Plasmalemmal Proteins of Cultured Vascular Endothelial Cells Exhibit Apical–Basal Polarity: Analysis by Surface-selective Iodination

William A. Muller and Michael A. Gimbrone, Jr.

Vascular Research Division, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Abstract. Vascular endothelium in vivo appears to function as a polarized epithelium. To determine whether cellular polarity exists at the level of the plasma membrane, we have examined cultured endothelial monolayers for evidence of differential distribution of externally disposed plasmalemmal proteins at apical and basal cell surfaces. Lactoperoxidase beads were used to selectively label the apical surfaces of confluent endothelial monolayers, the total surfaces of nonenzymatically resuspended cells, and the basal surfaces of monolayers inverted on poly-L-lysine–coated coverslips, while maintaining >98% viability in all samples. Comparison of the SDS PAGE radioiodination patterns obtained for each surface revealed a number of specific bands markedly enriched on either apical or basal surface. This polarized distribution involved membrane-associated as well as integral membrane proteins and was observed in several strains of bovine aortic endothelial cells, as well as in both primary and passaged human umbilical vein endothelial cells. In contrast, two morphologically nonpolarized cell types, bovine aortic smooth muscle and mouse peritoneal macrophages, did not display differential localization of integral membrane proteins. Polarized distribution of integral membrane proteins was established before the formation of a confluent monolayer. When inverted (basal-side-up) monolayers were returned to culture, the apical-side-up pattern was reexpressed within a few days. These results demonstrate that cell surface–selective expression of plasmalemmal proteins is an intrinsic property of viable endothelial cells in vitro. This apical/basal asymmetry of membrane structure may provide a basis for polarized endothelial functions in vivo.

VASCULAR endothelium is a simple squamous epithelium whose luminal (apical) surface faces the blood and whose abluminal (basal) surface faces the tissues of the body. Endothelial cells typically are flat and attenuated, and generally lack the morphologically apparent plasma membrane specializations and apical-basal asymmetry characteristic of many epithelia. In contrast, many physiological and biochemical studies have demonstrated that the luminal surface of endothelial cells can actively participate in a number of important processes, such as the transport of insulin (15) and low density lipoproteins (38), angiotensin metabolism (26), binding activation and inactivation of components of the coagulation cascade (reviewed in references 10, 21, and 25), and the localized adhesion of circulating blood leukocytes (12). In these roles, the endothelial cell interacts with specific cellular and molecular components of blood as well as those of subendothelial tissues. Moreover, it is becoming increasingly apparent that the endothelial cell surface can be dynamically modulated in response to certain pathophysiologic stimuli, including inflammatory stimuli and mediators such as endotoxin, lymphokines, and monokines (2, 3). These functionally apparent specializations of the endothelial surface thus belie its relative lack of morphologic specialization.

Simionescu and co-workers have approached the question of topographical specializations of the luminal endothelial cell surface by performing ultra-structural tracer perfusion studies in mice (33). In fenestrated visceral capillaries, they have defined differentiated microdomains, which are enriched in certain glycoconjugates that impart regional differences in surface charge along the blood front, as well as on luminal versus abluminal surfaces (34). The chemical nature of these microdomains has been assessed by selective enzymatic digestions (30). In other studies differences have been observed in the lateral mobility of membrane phospholipids of the apical and basal surfaces of endothelial cells under certain culture conditions (20), and, recently podocalyxin, a 140-kD sialoprotein originally described in rat glomerular epithelial cells, has been localized immunohistochemically to the luminal surface of endothelial cells in several organs (14). None of these studies, however, directly addressed whether these specializations reflect, or might contribute to, an apical–basal asymmetry of function in vascular endothelium.

In highly differentiated epithelial cells (e.g., renal tubule cells, hepatocyte), polarized functions often correlate with an asymmetric distribution of cell membrane proteins (structural membrane polarity) (1, 35). In the current study, we

have investigated whether structural membrane polarity exists in vascular endothelium. Using monolayers of endothelial cells cultured from different blood vessels and species as an in vitro model, we developed a method of selectively radiolabeling externally disposed membrane proteins of either the apical or the basal (substratum-attached) cell surface. By this approach we have been able to document consistent surface-specific patterns of integral membrane (as well as membrane-associated) proteins that appear to be intrinsic features of apical and basal endothelial cell surfaces. These findings provide strong evidence that vascular endothelium is indeed a polarized epithelium.

Materials and Methods

Materials

The following special reagents and materials were obtained from the sources indicated: EM-grade glutaraldehyde, osmium tetroxide, uranyl acetate, lead citrate, *n*-amyl acetate, nonflexible collodion, Ilford L-4 nuclear emulsion, copper EM grids, and carboxylate-modified polystyrene latex spheres (0.69-μm diam) (Polysciences Inc., Warrington, PA); *N*-hydroxysuccinimide and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (Aldrich Chemical Co., Milwaukee, WI); lactoperoxidase (LPO)[1] (Calbiochem-Behring Corp., La Jolla, CA); glucose oxidase type VII (GO), poly-L-lysine hydrobromide of 150–300 kD (PLL), Nonidet P-40 (NP-40), phenylmethylsulfonyl fluoride (PMSF), aprotinin, *o*-dianisidine, 3,3′-diaminobenzidine tetrahydrochloride (DAB), trypan blue dye, and high molecular weight PAGE protein standards (Sigma Chemical Co., St. Louis, MO); all other materials for SDS PAGE (Bio-Rad Laboratories, Richmond, CA); Triton X-114 (TX-114) (Fluka Biochemicals, Hauppague, NY); soybean trypsin inhibitor (SBTI) (Cooper Biochemical, Malvern, PA); LSM™ lymphocyte separation medium (LSM) (Litton Bionetics, Kensington, MD); and carrier-free $Na^{125}I$ (New England Nuclear, Boston, MA). Affinity-purified rabbit IgGs directed against human collagen types I, III, IV, and V and fibronectin were the generous gift of Dr. Joseph Madri (Yale University School of Medicine), and swine anti-rabbit IgG and rabbit peroxidase anti-peroxidase complexes were from DAKO (Santa Barbara, CA). All other chemicals were reagent grade or better.

Tissue culture reagents and supplies included Hanks' balanced salt solution without calcium or magnesium (HBSS), Dulbecco's phosphate-buffered saline (DPBS) containing 0.88 mM Ca^{++} and 0.49 mM Mg^{++}, Dulbecco's modified Eagle's medium (DME), Medium 199 (M199), calf serum (CS), and fetal calf serum (FCS) (Whittaker M. A. Bioproducts, Walkersville, MD); glass coverslips, 12-mm diam (Bellco Glass, Inc., Vineland, NJ); plastic coverslips, 15-mm diam (Lux Thermanox, Miles Scientific Div., Naperville, IL); standard tissue culture plastic petri dishes (Corning Glass Works, Corning, NY or Nunc, Roskilde, Denmark); 6- and 24-well culture plates and sterile pipettes (Costar, Cambridge, MA); standard and Primaria™ tissue culture dishes (Falcon Labware, Oxnard, CA); lyophilized human serum fibronectin (New York Center for Blood Research, New York, NY); gelatin (Difco Laboratories Inc., Detroit, MI); endothelial cell growth factor, culture grade (ECGF) (Meloy Laboratories Inc., Springfield, VA); heparin and porcine intestinal mucosa, reagent grade (Sigma Chemical Co.).

Cell Source and Culture

Bovine aortic endothelial cells (BAEC) were prepared from collagenase digests of calf thoracic aortic intima and passaged on uncoated tissue culture plastic or glass coverslips in DME supplemented with 10% calf serum (10% CS-DME) as previously described (8). The majority of this work was carried out using one strain (11-BAEC) between passages 16 and 39. However, several strains originally isolated by three different investigators were also tested. Strain JOS-BAEC was the generous gift of Dr. George King (Joslin Diabetes Center, Boston, MA). All BAEC strains used grew with a typical cobblestone morphology with rare or absent "sprout" cells. Bovine aortic smooth muscle cells (BASM) were cultured from explants of calf thoracic aortic media and passaged in 10% CS-DME.

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase perfusion of normal term umbilical cords as previously described (8). Primary cultures, pooled from three to five cords, were grown on uncoated tissue culture plastic in 20% FCS-M199. Passaged cultures were grown on gelatin (0.1%) or fibronectin (1.0 μg/cm²)-coated tissue culture plastic in 20% FCS-M199 supplemented with ECGF (50 μg/ml) and heparin (100 μg/ml).

Murine resident peritoneal macrophages were isolated from retired breeder Swiss-Webster mice (Taconic Farms, Inc., Germantown, NY) as previously described (18) and cultured in 10% FCS-M199.

Preparation of a Viable Population of Nonenzymatically Resuspended Cells

Confluent monolayers were washed three times in warm (37°C) HBSS, incubated for 5–10 min at 37°C in a CO_2 incubator in 30 μM EDTA in HBSS, and then washed with ice-cold HBSS. This treatment caused cells to round up slightly and retract from each other without actual detachment or loss of viability. The cells were gently scraped in 1–2 ml of HBSS on ice using a Teflon spatula, an equal volume of protease inhibitor solution (0.3 mg/ml SBTI and 1.4 trypsin inhibitor units [TIU]/ml aprotinin in DPBS) was added, and the cell suspension was gently pipetted several times in a siliconized pasteur pipette to help disaggregate residual cell clumps. The cell suspension was carefully pipetted onto a prechilled discontinuous Ficoll/hypaque gradient consisting of 4 ml of a 1.7:2.3 mixture of DPBS to LSM (ρ = 1.033) on top of 3 ml of undiluted LSM (ρ = 1.077). The gradient was centrifuged at 1,000 *g* for 5 min at 4°C, after which >85% of the cells were recovered at the upper interface with a viability of usually >99% (trypan blue dye exclusion). Dead or damaged cells that could not exclude metrizamide had their buoyant density artificially increased and therefore were enriched in the lower interface, which was discarded.

Inversion of Endothelial Cell Monolayers

To selectively radioiodinate the basal surface of living endothelial cells, a procedure was developed to invert the monolayer on a poly-L-lysine-coated coverslip (PLL-CS). Tissue culture clean glass or plastic coverslips were incubated with poly-L-lysine (1 mg/ml in HBSS) for 5 min at room temperature, followed by extensive washes in highly purified water (Millex System, 18 mega ohms) and air dried. PLL coverslips were generally used within a few days but could be stored for weeks at room temperature in a dry, dust-free environment.

Confluent monolayers of vascular endothelial cells in 35-mm diam petri dishes were washed three times in HBSS at 37°C. A PLL-CS was gently placed down onto the monolayer so that its edge rested against the wall of the petri dish. Several seconds later the coverslips were lifted up with fine forceps using the point of contact with the petri dish wall as a fulcrum, bringing an essentially confluent sheet of endothelial cells attached by their apical surfaces with their original basal surfaces now in contact with the surrounding medium (Fig. 1). These "inverted monolayers" were immediately placed, cell-side-up, in an ice-cold bath of protease inhibitors (0.3 mg/ml SBTI and 1.4 TIU/ml aprotinin in DPBS) which served to limit the effects of proteases liberated from any damaged cells as well as to quench excess poly-L-lysine charges. Inverted monolayers were washed in ice-cold DPBS, incubated with 0.1% trypan blue dye in DPBS (2 min on ice), examined microscopically, and cell viability assessed by lack of nuclear staining. Typically, three inverted monolayers could be prepared from a single 35-mm dish.

Cells in inverted monolayers retained viability (usually 95–99%) and cell–cell contact. Inverted monolayers typically contracted somewhat during their preparation; therefore, individual cells appeared slightly plumper by phase-contrast and electron microscopy. When inverted monolayers were returned to culture at 37°C in 10% CS-DME, they remained morphologically intact and viable for at least 2 wk.

To determine whether extracellular matrix (ECM) proteins remain associated with the basal surface, immunocytochemical localization of ECM proteins was performed on inverted monolayers using affinity-purified immunoglobulin directed against individual collagen types I, III, IV, and V and fibronectin. Inverted monolayers and the culture dish surface from which they were derived were fixed in acetone/methanol (1:1 vol/vol), incubated with the specific antibodies or nonimmune rabbit IgG (0.1–1 mg/ml,

1. *Abbreviations used in this paper*: AS, apical surface; BAEC, bovine aortic endothelial cell(s); BS, basal surface; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DPBS, Dulbecco's phosphate-buffered saline (with Ca^{++} and Mg^{++}); ECM, extracellular matrix; EM-ARG, electron microscopic autoradiography; GO, glucose oxidase; LPO, lactoperoxidase; LPO-latex, lactoperoxidase covalently bound to 0.7 μm polystyrene sphere; PLL, poly-L-lysine; SBTI, soybean trypsin inhibitor; TCS, total cell surface.

30 min, 25°C), washed extensively in HBSS + 0.2% gelatin, and bound rabbit IgG was visualized by standard peroxidase–anti-peroxidase staining. All of the ECM proteins examined were detectable on the basal surface of inverted monolayers as well as on the exposed culture dish surface. No staining was observed using nonimmune IgG at the same concentration. When the same antibodies were applied to the apical surface of paraformaldehyde-fixed nonpermeabilized BAEC monolayers, no staining was detected with any of the antibodies, except for a trace of fibronectin staining at the highest antibody concentration.

This inversion technique was used successfully on confluent monolayers of all five strains of BAEC tested, as well as on primary cultures of HUVEC. No special surface coating of the culture dish was found to be necessary. However, cells plated on fibronectin-, collagen-, or gelatin-coated dishes apparently adhered too firmly to these substrates, resulting in low yield and poor viability when the inversion procedure was attempted. Poor results were also obtained if the HBSS cooled much below 37°C, or if calcium- and magnesium-containing washing media were used. The inversion procedure did not work if: (*a*) uncoated coverslips (net negative charge) were used, (*b*) the PLL coverslip had been previously quenched in a protein-containing solution, or (*c*) subconfluent endothelial cell monolayers were used.

Covalent Coupling of LPO to Carboxylate-modified Polystyrene Latex Spheres (LPO-latex)

The synthesis, storage, handling, and assay of LPO-latex was performed as described in detail previously (18, 19). In brief, carboxylate-modified polystyrene latex spheres (<0.7-μm diam) are activated using a water-soluble carbodiimide and esterified with *N*-hydroxysuccinimide. In the subsequent step, primary amino groups of lactoperoxidase displace the ester, forming a stable amide bond. Enzymatic activity is assessed by a simple chromogenic assay (36). LPO-latex stock is stored in 50% glycerol at −20°C and will retain full activity for over a year (19).

Selective Radioiodination of the Apical, Basal, and Total Cell Surfaces

Radioiodination of the apical surface (AS), basal surface (BS), and total cell surface (TCS) of endothelial cells was performed in parallel on replicate cultured monolayers. The TCS iodination served as an internal control, since any integral membrane protein radioiodinated on either the AS or BS would also be expected to be labeled in the TCS preparation. For most experiments, one 35-mm petri dish of endothelial cells was used for the AS radioiodination, three to six coverslip preparations of inverted monolayers for the BS, and resuspended cells from two 60-mm petri dishes for the TCS. To minimize vesicular membrane movement and prevent phagocytosis of LPO-latex, all samples and solutions were kept at 0–4°C. A 1:400 dilution of LPO-latex stock (19) was freshly prepared in DPBS + 20 mM dextrose (PBS/glucose) and dispensed onto the DPBS-washed monolayers. 1.5 ml was pipetted onto right-side-up monolayers (35-mm dishes), and 0.4 ml per well was pipetted onto inverted monolayers in 16-mm wells. Resuspended cells recovered from the Ficoll/hypaque gradient were transferred to a clean 35-mm petri dish or 16-mm well, mixed with the appropriate volume of LPO-latex suspension, and allowed to settle (so that some LPO-latex would be under the cells) on ice before the centrifugation step.

The culture vessels containing the various cell preparations and LPO-latex were centrifuged (2,000 *g*, 5 min, 4°C) in microtiter plate carriers (Dynatech Laboratories, Alexandria, VA). This brought the beads down in intimate contact with the cell surface where they were adherent but did not cause detectable injury (18). This procedure also served to attach the resuspended cells to the plastic dish for ease in subsequent manipulations. The monolayers then were washed twice in DPBS and checked by phase microscopy.

Radioiodination then was carried out as previously described (18). Briefly, cells were incubated in an ice-water bath generally for 30 min, in a PBS/glucose solution containing 100–300 μCi/ml carrier-free $Na^{125}I$ and 12 μU GO. Iodination was stopped by aspiration of the reaction mixture, brief sequential washes with 0.02% NaN_3 in DPBS (to inactivate LPO), twice in 0.1 M NaI (in DPBS), and three times in DPBS. After the procedure, viability was assessed by trypan blue dye exclusion. Cells were then fixed for electron microscopic autoradiography (EM-ARG) or lysed for TCA precipitation and SDS PAGE analysis as described below.

Under our iodination conditions, incorporation of ^{125}I into TCA-precipitable material reached a plateau in 25–30 min (data not shown). To maintain optimum viability (>98% consistently, both immediately and 24 h after iodination) a low concentration of GO was used and iodination carried out on ice. As previously found for iodination of macrophages using LPO-latex (18), incorporation of ^{125}I was dependent on LPO and GO and blocked by NaN_3. Incorporation doubled if either the bead dose or the ^{125}I concentration was doubled. LPO-latex was some 50-fold more efficient at iodinating cell monolayers than an equal number of units of soluble LPO (data not shown).

Electron Microscopic Autoradiography (EM-ARG)

After iodination and washing cells were fixed in glutaraldehyde (2.5% in 0.1 M sodium cacodylate buffer pH 7.4) for 30 min on ice, stained with DAB-H_2O_2 10 min at room temperature (11) to visualize LPO activity and identify LPO-latex (18), and postfixed in 1% OsO_4 for 1 h on ice. Cells were then dehydrated in graded ethanols, removed from the culture dish with propylene oxide, and embedded in Epon.

EM autoradiograms were prepared by the flat substrate method of Salpeter and Bachman (27). Thin (1,000 Å) sections on collodion-coated slides were coated with a crystalline monolayer (purple interference color) of Ilford L.4 emulsion, exposed at 4°C, and developed with Kodak D-19 developer (Eastman Kodak Co., Rochester, NY).

Quantitative Analysis of EM-ARG

The probability circle method of Salpeter and McHenry (28) with a correction for "cross fire" (7) was used to localize the source of the radiolabel corresponding to particular silver grains. Around the center of each grain is drawn a circle which has a 50% probability of containing the source of that grain. For 1,000-Å sections, ^{125}I and Ilford L4 emulsion, the radius of the 50% probability circle is 1,350 Å (29). The grain is assigned to all structures (compartments) lying totally or partially within the circle. The probability of true radiolabel in a given compartment creating an exposed silver grain is proportional to the specific radioactivity of the compartment as well as the area that the compartment occupies in the cell profile exposed for autoradiography. To correct for the effect of the latter, a matrix of random points surrounded by 50% probability circles is placed over each autoradiogram and compartment(s) are assigned to each as for grains. The relative grain density is determined by dividing the percent of total grains assigned to a compartment by the percent of total points assigned to the compartment and is thus a measure of the concentration of radioactivity of each compartment. We have previously found (18) that the relative grain density of the nucleus is essentially a measure of background radiation in such preparations. We therefore define "Specific Grain Density" as the relative grain density of a given compartment minus the relative grain density of the nuclear compartment for that sample.

TCA Precipitation

For quantitation of iodide incorporation, duplicate or triplicate aliquots (10–100 μl) of detergent lysate were pipetted into 1.5-ml microfuge tubes containing 5 μl of normal calf or rabbit serum as carrier. The tubes were filled with ice-cold 10% TCA + 0.1 M KI and incubated at 0–4°C for ≥1 h. Tubes were then centrifuged (12,000 *g*, 5 min, 4°C), the pellets washed twice with TCA/KI, and the bottoms of the tubes cut off with a razor blade and counted in a gamma counter (model 8,000, Beckman Instruments, Inc., Palo Alto, CA).

To remove TX-114 from samples (see next section), the 1.5-ml microfuge tube containing the detergent phase of the TX-114 extract (50–100 μl) was filled with ice-cold 10% TCA + 0.1 M KI and incubated at 0–4°C for ≥1 h. Tubes were then centrifuged and washed as above, but the TCA pellet is subsequently resuspended in acetone (−20°C), re-centrifuged (12,000 *g*, 5 min, 4°C), and washed in cold acetone before counting and dissolution in SDS sample buffer.

Preparation of Samples for SDS PAGE

Integral Membrane Proteins. Two methods were used to eliminate non-integral membrane proteins from the iodinated samples.

(*a*) Elution with EDTA. In preliminary experiments, we found that a 10-min incubation at 37°C in 30–60 μM EDTA in HBSS (without calcium and magnesium) eluted virtually all iodinated non-integral membrane proteins that could be detected in our gel autoradiograms. This incubation was routinely performed during the preparation of our total cell surface iodination sample (see above and Results, Fig. 7) and was accomplished for the apical and basal surface samples by returning these cells to the incubator in 30 μM EDTA after iodination and washing. All samples were then lysed by scrap-

ing in 0.05% NP-40 in HBSS containing freshly added protease inhibitors (1–2 mM PMSF, 10–20 μg/ml leupeptin), vortexing for 10 s, and spinning out LPO-latex, nuclei, and insoluble material in a microfuge for 2 min. Aliquots of the supernatant were subjected to TCA precipitation. For SDS PAGE the supernatant was mixed with an equal volume of 2× SDS sample buffer and placed in boiling water for 5 min.

(*b*) Extraction with TX-114. A more efficient method for selectively extracting integral membrane proteins from cell lysates used TX-114 as described by Bordier (4). Briefly, after iodination and washing in DPBS, cells were lysed in 1% TX-114 (in 10 mM Tris 150 mM NaCl pH 7.4 + 1 mM PMSF + 0.32 TIU/ml aprotinin + 20 μg/ml leupeptin) by scraping and vortexing as described above. Generally 1 ml of detergent was used for each sample (apical, total, or basal). The lysate was centrifuged (12,000 *g*, 5 min, 4°C) in a microfuge tube, and the resulting supernatant centrifuged (12,000 *g*, 25 min, 4°C) in a clean tube. The second supernatant was carefully layered over a 0.3-ml sucrose cushion containing the same protease inhibitors in a microfuge tube, placed in a 37°C water bath for 5 min, then centrifuged at 300 *g* for 3 min at room temperature (4). The aqueous phase was aspirated and the detergent phase subjected to TCA precipitation. The washed TCA pellet was dissolved in SDS sample buffer and heated in boiling water for 5 min. This procedure yielded an efficient extraction of integral membrane proteins and therefore was used in the preparation of all SDS gels illustrated in this report except Fig. 7.

Total Membrane Proteins. To obtain a gel of the total membrane proteins (membrane-associated as well as integral membrane proteins) for each cell surface preparation, cells were lysed in NP-40 omitting the prior EDTA wash. Alternatively, cells were lysed in TX-114 as described but the second supernatant was mixed directly with 2× SDS sample buffer and boiled (i.e., no phase separation).

SDS PAGE

SDS sample buffer consisted of 2% SDS, 5% β-mercaptoethanol, 12% sucrose, and 0.01% bromphenol blue in 50 mM carbonate buffer, pH 8.6. Cell lysates and other liquid samples were mixed with an equal volume of 2× concentrated sample buffer.

Slab gels (4–11% or 5–15% linear acrylamide gradient) 16.5-cm long and 1.5-mm thick were prepared by the method of Neville and run using a discontinuous buffer system (22). Samples were subjected to electrophoresis at 16 mA constant current until the tracking dye reached the bottom of the gel. The gels were fixed, stained, destained, dried under vacuum, and subjected to autoradiography on x-ray film (Kodak XAR-5, Eastman-Kodak Co.) as previously described (18).

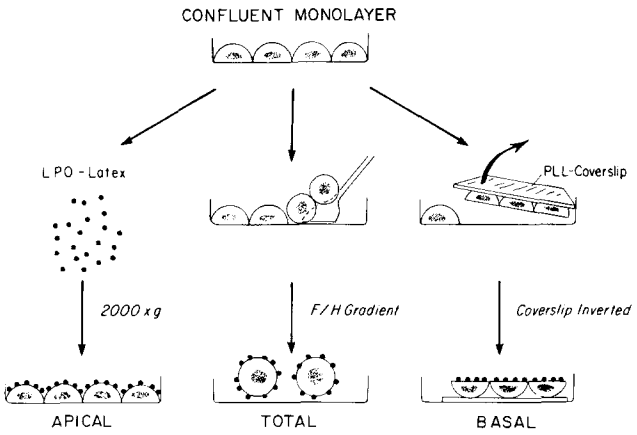

Figure 1. Method used to selectively radioiodinate the apical, basal, and total cell surfaces of cultured vascular endothelial cells. (See Materials and Methods for detailed description.) Apical surface (*APICAL*): LPO-latex are centrifuged onto washed monolayer. Total cell surface (*TOTAL*): Viable non-enzymatically resuspended cells, recovered from a discontinuous Ficoll-hypaque gradient (*F/H Gradient*), are mixed with LPO-latex and centrifuged. Basal surface (*BASAL*): Endothelial monolayer is inverted on poly-L-lysine-coated coverslip (*PLL-Coverslip*); after quenching, LPO-latex is centrifuged onto the original basal surface.

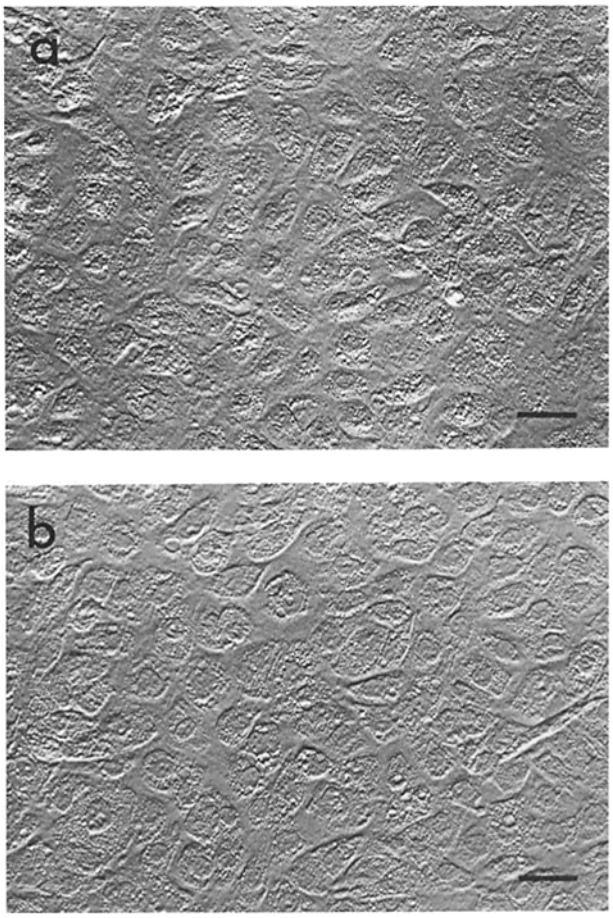

Figure 2. Photomicrographic comparison of intact apical surface and inverted basal surface of BAEC monolayers. The monolayers were prepared by our standard procedure up to the point of LPO-latex addition and then fixed in 2.5% glutaraldehyde in DPBS for 20 min at 4°C. Hoffman interference contrast optics reveal no discernable differences in the surfaces of the right-side-up (*a*) and inverted (*b*) monolayers. Bars, 10 μm.

Results

Inversion of Endothelial Cell Monolayers on Poly-L-Lysine-coated Coverslips

By use of the standard method detailed above (Fig. 1), basal-side-up or "inverted" monolayers of BAEC were routinely prepared with >95% initial viability; however, in the experiments reported here, only those preparations containing >98% viable cells were selected for radioiodination. By Hoffman interference contrast microscopy, the surfaces of both apical and basal (inverted) preparations appeared to be smooth, intact monolayers that were remarkably similar (Fig. 2). In particular, there were no cell fragments or visible extracellular matrix attached to the basal surface of inverted monolayers. Similarly, no cells, cellular debris, or membrane fragments were detectable on the bare areas of the petri dish from which these monolayers were derived. As discussed in Materials and Methods, collagen types I, III, IV, and V and fibronectin could be detected on the basal surface of inverted 11-BAEC monolayers (as well as on the bare areas of the petri dish) by use of affinity-purified specific IgGs in

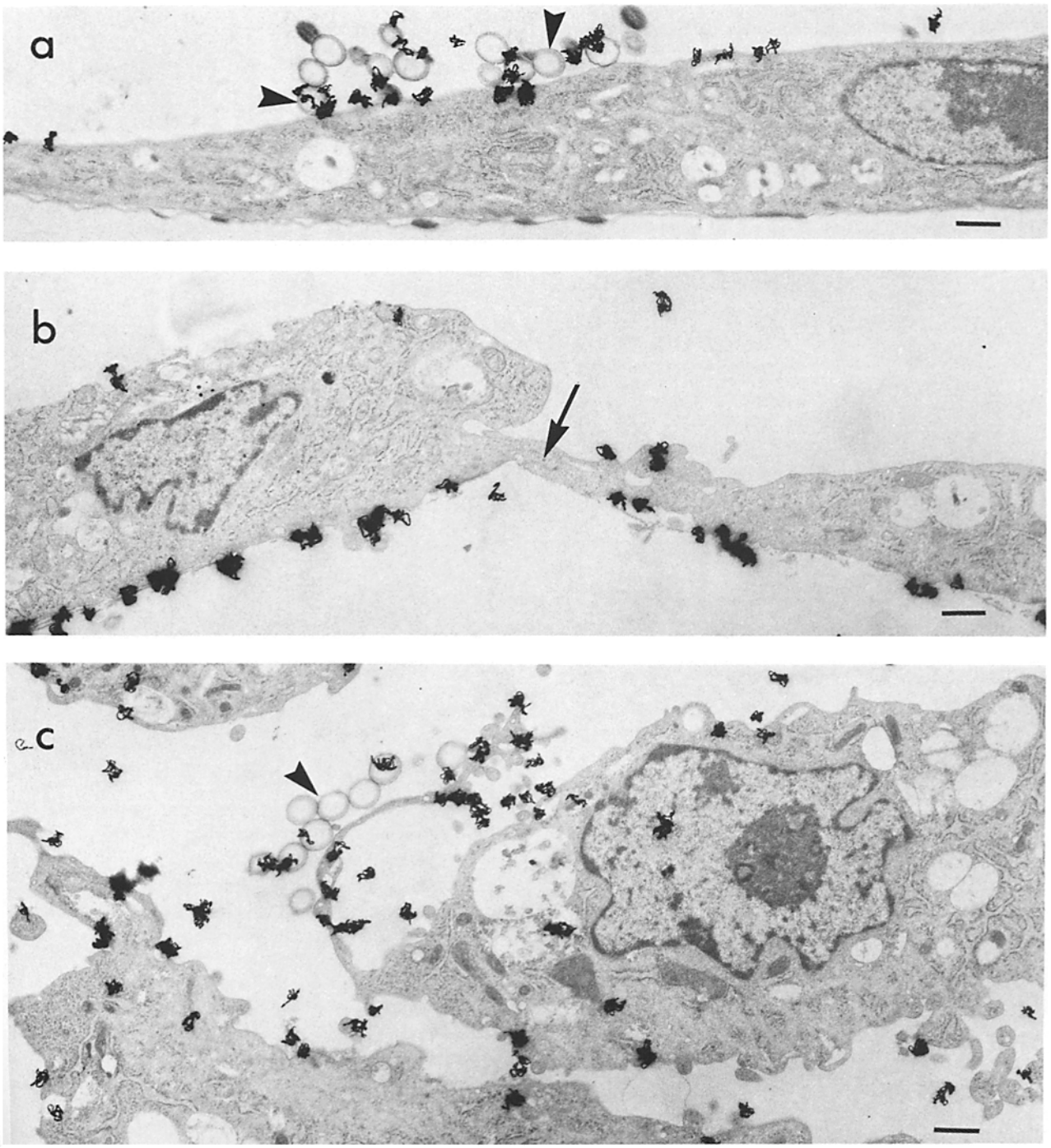

Figure 3. Representative electron microscopic autoradiograms illustrating the selective radioiodination of apical, basal, and total cell surfaces achieved using LPO-latex. Exposed grains are localized to the apical surface in the AS preparation (*a*) and to the basal surface in the BS preparation (*b*). In the TCS sample (*c*), grains appear distributed along the entire cell surface. See Table I for quantitative morphometric analysis. Most latex beads are dissolved during processing for electron microscopy; some residual LPO-latex beads are marked by the arrowheads in *a* and *c*. In this experiment the samples had been stained with DAB to identify sites of lactoperoxidase activity. Note that DAB reaction product outlines, and is restricted to, the rim of each LPO-latex bead. Arrow in *b* points to an intercellular junction which has been preserved in the inverted monolayer. Bars, 1 μm.

a sensitive peroxidase–anti-peroxidase immunocytochemical staining procedure. However, the presence of these matrix proteins did not interfere with our analysis of the integral membrane proteins of the endothelial cell plasmalemma (see below).

Selective Radioiodination of Apical, Basal, or Total Cell Surfaces

As schematically summarized in Fig. 1, LPO-latex was used to selectively radioiodinate the AS, BS, and TCS of cultured endothelial cells. The small size of the beads used (<0.7 μm)

Table I. Quantitative Autoradiographic Analysis of Cell Surface-selective Radioiodination Procedures

Preparation		Cellular Compartment		
		Plasmalemma	Cytoplasm*	Nucleus
Total cell surface	% Total grains (1,147)	83	12	6
	% Total points (1,027)	23	57	19
	Specific grain density‡	3.3	−0.1	0
		Plasmalemma§		
		Apical	Basal	(Indeterminate)
Apical surface	% Total grains (442)	77	7	(6)
	% Total points (777)	18	17	(2)
	Specific grain density	4.2	0.3	
	Surface-specific labeling ratio‖	14		
Basal surface	% Total grains (2,294)	3	81	(6)
	% Total points (990)	9	13	(3)
	Specific grain density	0.2	6.1	
	Surface-specific labeling ratio		30.5	

Radioiodinations of total cell surface, apical surface, and basal surface were carried out in parallel on replicate bovine aortic endothelial cell cultures, using our standard procedure. Quantitative analysis of EM autoradiograms was performed using the probability circle method (28) applied to randomly selected electron micrographs at 8,000× (see Materials and Methods for details). Numbers in parentheses indicate total number of grains or points counted for each surface preparation.

* Includes cytosol and all organelles other than nuclei. Most cytoplasmic grains were over vacuoles, many of which may have represented surface invaginations (6). Since this could not be verified in these specimens, these grains were assigned to the cytoplasmic compartment.

‡ A measure of specific activity of a compartment corrected for background grains defined as $\frac{\%\text{ total grains in compartment "x"}}{\%\text{ total points in compartment "x"}} - \frac{\%\text{ total grains in nucleus}}{\%\text{ total points in nucleus}}$

§ In the apical surface and basal surface preparations, 8–10% of the total grains were assigned to cytoplasm and 1–2% to nucleus. The majority of the remaining grains were associated with either apical or basal plasmalemma. In cross-sections of thin areas of the cell, where both apical and basal plasmalemmas fell within the probability circle for a given grain, that grain could not be uniquely assigned to either cell surface, and therefore was scored as "indeterminate."

‖ The ratio of specific grain density on the intended surface to the specific grain density on the opposite surface.

allowed many beads to come into direct contact with each cell, providing efficient yet localized catalysis. After centrifugation the beads remained on the cell surface with which they were originally brought in contact. When kept at ice temperature, endothelial cells did not phagocytose the beads.

EM-ARG confirmed that radiolabeling was restricted to the cell surfaces in contact with the LPO-latex beads (Fig. 3). (The vast majority of beads is dissolved during EM processing; several remaining beads are indicated by arrowheads in Fig. 3.) Quantitative morphometric analysis of the autoradiograms by the probability circle method (28) was performed on AS, TCS, and BS samples (Table I). In the TCS samples, 83% of the total grains were scattered randomly along the plasmalemma. When corrected for compartmental area, the plasmalemma had 12–18 times the specific radioactivity of any of the other cellular compartments (e.g., cytoplasm, nucleus). In the AS preparation, the specific grain density of the apical plasmalemma was at least 14 times that of the basal plasmalemma. Similarly, in the BS preparation, the specific grain density of the basal plasmalemma was more than 30-fold higher than that of the apical plasmalemma. In both the AS and BS preparations, ~6% of the grains fell over the peripheral regions of the cell, which were thinner than the resolution achievable with this technique; thus, these grains could not be uniquely assigned to any one compartment. In all preparations, every cell profile examined bore exposed silver grains on a plasmalemmal surface, indicating that our technique radiolabeled each cell.

Comparison of the Integral Membrane Proteins of Endothelial Cell Apical and Basal Surfaces

Autoradiography of SDS polyacrylamide gels was used to analyze the proteins iodinated on the AS, BS, and TCS. Fig. 4 (*left*) shows the results of a typical experiment in which equal amounts of TCA-precipitable radioactivity from each cell surface preparation were subjected to electrophoresis under reducing conditions. The iodination pattern observed for each surface was reproducible for a given strain of bovine aortic endothelial cells; each contained at least 15 major bands ranging in molecular weight from <20 kD to >200 kD.

Systematic comparison of radioiodination patterns of the apical and basal surfaces in more than 40 separate experiments revealed consistent differences. Thus, in the 11-BAEC strain (Fig. 4, *left*) there were several bands enriched in the apical pattern (at ~180, 140, 96, 86, 65, 57, and 36 kD) that consistently were absent, or nearly so, in the basal pattern. Similarly, there were bands in the basal pattern (at ~52, 46, 31, and 22 kD) that consistently were absent, or nearly so, in the apical pattern. Each of these specific surface-enriched proteins also was present in the TCS pattern. Moreover, the TCS pattern was nearly completely reconstituted by mixing equal samples of the AS and BS preparations (*lane AS + BS*).

Fig. 4 (*right*) compares the apical and basal surface iodination patterns of a different strain of BAEC (JOS-BAEC). Although there were minor differences in the overall patterns, several specific apical-enriched bands were clearly identifi-

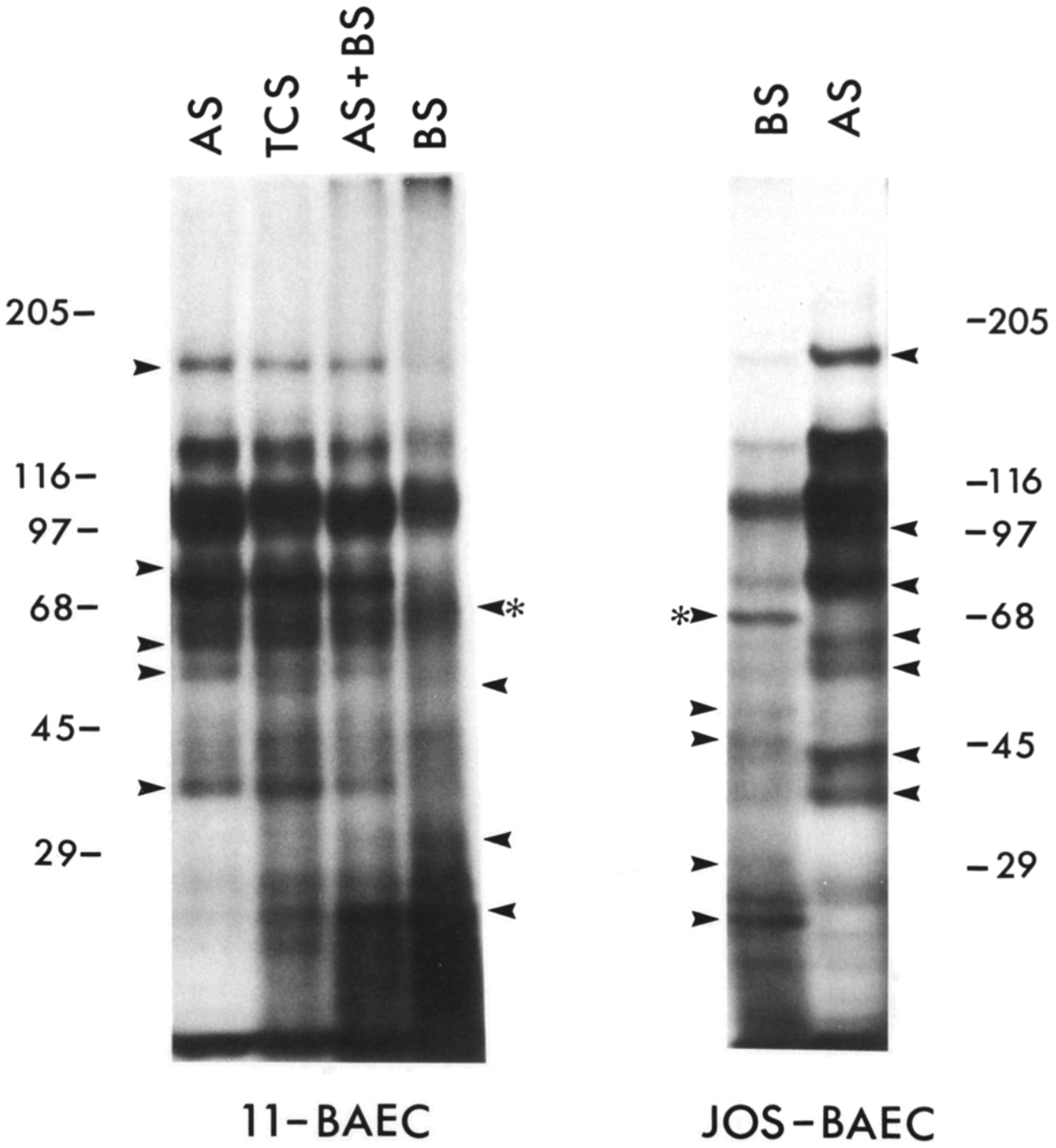

Figure 4. Comparison of radioiodinated integral membrane proteins of AS, BS, and TCS preparations from two different BAEC strains as analyzed by SDS PAGE and autoradiography. (*11-BAEC*): The designated lanes contain equal amounts of TCA-precipitable material (35,000 cpm) from TX-114 detergent phases of AS, TCS, and BS lysates. Arrowheads indicate certain bands that consistently appear to be specifically enriched in AS or BS, respectively, and are well-resolved in this autoradiogram. (Band indicated by asterisk probably represents BSA; its significance is discussed in text.) Note that all bands present in AS and BS patterns (except BSA) are represented in TCS pattern. Furthermore, the TCS pattern is closely approximated by mixing 17,500 cpm of AS lysate and 17,500 cpm of BS lysate (lane *AS + BS*). (*JOS-BAEC*): Analysis of AS and BS preparations of an independently isolated and cultured BAEC strain. Equal amounts of TCA-precipitable material were run in each lane. Although the iodination patterns of AS and BS differ slightly from those of 11-BAEC (*left*), apical and basal specific surface-enriched bands are present (*arrowheads*), including many of comparable molecular weight to those of the 11-BAEC strain (*left*). Molecular weights of protein standards ($\times 10^{-3}$) are indicated at left and right margins; 4–11% acrylamide gels; 51 h exposure (*left*); 66 h exposure (*right*).

able, including bands at ~180, 140, 96, 85, 65, 60, 45, and 40 kD, many of which appeared similar to those in 11-BAEC. Even more striking, the specific basal-enriched bands in this strain have molecular masses of ~52, 47, 30, and 24 kD, essentially identical to those seen in the 11-BAEC.

All of the bands in Fig. 4 represent integral membrane proteins as defined by two distinct methods. First, none of these bands were released into the supernatant when iodinated cells, washed as described in Materials and Methods, were subjected to three cycles of freezing and thawing in the presence of traditional chaotropic agents (including high and low ionic strength buffers, 1 mM EDTA, carbonate buffer pH 10.5, 0.5 M acetic acid, and 5% β-mercaptoethanol). However, all bands were solubilized by 0.05% NP-40, a nonionic detergent (not shown). Second, these polypeptides bound to, and partitioned with, the hydrophobic detergent TX-114 by the method originally described by Bordier (4) for the separation of integral membrane proteins from hydrophilic proteins. The only exception, noted by use of the TX-114 method, was a band co-migrating with bovine serum albumin (BSA) that appeared in the basal surface pattern (Fig. 4, band marked by asterisk). BSA has four known binding sites for the detergent (4, 17); therefore, it is incompletely separated from other soluble proteins. Note that BSA did not appear in the TCS pattern.

Several experiments were performed to rule out the possibility that the observed differences in surface iodination patterns were artifactually generated: (*a*) Iodination and processing of PLL-coated coverslips that had been incubated with serum-containing medium yielded no iodinated bands (other than BSA) that co-migrated with any of the bands in the AS, TCS, or BS patterns; (*b*) None of the bands in the surface-specific patterns corresponded to any of the enzymes or protease inhibitors added during the procedure (not shown); (*c*) Major cytoplasmic proteins, including actin and myosin, were not labeled (Fig. 4), consistent with restriction of radioiodination to the surfaces of intact viable cells; (*d*) When cells were nonenzymatically removed from the culture dish and the remaining dish surface was radioiodinated, the autoradiographic pattern obtained was a broad smear that did not resemble any of the cell surface patterns obtained (not shown); (*e*) When the apical surface of a monolayer was iodinated and then either resuspended (to simulate preparation of TCS; Fig. 5, *a* and *b*) or inverted (to simulate preparation of BS; Fig. 5, *c* and *d*) using our standard protocol, the resulting AS labeling pattern was unchanged; (*f*) Brief (30 s) exposure of cell cultures to PLL (1 mg/ml) followed by the usual "quenching" procedure did not change the subsequent iodination pattern of the apical surface (not shown). Thus, the experimental procedures used to produce resuspended and inverted preparations did not influence the final appearance of surface-specific iodination patterns; (*g*) The typical iodination patterns obtained were also unaffected by culturing cells on alternative substrata such as "Primaria," which has a net positive charge rather than standard tissue culture plastic, which has a net negative charge (not shown).

Therefore, each of the bands marked by arrowheads in Fig. 4 appears to represent an integral membrane protein that is markedly enriched in, or unique to, either the apical or the basal surface of cultured BAEC. The qualitative pattern of membrane polarity illustrated in Fig. 4 was consistently observed in more than 40 experiments performed with monolayers of the 11-BAEC strain at various passage levels (subcultures 16–39). In addition, a similar polarized expression of integral membrane proteins was documented in four other strains of BAEC. Moreover, when both primary and passaged cultures of human umbilical vein endothelium were examined by this technique, they also displayed several integral membrane proteins that appeared to be markedly enriched on either the apical or basal surface (Fig. 6).

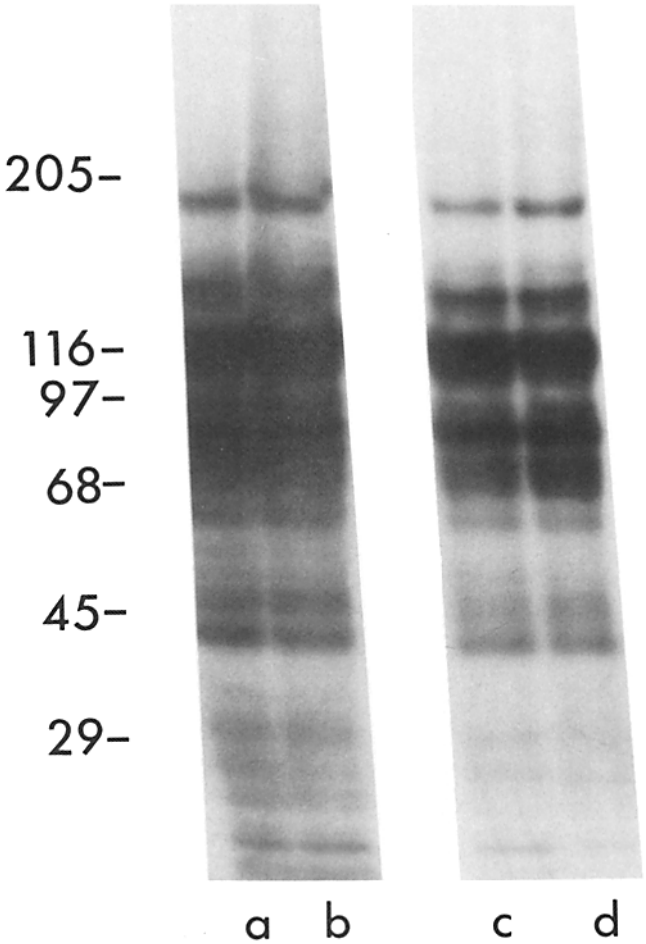

Figure 5. The apical surface-specific iodination pattern is not artifactually generated by the methods used to resuspend or invert BAEC monolayers. Confluent 11-BAEC monolayers were radioiodinated on the apical surface and lysed immediately in TX-114 (lane *a*) or non-enzymatically resuspended, harvested from the Ficoll-hypaque gradient, and lysed (lane *b*). In a separate experiment the monolayers were radioiodinated on the apical surface and lysed immediately in TX-114 (lane *c*) or inverted on PLL-CS by the standard procedure prior to lysis (lane *d*). In both comparisons, aliquots of lysate containing equal numbers of TCA-precipitable cpm were analyzed by SDS PAGE autoradiography. No changes were detected in the AS iodination pattern. Positions of molecular weight standards are indicated on the left. 4–11% gradient gel; lanes *a* and *b*, 32,000 cpm applied; lanes *c* and *d*, 16,000 cpm applied; 60 h exposure.

Comparison of Non-Integral Membrane Proteins Associated with Apical and Basal Surfaces

To examine whether there also was an asymmetric distribution of membrane-associated proteins on the surfaces of cultured BAEC, iodinated right-side-up and inverted mono-

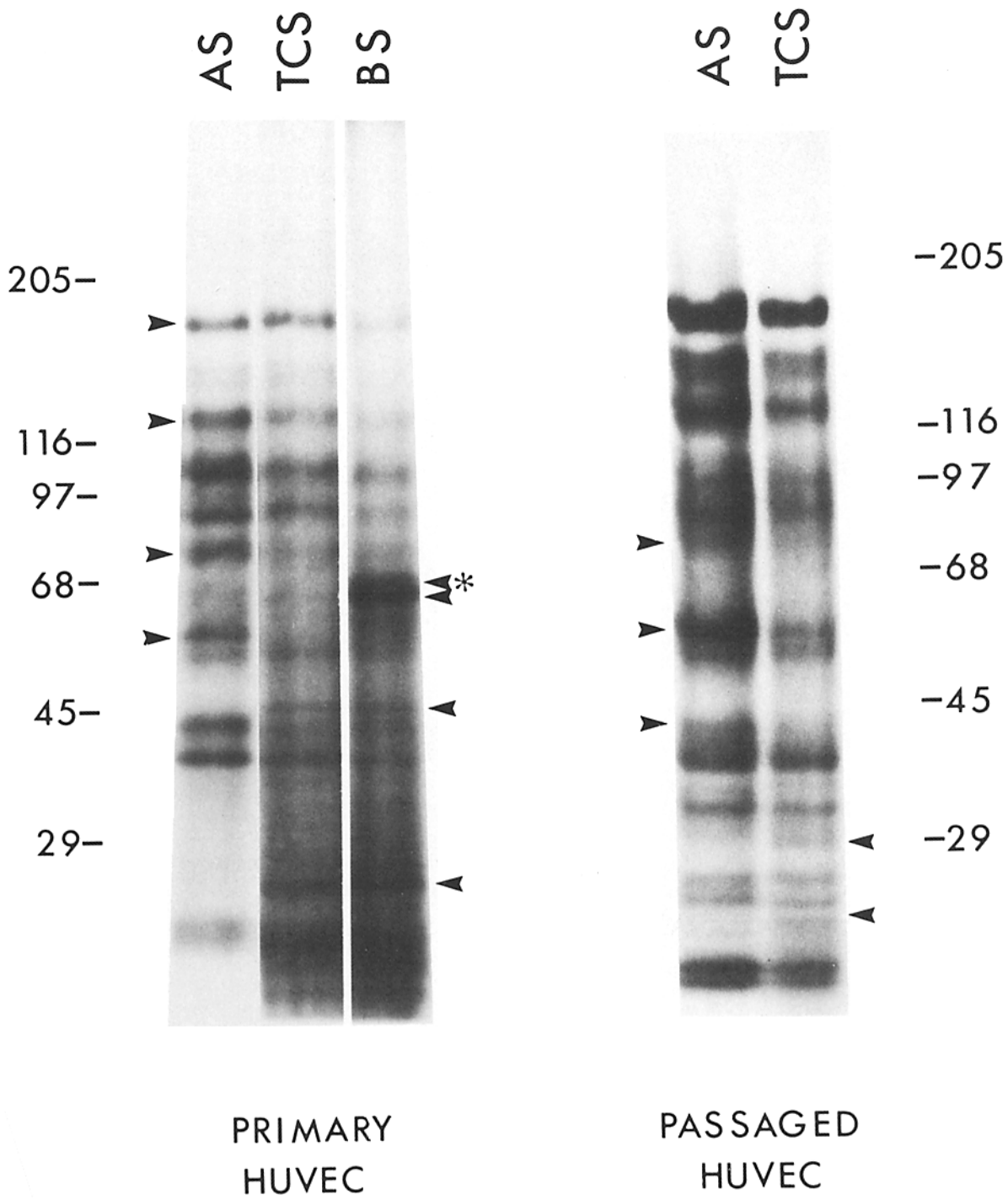

Figure 6. HUVEC also display structural polarity of integral plasmalemmal proteins. (*Left*) Primary cultures of HUVEC were subjected to surface-selective iodination, apical (*AS*), total (*TCS*), and basal (*BS*), using the same procedure as for BAEC. Iodinated integral membrane proteins extracted by TX-114 are compared. Proteins that appear markedly enriched in or unique to AS are marked by arrowheads on the left; those markedly enriched in or unique to BS are marked by arrowheads on the right. The band marked by the asterisk is BSA (see text for explanation). Essentially all of the bands visible in AS and BS patterns (except BSA) are present in TCS pattern. (*Right*) Passaged HUVEC (subculture 4) were iodinated on the AS and TCS and extracted with TX-114, as for primary cultures. With our current methodology we were unable to prepare inverted monolayers of passaged HUVEC. Therefore, specific AS-enriched proteins, indicated by arrowheads on the left, are inferred from bands markedly enriched in the AS pattern compared to the TCS pattern; specific BS-enriched proteins, indicated by the arrowheads on the right, are inferred from integral membrane proteins in the TCS pattern not visible in the AS pattern. Note that many of these specific surface-enriched proteins have the same M_r in primary as well as passaged cells. Positions of molecular weight standards are indicated at the margins. 4–11% gel. Primary HUVEC, 34,000 cpm/lane; passaged HUVEC, 40,000 cpm/lane.

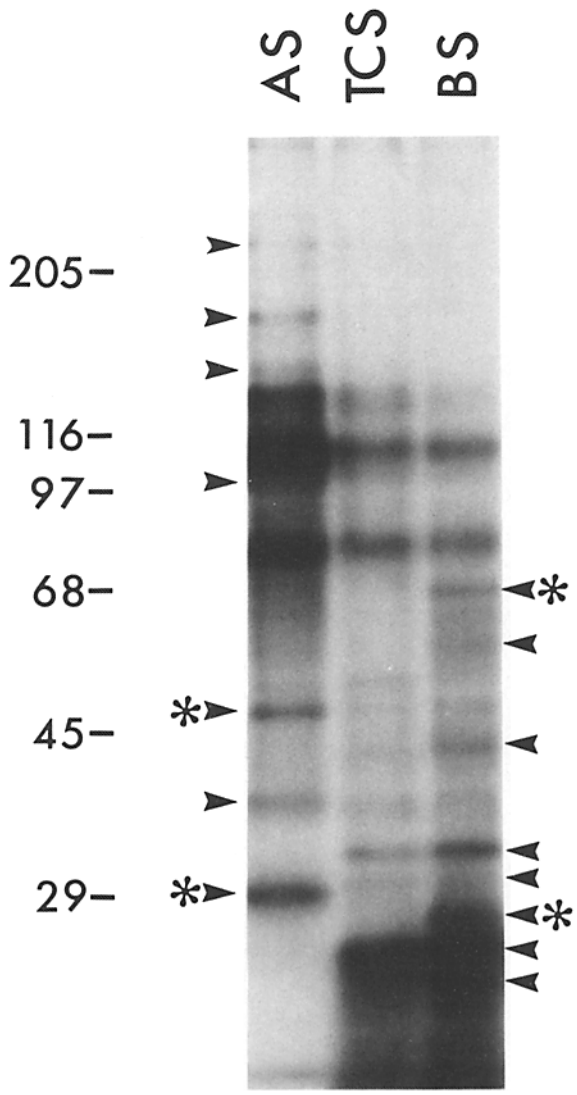

Figure 7. BAEC also display a polarized distribution of membrane-associated proteins. AS, BS, and TCS samples were prepared as usual but washed only in Ca^{++}- and Mg^{++}-containing buffers after iodination. (The TCS sample had been incubated in 30 μM EDTA in HBSS during harvest.) Samples were lysed in 0.05% NP-40 and aliquots containing equal numbers of TCA-precipitable cpm subjected to SDS PAGE on a 5–15% gel. Arrowheads to the left and right indicate proteins markedly enriched in or unique to the AS or BS, respectively. Those marked with asterisks represent non-integral membrane proteins which are partially or completely eluted by a 5-min incubation in 30 μM EDTA and are therefore markedly diminished or absent in the TCS pattern. Those specific surface-enriched bands resistant to EDTA represent the same integral membrane proteins seen in the TX-114 lysates of Fig. 4 on a 4–11% gel.

layers were processed for PAGE analysis without subjecting them to EDTA washing or TX-114 condensation (see Materials and Methods). Several non-integral membrane proteins remained selectively associated with either the apical or basal cell surface (Fig. 7). The bands marked by arrowheads in Fig. 7 represent proteins specific for a given surface. Those marked by asterisks are specific surface-enriched non-integral membrane proteins. Note that none of these non-integral membrane proteins appears prominently in the total cell surface pattern; presumably, they all partially or completely eluted off during the brief pre-flotation incubation of these cells in 30 μM EDTA. Many of the basal surface-associated proteins appear to be serum components.

The major band adherent to the apical surface has an apparent molecular mass of 30 kD on reducing gels, but 60 kD when not reduced (not shown). It was eluted by micromolar concentrations of EDTA and was more prominent in post-confluent than subconfluent monolayers. Thus, it most likely represents CSP-60, an endothelial-associated protein whose expression in culture is density-dependent, as originally described by Vlodavsky et al. (39) in cultured BAEC.

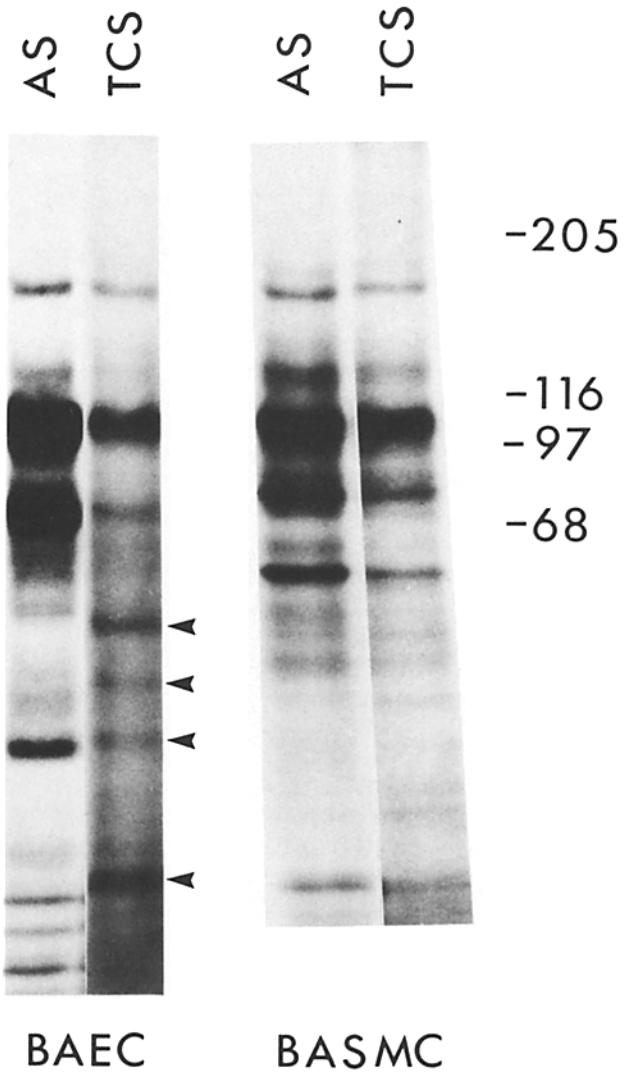

Figure 8. Bovine aortic smooth muscle cells (*BASMC*) do not display a differential distribution of plasmalemmal proteins. Cells were cultured at densities approximating a confluent monolayer. Apical surface (*AS*) and total cell surface (*TCS*) preparations were iodinated in parallel with 11-BAEC, and integral membrane proteins were analyzed by SDS PAGE autoradiography. Note that the AS and TCS patterns of BAEC are markedly different, reflecting the contribution of the basal surface-enriched proteins to the TCS pattern (*arrowheads*), whereas the AS and TCS patterns of BASMC are qualitatively similar. Molecular weights of protein standards ($\times 10^{-3}$) are indicated at the margins; 4–11% gels.

Structural Membrane Polarity Is an Intrinsic Property of Endothelial Cells

To test whether this observed polarity of plasmalemmal proteins was an intrinsic property of the endothelial cell rather than simply the consequence of its attachment to a culture dish, we compared cell surface iodination patterns of BAEC with those of bovine aortic smooth muscle cells derived from the same blood vessel as BAEC and serially passaged under similar in vitro conditions. Selective apical and total cell surface iodinations of bovine aortic smooth muscle cells cultured at a density that simulated a "confluent monolayer" was carried out in parallel to 11-BAEC monolayers. Fig. 8 compares apical and total surface iodination patterns of integral membrane proteins. In contrast to the differential cell surface patterns characteristically seen with 11-BAEC due to the presence of BS-enriched proteins in the TCS pattern (Fig. 8 arrowheads), a polarized pattern was not observed with vascular smooth muscle cells. No bands were identified in the smooth muscle TCS pattern that were not seen in the AS pattern, thus indicating a qualitatively symmetric expression of integral membrane proteins over the entire surface. A similar result was obtained using another nonpolarized cell type—mouse peritoneal macrophages (18; not shown).

Further experiments demonstrated that membrane polarity is not only an intrinsic property of cultured BAEC but also a dynamic one. When inverted monolayers were returned to culture, they remained morphologically intact and >98% viable for at least 2 wk. When the iodination patterns of the free surfaces of these inverted monolayers were se-

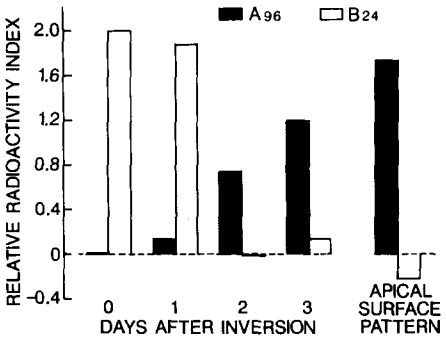

Figure 9. Inverted monolayers regain the apical surface radioiodination pattern when returned to culture. Confluent monolayers of JOS-BAEC were inverted and returned to culture for 1, 2, or 3 d, then iodinated in parallel with freshly inverted (day 0) or non-inverted (APICAL SURFACE PATTERN) monolayers and subjected to SDS PAGE. From the autoradiogram an AS-enriched band, a BS-enriched band and a symmetrically distributed reference band were chosen which could be most accurately excised from the gel and their radioactivity directly quantitated. On this particular gel, in contrast to Figs. 4 and 10, the most distinct AS band was the one at 96 kD (A96). The BS band used was the one with M_r 24 kD (B24). The change in radiolabeling of A96 and B24 with time was normalized and related to the reference band by the Relative Radioactivity Index = (CPM surface-enriched band ÷ CPM reference band) − 1. The absolute radioactivity of the reference band varied by <5% over the course of the experiment. Note the disappearance of B24 with time and the concomitant re-expression of A96 such that by 3 d the iodination pattern of the free surface of the inverted monolayer is qualitatively similar to the AS pattern of a non-inverted monolayer.

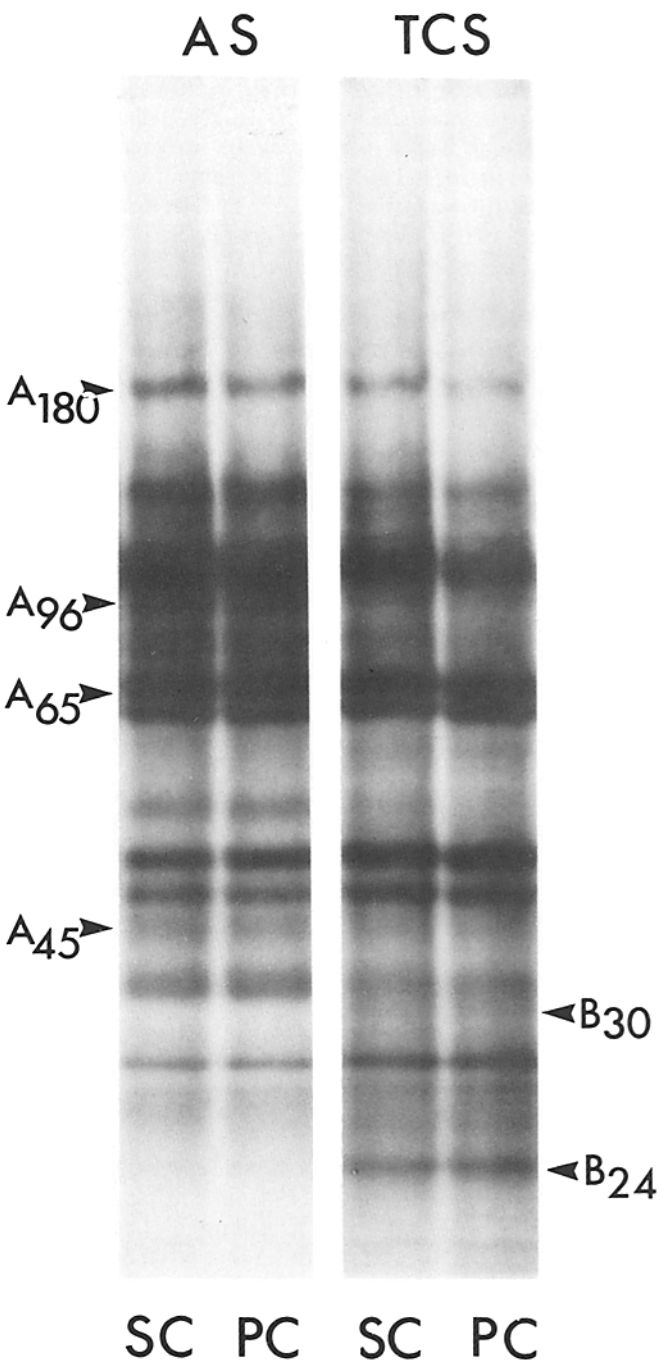

Figure 10. Integral membrane protein polarity is established before confluence of the monolayer. JOS-BAEC were plated at densities such that after 4 d in culture, cells were either 50% confluent (subconfluent, *SC*) or 1-d postconfluent (*PC*). Aliquots containing equal radioactivity in integral membrane proteins from apical surface (*AS*) and total cell surface (*TCS*) iodinations were compared by SDS PAGE autoradiography. AS and TCS samples from subconfluent monolayers closely resemble their postconfluent counterparts. Characteristic AS-enriched bands (four of which are indicated by arrowheads at left labeled with apparent molecular weights) are seen in the AS preparations, even at subconfluence. Similarly, two specific BS-enriched bands that stand out well in the TCS preparations (*arrowheads, right*) are seen at subconfluence. 4–11% gel. 2½-d exposure.

quentially examined a typical BS pattern was seen on day 0, but with time, specific BS-enriched proteins disappeared while specific AS-enriched bands appeared in the pattern and progressively increased in intensity. Fig. 9 illustrates the fate of two such bands in a representative experiment in which their relative radioactivity has been normalized to a symmetrically distributed reference band. The AS iodination pattern of a confluent non-inverted monolayer is stable for at least 1 wk in culture (not shown).

Endothelial Cell Membrane Polarity Is Established before Confluence

We compared the apical and total cell surface iodination patterns of BAEC as a function of culture density. Initial plating densities were adjusted so that after 4 d in culture cells were either ~50% confluent or 1 d postconfluent. As seen in Fig. 10, postconfluent monolayers (JOS-BAEC strain) displayed characteristics AS and TCS integral membrane protein iodination patterns. Cell-associated CSP-60 increased with confluence (not shown), as previously reported (13, 39). Of interest, however, at subconfluence the AS and TCS integral membrane protein iodination patterns were qualitatively similar to the corresponding patterns of confluent monolayers. Specific AS-enriched bands were present on the free surface of subconfluent monolayers (Fig. 10, arrowheads on left). Furthermore, the subconfluent TCS pattern contained specific BS-enriched bands not present in the AS pattern (Fig. 10, arrowheads on right) and was relatively depleted of specific AS-enriched bands. This indicated that the final polarized distribution of BAEC basal surface integral membrane proteins and most likely that of the apical surface integral membrane proteins as well was established before the formation of a confluent monolayer.

Discussion

The data presented in this paper demonstrate that cultured vascular endothelial cells possess a structurally polarized plasmalemma, which, by analogy to structurally more specialized epithelial cells (1, 35), may subserve polarized cell functions. Initial efforts were focused on integral membrane proteins rather than surface-associated proteins because any asymmetry in the disposition of the former could be taken as direct evidence for structural polarity of the plasmalemma. Both qualitative and quantitative surface-specific differences were observed. Because the efficiency of LPO-mediated iodination of a particular membrane protein can be affected by the local environment of the substrate (18), we cannot conclude that small differences in labeling observed with this method reflect actual differences in the density of that polypeptide on a given cell surface. Therefore, we have concentrated on the qualitative differences between the apical and basal surface and compared bands that appeared virtually restricted to one surface or the other. Reproducibly, in the 11-BAEC strain, certain polypeptides, with apparent molecular masses of ~180, 140, 96, 86, 65, 57, and 36 kD, were markedly enriched on the apical surface, while other polypeptides, with apparent molecular masses of 52, 46, 31, and 22 kD, were markedly enriched on the basal surface. In the JOS-BAEC strain a similar polarized distribution of integral membrane proteins was found. Moreover, the specific apical- and basal-enriched proteins had apparent molecular weights that were similar, but not identical to, those of their 11-BAEC counterparts (e.g., strain 11 bands B31 and B22 and strain JOS bands B30 and B24 [Fig. 4]). The functions of these specific surface-enriched integral membrane proteins are not yet known. However, the fact that asymmetry of integral membrane proteins exists demonstrates that these cultured vascular endothelial cells exhibit an apical/basal polarity of their plasmalemma. This phenomenon was not restricted to a certain species, vessel type, or passage level, since both primary and passaged human umbilical vein endothelial cells (Fig. 6), as well as several strains of BAEC (Fig. 4), all exhibited this polarity.

Several observations indicate that this structural polarity of the endothelial plasmalemma is not an artifact of our experimental system. First, cultured bovine aortic smooth muscle cells and mouse peritoneal macrophages (cells which are nonpolarized in vivo) both displayed a symmetric pattern of integral membrane proteins when analyzed by the same techniques (Fig. 8); thus, our methods themselves did not create asymmetric patterns. Second, the experimental procedures used to selectively radiolabel specific endothelial cell surfaces did not influence the final patterns obtained (Fig. 4, cf. lanes *AS + BS* vs. *TCS*; Fig. 5). Third, the iodination pattern of the inverted monolayer was relatively stable for at least 1 d in culture (Fig. 9). Therefore, it is unlikely that specific BS-enriched bands are the products of nonspecific proteolysis or protection therefrom.

The concept of structurally distinct domains along the endothelial plasma membrane is not new (33), yet compared to our knowledge of epithelial cells, relatively little is known about composition of the endothelial surface. In a series of in vivo ultrastructural studies, M. and N. Simionescu and their collaborators have extensively investigated the luminal surface of murine fenestrated endothelia, which exhibit morphologic specializations of their plasmalemma (e.g., diaphragms of vesicles and fenestrae) not seen in continuous endothelia of non-visceral vasculature. These specializations were found to have different overall anionic densities (34) and composition (30) as assessed by the binding of cationized ferritin perfused in situ. In a study of the abluminal surface, interstitially injected cationized ferritin bound in the same distribution as on the luminal surface with one major difference: it did not bind to the abluminal aspects of fenestral diaphragms (31). These studies established the concept of ionic charge microdomains on endothelium, some of which might be polarized with respect to the vascular lumen.

Recently, Horvat et al. (14) have used immunoelectron microscopy to demonstrate that podocalyxin, a 140-kD sialoprotein, appeared essentially restricted to the luminal surface of endothelial cells, where it was present in a patchy distribution (14) reminiscent of that of the CF binding sites (32). At present, it is not known if podocalyxin is an integral membrane protein synthesized by endothelial cells, or if it is produced elsewhere and adsorbed to the luminal endothelial surface from the blood. Immune precipitation with cross-reacting antibodies might help to determine whether our 140-kD apical surface-enriched band is the bovine equivalent of podocalyxin.

Cultured endothelial cells typically display density-dependent (contact) inhibition of cell division (13, 39), the mechanisms of which appear to be complex and are incompletely understood. Vlodavsky et al. (39) characterized a 60-kD pro-

tein (30 kD on reducing gels) that was associated with the plasma membranes of confluent monolayers of BAEC, but was not present on sparse, actively growing BAEC or bovine aortic smooth muscle cells. They postulated a role for this protein (which they named CSP-60) in the adaptation of a monolayer configuration by BAEC. A protein that appears to be CSP-60 by the above criteria behaves as a non-integral membrane protein selectively associated with the apical surface of BAEC (Fig. 7). Therefore, whatever physiologic role it plays in the stabilization of the cell monolayer or in mediating contact inhibition, it presumably functions at or between the apical surfaces of the cells. We find that the expression of this protein on the BAEC surface increases with increasing cell density (data not shown). However, the integral membrane proteins of the plasmalemma appear to be already polarized at a time when the cells are only 50% confluent (Fig. 10) and bear relatively little CSP-60 (13, 39, and our data [not shown]). Plasmalemmal polarity is therefore established independently of the degree of cell–cell contact or the status of growth inhibition.

When inverted BAEC monolayers were returned to culture, the integral membrane protein pattern of the free surfaces assumed an "apical" phenotype (Fig. 9). Reexpression of the full apical surface iodination pattern took 3–4 d, a time frame similar to that observed by Chambard et al. (5) for the reorientation of everted thyroid follicles implanted into a collagenous gel. This process seems slow compared with the rate at which most cells (37) can cycle large areas of membrane by vesicular transport and, in particular, with the rate of transcytosis in arterial endothelial cells (38). The prolonged time course of this process may be consistent with degradation and resynthesis of new surface-specific membrane proteins. It is also possible that the initial interaction with the PLL coverslip alters the kinetics of this process. Alternatively, the trace amounts of extracellular matrix remaining associated with the original basal aspect of the cell may stabilize that surface while new basement membrane components are being secreted and organized at the original apical surface. The morphology of cultured capillary endothelial cells is greatly affected by interactions with specific basement membrane components (16) and such interactions (or at least a surface for adhesion) may be crucial for the generation and maintenance of the polarized cell phenotype in endothelial cells, as they are in other epithelial cell systems (5, 24, 35).

Our radiolabeling procedure shows us the surface iodination pattern of the sum of the cells in the culture. It will be interesting to determine whether small subpopulations of cells with altered membrane polarity (or nonpolarized surfaces) exist, for example, at the growing edges of a wounded monolayer. Such studies may require direct visualization using antibodies directed against surface-specific components (see below).

Most of our present knowledge of cell polarity comes from studies on epithelial cells such as hepatocytes (1), thyroid parenchymal cells (5), and especially the Madin–Darby canine kidney (MDCK) renal tubular cell line (23, 24, 35). These studies involve methodologies very different from ours. Nonetheless, several differences between the physiology of our endothelial culture systems and other epithelial systems are worth pointing out. Epithelial cells feed from their basolateral surface (which in situ is the side facing their blood supply), whereas endothelial cells feed from their luminal (apical) surface. Thus, two sources of potential polarizing signals, blood-borne factors and the basal lamina, are on the same side for epithelial cells but on opposite sides for endothelial cells. There is evidence that both may influence epithelial cell polarity (5, 35). Another major difference between our system and most epithelial cell systems studied to date is that, under our culture conditions, the vascular endothelial cells do not form tight junctions, whereas the epithelial cells do. There is evidence that epithelial cells, like endothelial cells, do not require tight junctions to generate and maintain a polarized plasmalemma (24).

This demonstration of apical/basal polarity of the endothelial cell plasmalemma implies an ordered subcellular structure and raises several interesting questions: What factors influence the generation and maintenance of polarity in endothelial cells? What role do surface-specific cell components play in various physiologic functions and pathophysiologic responses of the endothelial cell? What is the cell surface distribution of receptors for various hormones, inflammatory mediators, and blood cells? Is this distribution altered as a result of ligand binding, in response to other stimuli, or in altered functional states (9)?

We are currently pursuing studies with monoclonal and polyclonal antibodies directed against defined cell surface structures. Several well-characterized enzymes such as angiotensin-I converting enzyme and carboxypeptidase N have been demonstrated immunochemically to be present on the endothelial plasmalemma in situ and in vitro (26), but their apical/basal distribution remains to be defined. Preliminary immune precipitation experiments using heterosera against angiotensin-I converting enzyme (in collaboration with Dr. Joseph Lanzillo, Tufts-NEMC, Boston) and insulin receptor ([15]; in collaboration with Dr. George King, Joslin Diabetes Center, Boston) suggest that both of these membrane proteins are located predominantly on the apical ("luminal") surface. Further studies are necessary to explore the regulation of the apical/basal expression of these functionally important molecules.

Many of the specialized roles served by vascular endothelium are now being studied at the level of molecular interactions at the cell surface. Previous work from other laboratories (14, 26, 33) has demonstrated topographical specializations along the luminal endothelial surface that may contribute to physiologic functions. Our study extends this knowledge with the demonstration that the vascular endothelial cell displays an apical/basal polarity of plasmalemmal proteins. Therefore a mechanism exists whereby the endothelial cell, in a manner similar to more structurally specialized epithelial cells, can selectively restrict particular functions to its luminal or abluminal surface. By use of the experimental approach described in this report, it should be possible to directly investigate the role that polarized distribution of plasmalemmal proteins may play in the biology and pathobiology of vascular endothelium.

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