EndoCAM: A Novel Endothelial Cell–Cell Adhesion Molecule

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Abstract. Cell–cell adhesion is controlled by many molecules found on the cell surface. In addition to the constituents of well-defined junctional structures, there are the molecules that are thought to play a role in the initial interactions of cells and that appear at precise times during development. These include the cadherins and cell adhesion molecules (CAMs). Representatives of these families of adhesion molecules have been isolated from most of the major tissues. The notable exception is the vascular endothelium. Here we report the identification of a cell surface molecule designated "endoCAM" (endothelial Cell Adhesion Molecule), which may function as an endothelial cell–cell adhesion molecule. EndoCAM is a 130-kD glycoprotein expressed on the surface of endothelial cells both in culture and in situ. It is localized to the borders of contiguous endothelial cells. It is also present on platelets and white blood cells. Antibodies against endoCAM prevent the initial formation of endothelial cell–cell contacts. Despite similarities in size and intercellular location, endoCAM does not appear to be a member of the cadherin family of adhesion receptors. The serologic and protease susceptibility characteristics of endoCAM are different from those of the known cadherins, including an endogenous endothelial cadherin. Although the precise biologic function of endoCAM has not been determined, it appears to be one of the molecules responsible for regulating endothelial cell–cell adhesion processes and may be involved in platelet and white blood cell interactions with the endothelium.

THE lining of blood vessels is formed by the close structural apposition of individual endothelial cells forming a polarized, nonthrombogenic, permeability barrier resting on an underlying basement membrane. This vascular lining is established during development by the formation of distinct junctional complexes between individual cells and between cells and their extracellular matrix. Morphological studies have demonstrated the presence of tight junctions, adherens junctions and gap junctions between adjacent endothelial cells (Schneeberger and Lynch, 1984; Franke et al., 1988), and immunochemical analysis has shown that integrins probably play a major role in anchoring the cells to their basement membrane (Albelda et al., 1989).

The assembly of cells into a flattened polarized layer of tissue requires specific cell–cell interactions. For example, in the developing mouse embryo, compaction must occur before blastomere polarity can develop and before the junctional complexes required for blastocyst formation can be assembled (Richa et al., 1985; Fleming and Johnson, 1988). In cultured epithelial cells, Pasdar and Nelson (1988) have shown that junctional complexes form rapidly after initial cell–cell contact. If epithelial cell–cell junctions are disrupted by lowering the Ca^{2+} concentration, the junctional complexes separate and disappear. Junctional complexes reappear as the cells reaccumulate cadherin-rich sites within the membrane. Their reformation can be blocked by antibodies against a member of the cadherin family (Behrens et al., 1985; Gumbiner et al., 1988). These and other studies imply that members of the cadherin family of receptors play a part in initiation, assembly, and maintenance of polarity of intact monolayers of epithelial cells (Gumbiner et al., 1988; Takeichi, 1988). Although the cadherins are important in the maintenance of cell–cell interactions, it is also possible that the calcium-independent adhesion proteins, represented by the neural cell adhesion molecule N-CAM,[1] may be involved (Edelman, 1988; Rutishauser and Jessel, 1988). Like the cadherins, N-CAM, originally described in nervous tissue, is expressed transiently in many tissues during embryonic development and is also thought to provide important information for cellular association.

Despite the widespread expression of CAMs and cadherins in most tissues, no molecules in endothelial cells have been described that might provide the positioning cues necessary for angiogenesis or the establishment of a polarized, nonpermeable vascular lining (Takeichi, 1988; Heimark and Schwartz, 1988; Hatta et al., 1985). Here, we report the identification of a cell surface molecule that may participate in such a function. The molecule, which we designate as en-

1. *Abbreviations used in this paper*: DFP, diisopropylfluorophosphate; endoCAM, endothelial Cell Adhesion Molecule; N-CAM, neural cell adhesion molecule; TNC, 0.01 M Tris acetate, pH 8.0, 0.5% NP-40, 0.5 mM Ca^{2+}; WGA, wheat germ agglutinin.

doCAM, is expressed on the surface of endothelial cells in culture and in situ, where it is found on both capillary and major vessel endothelium. EndoCAM is a 130-kD cell surface protein that differs from the cadherins both serologically and in its susceptibility to proteases. Although its precise biological functions have not been determined, antibodies against endoCAM block endothelial association, suggesting it is involved in the intercellular adhesion of endothelial cells. In addition, since this molecule appears on platelets and some white blood cells, it may also play a role in platelet-leukocyte-endothelial adhesion.

Materials and Methods

Cell Culture

Endothelial cells were isolated, cultured, and characterized from bovine fetal aortae, human adult iliac arteries or veins, or human umbilical veins as previously described (Rosen et al., 1983; Thornton et al., 1983; Jerrell et al., 1984; Albelda et al., 1989). Briefly, vessel segments were cannulated, incubated with 1 mg/ml collagenase for 10 min and the detached cells were plated into gelatinized tissue culture flasks. Bovine cells were cultured in F12 media supplemented with 10% heat-inactivated FBS. Human endothelial cells were cultured in medium 199 containing 15% heat-inactivated FBS, 75–100 μg/ml endothelial cell growth factor, 100 μg/ml heparin, and 2 mM L-glutamine. The media used for initial isolations also contained 100 μg/ml gentamycin and 4 μg/ml amphotericin B, however, no antibiotics were used after primary isolation. Endothelial cell identity was confirmed by morphology, by immunofluorescent staining for Factor VIII-related antigen, and by detection of angiotensin-converting enzyme activity (Rosen et al., 1983). Madin-Darby bovine kidney epithelial cells (MDBK cells) and human U937 cells were obtained from the American Type Culture Collection (Rockville, MD). All cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. At confluence, cells were subcultured by treatment with trypsin (0.25%)/EDTA (0.4%) in HBSS. All cultures were tested periodically and found to be free of mycoplasma infection.

Platelet Purification

Bovine blood, collected in 3.8% sodium citrate, was centrifuged at 750 *g* for 15 min to produce platelet-rich plasma (PRP). The pH of the PRP was adjusted to 6.5 with citric acid to prevent aggregation and the platelets pelleted by centrifugation at 2,500 *g* for 10 min. After washing, the platelet pellet was extracted as described below.

Antibodies

mAb to bovine endoCAM (Jaffe et al., 1987) was prepared as culture supernatant or from mouse ascites. Polyclonal antiserum to endoCAM was prepared as described below. An antiserum produced against a synthetic peptide containing the 20 carboxy-terminal amino acids of A-CAM was provided by Dr. Benjamin Geiger (Weizman Institute, Rehovot, Israel). This antisera cross-reacts with all of the known cadherins. A mAb against CD31 was generously provided by Dr. Jan Sixma (University Hospital, Utrecht, The Netherlands).

Cell Harvest and NP-40 Extractions

After achieving confluence in gelatinized 75-cm^2 tissue culture flasks, endothelial cells were harvested by scraping. Cell pellets of ~5 × 10^7 cells were frozen at −70°C. Membrane extracts were prepared by adding small amounts (i.e. two to three times the volume of the cell pellet) of 0.01 M Tris acetate, pH 8.0, 0.5% NP-40, 0.5 mM Ca^{2+} (TNC) with 2 mM PMSF to the pellet, pipetting on ice for 15 min, and then centrifuging for 30 min at 12,000 *g*. The resulting supernatant was designated the nonionic detergent extract and frozen at −70°C until used. Some extractions were performed in TNC with addition of 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 mM diisopropylfluorophosphate (DFP) (all purchased from Sigma Chemical Co., St. Louis, MO).

Purification of Bovine EndoCAM

Bovine endoCAM was purified using a combination of lectin affinity and mAb affinity chromatography techniques as previously described (Albelda et al., 1989). Briefly, a water jacketed column (1 × 30 cm) containing 1.5 ml of wheat germ agglutinin (WGA) coupled to Sepharose beads (E.Y. Laboratories, San Mateo, CA) was equilibrated in TNC buffer containing 0.15 M NaCl. Nonionic detergent extracts from bovine endothelial cells were applied to the column. The column was washed extensively with TNC/0.15 M NaCl. Bound material was eluted using 0.2 M *N*-acetyl-D-glucosamine in TNC buffer in 1 ml fractions, which were monitored for the presence of endoCAM by immunoblotting.

Fractions from the WGA column that contained endoCAM were pooled and applied to a water jacketed column containing 1 ml packed volume of anti-endoCAM mAb (Jaffe et al., 1987) coupled to cyanogen bromide sepharose (5 mg antibody/ml of Sepharose). After extensive washing with TNC buffer, bound material was eluted with 1-ml fractions of a solution of 50 mM diethylamine in 0.01 M Tris acetate, 0.5 mM Ca^{2+}, 0.05% NP40 adjusted to pH 11.5. Each fraction was neutralized, dialysed against TNC/0.15 M NaCl overnight at 4°C, and analyzed by SDS-PAGE followed by silver staining. When material was used for the blocking assay (see below), detergent was removed using SM 2 Biobeads (Bio-Rad Laboratories, Richmond, CA) as previously described (Knudsen et al., 1981).

Preparation of Polyclonal Anti-endoCAM Antiserum

Purified endoCAM obtained from the mAb column was injected into rabbits to prepare a monospecific polyclonal anti-endoCAM antiserum. Rabbits were initially injected subcutaneously with 100 μg of protein suspended in CFA. Subsequent injections of 50 μg of purified endoCAM suspended in incomplete adjuvant were made at 10-d intervals for a total of seven injections. Rabbit serum was tested for activity by immunoblotting and immunoprecipitation against bovine and human endothelial cell extracts.

Labeling of Cells

For ^{125}I-labeling, intact monolayers of cells in 75-cm^2 tissue-culture flasks were washed with PBS and exposed sequentially to 100 U/ml of lactoperoxidase (Sigma Chemical Co., St. Louis, MO), 1 mCi of carrier-free ^{125}I (Amersham Chemical Co., Arlington Heights, IL), and three, 40-μl aliquots of 0.06% hydrogen peroxide. The cells were harvested and extracted as described above.

For ^{35}S labeling, cells in 75-cm^2 flasks were washed with PBS and then exposed to DME without methionine in the presence of dialyzed 10% FBS for 1 h. Fresh media containing 250 μCi of [^{35}S]methionine (New England Nuclear, Boston, MA) was then added. After 24 h, the cells were harvested and extracted as described above.

Immunoprecipitation

Nonionic detergent extracts were preadsorbed for 30 min at 4°C with Protein A conjugated to Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ). The appropriate antibody was then added to the extract for 1 h at 4°C. Immunocomplexes were collected by precipitation with Protein A-Sepharose beads for 1 h at 4°C, washed five times with a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5% deoxycholate, and 0.1% SDS. The sample was then dissolved in electrophoresis sample buffer (62.5 mM Tris base, 2% SDS, 10% glycerol, pH 6.8), electrophoresed on 7% polyacrylamide gels and processed for autoradiography as described below.

Gel Electrophoresis and Immunoblotting

Samples were analyzed by SDS-PAGE using 6% polyacrylamide gels by the method of Laemmli (1970) without the use of reducing agents. Gels were dried and exposed to Kodak XR-5 x-ray film at −70°C, processed for Western immunoblotting or stained for protein using the silver nitrate reagent and methods previously described (Albelda et al., 1989). For immunoblot analysis, an alkaline phosphatase-coupled secondary antibody (Promega Biotec, Madison, WI) was used to detect specific antibody binding to proteins on nitrocellulose paper.

Trypsin and Calcium Sensitivities of Cell Adhesion Molecules

To test endoCAM sensitivity to degradation by varying concentrations of trypsin in the presence and absence of calcium ions, confluent 25-cm^2 flasks of bovine endothelial cells and bovine epithelial cells (MDBK cells) were incubated at 37°C for 15 min in 2.5 ml of one of the following four

solutions: (*a*) 1 mM EGTA (E); (*b*) 0.01% trypsin (Worthington Biochemicals, Freehold, NJ) plus 1 mM EGTA (TE); (*c*) 0.0001% trypsin plus 1 mM EGTA (LTE); or (*d*) 0.01% trypsin plus 10 mM $CaCl_2$ (TC) in 10 mM Hepes-buffered saline (Takeichi, 1977). 5 ml of 0.05% soybean trypsin inhibitor (Worthington Biochemicals) was added to each flask, cells were harvested, washed twice, and extracted in TNC/PMSF as described above. Extracts were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-endoCAM or anti-cadherin antibodies.

Bioassay

To test the effect of polyclonal anti-endoCAM antibodies on the establishment of cell–cell adhesion, bovine endothelial cells were treated for 3 min at 37°C with trypsin (0.25%)/EDTA (0.4%) and plated at a density of 7.5 × 10^4 cells/cm^2 into 2 cm^2 tissue culture wells in 1 ml of F12 media containing 10% serum. After allowing cell adhesion to proceed for 1 h, 700 μl of media was removed and varying amounts of preimmune rabbit serum or anti-endoCAM antisera were added. After 18 h, the cell layers were washed once and photographed.

Blocking Assay

To confirm the specificity of our polyclonal antibody effects, endothelial cells were observed in the presence of antibody plus highly purified endoCAM. Endothelial cells were plated as above. After 1 h, 850 μl of media was removed and replaced with 150 μl of material eluted from the mAb column that had been rendered detergent-free via treatment with Biobeads. As a control, buffer from a fraction of the column that contained no endoCAM by silver staining or immunoblotting was used. Anti-endoCAM antibody was added at a dilution of 1:50 and the cells observed and photographed after 18 h.

Immunofluorescence

Endothelial cells were plated at 4 × 10^4 cells/cm^2 on glass coverslips coated with 10 μg/ml of human fibronectin (Bethesda Research Laboratories, Bethesda, MD) or 1% gelatin in PBS (Difco Laboratories, Detroit, MI). After the cells had grown to confluence, fixation and staining was performed using previously described methods (Albelda et al., 1989). Briefly, cells were fixed with 3% paraformaldehyde for 20 min and then permeabilized with 0.5% NP40 for 1 min. After extensive washing, 50 μl of antibody or preimmune serum was added for 1 h, and after rinsing, the coverslips were stained with 50 μl of a 1:200 dilution of fluorescein-labeled anti-mouse or anti-rabbit antibodies for 1 h. Cells were viewed on a Zeiss phase-epifluorescent microscope using a 63× planapochromat oil-immersion lens NA 1.4 and photographed using Tri-X film at 400 ASA.

Results

Identification of an Endothelial Cell–Cell Adhesion Molecule (EndoCAM)

An mAb (D2) has been previously described which reacts with a 130-kD protein on the surface of bovine endothelial cells (Jaffe et al., 1987). The protein is found mainly at cell–cell borders and hence is a good candidate for a molecule which plays a role in establishing cell–cell interactions. To examine this possibility, the protein was purified from a nonionic detergent extract of bovine endothelial cells by a combination of wheat germ and mAb affinity chromatography. The purified protein was then injected into rabbits to produce a broad-spectrum anti-endoCAM antiserum. The specificity of this antiserum is shown in Fig. 1. ^{125}I-surface-labeled bovine endothelial cells were extracted with TNC and aliquots were immunoprecipitated with preimmune serum (Fig. 1, lane *B*), the polyclonal anti-endoCAM (Fig. 1, lane *C*), or the mAb D2 (Fig. 1, lane *D*). The polyclonal anti-endoCAM immunoprecipitated a single 130-kD protein that migrated in precisely the same region on SDS-PAGE as that precipitated by the D2 monoclonal antibody. A similar protein was immunoprecipitated from extracts of human adult endothelial cells (Fig. 1, lane *E*). Further evidence that these antibodies reacted with the same molecule was provided in immunoclearing experiments described below.

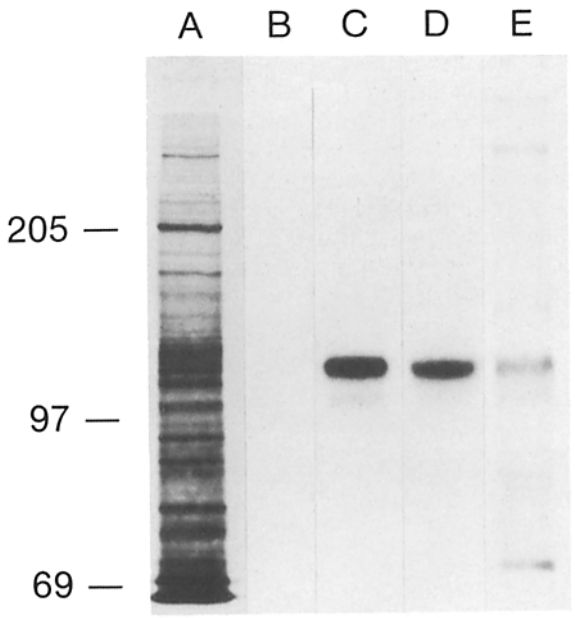

Figure 1. Immunoprecipitation of extracts from bovine and human endothelial cells using anti-endoCAM antibodies. Immunoprecipitates of nonionic detergent extracts from ^{125}I-labeled endothelial cells were isolated and subjected to SDS-PAGE and autoradiography. Lane *A*, unprecipitated extract from bovine endothelial cells; lane *B*, precipitate of bovine endothelial cell extract using preimmune control serum; lane *C*, immunoprecipitate of bovine endothelial cell extract using polyclonal anti-endoCAM; lane *D*, immunoprecipitate of bovine endothelial cell extract using monoclonal anti-endoCAM; lane *E*, immunoprecipitate of human endothelial cell extract using polyclonal anti-endoCAM. Molecular mass markers designated in kilodaltons are on the left.

EndoCAM Is Localized to Endothelial Cell–Cell Borders

To further confirm the fact that the polyclonal anti-endoCAM reacted with the same material as the mAb, endothelial cells were examined by immunofluorescent microscopy using both antibodies. As can be seen in Fig. 2 *A*, polyclonal anti-endoCAM reacted with a protein concentrated at the borders between closely apposed bovine endothelial cells. The concentrated staining was seen only at sites of cell–cell adhesion. Under identical conditions, preimmune serum from the same rabbit did not react with bovine endothelial cells (Fig. 2 *B*). The polyclonal antibody displayed similar reactivity with cultured human endothelial cells (Fig. 2, *C* and *D*). These patterns are identical to those seen in bovine endothelial cells stained with the monoclonal antibody D2 (Jaffe et al., 1987).

A similar staining pattern was noted in frozen sections of bovine tissue (Fig. 3). In this case, a section through the bovine thymus showed cells lining a small artery and a large vein. In both cases, the anti-endoCAM-stained material localized primarily between adjacent cells. Thus, endoCAM was found concentrated at intercellular contacts both in culture and within tissue. EndoCAM was expressed only by endothelial cells of the vessel lining. This is evident in Fig. 3 where no anti-endoCAM reaction was noted with mesenchy-

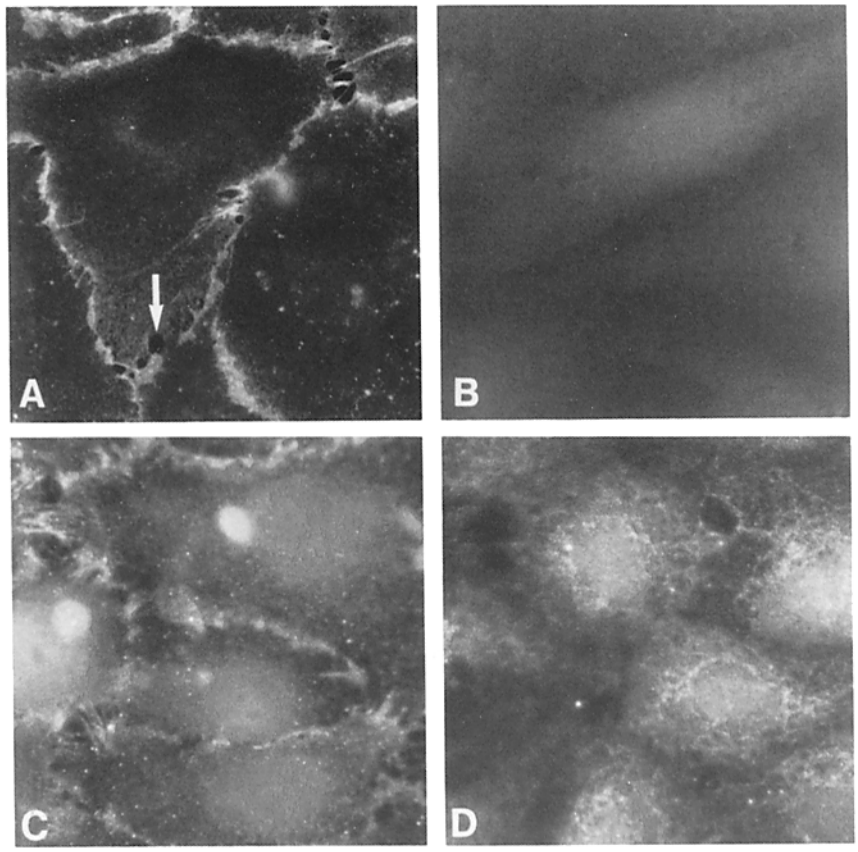

Figure 2. Localization of endoCAM on cultured endothelial cells by indirect immunofluorescence. Bovine aortic endothelial cells (*A* and *B*) or human umbilical vein endothelial cells (*C* and *D*) were grown to confluence on coverslips. The cells were fixed, permeabilized, and exposed to anti-endoCAM polyclonal antibody (*A* and *C*) or preimmune rabbit serum (*B* and *D*). The cells were then treated with FITC-conjugated anti-rabbit antibodies and photographed through a microscope equipped for epifluorescence (×1,600). *Arrow*: areas where the cells are not in contact show no immunofluorescence.

mal or smooth muscle cells of the artery. In sections from other bovine tissues, no material reacting with anti-endoCAM was seen on epithelial cells.

Requirement of EndoCAM for the Establishment of Initial Cell-Cell Interactions

The intercellular location of endoCAM suggested that it might function in the establishment or maintenance of endothelial cell–cell interactions. If this were the case, antibodies against endoCAM should prevent the association of endothelial cells with one another in much the same manner as anti-cadherins have been shown to interfere with epithelial cell–cell adhesion (Damsky et al., 1983). To examine this possibility, bovine endothelial cells were plated in the presence of preimmune serum (Fig. 4 *A*) or polyclonal anti-endoCAM (Fig. 4 *B*). The following day, the cultures were examined for the extent of cell–cell association that had occurred. In the presence of preimmune serum, the expected monolayer of closely associated endothelial cells was formed. In contrast, in the presence of anti-endoCAM, the monolayer was almost fibroblastic in appearance, showing no signs of the close cellular associations characteristic of endothelial cells. The antibody effect was completely reversible, thereby ruling out antibody-induced toxicity. To confirm the specificity of the effect of the polyclonal antibody, highly purified endoCAM (see Fig. 8 *E*) was added simultaneously with the anti-endoCAM antibody. The purified material inhibited the ability of the antibody to disrupt cell–cell interaction (Fig. 4 *C*). Control material from a similarly treated fraction of the mAb column which contained no purified endoCAM, did not block the anti-endoCAM effect (Fig. 4 *D*). These results indicate that endoCAM may be involved in the establishment of cell–cell interactions in endothelial cells.

Comparison of endoCAM to Other Cell Adhesion Molecules

Size, intercellular location, and involvement in cell–cell adhesion suggested that endoCAM might be similar to either the cadherins (Takeichi, 1988) or a molecule previously reported to be present on platelets, some white blood cells and at the intercellular junctions of endothelial cells that has subsequently been designated as CD31 (van Mourik et al., 1985; McMichael et al., 1987).

For the purpose of these comparisons, nonionic detergent extracts were prepared from bovine endothelial cells, bovine kidney epithelial cells (MDBK), and bovine platelets. The extracts were subjected to SDS-PAGE and immunoblotted using antisera reactive with the particular proteins in question. The monoclonal and polyclonal anti-endoCAM antibodies reacted strongly with 130- and 120-kD material in the extracts of endothelial cells (Fig. 5, *middle* and *right*). In contrast, these antibodies failed to react with any material in the MDBK extracts (Fig. 5, *middle* and *right*). In addition, the monoclonal anti-endoCAM antibody reacted with a protein of similar size in an extract of bovine platelets (Fig. 5, *middle*). The presence of cadherins in the various cell extracts was confirmed using an anti-cadherin antiserum produced against a peptide corresponding to the cytoplasmic

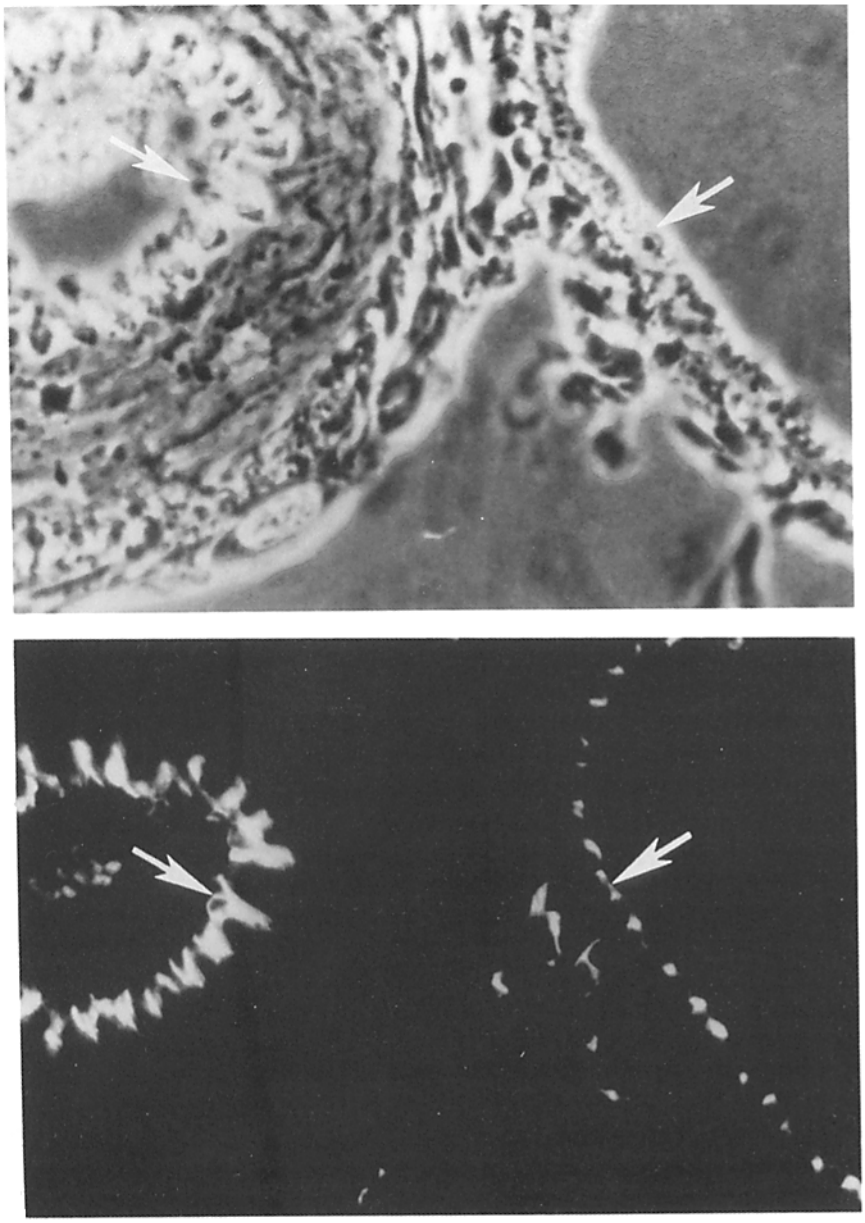

Figure 3. Localization of endoCAM in bovine tissue. Cryostat sections of bovine thymic tissue were labeled with endoCAM mAb and counterstained with FITC-conjugated anti-mouse antibodies. (*Top*) Phase micrograph of a thymic artery (*left*) and vein (*right*). (*Bottom*) immunofluorescent micrograph of same section demonstrating that endoCAM localized only to the endothelial layer of each vessel (*arrows*). No staining of smooth muscle, intima, or thymic parenchyma is seen.

domain of A-CAM or N-cadherin (Takeichi, 1988). The conserved nature of the cytoplasmic domain allows the antibody to react with all known cadherins. The anti-cadherin antibody reacted with molecules of similar size in extracts from both MDBK and endothelial cells (Fig. 5, *left*).

Both 130- and 120-kD material was detected in nonionic detergent extracts that reacted with anti-endoCAM. The amount of this material varied from preparation to preparation depending upon extraction conditions suggesting that it represented breakdown products of the 130-kD molecule. This was confirmed by comparing immunoblots of bovine endothelial cells extracted in the presence of PMSF alone or with a mixture of protease inhibitors (Fig. 6). The mixture of protease inhibitors markedly reduced the amount of 120-kD material reactive with either monoclonal or polyclonal endoCAM (Fig. 6, lane *A* and *B* vs. lane *C*).

Because both anti-cadherin and anti-endoCAM antibodies reacted with 130-kD proteins in endothelial cell extracts, it was impossible to determine if they were reacting with the same or different molecules, even though anti-endoCAM did not recognize any material in the MDBK extracts. Therefore, endoCAM was isolated from endothelial cells, and equal amounts of material were subjected to SDS-PAGE. Portions of the gel were either silver stained or transferred and subsequently immunoblotted using anti-endoCAM and anti-cadherin antibodies. As is shown in Fig. 7, both the polyclonal anti-endoCAM (Fig. 7 *A*) and monoclonal anti-endoCAM (Fig. 7 *B*) reacted with the purified endoCAM. No reactivity was seen using preimmune serum (Fig. 7 *C*) or anti-cadherin antibody (Fig. 7 *D*). Some presumed breakdown products were noted reacting with the polyclonal anti-endoCAM and to some extent with the monoclonal anti-endoCAM. This material was not visible in silver-stained preparations of the purified endoCAM (Fig. 7 *E*).

To further confirm that the anti-endoCAM and anti-cadherin antibodies were reacting with different molecules,

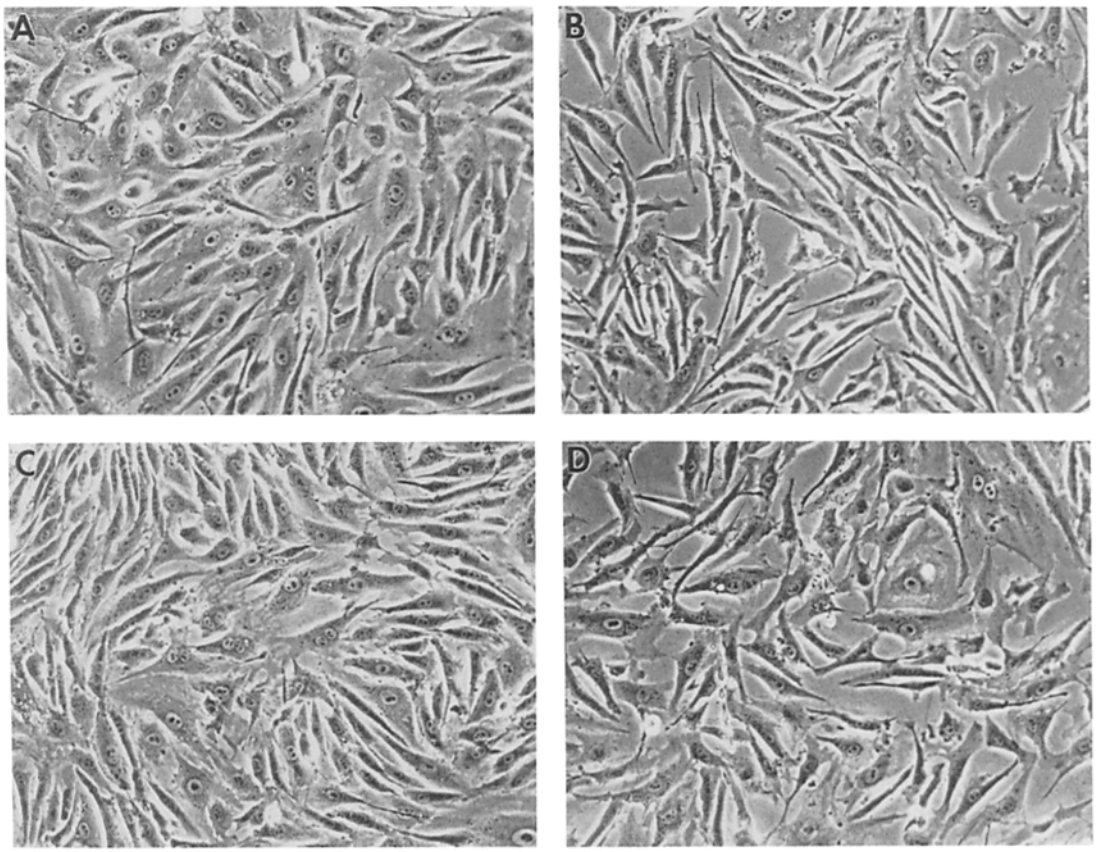

Figure 4. Effect of anti-endoCAM antibodies on the establishment of endothelial cell–cell interactions. Bovine aortic endothelial cells were plated in the presence of preimmune serum (*A*), or anti-endoCAM antiserum (*B*, *C*, and *D*). After 24 h, the cells were photographed. In the presence of preimmune serum (*A*), the cells adhere well and form a typical "cobblestone"-appearing monolayer. In contrast, in the presence of anti-endoCAM antibody (*B*), the endothelial cells adhere normally to the plate but fail to establish cell–cell contacts and take on a "fibroblastoid" appearance. When a preparation of endoCAM purified by monoclonal affinity column was added concurrently with the anti-endoCAM antiserum, the cell-cell inhibitory effect was blocked (*C*). Addition of material obtained from the same affinity column, yet lacking detectable endoCAM, had no blocking effects (*D*).

immunoclearing experiments were performed. Extracts from endothelial cells were exhaustively preadsorbed with the anti-endoCAM mAb or a control, irrelevant monoclonal antibody of the same IgG class (Fig. 8). Unadsorbed extracts and samples of the extract adsorbed with each of the antibodies were subjected to SDS-PAGE (nonreduced) and then immunoblotted with anti-endoCAM mAb (*left*), anti-endoCAM polyclonal antibody (*middle*) or anti-cadherin antibody (*right*). Preadsorbtion with anti-endoCAM mAb completely removed any material in the 120–130-kD region of the gel that reacted with either anti-endoCAM antibody (Fig. 8, *left* and *middle*), confirming that both the monoclonal and polyclonal antibody react against the same molecular species. The bands visible in lane *B* of the left panel of Fig. 8 do not represent endoCAM, but residual murine antibody identified by the secondary alkaline phosphatase-coupled anti-mouse IgG antibody used for the immunoblot analysis. In contrast to the total removal of endoCAM by the monoclonal antibody, material reactive with anti-cadherin antibody remained in all extracts (Fig. 8, *right*).

One characteristic feature of the cadherin family of cell adhesion proteins is a sensitivity to degradation by trypsin, which can be blocked by the addition of calcium ions to the reaction mixture (Takeichi, 1988). EndoCAM was examined for similar properties. Monolayers of bovine endothelial cells were exposed to EGTA, EGTA and trypsin, or trypsin plus Ca^{2+} for 15 min. The cells were then extracted with NP-40 and the extracts subjected to SDS-PAGE. Either endoCAM or the endogenous cadherin was identified using anti-endoCAM or anti-cadherin antibodies, respectively. As a positive control, monolayers of MDBK cells were exposed to identical conditions. The immunoblots of epithelial cell extracts with anti-cadherin antibody clearly showed proteins of 130 kD in EGTA and trypsin/calcium-treated cells (Fig. 9 *A*, lanes *E* and *TC*) but not in cells treated with either 0.01% or 0.0001% trypsin in the presence of EGTA (Fig. 9 *A*, lanes *TE* and *LTE*). Thus, as expected, the MDBK cadherin was protected from trypsin digestion by the presence of calcium. In contrast, the immunoblots of extracts from EGTA treated endothelial cells prepared using anti-endoCAM antibody (Fig. 9 *B*) showed that the 130- and 120-kD bands reactive with this antibody (Fig. 9 *B*, *E*), were sensitive to digestion by trypsin in the presence (Fig. 9 *B*, *TC*) and absence (Fig. 9 *B*, *TE*) of calcium. Unlike the epithelial cadherin, endoCAM was resistant to degradation by low concentrations of trypsin (0.0001%) in the presence of EGTA (Fig. 9 *B*, *LTE*). When the immunoblots of the endothelial cells were performed using anti-cadherin, somewhat different results were

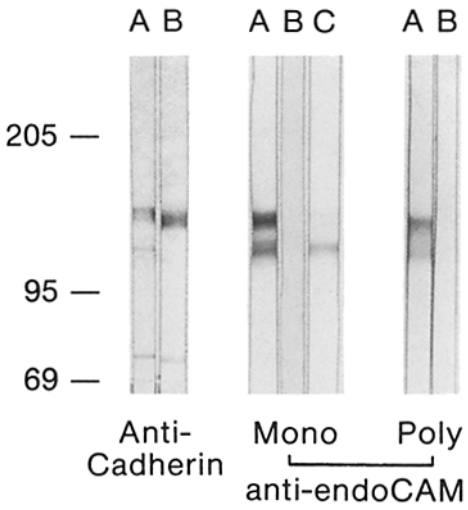

Figure 5. Immunoblot analysis of extracts of endothelial cells, epithelial cells and platelets using anti-endoCAM antibodies. Nonionic detergent extracts of bovine aortic endothelial cells (*A*), bovine kidney epithelial cells (*B*), or bovine platelets (*C*) were subjected to SDS-PAGE, transferred to nitrocellulose and then immunoblotted with an anti-pan-cadherin antibody (*left*), monoclonal anti-endoCAM antibody (*middle*) or polyclonal antibody against endoCAM (*right*). The anti-cadherin antibody recognizes a 130-kD protein on both endothelial and epithelial cells. In contrast, anti-endoCAM antibodies do not react with epithelial cells, but do recognize proteins of identical mass in endothelial and platelet extracts.

noted (Fig. 9 *C*). No material was visible after either trypsin/EGTA, low trypsin/EGTA or trypsin/CA^{2+} treatment. This suggests that the endothelial cell cadherin-like protein behaved in a manner similar to that reported for A-CAM

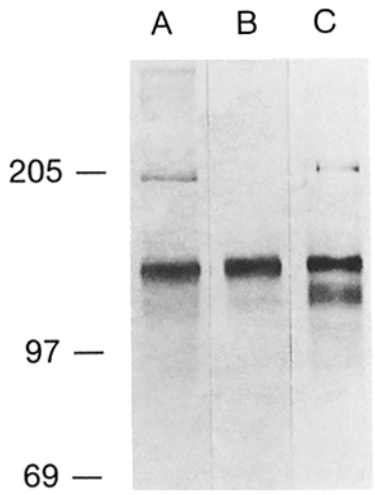

Figure 6. Effects of protease inhibitors on endoCAM integrity. Bovine endothelial cells were extracted in TNC buffer (see Materials and Methods) containing a mixture of protease inhibitors including 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 mM EDTA, and 5 mM DFP (lanes *A* and *B*) or 2 mM PMSF (lane *C*) and subjected to SDS-PAGE on 7% gels under nonreducing conditions and transferred to nitrocellulose for immunoblot analysis. Lanes *A* and *C*, immunoblot using polyclonal anti-endoCAM; lane *B*, immunoblot using monoclonal anti-endoCAM.

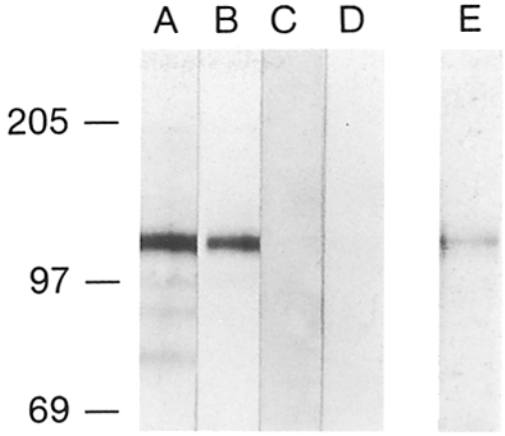

Figure 7. Immunoblot analysis of purified endoCAM using anti-cadherin and anti-endoCAM antibodies. EndoCAM was purified from bovine endothelial cells by lectin and antibody affinity chromatography. Equal amounts of material were subjected to SDS-PAGE on 7% gels under nonreducing conditions and silver stained or transferred to nitrocellulose for immunoblot analysis. Lane *A*, immunoblot using polyclonal anti-endoCAM; lane *B*, immunoblot using monoclonal anti-endoCAM; lane *C*, immunoblot using preimmune serum; lane *D*, immunoblot using anti-pan-cadherin antibody; lane *E*, silver stain of purified endoCAM.

(Volk and Geiger, 1986). The differential protease sensitivities of the epithelial and endothelial cadherins when compared with endoCAM, further document the differences between these proteins and endoCAM.

To explore the relationship of endoCAM to the protein identified by van Mourik et al. (1985) and the CD31 antigen, the ability of anti-endoCAM antibody to react with other cell types was studied. The anti-endoCAM mAb reacted with proteins of a similar size as seen in endothelial cells in the extracts from bovine platelets (Fig. 5, *middle*). A more precise comparison between endoCAM and the platelet protein reported by van Mourik et al. (1985) was not possible, as the mAb against this protein would not react with bovine material. However, by using an mAb against CD31 (generously provided by Dr. Jan Sixma), it was possible to more directly determine if CD31 and endoCAM represented similar molecules and at the same time determine if endoCAM was present on white blood cells. To accomplish this, human U937 cells were labeled with [^{35}S]methionine and immunoprecipitated with preimmune serum, anti-CD31 mAb antibody and polyclonal anti-endoCAM. Fig. 10 shows that U937 cells contain a 130-kD protein recognized by the anti-CD31 mAb (Fig. 10 *B*) and that a protein of the same size is identified with our anti-endoCAM antibody (Fig. 10 *C*). When the extracts were adsorbed with anti-CD31 antibodies before immunoprecipitation, no material reactive with either CD31 or endoCAM remained (data not shown). Thus, endoCAM and CD31 appear to be related.

Discussion

The endothelium is composed of a dynamic group of cells organized along the inner surface of blood vessels forming a polarized, nonthrombogenic, highly specialized mono-

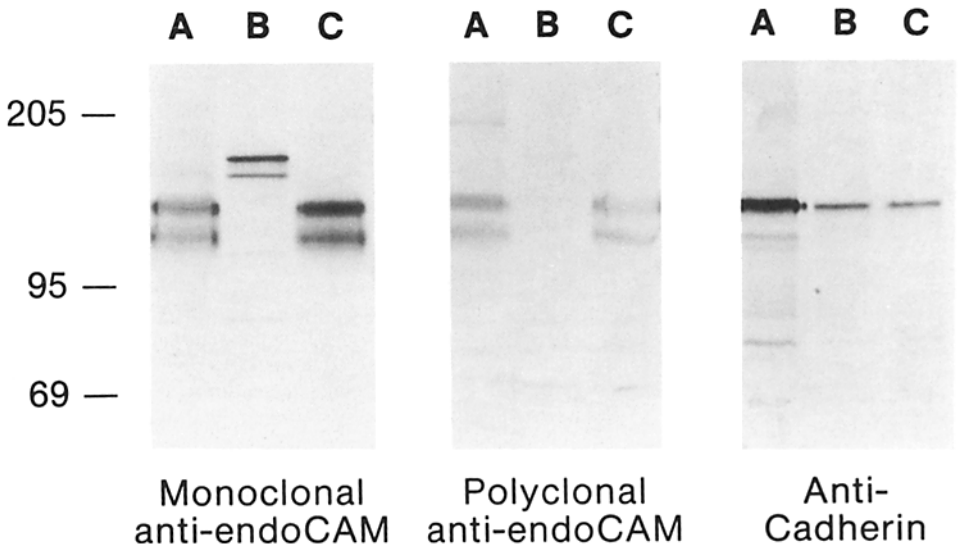

Figure 8. Immunoblot analysis of endothelial cell extracts preadsorbed with anti-endoCAM antibodies. Nonionic detergent extracts from bovine endothelial cells were exhaustively immunoprecipitated with a mAb against endoCAM or a nonreactive control mAb. The total cell extract (lanes marked *A*), the supernatants preadsorbed with endoCAM (lanes marked *B*), or the supernatants preadsorbed with irrelevant mAb (lanes marked *C*) were subjected to SDS-PAGE, and transferred to nitrocellulose. All lanes were immunoblotted with either monoclonal anti-endoCAM (*left*), polyclonal rabbit anti-endoCAM (*middle*) or a polyclonal rabbit anti-pan-cadherin (*right*). The two molecular higher weight bands seen in lane *B*, *left*, represent residual mouse antibodies reacting with the secondary rabbit-anti-mouse antibodies used in the immunoblot.

layer. They possess well-defined junctional complexes that prevent the free flow of fluids between cells, but which also allow intercellular communication. They interact with platelets and leukocytes at sites of injury and in some cases possess receptors required for lymphocyte homing (Harlan, 1985; Stoolman, 1989). Thus, endothelial cells must contain molecules which control endothelial cell interactions, initiate the formation of polarity and junctional complexes and react with other cell types. The molecule isolated here, designated endoCAM (endothelial Cell Adhesion Molecule) has properties that might be expected of a molecule involved in one or more of these processes.

EndoCAM is concentrated at sites of endothelial cell–cell junctions both in culture and in tissue (Figs. 2 and 3). Immunohistochemistry suggests that endoCAM is found on endothelial cells lining major vessels, as well as capillaries. In subconfluent cultures, endoCAM is seen by immunofluorescence to be uniformly distributed over the cell surface. After the monolayer has reached confluency, endoCAM is seen only at regions of cell–cell contact. The concentration of endoCAM in these regions appears to be a specific response to contact with other endothelial cells. In mixed cultures of epithelial and endothelial cells, the two cell types remain clearly segregated. However, at the point of endothelial–epithelial cell contact, endoCAM is not found, suggesting that the molecules responsible for initiating intercellular adhesion on each cell type are functionally distinct (Albelda, S. M., and L. Romer, unpublished observations).

EndoCAM is expressed at the cell surface, as evidenced by the fact that it could be labeled by lactoperoxidase-catalyzed iodination (Fig. 1) and appears to be an integral membrane protein as it could only be extracted using detergents. Its function as an intercellular adhesion molecule was suggested by the fact that the polyclonal antibody specific for endoCAM was able to prevent the association of endothelial cells into a typical cobblestone-like monolayer. This effect was blocked by purified endoCAM, but not with extracts from which endoCAM was absent (Fig. 4). A similar assay was used to detect the presence of cadherins on epithelial cells in culture (Damsky et al., 1983) and to implicate them in the initiation of cell–cell adhesion. These observations suggest that endoCAM is involved in initial endothelial cell–cell adhesion, although its precise role remains to be determined. Unlike anti-cadherin antibodies (Behrens et al., 1985), however, addition of anti-endoCAM antibodies do not disrupt confluent cell monolayers.

The relationship of endoCAM to other known cellular adhesion molecules is an important issue. EndoCAM is not a member of the integrin family, despite the fact that two immunoreactive bands are frequently seen in immunoblots (Fig. 6). It is not found in adhesion plaques (Fig. 2). It does not react with antibodies against any of the known integrin subunits (Albelda, S. M., and C. A. Buck, unpublished observations), and immunoprecipitation analysis does not reveal the presence of a second noncovalently associated subunit (Fig. 1).

Superficially, endoCAM resembles a cadherin. It is similar in size, intercellular location and possible biologic function. Anti-endoCAM antibodies, like anti-cadherin antibodies block cell–cell interactions. However, endoCAM appears to be distinct from known cadherins by several criteria. First, an antibody that recognizes all known cadherins did not react with purified endoCAM (Fig. 7). Second, complete removal of all endoCAM from endothelial cell extracts by immunoadsorption did not remove any significant amount of material reactive with the anti-pan-cadherin antibody (Fig. 8). Third, the two molecules exhibited different sensitivities to proteases (Fig. 9). Endothelial cells do, however, possess

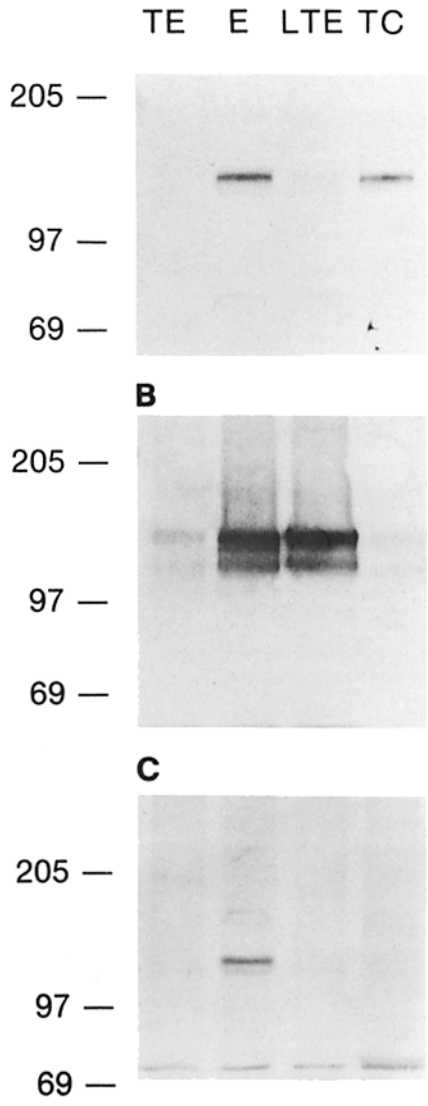

Figure 9. Protease sensitivities of endoCAM and cadherins. Bovine epithelial (MDBK) cells (*A*) or bovine endothelial cells (*B* and *C*) were exposed to 0.01% trypsin/EGTA (*TE*), EGTA (*E*), 0.0001% trypsin/EGTA (*LTE*) or trypsin plus calcium (*TC*) for 30 min, extracted with NP-40 and subjected to SDS-PAGE and immunoblot analysis. Group A, epithelial cell extracts immunoblotted with anti-pan-cadherin; group B, endothelial cells immunoblotted with anti-endoCAM; group C, endothelial cells immunoblotted with anti-pan-cadherin. Molecular mass markers in kilodaltons are designated on the left.

a protein that cross-reacts with the pan-cadherin antibody (Fig. 5). This finding supports a preliminary report by Heimark and Schwartz (1988) describing a potential endothelial cell cadherin. How endoCAM and the endothelial cadherins might function together to regulate endothelial cell–cell adhesion remains to be determined. It is possible that they work cooperatively in the formation of initial cell–cell contacts. Such cooperativity would resemble that reported for adhesive interactions occurring between T cells and their targets, where both integrins and T cell antigen recognition receptors must react with their respective ligands for effective binding to become established (Dustin and Springer, 1989). A similar interaction may occur in focal contact formation where proteoglycans and integrins must both bind to their respective ligands before a stable focal contact is formed (LeBaron et al., 1988).

The size, location, and tissue distribution of endoCAM also suggest that it may be related to a previously described cell surface protein common to endothelial cells, granulocytes, monocytes, some lymphocytes and platelets (Ohto et al., 1985; van Mourick et al., 1985; Goyert et al., 1986). This molecule has been designated CD31 (McMichael et al., 1987). Ohto et al., (1985) further suggest that CD31 is involved in endotoxin-stimulated granulocyte chemotaxis. Like CD31, endoCAM is found on platelets and white cells. Immunoblot analysis of bovine platelets using the anti-endoCAM mAb revealed a cross-reacting protein of similar size to endoCAM (Fig. 5). Like endoCAM, this protein was rapidly degraded from 130 to ~120 kD upon extraction. Similarly, both anti-endoCAM and anti-CD31 reacted with a 130-kD molecule on the surface of the human myelomonocytic cell line U937 (Fig. 10). These data suggest that endoCAM may also be related to the 135-kD protein described on human cells by Newman (1990) and Muller et al. (1989). This protein is an integral membrane protein restricted to the cell–cell borders of cultured human endothelial cells and endothelial cells in a variety of tissue sections, but which is also present on platelets and white blood cells. We have exchanged antibodies with these laboratories and shown that our polyclonal endoCAM antibody reacts with an identical protein in extracts of human endothelial cells. Although more rigorous analysis is still required to confirm the precise relationship of CD31 and endoCAM, it is clear that an endoCAM-like molecule exists on platelets and white blood cells, and that these molecules are serologically related.

Several other molecules of similar size and tissue distribution to endoCAM have been described. These include PADGEM, a platelet activation–dependent granule-external

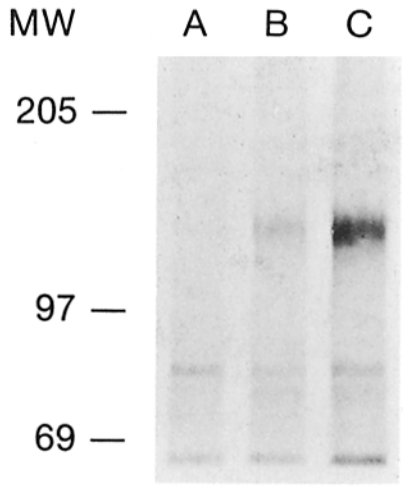

Figure 10. Immunoprecipitation of human U937 cells with anti-endoCAM and anti-CD31 antibodies. Human U937 cells were labeled with [^{35}S]methionine and immunoprecipitated with preimmune serum (lane *A*), a monoclonal antibody against CD31 (lane *B*), or anti-endoCAM polyclonal antibody (lane *C*). Both antibodies identify a protein migrating in the 130-kD region of the gel.

membrane protein also known as GMP-140 that is found on platelets, megakaryocytes, and endothelial cells (Johnston et al., 1989; Larsen et al., 1989). However, the distribution of PADGEM, i.e. within the Weibel-Palade bodies (Bonfanti et al., 1989), clearly differentiates it from endoCAM. Structurally, PADGEM is similar to the endothelial leukocyte adhesion molecule ELAM-1 (Bevilacqua et al., 1989) and the murine lymphocyte homing receptor MEL-14 (Siegelman et al., 1989). These molecules all mediate the interaction of leukocytes or platelets with endothelial cells and hence could be at least functionally related to endoCAM. Whether endoCAM is a member of this family of molecules remains to be determined. EndoCAM, however, is not restricted to certain types of endothelial cells as is MEL-14, and is constitutively expressed on endothelial cells, in contrast to ELAM-1 that is present only after endothelial cell activation, making it likely that endoCAM is distinct from these molecules.

In summary, the data presented here suggest that endoCAM functions in the endothelial cell-cell adhesion process and is likely related to the molecule defined by anti-CD31 antibodies. The presence of endoCAM on leukocytes and platelets and the implication of an endoCAM-related molecule in chemotaxis (Ohto et al., 1985) suggest possible additional functions. EndoCAM may well be among the many molecules involved in thrombogenesis and wound healing, as well as in leukocyte–endothelial cell interactions. Its precise role in these events and relationship to other molecules will be better understood once the complete cDNA sequence is known.

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Note Added in Proof. Newman et al. (1990) have recently cloned the human protein identified by anti-CD31 antibodies and found that it is a member of the immunoglobulin superfamily. In collaboration with this group, we have expressed the full-length cDNA encoding for PECAM-1 in COS-7 cells and found that the protein produced localizes at cell-cell borders and cross-reacts with the polyclonal endoCAM antibody described here. This protein thus represents the human homologue of endoCAM. (Newman, P. J., M. C. Berndt, J. Gorsk, G. C. White II, S. Lyman, C. Paddock, and W. A. Muller. 1990. Molecular cloning of PECAM-1 [CD31]: a novel member of the immunoglobulin gene superfamily expressed on human platelets and at endothelial cell intercellular junctions. *Science [Wash. DC]*. In press.)

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