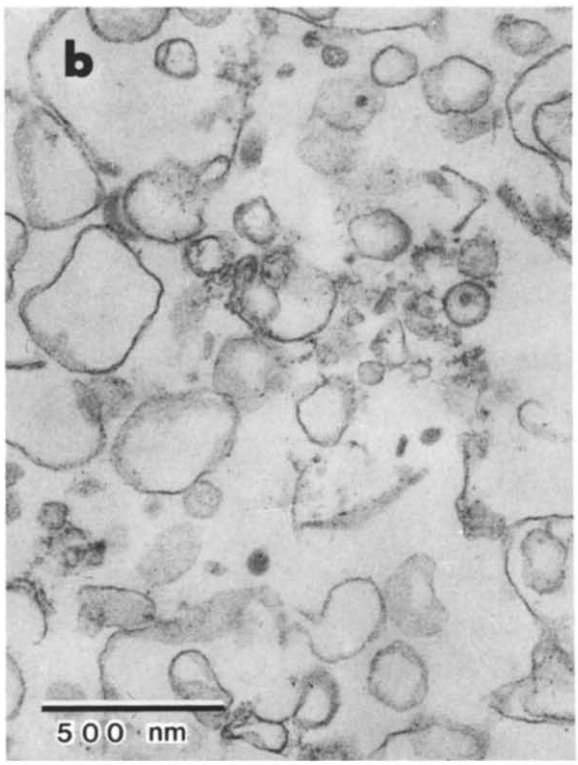


Fig. 3. Electron microscopy of BAEC membrane fractions. These micrographs show representative views of thin sections taken from the isolated fractions II and IV. (a) Fraction II, ×52000; b. Fraction IV, ×52000. Bar: 500 nm.

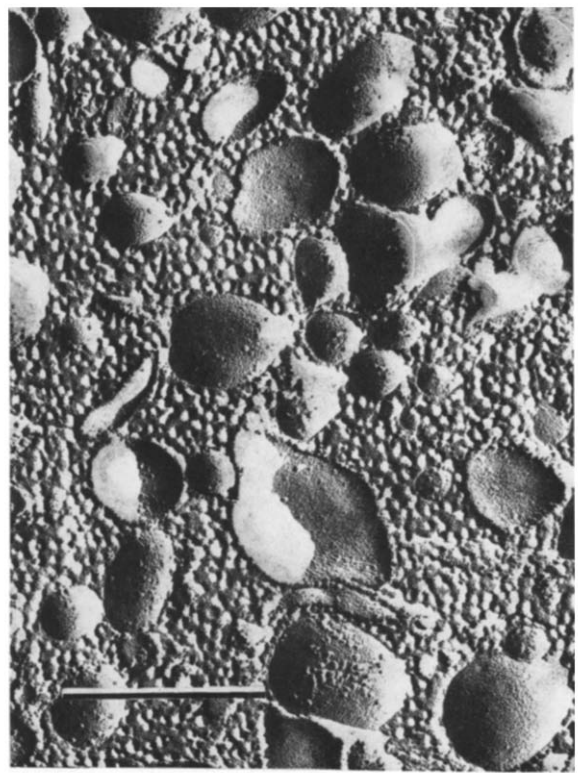


Fig. 4. Freeze-fracture electron microscopy of fraction II (luminal membranes). This micrograph shows isolated membranes which have formed spherical vesicles. The convex and concave fracture faces show an even distribution of particle density; ×53000. Bar: 500 nm.

eight of the 63 hybridomas were specific for endothelial cells. Two of these primary hybridomas were chosen; one positive clone from each of these primary hybridomas ($XIVC_6$ and XVD_2) was studied in detail. Using immunofluorescence microscopy, the supernatant from the clones reacted only with vascular endothelial cells in tissue sections from bovine thymus, liver, kidney, aorta and cornea (Fig. 5 and Table III). No discernible difference in the labeling pattern was observed between endothelial cells of capillaries, arteries and veins in these tissues.

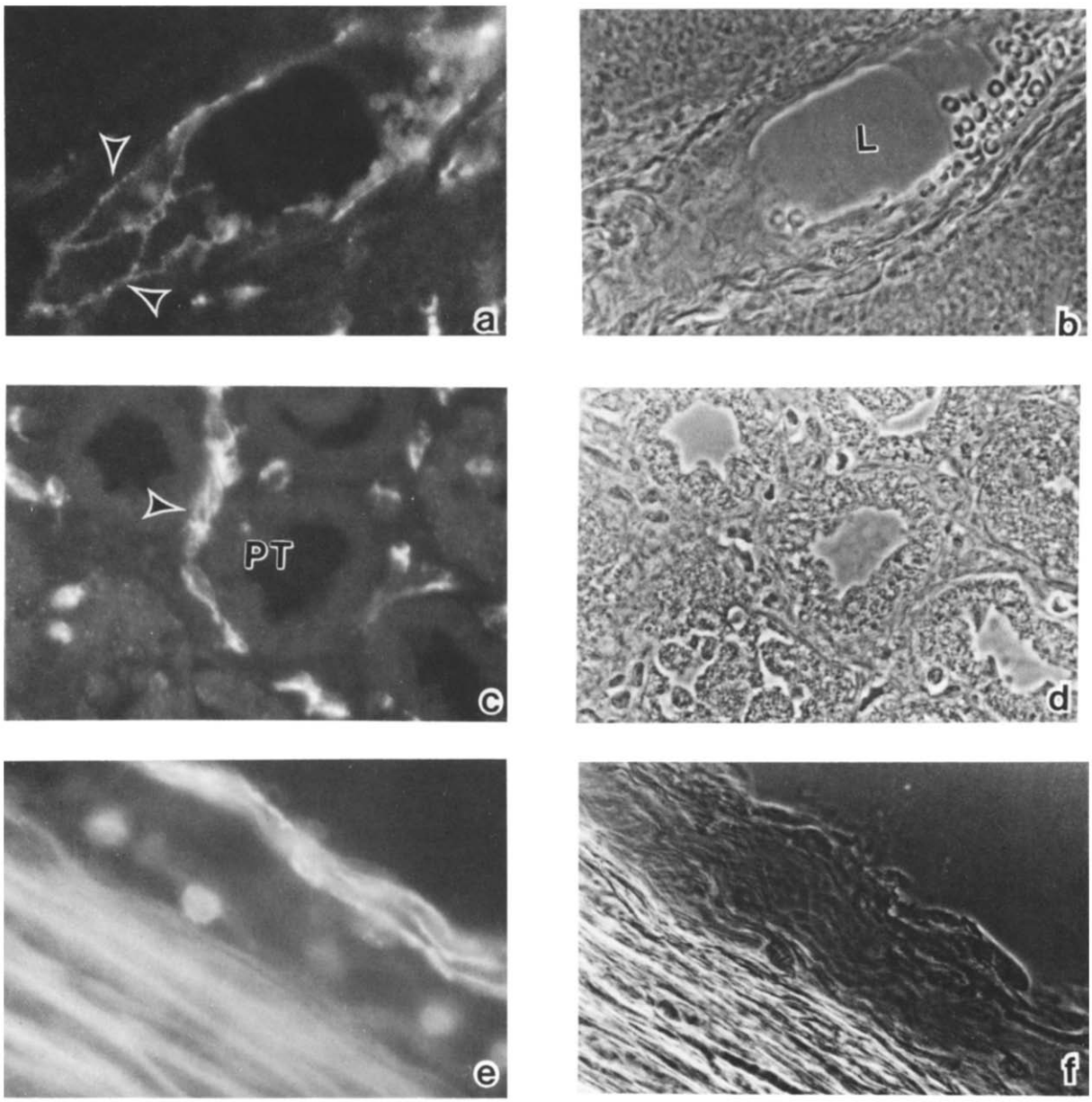


Fig. 5. Indirect immunofluorescence of bovine tissue labeled with Mab $XIVC_6$ and XVD_2 ($\times 300$). (a) Staining with Mab $XIVC_6$ shows labeled endothelial cells located in a thymic medullary venule cut tangentially. The lateral surfaces of the endothelial cells are well outlined (arrows). (b) Phase contrast of (a). L, lumen of the venule. (c) Section of the renal cortex demonstrating that the immunoreactivity of XVD_2 is confined to the capillary endothelium (arrows) and does not label the epithelium of the proximal tubules (PT). (d) Phase contrast of (c). (e) In a longitudinal section of the aorta, only the endothelial cells are labeled; again, the reactivity is very strong on the lateral cell surfaces. (f) Phase contrast of (e).

Using the ELISA assay, these antibodies showed a positive reaction for bovine aortic endothelial cells, but not for endothelial cells derived from human umbilical vein. Moreover, these antibodies did not react with bovine smooth muscle cells, fibroblasts, and corneal endothelial cells (Table

TABLE III

INDIRECT IMMUNOFLUORESCENCE OF ENDOTHELIAL CELLS IN BOVINE TISSUE SECTIONS

The immunofluorescence assay in tissue sections with Mab $XIVC_6$ and Mab XVD_2 was carried out as described in Materials and Methods.

Antibody	Thymus	Aorta	Kidney	Liver	Corneal
XIV_6	+	+	+	+	–
XVD_2	+	+	+	+	–

IV). The immunoglobulin subclasses for monoclonal antibodies $XIVC_6$ and XVD_2 were IgG_1 and IgG_{2B}, respectively.

Identification of antigen(s) on endothelial cells for Mab $XIVC_6$ and Mab XVD_2

In an effort to characterize the antigen, endothelial cells were metabolically labeled with ^{35}S-methionine for 18–24 h. Cells were solubilized with either 0.5% Triton X-100 or 0.5% Nonidet P-40 detergent. The supernatant was reacted with either Mab $XIVC_6$ or Mab XVD_2, followed by the addition of protein A-Sepharose. The precipitated sample was solubilized in 1% SDS and analyzed by SDS-polyacrylamide gel electrophoresis. The autoradiograph of the gels showed a major band of M_r 130 000 ± 5000 and two minor bands of M_r 120 000 and 110 000 ± 5000 with both antibodies (Fig. 6).

Localization of antigens of Mab $XIVC_6$ and Mab XVD_2 on the endothelial cell plasma membrane

To define whether these antibodies reacted with the plasma membranes of endothelial cells, luminal (fraction II) and abluminal (fraction IV) membrane domains were tested using Western blot analysis. As shown in Fig. 7, Mab XVD_2 exhibited two bands of M_r 130 000 ± 5000 and M_r 120 000 ± 5000 under reduced and non-reduced conditions. At equal protein concentrations of luminal and abluminal membranes, the XVD_2 antibody exhibited preferential reactivity toward the luminal membrane fraction (Fig. 7). Mab $XIVC_6$ did not show reactivity with either the luminal or abluminal membrane domain in Western blots of SDS solubilized membranes, suggesting that the epitope for Mab $XIVC_6$ is denatured under these conditions; in addition, this data shows that Mab $XIVC_6$ and Mab XVD_2 are directed toward different epitopes.

The two bands which reacted with the antibody in Western blots could have arisen as a result of the same epitope being present on two different proteins, as a result of proteolysis of the antigen

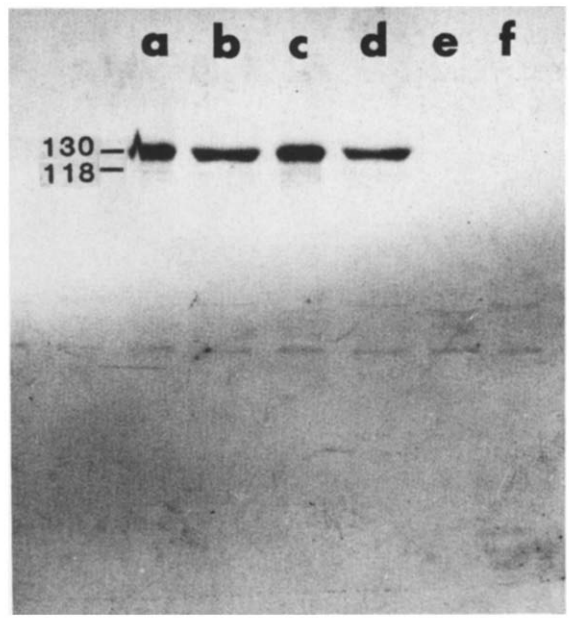


Fig. 6. Autoradiograph of SDS-polyacrylamide gel electrophoresis of endothelial cell proteins precipitated by the monoclonal antibodies $XIVC_6$ and XVD_2. Lanes (a) and (c): Non-reduced proteins precipitated by XVD_2 and $XIVC_6$, respectively. Lanes (b) and (d): Reduced proteins precipitated by XVD_2 and $XIVC_6$, respectively. Lanes (e) and (f): No proteins are evident when endothelial cells were precipiated with an antibody raised against an irrelevant viral protein.

TABLE IV

ELISA PROFILES OF BOVINE AND HUMAN CULTURED CELLS

ELISA was carried out as described in Materials and Methods.

Bovine cell type	Mab $XIVC_6$	Mab XVD_2
Smooth muscle	–	–
Fibroblasts	–	–
Corneal endothelial	–	–
Aortic endothelial	+	+
Human endothelial	–	–

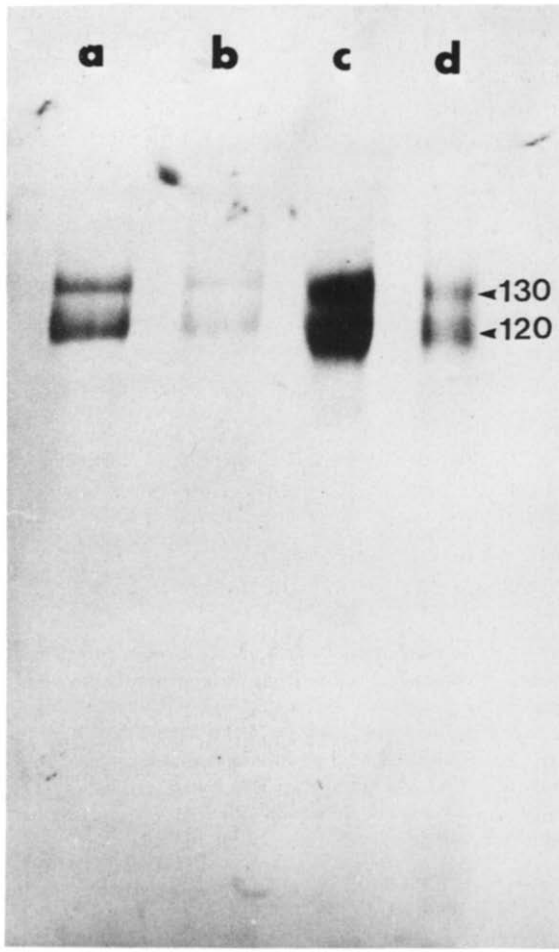


Fig. 7. Western blot of purified endothelial cell luminal and abluminal plasma membrane proteins reacted with antibody XVD_2. Reduced (lane a) and non-reduced (lane b) abluminal domain; reduced (lane c) and non-reduced (d) luminal domain. 50 μg protein was applied on to each lane.

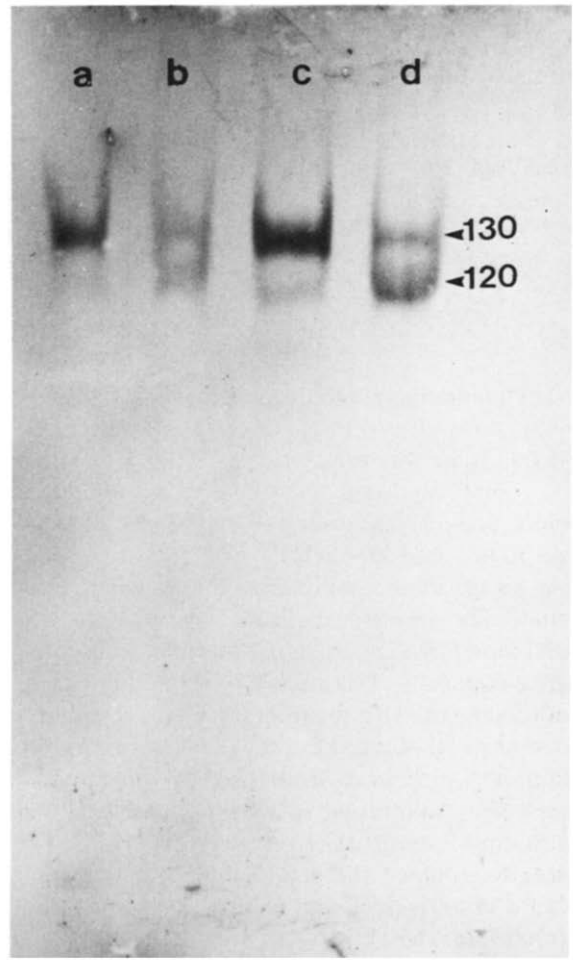


Fig. 8. Western blot of purified endothelial cell luminal and abluminal membrane proteins prepared in the presence and absence of protease inhibitors reacted with antibody XVD_2. Abluminal domain prepared in the presence (lane a) and absence (lane b) of PMSF-EDTA; luminal domain prepared in the presence (lane c) and absence (lane d) of PMSF-EDTA.

during membrane preparation, or because Mab XVD_2 was derived from two different clones. As shown in Fig. 8, when the membranes were prepared in the presence of the proteolytic inhibitors, phenylmethylsulfonyl fluoride and EDTA, only the band of M_r 130 000 ± 5000 was observed. This data indicates that the antigen which cross-reacts with Mab XVD_2 undergoes proteolytic cleavage during isolation of the plasma membrane.

Interaction of Mab $XIVC_6$ and Mab XVD_2 with human and bovine platelets

Recent studies of Fitzgerald et al. [46] have shown that polyclonal antibodies to the platelet glycoprotein IIb-IIIa complex, cross-react with two membrane proteins of human umbilical vein endothelial cells (M_r = 130 000 and 110 000), and bovine aortic endothelial cells (M_r = 135 000 and 105 000). It was therefore of interest to determine whether monoclonal antibodies XVD_2 or $XIVC_6$ cross-reacted with either human or bovine platelets.

Immunoprecipitation of Nonidet P-40 solublized human and bovine platelets, followed by

SDS-polyacrylamide gel electrophoresis, did not show any platelet antigen that cross-reacted with either Mab $XIVC_6$ or Mab XVD_2 (data not shown). Similarly, SDS-gel electrophoresis of human and bovine platelets, followed by transfer to nitrocellulose membranes, did not show any reactive band with Mab XVD_2 and Mab $XIVC_6$ (data not shown). These results reveal that Mab XVD_2 and Mab $XIVC_6$ are not directed agianst platelet proteins or, specifically, glycoproteins GP-IIb and GP-IIIa (M_r 130000 and 110000, respectively).

Discussion

Since the study of membrane-related phenomena requires the isolation of plasma membranes, a variety of techniques have been utilized for cell disruption and subcellular fractionation [47–51]. The results presented in this study describe a method for the isolation and separation of two topologically distinct membrane domains of vascular endothelial cells. Endothelial cells cultured in monolayer were disrupted by homogenization followed by differential centrifugation to remove nuclei, debris and mitochondria. The crude plasma membrane fraction was enriched in $(Na^+ + K^+)$-ATPase activity and alkaline phosphatase activity, and was further resolved on a discontinuous sucrose density gradient. This procedure resulted in the separation of two populations of membranes, the first enriched 8–10-fold in alkaline phosphatase activity and 12–16-fold in angiotensin-converting enzyme activity, with minor contamination of $(Na^+ + K^+)$-ATPase activity compared to the original homogenate. The second fraction was enriched 6–8-fold in $(Na^+ + K^+)$-ATPase activity, with slight contamination of alkaline phosphatase activity compared to the original homogenate. These membrane fractions were minimally contaminated with mitochondria, endoplasmic reticulum and Golgi complex, as shown by the low activity of the marker enzymes succinate dehydrogenase, glucose-6-phosphatase and thiamin pyrophosphatase, respectively. The yield of the membranes was 0.5 to 2% of the cellular protein and recovery of these membrane domains was relatively low (4 to 8%). Attempts to further purify membranes, either by plant lectin concanavalin A [52] or shallow gradient centrifugation, did not significantly increase the specific activity of plasma membrane markers. The plasma membrane fraction enriched in alkaline phosphatase and angiotensin-converting enzyme activities was assigned, a priori, to the luminal domain; and the membrane fraction enriched in $(Na^+ + K^+)$-ATPase was assigned as the abluminal domain, in analogy with the presence of these enzymes in epithelial cell domains corresponding to luminal and baso-lateral membrane domains, respectively [42,50]. However, these marker assignments may oversimplify the subcellular organization of biochemical constituents in the plasma membranes. Therefore, studies were carried out to label the cell surface of endothelial cells grown in monolayer with impermeable functional reagents [44,45], in an attempt to selectively label the cell surface which is exposed to the growth medium in contrast to the surface domain anchored to the substratum.

We utilized solid phase lactoperoxidase-catalyzed iodination, which preferentially iodinates tyrosine residues of proteins [44], to label endothelial cells growing in monolayer, followed by isolation and separation of the membrane domains on a discontinuous sucrose density gradient. The results show that most of the radioactivty was associated with fraction II (enriched in alkaline phosphatase activity), while less than 14% of the radioactivity was associated with membrane fraction IV (enriched in $(Na^+ + K^+)$-ATPase activity). However, when endothelial cells in suspension were iodinated, both fraction II and fraction IV incorporated radioactivity. The extent of iodination in fraction IV was significantly higher when cells were labeled in suspension compared to cells in monolayer. As angiotensin-converting enzyme has been shown to be associated with cultured endothelium [43], and the activity of this enzyme is highly enriched in fraction II, it is suggested that the luminal membrane domain is mainly associated with the presence of this enzyme. Since endothelial cells grow in monolayer and exhibit contact inhibition, one would expect the membrane surface facing the growth media to be more accessible to solid-phase radioiodination than the surface domain anchored to the substratum. Thus, we suggest that fraction II, enriched in alkaline phosphatase activity and angiotensin-con-

verting enzyme activity, corresponds to the luminal surface of the endothelium in vivo.

The membrane fraction IV, enriched in ($Na^+ + K^+$)-ATPase activity, exhibited less than 14% of the incorporation of ^{125}I when labeled in monolayer, indicating that this surface of the membrane is not easily accessible to radioiodination either because this membrane domain is in contact with the substratum, or because of membrane-membrane junctions between adjacent cells. Based on the available data, we suggest that fraction IV is the abluminal surface membrane domain corresponding to the baso-lateral membrane region enriched in ($Na^+ + K^+$)-ATPase activity described for epithelial cells [50,53].

Electron microscopy of sectioned and freeze-fractured membranes showed that the luminal and abluminal domains isolated in this study were in the form of vesicles varying in size from 100 to 400 nm in diameter. These membrane vesicles should be useful in studies of trnsport of nutrients since relatively little information is available regarding the energetics of transport of ions, metabolites, and macromolecules across the endothelium from the luminal side to the abluminal side.

In this study, we have also prepared two monoclonal antibodies, $XIVC_6$ and XVD_2, which recognize antigens specific for bovine vascular endothelial cells. Using fluorescence microscopy, both antibodies showed qualitatively equal reactivity with endothelial cells of capillaries, veins and arteries, and did not react with any other cell in any tissue tested (e.g., liver, kidney, aorta). Using the ELISA assay, these antibodies reacted with bovine vascular endothelial cells, but not with bovine corneal endothelial cells or human umbilical vein endothelial cells. These results suggest that the antigens recognized by these antibodies are not only cell-specific, but also species-specific.

These monoclonal antibodies, in an immunoprecipitation assay of ^{35}S-methionine-labeled bovine aortic endothelial cells, recongized an antigen of M_r 130 000 ± 5000, and two minor proteins of M_r 120 000 ± 5000 and M_r 110 000 ± 5000. These results indicate that the two antibodies either recognize the same epitope(s) on these three proteins, or that the two minor proteins are products of the major protein with M_r of 130 000.

Our studies on the localization of the antigens in Western blots showed that Mab XVD_2 reacted with two proteins, M_r 130 000 ± 5000 and 120 000 ± 5000, preferentially localized on the luminal membrane domain. Since there was also partial reactivity with abluminal membrane antigen(s), it is possible that either the abluminal membrane domain is contaminated with luminal membrane, or antigens are localized in the two domains. It is pertinent to mention that Hubbard et al. [54] have characterized five hepatocyte specific monoclonal antibodies; one was specific for the bile canalicular surface, three reacted predomaintely with the lateral and sinusoidal surface, and one monoclonal antibody reacted with all three doamins of hepatocytes, indicating that these antigens were differentially distributed on the plasma membrane of rat hepatocytes.

Mab $XIVC_6$ did not react with either luminal or abluminal membrane proteins in the Western blot assay, suggesting that the epitope against which this antibody is directed is denatured by SDS. These results indicate that the antibody is directed against a secondary or tertiary structure of the antigen. Since Mab XVD_2 shows reactivity in Western blots, while Mab $XIVC_6$ does not, it is reasonable to assume that these antibodies are directed toward two different epitopes.

Our studies of luminal and abluminal membranes prepared in the presence of proteinase inhibitors reveal that only one band of M_r 130 000 reacts with Mab XVD_2. This suggests that the two minor bands (M_r 120 000 and 110 000) observed in the immunoprecipitation assay are proteolytic degradation products of the major M_r 130 000 ± 5000 protein band.

The identity of this M_r 130 000 antigen is not known. It does not appear to be angiotensin-converting enzyme (M_r 130 000, [55]), since in preliminary experiments neither monoclonal antibody inhibited its activity when assayed using the radioactive substrate [^3H]hydroxybenzoyl-Phe-Ala-Pro [56], although such results would be expected if the antibodies are directed against epitopes of the enzyme removed from the active site. Since the antibodies do not react with human endothelial cells, the antibodies would also have to be directed against epitopes which would not be common to the bovine and human enzymes. Since neither

Mab $XIVC_6$ nor Mab XVD_2 precipitate any protein from human or bovine platelets, the M_r 130000 antigen recognized by these antibodies is not related to the platelet glycoprotein IIb which has a similar molecular weight [46].

The function of the M_r 130000 molecule remains to be elucidated. Monoclonal antibodies $XIVC_6$ and XVD_2, and others being characterized in our laboratory, will be tested for their effects on function, such as cell–cell interactions and transport of various substances. A monoclonal antibody which preferentially reacts with an antigen on the luminal membrane would be useful for monitoring the separation of the luminal membrane from other membrane domains of endothelial cells. In addition, these antibodies can be used as probes for determining specific protein–protein interactions which may occur in the adherence of platelets, lymphocytes or red blood cells to endothelial cells.

To our knowledge, this is the first report that describes the isolation and separation of luminal and abluminal membrane domains from endothelial cells. Further studies will involve immunoaffinity purification of membranes, along with the characterization of membrane antigens and their putative role in the adherence of blood cells.

Acknowledgements

This work was supported in part by National Institutes of Health grants HL-15162 (V.K.K.), EY-04130 (N.S.), and the California Foundation for Biochemical Research. We thank Ms. Ljilana Jakovljevic for her excellent technical assistance, and Diane Gegala for skillfully typing this manuscript.

References

1 Czervionke, R.L., Hoak, J.C. and Fry, G.L. (1978) J. Clin. Invest. 62, 847–856
2 Stamper, H.B., Jr. and Woodruff, J.J. (1976) J. Exp. Med. 144, 828–833
3 Hoover, R.L., Briggs, R.-T. and Karnovsky, M.J. (1978) Cell 14, 423–428
4 Lackie, J. and DeBono, D. (1977) Microvasc. Res. 13, 107–112
5 Gimbrone, M.A., Jr. and Buchanan, M.R. (1982) Ann. N.Y. Acad. Sci. 401, 171–183
6 Harlan, J.M. (1985) Blood 65, 513–525
7 Fraggiotto,, A., Ross, R. and Harker, L. (1984) Arteriosclerosis 4, 323–340
8 Hebbel, R.Y., Yamada, O., Maldow, C.F., Jacob, H.S., Whitem, J.G. and Eaton, J.W. (1980) J. Clin. Invest. 65, 154–160
9 Kramer, R.H. and Nicolson, G.L. (1979) Proc. Natl. Acad. Sci. USA 76, 5704–5757
10 Jones, P.A., Neustein, H.B., Gonzales, F. and Bogemann, E. (1981) Cancer Res. 41, 4613–4620
11 Alby, L. and Auerbach, R. (1984) Proc. Natl. Acad. Sci. USA 81, 5739–5743
12 Stern, D.M., Drillings, M., Nossel, H.L., Hurlet-Jensen, A., La-Gamma, K.S. and Owen, J. (1984) Proc. Natl. Acad. Sci. USA 81, 913–917
13 Heimark, R.L. and Schwartz, S. (1983) Biochem. Biophys. Res. Commun. 111, 723–731
14 Laug, W., Benedict, W., Tokes, Z.A. and Sorgente, N. (1980) J. Cell Biol. 84, 281–293
15 Busch, C. and Owern, W.G. (1982) J. Clin. Invest. 69, 726–729
16 Ross, R. (1981) Arteriosclerosis 1, 293–311
17 Dalgas, V.V., Zaikins, O.E., Bordarenko, M.V. and Repin, V.S. (1982) Diabetologia 22, 338–343
18 Simionescu, M., Simionescu, N. and Palade, G.E. (1982) Ann. N.Y. Acad. Sci. 401, 9–24
19 Horvat, R., Hovorka, A., Dekan, G., Poczewski, H. and Kerjaschki, D. (1986) J. Cell Biol. 102, 484–491
20 Eisenstein, R., Kuettner, K.E., Neopolitan, C., Soble, L.W. and Sorgente, N. (1975) Am. J. Pathol. 81, 337–348
21 Rudnick, G., Bencuya, R., Nelson, P.J. and Zitro, R.A. (1981) Mol. Pharmacol. 20, 118–123
22 Hubscher, G. and West, G.R. (1965) Nature 205, 799–800
23 King, T.E. (1967) Methods Enzymol. 10, 322–331
24 Meldolesi, J., Jamieson, J.D. and Palade, G.E. (1971) J. Cell Biol. 49, 150–158
25 Grau, E.M., Marathe, G.V. and Tate, S. (1979) FEBS Lett. 98, 91–95
26 Castillo, J.R.D. and Robinson, J.W.L. (1982) Biochim. Biophys. Acta 688, 45–56
27 Haggerty, D.F., Kalra, V.K., Popjak, G., Reynold, E.E. and Chiappeli, F. (1978) Arch. Biochem. Biophys. 189, 51–62
28 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
29 Beaugé, L., Berberian, G. and Campos, M. (1984) Biochim. Biophys. Acta 773, 157–164
30 Cushman, D.W. and Cheung, H.H. (1971) Biochem. Pharmacol. 20, 1637–1648
31 Phillips, D.R. and Morrison, M. (1975) Biochemistry 10, 1766–1771
32 Galfre, G., Howe, S.C., Milstein, C., Butcher, G.W. and Howard, J.C. (1977) Nature 266, 550–552
33 Olsson, L. and Kaplan, H. (1983) Methods Enzymol. 92, 2–16
34 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354
35 Tsang, V.C., Peralta, M. and Simons, R. (1983) Methods Enzymol. 92, 377–391

36 Hyatt, M.A. (1972) Basic Electron Microscopy Techniques, Van Nostrand Reinhold Co., New York
37 Koehler, J.K. (1972) in Principles and Techniques of Electron Microscopy. Vol. 2: Biological Applications (Hyatt, M.A., ed.), pp. 52–98, Van Nostrand Reinhold, New York
38 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–507
39 Jain, S. (1984) J. Biol. Chem. 259, 3391–3394
40 Marinetti, G.V. (1962) J. Lipid Res. 3, 1–20
41 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
42 Fujita, M., Matsui, H., Nagano, K. and Nakao, M. (1972) Biochim. Biophys. Acta 274, 336–367
43 Cushman, D.W. and Cheung, H.H. (1971) Biochem. Pharmacol. 20, 1637–1648
44 Phillips, D.R. and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284–289
45 Hubbard, A.L. and Cohn, Z.A. (1972) J. Cell Biol. 55, 390–405
46 Fitzgerald, L.A., Charo, I.F. and Phillips, D.R. (1985) J. Biol. Chem. 260, 10893–10896
47 Mircheff, A.K. (1983) Am. J. Physiol. 244, 6347–6356
48 DePierre, J.W. and Karnovsky, M.L. (1973) J. Cell Biol. 56, 275–303
49 Sachs, G. and Kinne, R. (1978) in Physiology of Membrane Disorders, (Andreoli, T.E., Hoffman, J.F. and Fanestill, D.D., eds.), pp. 95–105, Plenum Press, New York
50 Murer, H. and Kinne, R. (1980) J. Mol. Biol. 55, 81–95
51 Meier, P.J., Sztul, E.S., Reuben, A. and Boyer, J.L. (1984) J. Cell Biol. 98, 991–1000
52 Lindsay, J.G., Reid, G.P. and D'Souza, M.P. (1981) Biochim. Biophys. Acta 640, 791–801
53 Lewis, B.A., Elkin, A., Mitchell, R.H. and Coleman, R. (1975) Biochem. J. 152, 71–84
54 Hubbard, A.L., Bartles, J.R. and Braiterman, L.T. (1985) J. Cell Biol. 100, 1115–1125
55 Friedland, J., Silverstein, E., Druker, M. and Setton, C. (1981) J. Clin. Invest. 67, 1151–1160
56 Ryan, J.W., Chung, A., Martin, L.C. and Ryan, U.S. (1978) Tissue Cell 10, 555–562