Cloning from Purified High Endothelial Venule Cells of Hevin, a Close Relative of the Antiadhesive Extracellular Matrix Protein SPARC

Jean-Philippe Girard and Timothy A. Springer
The Center for Blood Research
and Department of Pathology
Harvard Medical School
Boston, Massachusetts 02115

Summary

High endothelial venules (HEV) in lymphoid tissues support high levels of lymphocyte extravasation from the blood. We purified high endothelial cells from human tonsils by immunomagnetic selection with MECA-79 MAb to construct an HEV cDNA library. Differential screening of this library using cDNA probes from HEV (plus) or flat-walled vessel (minus) endothelial cells allowed us to characterize a novel human cDNA expressed to high levels in HEV. The cDNA encodes a secreted acidic calcium-binding glycoprotein of 664 aa residues, designated hevin, exhibiting 62% identity with the antiadhesive extracellular matrix protein SPARC, over a region of 232 aa spanning more than four fifths of the SPARC coding sequence. The primary structure and sequence of hevin are similar to SPARC-like proteins from rat and quail, called SC1 or QR1. Hevin could contribute to the induction or maintenance of features of the HEV endothelium that facilitate lymphocyte migration.

Introduction

Patrolling the body in search of foreign antigen, lymphocytes continuously recirculate from blood, through lymphoid and other tissues, into the lymphatics, and back to the blood. This process, called lymphocyte recirculation, allows the dissemination of the immune response throughout the body and, thus, provides an effective immune surveillance for foreign invaders (Yednock and Rosen, 1989; Picker and Butcher, 1992). In human secondary lymphoid organs, such as lymph nodes, tonsils, adenoïds, appendix, and Peyer's patches, lymphocyte adherence and trans-endothelial migration occur at specialized postcapillary vascular sites called high endothelial venules (HEV). In contrast with the endothelial cells from other vessels, the high endothelial cells of HEV have a plump, almost cuboidal, appearance and are able to support high levels of lymphocyte extravasation (Marchesi and Gowans, 1963; Anderson et al., 1976). In chronic inflammation, the activated endothelium of nonlymphoid tissues has an HEV-like morphology and exerts functions in lymphocyte recruitment from the blood, comparable to high endothelium in lymphoid tissues (Freemont, 1966). The plump morphology of endothelial cells from HEV, together with their striking plasticity, could represent a special adaptation to minimize the loss of blood fluid during lymphocyte transmigration (Schoell, 1972). At the ultrastructural level, high endothelial cells are characterized by a prominent Golgi complex, abundant polyribosomes, and rough endoplasmic reticulum, revealing an intense biosynthetic activity not observed in "flat" endothelial cells (Anderson et al., 1976; Freemont and Jones, 1983). Another typical feature of HEV, revealed by ultrastructural studies, is the existence of discontinuous "spot-welded" junctions between adjacent high endothelial cells that resemble desmosomes (Anderson et al., 1976; Freemont and Jones, 1983). This latter characteristic is very important, since the absence of continuous "tight" junctions in HEV is likely to be one of the factors allowing massive lymphocyte migration (Anderson and Shaw, 1993).

It was originally thought that a single receptor-counter-receptor pair would explain the specificity of lymphocyte migration through HEV. However, recent studies suggest that the molecular mechanisms conferring this specificity are more complex, with many different molecules involved in a multistep process (Springer, 1994). The initial interaction of lymphocytes with HEV in vivo under flow results in a loose transient adhesion, known as lymphocyte rolling (Bargatz and Butcher, 1993). In vitro studies have shown that lymphocyte L-selectin binds to the peripheral node addressin, defined by MECA-79 monoclonal antibody (Ab) (Berg et al., 1991), and to its components, GlyCAM-1 (Lasky et al., 1992) and CD34 (Baumhueter et al., 1993), as well as to the mucosal addressin MadCAM-1 (Berg et al., 1993), and that this interaction can mediate rolling (Berg et al., 1993) (Lawrence et al., submitted). Lymphocytes rolling on HFV arrest in a process that is dependent on G protein–coupled receptors on lymphocytes (Bargatz and Butcher, 1993) and that likely involves chemotactic-stimulated activation of firm adhesion through integrins (Springer, 1994). Lymphocyte migration through HEV is very efficient, since it has been estimated that as many as 1.4 x 10^9 lymphocytes extravasate from the blood into a single lymph node (via HEV) every second (Cahill et al., 1976). The entire process of lymphocyte sticking to HEV (rolling, activation, strong adhesion) takes only a few seconds (Bargatz and Butcher, 1993), while the transendothelial migration and the passage of lymphocytes through the HEV basement membrane occur in about 10–15 min (Smith and Ford, 1983).

HEV are likely to express many adhesion molecules and extracellular matrix proteins that facilitate the migration of lymphocytes between high endothelial cells and, thus, allow lymphocyte emigration in HEV to be so efficient. However, these molecules remain poorly characterized. Studies of the specialization of the HFV endothelium have been hampered by the fact that the lymphoid tissue environment is required to induce and maintain the HEV phenotype (Hendriks and Eestermans, 1983; Mebius et al., 1991b) and that high endothelial cells very rapidly lose their specialized phenotype when they are grown in vitro (Mebius et al., 1991a). To characterize further the specialization of the HEV endothelium at the molecular level, we designed a new strategy to isolate HEV-specific genes. We purified high endothelial cells from human tonsils by
Figure 1. Schematic of the Strategy Used to Purify High Endothelial Cells from Human Tonsils

**Results**

**Purification of High Endothelial Cells from Human Tonsil**

We purified high endothelial venule cells from human tonsils in four steps (Figure 1). Tonsils were minced finely with scissors on a steel screen under frequent flushing with media to remove most lymphocytes. To remove lymphocytes trapped between or adherent to stromal elements, stromal elements remaining on the screen were collected, incubated with collagenase under mild conditions, and the released lymphocytes were removed with a secondary screening step. The stromal elements were then subjected to strong collagenase digestion to obtain a single cell suspension, and stromal cells that passed through the screen were collected. Indirect immunofluorescence staining with the HEV-specific MAb MECA-79 showed that this tonsillar stromal cell suspension contained 1% of high endothelial cells (Figure 2a). We estimated that high endothelial cells accounted for 0.025% or less of the total cells from human tonsils, and that a 40-fold enrichment in high endothelial cells was obtained (Table 1). Immunomagnetic selection was used to separate further HEV endothelial cells from other tonsillar stromal cells. To select high endothelial cells, we used the MAb MECA-79 (provided by Dr. E. Butcher), which recognizes HEV ligands for lymphocyte L-selectin (Berg et al., 1991) and inhibits lymphocyte homing to lymph node in vivo (Streeter et al., 1988). MECA-79 was a suitable reagent for high endothelial cell purification, since it reacted strongly with all HEV present in human tonsils and did not cross-react with other cells (Michie et al., 1993). We labeled the tonsillar stromal cell suspension with MAb MECA-79 and paramagnetic beads, selected the magnetic high endothelial cells with the Magnetic Cell Sorting system, and monitored the separation by flow cytometry (Figure 2a). In a typical experiment, about $3.7 \times 10^6$ high endothelial cells were obtained in one preparation from four tonsils (Table 1). Under fluorescence microscopy, the purified high endothelial cells appeared as isolated single cells or small clusters of 2–10 cells (Figure 2c). They were heterogeneous in size, ranging from 15–20 μm in diameter (Figure 2d). Some of them retained a “cuboidal” morphology (Figure 2e). The enrichment in high endothelial cells obtained after the entire procedure was at least 2400-fold (Table 1). The typical purity of the HEV cells was 60%–85%, with a range of 50%–90%, which was sufficient for most purposes. We repeated the purification procedure seven times to obtain enough magnetic cells ($4.1 \times 10^7$) for mRNA isolation, cDNA library construction, and cDNA probe synthesis.

**Isolation of an HEV-Specific cDNA (Hevin) by Differential Hybridization**

mRNA from the purified HEV endothelial cells was used to construct a cDNA library in the IZAPII vector. To isolate HEV-specific cDNAs, we screened duplicate filters from the cDNA library by differential hybridization. HEV cDNA was used as the plus probe. Human umbilical vein endothelial cells (HUVEC) were used as a source of flat endothelial cells. Since most of the cells contaminating the high endothelial cell preparation appeared to be leukocytes (ICAMB'), the minus probe was prepared from a mixture of 20% mRNA from tonsil leukocytes and 80% HUVEC mRNA. Duplicate filter lifts from the HEV cDNA phage library (15,000 plaques plated at low density) were screened with the plus and minus probes to isolate clones specifically detected with the HEV cDNA probe (Figure 3a). Se-
Hevin, a New SPARC-Like Protein from Human HEV

Figure 2. Purification of Human High Endothelial Cells by Immuno-magnetic Selection with the HEV-Specific MAb MECA-79
(a) Immunofluorescent flow cytometry of tonsillar stromal cells (after step 3 in Figure 1) and magnetic cells (after step 4 in Figure 1) stained with MECA-79 MAb. Cells were stained with MECA-79 for 15 min at 4°C and binding was detected with FITC-conjugated anti-rat \( \kappa \) light chain MAb.
(b) Immunofluorescence staining of human tonsil frozen sections with HEV-specific MAb MECA-79. Frozen sections of human tonsils (8 \( \mu \)m, fixed with acetone) were stained with MECA-79 MAb for 1 hr in a moist chamber at room temperature and binding was detected with FITC-labeled secondary antibodies. Scale bar, 30 \( \mu \)m.
(c–e) Fluorescence microscopy of same preparation as shown in (a), stained with MECA-79 and FITC-labeled secondary antibodies. The purified high endothelial cells are shown at low (c: scale bar, 30 \( \mu \)m), intermediate (d: scale bar, 10 \( \mu \)m), or high magnification (e: scale bar, 5 \( \mu \)m).

Figure 2. Purification of Human High Endothelial Cells by Immuno-magnetic Selection with the HEV-Specific MAb MECA-79
(a) Immunofluorescent flow cytometry of tonsillar stromal cells (after step 3 in Figure 1) and magnetic cells (after step 4 in Figure 1) stained with MECA-79 MAb. Cells were stained with MECA-79 for 15 min at 4°C and binding was detected with FITC-conjugated anti-rat \( \kappa \) light chain MAb.
(b) Immunofluorescence staining of human tonsil frozen sections with HEV-specific MAb MECA-79. Frozen sections of human tonsils (8 \( \mu \)m, fixed with acetone) were stained with MECA-79 MAb for 1 hr in a moist chamber at room temperature and binding was detected with FITC-labeled secondary antibodies. Scale bar, 30 \( \mu \)m.
(c–e) Fluorescence microscopy of same preparation as shown in (a), stained with MECA-79 and FITC-labeled secondary antibodies. The purified high endothelial cells are shown at low (c: scale bar, 30 \( \mu \)m), intermediate (d: scale bar, 10 \( \mu \)m), or high magnification (e: scale bar, 5 \( \mu \)m).

Figure 2. Purification of Human High Endothelial Cells by Immuno-magnetic Selection with the HEV-Specific MAb MECA-79
(a) Immunofluorescent flow cytometry of tonsillar stromal cells (after step 3 in Figure 1) and magnetic cells (after step 4 in Figure 1) stained with MECA-79 MAb. Cells were stained with MECA-79 for 15 min at 4°C and binding was detected with FITC-conjugated anti-rat \( \kappa \) light chain MAb.
(b) Immunofluorescence staining of human tonsil frozen sections with HEV-specific MAb MECA-79. Frozen sections of human tonsils (8 \( \mu \)m, fixed with acetone) were stained with MECA-79 MAb for 1 hr in a moist chamber at room temperature and binding was detected with FITC-labeled secondary antibodies. Scale bar, 30 \( \mu \)m.
(c–e) Fluorescence microscopy of same preparation as shown in (a), stained with MECA-79 and FITC-labeled secondary antibodies. The purified high endothelial cells are shown at low (c: scale bar, 30 \( \mu \)m), intermediate (d: scale bar, 10 \( \mu \)m), or high magnification (e: scale bar, 5 \( \mu \)m).
human tonsils. The hevin probe selectively hybridized with HEV (Figure 4a). The dense cellular reaction product was confined to the cytoplasmic region of high endothelial cells (Figures 4b and 4c). No hybridization signal was detected with thin-walled vessels within tonsil (Figure 4d), or with any other cells within lymphoid or epithelial areas in tonsil (Figures 4e and 4f). An IgA probe was used as a control. It hybridized intensely with single scattered cells that were numerous near the crypt epithelial areas of the tonsil and are likely to be IgA-secreting plasma cells (Figure 4f). The IgA signal was detected after 15 min, whereas the hevin signal was detected after 4 hr. Typically, 40% or more of the total protein synthesis in myeloma cells is devoted to the production of immunoglobulin chains (Harlow and Lane, 1988), and an estimate that heavy and light chain mRNA comprises 1%-5% of total cellular mRNA is unlikely to be far off (Dr. C. Milstein, personal communication). No nonspecific hybridization of the IgA probe with HEV was detected, and even after prolonged exposure with the hevin cDNA probe, no cells other than HEV were stained, showing that within human tonsills, hevin is specifically expressed in HEV.

**Nucleotide Sequence Analysis of Hevin cDNA**

Three essentially full-length hevin cDNA clones were isolated. The longest cDNA, which we sequenced on both strands, predicts an mRNA of 2645 bases (Figure 5a). The hevin cDNA consists of 198 nt of 5' noncoding sequence, a single long open reading frame of 1992 nt encoding a putative protein of 664 aa with a calculated molecular mass of 75.2 kDa, and 455 nt of 3' noncoding sequence. A good translational start site context (Kozak, 1984) surrounds the putative ATG initiation codon, and a consensus polyadenylation sequence AATAAA is found 16 nt upstream of the poly(A) tail.

The deduced amino acid sequence reveals that the hevin protein is composed of four regions (Figure 5b). The extreme N-terminal portion is predicted to be a signal peptide. It contains a hydrophobic stretch of 21 aa residues ending with a consensus signal peptidase cleavage site (von Heijne, 1986). From the beginning of the mature polypeptide to residue 432, the sequence of hevin is highly acidic. This acidic domain contains 26% glutamic acid and aspartic acid residues. There are short tracts of glutamic acid (EEEEE, residues 201-205) and aspartic acid (DDDDGDDDD, residues 338-347). A third region of hevin, comprised of residues 433-516, is characterized by the presence of 11 cysteine residues (cysteine-rich domain). A fourth C-terminal region contains a 12 aa segment that is homologous to the calcium-binding loops of the EF-hand structures found in the calmodulin family of proteins (Kretzinger, 1980). The presence of a putative signal peptide and the lack of an internal hydrophobic membranespanning domain (Kyte–Doolittle predictions; Figure 5c) suggest that the hevin protein is secreted. The protein sequence contains seven Asn-X-Ser/Thr potential N-linked glycosylation sites (Figure 5a), consistent with the hypothesis that hevin may be glycosylated.

**Comparison of Hevin with the Extracellular Matrix Protein SPARC and Other SPARCLike Proteins**

The hevin sequence was compared with all sequences present in the National Center for Biotechnology Information nonredundant protein database using the BLASTP program (Altschul et al., 1990). This search revealed striking similarities between hevin and the antiadhesive ECM protein SPARC (Figure 6). SPARC is a secreted calcium-
binding glycoprotein, widely expressed during development and tissue remodeling, that inhibits cell spreading and induces cell rounding by disrupting focal adhesions (Sage et al., 1989b; Murphy-Ullrich et al., 1991). The region of sequence similarity corresponds to the C-terminal part of hevin and most of the SPARC coding sequence (Figure 6a). The database search also revealed that hevin is closely related to two other SPARC-like proteins: SC1, a rat glycoprotein of 118/120 kDa expressed to high levels by many neurons in the brain (Johnston et al., 1990), and QR1, an embryonic quail protein expressed by glial cells in the neuromesencephalon (Guermah et al., 1991). The 232 C-terminal amino acids of hevin (from amino acid 433 to the C-terminal end) show respectively, 91%, 73%, and 82% identity to the homologous portions of rat SC1, quail QR1, and human SPARC (Figure 6b). The N-terminal part of the four proteins is the most divergent, although a significant homology is observed between the 432 N-terminal amino acids of hevin and the 402 N-terminal residues of rat SC1 (53% identity). SPARC has a short N-terminal extension.
Figure 6. Comparison of Hevin Deduced Amino Acid Sequence with SPARC and SPARC-Like Proteins, SC1 and QR1
(a) Schematic representation of hevin, SPARC (Villarreal et al., 1989), SC1 (Johnson et al., 1990), and QR1 (Guermah et al., 1991) proteins. The number of amino acid residues and the predicted isoelectric points of each protein are indicated. The percentage of amino acid sequence identity between domains of hevin and corresponding domains of SPARC, SCI, and QR1 is shown. Horizontal hatched lines, signal peptides; shaded boxes, acidic domains; diagonal hatched lines, cysteine-rich domains; closed boxes, EF-hand calcium-binding domains.
(b) Amino acid sequence alignment of the C-terminal portions of human hevin, rat SCI, quail QRI, and human SPARC. Dashed lines indicate amino acid identities with hevin. Dots represent gaps introduced to align sequences.

that is similar to the long N-terminal domain of hevin, SCI, and QRI, in the sense that all contain clusters of acidic residues that impart an overall acidic pl to the proteins. Interestingly, the 14 cysteine residues found in SPARC are all present at homologous positions in hevin, SCI, and QRI. These include the 11 cysteine residues of the cysteine-rich domains that are similar to follistatin-like modules involved in cytokine binding (Lane and Sage, 1994). Finally, the EF-hand calcium-binding motif at the C terminus is present in all four proteins, suggesting an important role for calcium in the function of these proteins.

Evolutionary Conservation of Hevin
To characterize further the relationship of hevin with the two other SPARC-like proteins, rat SCI and quail QRI, we performed cross-species Southern blots under moderate stringency hybridization conditions. The full-length hevin cDNA probe detects four fragments in human and monkey genomic DNA digested with EcoRI (Figure 7), one or two EcoRI fragments in rat, mouse, dog, and cow genomic DNA, and no fragment in rabbit, chicken, or yeast DNA. The 8 kb EcoRI fragment detected in the dog genome by the hevin cDNA probe is also detected by a rat SCI cDNA probe (data not shown); close comparison of rat and human hybridizing fragments was hindered because only a short rat SCI cDNA probe was available that encodes amino acids 341-441, which contains little of the highly conserved region. The results suggest that hevin has been conserved in evolution and might be the human homolog of rat SCI.

Distribution of Hevin mRNA Transcripts in Human Tissues
Since SCI is expressed in rat brain and heart (Johnston et al., 1990), we investigated further the expression of hevin in the human body. Hevin mRNA expression in 17 different human tissues was examined by Northern blot (Figure 8a). A single mRNA species of 2.7 kb, correspond-
Hevin, a New SPARC-Like Protein from Human HEV

Figure 8. Northern Blot of Hevin cDNA with mRNA from Different Human Tissues

Each lane contains approximately 2 μg of poly(A)+ RNA isolated from the indicated human tissues.

(a) The blot was hybridized, under high stringency conditions, with a 32P-labeled probe encompassing the 5' end of the HEVIN cDNA (1.1 kb SacI fragment).

(b) After the removal of bound probe, the blot was reprobed with a 32P-labeled human SPARC cDNA fragment.

Discussion

A Novel Strategy to Characterize the Specialization of HEV at the Molecular Level

HEV represent one of the most striking examples of endothelial differentiation. However, despite intensive efforts, the MECA-79 carbohydrate determinant is the only marker thus far described that is specific for HEV endothelium, and no gene specifically expressed in high endothelial cells has been described. CD34 is expressed on many types of endothelial cells (Fina et al., 1990), and GlyCAM-1 is abundantly expressed in epithelial cells of the lactating mammary gland as well as on HEV (Dowbenko et al., 1993). Characterization of HEV is difficult, because they are a very minor component of lymphoid tissues, and thus far have only been partially purified by enrichment for adherent cells from lymph nodes (Agar and Mistry, 1990) or density gradient centrifugation (Chin et al., 1992). Furthermore, HEV rapidly lose their specialized phenotype when isolated from the lymphoid tissue environment, even when this only consists of surgically severing the afferent lymphatics while leaving the vascular blood supply intact (Mebius et al., 1991b, 1991a). In this study, we have purified HEV cells almost to homogeneity. The cells were purified about 2,400-fold using differential collagenase digestion and immunomagnetic isolation with the MECA-79 MAb. The preparations were, on average, at least 60% MECA-79+ as shown by immunofluorescence flow cytometry, and up to 90% purity was achieved in one preparation. About 4 x 10^6 HEV cells could be obtained in one preparation from four tonsils. No in vitro culture was used during preparation, ensuring no alteration in phenotype. This method for purifying HEV cells may find many further applications, including studying the function of HEV cells, and characterizing the conditions required for maintenance of the differentiated phenotype and growth of HEV cells.

From the purified HEV cells, we obtained sufficient mRNA to construct a cDNA library and synthesize cDNA probes for hybridization. We used a novel strategy of differential hybridization to isolate cDNA for messages expressed in plump endothelial cells of HEV but not in flat endothelial cells of the human umbilical vein. We have illustrated this strategy with one of five different cDNAs isolated, that was by far the best expressed and the only one with homology to genes in the current sequence databases. Further work is required to confirm whether the other four mRNAs are specifically expressed in HEV. The cDNA we studied here encodes a 664 aa protein, desig-
nated hevin, that is structurally related to the antiadhesive ECM protein SPARC.

Hevin, a Novel Human SPARC-Like Protein
Unlike many other ECM-associated proteins, SPARC does not have any adhesive properties and, for instance, is not able to support endothelial cell attachment in vitro (Sage and Bornstein, 1991). In contrast, its antiadhesive properties are impressive (Lane and Sage, 1994); SPARC inhibits endothelial cell spreading, induces cell rounding in well-spread endothelial cells (Sage et al., 1989b), and negatively modulates cell–substrate adhesion by stimulating the disruption of focal adhesions (Murphy-Ullrich et al., 1991). The high degree of homology between hevin and SPARC, which exhibit 62% amino acid sequence identity over more than four fitting of the SPARC coding sequence, predicts that hevin is likely to have similar antiadhesive properties. This possibility is further supported by the fact that the region of homology includes the C-terminal EF-hand calcium-binding domain, which allows specific interaction of SPARC with endothelial cells (Yost and Sage, 1983) and mediates many of the antiadhesive effects of SPARC (Lane and Sage, 1990). Scanning electron microscopy studies have revealed striking effects of SPARC on endothelial cell morphology and intercellular gap formation (Goldblum et al., 1994): endothelial cells exposed to SPARC remain attached to the substrate but become rounded and assume a plump morphology with marked separation from neighboring cells (discontinuous junctions). Interestingly, the plump morphology of the endothelial cells and the discontinuous junctions between adjacent endothelial cells are two prominent features of the specialized endothelium of HEV (Anderson and Shaw, 1993). These latter results, together with the high expression of hevin mRNA in HEV and the strong homology between hevin and SPARC, lead us to propose that hevin could be one of the factors contributing to the plump morphology of high endothelial cells and the presence of discontinuous junctions between high endothelial cells. Further studies, including expression of hevin, characterization of hevin at the protein level, and functional studies, are required to test this proposal.

Although SPARC is expressed transiently in a wide range of cell types and tissues during development, in adults its expression is limited to tissues that are actively remodeling their matrix, where cells are dividing or migrating (Lane and Sage, 1994). The highest levels of SPARC mRNA are found in decidual cells of the endometrium, the steroid-producing Leydig cells of the testis, and the granulosa cells of the ovaries (Holland et al., 1990). SPARC expression is clearly associated with epithelia exhibiting high rates of turnover (gut, skin, glandular tissue). In newborn thymus, SPARC is associated with epithelial processes that provide support for T cell populations (Sage et al., 1989a). In the adult, SPARC is not expressed in endothelial cells (except in endothelial cells associated with angiogenesis in vivo); however, its synthesis is rapidly induced in endothelial cells grown in vitro, as exemplified here with HUVEC. In contrast, hevin mRNA is expressed constitutively in HEV endothelial cells in vivo but is not detected in HUVEC grown in vitro.

The high homology between human hevin and human SPARC indicates that hevin is clearly a SPARC-like protein. However, hevin is more closely related to two other nonhuman SPARC-like proteins than it is to human SPARC. The primary structure of these two SPARC-like proteins, rat SC1 and quail QR1, is very similar to that of hevin: in addition to the 232 aa C-terminal part homologous to SPARC, all these proteins have a long N-terminal domain of approximately 400 aa that is highly acidic (Figure 8). The presence of a putative signal peptide consisting of a hydrophobic stretch of 17–21 aa residues and the lack of an internal hydrophobic transmembrane domain suggest, as has been shown for SPARC, that these three SPARC-like proteins are secreted. One question that remains to be solved is whether these three SPARC-like proteins, which have been described in different species, are species homologs or are different members of the family of SPARC-like proteins. Hevin and quail QR1 are unlikely to be species homologs, since QR1 has low sequence similarity with hevin in the long N-terminal part and its expression appears to be restricted to glial cells in the quail neuroretina. In contrast, hevin and rat SC1 show many homologies: their N-terminal parts exhibit 53% identity, they are both expressed to high levels in the brain and the heart and not expressed or expressed in low levels in kidney and liver, and the hevin cDNA probe detects the same dog EcoRI genomic DNA fragment as the SC1 cDNA probe. All together, these data suggest that hevin could be a human homolog of rat SC1 and are consistent with hevin being not a true HEV-specific protein but rather a major component of the ECM expressed in many tissues of the human body, including brain and heart. Interestingly, SC1 has been shown to be expressed strongly in Bergman glial cells and their radial fibers in the developing rat cerebellum, at a time when granule cells are migrating along these radial fibers, suggesting SC1 may play a role in cell migration (Mendle et al., 1994). Moreover, unlike many other ECM molecules, SC1 is widely expressed in the adult brain and is present in many types of neurons (Johnston et al., 1990).

Possible Functions of Hevin in Lymphocyte Migration through HEV

The high expression of hevin mRNA in HEV, together with its strong homology with SPARC, suggest different ways by which hevin could facilitate lymphocyte migration through HEV. We estimated that high endothelial cells from human tonsils expressed at least 10-fold higher levels of hevin mRNA than CD34 mRNA, although CD34, one of the ligands for lymphocyte L-selectin (Baumhueter et al., 1993), is itself abundantly expressed in human tonsil and murine peripheral lymph node HEV (Girard and Springer, 1994; Baumhueter et al., 1993). The abundance of the hevin mRNA in HEV is further interfered from the cloning of the hevin cDNA by differential screening, a method that allows only the detection of abundant mRNAe comprising more than about 0.05% of the total mRNA (Sambrook et al., 1989), and ready detection of hevin mRNA in Northern blots of lymphoid tissue, despite expression in a small subset of cells. If hevin has the same...
antiadhesive effects on endothelial cells as SPARC, it could facilitate the migration of lymphocytes through HEV by promoting the formation of intercellular gaps and discontinuous junctions between high endothelial cells. Alternatively, hevin could act on lymphocytes and modulate lymphocyte adhesion—deadhesion during transendothelial migration. Independently of its potential role as an adhesion modulator, hevin could also be involved in the presentation of adhesion-inducing cytokines to the lymphocytes rolling on or migrating across HEV. It has been hypothesized that proadhesive chemokines that mediate arrest of the rolling lymphocytes have to be immobilized on the luminal surface of endothelial cells to avoid being washed away by the blood flow (Rot, 1992; Tanaka et al., 1993b).

Proteoglycans that contain long hydrophilic, acidic glycosaminoglycan side chains have been proposed as important contributors for the immobilization of the basic chemokine MIP1-β on the luminal surface of HEV (Tanaka et al., 1993a). However, other molecules could be involved in this process. We would like to propose hevin as another good candidate for cytokine binding and immobilization on the luminal surface of HEV. Hevin could bind basic chemokines via its long N-terminal acidic domain or via its cysteine-rich follistatin-like module, which binds cytokines in other proteins (Pathy and Nikolics, 1993), while being retained on the surface of high endothelial cells via its SPARC-like C-terminal EF-hand calcium-binding domain, which mediates specific interaction of SPARC with endothelial cells (Yost and Sage, 1993). It will be important to test this speculation by expressing the hevin protein, and testing its affinity for chemokines and its effects on chemokine immobilization on the surface of endothelium.

Similarly, recognition of a role for hevin in the induction or the maintenance of the differentiated phenotype of HEV will require further studies.

**Experimental Procedures**

**Preparation of Stromal Cell Suspensions from Human Tonsil**

The tonsillar stromal cell suspensions are prepared following the protocol presented in Figure 1. After digestion of blood clots, fresh human tonsils (Children's Hospital, Boston, Massachusetts) are minced finely with scissors on a stainless steel screen (200 mesh = 74 μm, Type 316, Tylinter, Mentor, Ohio) under frequent flushing with RPMI 1640 medium to eliminate recirculating lymphocytes that pass through the screen. Stromal elements remaining on the screen are collected and incubated 15 min at 37°C in RPMI 1640 containing 0.5% collagenase (collagenase I, 187 U/mg; Worthington Biochemical Corporation, Freehold, New Jersey) and 2 μg/ml DNAase I (Gibco Chemical Company, St. Louis, Missouri). After this mild collagenase treatment, the stromal elements suspension is placed on the steel screen and subjected to flow hemacytometer for the determination of cell counts, and the numbers of lymphocytes, and the number of endothelial cells are calculated from these counts, and the number of endothelial cells are calculated from these counts.

**Immunomagnetic Selection of High Endothelial Cells**

To enrich for high endothelial cells, we used the method of Magnetic Cell Sorting (Miltenyi Biotech, Sunnyvale, California). In brief, tonsillar stromal cells are stained with the HEV-specific antibody MECA-79.
New York). The MEGA-79 (HEV-depleted) tonsillar stromal cell cDNA probe was prepared in parallel using the same reagents.

DNA Sequencing

A nested series of deletions in the hevin cDNA was generated in both directions with the Erase A Base system (Promega Corporation, Madison, Wisconsin). Subclones containing progressive unidirectional deletions covering the complete hevin cDNA (2645 bp) were sequenced with an ABI 373A automated DNA sequencer. Analysis of DNA se-
quences was carried out on a VAX computer using the Genetics Com-
puter Programing group of the Wisconsin Genetics Group (Devereux et al., 1994). The program BLASTP (Altschul et al., 1990) was used to compare the hevin-deleted amino acid sequence with all sequence present in the National Center for Biotechnology Information nonre-
dundant protein database.

In Situ Hybridization

Fresh human tonsils were cut in small pieces, embedded in OCT medium, quick-frozen in isopentane on dry ice, and stored at -80°C. Cryostat sections (8 µm) were collected on slides, fixed in freshly pre-
pared 4% paraformaldehyde in PBS at room temperature for 15 min, washed in 3 x PBS, then twice in 1 x PBS, dehydrated in 30%, 50%, 80%, 95%, 100% ethanol (2 min each), air dried, and stored at ~80°C with desiccant. Sections were then equilibrated to room temperature, placed in moist chambers, and prehybridized at 42°C for 3 hr in 50% formamide, 5 x SSC, 10 x Denhardt's solution, 2% SDS, 100 µg/ml herring sperm DNA. After prehybridization, buffer was drained off, and 50 µl of the same solution, containing 100 ng digoxigenin-labeled probe, was pipetted onto the slides. After hybridization for 12-16 hr at 42°C in moist chambers, slides were washed at room temperature in 2 x SSC, 0.05% SDS (two times for 20 min). To remove unspecifically bound probe, slides were incubated at 50°C in 0.1 x SSC, 0.1% SDS (two times for 30 min). The detection of the digoxigenin-labeled probe was performed according to the instructions of the manufacturer (nu-
cleic acid detection system Genius 3, Boehringer-Mannheim Corpora-
tion, Indianapolis, Indian). The probes used for in situ hybridization, a 0.8 kb BamHI fragment containing the 5' part of the hevin cDNA, and a 0.6 kb EcoRI-XhoI fragment corresponding to the 3' end of IgA cDNA, were labeled with digoxigenin-DUTP (Boehringer-Mannheim Corporation, Indianapolis, Indian) by random priming (GIBCO BRL, Grand Island, New York).

Southern Blots

A zoo blot (Clontech, Palo Alto, California), containing 4 µg EcoRI-
digested genomic DNAs from different species was hybridized at 85°C for 18 hr in 5 x SSC, 10 x Denhardt's solution, 2% SDS, 100 µg/ml herring sperm DNA, with a full-length hevin cDNA (2.6 kb) probe labeled by random priming (GIBCO BRL, Grand Island, New York). The blot was then washed sequentially with 2 x SSC, 0.1% SDS at room temperature (two times for 30 min) and 2 x SSC, 0.1% SDS at 65°C (two times for 30 min).

Northern Blots

Blots of poly(A) RNA from multiple human tissues were purchased from Clontech (Palo Alto, California). Human lymph node poly(A) RNA (2 µg, Clontech, Palo Alto, California) and HUVEC poly(A) RNA (0.6 µg, purified with the mRNA isolation kit from Stratagene (La Jolla, California), were fractionated on a 1% agarose gel containing 2.2 M formaldehyde, stained with ethidium bromide, and transferred to a

filter at -80°C for 3 hr. A 1 kb SacI fragment, containing the 5' part of the hevin cDNA, and a 0.5 kb EcoRI fragment, corresponding to the 5' end of the human SPARC cDNA (Villarreal et al., 1989), were labeled by random priming (GIBCO BRL, Grand Island, New York) and used as probes in the Northern blots.

Acknowledgments

We are grateful to Dr. E. Butcher (Stanford, California) for his generous gift of MAb MEGA-79. We thank Drs. I. Brown (University of Toronto, Toronto, Canada), G. Long (University of Vermont, Burlington, Ver-
mont), and D. Tenen (Beth Israel Hospital, Boston, Massachusetts) for their gifts of SCI, SPARC, and CD34 cDNAs, respectively. Special thanks to R. Clark for her gift of tonsil sections, and Dr. L. Picker for suggestions on mincing and screening lymphoid tissue. J.-P. Girard was a recipient of Association pour la Recherche sur le Cancer and Human Science Frontier Program fellowships. This work was sup-
ported by grants from Human Science Frontier Program (to J. P. Girard) and National Institute of Health grant CA-31795 (to T. A. Springer).

Received October 20, 1994; revised December 5, 1994.

References


Freemont, A. J. (1988). Functional and biosynthetic changes in endo-
thelial cells of vessels in chronically inflamed tissues: evidence for endothelial control of lymphocyte entry into diseased tissues. J. Pathol. 155, 225-230.

Freemont, A. J., and Jones, C. J. P. (1983). Light microscopic, histo-


