Identification of a Ca²⁺-dependent Cell–Cell Adhesion Molecule In Endothelial Cells

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Abstract. Confluent cultures of aortic endothelial cells contain two different cell–cell adhesion mechanisms distinguished by their requirement for calcium during trypsinization and adhesion. A hybridoma clone was isolated producing a monoclonal antibody Ec6C10, which inhibits Ca²⁺-dependent adhesion of endothelial cells. There was no inhibition of Ca²⁺-independent adhesion of endothelial cells and only a minor effect on Ca²⁺-dependent adhesion of smooth muscle cells. Immunoblotting analysis shows that the antibody Ec6C10 recognizes a protein in endothelial but not epithelial cells with an apparent molecular weight of 135,000 in reducing conditions and 130,000 in nonreducing conditions. Monoclonal antibody Ec6C10 reacts with an antigen at the cell surface as shown by indirect immunofluorescence of confluent endothelial cells in a junctional pattern outlining the cobblestone morphology of the monolayer. Removal of extracellular calcium increased the susceptibility of the antigen recognized by antibody Ec6C10 to proteolysis by trypsin. The role of the Ca²⁺-dependent cell adhesion molecule in organization of the dense peripheral microfilament band in confluent endothelial cells was examined by adjusting the level of extracellular calcium to modulate cell–cell contact. Addition of the monoclonal antibody Ec6C10 at the time of the calcium switch inhibited the extent of formation of the peripheral F-actin band. These results suggest an association between cell–cell contact and the peripheral F-actin band potentially through the Ca²⁺-dependent CAM.

THE vasculature originates from the mesodermal germ layer with the differentiation of angioblasts into endothelial cells (8, 33, 52). Initially, these cells are separate from one another implying that some mechanism exists for association of individual cells into vessels. Once the primitive vasculature is formed, the network of blood vessels grows by extension and interconnection to form a network of continuous channels (1). Maintenance of a continuous morphology during the formation and regeneration of the complex vascular network implies the presence of some kind of specific intercellular adhesive mechanism. Molecules of both the calcium-dependent and calcium-independent cell adhesion mechanisms (CAMs)[1] have recently been identified in various cell types, other than vascular endothelial cells and have been implicated in embryonic development in construction of tissues and in maintenance of cellular integrity (10, 43). The Ca²⁺-dependent CAMs include the liver cell adhesion molecule (L-CAM) (16), uvomorulin (35, 49), E-cadherin (56), the cell adhesion molecule 120/80 (9), A-CAM (50), N-cadherin (19), and P-cadherin (31). The similarity in their biochemical characteristics and tissue distribution has demonstrated that they represent a family of related proteins (10, 43).

The type of intercellular junction present in the endothelium of different vessels determines the permeability properties of the vasculature (40). The junctional complex in the endothelium of the aorta has been shown to be a composite of occluding junctions with gap junctions (26). Ultrastructural studies of cell junctions in epithelial cells have localized uvomorulin (4) and A-CAM (50) to distinct plaque-like structures of adherens junctions, which are associated with F-actin filaments. Although no plaque structure is found in endothelium, the cytoplasmic aspect of endothelial junctions contain a dense peripheral band of actin microfilaments associated with vinculin (54) and plakoglobin (12). Each of these proteins has also been found to be associated with the adherens junction or desmosome structures of epithelial cells (12, 55). Cultured aortic endothelial cells show both Ca²⁺-dependent and Ca²⁺-independent intercellular adhesion mechanisms. The present study is concerned with comparison of the cell surface Ca²⁺-dependent cell adhesion molecule of aortic endothelial cells to those described in other tissues. The Ca²⁺-dependent CAM in the endothelium shows similarities to other Ca²⁺-dependent adhesion molecules of the cadherin family, but appears to be uniquely present in endothelium.

A preliminary account of this work has appeared in abstract form (Heimark, R. L., G. Misselbeck, and S. M. Schwartz. 1986. *J. Cell Biol.* 103:195a [Abstr.]).

1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cells; CAM, cell adhesion molecule; CMF/PBS, Ca²⁺/Mg²⁺–free PBS; CMPS, Ca²⁺/Mg²⁺–free Hepes-buffered saline; MDBK, Madin-Darby bovine kidney.

Materials and Methods

Cells and Cell Culture

Endothelial cells were isolated from either adult or fetal (48-cm crown to rump) bovine aortas and grown in culture (passage 3-12) as previously described in Waymouth's complete medium containing 10% heat inactivated FBS (15). For certain experiments endothelial cells were grown on cell culture plates coated with a collagen film that was prepared as described by the manufacturer (Collagen Corporation, Palo Alto, CA). Smooth muscle cells were isolated from explants of fetal bovine aorta and were used between passage 2 and 7 (37). Sp2/0-Ag14 myeloma cells, hybridoma ACT I and anti-*Dictyostelium* actin (IgG₁), F9 mouse embryonal carcinoma cells, Madin-Darby bovine kidney cells (MDBK) and MDCK were obtained from American Type Culture Collection.

Preparation of Polyclonal and Monoclonal Antibodies

Antisera to endothelial cell surface antigens were produced in rabbits by alternate intravenous and subcutaneous injections every 2 wk of cells (2 × 10⁷) harvested gently with a cell scraper. Immunoglobulin and Fab' fragments were prepared as described by Brackenbury et al. (5).

Monoclonal antibodies were produced by the method of Kennett et al. (23). BALB/c mice were immunized intraperitoneally with scraped confluent endothelial cells (10⁷). Immune spleens were removed and fused with Sp2/0-Ag14 myeloma cells with PEG 1450 (Eastman Kodak Co., Rochester, NY). The fused cells were selected in 96-well culture plates in Iscove's DME containing 15% FBS (Hyclone Laboratories, Logan, UT) supplemented with hypoxanthine/aminopterin/thymidine. Hybridomas were screened for the production of antibodies in a sequential series of assays described below. After screening, selected hybridomas were subcloned twice by limiting dilution with BALB/c peritoneal macrophages and grown in ascites in pristane-primed BALB/c mice. Immunoglobulin subtyping was performed after the suggested protocol from Serotec (Indianapolis, IN).

Solid-phase ELISA. Hybridoma culture supernatants that reacted with the cell surface of endothelial cells were identified by a solid-phase assay. Endothelial cells were grown to confluence in 96-well plates, washed with Ca²⁺/Mg²⁺-free PBS (CMF/PBS) and fixed with 3% p-formaldehyde in CMF/PBS containing 1% sucrose for 15 min. The plates were blocked with 2% BSA for 1 h at 37°C and then reacted with culture supernatants from the hybridomas for 1 h at room temperature. The monolayers were washed with CMF/PBS containing 0.1% BSA and 0.05% Tween 20 and reacted with peroxidase-conjugated rabbit anti-mouse IgG (H and L chains, Hyclone Laboratories).

Immunoblotting. Each of the hybridomas producing antibodies to cell surface proteins was tested for its reactivity with membrane polypeptides that had been separated by SDS-PAGE (25) and then electrophoretically transferred to nitrocellulose (45). Membrane samples were solubilized with 2% SDS sample buffer containing 5 mM EDTA with or without 2-mercaptoethanol. The nitrocellulose was blocked with 5% nonfat dry milk in TBS (pH 8.0), containing 0.05% Tween 20, and then reacted with the hybridoma culture supernatants for 1.5 h at room temperature. The blot was washed with TBS containing 0.05% Tween 20 and antigens were detected with alkaline phosphatase-conjugated anti-mouse IgG (Promega Biotec, Madison, WI). The filters were stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indol-phosphate. Membranes were prepared from confluent endothelial, MDCK or MDBK cell cultures as described by Magargal et al. (27) and collected as a postmitochondrial pellet. Protein concentrations were measured by using the BCA assay procedure (41) (Pierce Chemical Co., Rockford, IL), with BSA as a standard.

For quantitative immunoblotting, equal amounts of cell protein were analyzed by SDS/PAGE and transferred to nitrocellulose. Monoclonal antibody Ec6C10 was purified to homogeneity on Affi-Gel protein A (Bio-Rad Laboratories, Richmond, CA) and labeled with ¹²⁵iodine using iodogen by the method suggested by the manufacturer (Pierce Chemical Co., Rockford, IL). Typical specific activities were 5 × 10⁵ cpm/µg IgG. After blocking, the immunoblots were reacted with 1 × 10⁶ cpm in 5% nonfat dry milk in TBS with 0.05% Tween 20 for 1 h. Autoradiography was carried out at −70°C using a Lightning Plus intensifying screen (DuPont Co., Wilmington, DE).

Cell Adhesion Assay. Hybridoma supernatants recognizing membrane antigens in the molecular weight range of 125,000-145,000 were then dialyzed against CMPS and screened for neutralization in the adhesion assay. Confluent cultures were dispersed using modifications of the methods described by Urushihara et al. (47) with the following treatments: (a) the cul-

tures were rinsed three times with Ca²⁺/Mg²⁺-free Hepes-buffered Puck's saline, pH 7.4 (CMPS), and incubated with a solution containing 200 µg/ml trypsin (199 U/mg; Cooper Biomedical Inc., Malvern, PA) and 1 mM CaCl₂; (a) alternatively incubated with a solution containing 200 µg/ml trypsin and 1 mM EGTA; or (c) incubated in 2 µg/ml trypsin in CMPS containing 1 mM EGTA. After 15 min at 37°C, an equal volume of 0.5 mg/ml soybean trypsin inhibitor was added and the suspension centrifuged. The pellet was washed and dispersed into single cells with a Pasteur pipet. In some experiments, 1 mM EDTA was substituted for EGTA.

To assay adhesion, the cells (1.9 × 10⁶) were suspended in 2 ml of the test medium. In some experiments, the cells were preincubated (15 min at 4°C) with anti-Ec Fab' fragments or dialyzed hybridoma supernatants. Calcium or EGTA (1 mM) was added and cell suspensions were shaken (90 rpm) at 37°C. Aliquots were removed and diluted immediately in Isoton II (Coulter Electronics Inc., Hialeah, FL) containing 1.0% glutaraldehyde and then counted (counter made by Coulter Electronics, Inc.).

Solid-phase Neutralization Assay

A solid-phase neutralization assay was an adaptation of the procedure described by Urushihara and Yamada (46). For the solid-phase neutralization assay, 600 µg of endothelial membranes were applied to an 8-cm well and subjected to SDS-PAGE 7.5% (11 × 14 cm) (25) and transferred electrophoretically to nitrocellulose (45). The blot was cut into 5-mm strips corresponding to molecular weight regions, then cut into pieces and blocked with 5% BSA in CMF/PBS at 4°C overnight. Each fraction received 320 µg of anti-Ec Fab' in 250 µl for 2.5 h at 4°C. The supernatant (200 µl) was removed and added to the aggregation assay in the presence of 1 mM CaCl₂ with 6.25 × 10⁵ cells dissociated by trypsinization (200 µg/ml in the presence of 1 mM CaCl₂). Neutralization was calculated according to the formula:

$$\% \text{ Neutralization} = 100 \times (\Delta\text{aggregation}_{\text{anti-Ec+membrane}}^{-\Delta}$$

$$\text{aggregation}_{\text{anti-Ec}}/ \Delta \text{ aggregation}_{\text{control}}).$$

Immunocytochemical Localization

Cells were grown on gelatin-coated glass coverslips until confluent, were washed and fixed for fluorescent labeling. Confluent cultures were fixed with 3% p-formaldehyde in CMF/PBS with 1% sucrose for 10 min at room temperature. Alternatively, they were permeabilized by treatment with 0.5% Triton X-100 in 10 mM Pipes (pH 6.8), 50 mM NaCl, 300 mM sucrose and 3 mM MgCl₂ (11) for 2 min at 4°C and then fixed. The cell layers were rinsed with PBS and incubated with CMF/PBS with 50 mM glycine, and then blocked with 1% BSA and 2% normal goat serum in CMF/PBS for 30 min. Monoclonal antibodies (diluted 1:20) were incubated with cells for 45 min, the cell layers were washed and biotinylated goat anti-mouse IgG (Bethesda Research Laboratories, Gaithersburg, MD) was added for 30 min. FITC-streptavidin was added for an additional 30 min (Bethesda Research Laboratories). After extensive washing, the coverslips were mounted in 90% glycerol in PBS containing 0.1% n-propyl gallate. They were examined on a Leitz fluorescence microscope equipped with epifluorescent illumination and either 40× or 100× objectives and photographed with Illford HP5 film (ASA 400).

Bovine carotid and intercostal arteries were freshly removed at slaughter, cut into small pieces, and briefly fixed in 3% p-formaldehyde in CMF/PBS. Vessel pieces were embedded in OCT. (Miles Ames Div., Elkhart, IN) and rapidly frozen. Frozen sections (4-8 µm) were applied to gelatin-coated slides, permeabilized in −20°C methanol for 10 min, and washed in CMF/PBS containing Levamisole (Vector Laboratories, Burlingame, CA). To prevent nonspecific binding, the tissues were then preincubated with 2% BSA and 5% normal horse serum. Culture supernatant from Ec6C10 was used at a dilution of 1:50 for 30 min. After three washes in CMF/PBS, the tissues were exposed to biotinylated horse anti-mouse IgG for 30 min (Vector Laboratories). The alkaline phosphatase labeling was carried out using an avidin-alkaline phosphatase complex (as described by Vector Laboratories) and visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Some sections were stained with hematoxylin.

Ca²⁺ Switch Assay

Confluent cultures were trypsinized (200 µg/ml + 1 mM EDTA in CMPS) for 5 min, centrifuged, and plated on gelatin-coated glass coverslips at 1.5 × 10⁵ cells/cm² in medium 199 with 10% dialyzed FBS and 2 mM EGTA. This low calcium-medium was shown by Shasby and Shasby (39) to contain

~5 μM calcium. After 15 h the low Ca^{2+} medium was removed and medium 199 with 10% FBS (normal Ca^{2+} medium) was added, restoring the calcium level to 1.8 mM. Plating efficiency was >90% in both low Ca^{2+} and normal Ca^{2+} medium (not shown). At the same time 2-d culture supernatants were added from Sp2 or ACT I to control cells or from hybridoma Ec6C10. Heat-inactivated clarified ascites from an anti-bovine class II MHC monoclonal antibody (TH14B, IgG_{2a}) (Veterinary Medical Research & Development, Pullman, WA), which binds to the endothelial cell surface as shown by a solid-phase ELISA, was used as a control cell surface binding antibody. Visualization of microfilaments was with rhodamine-labeled phalloidin (0.17 μM) (Molecular Probes, Eugene, OR) for 20 min as described previously (54).

Inhibition of DNase I (2 μg/ml) (3071 U/mg; Organon Teknika-Cappel, Malvern, PA) activity to quantitate G-actin was measured on a Gilford System 2600 spectrophotometer according to the method of Blickstad et al. (3). The cell layer was extracted with SF buffer (2), (0.5% Triton X-100, 100 mM NaF, 50 mM KCl, 2 mM $MgCl_2$, 10 mM potassium phosphate (pH 7.0), 0.2 mM DTE, 1 M sucrose, and 1 mM DFP), which previously was shown to stabilize F-actin, for 2 min at 4°C. The nonionic detergent insoluble cell layer was scraped in fresh 1.5 M guanidine hydrochloride, 1 M sodium acetate, 1 mM ATP, 20 mM ATP, 20 mM Tris, and 6 mM $CaCl_2$ (pH 7.5), to dissociate the F-actin and syringed to reduce viscosity. Rabbit skeletal muscle actin (Sigma Chemical Co., St. Louis, MO) was used as a G-actin standard.

Results

Cell-Cell Adhesion Mechanisms of Endothelial Cells

Extracellular calcium has been shown to play an essential role in maintaining integrity of the monolayer of large vessel and microvascular endothelial cells (13, 23, 39). To characterize the intercellular adhesion mechanisms of aortic endothelial cells, confluent cultures were dissociated by treatment with trypsin in the presence or absence of calcium and allowed to reassemble in a cell adhesion assay (Fig. 1). This approach, using the trypsin sensitivity of cell adherence systems, has been described previously for other epithelial cells (6, 28, 47). Treatment of confluent endothelial cultures with trypsin at a concentration of 200 μg/ml in the presence of EGTA totally abolished cell adhesion assayed in the presence or absence of calcium. When calcium (1 mM) was added to the trypsin solution, it protected a Ca^{2+}-dependent adhesion process (Fig. 1 A). Retrypsinization of cells showing Ca^{2+}-dependent adhesion in the presence of EGTA, even with trypsin concentrations at 2 μg/ml, abolished all of the adhesion activity (not shown). This implies an intercellular adhesion mechanism that requires calcium for adhesion and is also protected by calcium from digestion by trypsin. Substitution of magnesium (1 mM) during trypsinization was only partially as effective as calcium in promoting adhesion and was unable to promote adhesion of endothelial cells dissociated in the presence of calcium (not shown). A specificity for calcium has been shown for intercellular adhesion of Chinese hamster fibroblasts (42) and in reformation of occluding junctions in epithelial cells (29). When confluent cells were dispersed with a low concentration of trypsin (2 μg/ml) in the presence of EGTA or EDTA, they were able to assemble extensively in the cell adhesion assay (Fig. 1 B). Addition of calcium had no effect on adhesion in this case. Dispersion of confluent endothelial cells by the above treatments demonstrates the presence of both a Ca^{2+}-dependent and a Ca^{2+}-independent cell adhesion mechanism. The differences in trypsin sensitivity of the two adhesion mechanisms imply that different cell surface components may be responsible for each adhesion mechanism.
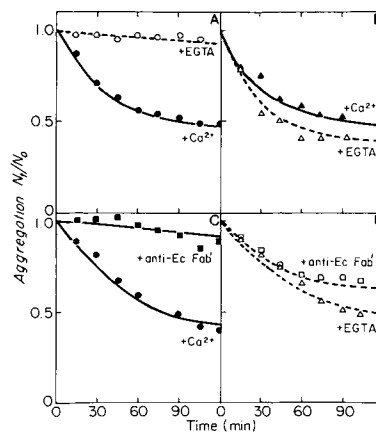

Figure 1. Cell adhesion mechanisms of aortic endothelial cells. Confluent cultures were dispersed with trypsin at the concentrations indicated in the presence or absence of 1 mM Ca^{2+}. The rate of adhesion (1.9 × 10^6 cells) was measured using a Coulter counter and is represented by the decrease in particle number, N_t/N_0. A typical assay for aggregation of fetal endothelial cells dispersed with (A) 200 μg/ml trypsin + 1 mM $CaCl_2$ and aggregation in medium containing 1 mM $CaCl_2$ (—●—) or in 1 mM EGTA (— —○—); and (B) 2 μg/ml trypsin + 1 mM EGTA and aggregation in medium containing 1 mM $CaCl_2$ (—▲—) or 1 mM EGTA (— —△—). Aggregation in the presence of anti-Ec Fab' or nonimmune Fab' fragments (1.8 mg) of adult endothelial cells dispersed with (C) 200 μg/ml trypsin + 1 mM $CaCl_2$ and aggregation in medium containing 1 mM $CaCl_2$ + nonimmune Fab' (—●—) or 1 mM $CaCl_2$ + anti-EcFab' (—■—), or (D) 2 μg/ml trypsin + 1 mM EGTA and aggregation in medium containing 1 mM EGTA + nonimmune Fab' (— —△— —) or 1 mM EGTA + anti-Ec Fab' (— —□—). For Ca^{2+}-dependent adhesion, the mean aggregation index, N_{60}/N_0, for five experiments was 0.55 ± 0.03; with nonimmune Fab', it was 0.54 ± 0.02; and, with anti-Ec Fab' it was 0.91 ± 0.03. For Ca^{2+}-independent adhesion, the mean aggregation index was 0.52 ± 0.04; with nonimmune Fab', it was 0.55 ± 0.04; and, with anti-Ec Fab', it was 0.66 ± 0.05.

The assembly of endothelial cells dispersed with 200 μg/ml trypsin in the presence of 1 mM calcium was almost completely inhibited by addition of anti-Ec Fab' fragments prepared from a polyclonal antiserum raised against intact endothelial cells (Fig. 1 C). The inhibition of Ca^{2+}-dependent adhesion by anti-Ec Fab' fragments was linear with concentration and reached a plateau value at 2.0 mg. However, cells dispersed with 2 μg/ml trypsin in 1 mM EGTA showed adhesion to a similar extent in the presence or absence of the same concentration of anti-Ec Fab' fragments (Fig. 1 D). The Ca^{2+}-dependent CAM is apparently more antigenic than the Ca^{2+}-independent. These results suggest that the assembly of endothelial cells by two adhesion mechanisms are mediated by components of different immunological specificities. Fab' fragments prepared from nonimmune rabbit serum were without effect in either adhesion system and the aggregation index (N_{60}/N_0) was 0.54 and 0.52 for Ca^{2+}-dependent adhesion and Ca^{2+}-independent adhesion, respectively.
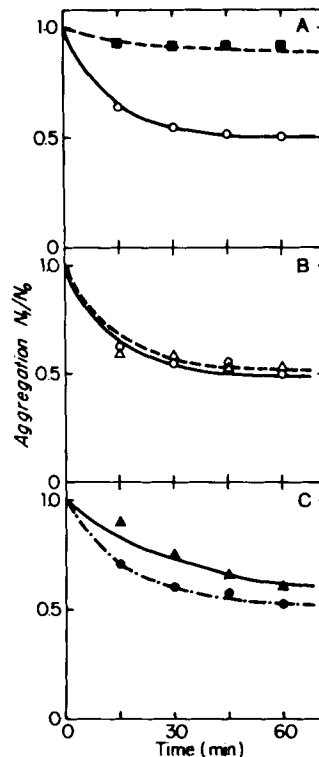
Fractionation of Ca^{2+}-dependent Adhesion Activity by SDS-PAGE

To determine the molecular weight of the membrane protein antigen, an endothelial plasma membrane preparation was
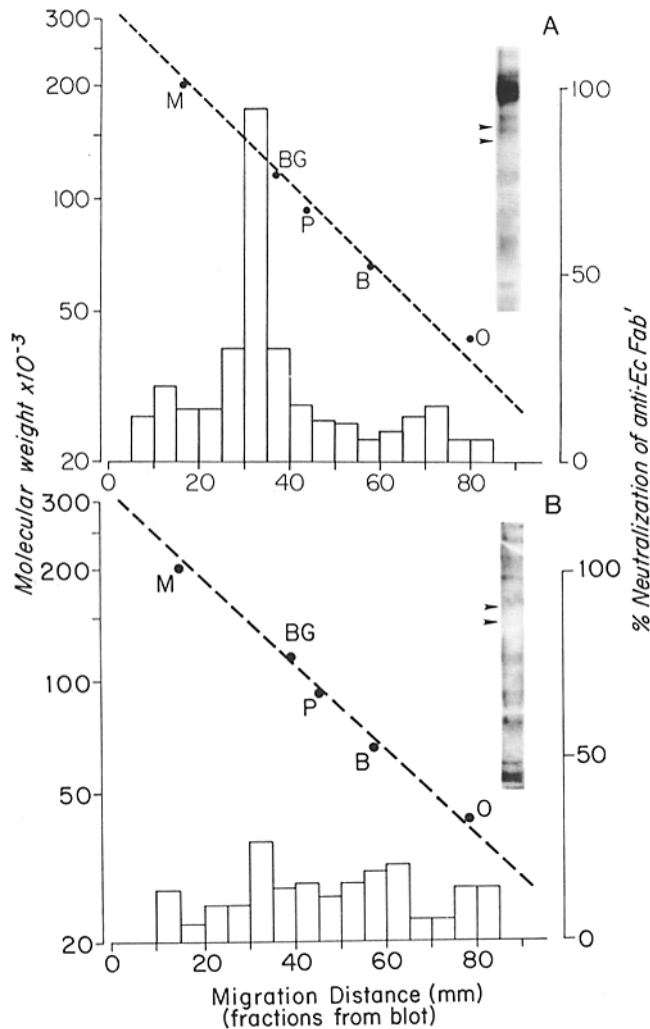
Figure 2. Size fractionation of the Ca^{2+}-dependent cell adhesion activity from endothelial membranes by neutralization of anti-Ec Fab'. Endothelial cell membranes were solubilized in 2% SDS sample buffer in the absence of reducing agents, subjected to SDS-PAGE (7.5%), and electrophoretically transferred to a nitrocellulose membrane. Endothelial cell membranes from control cells (A) or from cells sequentially treated with 200 $\mu g/ml$ trypsin + 1 mM $CaCl_2$ and then with 2 $\mu g/ml$ trypsin + 1 mM EGTA (B). The nitrocellulose membranes were blocked, cut into 5-mm strips, and each was assayed for anti-Ec Fab' neutralizing activity in the Ca^{2+}-dependent adhesion assay. The inset is an immunoblot reacted with anti-Ec IgG and the ^{125}I-labeled protein A. Arrows indicate molecular weight region of neutralizing activity between 125-145 kD. The molecular weight markers are M, myosin (200 kD); BG, β-galactosidase (116 kD); P, phosphorylase b (94 kD); B, BSA (68 kD); O, ovalbumin (43 kD).

Figure 3. Cell adhesion of aortic endothelial or smooth muscle cells in the presence of monoclonal antibody Ec6C10. Dissociated cells were allowed to reassemble in the presence of dialyzed control 2-d culture medium from Sp2 or monoclonal antibody Ec6C10. Confluent cells were dissociated in the presence of 200 $\mu g/\mu l$ trypsin containing 1 mM $CaCl_2$ (Ca^{2+}-dependent) or 2 $\mu g/ml$ trypsin containing 1 mM EGTA (Ca^{2+}-independent), and were incubated 15 min at 4°C with the supernatants; assembly was initiated by placing the cells at 37°C and adjusting the reaction to 1 mM $CaCl_2$ or 1 mm EGTA. Representative time courses of aggregation are shown from three independent experiments: (A) Ca^{2+}-dependent aggregation of endothelial cells after addition of 1 mM $CaCl_2$ in the presence of Sp2 medium (—O—) $N_{60}/N_0 = 0.54 \pm 0.04$, or Ec6C10 medium (— —■—) $N_{60}/N_0 = 0.99 \pm 0.07$. (B) Ca^{2+}-independent aggregation of endothelial cells after addition of 1 mM EGTA in the presence of Sp2 medium (—O—) $N_{60}/N_0 = 0.51 \pm 0.1$, or Ec6C10 medium (— —△— —) $N_{60}/N_0 = 0.54 \pm 0.1$. (C) Ca^{2+}-dependent aggregation of smooth muscle cells after addition of 1 mM $CaCl_2$ in the presence of Sp2 medium (·—●—·—) $N_{60}/N_0 = 0.53 \pm 0.05$, or Ec6C10 medium (—▲—) $N_{60}/N_0 = 0.67 \pm 0.05$.

subjected to SDS-PAGE (25) and transferred to a nitrocellulose membrane (45) (Fig. 2 A). The membrane was cut transversely into strips representing different molecular weight classes and the pieces were assayed for neutralizing activity with anti-Ec Fab' fragments in the Ca^{2+}-dependent cell adhesion assay. Only one region of neutralizing activity was found. This region was in the molecular weight range of 125,000-145,000. No activity was found on membranes prepared from endothelial cells which had been treated with trypsin in the presence of EGTA, which abolishes the cal-

cium-dependent adhesion (Fig. 2 B). Endothelial membrane fractions were subjected to SDS-PAGE and transferred to nitrocellulose, and the cellular components that reacted with anti-Ec immunoglobulins were detected with ^{125}I-labeled protein A (Fig. 2). A number of proteins are detected by the anti-Ec antibody, but one component with an apparent molecular weight of 135,000 was present only in membranes from endothelial cells containing the calcium-dependent cell adhesion system.

Inhibition of Ca^{2+}-dependent Adhesion in Endothelium by Monoclonal Antibody Ec6C10

The approach used to identify hybridoma cultures producing antibodies to the cell surface component involved in Ca^{2+}-

dependent intercellular adhesion of endothelial cells consisted of the following multiple step procedure: (a) all cultures were tested for production of antibodies that bind to the endothelial cell surface by a solid-phase ELISA; (b) hybridoma cultures positive in the ELISA assay were tested by
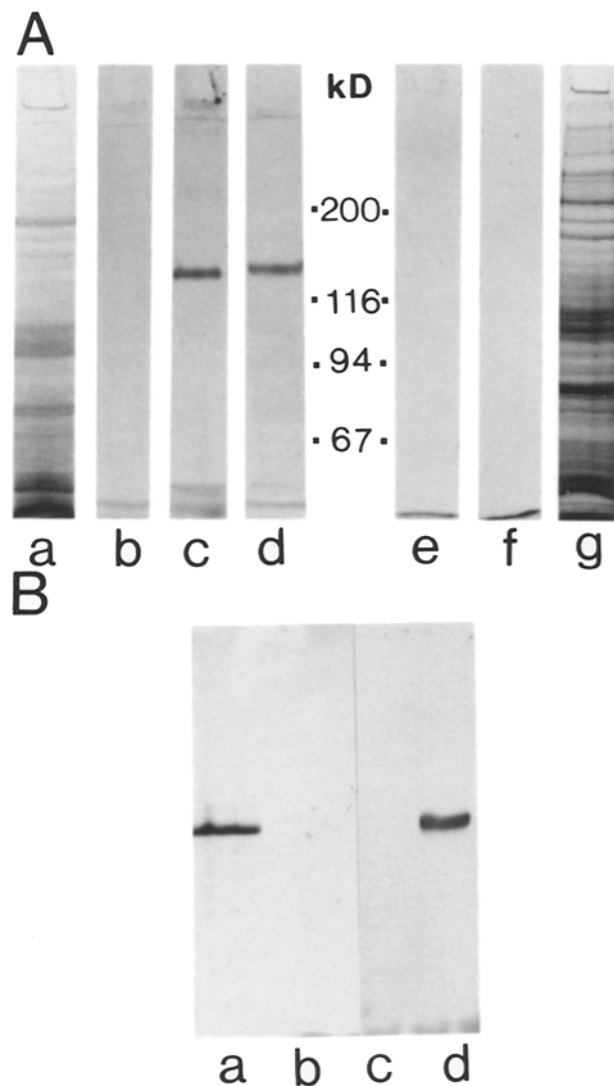
Figure 4. Immunoblotting analysis of membrane proteins recognized by antibody Ec6C10. (A) Membranes were prepared from confluent cultures of bovine aortic endothelial cells, MDBK cells, or MDCK cells. 50 μg of membrane protein were electrophoresed on 7.5% polyacrylamide SDS gels and then electrophoretically transferred to nitrocellulose, or 25 μg were analyzed on a separate gel and stained with Coomassie blue G-250. (Lane a) Nonreduced sample from endothelial cells stained with Coomassie blue; (lane b) nonreduced sample from endothelial cells stained with Sp2 supernatant; (lane c) nonreduced sample from endothelial cells stained with mAb Ec6C10; (lane d) reduced sample from endothelial cells stained with mAb Ec6C10; (lane e) nonreduced sample from MDCK cells stained with mAb Ec6C10; (lane f) nonreduced sample from MDBK cells stained with mAb Ec6C10; (lane g) nonreduced sample from MDBK cells stained with Coomassie blue. (B) Cell lysates of BAE or F9 cells were made by scraping the cells into SDS sample buffer, boiling, and syringing. 75 μg of total protein were applied to each lane. (Lane a) F9 cells stained with rabbit antiuvomorulin; (lane b) bovine aortic endothelial cells (BAEC) stained with antiuvomorulin; (lane c) F9 cells stained with mAb Ec6C10; (lane d) BAEC stained with mAb Ec6C10.

SDS-PAGE of endothelial membranes and immunoblotted; and (c) cultures positive for their ability to react to membrane proteins in the molecular weight range of 125,000–145,000, implicated from the data in the solid-phase neutralization assay, were subsequently screened and tested for inhibition of the intercellular adherence assay. As described here, Ca^{2+}-dependent cell adhesion was the only activity present after treatment of BAE cells with 200 μg/ml trypsin in the presence of calcium. Ca^{2+}-independent adhesion was lost under these conditions and was displayed by cells after treatment with 2 μg/ml of trypsin in the absence of calcium. The data depicted in Fig. 3 shows a monoclonal antibody (Ec6C10; isotype IgG_1) we identified that could inhibit Ca^{2+}-dependent adherence of endothelial cells but had no effect on Ca^{2+}-independent adherence. At an antibody concentration that abolished Ca^{2+}-dependent adherence of endothelial cells, there was only a minimal effect on Ca^{2+}-dependent adherence of aortic smooth muscle cells. Control culture medium (Sp2) had no effect on any of the cell adhesion assays.

In a solid-phase ELISA, there was minimal binding of antibody Ec6C10 to confluent cultures of bovine smooth muscle cells permeabilized with 0.5% NP-40 or nonpermeabilized and only background levels with MDBK or MDCK cells (not shown). In MDCK cells, Gumbiner and Simons (17) have shown that the Ca^{2+}-dependent CAM, uvomorulin, is localized to the lateral cell surface and functions in tight junction formation. Immunoblot analysis on endothelial membranes after SDS-PAGE was performed to determine which of the membrane polypeptides is recognized with monoclonal antibody Ec6C10 (Fig. 4 A). The antigen identified by this antibody was a single molecule with an apparent molecular weight of 130,000 under nonreducing conditions (lane c). In the presence of the reducing agent, 2-mercaptoethanol, the apparent molecular mass was slightly but consistently higher at 135 kD (lane D). This antigen does not appear to be a major Coomassie blue staining polypeptide of membrane proteins. There was no reactivity of monoclonal antibody Ec6C10 with membranes prepared from confluent cultures of either MDCK or MDBK cells (lanes e and f).

Uvomorulin is a well characterized member of the family of Ca^{2+}-dependent CAMs and has been shown to be restricted to epithelial cells (35, 48, 49). Immunoblot analysis (Fig. 4 B) with rabbit antiuvomorulin on F9 mouse embryonal carcinoma cells shows a band which migrates with an apparent molecular mass of 120 kD (lane a). There was no staining of BAE cells with polyclonal antiuvomorulin (lane b). The antiuvomorulin identified a band in MDCK cells and only weakly identified a band in MDBK cells (not shown). Immunoblotting of cell lysates from BAEC and F9 cells with antibody Ec6C10 showed staining of BAE cells (lanes c and d). The band migrated more slowly in SDS-PAGE than uvomorulin seen in F9 cells with an apparent molecular weight of 135,000.

Trypsin Sensitivity of the Cadherin-like Ec6C10 Antigen

The Ca^{2+}-dependent cell adhesion molecules described by Takeichi (43) show a characteristic sensitivity to trypsinization where the presence of calcium preserves the molecule, but trypsin in the absence of extracellular calcium cleaves the
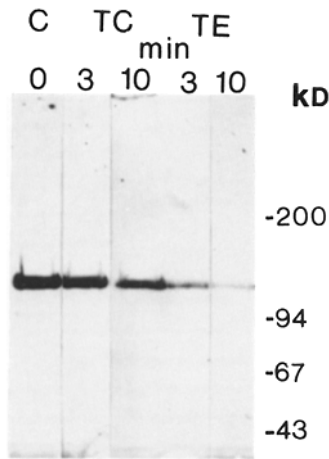
Figure 5. Immunoblot with Ec6C10 of endothelial cells treated with trypsin in the presence or absence of calcium. Endothelial cells were grown to confluence on a film of collagen and treated with trypsin (100 μg/ml) as described by Volk and Geiger (50). Incubation of cells for 10 min in 10 mM Hepes (pH 7.4), buffered DME with trypsin and 5 mM EGTA (*TE*) or trypsin in a final concentration of 5 mM Ca^{2+} (*TC*). The reaction was stopped with addition of 1 mg/ml soybean trypsin inhibitor. The cells were scraped, an aliquot was removed for determination of cell number, lysed with SDS sample buffer containing 5 mM EDTA, boiled, and immunoblotted with Ec6C10. Control cells (*C*) were incubated in Hepes-buffered DME containing EGTA alone and lysed with SDS sample buffer. Samples were loaded according to equal cell number.

molecule from the cell surface. This reactivity is shown in Fig. 5 with trypsin in the presence of calcium (TC) or EGTA (TE). After various incubation periods, the reaction was stopped, cells were solubilized in SDS sample buffer containing 5 mM EDTA and equal numbers of cells were analyzed by SDS-PAGE. Loss of the 135-kD antigen identified by immunoblotting with Ec6C10 was more rapid in the presence of trypsin and EGTA. A major fragment released by trypsinization in EGTA has an approximate molecular weight of 28,000 when fractionated on a Sephacryl S200 column and would run with the dye front of the gel (not shown). The antigen detected by antibody Ec6C10 in the TC condition was severalfold less susceptible to trypsin. We have tentatively termed this endothelial molecule vascular or V-cadherin according to the functional subdivisions of cadherins described by Hatta et al. (19).

Immunolocalization of the Ec6C10 Antigen to Cell–Cell Borders

The distribution of the antigen identified by monoclonal antibody Ec6C10 is shown in Fig. 6 in confluent endothelial
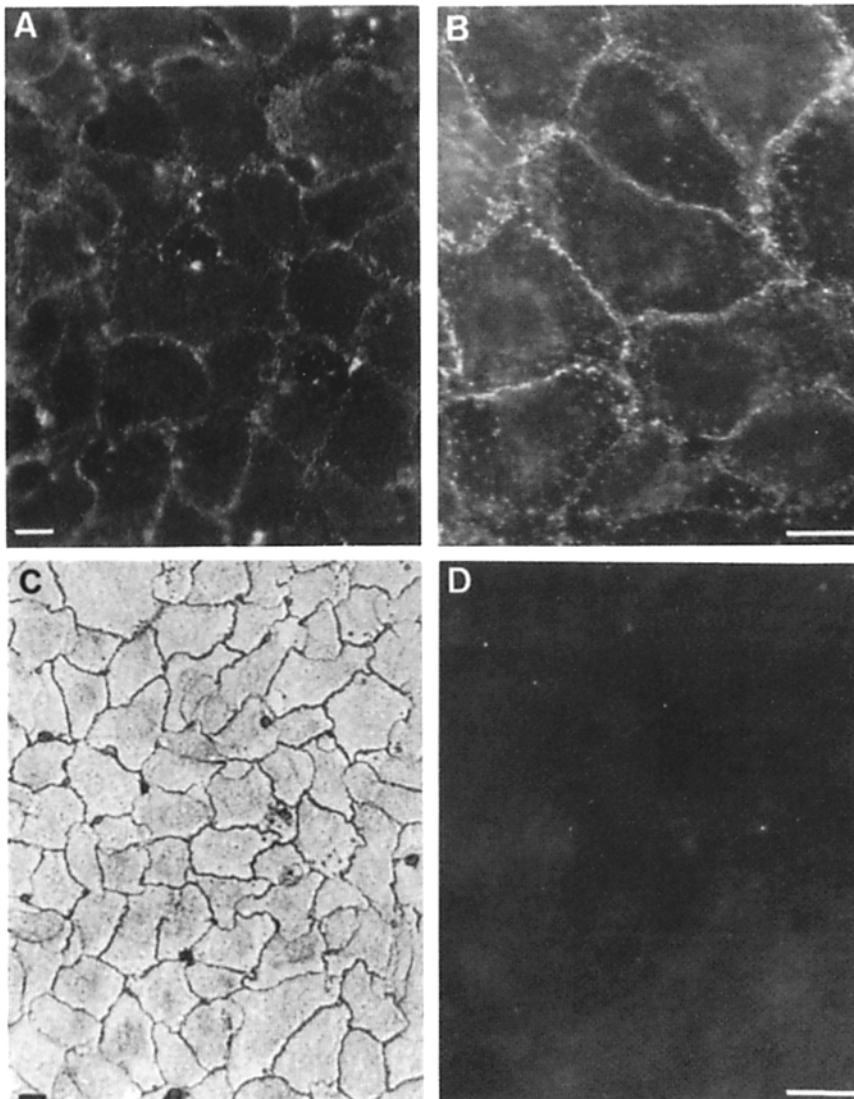
Figure 6. Indirect immunofluorescence of antibody Ec6C10 in confluent bovine endothelial cells. Confluent cultures were fixed with 3% p-formaldehyde in CMF/PBS and incubated with mAb Ec6C10 (*A*) or (*D*) incubated with Sp2 culture supernatant. Alternatively, cultures were first permeabilized by treatment with 0.5% Triton X-100 in 10 mM Pipes (pH 6.8), 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂ for 2 min followed by fixation before antibody staining with mAb Ec6C10 (*B*); silver nitrate staining (13) of endothelial cells grown to confluence on a film of collagen (*C*). Bar, 20 μm.
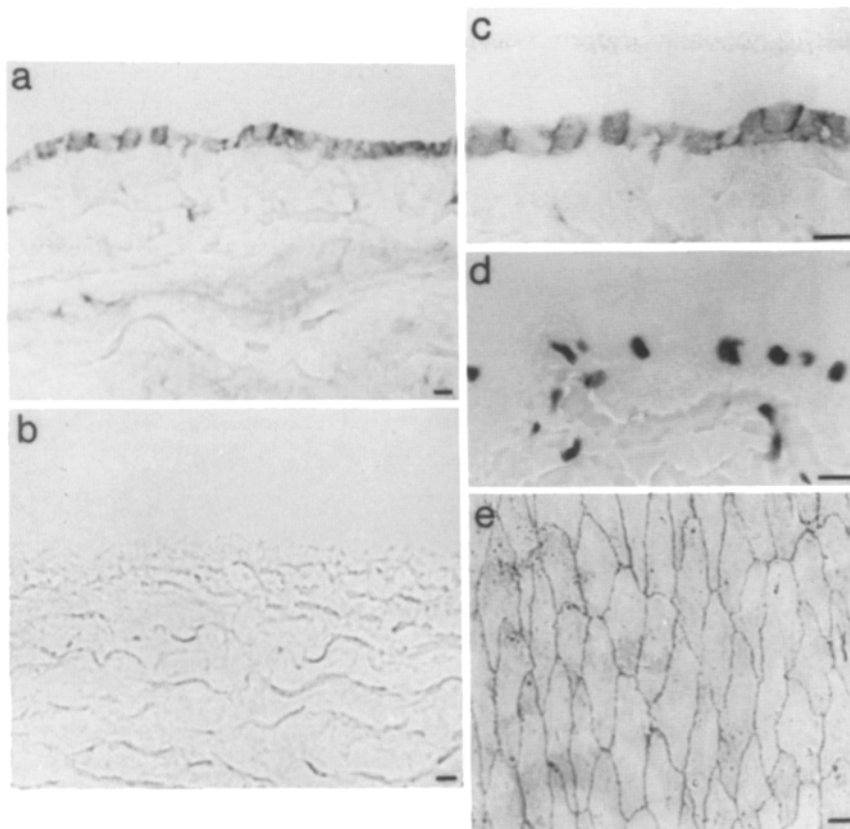
Figure 7. Localization of the antigen recognized by Ec6C10 in frozen sections of bovine vessels. Cross sections of intercostal arteries were stained with Ec6C10 (*a*) and (*c*), in an adjacent section with hematoxylin to identify nuclei (*d*), and with ACT I (*b*). For comparison, *e* shows *en face* staining with silver nitrate of cell borders in a carotid artery. ACT I culture supernatant was used as a negative control secreting an antibody of the same subclass as Ec6C10. Bar, 20 μm.

cells. It was localized to the lateral cell surface of intercellular boundaries as identified by indirect immunofluorescence (Fig. 6 *A*). Antibody Ec6C10 labeled the surface of confluent cells when intercellular junctions were kept intact. The immunofluorescent pattern of the antigen is similar to the silver nitrate staining of cell junctions, showing the typical cobblestone morphology of aortic endothelial cells (Fig. 6 *C*). Bovine microvascular endothelial cells cultured on treated amnion show a similar pattern of silver nitrate staining, and they display a transendothelial electrical resistance suggesting tight junction formation (13). Furie et al. (13) have described the ultrastructural localization of the silver granules deposited by this technique to intercellular spaces and subjunctional areas. To determine the spatial distribution of the total cellular antigen, cell membranes were permeabilized with 0.5% Triton X-100 in a cytoskeletal stabilization buffer (11) before fixation (Fig. 6 *B*). The immunofluorescent staining remained associated with the cell layer and was more intense and concentrated at cell–cell boundaries. The morphology of the intercellular immunofluorescent staining shows a beaded pattern. This is a variation of the immunofluorescent pattern of other Ca^{2+}-dependent CAMs, such as A-CAM, in epithelial cells (17, 22, 50). The antigen recognized by monoclonal antibody Ec6C10 is a surface component concentrated at the lateral plasma membrane of confluent endothelial cells. The cell surface of cells not in contact with other cells was weakly stained in a nonorganized pattern (not shown).

Immunostaining of frozen cross sections of bovine vessels shows that the distribution of the antigen recognized by Ec6C10 is restricted in the endothelium (Fig. 7, *a* and *c*).

Monoclonal antibody staining was localized to the endothelium and there was only rare labeling in the media. The occasional immunostaining seen in the media of the vessel was not found in cultured bovine smooth muscle cells. The staining in the endothelium was concentrated at the lateral surface where the cells come into contact and is reminiscent of the pattern of silver staining. The antigen does not appear to be concentrated at any position on the lateral cell surface. For comparison *en face* staining of bovine vessels with silver nitrate shows a decoration of cell borders (36) with the endothelial cells positioned along the axis of the vessel (Fig. 7 *e*).

Immunoblot Analysis with Ec6C10 after Induction of Cell–Cell Contacts

The integrity of both epithelial (17, 29, 30, 55) and endothelial (24, 39) intercellular junctions has been shown to be affected by the level of extracellular calcium ions. Depletion of extracellular calcium by switching monolayers of endothelial cells to low Ca^{2+}-containing medium resulted in loss of cell–cell contact followed by loss of transendothelial electrical resistance (13, 39). Ca^{2+} switch assays have been used to follow the assembly of the different polypeptides associated with desmosomes (34) and adherens junctions in MDCK cells (18) by modulating the absence or presence of cell–cell contact through adjusting the level of extracellular calcium. Using this approach to follow the reappearance of the vascular cadherin, confluent endothelial cells dispersed with trypsin (200 μg/ml) containing 1 mM EDTA were replated in low Ca^{2+} medium at confluent density (1.5 × 10⁵
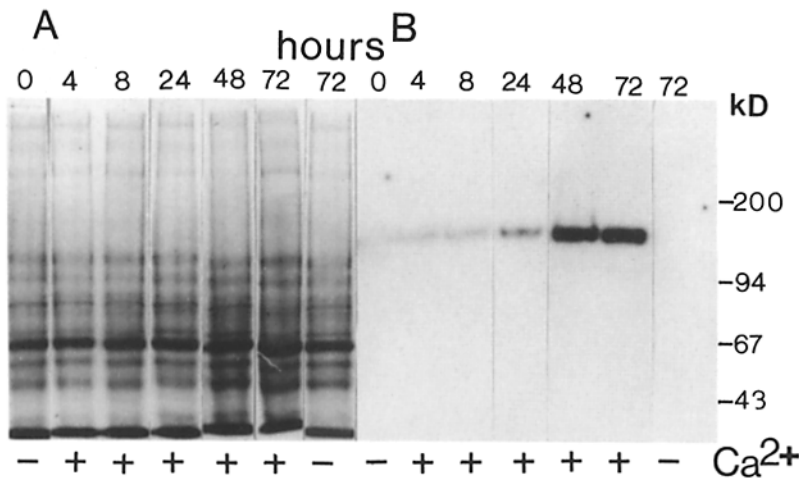
Figure 8. Expression of the antigen recognized by antibody Ec6C10 after induction of cell–cell contact. Endothelial monolayers were trypsinized (200 μg/ml + 1 mM EDTA) and plated in low-calcium medium at confluent density (1.5 × 10^5 cells/cm²). After 15 h normal calcium medium (+) or low-calcium (−) was added and the cells were harvested at various times by scraping into SDS sample buffer, boiling, and syringing. Samples were analyzed according to equal amounts of protein on SDS-PAGE and either stained with (*A*) Coomassie blue or (*B*) transferred to nitrocellulose and incubated with ¹²⁵I-labeled Ec6C10.

cells/cm²). After 15 h, the cells were attached and spread, and displayed a disorganized cell layer with overlapping processes. Immunoblotting of equal amounts of total cellular protein after addition of normal Ca²⁺ medium reveals a time dependent increase in the antigen recognized by ¹²⁵I-labeled Ec6C10 (Fig. 8). A low level of antigen detected by ¹²⁵I-Ec6C10 binding (21% of the plateau level) was observed with cells in low Ca²⁺ medium after 15 h, but was decreased somewhat by 72 h. As shown in Fig. 8, the reappearance of the antigen identified by monoclonal antibody Ec6C10 required addition of normal calcium containing medium. In the presence of normal calcium this increased ∼50% by 24 h and had reached a plateau at 48 h. Immunoblotting with a rabbit polyclonal antibody recognizing the beta₃-chain of the vitronectin receptor (7), which also binds divalent cations to identical blots cells after the switch to normal calcium showed no change in antibody binding at 48 h (not shown). Extracellular calcium or cell–cell contact may act to stabilize the Ec6C10 antigen. The level of extracellular calcium has been shown to be important in the organization of cell–cell adhesion molecules at cell junctions (22, 50, 55). Immunofluorescence studies show a rapid loss of junctional localization with the removal of calcium.

Effect of Antibody Ec6C10 on Formation of the Peripheral Microfilament Band during Induction of Cell Contacts

The localization of F-actin microfilaments in endothelial cells demonstrates two distinct patterns: (*a*) a series of stress fibers at the ventral aspect, and (*b*) a peripheral F-actin band at intercellular junctions (54). Organization of the peripheral actin microfilament band has been shown by Wong and Gottlieb (53, 54) to be dependent on the degree of cell–cell contact in the endothelium. Removal of extracellular calcium results in retraction of the peripheral actin microfilament band towards the center of the cell. Subsequent reincubation in normal Ca²⁺ medium resulted in rapid assembly of cell–cell contacts and reestablishment of the dense peripheral microfilament band (39).

To determine the role of the Ca²⁺-dependent CAM on events associated with endothelial cell–cell contact, we followed organization of the dense peripheral F-actin band in confluent cells in a Ca²⁺ switch assay. Cells were plated in

low Ca²⁺-medium and normal Ca²⁺-medium was added in the presence of monoclonal antibody (2 μg/ml) Ec6C10, which blocked Ca²⁺-dependent adhesion in the assembly assay, or monoclonal antibody ACT I supernatant as a control. Fluorescent staining of F-actin microfilaments using rhodamine phalloidin was used to follow the organization of the peripheral bundles of microfilaments. At the time of the calcium switch, there was a general low level of fluorescence seen in the regions of cell–cell junctions with some focal sites of fluorescence (Fig. 9, *A*). Stress fiber microfilament bundles were randomly distributed throughout the central region of the cells. By 6 h after switching to normal calcium medium, an increase in fluorescent labeling at sites of cell–cell contact was seen in the majority of the control cells (not shown). A diffuse continuous band had formed at the periphery where endothelial cells were in contact, suggesting that the actin microfilaments had started to organize into the peripheral F-actin band. After 24 h in normal calcium medium (Fig. 9, *B* and *G*), the recovery of the peripheral F-actin band was well organized, much broader, and had started to look like the phalloidin staining pattern of a well-established monolayer of endothelial cells (54). Fine microfilament bundles extend from the dense peripheral band toward the cell border. Addition of antibody Ec6C10 to the recovery phase with normal Ca²⁺ medium inhibited the formation of the peripheral microfilament dense band (*C* and *H*). Only a limited number of filaments were formed in the presence of antibody Ec6C10. There was no evidence of toxicity to the cells or of cell detachment with addition of monoclonal antibody Ec6C10. Addition of a cell surface binding monoclonal antibody recognizing bovine class II MHC (TH14B) at 2 μg/ml did not have any effect on F-actin distribution (not shown). These results suggest that monoclonal antibody Ec6C10 perturbs organization of the peripheral microfilament band when calcium concentration is adjusted to allow cell–cell contact and expression of the Ca²⁺-dependent CAM. This suggests that the Ca²⁺-dependent CAM might play a role in endothelial permeability.

Quantitation of the F-actin content of confluent endothelial cells before and after the Ca²⁺ switch in the presence of increasing Ec6C10 concentration is shown in Table I. 24 h after the cells were switched to normal calcium medium, the cell layer was extracted first with 0.5% Triton X-100 in SF
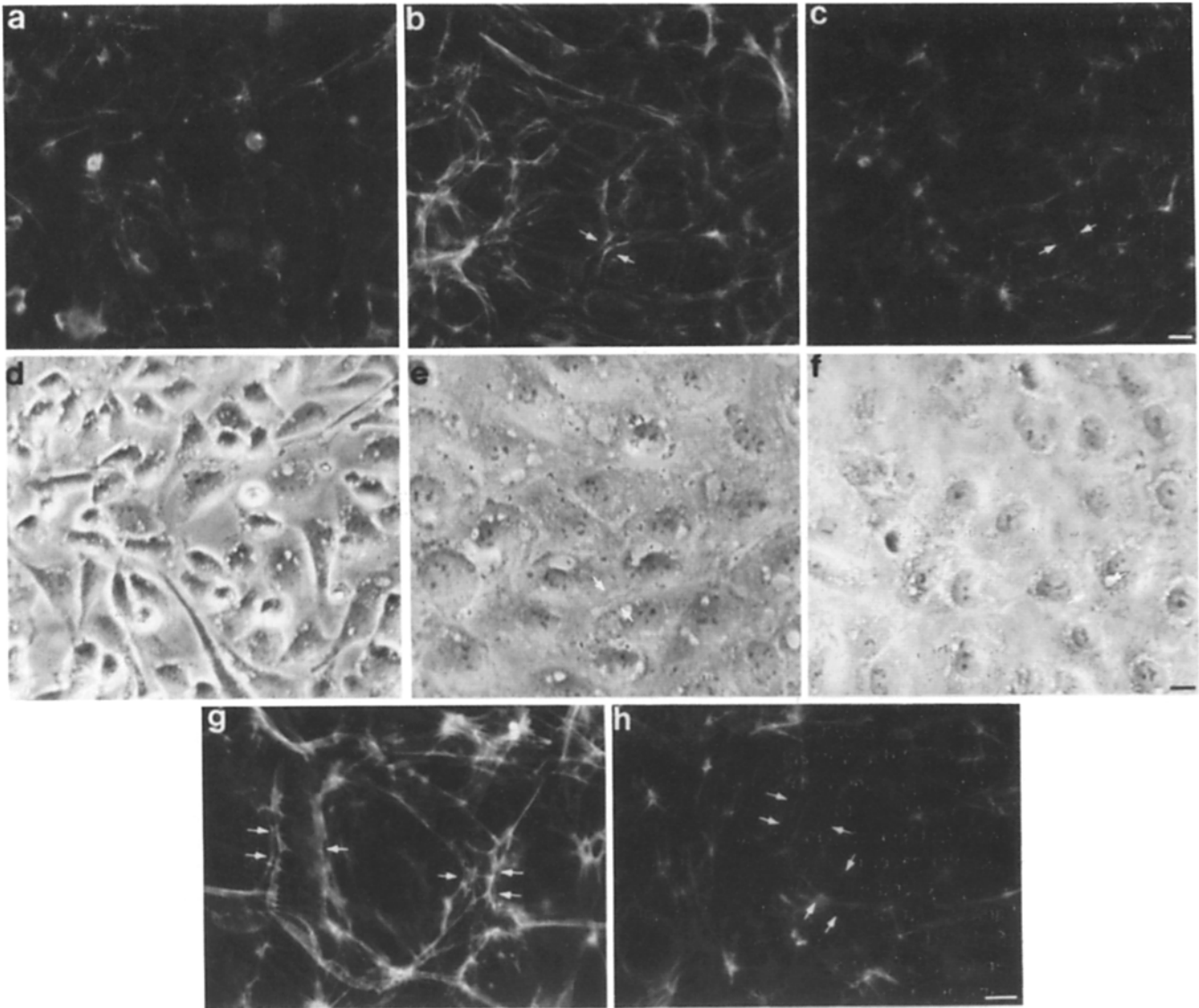
Figure 9. Effect of antibody Ec6C10 on the peripheral microfilament band during formation of cell–cell contacts. Endothelial cells were plated in low Ca^{2+} medium on coverslips at confluent density (1.5 × 10⁵ cells/cm²) for 15 h. Cells were then switched to normal Ca^{2+} medium in the presence of control anti–ACT I conditioned medium or Ec6C10 conditioned medium. Cells were fixed at various times after adding normal Ca^{2+} medium with 3% p-formaldehyde, then permeabilized with 0.1% Triton X-100 in PBS and incubated with rhodamine-phalloidin. *d, e,* and *f* are the corresponding phase-contrast photomicrographs for the fluorescent photomicrographs *a, b,* and *c,* respectively. (*a* and *d*) Cells maintained for 15 h in low Ca^{2+} medium; (*b, e,* and *g*) cells 24 h in normal Ca^{2+} medium with anti–ACT I monoclonal antibody (2.0 μg/ml of IgG); (*c, f,* and *h*) cells 24 h in normal Ca^{2+} medium with Ec6C10 (2.0 μg/ml). *G* and *H* were photographed at 100× magnification. Arrows indicate the phalloidin staining of microfilaments between cells. The photomicrographs shown were taken at a constant exposure time. Bar, 20 μm.

buffer (2) to remove G-actin. F-actin was then dissociated with guanidine hydrochloride and the G-actin content determined using the DNase I assay (3). The maximum inhibition achieved by antibody Ec6C10 was 30% of the control F-actin content at 24 h after the Ca^{2+} switch, and, as shown above, this represents a limited organization of the peripheral F-actin band. Studies by Wong and Gotlieb (54) showing that the F-actin microfilaments of the dense peripheral band are more sensitive to cytochalasin B, suggesting they represent a different population than the F-actin of stress fibers. Induction of endothelial migration by mechanical wounding shows also a rapid change in the state of actin polymerization by

a decrease in F-actin content (14) with the loss of the dense peripheral band (53, 54). An association with cytoskeletal elements has been reported in the literature for other Ca^{2+}-dependent CAMs. Using immunofluorescence techniques a co-distribution is seen between peripheral actin microfilaments and A-CAM (50) and with P-, E-, and N-cadherin (22).

Discussion

Confluent cultures of aortic endothelial cells dissociated by trypsinization in the presence or absence of calcium reassemble with distinct cell adhesion mechanisms. Two distinct

Table I. Effect of Monoclonal Antibody Ec6C10 on the F-actin Content of Endothelial Cells in a Calcium Switch Assay

Treatment	μg F-actin / 10^6 cells	Relative F-actin content
Control monolayer	15.9 ± 0.9	1.2
Low Ca^{2+} medium	5.8 ± 0.6	0.4
+ Normal Ca^{2+} medium	13.3 ± 0.9	1.0
+ Normal Ca^{2+} + 2 μg/ml Ec6C10	11.6 ± 0.9	0.9
+ Normal Ca^{2+} + 5 μg/ml Ec6C10	9.8 ± 0.7	0.7
+ Normal Ca^{2+} + 12.5 μg/ml Ec6C10	10.0 ± 0.3	0.7
+ Normal Ca^{2+} + 2 μg/ml TH14B	13.7 ± 0.8	1.0

Confluent cultures of BAEC were harvested with 200 μg/ml trypsin containing 1 mM EDTA and were plated confluent density (1.5 × 10^5 cell/cm²) in 60-mm dishes in M199 medium containing 10% FCS and 2 mM EGTA (low-calcium medium). After 15 h the medium was switched to normal-calcium medium in the absence or presence of protein A-purified Ec6C10. After 24 h, the cell layers were washed with PBS, and placed on ice. The cells were treated with SF buffer (2), to remove monomeric actin for 2 min at 4°C. The detergent insoluble cell layer was taken up in fresh 1.5 M guanidine hydrochloride, 1 M sodium acetate, 1 mM ATP, 20 mM Tris and 6 mM $CaCl_2$ (pH 7.5), syringed, and the depolymerized F-actin assayed for the inhibition of DNase I (2 μg/ml) by G-actin (3). The data shown is the mean of three independent experiments.

modes of intercellular adhesion exist for endothelial cells, one Ca^{2+}-dependent and the other Ca^{2+}-independent. The Ca^{2+}-dependent CAM in endothelium we have described has an apparent molecular weight of 135,000. In recent studies, we have localized the Ec6C10 determinant to the NH_2 terminus 15 kD of the antigen that shows moderate sequence homology to the NH_2 terminus of the precursor region of the cadherin family (43). Cell surface labeling and immunoprecipitation shows a similar pattern to immunoblotting identifying a single chain polypeptide. A number of related Ca^{2+}-dependent cell adhesion molecules have been described in other epithelia (16, 19, 31, 35), and our results suggest a similar molecule in endothelium. The vascular endothelium is somewhat unique among mammalian epithelia because it is derived from the mesoderm and lacks desmosomes and the keratin class of intermediate filaments. It is, therefore, not surprising that uvomorulin, L-CAM, or the other cadherins have not been detected in vascular endothelial cells (10, 43). We also find that a polyclonal antibody to uvomorulin (48) does not react with aortic endothelium and that monoclonal antibody Ec6C10 does not recognize an antigen in epithelial cells, such as MDCK or MDBK cells, expressing uvomorulin. A-CAM appears to be the most similar Ca^{2+}-dependent CAM in terms of molecular weight, but it also has not been found in endothelium (50). Immunohistochemical labeling with Ec6C10 of sections of bovine tissues showed staining of both large and microvascular endothelium was found with no staining of smooth muscle or other cells. Analysis of bovine lung membranes by SDS-PAGE and immunoblotting with Ec6C10 showed that the antigen in vivo displayed an apparent molecular weight of 130,000 under nonreducing conditions, which is identical to that seen in culture (not shown).

The Ca^{2+}-dependent CAMs have been postulated as playing a critical role in the developing embryo and in maintaining integrity in the adult (10, 43). The distribution of L-CAM (44) and P- and E-cadherin (31) during organogenesis shows that they appear and disappear at various stages of develop-

ment. Antibodies to uvomorulin or E-cadherin block the compaction of preimplantation mouse embryos and interfere with cell-cell interaction of embryonic hepatocytes and MDCK cells (35, 48, 49, 54). Antibodies to L-CAM block the aggregation of embryonic liver cells (16). Uvomorulin and L-CAM are widely distributed in many adult epithelia where uvomorulin is localized to intermediate junctions (3, 50) of intestinal epithelial cells. In addition, antibodies to uvomorulin block not only the reformation of occluding junctions in MDCK epithelial cells (17), but also the assembly of desmosomes and adherens junctions (16). Apparently, the cellular adhesions of uvomorulin function in the formation of all cell junction contacts. A-CAM, a 135-kd protein, is also associated with adherens junctions in certain epithelia (50). Studies by Volk and Geiger (51) show that addition of Fab' fragments of A-CAM perturb microfilament bundle assembly in contrast to the intact IgG that renders the junctions Ca^{2+}-independent. They have suggested that the organization of microfilament bundles at intercellular sites is mediated through interactions at sites of cell-cell contact by CAMs as cell surface receptors (55). The role of calcium appears to be in stabilizing the organization of junctional components in epithelial cells (17, 18, 22, 34, 50). The presence of extracellular calcium may function in this process by allowing either a stable association of the cell junction or a functional conformation of the CAM. Other evidence for a contact receptor mechanism is suggested by studies of Painter et al. (32) showing the cytoskeletal association with the substrate adhesive proteins GpIIb/IIIa after Con A stimulation of platelets.

The existence of a similar system for endothelium is of particular interest because the monolayer configuration is a key parameter of the endothelial phenotype and a requirement for many of the transport properties associated with endothelial function. Shasby and Shasby (39) have shown that calcium has an important role in maintaining endothelial integrity and shape. Intercellular junction formation can be analyzed in endothelial cells since the degree of cell-cell contact can be modulated by adjusting the Ca^{2+} concentration of the growth media (24, 39). Confluent endothelium switched to growth medium containing low Ca^{2+} exhibit little cell-cell contact and no evidence of junctional complex formation (39). Subsequent addition of complete growth medium containing 1.8 mM Ca^{2+} results in rapid induction of cell-cell contact throughout the monolayer and establishment of the dense peripheral band of F-actin filaments characteristic of the confluent monolayer. In confluent endothelium vinculin is colocalized with the peripheral F-actin band at sites of cell-cell contact in a punctate distribution (54) similar to that of other adherens type junctions (50). The beaded immunofluorescent staining pattern for the antigen identified by Ec6C10 is reminiscent of the pattern for vinculin in cultured endothelium (54). In the present experiments, inclusion of monoclonal antibody Ec6C10 in normal calcium medium at the time of the Ca^{2+} switch inhibited formation of the peripheral microfilament band during the recovery phase apparently blocking cell-cell contact. The recovery of the peripheral F-actin band and the antigen recognized by Ec6C10 is slower than F-actin and uvomorulin in epithelial cells (18) suggesting a potential stabilizing role for desmosomes. Among the agents able to stimulate growth of confluent endothelial monolayers are those that disrupt the continuity of monolayer structure (38). This evidence suggests

that, at saturation density, intercellular adhesions may also function to regulate cell growth (20). We would speculate that modulation of expression of cell-cell adhesion molecules would be an important aspect in angiogenesis (1).

Note Added in Proof. While this manuscript was in preparation another antigen was described at the cell borders of human umbilical vein endothelial cells (30a) using monoclonal antibody hec7. Preliminary studies have shown that the antigens recognized by Ec6C10 and hec7 are different in molecular weight and amino-terminal sequence (data not shown).

References

1. Ausprunk, D. H., and J. Folkman. 1977. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc. Res.* 14:53–65.
2. Blickstad, I., and L. Carlsson. 1982. On dynamics of the microfilament system in HeLa cells. *J. Cell Biol.* 93:122–128.
3. Blikstad, I., F. Markey, L. Carlsson, T. Persson, and U. Lindberg. 1978. Selective assay of monomeric and filamentous actin in cell extracts, using inhibition of Deoxyribonuclease I. *Cell.* 15:935–943.
4. Boller, K., D. Vestweber, and R. Kemler. 1985. Cell adhesion molecule uvomorulin is localized in the intermediate junctions of adult intestinal epithelial cells. *J. Cell Biol.* 100:327–332.
5. Brackenbury, R., J.-P. Thiery, U. Rutishauser, and G. M. Edelman. 1977. Adhesion among neural cells of the chick embryos. I. An immunological assay for molecules involved in cell-cell binding. *J. Biol. Chem.* 252:6835–6840.
6. Brackenbury, R., U. Rutishauser, and G. M. Edelman. 1981. Distinct calcium-independent and calcium-dependent adhesion systems of chicken embryo cells. *Proc. Natl. Acad. Sci. USA.* 78:387–391.
7. Chen, C. S., P. Thiagarajan, S. M. Schwartz, J. M. Harlan, and R. L. Heimark. 1987. The platelet glycoprotein IIb/IIIa-like protein in human endothelial cells promotes adhesion but not initial attachment to extracellular matrix. *J. Cell Biol.* 105:1885–1892.
8. Coffin, J. D., and T. J. Poole. 1988. Embryonic vascular development: identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development (Camb.).* 102:735–748.
9. Damsky, C. H., J. Richa, D. Solter, K. Knudsen, and C. A. Buck. 1984. Identification and purification of a cell surface glycoprotein mediating intercellular adhesion in embryonic and adult tissue. *Cell.* 34:455–466.
10. Edelman, G. M. 1988. Morphoregulatory molecules. *Biochemistry.* 27:3533–3543.
11. Fey, E. G., K. M. Wan, and S. Penman. 1984. Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three-dimensional organization and protein composition. *J. Cell. Biol.* 98:1973–1984.
12. Franke, W. W., H.-P. Kapprell, and P. Cowin. 1987. Immunolocalization of plakoglobin in endothelial junctions: Identification as a special type of *Zonulae adherentes. Biol. Cell.* 59:205–218.
13. Furie, M. B., E. Cramer, B. L. Naprstek, and S. C. Silverstein. 1984. Cultured endothelial cell monolayers that restrict the transendothelial passage of macromolecules and electrical current. *J. Cell Biol.* 98:1033–1041.
14. Gabbiani, G., F. Gabbiani, R. L. Heimark, and S. M. Schwartz. 1984. Organization of actin cytoskeleton during early endothelial regeneration *in vitro. J. Cell Sci.* 66:39–50.
15. Gajdusek, C. M., and S. M. Schwartz. 1984. Technique for cloning bovine aortic endothelial cells. *In Vitro (Rockville).* 19:394–402.
16. Gallin, W. J., G. M. Edelman, and B. A. Cunningham. 1983. Characterization of L-CAM, a major cell adhesion molecule from embryonic liver cells. *Proc. Natl. Acad. Sci. USA.* 80:1038–1042.
17. Gumbiner, B., and K. Simons. 1986. A functional assay for proteins involved in establishing an epithelial occluding barrier: identification of a uvomorulin-like polypeptide. *J. Cell Biol.* 102:457–468.
18. Gumbiner, B., B. Stevenson, and A. Grimaldi. 1988. The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.* 107:1575–1587.
19. Hatta, K., T. S. Okada, and M. Takeichi. 1985. A monoclonal antibody disrupting calcium-dependent cell-cell adhesion of brain tissues: possible role of its target antigen in animal pattern formation. *Proc. Natl. Acad. Sci. USA.* 82:2789–2793.
20. Heimark, R. L., and S. M. Schwartz. 1985. The role of the membrane-membrane interactions in the regulation of endothelial growth. *J. Cell*

Biol. 100:1934–1940.
21. Deleted in proof.
22. Hirano, S., A. Nose, K. Hatta, A. Kawakami, and M. Takeichi. 1987. Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. *J. Cell. Biol.* 105:2501–2510.
23. Kennett, R. H., K. A. Denis, A. S. Tung, and N. R. Klinman. 1977. Hybrid plasmacytoma production: fusion adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. *Curr. Top. Microbiol. Immunol.* 81:77–93.
24. Kern, D. F., and A. B. Malik. 1985. Microvascular albumin permeability in isolated perfused lung: effects of EDTA. *J. Appl. Physiol.* 58:372–375.
25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
26. Larson, D. M., and J. D. Sheridan. 1982. Intercellular junctions and transfer of small molecules in primary vascular endothelial cultures. *J. Cell Biol.* 92:183–191.
27. Magargal, W. W., E. S. Dickinson, and L. L. Slakey. 1978. Distribution of membrane marker enzymes in cultured arterial endothelial and smooth muscle cells. *J. Biol. Chem.* 253:8311–8318.
28. Magnani, J. L., W. A. Thomas, and M. S. Steinberg. 1981. Two distinct adhesion mechanisms in embryonic neural retina cells. I. A kinetic analysis. *Dev. Biol.* 81:96–105.
29. Martinez-Palomo, A., I. Meza, G. Beaty, and M. Cereijido. 1980. Experimental modulation of occluding junctions in a cultured transporting epithelium. *J. Cell Biol.* 87:736–745.
30. Meza, I., G. Ibarra, M. Sabanero, A. Martinez-Palomo, and M. Cereijido. 1980. Occluding junctions and cytoskeletal components in a cultured transporting epithelium. *J. Cell Biol.* 87:746–754.
30a. Newman, P. J., M. C. Berndt, J. Gorski, G. C. White, S. Lyman, C. Paddock, and W. A. Muller. 1990. PECAM-1 (CD31) Cloning and the relation to adhesion molecules of the immunoglobulin gene superfamily. *Science (Wash. DC).* 247:1219–1222.
31. Nose, A., and M. Takeichi. 1986. A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *J. Cell Biol.* 103:2649–2658.
32. Painter, R. G., M. Ginsberg, and B. Jacques. 1982. Concanavalin A induces interactions between surface glycoproteins and the platelet cytoskeleton. *J. Cell Biol.* 92:563–573.
33. Pardanaud, L., C. Altman, P. Kitos, F. Dieterlen-Lievre, and C. A. Buck. 1987. Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development (Camb.).* 100:339–349.
34. Pasdar, M., and W. J. Nelson. 1988. Kinetics of desmosome assembly in Madin–Darby canine kidney epithelial cells: temporal and spatial regulation of desmoplakin organization and stabilization upon cell-cell contact. *J. Cell Biol.* 106:677–685.
35. Peyrieras, N., F. Hyafil, D. Louvard, H. Pleogh, and F. Jacob. 1983. Uvomorulin: a nonintegral membrane protein of early mouse embryo. *Proc. Natl. Acad. Sci. USA.* 80:6274–6277.
36. Reidy, M. A., and S. M. Schwartz. 1981. *En face* morphology of endothelial junctions. *J. Ultrastruct. Res.* 75:363–367.
37. Ross, R. 1971. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell Biol.* 50:172–186.
38. Schwartz, S. M., C. M. Gajdusek, and S. C. Seldin. 1981. Vascular wall growth control: the role of the endothelium. *Arteriosclerosis.* 1:107–161.
39. Shasby, D. M., and S. S. Shasby. 1986. Effects of calcium on transendothelial albumin transfer and electrical resistance. *J. Appl. Physiol.* 60:71–79.
40. Simionescu, M., N. Simionescu, and G. E. Palade. 1976. Segmental differentiation of cell junctions: arteries and veins. *J. Cell Biol.* 68:705–723.
41. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76–85.
42. Takeichi, M. 1977. Functional correlation between cell adhesive properties and some cell surface proteins. *J. Cell Biol.* 75:464–474.
43. Takeichi, M. 1988. The cadherins: Cell-cell adhesion molecules controlling animal morphogenesis. *Development (Camb.).* 102:639–655.
44. Thiery, J. P., A. Delouvee, W. J. Gallin, B. A. Cunningham, and G. M. Edelman. 1984. Ontogenetic expression of cell adhesion molecules: L-CAM is found in epithelia derived from the three primary germ layers. *Dev. Biol.* 102:61–78.
45. Towbin, H. T., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
46. Urushihara, H., and K. M. Yamada. 1986. Evidence for involvement of more than one class of glycoprotein in cell interactions with fibronectin. *J. Cell. Physiol.* 126:323–332.
47. Urushihara, H., H. S. Ozaki, and M. Takeichi. 1979. Immunologic detection of cell surface components related with aggregation of chinese hamster and chick embryonic cells. *Dev. Biol.* 70:206–216.
48. Vestweber, D., and R. Kemler. 1984. Rabbit antiserum against a purified surface glycoprotein decompacts mouse preimplantation embryos and reacts with specific adult tissues. *Exp. Cell Res.* 152:169–178.

49. Vestweber, D., and R. Kemler. 1985. Identification of a putative cell adhesion domain of uvomorulin. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:3393-3399.

50. Volk, T., and B. Geiger. 1986. A-CAM: a 135-kD receptor for intercellular adherens junctions. I. Immunoelectron microscopic localization and biochemical studies. *J. Cell Biol.* 103:1441-1450.

51. Volk, T., and B. Geiger. 1986. A-CAM: a 135-kD receptor for intercellular adherens junctions. II. Antibody mediated modulation of junction formation. *J. Cell Biol.* 103:1451-1464.

52. Wagner, R. C. 1980. Endothelial cell embryology and growth. *Adv. Microcirc.* 9:45-75.

53. Wong, M. K. K., and A. Gotlieb. 1984. *In vitro* reendothelization of a single-cell wound. Role of microfilament bundles in rapid lamellipodia mediated wound closure. *Lab. Invest.* 51:75-81.

54. Wong, M. K. K., and A. I. Gotlieb. 1986. Endothelial cell monolayer integrity. I. Characterization of dense peripheral band of microfilaments. *Arteriosclerosis.* 6:212-219.

55. Volberg, T., B. Geiger, J. Kartenbeck, and W. W. Franke. 1986. Changes in membrane-microfilament interaction in intercellular adherens junctions upon removal of extracellular Ca^{2+} ions. *J. Cell Biol.* 102:1832-1842.

56. Yoshida-Noro, C., N. Suzuki, and M. Takeichi. 1984. Molecular nature of the calcium-dependent cell-cell adhesion system in mouse teratocarcinoma and embryonic cells studied with a monoclonal antibody. *Dev. Biol.* 101:19-27.