

Lung Endothelial Dipeptidyl Peptidase IV Is an Adhesion Molecule for Lung-metastatic Rat Breast and Prostate Carcinoma Cells

Robert C. Johnson, Duzhang Zhu, Hellmut G. Augustin-Voss, and Bendicht U. Pauli

Cancer Biology Laboratories, Department of Pathology, Cornell University College of Veterinary Medicine, Ithaca, New York 14853

Abstract. Attachment of circulating tumor cells to endothelial cell adhesion molecules restricted to select vascular compartments is thought to be responsible for site-specific metastasis. Lung-metastatic rat R3230AC-MET breast and RPC-2 prostate carcinoma cells bound outside-out endothelial cell membrane vesicles, prepared by perfusion of the rat lung vasculature with a low-strength formaldehyde solution, in significantly higher numbers than their nonmetastatic counterparts R3230AC-LR and RPC-LR. In contrast, vesicles derived from the vasculature of a nonmetastasized organ (e.g., hind leg muscle) showed no binding preference for either of the four tumor cell lines. Lung-derived endothelial vesicles were used here to generate mAbs against lung endothelial cell adhesion molecules. The first group of mice were actively immunized against lung endothelial vesicles, whereas the second group was injected with syngeneic mouse antiserum against leg endothelial vesicles before active immunization

with lung endothelial vesicles. 17 hybridoma supernatants obtained from the two fusions bound lung vesicles with at least a 10-fold higher affinity than leg vesicles. Seven (four obtained by a passive/active immunization protocol) stained rat capillary endothelia. One mAb, mAb 8.6A3, inhibited specific adhesion of lung-derived vesicles to lung-metastatic breast and prostate carcinoma cells. Purification of the antigen (endothelial cell adhesion molecule) from rat lung extracts revealed a protein with a 110-kD mol wt. NH₂-terminal sequencing established identity with dipeptidyl peptidase IV which had been reported to serve as a fibronectin-binding protein. These results indicate that vesicles obtained from in situ perfused organs are a convenient immunogen for the production of antibodies to compartment-specific endothelial cell surface molecules, and reinforce the concept that endothelial cell surface components are selectively recognized by circulating cancer cells during metastasis formation.

METASTASIS is the process by which blood-borne cancer cells establish new tumor colonies in secondary organs. The selection of target organs for metastasis occurs in nonrandom fashion and is dictated by compatible tumor cell and host cell characteristics (for a review, see references 6, 17, 30). The initiating step for arrest at a preferred, secondary location is adhesion to endothelium which is believed to be mediated by tumor cell surface molecules that recognize components on the endothelial luminal surface of select vascular branches (2, 5, 12, 19–21, 26). This premise is supported by recent work in our laboratory detailing the isolation and characterization of the 90-kD lung-specific, melanoma cell-binding endothelial cell adhesion molecule Lu-ECAM-1 (37). Lu-ECAM-1 is constitutively expressed on endothelia of pleural and subpleural capillaries and venules and, to a lesser extent, other pulmonary venules and veins. Its expression in these blood vessels

correlates closely with the topographical distribution of B16-F10 melanoma lung metastases. Anti-Lu-ECAM-1 mAbs inhibit colonization of the lungs by lung-metastatic B16 melanoma cells, but have no effect on the colonization of the lungs by other types of lung-metastatic cancers (e.g., KLN205 squamous carcinoma cells) or on the number of liver colonies produced by liver-metastatic B16-L8-F10 melanoma cells (36, 37). This work supports earlier site-specific, tumor cell adhesion data in other laboratories using isolated microvascular endothelia from various organs. Although molecular details have not been revealed in these studies, cancer cells have been reported to preferentially bind to microvascular endothelium isolated from the metastasized organ (1, 3, 18, 27). For example, lung-metastatic B16-F10 melanoma cells and RAW117-L17 lymphoma cells adhere preferentially to monolayers of lung microvascular endothelial cells, while brain-metastatic glioma cells, liver-metastatic RAW117-H10 and MB6A lymphoma cells, and ovary-metastatic teratoma cells bind preferentially to brain-, liver-, and ovary-derived microvascular endothelia, respectively.

Address correspondence to Dr. Pauli at Cancer Biology Laboratories, Department of Pathology, Cornell University College of Veterinary Medicine, Ithaca, NY 14853.

Our objective in this study was to expand our initial work on melanoma lung metastasis (36, 37) and to investigate whether some of the more ubiquitous, epithelial-derived cancers that consistently metastasize to the lungs, i.e., breast and prostate cancers, recognize and adhere to cancer type-specific or common endothelial cell adhesion molecules of the lung vasculature (20). To achieve this goal, we relied on a method that circumvented the use of cumbersome cell isolation techniques in the identification of such endothelial cell adhesion molecules and in the performance of tumor cell/endothelial cell adhesion assays (11, 21). Outside-out membrane vesicles that are representative of luminal membranes of lung microvascular endothelium and that have been shown to preferentially bind to lung-metastatic cancer cells were obtained by perfusing the lungs with a low-strength formaldehyde solution as previously described (11). Endothelial membrane vesicles were then employed in the production of mAbs against lung endothelial determinants, using standard active or passive/active immunization protocols (35). We report here on a mAb that inhibits the selective adhesion of lung endothelial cell-derived vesicles to lung-metastatic breast and prostate carcinoma cells. This antibody is used to purify and characterize a lung endothelial cell surface glycoprotein, identified as dipeptidyl peptidase IV.

Materials and Methods

Cell Cultures

R3230AC rat mammary carcinoma cells (R3230AC-MET and R3230AC-LR) were obtained from Dr. J. A. Kellen, Sunnybrook Medical Center, University of Toronto, Toronto, Ontario, Canada (16, 23). The R3230AC-MET carcinoma cell line was selected *in vivo* for high lung colonization. The [Con A and WGA]-resistant variant R3230AC-LR was nonmetastatic. A high lung-metastatic rat prostate carcinoma cell line designated RPC-2 was isolated from the *in vivo* transplantable Dunning R3327 prostatic carcinoma MatLyLu obtained from Dr. J. T. Isaacs, Johns Hopkins Oncology Center, Baltimore, MD (10). The RPC-LR cell line was developed as a [Con A and WGA]-resistant variant of RPC-2 and was found to be nonmetastatic. All tumor cells were maintained in Rosewell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% heat-inactivated FBS (Gibco Laboratories, Grand Island, NY). Aortal endothelial cells were isolated and grown as described elsewhere (35, 37).

Preparation of Endothelial Cell Membrane Vesicles

Outside-out, luminal endothelial cell membrane vesicles were prepared from the microvasculature of rat lungs as reported earlier (11). In brief, 3-mo old, male Sprague-Dawley rats were injected intraperitoneally with 0.5 ml of 20% sodium citrate, sacrificed with an overdose of sodium phenobarbital, and prepared for immediate organ perfusion. Lung perfusates entered through the pulmonary artery and exited from the left heart atrium. The lung vascular bed was first flushed with PBS, pH 7.4, containing 1 mM CaCl₂ and 0.5 mM MgCl₂ (PBS-CM)¹ at a flow rate of 7 ml/min for 20 min at 37°C. Lungs were inflated periodically through the trachea during the washing procedure to improve removal of blood components. Flushing was followed by perfusion of the vasculature with 100 mM paraformaldehyde and 2 mM DTT in PBS-CM at a flow rate of 0.25 ml/min for 4 h at 37°C. Perfusates were centrifuged at 200 g to remove whole cell contaminants. Vesicles were then collected by high speed centrifugation (30,000 g; 1 h; 4°C). Vesicles were washed three times in PBS-CM containing 0.2 mM phenylmethyl-sulfonylfluoride and immediately used in the outlined experiments.

Control endothelial cell membrane vesicles were prepared in a similar fashion from the vasculature of rat hind legs. Leg perfusates entered through

the common iliac artery and exited via the iliac vein. Composition of the perfusion fluid, perfusion flow rate and time, and endothelial vesicle collection were as described above. The following reasons were behind the selection of hind leg endothelial membrane vesicles as controls in our experiments: (a) the hind leg vasculature provided a large endothelial luminal surface that allowed the harvest of large numbers of endothelial vesicles; (b) the hind leg vasculature was lined with continuous endothelium preventing edema formation and loss of endothelial vesicles into the interstitium during the perfusion process; (c) the hind leg was rarely metastasized by blood-borne cancer cells; and (d) there were little or no technical difficulties associated with the perfusion of the hind leg vasculature. Select experiments also included control endothelial cell membrane vesicles derived from monolayers of cultured aortic endothelium.

Iodination of Endothelial Cell Membrane Vesicles

Vesicle-associated membrane proteins were labeled by lactoperoxidase-catalyzed iodination, essentially as described by Soule et al. (31). Briefly, 200 μ l of 200 mM phosphate buffer, pH 7.3, containing 0.2 mCi Na¹²⁵I, 200 μ l of Enzymobead reagent (BioRad Laboratories, Richmond, CA), and 100 μ l of 1% β -D-glucose were added to 500 μ l (50 μ g protein/ml) of endothelial cell membrane vesicles in PBS-CM. Vesicles were incubated in this mixture for 30 min at room temperature. The reaction was stopped by removing Enzymobeads by centrifugation (100 g; 10 min; 4°C). Vesicles were washed four times (each wash 10 min) in PBS-CM to remove all unbound ¹²⁵I and suspended in 1 ml of PBS-CM.

Tumor Cell/Vesicle Binding Assay

R3230AC carcinoma cell variants were seeded into wells of 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) so that confluent monolayers of equal tumor cell numbers and surface area were present after 48 h of incubation at 37°C (11). Tumor cells were then washed with RPMI-1640 medium and nonspecific binding sites blocked by incubation with 0.4% BSA in RPMI-1640 medium for 30 min at 37°C. 40 ml of vesicle suspension ($\sim 2.5 \times 10^4$ vesicles yielding 100,000 cpm; 8.5×10^4 vesicles/cm² of tumor cell surface) were added to each of three wells and the plates centrifuged at 200 g for 5 min at room temperature. After 30 min of incubation at 37°C, cells were washed three times with medium, solubilized with 1% SDS in H₂O and counted in a gamma counter. The anchorage-independent rat prostate carcinoma (RPC) carcinoma cell variants were incubated with endothelial membrane vesicles in suspension. Briefly, RPC cells were washed first in RPMI-1640 medium supplemented with 0.4% BSA, then aliquots of 100 μ l containing 5×10^4 tumor cells were mixed with 70 μ l of ¹²⁵I-labeled vesicles to yield $\sim 8.5 \times 10^4$ vesicles/cm² of tumor cell surface (assuming that RPC cells are spheres of 18- μ m diam). The tumor cell/vesicle mixture was incubated for 30 min at 37°C. After removing unbound vesicles by gentle washing, tumor cells and bound vesicles were solubilized by adding 100 μ l of 1% SDS in H₂O and counted in a gamma counter. Data are presented as percentages of the total cpm in 40 μ l of vesicle suspension, or as relative percentages setting binding of lung-derived endothelial membrane vesicles to the high lung-metastatic tumor variants (R3230AC-MET; RPC-2) as 100%.

In binding inhibition experiments, mAb 8.6A3 was added to iodinated vesicles at the final concentrations of 100 μ g/ml and 10 μ g/ml. All antibody/vesicle mixtures including control vesicles without antibody were agitated overnight at 4°C. Vesicles were then washed three times in RPMI-1640 medium. The remainder of the assay was carried out as described above. mAb 7.3D5 which stained both rat lung and leg microvessels was used as a control antibody at the same concentrations as mAb 8.6A3.

Antibody Production

Active Immunization Procedure. Female Balb/c mice, 8–10-wk old, were immunized with vesicles obtained from perfused lungs. Each mouse was injected intraperitoneally with 0.25 ml of vesicle suspension (100 μ g protein) emulsified in complete Freund's adjuvant. Mice were reinoculated 4 wk later with 0.25 ml of the same antigen emulsified in incomplete Freund's adjuvant. 6 wk after the initial immunization and 3 d before fusion of mouse splenocytes with Sp2/O/Ag14 myeloma cells (American Type Culture Collection, Rockville, MD) the mouse with the highest titer for lung endothelial derived vesicles was injected intrasplenically with 0.1 ml of antigen (50–100 μ g/ml) in PBS. Fusion procedures, hypoxanthine-aminopterin-thymidine (HAT) medium preparation and selection, cloning of selected hybridomas, and immunoglobulin subclass determination were as described in detail elsewhere (34, 38).

1. **Abbreviations used in this paper:** PBS-CM, 1 mM CaCl₂ and 0.5 mM MgCl₂; RPC, rat prostate carcinoma.

Passive/Active Immunization Procedure. In an alternative immunization protocol, passive immunization of mice with mouse antiserum directed against endothelial membrane vesicles from perfused rat hind leg musculature preceded active immunization with vesicles derived from in situ perfused rat lungs (11). Antiserum was produced by injecting female Balb/c mice with a suspension of rat leg-derived endothelial membrane vesicles (150 μg protein) on weeks 0, 4, and 6 as described above (35). Antiserum that had a titer of at least 1:1,000 was then injected intravenously into a second set of Balb/c mice (100 μl per mouse). 5 min later, these mice were actively immunized with vesicles derived from rat lung endothelium emulsified in Freund's complete adjuvant. Subsequent inoculations with antigen were as described above.

Screening of Hybridoma Supernatants. Hybridoma supernatants were initially screened for relevant mAbs against lung-derived vesicles in an ELISA. Strongly positive supernatants were then further tested in differential ELISAs against both lung- and leg-derived vesicles (35). Vesicles (1 μg /well) were centrifuged at 2,500 g for 30 min onto poly-L-lysine (MW >300,000; 0.1 mg/ml; Sigma Chemical Co., St. Louis, MO) coated 96-well plates (Immobilon, Dynatech, Alexandria, VA). Wells were blocked with 0.2% gelatin in PBS for 1 h at room temperature. Washed vesicles were then incubated with 25 μl of hybridoma supernatant for 1 h at room temperature. Plates were washed three times with 0.1% gelatin in PBS and incubated for 1 h at room temperature with 50 μl of goat anti-mouse IgG F(ab')₂-peroxidase conjugate (Cappel Laboratories, Malvern, PA) diluted 1:1,000 in PBS containing 10% heat-inactivated normal goat serum (35). The plates were washed four times and incubated with 75 μl of substrate solution consisting of 4 mg ortho-phenylenediamine (Sigma Chemical Co.), 4.0 μl of 30% H₂O₂ in 10 ml of 0.1 M citrate buffer, pH 4.5, for 15 min at room temperature in the dark. The reaction was stopped by adding 50 μl of 2.5 M H₂SO₄ and immediately read at 490 nm in a MicroELISA plate reader (Bio-Tek Instruments, Winooski, VT).

Immunohistochemistry

Serial, 3- μm thick sections were prepared from normal rat organs perfused with 4% formaldehyde in PBS, pH 7.4, and embedded in paraffin. Deparaffinized sections were stained by an indirect immunoperoxidase technique utilizing a histostain kit from Zymed Labs, Inc. (South San Francisco, CA). Briefly, deparaffinized tissue sections were treated with 0.3% H₂O₂ in methanol for 15 min at 4°C, then blocked with 10% normal rabbit serum for 10 min at room temperature. Sections were incubated with primary antibody (hybridoma supernatant) for 30 min at 37°C, followed by incubations with biotinylated rabbit anti-mouse Ig (1:1000 in PBS) and streptavidin-peroxidase conjugate, each for 10 min at room temperature (37). Antibody binding was visualized by adding the peroxidase substrate 3-amino-9-ethylcarbazole. Between each of these steps and after the development of color, sections were washed three times with PBS, 5 min each. Stained tissue sections were examined with a light microscope. Control sections were stained in the absence of primary antibody (hybridoma supernatant).

Immunoaffinity Purification

40 rat lungs were homogenized (Polytron^R, Kinematica, Brinkman Instruments, Westbury, NY) and washed extensively by centrifugation until the supernatant was clear (free of blood). Homogenates were extracted overnight at 4°C with 60 ml of lysis buffer: 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM benzamidine chloride, 1 mM PMSF, 30 μg /ml DNase, 2 μg /ml leupeptin, 0.27 TIU/ml aprotinin, 1% NP-40, pH 7.4. The lysates were first prepared on a 1-ml column containing nonimmune mouse IgG immobilized on protein G-Sepharose 4FF, then directly applied onto a second 1-ml column of mAb 8.6A3 coupled to protein G-Sepharose 4FF (37). Both columns had been previously equilibrated with lysis buffer and were run at a flow rate of 0.5 ml/min. The second column was washed with 30 ml of each of the following: (a) lysis buffer; (b) 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.2% NP-40; (c) 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40; (d) 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 0.1% SDS, 0.5% deoxycholate; and (e) 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, with 10 mM CHAPS (Sigma Chemical Co.). Bound molecules were eluted with 200 mM glycine, pH 2.8, 150 mM NaCl, 10 mM CHAPS. Fractions of 1 ml were collected into tubes containing 0.1 vol of 1 M Tris, pH 11 (to yield a final pH of 8.0). Fractions were analyzed by SDS-PAGE (8% polyacrylamide) and visualized by silver staining.

Purified sample was electrophoresed in 8.0% polyacrylamide. The protein was electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA) in 20% methanol transfer buffer

at 300 A for 1 h. The transfer was confirmed with Coomassie blue stain after destaining with 10% acetic acid. Automated Edman degradation of protein was performed using an Applied Biosystems Inc. (model 470A) gas-phase sequencer with an on-line (model 120A) phenylthiohydantoin derivative analyzer. The amino acid sequence was compared using FASTA software and the SWISS-PROT database (24).

Scanning Electron Microscopy

R3230AC-MET and R3230AC-LR carcinoma cells were grown on Thermanox^R coverslips (Lux Scientific Corp., Thousand Oaks, CA) placed into wells of 96-well plates and a binding assay was performed with lung-derived endothelial cell membrane vesicles as described above (11). Wells were washed three times with medium and once with 100 mM cacodylate buffer. Cells were fixed with 2% glutaraldehyde in 100 mM cacodylate buffer, pH 7.3, dehydrated in graded ethanol solutions, then critical point dried in a Polaron Jumbo critical point dryer (Polaron, Watford, England). Coverslips containing the critical point dried cells were attached to metal stubs with silver paint and sputter-coated with a thin layer of carbon in an Edwards evaporator (Manor Royal, Crawley, Sussex, England). Specimens were examined in a Jeol SEM 35CF scanning electron microscope.

Binding of mAb 8.6A3 to the surface of lung-derived endothelial membrane vesicles was visualized by a scanning electron microscopic immunogold technique as described in detail by Johnson et al. (11). In brief, endothelial vesicles immobilized on Thermanox^R coverslips were incubated with mAb 8.6A3 (hybridoma supernatant) for 1 h at room temperature, then washed four times with RPMI-1640 and incubated for 30 min with goat anti-mouse IgG conjugated to 30 nm gold particles (Vector Laboratories, Burlingame, CA) diluted 1:10 in RPMI-1640 containing 10% heat-inactivated, normal goat serum. After washing, vesicles were processed for scanning electron microscope examination as described above.

Results

Attachment of Lung Endothelial Cell Vesicle to Lung Metastatic Cancer Cells

Tumor cells with high lung-metastatic potential were tested for their capacity to bind lung- and leg-derived endothelial cell membrane vesicles. Tumor cells selected in vivo for high lung colonization (R3230AC-MET; RPC-2) bound significantly more lung endothelial vesicles than their nonmetastatic counterparts (R3230AC-LR; RPC-LR). At an approximate concentration of 8.5×10^4 vesicles/cm² of tumor cell surface, lung-metastatic R3230AC-MET bound $14.0 \pm 1.9\%$ and RPC-2 $52.6 \pm 5.3\%$ of the lung-derived endothelial cell membrane vesicles, whereas the nonmetastatic R3230AC-LR and RPC-LR only bound $6.5 \pm 1.0\%$ and $36.0 \pm 3.7\%$, respectively (Fig. 1, A and B). Control leg musculature-derived endothelial cell membrane vesicles adhered to lung-metastatic and nonmetastatic cells in comparable numbers. R3230AC-MET and R3230AC-LR bound $7.1 \pm 1.2\%$ and $5.8 \pm 1.1\%$ leg vesicles, whereas RPC-2 and RPC-LR bound $27.1 \pm 2.7\%$ and $33.4 \pm 3.7\%$, respectively. Vesicles derived from cultured aortal endothelial cells bound in equal numbers to R3230AC-MET (6.5 ± 1.0) and R3230AC-LR (5.8 ± 0.8) carcinoma cells as leg-derived vesicles (Fig. 1 A). Vesicle binding results correlated well with the ability of breast and prostate tumor cell lines to produce lung colonies after tail vein inoculation (11, 23). Rat R3230AC-MET mammary carcinoma cells and RPC-2 prostatic carcinoma cells both produced numerous lung colonies 3 wk after tail vein inoculation of eight rats/tumor cell line with 1×10^6 tumor cells/rat. R3230AC-MET carcinoma cells generated 204 (176–231) colonies, whereas the prostatic carcinoma cell line formed 347 (168→400) colonies. In contrast, the lectin resistant cell variants R3230AC-LR and RPC-LR were unable to form experimental lung metastases.

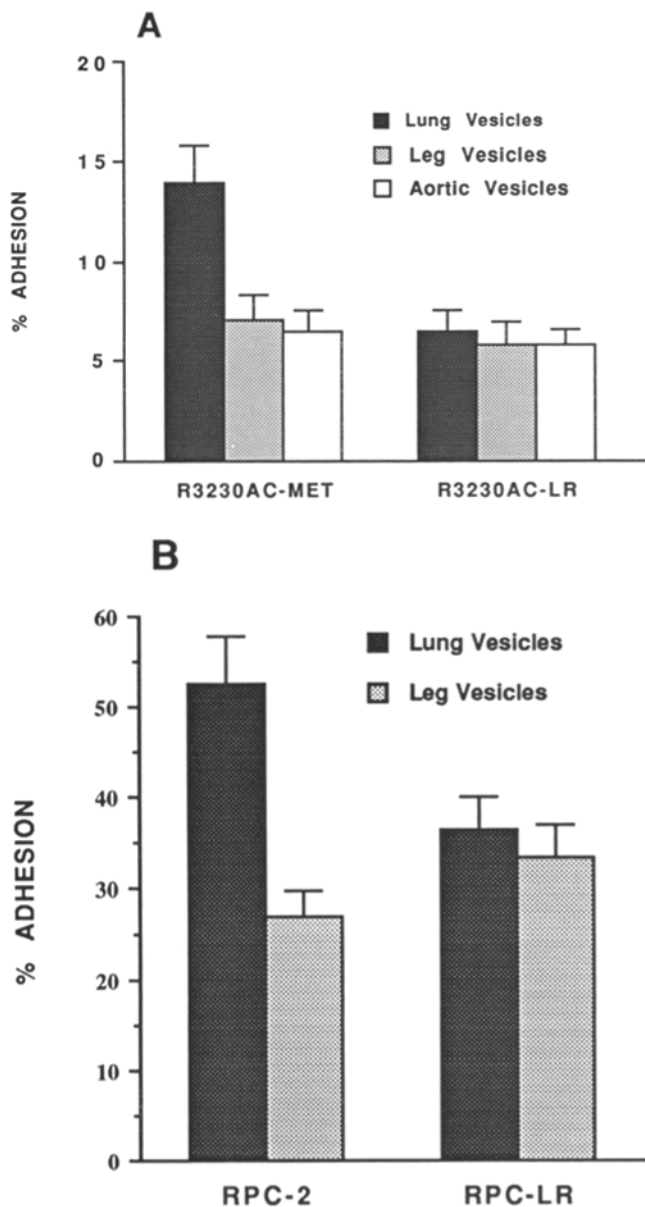


Figure 1. Preferential attachment of lung-derived endothelial cell membrane vesicles to lung-metastatic tumor cells. Lung-metastatic rat R3230AC-MET breast (A) and RPC-2 prostate (B) carcinoma cells selected for high lung colonization bind significantly more lung-derived endothelial membrane vesicles (dark bars) than their nonmetastatic counterparts R3230AC-LR and RPC-LR. In contrast, neither of the four tumor cell lines shows any binding preference for leg-derived endothelial membrane vesicles (light bars). The graphs represent means and standard deviations from six (A) and two (B) separate experiments with triplicate determinations in each experiment. Binding of lung endothelium-derived membrane vesicle to low and high metastatic tumor cells are compared by *t* test: [R3230AC-MET vs R3230AC-LR and RPC-2 vs RPC-LR; $p < .001$].

The preferential binding of lung-derived endothelial vesicles to lung-metastatic R3230AC-MET carcinoma cells was confirmed by scanning electron microscopy. R3230AC-MET carcinoma cells often bound several lung-derived vesicles (Fig. 2 A). Vesicle binding occurred throughout the exposed

tumor cell surface and was often mediated by microvilli (Fig. 2 B). A slight binding preference was observed for the marginal zones of R3230AC-MET cells. In contrast, non-metastatic R3230AC-LR carcinoma cells bound few, if any, lung-derived endothelial vesicles (Fig. 2 C). Endothelial vesicles were easily distinguished from occasional tumor cell membrane plebs with immunogold staining using mAb 8.6A3 (Fig. 2 D).

Generation and Selection of mAbs against Lung-derived Endothelial Cell Adhesion Molecule

Immunization and Initial Selection. Two fusions were performed, the first following standard, active immunization, the second a passive/active immunization protocol. Hybridoma clones developed in 404 wells of the two fusions (35%) (Table I). Of these, 81 (20%) were positive for lung-vesicle binding with the majority of these clones being derived from the passive/active protocol. 13 of the 81 clones that were positive for lung vesicle binding were also ELISA-positive for leg vesicle binding. The majority of these 13 clones were from the active immunization protocol. In the final analysis, 68 of all clones (16.8%) were positive for lung-vesicle binding only and the majority of these (49) were acquired through the passive/active procedure. This finding indicates that the latter is the most efficient protocol in obtaining antibodies which are specific to endothelial cells in a given tissue.

Secondary Selection by Immunohistochemistry. Supernatants with the highest ELISA absorbance ratios (≥ 10) of lung/leg vesicle binding were further selected by the staining of 3 μm thick sections from perfusion-fixed rat lungs and leg muscle (Table II). A total of 17 clones were analyzed, 11 from the active immunization group, 6 from the passive/active immunization group. Seven of the hybridoma supernatants stained lung capillary endothelial cells. Four of these were from the passive/active protocol (Table II). Selective staining of lung capillary endothelium was observed with supernatants 7.5E6, 8.4B7, 8.5F6, and 8.6A3. Supernatant 8.6A3 revealed the strongest staining reaction (Fig. 3 A), while supernatants 7.5E6 yielded mild to moderate and 8.4B7 and 8.5F6 mild staining reactions. Arteries, arterioles, and venules were not stained by these supernatants nor were bronchial epithelia, pneumocytes, and smooth muscle cells. Furthermore, these supernatants failed to stain vascular endothelium of the hind leg musculature. Supernatants 7.6B7 and 8.3E1 stained lung capillary endothelium in addition to bronchial epithelium, pneumocytes, and smooth muscle cells. Finally, supernatant 7.3D5 stained strongly lung capillary endothelium and weakly capillary endothelium of hind leg musculature, but not endothelia of any other vessel calibers. The staining reaction of 7.3D5 with lung capillary endothelium was of similar intensity as that of 8.6A3 (Fig. 3 B). The relatively high number of mAbs that stained neither lungs nor leg muscle appears to be related to denaturation of antigens during tissue fixation and processing.

Final Selection in Tumor Cell/Endothelial Vesicle Binding Inhibition Assay. Hybridoma cells producing supernatants which unequivocally stained rat lung endothelium (7.3F3; 8.4B7; 8.5F6; and 8.6A3) were cloned by limiting dilution before subjection to a final selection in a tumor cell/endothelial vesicle binding inhibition assay. mAb 8.6A3

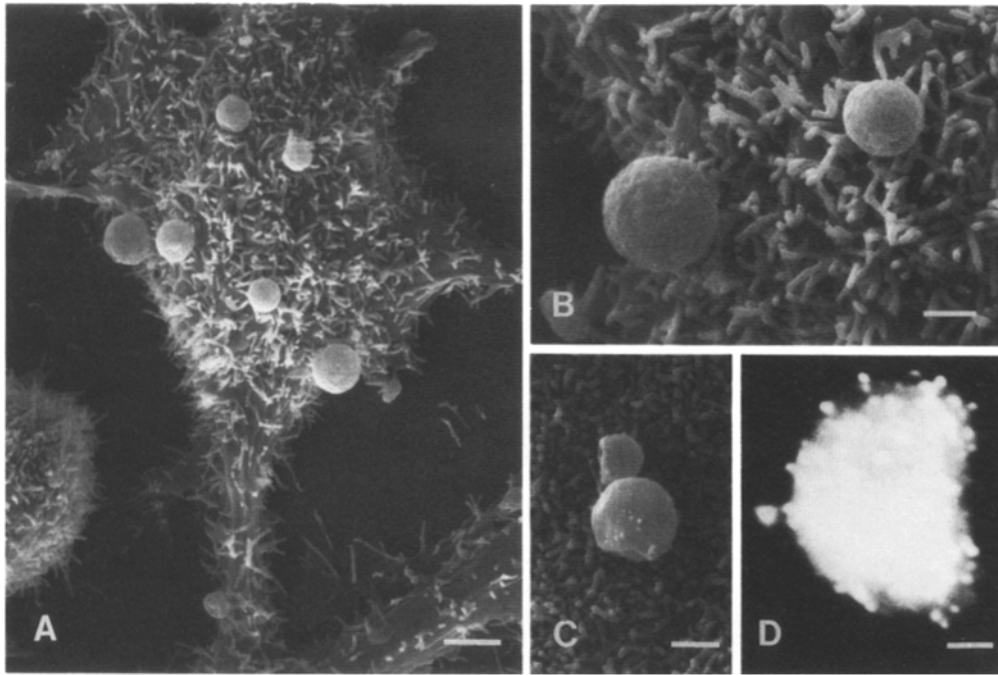


Figure 2. Scanning electron micrograph depicting endothelial membrane vesicles binding to R3230AC breast carcinoma cells. (A) Lung-metastatic R3230AC-MET carcinoma cell exhibits numerous bound lung-derived endothelial membrane vesicles. (B) Vesicle binding appears to be mediated by microvilli. (C) R3230AC-LR carcinoma cell shows only an occasionally bound lung-derived endothelial membrane vesicle. (D) Binding sites for mAb 8.6A3 on lung-derived endothelial cell membrane vesicle are visualized by immunogold staining. Bars: (A and D) 2 μ m; (B and C) 1 μ m.

(10 μ g/ml) caused a significant reduction in the binding of lung endothelial vesicles to lung-metastatic R3230AC-MET breast carcinoma cells (36% vesicle binding inhibition) and RPC-2 prostate carcinoma cells (34%) (Fig. 4). The blocking extent was to a level comparable with the binding of lung-derived vesicles to the nonmetastatic cell lines R3230AC-LR and RPC-LR. A concentration of 100 μ g/ml of mAb 8.6A3 did not cause further reduction in binding. There was a moderate reduction of lung vesicle binding to the non-metastatic rat mammary carcinoma cell line R3230AC-LR. The mAb had no significant effect on leg vesicle adhesion to either of the four cell lines. Flow cytometric analysis of lung endothelial vesicles incubated with mAb 8.6A3 revealed labeling of 60% of the vesicles indicating that the vesicles were reflective of the microvascular luminal surface in the lung (11). mAbs 7.3F3, 8.4B7, and 8.5F6 failed to cause significant reduction in selective adhesion (data not shown). Control mAb 7.3D5, which had been shown to stain lung capillary endothelium with similar intensity as 8.6A3 had no effect on the binding of lung- or leg-derived endothelial vesicles to either lung-metastatic or non-metastatic breast and prostate carcinoma cell variants (Fig. 4).

Immunohistochemistry with mAb 8.6A3

Tissue distribution of the lung capillary endothelial cell

Table I. Generation of mAbs Against Lung-derived Endothelial Cell Luminal Membrane Vesicles

Immunization	Growth per 576 wells	ELISA Positive		Lung vesicle hybridoma
		Lung-V	Leg-V	
Active	215	30	11	19
Passive/active	189	51	2	49
Totals	404	81	13	68

adhesion molecule was performed by immunohistochemistry using mAb 8.6A3. Stained tissues were the lungs, brain, heart, small intestine, kidney, liver, skin, spleen, lymph node, and thymus. In addition to lung capillaries, the antibody bound endothelia of medium-size to large splenic venules (Fig. 5 A) and the vasa recta of the kidney medulla (Fig. 5 B). There was no staining of endothelium in the remaining organs. However, the antibody also stained bile canaliculi in

Table II. Selection of Lung-specific mAbs

Immunization protocol	Hybrid No.	Absorbance at 490 nm		Ratio Lung-V/leg-V	Rat Lung/leg*	
		Lung-V	Leg-V			
Active	7.1B2	2.251	0.005	450	‡	
	7.2B9	1.559	0.056	28	—	
	7.3D5	1.331	0.015	89	Ec,L‡	
	7.4G2	1.484	0.002	742	—	
	7.6B7	2.085	0.011	189	Ec,B,P,S	
	7.6C10	1.717	0.015	114	—	
	7.2F4	1.622	0.009	180	—	
	7.2F8	2.123	0.018	118	—	
	7.2H11	2.344	0.005	469	—	
	7.3F3	2.387	0.032	75	Ec	
	7.5E6	1.804	0.003	601	—	
	Passive/active	8.3E1	2.511	0.010	251	Ec,Ev,S,B
		8.4A8	1.163	0.014	83	—
8.4B7		1.725	0.028	62	Ec	
8.4F10		1.753	0.057	31	—	
8.5F6		0.574	0.056	10	Ec	
8.6A3		1.784	0.020	89	Ec	

* Immunohistochemical staining of rat lung and skeletal muscle tissue sections.

‡ Negative staining reaction.

§ Positive staining reaction: Ec, lung capillary endothelia; Ev, lung venule endothelia; L, leg small venule/arteriolar endothelia; B, bronchial epithelia; P, pneumocytes; S, smooth muscle.

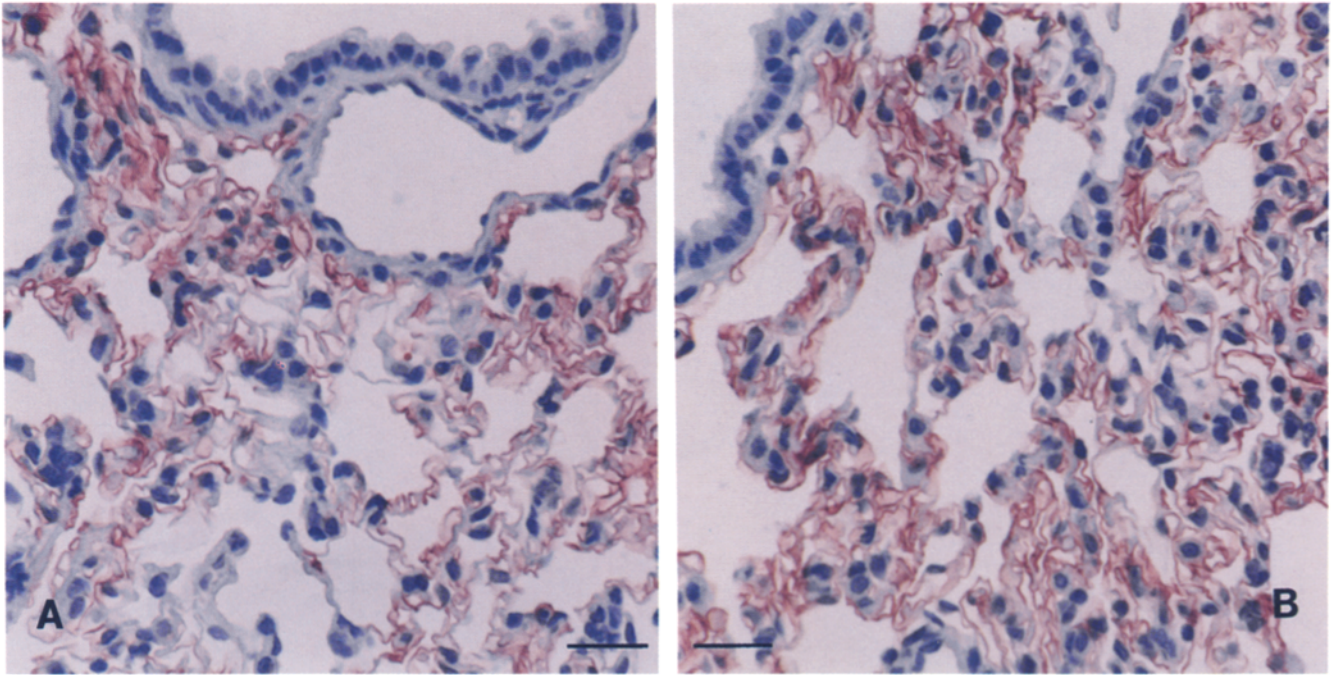


Figure 3. Indirect immunoperoxidase staining of rat lungs with mAb 8.6A3 (A) and mAb 7.3D5 (B): Both antibodies stain lung capillary endothelia, but only mAb 7.3D5 also stains capillary endothelia from hind leg musculature. No staining reaction is observed on endothelia of other lung and leg vessels and tissue components. The magnification is 480 \times .

the liver (Fig. 5 C) and the brush borders of kidney tubular epithelia (Fig. 5 D) and small intestinal villi. No staining of any structures was observed in the remaining organs. Control slides lacking primary antibody were negative.

Antigen Purification and NH₂-terminal Sequencing

mAb 8.6A3 was used to purify the endothelial cell adhesion molecule by immunoaffinity chromatography of detergent

extracts from washed, homogenized rat lungs. Evaluation of the sequential fractions eluted from the column by acidic solutions disclosed a protein with a relative mobility on SDS-PAGE of 110 kD under reducing and nonreducing conditions (Fig. 6). Although the elution buffer contained detergent, the protein exhibited a tendency to aggregate under nonreducing conditions, a typical characteristic of many membrane proteins. NH₂-terminal sequencing of 30 amino acids of the purified protein revealed 100% identity with the integral

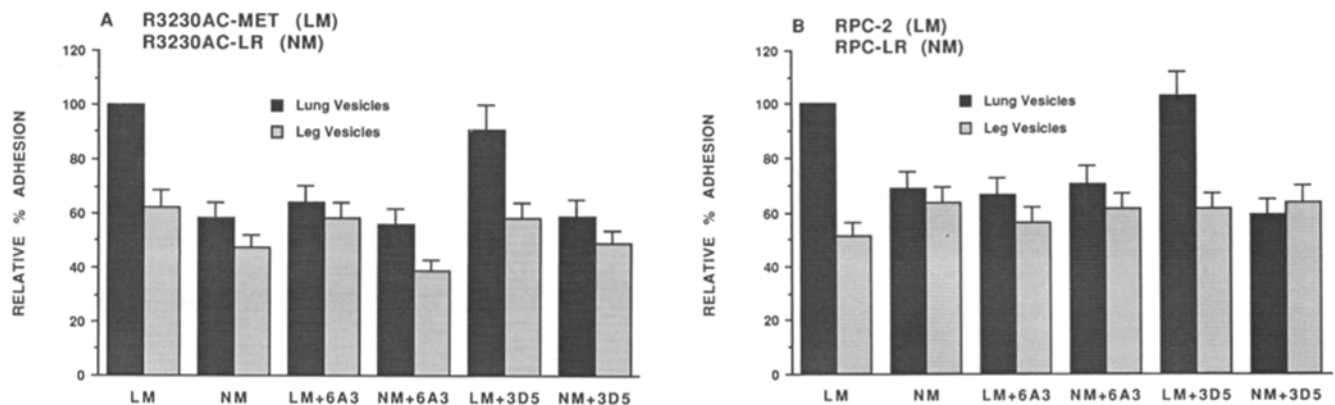


Figure 4. Inhibition of preferential attachment of lung-derived endothelial membrane vesicles to lung-metastatic breast (A) and prostate (B) carcinoma cells using mAb 8.6A3. mAb 8.6A3 (6A3) reduces adhesion of lung-derived endothelial membrane vesicles (dark bars) to lung-metastatic R3230AC-MET and RPC-2 carcinoma cells to similar levels as “unspecific” (leg-derived) endothelial membrane vesicles bind to these cell lines. Control mAb 7.3D5 (3D5) has no effect on endothelial membrane vesicle binding to lung-metastatic carcinoma cells. Leg-derived endothelial membrane vesicles bind high lung-metastatic and nonmetastatic breast and prostate carcinoma cells in similar numbers. mAbs 6A3 and 7D5 have no significant effect on the binding of leg-derived vesicles to either of the four cell lines, with the possible exception of a further reduction in the binding of these vesicles to RPC-LR by mAb 6A3. The graphs represent means and standard deviations from five (A) and two (B) separate experiments with triplicate determinations in each experiment. Binding of lung-derived endothelial membrane vesicles to high metastatic breast and prostate carcinoma cells and binding after incubation with mAb 6A3 are compared by *t* test: [LM vs LM + 6A3: $p < .01$].

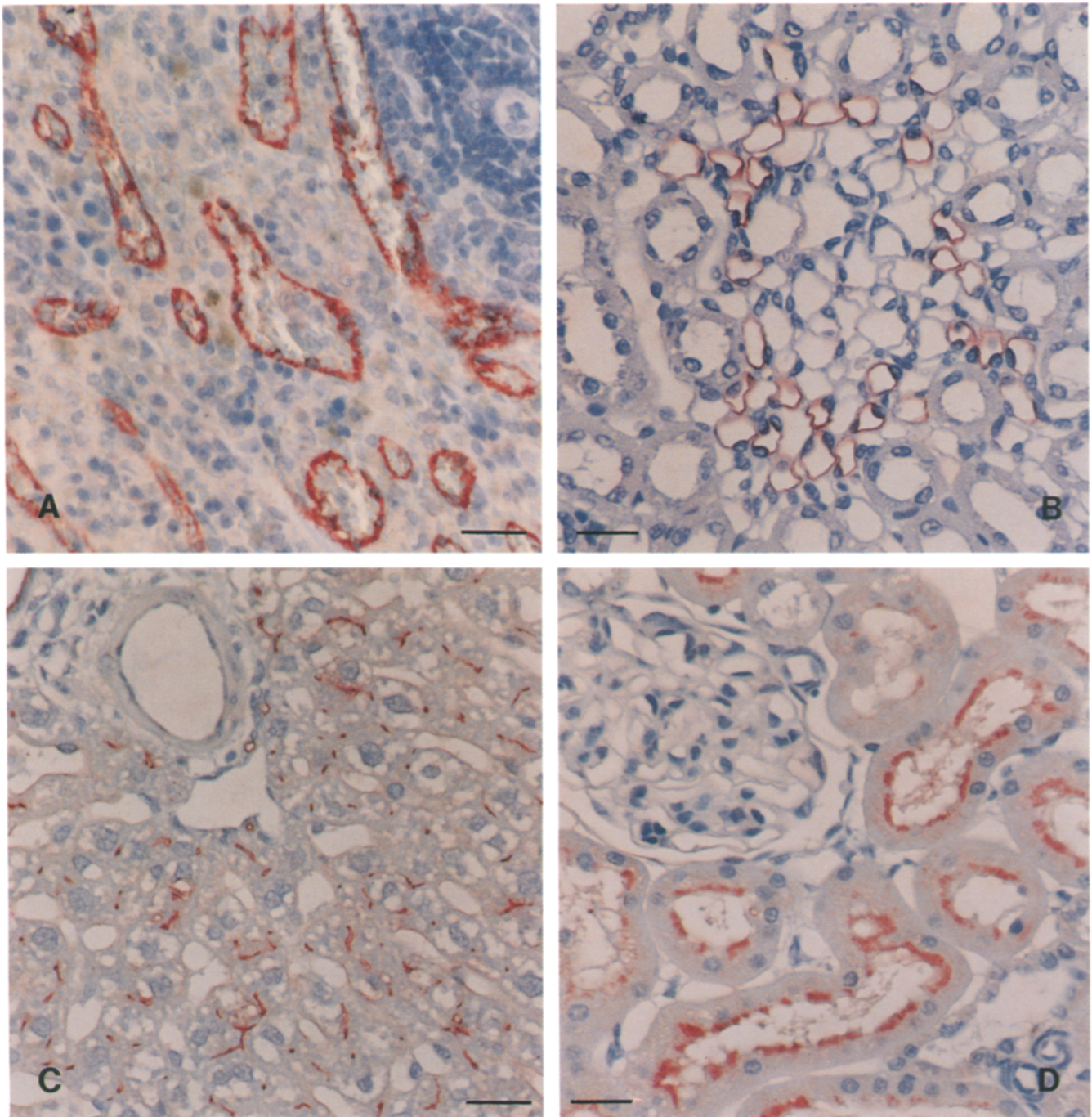


Figure 5. Indirect immunoperoxidase staining of rat tissues with mAb 8.6A3. Positive staining is observed on the surface of endothelia of medium-size to large splenic venules (*A*), endothelia of the vasa recta of the renal medulla (*B*), bile canaliculi (*C*), and kidney proximal tubular epithelium (*D*). Bar, 20 μm .

membrane glycoprotein dipeptidyl peptidase IV (DPP IV). The results described herein suggest a previously unrecognized role for this molecule.

Discussion

Outside-out vesicles derived from the luminal membrane of lung microvascular endothelium were used here as tools in identifying and, eventually, purifying an endothelial cell adhesion molecule that promotes binding of lung-metastatic

breast and prostate carcinoma cells. Our strategy was to first produce mAbs against membrane vesicles shown by ultrastructural immunocytochemistry to provide perfect representatives of lung microvascular endothelium in vivo (11, 15, 28). Both active and passive/active immunization schedules provided mAbs that were directed against endothelial cell surface molecules of select vascular compartments of the lungs. However, the efficiency with which such antibodies were generated was greater in the fusion group that was immunized by the passive/active protocol. mAbs that highlighted

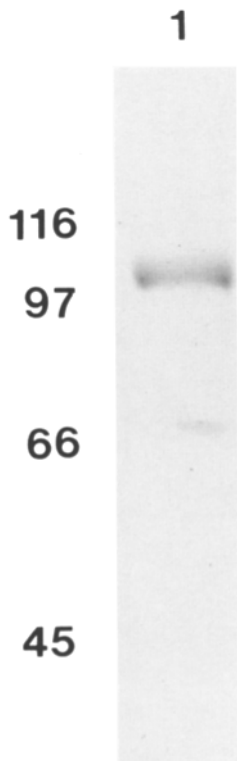


Figure 6. Silver stained SDS-polyacrylamide gel (8%; nonreducing) shows affinity purified 110 kD DPP IV isolated from detergent-extracts of rat lungs.

lung capillary endothelia were endothelial- and organ-specific, but only with respect to the two organs from which endothelial membrane vesicles had been derived, namely hind leg musculature and lungs. Further testing by immunohistochemistry revealed that these antibodies were neither endothelial- nor organ-specific, although staining was restricted to distinct tissue and vessel compartments. These results are in agreement with those reported by Auerbach et al. (4), who employed cultured microvascular endothelial cells isolated from various organs including the lungs as immunogens in the generation of mAbs against endothelial cell surface molecules.

The distribution of the endothelial cell determinants that were recognized by mAbs generated against endothelial membrane vesicles obtained by *in situ* lung perfusion was quite different from that previously observed with mAbs raised against endothelial cell membrane vesicles derived from organ-specifically modulated aortal endothelial cells (22, 35). While the present set of mAbs was almost exclusively directed against endothelial cell epitopes that were expressed in lung capillaries, mAbs raised against vesicles from lung matrix-modulated endothelial cells almost always identified epitopes on venular endothelia (35, 37). This finding might be explained by the predominance of capillary endothelium-derived vesicles in standard *in situ* vesicle preparations, since the capillary bed provides by far the largest luminal surface area of the lung vasculature (32). In contrast, modulation for lung specificity of vascular endothelium by lung matrix-derived extracts delivered mostly mAbs that were directed against endothelial cell epitopes of blood vessels in which the endothelium was readily exposed to pockets of organ-specific matrix, *i.e.*, venules (35). In this context, it

is also noteworthy that neither in the present nor in the previous series of fusion experiments conducted in our laboratory, we were able to produce mAbs that recognized endothelial cell epitopes displayed on the arterial segment of the lung vasculature (35). For the present study, this can again be explained by an underrepresentation of endothelial cell vesicles derived from the arterial segment of the lung vasculature in standard *in situ* lung vesicle preparations. Similar arguments can be made when matrix-modulated endothelial cells are used for the production of mAbs. The arterial matrix constitutes only a minor part of lung-derived extracellular matrix, making an induction of the arterial endothelial cell phenotype difficult, if not impossible. In summary, the methodologies established in our laboratories for the generation of mAbs against organ-specific endothelial cell surface epitopes provide unique instruments with which we have been able to generate antibodies that are directed either against endothelia from the capillary network or against endothelia from the venular branches of the organ vasculature.

The mAb (8.6A3) that inhibited adhesion of lung-derived endothelial membrane vesicles to lung-metastatic R3230AC-MET breast and RPC-2 prostate carcinoma cells was found to be directed against a unique lung endothelial cell adhesion molecule, identified as dipeptidyl peptidase IV (DPP IV). While this sialoglycoprotein has been extensively investigated with respect to its enzymatic activity (14), little has been published on its adhesion properties though it is recognized as a fibronectin-binding protein (8, 24). Preliminary work in our laboratory indicates that this binding property of DPP IV may be responsible for the attachment of lung-metastatic rat R3230AC-MET breast and RPC-2 prostate carcinoma cells (20). This hypothesis is supported by the finding that the lung-metastatic carcinoma cell lines R3230AC-MET and RPC-2 contain significantly higher numbers of cells that are decorated with fibronectin than their nonmetastatic (Con A and WGA)-resistant counterparts R3230AC-LR and RPC-LR. It appears that fibronectin is bound to and stored on cancer cell surfaces, at least in part, by adhering to β_1 and β_3 integrins, both significantly upregulated on lung-metastatic relative to nonmetastatic tumor variants (Johnson, R. C., and B. U. Pauli, manuscript in preparation). These preliminary observations suggest that tumor cell surface-associated fibronectin may serve as an intermediary adhesion molecule between tumor cell integrins and capillary endothelium DPP IV.

The expression of DPP IV on lung capillary endothelia as well as on endothelia of the renal vasa recta and splenic venules (9, 13) contrasts with the selective colonization of the lungs by R3230AC-MET breast and RPC-2 prostate carcinoma cells. Although this apparent discrepancy raises questions concerning the proposed involvement of DPP IV in lung metastasis, we believe our data merely reinforce an "old" concept that successfully metastatic tumor cells must complete all steps of the metastatic cascade in order to give rise to secondary tumor growth. Thus, lack of metastases in kidney and spleen may well be related to "infertility" of the kidney and spleen microenvironments in promoting growth of extravasated tumor cells to noticeable colonies (for a review, see references 6, 17, 30, 33). Alternatively, tumor cells inoculated via the tail vein may not reach spleen and kidney

in sufficient numbers to generate experimental metastases in these organs. This hypothesis draws support from observations in the mouse where tail vein inoculation of 2×10^5 radiolabeled, lung-metastatic B16-F10 melanoma cells results in the dissemination of a maximum of 2,000 and 3,000 cells to either of these two organs, while the lungs are showered with a cell number that is close to that of the inoculum (7, 36).

Data published by our laboratory and those of others suggest that expression of constitutive endothelial cell adhesion molecules is exclusive in its distribution, occurring only on endothelia of select vascular branches in distinct organ sites (5, 20, 37). These adhesion molecules are recognized by blood-borne cancer cells to promote arrest and subsequent extravasation at the secondary target organ. The application of in situ-derived endothelial cell membrane vesicles for the identification of such compartment-specific vascular molecules avoids the problems encountered with cultured, organ-derived microvascular endothelial cells and, thus, adds a new and powerful dimension to our pursuit of elucidating the role of endothelial cell adhesion molecules in site-specific metastasis. However, at the present writing this system has only been characterized to allow the systematic analysis of lung-derived endothelial cell adhesion molecules in metastasis of the lungs, the sites most frequently targeted by blood-borne cancer cells. Attempts to include the liver in such studies so far have failed due to massive edema and accompanying loss of endothelial vesicle across the discontinuous endothelium of the liver sinusoids. Large scale perfusion experiments are currently in progress to explore the usefulness of various polysaccharide and protein additives to the perfusion fluid in order to better control edema formation and vesicle loss. Whether this system can be further expanded to include organs with endarterial blood supply such as brain and kidney remains to be seen. Initial perfusion experiments seem to indicate that endothelial vesicle harvest from such organs is satisfactory only during the early, less productive perfusion phase, but ceases as continued perfusion with the aldehyde solution leads to increasing vascular and tissue rigidity and associated occlusion of capillary lumina with vesicle clusters.

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