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Modulation of Endothelial Cell Adhesion by Hevin, an Acidic Protein Associated with High Endothelial Venules*

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High endothelial venules (HEV) are specialized plump postcapillary venules in lymphoid tissues that support high levels of lymphocyte extravasation from the blood. We have recently identified a novel human transcript, expressed to high levels in HEV, that encodes a secreted, acidic protein closely related to the anti-adhesive extracellular matrix protein known as BM-40, osteonectin, and SPARC (secreted protein acidic and rich in cysteine). Here, we show that this protein, designated hevin, is associated with basal, lateral, and apical surfaces of HEV cells, and unlike MECA-79 antigen, is not expressed on the underlying basement membrane. In contrast to fibronectin or other adhesive extracellular matrix proteins, purified hevin does not support endothelial cell adhesion *in vitro*. Moreover, addition of soluble exogenous hevin inhibits attachment and spreading of endothelial cells on fibronectin substrates. Hevin-treated cells do not form focal adhesions and exhibit a rounded morphology. Together, these results suggest that hevin is an abundant extracellular protein that modulates high endothelial cell adhesion to the basement membrane.

Lymphocytes continuously recirculate between the blood and lymphatic systems, thereby providing an effective immune surveillance for foreign invaders (1). In lymphoid organs, high numbers of lymphocytes leave the blood by recognizing and migrating through specialized postcapillary venules called high endothelial venules (HEV)[1] (for a review, see Ref. 2). The endothelial cells of HEV are called high endothelial cells by reference to their typical plump, almost cuboidal, morphology, very different from the flat appearance of endothelial cells that line other vessels. Another important feature of HEV endothelium is the expression of sialomucin counter-receptors for lymphocyte L-selectin (3–5), that are important in the initial step of lymphocyte binding to HEV and are decorated with sulfated oligosaccharides recognized by L-selectin and the HEV-specific mAb MECA-79 (6–8).

Although the molecular mechanisms involved in the induction and maintenance of the specialized morphology and phenotype of HEV have not yet been identified, local microenvironmental factors, such as extracellular matrix (ECM) molecules and cytokines associated with the immune response, are likely to play an important role (2). The ECM is an important component of the cellular environment, which plays a key role in the modulation of cell shape (9), cell differentiation, and tissue-specific gene expression (10). Endothelial cells are separated from adjacent connective tissue by a specialized sheet of ECM, known as the basement membrane, that contains adhesive ECM proteins such as laminin, collagen IV, and fibronectin. The ECM composition of the basement membrane has been shown to influence both endothelial cell morphology and differentiation (11, 12).

Two antiadhesive proteins of the ECM, thrombospondin (TSP) and SPARC (BM-40), have been shown to modulate the adhesion of endothelial cells to ECM and substratum (13). TSP substrates support the attachment of some endothelial cells but not cell spreading or formation of stable cell-substrate adhesion plaques or focal adhesions, while soluble TSP inhibits focal adhesion formation in cells seeded on fibronectin substrates (14). SPARC is an acidic Ca^{2+}-binding glycoprotein (15) that regulates endothelial cell shape and barrier function (16) by inhibiting cell spreading (17) and modulating focal adhesion disassembly (18). Although the precise mechanisms by which SPARC regulates endothelial cell adhesion are not well understood, a Ca^{2+}-binding EF hand, located in the carboxyl-terminal part of the protein, has been shown to have both antispreading (19) and focal adhesion-labilizing activity (20). Furthermore, this region binds to collagen IV (21).

We have recently characterized a novel human cDNA encoding an acidic putative calcium-binding protein, designated hevin, that exhibits 62% identity with SPARC over a region of 232 amino acids spanning more than four-fifths of the SPARC coding sequence (22). The greatest difference between the proteins is in the highly acidic amino-terminal domain of hevin (26% glutamic acid and aspartic acid residues), which is considerably larger (432 residues) than the corresponding domain in SPARC (71 residues). *In situ* hybridization analysis revealed that hevin mRNA is expressed to high levels in HEV from human lymphoid tissues. Although hevin mRNA is detected only in HEV in tonsil and is absent from human umbilical vein endothelial cells, Northern blots show that hevin is expressed in other tissues, including brain and heart, in a pattern distinct from SPARC. In view of its strong homology with the antiadhesive ECM protein SPARC and its high expression in HEV, hevin is a good candidate for an ECM protein that may facilitate lymphocyte migration by modulating high endothelial cell adhesion and phenotype. In this study, we have investigated

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[1] The abbreviations used are: HEV, high endothelial venules; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cells; SPARC, secreted protein acidic and rich in cysteine; TSP, thrombospondin; M199, Medium 199; FBS, fetal bovine serum; CPAE, calf pulmonary arterial endothelial cells; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; CHO, Chinese hamster ovary; α-MEM, α minimal essential medium; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

the effects of hevin on endothelial cell adhesion *in vitro*. We found that hevin is antiadhesive and inhibits both endothelial cell attachment and spreading on fibronectin substrates. We also show that hevin is associated with basal, lateral, and apical surfaces of high endothelial cells *in vivo* but not with the underlying basement membrane. Together, these results suggest that the function of hevin could be to modulate high endothelial cell adhesion to the basement membrane.

MATERIALS AND METHODS

Endothelial Cell Culture—Isolation and culture of human umbilical vein endothelial cells (HUVEC) was essentially as described previously (34). Passage 1–3 HUVEC were grown in Medium 199 (M199) (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated, low endotoxin FBS (Sigma), 100 μg/ml heparin (Sigma), 100 μg/ml endothelial mitogen (Biomedical Technologies, Inc., Stoughton, MA), 5 mM L-glutamine, 25 mM HEPES, and 50 μg/ml gentamicin at 37 °C, 5% CO_2. Bovine pulmonary artery endothelial cells (CPAE) were obtained from the American Tissue Culture Collection and grown in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD) containing 20% FBS (Sigma). The spontaneously transformed HUVEC-derived cell line ECV 304 (35) was cultured in M199 (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (Sigma).

Antibody Production, Immunoblotting, and Indirect Immunofluorescence Microscopy—Rabbit polyclonal antibodies against peptide AR-LLSDHSKPTAET corresponding to amino acids 21–34 of the hevin sequence (22) were produced by using multiple antigenic peptide technology (36) (Research Genetics, Huntsville, AL). Antibodies were affinity-purified on a multiple antigenic peptide affinity column prepared by coupling 5 mg of multiple antigenic peptide to 10 ml of carboxyl-activated support (Affi-Gel 10, Bio-Rad) in 50 ml of dry Me_2SO plus 100 μl of dry triethylamine for 16 h on a rocking shaker at room temperature. After washing with Me_2SO (3 × 50 ml), 1 M acetic acid (4 × 50 ml), and distilled water, the affinity support was poured into a column (1 ml bed volume) and equilibrated with 5 × PBS. Crude antisera (20 ml) was filtered, diluted 1:1 with 10 × PBS, and passed over the peptide affinity column 2 times at 0.5 ml/min. After washing with 50 ml of 5 × PBS, antibodies were eluted using 10 ml of 100 mM citrate, pH 2.5, into a tube containing 5 ml of 1 M Tris, pH 8.8. The affinity-purified hevin-peptide antibodies were then dialyzed against 1 × PBS at 4 °C for 16 h and concentrated to 400 μl by Centricon 100. The affinity-purified hevin-peptide antibodies were used for immunoblotting and immunohistochemistry at 1:20 and 1:5 dilutions, respectively. Western blotting (37) and detection was achieved with an enhanced chemiluminescence kit (Amersham Corp.). For immunohistochemistry, 8-μm acetone-fixed frozen sections of human tonsils were double stained in PBS, 1% BSA with affinity-purified antibodies against hevin peptide and monoclonal antibody MECA-79 (23) (rat IgM diluted to 1:5, kindly provided by Dr. Eugene Butcher, Stanford, CA) or monoclonal antibody against human fibronectin (mouse IgG1 diluted to 1:50, AMAC, Westbrook, ME), for 1 h in a moist chamber at room temperature, washed with PBS (3 × 10 min), incubated 30 min in PBS, 1% BSA with Texas Red-labeled donkey anti-rabbit Ig (1:50, Amersham Corp.), and FITC-labeled mouse anti-rat κ light chain (AMAC, Westbrook, ME) or FITC-labeled goat anti-mouse IgG (Zymed, San Francisco, CA), washed again with PBS (3 × 10 min) and then coverslipped using fluoromount-G medium (Fisher Scientific, Pittsburgh, PA) and viewed with a Zeiss microscope (Carl Zeiss, Thornwood, NY). Photographs were taken using a Nikon microflex UFX-IIA (Nikon, Garden City, NY) and Kodak T-Max P3200 film.

Production of Recombinant Hevin in CHO Cells—The glutamine synthetase expression system (25) was used to express hevin in CHO cells. A hevin cDNA 2.6-kilobase pair *Eco*R I-*Xho*I fragment was blunt-ended with Klenow DNA polymerase and blunt-ligated into the unique *Xho*I and *Not*I sites filled in with Klenow in the polylinker of the expression vector pBJ5-GS (26). The expression vector was transfected in CHO-K1 cells with calcium phosphate. Briefly, a calcium phosphate/DNA precipitate containing 20 μg of DNA was added to CHO cells in 15 ml of fresh α-MEM medium (Life Technologies, Inc.) with 10% dialyzed FBS (Sigma). The next day, the cells were washed and resuspended in α-MEM medium with 10% dialyzed FBS and 25 μM methionine sulfoximine. After 2 weeks, individual clones were picked and expanded. Clones secreting hevin were identified by SDS-PAGE of conditioned serum-free media after metabolic labeling with [^{35}S]methionine and cysteine. Exponentially growing cells in 96-well plates were starved for 45 min in serum-free Dulbecco's modified Eagle's medium minus methionine and cysteine (Life Technologies, Inc.) and then incubated in 100 μl of the same medium supplemented with 250 μCi/ml ^{35}S-protein labeling mix (DuPont NEN) for 4 h. Proteins secreted in conditioned media (25 μl) were fractionated by SDS-PAGE under reducing conditions and analyzed by fluorography. As a control, the hevin protein was also produced by *in vitro* translation of the hevin cDNA using the TNT-coupled reticulocyte lysate system (Promega).

Purification of Hevin Protein—CHO-hevin transfectants (clone 152-G11) were grown in α-MEM medium with 10% dialyzed FBS and 25 μM methionine sulfoximine in roller bottles until subconfluent. The cells were then washed twice in serum-free medium and incubated for 3 days in serum-free α-MEM supplemented with 25 μM methionine sulfoximine. Conditioned medium (250 ml) was collected, adjusted to pH 6, and fractionated on a HiTrap Q column (Pharmacia Biotech Inc.) in buffer bis-Tris 20 mM, pH 6, using the fast protein liquid chromatography system (Pharmacia). Proteins retained on the column were eluted using a 0.2–0.5 M NaCl salt gradient. The hevin protein was found to elute as a single peak at 0.3 M NaCl. Typically, ~3 mg of hevin (quantified by bicinchoninic acid assay, Pierce, Rockford, IL) was obtained from 250 ml of supernatant.

Endothelial Cell Attachment Assay—Endothelial cell attachment assays were performed as described previously (29) with minor modifications. Purified human fibronectin, tenascin, and thrombospondin were purchased from Life Technologies, Inc. (Gaithersburg, MD). The entactin-collagen IV-laminin cell attachment matrix, derived from Englebreth-Holm-Swarm mouse tumor, was obtained from Promega (Madison, WI). The ECM proteins (10 μg/ml) diluted in PBS containing 1 mM Ca^{2+} and Mg^{2+} (PBS^+) were immobilized onto nontissue culture-treated Linbro/Titertek 96-well plates (Flow Laboratories, McLean, VA) by incubating overnight at 4 °C. The unbound sites were then blocked in 1% BSA, PBS^+ for 1 h at 37 °C. The endothelial cells were resuspended in serum-free growth medium, M199 or Dulbecco's modified Eagle's medium, at a concentration of 10^6 cells/ml, and 100 μl were added to each well. The plates were incubated for 3 h at 37 °C, 5% CO_2 and then washed twice with PBS^+ to remove the unbound cells. The adherent cells were fixed with 3.7% formaldehyde containing 0.5% crystal violet for 15 min, gently washed twice with PBS^+, and quantitated by measuring the absorbance at 620 nm on a Titertek Multiskan MCC/340 (Flow Laboratories, McLean, VA) microtiter plate reader.

Effects of Hevin on Endothelial Cell Adhesion and Spreading—Cells were trypsinized, washed in media containing 10% FBS, and resuspended in M199 or Dulbecco's modified Eagle's medium serum-free media at 5 × 10^5 cells/ml. Cell suspensions (100 μl) were added together with protein solutions (100 μl in PBS^+) in 96-well nontissue culture-treated Linbro Titertek plates coated with human fibronectin (1 μg/ml for 2 h at 37 °C). Purified human hevin, tenascin, SPARC (Haematologic Technologies Inc., Essex Junction, VT), or thrombospondin were used at a final concentration of 10 μg/ml while BSA was used at a final concentration of 100-5000 μg/ml. Conditioned media from CHO-hevin and -control transfectants were 10 μl of serum-free conditioned media concentrated 20-fold with Centricon 30, added to 90 μl of PBS^+, to yield a final concentration in the assay of 1-fold. After 3 h at 37 °C, 5% CO_2, the wells were gently washed twice with PBS^+, and adherent cells were fixed with 3.7% formaldehyde containing 0.5% crystal violet. After two more washes in PBS^+, adherent cells were quantitated by measuring the absorbance at 620 nm on a microtiter plate reader. Cell spreading was assessed by visual inspection using an inverted Nikon Diaphot microscope and photographed with Kodak T-Max 400 film. Flattened cells with diminished cellular refractility were classified as spread, rounded cells with short processes in the initial stages of spreading as unspread, and highly refractile cells with no apparent processes as round.

Analysis of Focal Adhesion Formation—Glass coverslips (18-mm diameter) were placed in 6-well plates and incubated for 2 h at 37 °C in PBS^+ containing 2 μg/ml highly purified human fibronectin (Life Technologies, Inc.). Wells were washed twice with PBS^+ before the addition of soluble proteins (hevin or BSA at 10 μg/ml) and freshly trypsinized endothelial cells (2 × 10^5 cells/well) in serum-free M199 media. HUVEC and ECV304 cells were allowed to attach and spread at 37 °C, 5% CO_2 for 4 or 16 h, respectively. Wells were then washed twice with PBS^+ to remove nonadherent cells, and cells attached to the coverslips were fixed for 15 min at room temperature in 3.7% formaldehyde in PBS^+, followed by two washes in PBS^+ and a 10-min incubation in 50 mM NH_4Cl. Cells were permeabilized by treatment with 0.2% Triton X-100 for 5 min at room temperature, washed twice with PBS^+, and incubated 30 min with 1% BSA in PBS^+ to block nonspecific binding sites. Indirect immunofluorescence staining for vinculin was then performed as described above with monoclonal anti-human vinculin antibody hVIN-1 (mouse IgG1 diluted to 1:100, Sigma) and Texas Red-labeled sheep

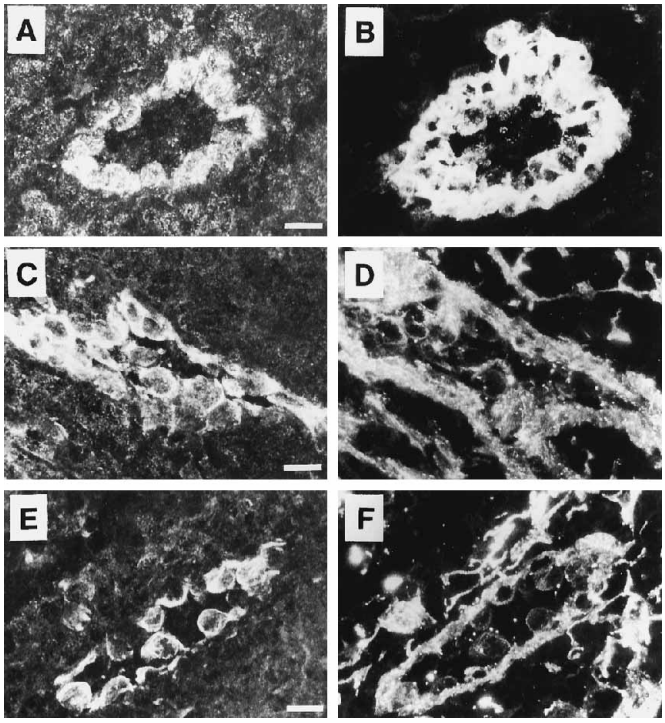

FIG. 1. **Immunofluorescence staining of human tonsil frozen sections with hevin-peptide antibodies.** Frozen sections of human tonsils (8 μm, fixed with acetone) were double-stained with affinity-purified hevin-peptide antibodies (*A*, *C*, and *E*) and HEV-specific monoclonal antibody MECA-79 (*B*) or monoclonal antibody against human fibronectin (*D* and *F*) for 1 h in a moist chamber at room temperature and binding was detected with FITC- and Texas Red-labeled secondary antibodies. *Bar*, 20 μm.

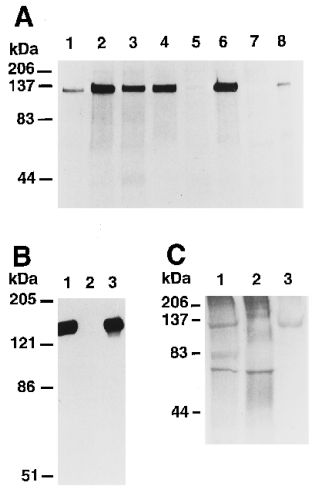

FIG. 2. **Expression and purification of hevin protein.** *A*, hevin protein secreted by CHO cells. CHO-hevin (*lanes 1-6*) or -control (*lane 7*) clonal transfectants were labeled for 4 h with [^{35}S]cysteine and methionine, and 25 μl of conditioned media was subjected to 10% SDS-PAGE under reducing conditions and fluorography. Hevin translated *in vitro* was used as a control (*lane 8*). *B*, immunodetection of hevin protein in CHO-hevin but not CHO-control transfectants. Media conditioned by CHO-hevin (*lane 1*) or CHO-control (*lane 2*) transfectants (10 μl of serum-free conditioned media concentrated 20-fold) and purified hevin protein (1 μg, *lane 3*) were fractionated by 10% SDS-PAGE under reducing conditions, and proteins were immunoblotted with affinity-purified hevin-peptide antibodies. *C*, purification of hevin. Conditioned media (10 μl of serum-free conditioned media concentrated 20-fold) from CHO-hevin (*lane 1*) or CHO-control (*lane 2*) transfectants and hevin protein purified from conditioned media by anion-exchange chromatography (1 μg, *lane 3*) were fractionated by 10% SDS-PAGE under reducing conditions and subjected to silver staining.

anti-mouse Ig (diluted to 1:50, Amersham Corp.). FITC-labeled phalloidin (diluted to 0.5 μg/ml, Sigma) was added to the Texas Red-labeled antibody to stain for F-actin.

RESULTS

Hevin Is Associated with the Surface of HEV Cells in Vivo—To determine whether hevin is expressed in the basement membrane or associated with the cell surface of HEV *in vivo*, we stained human tonsil sections with affinity-purified polyclonal antibodies directed against a peptide corresponding to residue 21–34 of the hevin protein (Fig. 1, *A*, *C*, and *E*). Double labeling with the HEV-specific rat mAb MECA-79 (23, 24) (Fig. 1*B*) revealed that the hevin-peptide antibodies stain MECA-79$^+$ HEV exhibiting plump endothelium (Fig. 1, *A* and *B*). Strikingly, whereas MECA-79 gave strong staining of both HEV cells and basement membrane (Fig. 1*B*), hevin staining was primarily associated with the HEV cells (Fig. 1*A*). Hevin was found on the basal surface of HEV apposed to the substratum, but it also appeared to be exposed on the lateral surface between high endothelial cells and on the lumenal surface in contact with blood. In contrast, fibronectin was primarily found in the basement membrane of HEVs, although low levels were also detected on the surface of some HEV cells (Fig. 1, *D* and *F*). Double labeling with hevin-peptide antibodies showed colocalization of hevin with fibronectin on the surface of high endothelial cells (Fig. 1, *C* and *E*).

Hevin Is Not a Substrate for Endothelial Cell Attachment and Spreading—Since hevin is associated with the surface of high endothelial cells *in vivo*, we decided to investigate the effects of exogenous hevin on endothelial cell adhesion *in vitro*. To obtain sufficient amounts of hevin for *in vitro* analysis, we expressed the hevin cDNA in CHO cells using the glutamine synthetase expression and amplification system (25, 26). Colonies resistant to the drug methionine sulfoximine were selected and analyzed for hevin expression. Since the hevin cDNA is predicted to encode a secreted protein, we analyzed conditioned media after metabolic labeling with [^{35}S]methionine and cysteine. We found that 14 out of 15 clones secrete various amounts of a major protein of about 130 kDa after fractionation by SDS-PAGE under reducing conditions (Fig. 2*A*, representative supernatants in *lanes 1-6*). This protein is not secreted by CHO cells transfected with the vector alone (*lane 7*). Immunoblotting reveals that the hevin-peptide antibodies recognize this protein of 130 kDa in CHO-hevin supernatants but do not react with CHO-control supernatants (Fig. 2*B*, *lanes 1* and *2*), thereby establishing the identity of this secreted protein of 130 kDa with hevin. The predicted molecular mass of the hevin protein is 75.2 kDa. To examine the basis for this discrepancy, we produced an *in vitro* translated protein in rabbit reticulocyte lysate. We found that the protein produced by *in vitro* translation of the hevin cDNA, in a system in which *N*-linked glycosylation is absent, has the same apparent molecular mass of 130 kDa as the protein secreted by CHO transfectants (Fig. 2*A*, *lane 8*). This suggests that the difference between the apparent and the predicted mass of the hevin protein is not due to glycosylation or other post-translational processing events and is more likely to result from aberrant migration of the protein on SDS-PAGE. This abnormal migration is probably due to the large amino-terminal acidic domain, since a similar phenomenon has been described for other acidic proteins (27).

Silver staining after fractionation by SDS-PAGE under reducing conditions revealed that the hevin protein is a major component secreted by CHO-hevin (Fig. 2*C*, *lane 1*) but not by CHO-control (*lane 2*) transfectants. To purify hevin from conditioned media, we took advantage of the acidic isoelectric point predicted for the hevin protein (pI = 4.5). After 3 days of culture in media without serum, we collected supernatants from the CHO-hevin transfectants and separated the hevin

protein from other components of the conditioned media by anion-exchange chromatography at pH 6 on HighTrap Q. Under the conditions used, the hevin protein was retained on the column and eluted as a single 130 kDa band almost devoid of contaminants (Fig. 2*C*, *lane 3*). Starting with 250 ml of conditioned media, this strategy allowed us to obtain ~3 mg of pure protein in an intact native form appropriate for functional studies.

Hevin was tested for its ability to mediate attachment of human and bovine endothelial cells relative to substrates composed of ECM proteins, which are known to support endothelial cell adhesion. Since it was not possible to obtain sufficient purified HEV cells from tonsils for these studies, we used HUVEC as a source of human endothelial cells. We also used CPAE cells, which are sensitive to the antiadhesive effects of SPARC (16) and have been shown to express heparin-like ligands for L-selectin (28). Individuals wells of a 96-well tissue culture dish were incubated for 18 h at 4 °C with 10 μg/ml of each ECM molecule. Cells were then added to each well in serum-free medium and allowed to attach for 3 h at 37 °C. To quantitate cell attachment, a colorimetric assay was used (29). The A_{620}, a measure of crystal violet staining of cells bound to the substrate, correlates well with the visual scoring and allows a quantitative analysis of cell attachment (Fig. 3). We observed efficient attachment and spreading of both HUVEC (Fig. 3*A*) and CPAE cells (Fig. 3*B*) on substrates coated with fibronectin (Fig. 3), collagen 1, or a basal lamina ECM preparation containing entactin, collagen IV, and laminin (data not shown). We also observed efficient attachment and spreading of HUVEC and CPAE on substrates coated with tenascin, which is in agreement with other studies that have revealed that tenascin is not anti-adhesive for endothelial cells but promotes endothelial cell attachment mediated by $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins (29). In contrast, substrates coated with hevin did not promote any attachment after 3 h of plating at 37 °C. Since the adhesive activity of tenascin has previously been shown to depend on the coating conditions (30), we coated hevin on plastic at different concentrations (1–20 μg/ml), different temperatures (4 or 37 °C) and for varying periods of time (1, 2, or 18 h). Under all conditions examined, including at 10 μg/ml and 4 °C for 18 h, which for tenascin allows maximal attachment of endothelial cells, hevin had no adhesive activity (Fig. 3, *A* and *B*). The lack of adhesive activity of the native hevin protein is not due to inefficient coating of hevin on plastic, since when coated under the same conditions hevin inhibits attachment of CPAE cells to plastic (see below). Finally, another anti-adhesive ECM protein, TSP, was tested in the endothelial cell attachment assay. TSP supported attachment of some HUVEC and CPAE cells but was not as effective as fibronectin, collagen 1, basal lamina ECM, or tenascin substrates (Fig. 3, *A* and *B*). Moreover, in contrast to the other substrates, HUVEC and CPAE did not spread on TSP substrates.

Hevin Inhibits Endothelial Cell Attachment and Spreading on Fibronectin Substrates—We tested the effect of soluble hevin on attachment of endothelial cells to plastic substrates or substrates coated with fibronectin or basal lamina ECM. Addition of soluble hevin at a concentration of 10 μg/ml at the time of cell plating resulted in a 90% reduction of CPAE cell attachment to plastic, while BSA at 100 μg/ml had little effect (Fig. 4*A*). Soluble hevin inhibited attachment of CPAE cells on a fibronectin substrate in a concentration-dependent manner (Fig. 4*B*). In contrast, BSA at concentration of 5000 μg/ml had little effect. Similarly, addition of 1–10 μg/ml soluble hevin inhibited HUVEC attachment to fibronectin-coated plates (Fig. 4*C*). Hevin at a final concentration of 10 μg/ml was also able to reduce significantly the attachment of HUVEC to plates coated

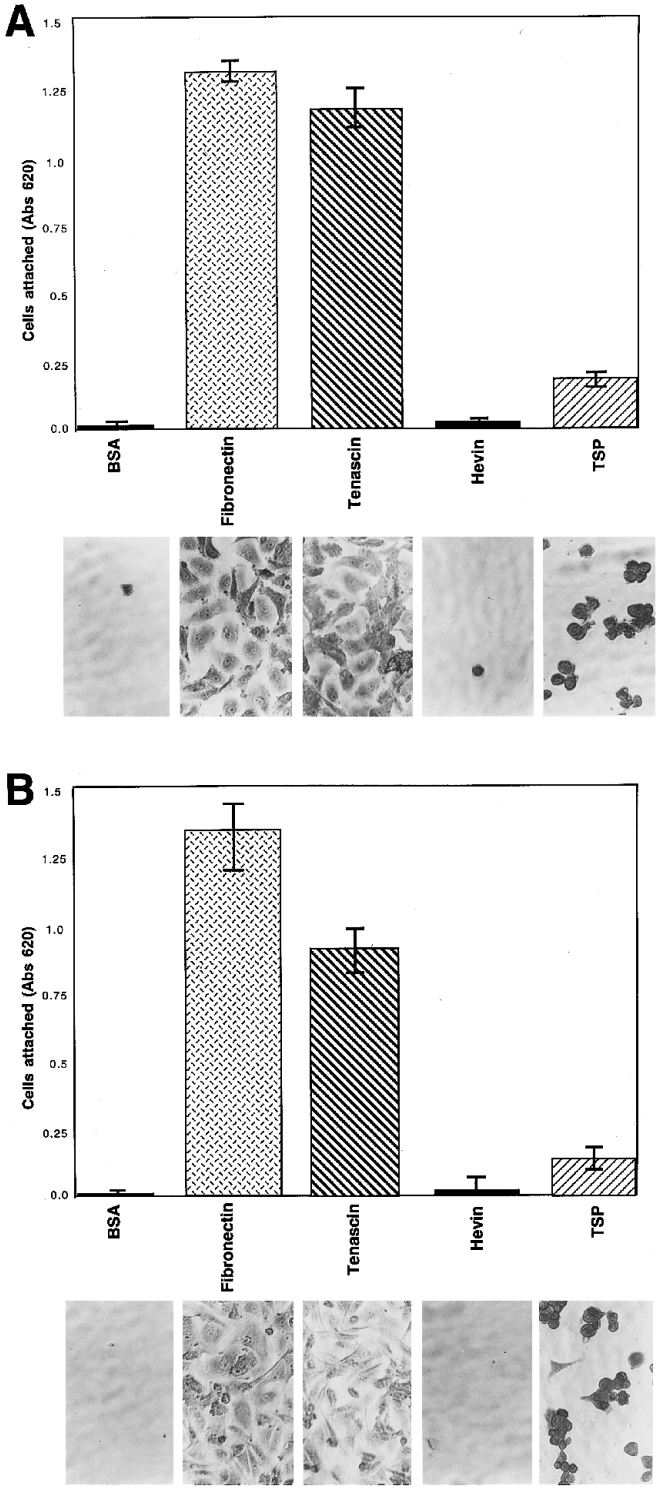

FIG. 3. **Hevin substrates do not support endothelial cell attachment and spreading.** Adhesion of HUVEC (*A*) and CPAE cells (*B*) to substrates coated with BSA, fibronectin, tenascin, hevin or TSP. BSA (1 mg/ml) and purified ECM proteins (10 μg/ml) were coated overnight at 4 °C. 10^5 cells were added to each well in serum-free medium and allowed to attach for 3 h at 37 °C, 5% CO_2. Cell attachment was quantitated by a colorimetric assay. The A_{620} correlates with the number of cells bound to the substrate. Cell attachment was also assessed using an inverted microscope, and representative photomicrographs are shown of cells on each substrate. Results are mean and standard deviations of triplicate determinations.

with a basal lamina ECM preparation containing entactin, collagen IV, and laminin (Fig. 4*D*). Cells from an HUVEC-derived cell line, ECV304, were also tested and found to be even

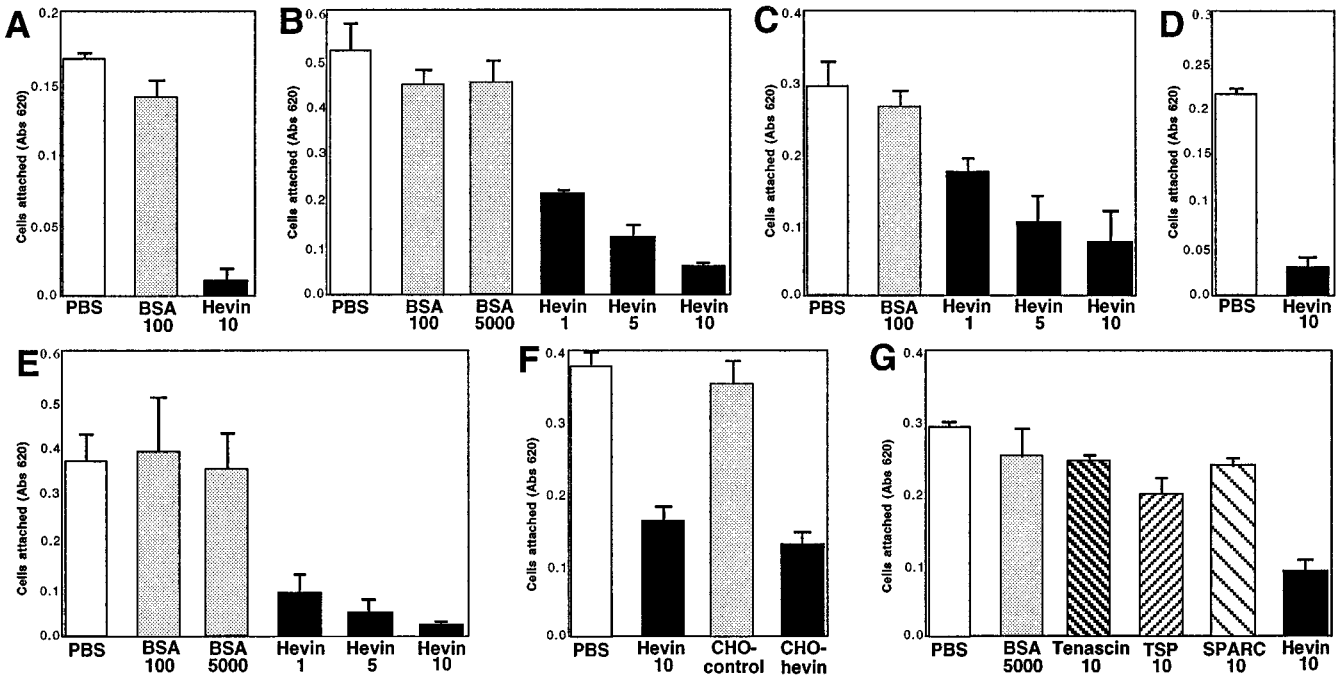

FIG. 4. **Hevin inhibits endothelial cell adhesion.** *A*, CPAE adhesion to plastic; *B*, CPAE adhesion to fibronectin; *C*, HUVEC adhesion to fibronectin; *D*, HUVEC adhesion to a basal lamina ECM preparation containing entactin, collagen IV, and laminin. *E*, ECV 304 cell adhesion to fibronectin; *F*, effects of conditioned media from CHO-hevin or -control transfectants on HUVEC adhesion to fibronectin; *G*, effects of antiadhesive ECM proteins on HUVEC adhesion to fibronectin. Soluble proteins were added together with 5×10^4 cells at the time of seeding on plates coated with 1 μg/ml purified fibronectin or 2 μg/ml basal lamina ECM. Soluble proteins included hevin (1–10 μg/ml), BSA (10–5000 μg/ml), tenascin, SPARC, or thrombospondin (10 μg/ml) or conditioned media from CHO-hevin and -control transfectants (10 μl of serum-free conditioned media concentrated 20-fold and diluted 20-fold in the assay to a final concentration of 1-fold). Cell attachment was quantitated by a colorimetric assay. The A_{620} correlates with the number of cells remaining bound to the substrate. Results are means of two independent experiments performed in triplicate with the larger of the S.D. for each point from the two experiments.

more sensitive to the anti-adhesive effects of hevin than HUVEC or CPAE cells (Fig. 4*E*). To eliminate the possibility that antiadhesive effects could be due to a minor contaminant secreted by CHO cells and copurifying with hevin, we tested conditioned media from CHO-control and -hevin transfectants in the HUVEC adhesion assay. We found that media from CHO cells transfected with the vector alone (CHO-control) had no anti-adhesive activity (Fig. 4*F*). In contrast, conditioned media from CHO transfectants secreting hevin (CHO-hevin) inhibited attachment of HUVEC to fibronectin equivalently to purified hevin (Fig. 4*F*). Together, these experiments show that hevin, acting as a soluble ligand, has strong inhibitory effects on the attachment of both human and bovine endothelial cells to fibronectin. We then addressed the specificity of the antiadhesive effect of hevin on endothelial cell adhesion to fibronectin by testing other antiadhesive ECM proteins. While tenascin and SPARC at a concentration of 10 μg/ml had minimal effects on HUVEC attachment to fibronectin, TSP consistently inhibited attachment by 20% (Fig. 4*G*). Similar results were obtained when the effects of tenascin, SPARC, and TSP on CPAE attachment to fibronectin were analyzed (data not shown). Therefore, among the antiadhesive ECM proteins, hevin appears to have the strongest inhibitory effect on endothelial cell adhesion to fibronectin.

Strong adhesion of endothelial cells to fibronectin substrates develops over time because of increases in cell area (spreading) and the active formation of focal adhesions (31). One mechanism by which hevin could inhibit endothelial cell adhesion to fibronectin is by interfering with these processes. To test for the first possibility, the effects of soluble hevin on endothelial cell spreading were analyzed. HUVEC that attached to fibronectin substrates in the presence of BSA started to spread after 1 h, and most of the cells were spread after 3 h at 37 °C (Fig. 5*A*). In contrast, cells that received soluble hevin at 10 μg/ml, retained a rounded morphology on fibronectin-coated plates and did not spread after 3 h at 37 °C (Fig. 5*B*). The antispreading effect of hevin was found to be specific and not due to a minor contaminant secreted by CHO cells, since HUVEC incubated in the presence of conditioned media from CHO-hevin transfectants exhibited a rounded morphology, while HUVEC plated in the presence of conditioned media from CHO-control transfectants underwent extensive spreading during a 3-h incubation period (data not shown).

To further characterize the anti-adhesive activities of the hevin protein, we analyzed the formation of focal adhesions in HUVEC seeded on fibronectin substrates in the presence or absence of hevin. In the absence of hevin, staining for vinculin revealed that HUVEC formed many focal adhesions, which were present both over the central cell body and at the edges of the cells (Fig. 5*C*). Double-staining for F-actin revealed prominent stress fibers that traversed the cell body (Fig. 5*E*). In contrast, there was no formation of focal adhesions in hevin-treated cells, and the staining for vinculin was diffuse throughout the cytoplasm (Fig. 5*D*). Double staining for F-actin revealed the absence of prominent actin-containing stress fibers (Fig. 5*F*). Inhibition of spreading and focal adhesion formation by soluble hevin was also observed with the HUVEC-derived cell line ECV 304. Although spreading and focal adhesion formation of ECV 304 cells on fibronectin substrates required longer time than for HUVEC, essentially identical results were obtained. BSA-treated cells contained prominent actin stress fibers terminating at vinculin-positive focal adhesions (Fig. 5, *G* and *I*). In contrast, hevin-treated ECV 304 cells failed to spread and did not form focal adhesions (Fig. 5, *H* and *J*). These results demonstrate that hevin is able to inhibit both endothelial cell spreading and focal adhesion formation on fibronectin substrates.

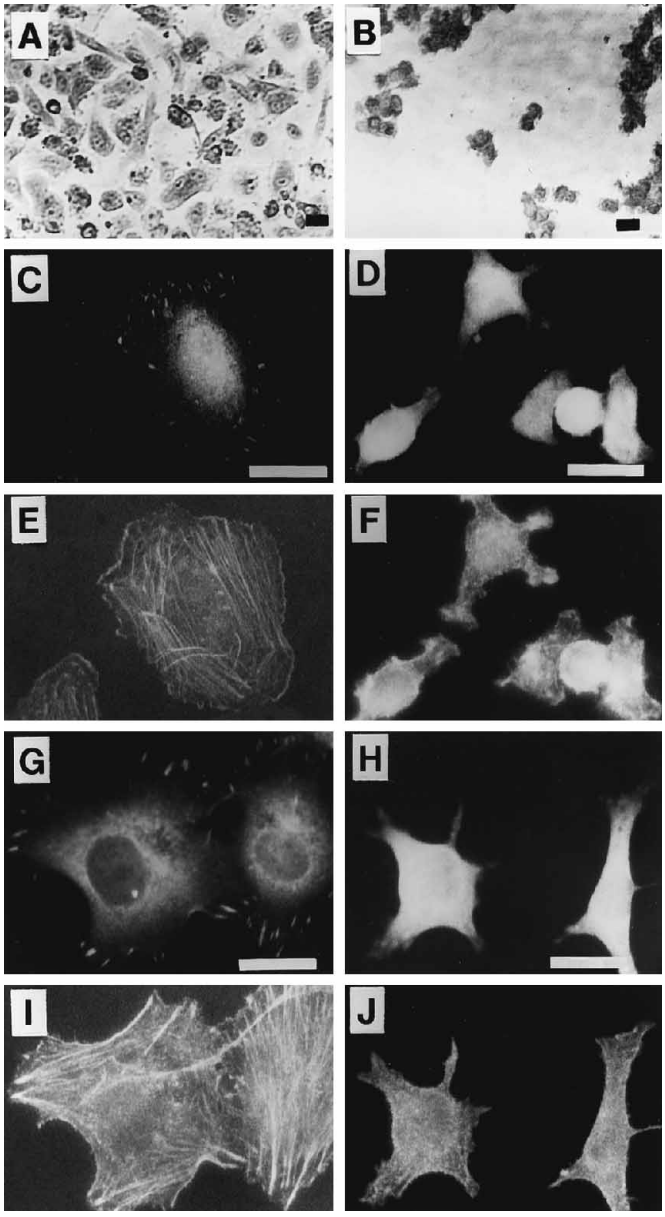

FIG. 5. **Hevin inhibits endothelial cell spreading and focal adhesion formation on fibronectin substrates.** *A*, spreading of HUVEC for 4 h on fibronectin in the presence of 10 μg/ml BSA; *B*, hevin at a concentration of 10 μg/ml inhibits HUVEC spreading on fibronectin; *C–F*, localization of vinculin (*C* and *D*) and F-actin (*E* and *F*) in HUVEC treated with 10 μg/ml BSA (*C* and *E*) or hevin (*D* and *F*); *G–J*, localization of vinculin (*G* and *H)* and F-actin (*I* and *J*) in ECV 304 cells treated with 10 μg/ml BSA (*G* and *I*) or hevin (*H* and *J*) and allowed to spread for 16 h. BSA-treated HUVEC or ECV 304 cells have numerous actin-containing stress fibers and vinculin-containing plaques terminating at the end of these stress fibers. Hevin-treated cells exhibit a primarily diffuse distribution of vinculin and do not have prominent stress fibers. *Bar*, 25 μm.

DISCUSSION

Hevin Is a Novel Member of the Family of Adhesion-modulating Proteins—In this study, we show that hevin not only fails to serve as a substrate for endothelial cell attachment and spreading, but also that soluble hevin added to cells at the time of seeding inhibits endothelial cell attachment, spreading, and focal adhesion formation on plastic and on fibronectin substrates. These results suggest that hevin is a novel member of the family of adhesion-modulating proteins that includes SPARC, thrombospondin, and tenascin (13). These antiadhesive extracellular proteins are expressed to high levels in tissues with a high proportion of dividing or migrating cells. In accordance with this generalization, hevin is expressed to high levels in HEV, where large scale lymphocyte emigration is occurring.

The results presented here, together with our previous work (22), clearly show that hevin is related to SPARC both structurally and functionally. However, the two proteins are likely to have distinct, rather than overlapping, physiological roles. The tissue distribution of hevin mRNA is clearly different from that of SPARC (22). For example, hevin is expressed to high levels in brain and low levels in placenta and testis, while SPARC has an opposite expression pattern in these tissues. In lymphoid tissues, both proteins are expressed; however, they have a different cellular distribution. Hevin is expressed in HEV, while SPARC is not detected in HEV but can be found in scattered cells of the tonsils.[2]

We compared hevin with other proteins for inhibition of endothelial cell adhesion. TSP slightly reduces the number of endothelial cells attaching to a fibronectin substrate. These findings are in agreement with previous studies that have revealed that TSP inhibits both endothelial cell adhesion and focal adhesion formation on fibronectin substrates (14, 32). Since there are no structural similarities between hevin and TSP, it is likely that these two proteins exert their antiadhesive effects by different mechanisms. Although the heparin-binding domain of TSP has been shown to have focal adhesion labilizing activity (33), the molecular mechanisms by which TSP inhibits focal adhesion formation remain to be characterized. Tenascin has also been shown to modulate focal adhesion formation in endothelial cells seeded on fibronectin substrates (18). However, tenascin is primarily adhesive for endothelial cells, since human endothelial cells have been shown to use $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins to attach and spread on tenascin substrates (29).

A Role for Hevin in the Control of HEV Specialization and Lymphocyte Migration?—It is not yet known whether HEV-derived ECM can induce HEV characteristics in non-HEV endothelial cells. Hevin could be one of the factors important for the induction of specialized properties of HEV cells. Hevin is expressed to high levels in HEV and associated with the cell surface on both basal, lateral, and lumenal sides of HEV. Therefore, hevin is ideally located to modulate adhesion of HEV cells to fibronectin, collagen, and other ECM proteins of the basal lamina. Inhibition of high endothelial cell adhesion to ECM proteins by the antiadhesive hevin may facilitate lymphocyte migration through HEV. One of the current mysteries of lymphocyte homing is how a lymphocyte that is rolling on a HEV is stimulated to develop firm adhesion through lymphocyte function-associated antigen-1 (CD11a/CD18) and arrest, a step that precedes emigration through the HEV. The finding of hevin on the lumenal surface of HEV suggests that it might also participate in this step, either directly, or indirectly. The negatively charged acidic domain of hevin might act analogously to negatively charged heparan sulfate proteoglycan side chains and allow hevin to present basic activating proteins to rolling lymphocytes. To determine the physiologic role of hevin *in vivo*, it will be important to knock out the hevin gene or carry out studies with function-blocking antibodies.

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