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SEGMENTAL DIFFERENTIATION OF PERMEABILITY, PROTEIN GLYCOSYLATION, AND MORPHOLOGY OF CULTURED BOVINE LUNG VASCULAR ENDOTHELIUM+

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SUMMARY: The barrier function, surface biochemistry, and morphology of confluent monolayers of endothelial cells isolated from different segments of the bovine lung vasculature [microvessels (BLMVEC), vein (BPVEC) and artery (BPAEC)] were grown in culture and compared. A number of common cell surface proteins were identified along with two proteins of 46 and 48 kDa found exclusively on BPVEC. Lectin affinity chromatography revealed multiple glycosylation differences. The lectins, *Arachis hypogaea* (AHA) and *Lycopersicum esculentum* (LEA) agglutinins, interacted with several glycoproteins of BLMVEC but not of BPAEC. *Bandeiraea simplicifolia* (BS-1) and *Caragana arborescens* (CAA) agglutinins recognized several glycoproteins of BPVEC and BPAEC but not BLMVEC. Permeabilities were much lower for BLMVEC than BPAEC or BPVEC monolayers, with a range of about 16-fold less for sucrose to 2-fold less for albumin. Electron microscopy revealed that BLMVEC have a greater surface density of plasmalemmal vesicles (~4-fold) and more extensively developed intercellular junctions with more focal membrane adhesion sites per junction (~9-fold) than the other cells. We conclude that: i) BLMVEC monolayers form a much more restrictive barrier to molecular transport as a result of the tighter junctional formation; and ii) endothelial surface glycoproteins may be differentially glycosylated depending on their segmental location within the vasculature.

The attenuated monolayer of endothelial cells lining the vasculature is a critical mediator of a number of important cardiovascular functions including transvascular water and solute exchange, thrombosis, coagulation, inflammation, and immune response. Although the endothelium throughout the vasculature of the body has many common features, it is not homogeneous in character. Differences in ultrastructure, surface properties (surface charge, microdomain glycosylation), stimuli responses, and permeabilities have been observed for endothelium in different segments of the tissue vasculature (for reviews 5,15). Very little is known about possible segmental differences in the endothelial

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expression of specific surface proteins, especially in the lung vasculature. Direct examination of segmental differences in protein expression would be quite difficult *in situ*. Therefore, we have chosen to examine possible differences in protein expression and glycosylation *in vitro* using bovine endothelial cell monolayers derived from pulmonary arteries (BPAEC), microvessels (BLMVEC), and veins (BPVEC). Although it is not clear that segmental differentiation will persist *in vitro*, recent experiments suggest that endothelial cells tend to express similar plasmalemmal proteins *in situ* as well as in culture (9). We also have compared the barrier function and morphology of these three types of endothelial cell monolayers grown similarly in culture.

METHODS

Endothelial cell isolations and growth in culture. Bovine endothelial cells were isolated from pulmonary artery (BPAEC) and vein (BPVEC) as in (12,13) and from the lung microvessels (BLMVEC) as in (4). These isolation procedures were performed on the lung tissue of 4 different cows with similar yields and results. When the isolated BPAEC, BLMVEC and BPVEC became confluent, some of the cells were passaged to fibronectin-coated 75 cm^2 flasks and grown in 20% fetal calf serum in Dulbecco's modified Eagle's medium (DMEM) as in (13) whereas the remainder were frozen in liquid nitrogen until use. All three types were characterized as endothelial in origin by the uptake of acetylated low density lipoprotein and presence of established markers including Factor VIII - related antigen and angiotensin-converting enzyme as described (2,8,10).

Permeability measurements of endothelial cell monolayers. The in vitro system used for measuring the transendothelial flux of tracer molecules across BPAEC, BLMVEC and BPVEC monolayers has been described in (12,13. The tracer is added to the luminal compartment and the radiolabeled material cleared from the luminal to the abluminal compartment was measured as a function of time (1). DMEM with 20 mM HEPES and 5 mg/ml BSA is present in both compartments. The transendothelial permeability of each tracer was calculated with correction for the permeability of the coated filters as in (13).

Tracer molecules. D-[1-^{14}C]-mannitol, [^{14}C(U)]-sucrose and [carboxyl-^{14}C]-inulin-carboxyl were obtained from NEN-Du Pont Co (Boston, MA) and handled as reported (12,13). Cytochrome C (Sigma) samples were measured spectrophotometrically (18) using a DU-40 Spectrophotometer. All other molecules were radioiodinated with Na^{125}I using various procedures as repored in (1,12,13).

Surface radiolabelling of endothelial cell surface glycoproteins. After extensive washing with DMEM at 37°C, confluent BLMVEC, BPAEC, and BPVEC monolayers were radioiodinated using lactoperoxidase and hydrogen peroxide as in (15). The cells were lysed and their proteins solubilized in 5% Triton X-100 and 1% SDS in PBS at 4°C. After 10 min on ice, the cell lysate was collected and centrifuged for 10 min at 13,000 x g. The supernatants and pellets were processed for SDS-PAGE as in (9).

Lectin affinity chromatography. The radioiodinated cell lysates were used for batch lectin affinity chromatography using the following biotinylated lectins: *Arachis hypogaea* (PNA), *Bandeiraea simplicifolia* (BS-1), *Caragana arborescens* (CAA), *Concanavalia ensiformis* (Con A), *Erythrina cristagalli* (ECA), *Glycine max* (SBA), *Lycopersicon esculentum* (LEA), *Limax flavus* (LFA), *Ricinus communis* (RCA), *Triticum vulgare* (WGA), succinylated WGA (sWGA), and *Ulex europaeus* (UEA) agglutinins. The specificities of these lectins have been tabulated in (15). After incubating these lectins with the cell lysates, the lectin-glycoprotein complexes were precipitated and processed for SDS-PAGE as in (9).

Electron microscopy of endothelial cell monolayers. BPAEC, BLMVEC and BPVEC monolayers were washed twice with DMEM at 37°C and then processed for electron microscopy through the following steps: i) 3% glutaraldehyde (vol/vol) in 0.1 M cacodylate buffer (pH 7.2), 1 x 1hr; ii) 0.2 M cacodylate wash, 3x5min; iii) 2% OsO_4 in 0.1 M cacodylate, 1 x 1hr; iv)

DDW wash, 3 x 5 min; v) 2 % aqueous uranyl acetate (wt/vol), 1 x 1hr; vi) DDW wash, 3 x 5 min; vii) graded ethanol dehydration. Each culture was then embedded and sectioned equivalently for transmission electron microscopy. Ultrathin sections were of silver-gold interference color corresponding to a thickness of 80-90 nm. Every effort was made to prepare sections of equal thickness consistently. Electron micrographs were taken of intercellular junctions and of the anuclear regions of the cells. The number of uncoated vesicles on the luminal and abluminal cell membranes (not overlying the nucleus) was counted on each micrograph and the length of the plasmalemma was measured and converted to the actual length of the cell membrane (in μm). Noncoated plasmalemma vesicles were counted only if they: i) fit the appropriate size range (<80nm), ii) were directly attached to the cell membrane, were within 100nm of the cell surface, or were part of a series of vesicles forming a racemose structure that clearly was attached to the cell surface, and iii) clearly did not have evidence of an electron dense , fuzzy coat characteristic of coated vesicles. For each micrograph, the number of vesicles per μm of cell membrane was calculated. For each micrograph showing an intercellular junction, the actual length of the junction in the plane of the micrograph was calculated. In addition, the number of focal membrane contacts between cells (also called cell-to-cell membrane adhesion sites) per junction was counted. To be considered a focal contact, evidence of membrane fusion along with an adjacent region of electron dense fibrillar network had to be found.

RESULTS

Analysis of surface radiolabeled cell monolayers. Endothelial surface proteins of the intact cell monolayers were radioiodinated using lactoperoxidase and then solubilized. Proteins from both the detergent soluble phase (supernatant) and the insoluble fraction (pellet) were processed for SDS-PAGE followed by autoradiography. As shown in Figure 1A, we identified in both fractions a number of cell surface proteins which are common to the three types of endothelial cell monolayers. Comparison of the protein profiles of the detergent-soluble phase shows that most of the radiolabeled proteins were common; however, quantitative differences in certain surface radiolabelled proteins were found. Bands at 110 kDa and 140 kDa were more prominent in the BPAEC lane while two lower bands at 46 and 48 kDa were found only in the BPVEC lane. In the pellet fraction, a 110 kDa band was again more distinct in the BPAEC lane while in the BLMVEC lane, a band at 160 kDa was much more prominent than in the other lanes. Several proteins were significantly enriched in either the pellet fraction or the detergent-soluble fraction. The bands at 48, 46, and 43 kDa were not found in the pellet fraction while bands at 220, 180, and 160 kDa were only present in the pellet fraction. Proteins specific for microvascular endothelium were not detected. However, it does appear that the 46 and 48 kDa proteins are specific for BPVEC.

Characterization of cell membrane-associated glycoproteins. The soluble-fraction of the cell lysates was subjected to lectin precipitation and analyzed by SDS-PAGE and autoradiography. Figure 1 shows that each lectin recognized a subset of the total proteins and the patterns of the precipitated glycoproteins were different both qualitatively and quantitatively from one lectin to another. Within each cell type, there were distinct differences in the glycosylation of the membrane-associated glycoproteins. Several of the lectins clearly interacted differently with the surface glycoproteins of the microvessel endothelium than those of the large vessel endothelia. AHA and LEA interacted with several radioiodinated glycoproteins of BLMVEC but not with any radiolabelled surface

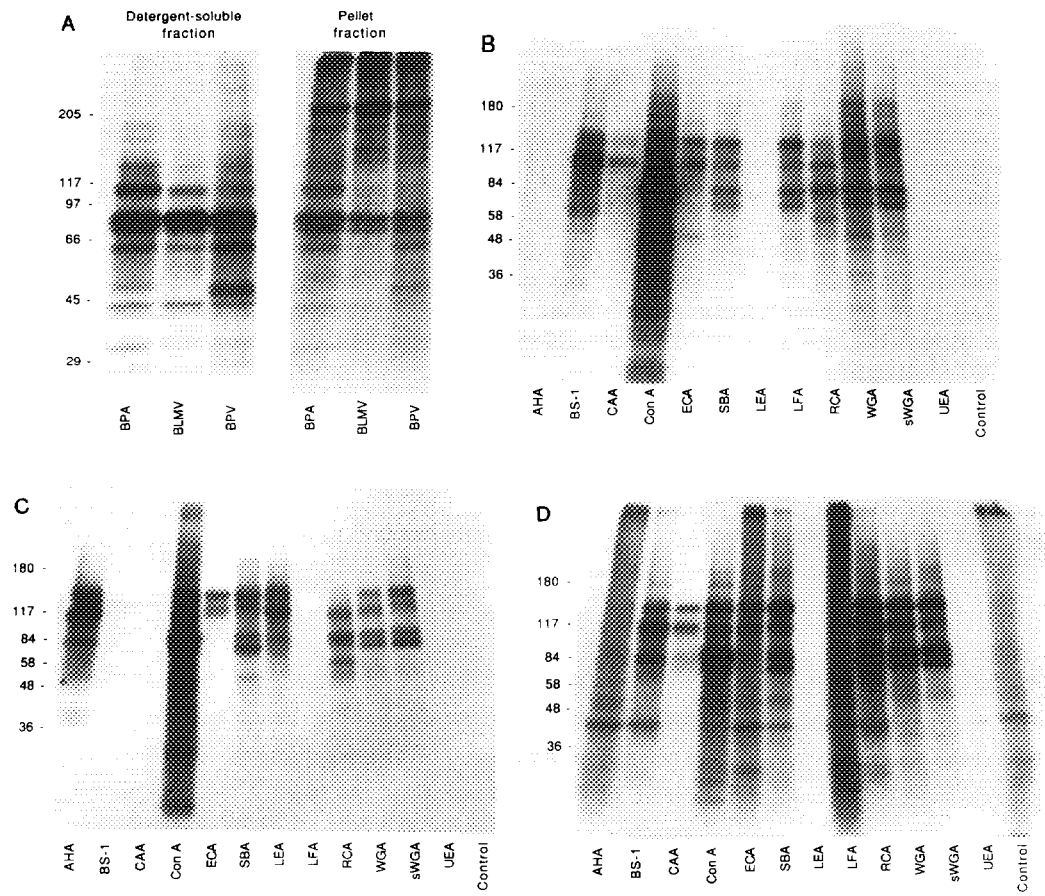

Figure 1. Analysis of radioiodinated endothelial cell surface proteins. Confluent monolayers of the indicated cells were radioiodinated and the cells were lysed by adding cold PBS containing 5% Triton X-100 and 1% SDS. (A) The proteins from the detergent soluble phase (equivalent protein of ~50 μg per gel lane) and the insoluble pellet (~10 μg/ gel lane) were processed for SDS-PAGE and autoradiography. (B-D) The detergent-soluble phase of the cell lysates was precipitated with the indicated biotinylated lectins and processed for SDS-PAGE and autoradiography. B: BPAEC monolayer; C: BLMVEC monolayer; and D: BPVEC monolayer.

glycoproteins of BPAEC. The glycoproteins of BPVEC also did not bind LEA and only interacted mildly with AHA (primarily the 46 kDa glycoprotein discussed above that appears to be specific for these cells). The opposite was true for BS-1 and CAA which recognized several glycoproteins of BPVEC and BPAEC but not BLMVEC. These results indicate that, although most of the radioiodinated proteins were common to all three cell types, there were notable differences in their glycosylation. Endothelial plasmalemmal glycoproteins appear to be differentially glycosylated depending on the vascular segment of origin of the cells within the pulmonary vasculature. Since recent experiments indicate that endothelium can express similar plasmalemmal glycoproteins *in situ* and in culture with similar lectin binding profiles (9), our findings suggest that segmental differential glycosylation of surface proteins may exist within the vasculature *in vivo*.

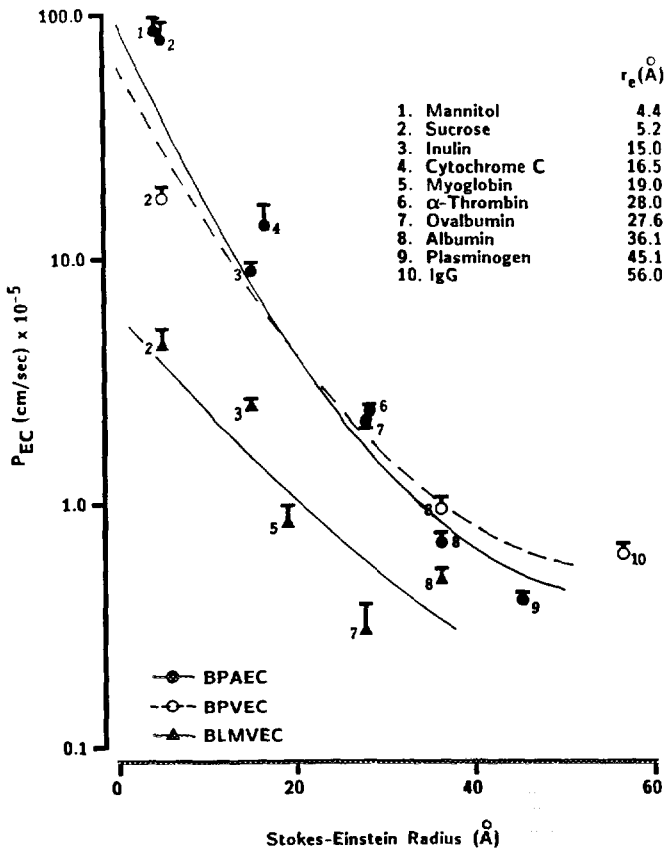

Figure 2. Permeability comparison for BLMVEC, BPAEC and BPVEC monolayers to molecules of different sizes. Endothelial permeability (P_{EC}) of the indicated cell monolayers are plotted on a logarithmic scale as a function of the Stokes-Einstein radius (r_e) of the tracer molecule. Bars indicate ± SEM. For each endothelial cell type, these data represent a composite of several different batches of cells.

Barrier function of endothelial cell monolayers. The permeabilities of BLMVEC monolayers were compared to BPAEC and BPVEC monolayers. For a wide range of molecular sizes (182 - 66,000 Daltons) studied, permeabilities were lower for BLMVEC than BPAEC or BPVEC as shown in Figure 2. The permeability of ^{125}I-albumin to the BLMVEC monolayers was about 2-fold less than the BPAEC value. However, BLMVEC were 15.5-fold and 3.5-fold less permeable to ^{14}C-sucrose and ^{14}C-inulin, respectively, than BPAEC.

Comparative differences in structural components important in transport. Electron micrographs taken of the three-types of endothelial cell monolayers at confluency were used to assess possible differences between the cells with respect to structures believed to be important in transendothelial transport, namely plasmalemmal vesicles and intercellular junctions. It was clear that: i) the plasmalemmal vesicles appeared to be of similar size for all three endothelial cells; ii) the number of plasmalemmal vesicles on the cell surface were significantly greater for BLMVEC than the other cells; and iii) the number of focal contacts between the cells (ie., cell-to-cell membrane adhesion sites) in the intercellular junctions were greater for the BLMVEC than the BPAEC and BPVEC. Quantitative estimations were

Table I. Morphometric analysis of important structures involved in the transport of molecules across endothelium. The three indicated endothelial cell types were examined by electron microscopy. The number of plasmalemmal vesicles per μm, the number of focal contacts (membrane adhesion sites) per either junction or μm, and the path length in μm per junction are given in each column, respectively. The average value is given for all fields examined along with the standard deviation. These results represent a composite from several different cell isolates (at least two) which gave very similar findings and therefore, were combined for each cell type.

Cell type	# vesicles[a] μm	#focal contacts[b] junction	#focal contacts μm	Path length μm junction
BLMVEC	3.32 ± 2.21*	5.0 ± 2.8*	9.0 ± 5.4*	7.8 ± 5.6
BPAEC	0.84 ± .92	0.59 ± .74	1.4 ± 1.7	6.4 ± 2.6
BPVEC	1.05 ± 1.30	0.11 ± 0.29	0.21 ± 0.52	2.1 ± 3.4
R	3.95	8.47	6.43	1.22

a - ≥ 100 μm of cell membrane examined.
b - 30 intercellular junctions examined.
* - Statistically different from BPAEC (P >0.95 as per unpaired *t* test analysis).
R - ratio of values from BLMVEC to BPAEC.

performed (see Table 1) and the density of vesicles and junctional focal adhesion sites were 4.0- and 8.5-fold greater, respectively, in BLMVEC than BPAEC. The average path length of the junctional pathway was only 22% greater for the BLMVEC than BPAEC. Figure 3 shows the distribution profile of the plasmalemmal vesicles for the three types of endothelial monolayers. The BLMVEC had a much greater frequency of cells with a high population of plasmalemmal vesicles than the other endothelial cells. Greater than 50% of the BLMVEC fields examined and less than 10% of the BPAEC and BPVEC fields had greater than 3 vesicles/μm. Most micrographs of the BPAEC and BPVEC showed less than 1 vesicle/μm.

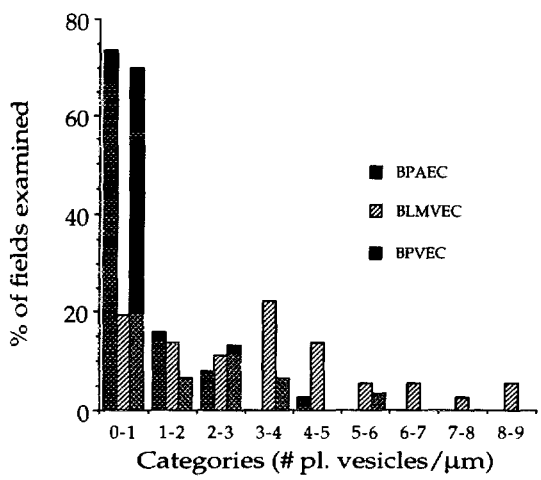

Figure 3. Percentage distribution profile for the frequency of plasmalemmal vesicles on the endothelial cell membrane. The BPAEC, BLMVEC, and BPVEC monolayers were examined by electron microscopy and the number of vesicles per μm was counted for each micrograph. The Y axis gives the percentage of all of the fields examined that had the number of vesicles indicated on the x-axis. At least three different cultures for each type of endothelium were examined.

About 70% of the BPAEC and BPVEC micrographs had between 0 and 1 vesicle/μm whereas more than 80% of the BLMVEC fields had between 1 and 9 vesicles/μm.

DISCUSSION

Comparative morphology in culture. As observed in our previous work (7), the organization of endothelial cell monolayers grown in culture differs significantly from that found *in vivo* as a result of phenotypic drift. All three endothelial cell types have been isolated from vascular beds provided with a continuous endothelium. Like most endothelium of this type, the dominent feature of pulmonary vascular endothelium is a large population of plasmalemmal vesicles associated with the luminal or abluminal plasmalemma or apparently free in the cytoplasm. Furthermore, this endothelium forms a continuous monolayer of attenuated cells that are interconnected via elaborately developed intercellular junctions with many regions of focal contact or apparent membrane fusions between neighboring cells. These cell-to-cell membrane adhesion sites within junctions are apparent by transmission electron microscopy and form a series of linear arrays that surround the cells as revealed by freeze-fracture (for review, 15). The architectural complexity of these junctions appears to provide a "small-pore" pathway resulting in restrictive barrier to the paracellular transport of water and other small molecules across the endothelium while the abundance of plasmalemmal vesicles may provide a "large-pore" pathway for the transport of macromolecules by vesicular shuttling and/or transient formation of transendothelial channels (5,6,11,15).

The endothelial cells derived from the lung microvasculature exhibit distinct differences from cultured endothelial cells derived from large pulmonary vessels, the pulmonary artery and vein. Morphological examination using electron microscopy revealed that BLMVEC maintained a phenotype in culture much more consistent with the known characteristics of this continuous endothelium *in vivo*. They have a significantly greater population of plasmalemmal vesicles than either BPAEC or BPVEC and the distribution profile for the plasmalemmal vesicles showed a much greater frequency of membrane regions with a high density of vesicles. However, it is clear that the number of vesicles is still less than observed *in vivo.* In addition, although all three endothelial cell types appear to form confluent monolayers when grown in culture, closer scrutiny of the intercellular junctions by electron microscopy revealed distinct differences in their development. The intercellular junctions were much more elaborate and complex for BLMVEC monolayers with almost a 10-fold greater number of membrane focal adhesion sites than either the BPAEC or BPVEC. This greater degree of junctional complexity should create a more restrictive barrier to the transport of molecules across intercellular junctions. It is unclear if these morphological differences reflect modification occurring during isolation and growth in culture, or if the changes are attributable to physical differences between the cell environments found *in vivo* and *in vitro* (eg., the lack of a pressure differential across the monolayer or fluid shear stresses).

Permeability differences. The permeabilities of the BLMVEC monolayers were much more restrictive to molecular transport than the BPAEC and BPVEC., apparently consistent with a "tightening" of the intercellular junctions which restricts the paracellular transport pathway responsible for small solute flux. This functional assessment correlates well with our ultrastructural assessment of the tight junctions. The greater development of the junction achieved in the BLMVEC monolayers appears to form a tighter monolayer that reduces the paracellular transport of these molecules. Although the microvessel monolayers were also more restrictive to the larger macromolecules tested, the relative change was not as great for the large molecules as the smaller ones. This suggests that possibly the "large pore" pathway was not altered in the same manner or at least to the same degree as the "small pore" pathway. Ultrastructurally, the microvessel cells had a greater number of plasmalemmal vesicles whose proposed involvement in macromolecular transport would suggest a potential increase in the observed transport via the transcellular pathway. Transport studies using endothelial cell monolayers from large vessel preparations revealed molecular permeabilities that far exceed those observed *in situ* (3). This "leakiness" of the monolayers has been attributed to paracellular transport of macromolecules via intercellular junctions which develop incompletely *in vitro* . The large vessel endothelial cells did not have many plasmalemmal vesicles which is expected to reduce the degree of transcellular vesicular transport. From the small solute transport data and from the ultrastructural studies, it is clear that the well-known "leakiness" of endothelial cell monolayers derived from large vessel preparations was much less in the microvessel monolayers. It would appear that this reduction in paracellular transport in the BLMVEC monolayers was sufficient to more than offset the expected increase in transcellular transport via the greater number of plasmalemmal vesicles.

Differences at the molecular level. Multiple segmental differences in protein expression and surface glycoprotein glycosylation were noted and may play a role in the differential development of the endothelial cell monolayer in culture and ultimately, in the observed functional and structural differences between the cells. Obviously, functional correlations are likely in terms of endothelial cell surface interactions with blood cells or molecules; however, at this time, a precise statement of the likely candidates seems fruitless in lieu of the great number of possible different functions attributable to endothelial surface interactions. For example, specific carbohydrate recognition are frequently involved in cell-cell interactions including the interaction of myeloid and tumor cells with vascular endothelium (16). Future work devoted to the development of antibodies specific for these endothelial surface glycoproteins may aid in our understanding of the basis of these observed differences. Futhermore, such probes may prove to be specific to endothelial segments of the vasculature which could prove to be very helpful in isolating endothelial cells with improved fidelity from specific segments of the vasculature.

REFERENCES

1. Cooper, J.A., Del Vecchio, P. J., Minnear, F.L., Burhof, K.E. Selig, W.M., Garcia, J.G.N. and Malik, A.B. (1987) J. Appl. Physiol., 62: 1076-1083

2. Del Vecchio, P. J., Ryan, J.W., Chang, A. and Ryan, U.S. (1980) Biochem. J. 186: 605-608
3. Del Vecchio, P. J., Siflinger-Birnboim, A., Shepard, J.M., Bizios, R., Cooper, J.A. and Malik, A.B. (1987) Fed. Proc. 46: 2511-2515
4. Del Vecchio P. J., Siflinger-Birnboim, A., Belloni, P. N., Holleran, L. A., Lum, H. and A. B. Malik (1992) In Vitro Cell. Dev. Biol., 28: 711-5
5. Palade, G. E. The microvascular endothelium revisited. In *Endothelial Cell Biology*, ed. Simionescu, N. & Simionescu, M., pp. 3-22. Plenum Press, New York, 1988
6. Renkin, E. M. Transport pathways and processes. In *Endothelial Cell Biology*, ed. Simionescu, N. & Simionescu, M., pp. 51-68. Plenum Press, New York, 1988
7. Schnitzer, J.E., W. W. Carley, and G. E. Palade (1988) Am. J. Physiol. 254: H425-437
8. Schnitzer, J.E., W. W. Carley, and G. E. Palade (1988) Proc. Natl. Acad. Sci. USA 85: 6773-6777
9. Schnitzer, J.E., C.-P. J. Shen, and G. E. Palade (1990) Eur. J. Cell Biol. 52: 241-251
10. Schnitzer, J. E. (1992) Am. J. Physiol., 262: H246-H254
11. Schnitzer, J.E. (1993) Tr. Cardiovasc. Med. 3: 124-130
12. Siflinger-Birnboim, A., Del Vecchio, P. J., Cooper, J.A., Blumenstock, F. A., Shepard, J.M. and Malik, A. B. (1987) J. Cell Physiol. 132: 111-117
13. Siflinger-Birnboim, A., Del Vecchio, P.J., Lum, H. and Malik, A.B. (1988) Microvasc. Res. 36: 216-227
14. Siflinger-Birnboim, A., J. E. Schnitzer, H. Lum, F. A. Blumenstock, C.-P. J. Shen, P. J. Del Vecchio and A. B. Malik. (1991) J. Cellular Physiol., 149: 575-584
15. Simionescu, M. and Simionescu, N. (1986) Ann. Rev. Physiol. 48: 279-293
16. Walz, G., Aruffo, A., Kolanus, W., Bevilacqua, M., and Seed, B. (1990) Science, 250: 1132-1135