ELSEVIER

Brain Research 747 (1997) 103–113

BRAIN RESEARCH

Research report

Modulation of cellular adhesion in bovine brain microvessel endothelial cells by a decapeptide

Dhananjay Pal, Kenneth L. Audus, Teruna J. Siahaan *

Department of Pharmaceutical Chemistry, University of Kansas, 2095 Constant Avenue, Lawrence, KS 66047, USA

Accepted 8 October 1996

Abstract

The importance of cell adhesion molecules in maintaining the cellular integrity of the endothelial layer is well recognized, yet their exact participation in regulating the blood–brain barrier (BBB) is poorly understood. Both Ca^{2+}-dependent and Ca^{2+}-independent cell adhesion molecules are found in endothelial cells. In this study, we used immunofluorescence, ELISA, Western blot and cell adhesion assay to identify a Ca^{2+}-dependent cell adhesion molecule, E-cadherin, in bovine brain microvessel endothelial cells (BBMECs). Monoclonal anti-E-cadherin antibody specifically interacted with cultured BBMECs and decorated the cellular junctions with a series of punctate fluorescence spots as seen by indirect immunofluorescence using a confocal microscope. The intensity of these fluorescence spots increased after brief treatment with hIFN-γ or CPT-cAMP. In the cellular extract of BBMECs, a 120 kDa protein was immunoprecipitated with anti-E-cadherin antibody. BBMECs did not react with anti-N-cadherin antibody, but recognized the FITC-labeled LRAHAVDVNG-NH_2, a decapeptide generated from the EC-1 domain of N-cadherin, which decorated the lateral margins of the cells with fluorescence spots. A concentration-dependent binding of this decapeptide was also observed in the flow cytometry assay. BBMECs dissociated with trypsin plus Ca^{2+} were able to reaggregate only in the presence of Ca^{2+}. However, such cell-cell aggregations of BBMECs were prevented by the presence of either anti-E-cadherin antibody or the decapeptide in the assay medium. These results confirm that BBMECs possess a distinct Ca^{2+}-dependent cell adhesion mechanism that can be modulated by the decapeptide. This modulation of cell-cell adhesion in BBMECs by the decapeptide is thought-provoking for creating channels for paracellular drug delivery across the BBB.

Keywords: Bovine brain microvessel endothelial cells (BBMECs); E-cadherin; Cell adhesion molecules; Ca^{2+}-dependent; Decapeptide; Immunofluorescence; Cell-cell adhesion

1. Introduction

The selective permeability barrier between the blood and the brain is maintained by a fine structure called the blood–brain barrier (BBB). This barrier is made up of very tightly apposed endothelial cells of the brain capillaries and is relatively impermeable to most proteins and peptides and a variety of hydrophilic drugs. Ultrastructural studies have shown that tight junctions are also present between the adjacent endothelial cells, but are not identical to epithelial tight junctions [11,33]. Although the endothelial cells lack desmosomes, which are prominent in epithelial cells, they do exhibit zonula adherens-type junctions [9,12]. Moreover, the type of intercellular tight junctions present in the endothelium of brain capillaries differs from those of most peripheral blood vessels. Despite this structural diversity in cell-cell junctions, this association between endothelial cells definitely contributes to the physical integrity of the tissue and limits the bidirectional passage of substances between blood and the brain. The cell junctions are made up of cell adhesion molecules (CAMs) and have been found in various cell types.

The mechanism of cell-cell adhesion can be Ca^{2+}-dependent or Ca^{2+}-independent. The Ca^{2+}-dependent cell-cell adhesion is mediated by glycoproteins called cadherins. Cadherins are a family of transmembrane proteins that localize in the zonula adherens of a wide variety of cells and mediate Ca^{2+}-dependent cell-cell adhesion [38,39]. Cadherins promote cell-cell adhesion through homophilic interactions and are believed to be a critical regulator of epithelial cellular junction formation. The association of cadherins with cytoplasmic catenins is also necessary for cell-cell adhesion [29,30]. The classical E-, N- and P-

* Corresponding author. Fax: +1 (913)-864-5736; E-mail: Siahaan@smissman.hbc.ukans.edu

PII S0006-8993(96)01223-1

cadherins, along with 30 others recently identified, possess some similar structural and functional domains for Ca^{2+}-dependent adhesive properties. Each cadherin has three domains – extracellular, transmembrane and intracellular. Most cadherins have four to five extracellular repeats (EC-1 to EC-5 or CAD-1 to CAD-5) [28]. The EC-1 repeat at the N-terminus is required for specific homophilic interactions between cadherins [27]. Most cadherins also have a common cell-adhesion recognition sequence, histidine-alanine-valine (HAV) in the EC-1 domain, which is very important for specific homophilic interactions [27]. The presence of the HAV sequence is uniquely conserved through histogenesis, development and evolution of organisms. Synthetic peptides containing the HAV sequence are found to inhibit the compaction of mouse embryos and neurite growth through the inhibition of E- and N-cadherin interactions, respectively [8]. Similarly, antibodies generated against the extracellular domain of cadherins are capable of inhibiting the resealing of cellular junctions [36] and cadherin-mediated cell-cell adhesion [15,24]. Recently, we have shown that immobilized decapeptides generated from the sequences of the EC-1 to EC-3 regions of E-cadherin and the EC-1 of N-cadherin display antigenic reactivity to anti-E-cadherin antibodies [21,22].

In this work, we were interested in evaluating the role of cadherin in the cellular junctions of the BBB. An in vitro system consisting of bovine brain microvessel endothelial cells (BBMECs) [3–5] was used for the BBB model in this study. Since this barrier presents a formidable obstacle to the delivery of therapeutic agents to the brain, new information related to structure, association and function of cadherins in BBB may help in designing improved methods for paracellular drug delivery by regulating cellular junctions. We have generated multiple evidence by immunolocalization, immunoprecipitation followed by Western blotting, ELISA, flow cytometry and cell-adhesion assay to demonstrate that E-cadherin is predominant in adhesion processes in BBMECs. An FITC-labeled HAV-peptide (FITC-LRAHAVDVNG-NH_2) can also bind to E-cadherin, and this decapeptide can inhibit cell-cell adhesion mediated by E-cadherin in BBMECs.

2. Materials and methods

2.1. Cell isolation and culture

The procedure used for isolation and culture of BBMECs has been described in detail by Audus and Borchardt [3,4]. In brief, BBMECs were isolated from the gray matter of cerebral cortices. For each isolation, two bovine brains were collected from a local slaughterhouse. Subsequently, all procedures for isolation were performed under aseptic conditions using a sterile hood and materials. The meninges and all other large blood vessels were removed; the cortical gray matter was scraped, minced into small pieces (1–2 mm) and digested with protease 0.5% (Dispase; Sigma, P-3417) in minimum essential medium (MEM) for 3 h at 37°C. The digested gray matter was then centrifuged with 13% dextran (1/2 gray matter and 1/2 dextran, v/v) at 7730 rpm at 4°C for 10 min. A large red pellet containing the microvessels was collected and then resuspended in 20 ml MEM containing collagenase and protease (1.0 mg/ml) and incubated for 4 h at 37°C in a continuously shaking water bath. The digested product was centrifuged at 2000 rpm, room temperature (RT) for 10 min; the pellet was resuspended in 8 ml MEM and layered in four pre-made Percoll® (Sigma, P-1644) gradients. After centrifugation at 3500 rpm at 4°C for 10 min, the endothelial cells were collected from the second layer (below the white fatty layer). These microvessel fragments were then rinsed with culture medium and stored in freezing medium containing 35% MEM, 35% F-12 (Ham), 20% horse serum and 10% DMSO at −80°C. Prior to culture, frozen BBMECs were thawed at 37°C, rinsed 3 times and resuspended in culture medium containing 45% v/v MEM, 45% v/v F-12 nutrient mixture (Ham), 10% v/v horse serum, 100 μg/ml heparin, 2.5 μg/ml amphotericin-B, 50 μg/ml Polymyxin-B, 100 μg/ml penicillin-G and 100 μg/ml streptomycin; 50 000 cells/cm^2 were seeded in culture plates that had been precoated with rat-tail collagen and human fibronectin (25 μg/ml).

For immunofluorescence localization of E-cadherin or for localization of HAV peptides, BBMECs were grown on precoated glass coverslips or precoated polycarbonate membranes (Costar-110412). The culture plates were then incubated for 3 days in the plating medium at 37°C with 5% CO_2 and 95% humidity. The cells were fed with the aforementioned medium without amphotericin B and Polymyxin-B every other day until formation of a confluent monolayer.

2.2. Immunofluorescence localization of cadherin

Confluent monolayers of BBMECs grown on coverslips or polycarbonate membranes were washed 3 times in PBS and then fixed in 4% paraformaldehyde in PBS for 20 min at 4°C. Following fixation, the cells were washed 3 × 10 min in PBS. The cells were permeabilized with 2 mM EGTA in PBS for 10 min followed by 2 mM $CaCl_2$ in PBS for another 10 min at RT. The cells were washed again in PBS for 5 min and were used immediately or stored for a maximum of 7 days at 4°C for subsequent use for immunolocalization of cadherin. The cells were washed once again in 100 mM Tris (pH 7.4) buffered saline (TBS) for 5 min at RT and then incubated with 3% BSA in TBS for 1 h at 37°C to block non-specific binding. After 3 × 5 min wash in TBS, the cells were incubated with monoclonal antibodies against E-cadherin (Uvomorulin, U-3254, Sigma) 1:100 in TBS plus 0.05% Tween-20 (TBST) containing 2 mM $CaCl_2$ for 2 h at 37°C. Following incubation with antibodies, the cells were thoroughly washed (3 × 15

min) in TBST. They were incubated with anti-rat IgG-FITC (1:64) in TBST for 1 h at 37°C or RT and then washed 3×15 min in TBST. The coverslips or polycarbonate membrane having cell monolayers were mounted on glass slides with 50% glycerol in PBS and photographed under epifluorescence and confocal microscope. For some experiments, the live cells were exposed to anti-E-cadherin antibody at 37°C for 1–2 h without permeation; after washing in PBS, they were fixed in paraformaldehyde at 4°C.

2.3. Localization of synthetic peptides

The procedures used for immunofluorescence of E-cadherin were followed for the localization of HAV peptides. Instead of antibodies, an FITC-labeled HAV peptide (FITC-LRAHAVDVNG-NH_2) was used. Both fixed and unfixed BBMECs were incubated with FITC-labeled HAV peptides at 37°C for 1–2 h. The rest of the procedure was essentially the same as that described for immunofluorescence.

2.4. Enzyme linked immunosorbent assay (ELISA)

Confluent monolayers of BBMECs grown in 24-well culture plates were briefly washed in PBS and then fixed in 4% paraformaldehyde for 20 min at 4°C. They were washed 3×15 min in PBS and permeabilized in 2 mM EGTA in PBS followed by 2 mM $CaCl_2$ at RT for 10 min each. After a brief wash in PBS, the cells were either used immediately or stored at 4°C for the rest of the ELISA. Horseradish peroxidase (HRP) was coupled to monoclonal anti-E-cadherin (Uvomorulin-rat IgG-1 isotype, Sigma) to give anti-E-cadherin-HRP conjugate (for details see ref. [21]) and stored at -20°C in small aliquots. Prior to use, the anti-E-cadherin-HRP conjugate was diluted (1:740) in PBS. The cells were incubated with blocking agents (1% goat serum + 2% BSA) in PBS for 60 min at 37°C and washed twice in PBS for 10 min. Then, 400 μl of diluted anti-E-cadherin-HRP conjugate was added to each well, with the control wells receiving only HRP-conjugated rat IgG rather than E-cadherin antibody. These were incubated for 60 min at 37°C and then washed 3 times in PBS-Tween-20 for 15 min each. To develop color, the substrate for HRP (200 μl TMB, Pierce) was added to each well, allowed to react for 15–20 min, and stopped with the addition of 200 μl 1 M H_2SO_4 to each well. To read final reaction products, 300 μl from each well were transferred to ELISA plates and absorbance was recorded at 450 nm wavelength.

2.5. Flow cytometry

Confluent monolayers of BBMECs grown in 100 mm $\times$ 25 mm plates were rinsed in PBS and trypsinized (200 μg/ml PBS with 1 mM $CaCl_2$) for 10 min at 37°C. BBMECs were collected by centrifugation at 1800 rpm for 5 min. The cells were resuspended in PBS-$CaCl_2$ containing soybean trypsin inhibitor (0.5 mg/ml) and incubated for 10 min at 37°C followed by a thorough dispersion of cells using a plastic Pasteur pipette. The cells were centrifuged at 1800 rpm for 5 min at RT. The pellet was resuspended in PBS and then fixed in 4% paraformaldehyde in PBS for 20 min at RT. Dispersed BBMECs were washed 3 times in PBS and once in TBS. The cells were resuspended in TBS containing 2 mM $CaCl_2$ (TBSC) and 3% BSA and incubated for 60 min at 37°C to block non-specific binding. The BBMECs were washed twice in TBS, resuspended in TBSC and the concentration was adjusted (2×10^6 cells/ml). This cell suspension was then aliquoted, 500 μl each, into microfuge tubes. The aliquoted cells were incubated for 2 h at 37°C with (i) rat IgG-FITC (1:100), and (ii) FITC-labeled peptide at different concentrations (1.0, 0.5, 0.25 mg/ml). After 3×10 min washes in TBST, fluorescence intensity was measured in a FACSCAN (Becton-Dickinson), which scanned the fluorescence intensity of 10 000 cells and reported an averaged result.

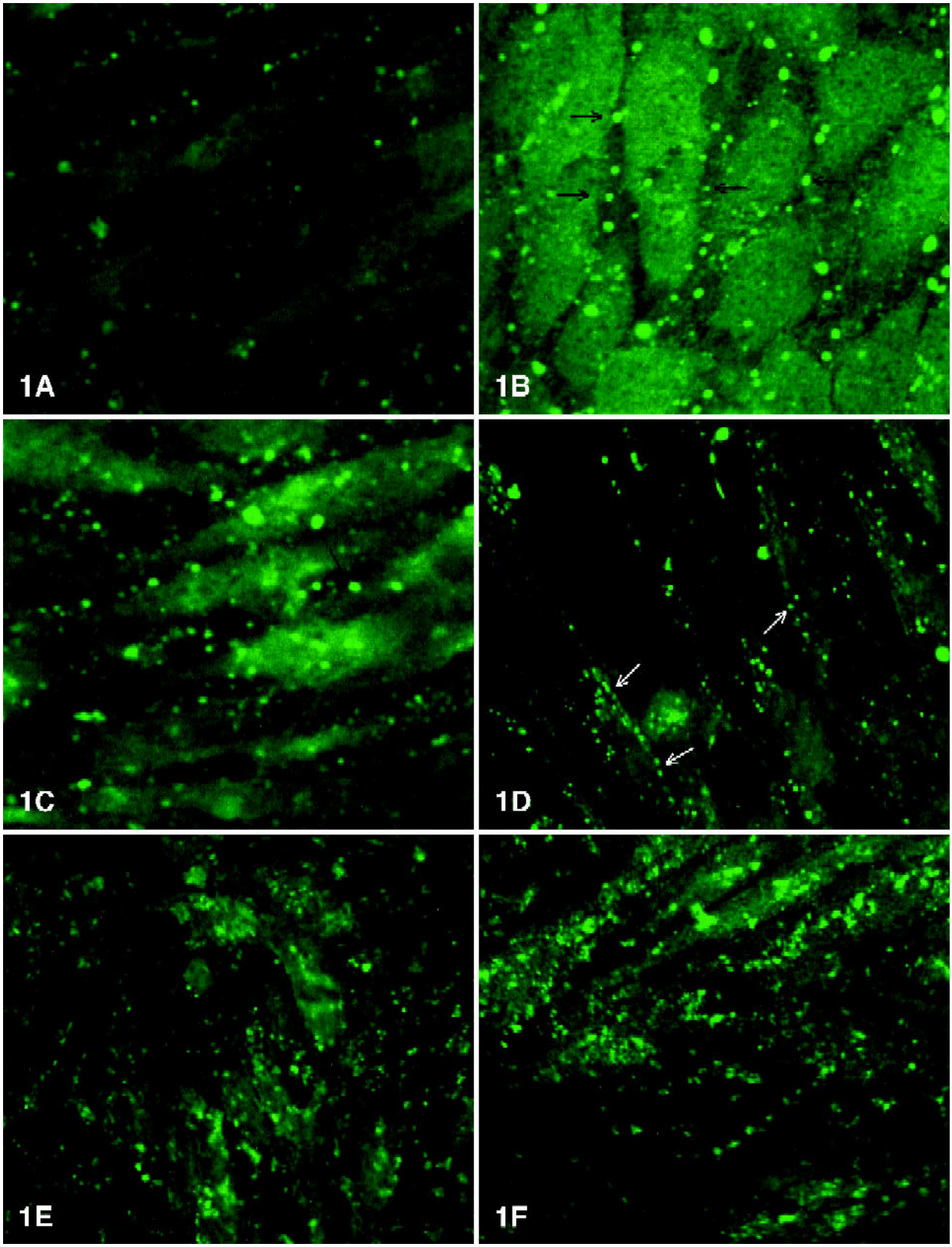
2.6. Cell adhesion assay

Using either trypsin-calcium or trypsin-EGTA, BBMECs were harvested from a primary culture of confluent monolayers grown in 100 mm $\times$ 25 mm plates. The cells were rinsed 3 times (3×5 min) with Ca^{2+}/Mg^{2+}-free HEPES buffered Puck's saline (CMPS) and then incubated with 200 μg/ml trypsin containing 1 mM $CaCl_2$ or 2 mM EGTA in CMPS for 10 min at 37°C. The cells were dispersed thoroughly with a plastic Pasteur pipette and centrifuged at 1800 rpm at RT for 5 min. After removing the supernatant, the soybean trypsin inhibitor (TI, 0.5 mg/ml) in CMPS was added to the pellet. The pellet was resuspended and incubated for 10 min. This was centrifuged at 1800 rpm for 5 min at RT and the TI was removed. The cells were resuspended in CMPS, the concentration was adjusted to 1×10^6 cells/ml and 1 ml aliquots were added into microfuge tubes for each test. To assay cell adhesion, the BBMECs were centrifuged at 1800 rpm for 5 min, the supernatant was removed, and they were resuspended in 1 ml of test medium, plated in a 12-well plate and incubated for 60 min at 37°C. The BBMECs were dissociated with trypsin-calcium and incubated with the following test media: (1) CMPS; (2) CMPS + 2 mM $CaCl_2$; (3) CMPS + 2 mM $CaCl_2$ + 1:100 anti-E-cadherin antibody; and (4) CMPS + 2 mM $CaCl_2$ + HAV-peptide 1.0 mg/ml, whereas cells dispersed with trypsin + EGTA were incubated with (5) CMPS and (6) CMPS + 2 mM $CaCl_2$.

2.7. Immunoprecipitation and Western blotting

Confluent BBMECs grown in two 100 mm $\times$ 20 mm dishes were washed 2 times with PBS and then harvested

using a cell scraper. The cells were collected by centrifugation and lysed with immunoprecipitation (IPP) buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM Na_3VO_4, 5 mM NaF, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupectin for 10 min on ice. The cells were ultrasonicated for a few seconds and then centrifuged at 12 000 rpm for 30 min at 4°C. The supernatant was collected, stored at



−20°C and subsequently used for immunoprecipitation. This BBMEC extract (150 μl) along with 250 μl of IPP buffer containing 1 mM $CaCl_2$ was immunoprecipitated with either anti-E-cadherin antibody (Sigma), anti-N-cadherin or anti-cadherin-5 antibodies (Transduction Laboratories) overnight at 4°C. These were then incubated with protein A-Sepharose for 2 h at 4°C. The Sepharose beads containing antigen-antibody complexes were collected by centrifugation, washed 3 times with IPP buffer, resuspended in 50 μl SDS sample buffer (62 mM Tris, pH 6.6, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.1% bromophenol blue) and then boiled for 5 min. All of these immunoprecipitated samples and molecular weight protein markers were then separated by SDS polyacrylamide gel electrophoresis. The protein markers and the samples were electrophoretically transferred to a nitrocellulose membrane. The portion of the blot containing cadherin samples was incubated in a blocking buffer containing 5% non-fat dry milk and 3% BSA in TBS for 1 h at RT. The blot containing immunoprecipitated E-cadherin was then incubated with anti-E-cadherin (Sigma) antibody coupled to HRP for 2 h at RT. Other immunoprecipitated samples were incubated with respective primary antibodies (2 h) followed by 4 × 10 min washing in TBST and then a 1 h incubation in secondary antibodies conjugated with HRP. After extensive washing in TBST, these blots were developed using an ECL Kit and Hyper-film (Amersham Life Science).

3. Results

3.1. Immunofluorescence localization of cadherin

Under a phase-contrast microscope, the confluent monolayer of BBMECs in primary culture exhibited two polymorphic cells – fusiform and polygonal; the closely apposed fusiform cells were predominant, with few colonies of polygonal cells.

Confluent monolayers of BBMECs reacted positively with anti-E-cadherin antibody and negatively with anti-N-cadherin or anti-cadherin-5 antibodies. A series of fluorescence punctate spots around the margins of BBMECs was observed in response to anti-E-cadherin antibody (Fig. 1A). The intensity and number of fluorescence spots varied from batch to batch of BBMECs. Interestingly, the immunoreactivity of BBMECs to E-cadherin differed when the cells were pre-confluent. The intensity and number of fluorescence spots were also increased when the BBMECs were exposed to 100 ng/ml human gamma interferon (hIFN-γ) (Fig. 1B) or 250 μM CPT-cAMP (Fig. 1C) for 2 h to 3 days. However, there was no appearance of a continuous fluorescence border around the individual cells. In contrast, when the same antibody was applied to cultured MDCK cells, a continuous thick fluorescence border outlining the cell margins was evident. These latter cells were used as a positive control.

For routine immunofluorescence localization, paraformaldehyde-fixed BBMECs were permeabilized with EGTA before application of primary antibody. In some tests, the primary antibody was directly added to living BBMECs in culture with Puck's saline containing calcium and was incubated for 2 h without permeation. The intensity as well as the distribution of cadherin labeling was slightly different. The internalization of cadherin was evidenced by more intracellular labeling (Fig. 1E). Immunolocalization of cadherin in fixed BBMECs without permeation was not possible. Cadherin is located at the junctions between cells, and the antibodies were apparently unable to reach the target in the tight junctions of fixed BBMECs. Antibodies somehow managed to reach and decorate the target antigen in living cells, perhaps as a result of unique antibody interactions with the dynamic intercellular junctions characteristic of living BBMECs.

3.2. Immunoprecipitation and Western blotting

Immunoprecipitation followed by Western blotting analysis also indicated that BBMECs contain a protein of 120 kDa that is recognized by anti-E-cadherin antibody (Fig. 2). Apart from the 120 kDa band, there is another protein of 96 kDa that also co-immunoprecipitates with anti-E-cadherin antibody. No other protein in the BBMEC extract was detected by immunoprecipitation either with anti-N-cadherin antibodies or anti-cadherin-5 antibodies. This result also confirms our immunofluorescence localiza-

Fig. 1. A–D: immunofluorescence localization of E-cadherin in cultured BBMECs. The cultures of BBMECs were fixed in cold buffered paraformaldehyde and permeabilized with EGTA before adding anti-E-cadherin antibody or FITC-labeled peptide. The cells were examined and photographed under a confocal microscope. A series of green punctate spots represents indirect immunofluorescence of E-cadherin or direct fluorescence from the binding of FITC-labeled decapeptide. A: a series of fluorescence punctate spots (arrows) is seen in the lateral border of untreated cells. B: hIFN-γ-treated cells clearly show increased immunolabeling as evident from intensified fluorescence spots. C: CPT-cAMP-treated cells also indicate increased immunolabeling and the intensity of immunofluorescence is comparable to that of hIFN-γ-treated cells. D: fluorescence localization of FITC-labeled decapeptide in untreated BBMECs. A series of punctate fluorescence spots in the lateral margins of the cells is quite comparable to that of immunolabeling of E-cadherin of untreated cells. E–F: the cultures of BBMECs were incubated either with anti-E-cadherin or with FITC-labeled decapeptide at 37°C for 2 h. Subsequently, the cultures were washed with cold PBS and fixed in cold paraformaldehyde. An FITC-labeled secondary antibody was added to the anti-E-cadherin incubated group. E: this shows more intracellular localization of E-cadherin, indicating internalization of the protein at 37°C. Compare with A where cadherin is localized primarily in the lateral margins of the cells. F: this photograph shows internalization of FITC-labeled decapeptide. This is clearly distinct from D, where labeled peptide is distributed along the borders of the cell. Bar, 10 μm.

tion study in which BBMECs responded positively to anti-E-cadherin and negatively to anti-N-cadherin and anti-cadherin-5 antibodies.

3.3. Localization of LRAHAVDVNG-NH_2

The localization of FITC-labeled peptides on the primary cultures of BBMEC monolayers essentially followed the same pattern as found with anti E-cadherin antibody. A series of punctate fluorescence spots of FITC-labeled LRAHAVDVNG-NH_2 peptide were found mainly along the boundaries between the BBMECs (Fig. 1D). However, the binding of FITC-labeled peptides to BBMECs varied with the concentration of peptide. BBMECs incubated with high concentrations of FITC-labeled peptide (1.0 mg/ml), although resulting in more binding, produced more nuclear and cytoplasmic staining. In fact, the use of a high concentration of FITC-labeled peptide in paraformaldehyde-fixed BBMECs often generated more background staining. In contrast, background staining by the FITC-labeled peptide was substantially reduced if living BBMECs were first incubated with the labeled peptide and then fixed in paraformaldehyde. Freshly dissolved peptide was essential for good localization of the peptide. The use of a solution phase-stored peptide for binding often resulted poor localization and inconsistency in binding. EGTA-mediated permeation was omitted for peptide localization in living cells. There were no discernible differences in the localization of peptides at the junctions of permeabilized, fixed cells and that in unfixed, unpermeabilized cells. Incubation of living BBMECs with FITC-labeled peptide at 37°C resulted in some internalization of the compound. Internalization of the FITC-labeled peptide was manifested as an increased punctate fluorescence in the cytoplasm of the cells (Fig. 1F).

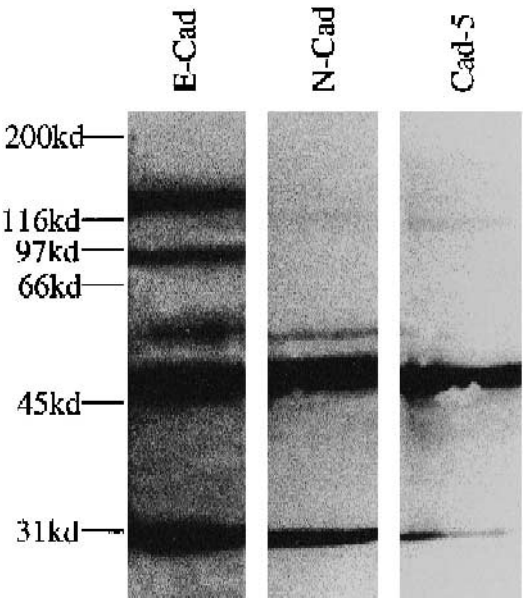


Fig. 2. Western blot analysis.

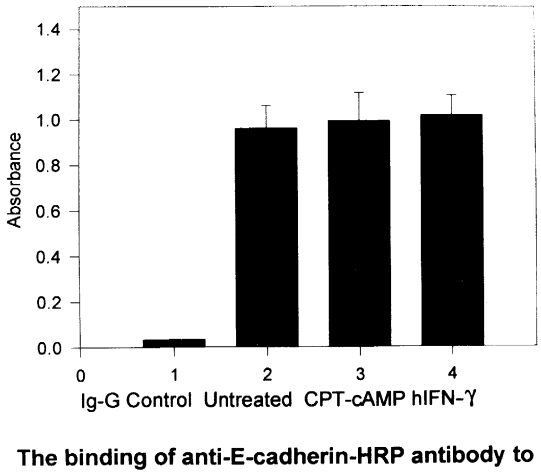


Fig. 3. ELISA: binding of HRP conjugated anti-E-cadherin antibody in the monolayer of BBMECs.

3.4. ELISA

The BBMECs responded positively to ELISA. We used a direct ELISA in which the anti-E-cadherin antibody was labeled with HRP through glutaraldehyde conjugation. In this procedure, the use of a secondary antibody was eliminated, reducing the background staining. The BBMECs were treated with hIFN-γ or CPT-cAMP for 2 h to 3 days.

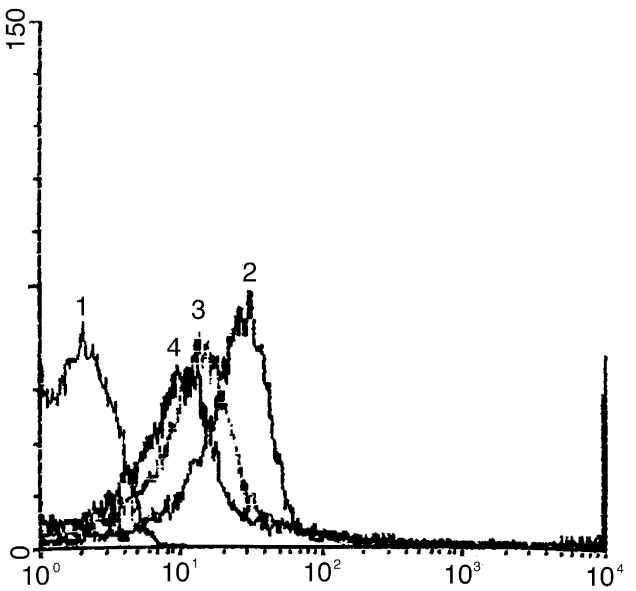


Fig. 4. Flow cytometry assay of concentration-dependent binding of FITC-labeled decapeptide to BBMECs: 1, IgG-FITC; 2, 1.0 mg peptide/ml; 3, 0.5 mg peptide/ml; and 4, 0.25 mg peptide/ml. In the graph, fluorescence peaks shifted toward the right indicate more fluorescence. The fluorescence peak in the extreme right corresponds to the highest concentration of peptide used and so on.

However, there was no appreciable increase in the ELISA reading in these different treatment groups (Fig. 3).

3.5. Flow cytometry

Trypsin-calcium dissociated BBMECs were found to bind FITC-labeled HAV peptide. The anti-E-cadherin antibody also binds to these BBMECs (data not shown). The fluorescence intensity of 10 000 cells was recorded with a FACSCAN and is presented in Fig. 4. The fluorescence peaks that are shifted toward the right indicate more fluorescence. The fluorescence peak at the extreme right was generated following the incubation of BBMECs with 1.0 mg/ml FITC-labeled peptide while peaks toward the

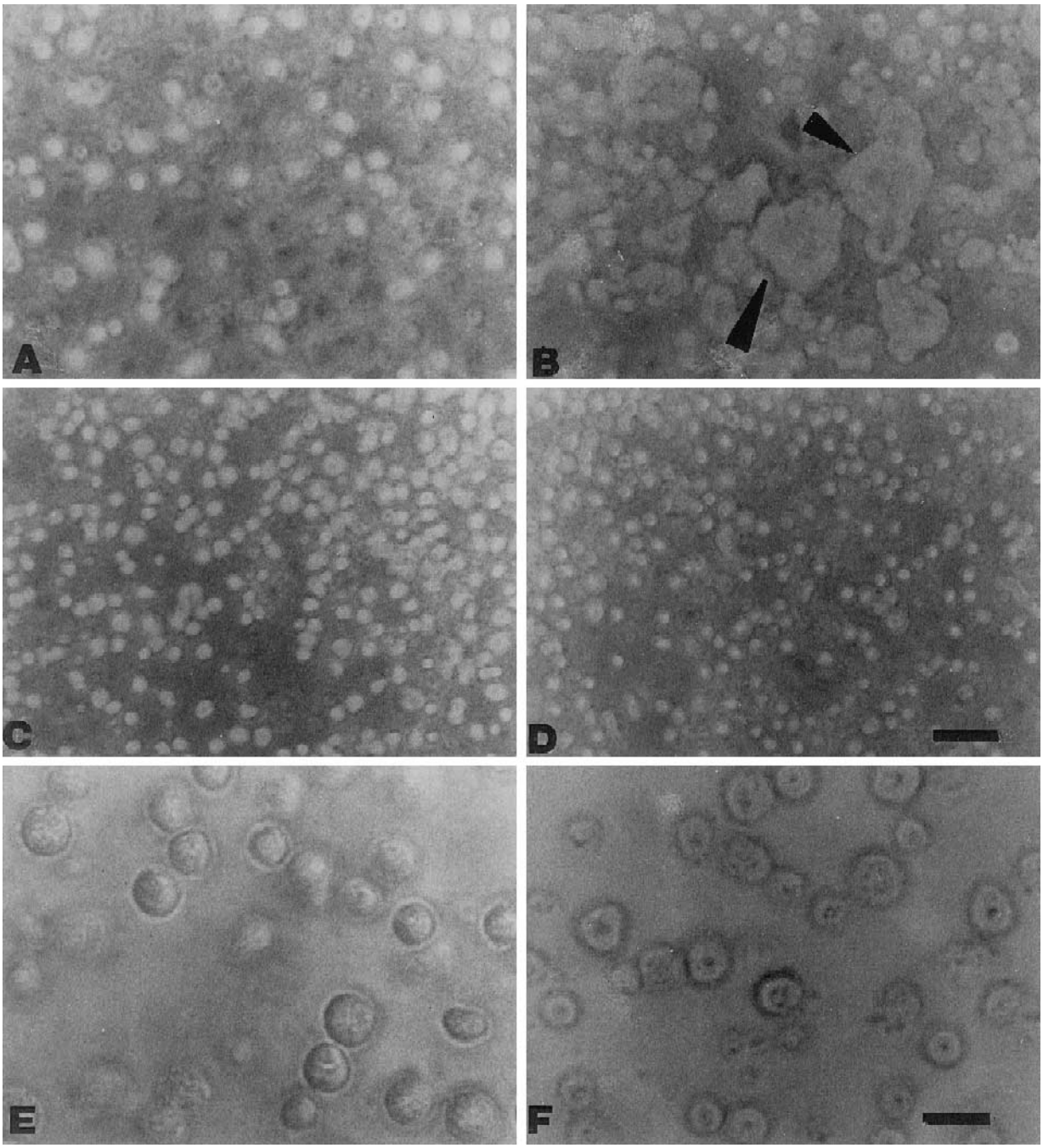


Fig. 5. A–D: cell-cell adhesion. Monolayers of BBMECs were dissociated with trypsin-calcium in CMPS and then allowed to aggregate in the following media at 37°C for 60 min. A: only CMPS: no sign of aggregation. B: CMPS + 2 mM Ca^{2+}: calcium-promoted aggregation (arrow) of BBMECs. C: CMPS + 2 mM Ca^{2+} + anti-E-cadherin antibody: cadherin antibody inhibited the cadherin-mediated, calcium-dependent cell-cell aggregation. D: CMPS + 2 mM Ca^{2+} + decapeptide: HAV-containing decapeptide also inhibited the cell-cell aggregation of BBMECs; this is comparable to that seen in Fig. 5C. Bar, 50 μm. E–F: monolayers of BBMECs were dissociated with trypsin-EGTA and then allowed to aggregate at 37°C for 60 min in the presence or absence of calcium. E: only CMPS: no cell-cell aggregation. F: CMPS + 2 mM Ca^{2+}: also no cell-cell aggregation. Bar, 20 μm.

left were the result of lower concentrations of peptide. From the statistical analysis, the values of the arithmetic median of fluorescence intensity representing 25, 13.5 and 9.9 correspond to 1.0, 0.5, and 0.25 mg/ml peptide concentration, respectively. These results suggest that binding of FITC-labeled HAV peptide to BBMECs is concentration dependent and directly proportional to the added concentration of labeled peptide.

3.6. Cell-cell adhesion

To characterize the intercellular adhesion of BBMECs, confluent monolayers were trypsinized in the presence or absence of Ca^{2+} and then the BBMECs were allowed to aggregate for 60 min at 37°C. Treatment of confluent monolayers of BBMECs with trypsin (200 μg/ml) in the presence of 1 mM calcium and subsequent incubation of cells in CMPS containing 2 mM Ca^{2+} promoted aggregation of cells (Fig. 5B). In contrast, no cell aggregation was observed when these Ca^{2+}-trypsinized cells were incubated in only CMPS (Fig. 5A). However, BBMECs dissociated with the same concentration of trypsin but in the presence of 2 mM EGTA instead of Ca^{2+} were unable to promote aggregation regardless of whether or not the incubation medium contained Ca^{2+} (Fig. 5E,F). Addition of Ca^{2+} in the later stages of the adhesion assay had no effect unless it was done during trypsin digestion for cellular dissociation. These results imply that a specific calcium-dependent adhesion mechanism exists in BBMECs and that it is protected by Ca^{2+} from cleavage by trypsin. Cadherin-mediated cell-cell adhesion in BBMECs was examined by the following experiments: (1) BBMECs were dissociated with trypsin in the presence of Ca^{2+} and then allowed to reaggregate in CMPS with 2 mM $CaCl_2$ plus monoclonal antibodies against E-cadherin in a 1:100 dilution (Fig. 5C); and (2) HAV peptide (LRAHAVDVNG-NH_2, 2 mg/ml) was added to the incubation medium (Fig. 5D) instead of antibodies. In both experiments, the reaggregation of BBMECs was inhibited by the anti-E-cadherin antibodies as well as by the synthetic peptide even though the incubation medium contained calcium. This result further confirmed that specific peptide sequences, like antibodies, are equally effective in preventing reaggregation of BBMECs. This peptide (LRAHAVDVNG-NH_2) is also recognized by anti-E-cadherin antibody [21].

4. Discussion

Endothelial cells, both in culture and in situ, possess several types of intercellular adhesion molecules, and these are involved in the formation and maintenance of the endothelial barrier [2,18,19,32,34]. The importance of cell adhesion molecules and their specific implications on cell-cell adhesion has been extensively investigated in various tissue systems, yet their exact role in governing endothelial adhesion and, specifically, their intimate association in BBB are poorly characterized. In this study, we have demonstrated a specific Ca^{2+}-dependent cell adhesion mechanism present in the microvessel endothelial cells of bovine brain and also identified a peptide that inhibits endothelial cell adhesion.

Under our culture conditions, $>$ 99% of the BBMECs in the primary culture stained positive for factor VIII, a specific marker for endothelial cells [14]. The cultured BBMECs retained the characteristics of non-cultured BBMECs, such as tight intercellular junctions, no fenestra, few pinocytotic vesicles and an abundance of mitochondria, marker enzymes, transport systems and functional polarity [4,14].

We have presented evidence that an antigen recognized by anti-E-cadherin antibody is present on BBMECs. Following immunofluorescence localization, a series of punctate fluorescence spots around the cell margins of BBMECs was observed. Such fluorescence localization was not detectable with anti-N-cadherin or anti-cadherin-5 antibodies; this was further confirmed by immunoprecipitation and Western blotting analysis. Two protein bands of 120 and 96 kDa were found in the BBMEC extract after immunoprecipitation and Western blotting with anti-E-cadherin antibody. The 120 kDa band was the mature form of E-cadherin, whereas the 96 kDa band could be an intermediate form resulting from incomplete glycosylation. A 96 kDa protein, which binds non-specifically to the antibody and co-immunoprecipitates with plakoglobin, was also demonstrated in MDCK cells [17]. Moreover, our ELISA results with anti-E-cadherin-HRP conjugate also demonstrated the presence of E-cadherin in BBMECs. The control wells that received rat IgG-HRP did not develop color. All of the evidence generated in this study strongly advocates the presence of E-cadherin in BBMECs.

Type-1 endothelial cells isolated from developing bovine corpus luteum reacted with antiserum against E-cadherin whereas type-5 endothelial cells responded to anti-N-cadherin labeling [10]. An endothelial-specific cadherin (7B4 antigen) was isolated from human umbilical vein endothelial cells (HUVECs) [19], and its cDNA sequence is identical with that of cadherin-5 cloned by Suzuki et al. [35]. The expression of a few other cadherins has also been described in the intercellular junction of endothelial cells, such as V-cadherin in bovine aortic endothelial cells [16], cadherin-5 in human placenta [35], and pan-cadherin in HUVECs [6]. In contrast to the distribution of pan-cadherin in the junctional region of HUVECs, a largely diffuse N-cadherin labeling was found over the entire cell surface with transient enrichment at the cell-cell contact zones [32]. Using PCR amplification, Liaw et al. [20] have cloned two species of cadherins from bovine aortic endothelial cells. The major species is almost identical to chicken N-cadherin, while the minor corresponds to mouse P-cadherin. Interestingly, this bovine N-cadherin was widely distributed among neuronal and non-neuronal tis-

sues, while P-cadherin was abundant in the kidney but undetectable in the placenta. Although endothelial cells of BBMECs contain only E-cadherin, the evidence suggests that endothelial cells of different origins possess different types of cadherin.

Treatment of endothelial cell monolayers with CPT-cAMP or hIFN-γ resulted in upregulation of E-cadherin expression at the cellular junctions of BBMECs. Possibly the cells acquired more stable intercellular contacts after such treatment. The distribution of E-cadherin immunostaining along the borders was also upregulated during induction of high resistance tight junctions in BBMECs by cAMP treatment [31]. The increased intensity of fluorescence labeling might be the result of reorganization of actin filaments. The phenotypic modulatory effect of IFN-γ was seen in other endothelial cells [13]. Rearrangement of actin and cytokeratin bundles coupled with reinforced E-cadherin expression along the cell margins was observed in IFN-γ-treated MVEC type-1, while untreated cells did not display any E-cadherin localization [10]. Cytoskeletal reorganization as well as redistribution of focal contact proteins were found in cultured HUVECs after treatment of phorbol ester and several cytokines including IFN-γ. Several morphological changes were easily observed by indirect immunofluorescence, but no quantitative differences in specific cytoskeletal proteins were detected by immunoblot or ^{35}S-labeling experiments [25]. Similarly, in the present experiments, the fluorescence localization of E-cadherin in the junctional region was significantly enhanced after cAMP or hIFN-γ treatment of the BBMECs, while the ELISA reading for E-cadherin remained unchanged. This result, along with previous reports, indicates that cytoskeletal reorganization occurs in endothelial cells after cytokine treatment or elevation of intracellular cAMP. Coupling and uncoupling of tight junctions are definitely dynamic processes and perhaps require some balanced adjustment of cytoskeletal structures. Cadherins mediate homophilic cell-cell contact in the presence of calcium [38,39]. Depletion of Ca^{2+} in the culture medium resulted in endocytosis of junctional E-cadherin in MDCK cells, while induction of Ca^{2+} channels by cAMP caused redistribution of cadherin at the junctions [7]. Therefore, upregulation of Ca^{2+}-current by cAMP and possibly by IFN-γ may be a plausible explanation for the upregulation of cadherin distribution in the cellular junctions of BBMECs.

This laboratory has generated several peptides from the EC-1 to EC-3 domains of E-cadherin and the EC-1 domain of N-cadherin [21–23]. Here, we have investigated the synthetic peptide sequence (LRAHAVDVNG-NH_2) from the EC-1 domain of N-cadherin for two reasons: (1) this peptide was reported to inhibit the compaction of mouse embryo and neurite outgrowth [8]; and 92) it was recognized by anti-E-cadherin antibody during peptide-epitope mapping [21]. As in E-cadherin immunostaining, this FITC-labeled synthetic peptide was found to localize along the margins of the BBMECs. Although an anti-N-cadherin antibody did not positively react to BBMECs, the decapeptide (LRAHAVDVNG-NH_2) generated from the EC-1 domain of N-cadherin was able to bind BBMECs because this peptide shares the conserved sequence His-Ala-Val (HAV), which is present in both E- and N-cadherin. Another peptide derived from E-cadherin sequences showed greater affinity for anti-E-cadherin antibody during epitope mapping [21]. The E-cadherin antibody, in fact, recognizes some common epitopes present in both E- and N-cadherin [21,22]. It has been previously demonstrated that at least part of the cadherin specificity among different subclasses is determined by the N-terminal region containing the HAV tripeptide sequence [27]. The binding of the decapeptide to BBMECs also indicated that cells expressing a specific cadherin may not react to non-specific antibodies made against different cadherins, but can react to conserved peptide sequences generated from a different subset of cadherins.

We tested FITC-conjugated HAV-peptide binding in both permeabilized, fixed and unpermeabilized, unfixed cells. Permeation did not alter peptide binding, but the antibody binding was substantially increased as evidenced from the increased fluorescence compared to that of unpermeabilized cells. This suggested that small molecules such as peptides can permeate the tight junctions more easily than large IgG molecules. Results from flow cytometry indicated that the binding of FITC-conjugated peptide to BBMECs depends on the concentration of the peptide. This result again suggested that a peptide derived from an N-cadherin sequence can bind to the cells expressing only E-cadherin.

Confluent BBMECs dissociated with trypsin plus Ca^{2+} were able to reaggregate only in the presence of Ca^{2+}, while those cells dissociated with trypsin plus EGTA failed to reaggregate even in the presence of calcium. This finding suggested that a distinct Ca^{2+}-dependent cell adhesion mechanism is present in BBMECs and that the mechanism is protected by Ca^{2+} from cleavage by trypsin. Moreover, the reaggregation of BBMECs was also inhibited by the anti-E-cadherin antibody. These results implied that the Ca^{2+}-dependent cell adhesion mechanism present in BBMECs is mediated by E-cadherin. In fact, trypsin in the absence of Ca^{2+} completely destroyed the cadherin-mediated cell-cell adhesion in other epithelial cells [36]. Truly, the mechanism of cell-cell adhesion is very complex; several adhesion molecules, various factors and multiple mechanisms are likely involved [37,39]. Both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms of intercellular adhesion exist in endothelial cells [6,16]. Generally, Ca^{2+}-dependent cell-cell adhesion is mediated by a family of transmembrane glycoproteins, namely the cadherins [37,39], whereas Ca^{2+}-independent cell adhesion occurs primarily through IgG superfamilies such as PECAM or endo-CAM [1,6,26,34]. Since trypsin-EGTA-treated BBMECs were unable to aggregate, it appears that a Ca^{2+}-independent cell adhesion mechanism may not exist in BB-

MECs. However, further investigation is needed to confirm that a Ca^{2+}-independent cell adhesion mechanism does not exist in the endothelial cells of brain.

The mechanism of cell-cell adhesion is very complex where more than one CAM and several cytoskeletal proteins are involved. A cascade of events and the combined actions of several molecules are necessary to determine the adhesive specificity of cells. The decapeptide generated from N-cadherin inhibited the neurite outgrowth and the compaction of mouse embryo through N-cadherin and E-cadherin, respectively [8]. Similarly, the aggregation of BBMECs was also interrupted by the same decapeptide, possibly by blocking the action of E-cadherin. There are at least three potential cell adhesion recognition sequences in the EC-1 domain of cadherins, including PPI, GAD and HAV [8,15]. The ability of this HAV-containing decapeptide to prevent E-cadherin-mediated cell-cell adhesion suggests that it utilizes one of the common cell adhesion recognition sequence [8]. Although the HAV sequence plays a regulatory role in mediating cadherin function, the residues flanking HAV (particularly the SSNG sequence in E-cadherin and the DVNG sequence in N-cadherin) influence their selectivity [8,21,23]. Recently, another synthetic HAV-containing decapeptide (REMHAVSRVQ) derived from the sequences of extracellular superoxide dismutase B (ECSOD-B) was reported to inhibit cadherin-mediated cell-cell adhesion [40]. Moreover, the presence of a conserved HAV sequence was also found in the CAM homology domain of fibroblast growth factor receptor (FGFR); the two peptides (KMEKKLHAVPAAK and KMEKRLHAVPAAN) having HAV sequence derived from the CAM homology domain of FGFR-1 and FGFR-2, respectively, were shown to inhibit neurite outgrowth stimulated by N-cadherin [41]. Originally, cadherins were thought to use only homophilic interactions, but recent evidence suggests that cadherins may be involved in heterophilic interactions, such as the interactions between the E-cadherin and integrins ($\alpha E\beta 7$ and $\alpha M290\beta 7$) and the interactions between N-cadherin and FGFR [39,41]. Using peptide-epitope mapping, our laboratory has identified several peptides from the EC-1, EC-2, and EC-3 domains of E-cadherin and the EC-1 domain of N-cadherin. Many of these peptides [21], along with the decapeptide-LRAHAVDVNG-NH_2, were recognized by anti-E-cadherin antibody as seen in an immobilized peptide-ELISA (unpublished data). Particularly, the HAV-containing 20 amino acid peptide generated from the sequence of the EC-1 domain of human E-cadherin appeared to be very potent in inhibiting the aggregation of BBMECs (unpublished data). The present results, along with previous reports, again indicate the significance of the HAV sequence in E-cadherin-mediated cell-cell adhesion. From this study, we conclude that cell-cell adhesion in BBMECs is regulated by a Ca^{2+}-dependent adhesion molecule, namely, E-cadherin. The HAV-containing peptide(s) derived from the EC-1 domain of E- or N-cadherin can modulate cell-cell adhesion in BBMECs. In this BBMEC model, the decapeptide possibly binds to the site present in E-cadherin and thus inhibits the cell-cell adhesion through cadherin-cadherin homophilic interaction. Alternatively, this HAV-containing decapeptide may operate through an indirect pathway by interfering in the function of FGFR, which was reported to influence the function of several CAMs [41]. We believe that this modulation can help in uncoupling the cellular tight junctions and thus create channels for paracellular drug delivery across the BBB. This understanding will enable us to design specific peptides as adjuvants for administering potential therapeutic agents to the brain.

Acknowledgements

This work was supported by grants from The University of Kansas GRF FY94, Alzheimer's Association (IIRG-94-097) and the National Science Foundation (EHR 92-5223). We thank Nancy Harmony for her help in preparing this manuscript.

References

[1] Albelda, S.M., Oliver, P., Romer, L. and Buck, C.A., Endo CAM: a novel endothelial cell-cell adhesion molecule, *J. Cell Biol.*, 110 (1990) 1227–1237.

[2] Albelda, S.M., Muller, W.A., Clayton, A.B. and Newman, P.J., Molecular and cellular properties of PECAM-1 (endo CAM/CD31): a novel vascular cell-cell adhesion molecule, *J. Cell Biol.*, 114 (1991) 1059–1068.

[3] Audus, K.L. and Borchardt, R.T., Characterization of an *in vitro* blood–brain barrier model system for studying drug transport and metabolism, *Pharmaceut. Res.*, 3 (1986) 81–87.

[4] Audus, K. L. and Borchardt, R.T., Bovine brain microvessel endothelial cell monolayers as a model system for the blood–brain barrier, *Ann. NY Acad. Sci. USA*, 507 (1987) 9–18.

[5] Audus, K.L., Bartel, R.L., Hidalgo, I.J. and Borchardt, R.T., The use of cultured epithelial and endothelial cells for drug transport and metabolism studies, *Pharmaceut. Res.*, 7 (1990) 435–451.

[6] Ayalon, O., Sabanai, H., Lampugnani, M.G., Dejana, E. and Geiger, B., Spatial and temporal relationships between cadherins and PECAM-1 in cell-cell junctions of human endothelial cells, *J. Cell Biol.*, 126 (1994) 247–258.

[7] Barres, B.A., Chun, L.L. and Corey, D.P., Calcium current in cortical astrocytes: induction by cAMP and neurotransmitter and permissive effect of serum factors, *J. Neurosci.*, 9 (1989) 3169–3175.

[8] Blaschuk, O.W., Sullivan, R., David, S. and Pouliot, Y., Identification of a cadherin cell adhesion recognition sequence, *Dev. Biol.*, 139 (1990) 227–229.

[9] Cowin, P., Kapprell, H.P., Franke, W.W., Tamkun, T. and Hynes, R.O., Plakoglobin: a protein common to different kinds of intercellular adhering junctions, *Cell*, 46 (1986) 1063–1073.

[10] Fenyves, A.M., Behrens, J. and Spanel-Borowski, K., Cultured microvascular endothelial cells (MVEC) differ in cytoskeleton expression of cadherins and fibronectin matrix: a study under the influence of interferon-γ, *J. Cell Sci.*, 106 (1993) 879–890.

[11] Franke, W.W., Kapprell, H.P. and Cowin, P., Immunolocalization of

plakoglobin in endothelial junctions: identification as a special type of zonulae adherents, *Biol. Cell*, 59 (1987) 205–218.
[12] Franke, W.W., Goldschmidt, M.D., Zimbelmann, R., Mueller, H.M., Schiller, D.L. and Cowin, P., Molecular cloning and amino acid sequence of human plakoglobin, the common junctional plaque protein, *Proc. Natl. Acad. Sci. USA*, 86 (1989) 4027–4031.
[13] Friesel, R., Komoriya, A. and Maciag, T., Inhibition of endothelial cell proliferation by gamma interferon, *J. Cell Biol.*, 104 (1987) 689–696.
[14] Guillot, F.L., Audus, K.L. and Raub, T.J., Fluid phase endocytosis by primary culture of bovine brain microvessels endothelial cell monolayers, *Microvasc. Res.*, 39 (1990) 1–14.
[15] Hatta, K., Nose, A., Nagafuchi, A. and Takeichi, M., Cloning and expression of cDNA encoding a neural calcium dependent cell adhesion molecules, its identity in the cadherin gene family, *J. Cell Biol.*, 106 (1988) 873–881.
[16] Heimark, R.L., Degner, M. and Schwartz, S.M., Identification of a Ca^{2+} dependent cell-cell adhesion molecule in endothelial cells, *J. Cell Biol.*, 110 (1990) 1745–1756.
[17] Hinck, L., Nathke, I.S., Papkoff, J. and Nelson, W.J., Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly, *J. Cell Biol.*, 125 (1994) 1327–1340.
[18] Lampugnani, M.G., Resnati, M., Dejana, E. and Marchisio, P.C., The role of integrins in the maintenance of endothelial monolayer integrity, *J. Cell Biol.*, 112 (1991) 479–490.
[19] Lampugnani, M.G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L.P. and Dejana, E., A novel endothelial-specific membrane protein is a marker of cell-cell contacts, *J. Cell Biol.*, 118 (1992) 1511–1522.
[20] Liaw, C.W., Cannon, C., Power, M.D., Kiboneca, P.K. and Rubin, L.L., Identification and cloning of two species of cadherins in bovine endothelial cells, *EMBO J.*, 9 (1990) 2701–2708.
[21] Lutz, K.L. and Siahaan, T.J., E-cadherin peptide sequence recognition by anti-E-cadherin antibody, *Biochem. Biophys. Res. Commun.*, 211 (1995) 21–27.
[22] Lutz, K.L. and Siahaan, T.J., Identification of cadherin sequences presumably responsible for the regulation of cell-cell adhesion in tight junctions. In: P.T.P. Kaumaya and R.S. Hodges (Eds.), *Peptides: Chemistry, Structure and Biology*, Mayflower Scientific, Kingswinford, 1996, pp. 168–169.
[23] Lutz, K.L., Jois, S.D.S. and Siahaan, T.J., Secondary structure of the HAV peptide which regulates cadherin-cadherin interaction, *J. Biomol. Struct. Dyn.*, 13 (1995) 447–455.
[24] Miyatani, S., Shimamura, K., Hatta, M., Nagafuchi, A., Nose, A., Matsunga, M., Hatta, K. and Takeichi, M., Neural cadherin: role in selective cell-cell adhesion, *Science*, 245 (1989) 631–635.
[25] Molony, L. and Armstrong, L., Cytoskeletal reorganization in human umbilical vein endothelial cells as a result of cytokine exposure, *Exp. Cell Res.*, 196 (1991) 40–48.
[26] Newman, P.J., Berndt, M.C., Gorsky, J., White, G.C., Paddock, L.S. and Muller, W.A., PECAM-1 (CD31): cloning and relation to adhesion molecules of the immunoglobulin gene superfamily, *Science*, 247 (1990) 1219–1222.
[27] Nose, A., Tsuji, K. and Takeichi, M., Localization of specificity determining sites in cadherin cell adhesion molecules, *Cell*, 61 (1990) 147–155.
[28] Overduin, M., Harvey, T.S., Bagby, S., Tong, K.I., Yau, P., Takeichi, M. and Ikura, M., Solution structure of epithelial cadherin domain responsible for selective cell adhesion, *Science*, 67 (1995) 386–389.
[29] Ozawa, M. and Kemler, R., Molecular organization of the Uvomorulin-catenin complex, *J. Cell Biol.*, 116 (1992) 989–996.
[30] Ozawa, M., Baribault, H. and Kemler, R., The cytoplasmic domain of the cell adhesion molecule Uvomorulin associates with three independent proteins structurally related in different species, *EMBO J.*, 8 (1989) 1711–1717.
[31] Rubin, L. L., Hall, D.E., Portex, S., Barbu, K., Cannon, C., Horner, H.C., Janatpour, M., Liaw, C.W., Manning, K., Morales, J., Tanner, L.I., Tomaselli, K.J. and Bard, F., A cell culture model of the blood–brain barrier, *J. Cell Biol.*, 115 (1991) 1725–1735.
[32] Salomon, D., Ayalon, O., Patel-king, R., Hynes, R.O. and Geiger, B., Extrajunctional distribution of N-cadherin in cultured human endothelial cells, *J. Cell Sci.*, 102 (1992) 7–17.
[33] Schneeberger, E.E. and Lynch, R.T., Tight junctions, their structure, composition and function, *Circ. Res.*, 55 (1984) 723–733.
[34] Simmons, D.L., Walker, C., Power, C. and Pigott, R., Molecular cloning of CD-31, a putative intercellular adhesion molecule closely related to carcino-embryonic antigen, *J. Exp. Med.*, 171 (1990) 2147–2152.
[35] Suzuki, S., Sano, K. and Tanihara, H., Diversity of cadherin family: evidence of eight new cadherins in nervous tissue, *Cell Regul.*, 2 (1991) 261–270.
[36] Takeichi, M., The cadherins: cell-cell adhesion molecules controlling animal morphogenesis, *Development*, 102 (1988) 639–655.
[37] Takechi, M., Cadherins: a molecular family important in selective cell-cell adhesion, *Annu. Rev. Biochem.*, 59 (1990) 237–252.
[38] Takeichi, M., Cadherin, cell adhesion receptor as a morphogenic regulator, *Science*, 251 (1991) 1451–1455.
[39] Takeichi, M., Morphogenetic roles of classic cadherins, *Curr. Opin. Cell Biol.*, 7 (1995) 619–627.
[40] Willems, J., Bruyneel, E., Noe, V., Slegers, H., Zwijsen, A., Mege, R.M. and Marrel, M., Cadherin-dependent cell aggregation is affected by decapeptide derived from rat extracellular superoxide dismutase, *FEBS Lett.*, 363 (1995) 289–292.
[41] Williams, E.J., Furness, J., Walsh, F.S. and Doherty, P., Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin, *Neuron*, 13 (1994) 583–594.