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

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Summary

High endothelial venules (HEV) in lymphoid tissues support high levels of lymphocyte extravasion 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, adenoids, appendix, and Peyer's patches, lymphocyte adherence and transendothelial 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 extravasion (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, 1988). 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 (Schoefl, 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–counterreceptor 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 (Bargatze and Butcher, 1993). In vitro studies have shown that lymphocyte L-selectin binds to the peripheral node addressin, defined by MECA-79 monoclonal antibody (MAb) (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 HEV arrest in a process that is dependent on G protein–coupled receptors on lymphocytes (Bargatze and Butcher, 1993) and that likely involves chemoattractant-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 1.4×10^4 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 (Bargatze 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 HEV 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

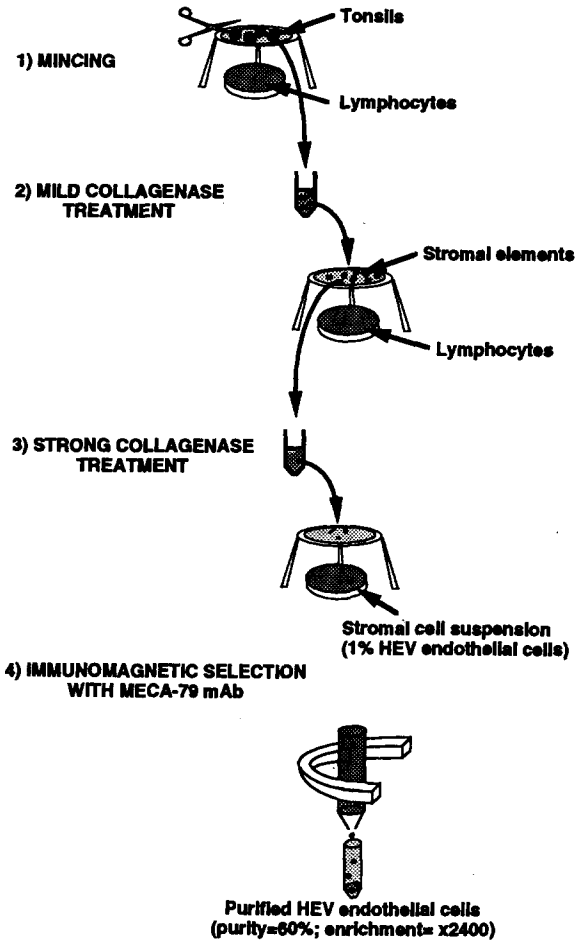


Figure 1. Schematic of the Strategy Used to Purify High Endothelial Cells from Human Tonsils

immunomagnetic selection with the HEV-specific antibody MECA-79 (Streeter et al., 1988) and constructed a cDNA library with mRNA isolated from the purified high endothelial cells. Then, we screened this library by differential hybridization with HEV cDNAs as the positive probe and umbilical vein flat-walled endothelial cDNAs as the negative probe. This strategy allowed us to characterize a novel human cDNA that encodes a 664 aa protein related to the antiadhesive extracellular matrix (ECM) secreted protein acidic and rich in cysteine (SPARC) (Lane and Sage, 1994). Since the mRNA is abundant in HEV and absent from flat-walled endothelium, and encodes a protein homologous to an ECM protein, we designate it hevin. We discuss potential roles of hevin in differentiation of the HEV endothelium and lymphocyte migration through HEV.

Results

Purification of High Endothelial Cells from Human Tonsils

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 3.7×10^6 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%–65%, with a range of 50%–90%, which was sufficient for most purposes. We repeated the purification procedure seven times to obtain enough magnetic cells (4.1×10^7) 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 λZAPII 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 (ICAM-3$^+$), 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-

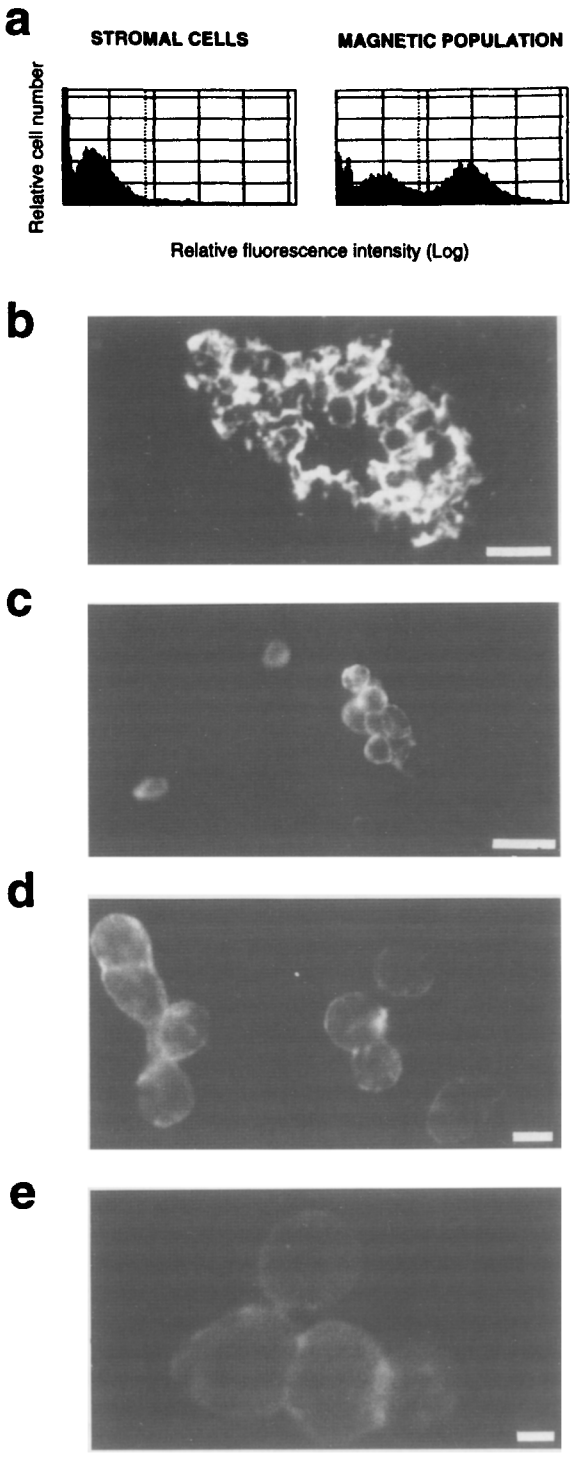


Figure 2. Purification of Human High Endothelial Cells by Immunomagnetic Selection with the HEV-Specific MAb MECA-79

(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 κ light chain MAb.

(b) Immunofluorescence staining of human tonsil frozen sections with HEV-specific MAb MECA-79. Frozen sections of human tonsils (8 μm, 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 μm.

(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 μm), intermediate (d: scale bar, 10 μm), or high magnification (e: scale bar, 5 μm).

quencing revealed that a significant fraction of the differentially hybridizing clones encoded immunoglobulin chains, suggesting that plasma cells were a significant contamination in the magnetic fraction. Since contaminating cells in the MECA-79$^+$ magnetic fraction were likely also to be present in the MECA-79$^-$ nonmagnetic fraction of tonsillar stromal cells, a secondary screen was carried out. A plus cDNA probe was prepared from a highly purified magnetic tonsillar stromal cell fraction that contained 90% MECA-79$^+$ cells and a minus cDNA probe was prepared from the MECA-79$^-$ (HEV-depleted) tonsillar stromal cells. Of five cDNA clones that differentially hybridized with the HEV probe versus HUVEC probe and represented novel human sequences, one also differentially hybridized in the second screen with the MECA-79$^+$ and not the MECA-79$^-$ tonsillar stromal cell cDNA probes, whereas the four other cDNA clones were not sufficiently abundant to be detected by the limited amount of HEV (90% purity) cDNA probe available. We report here on the clone that is by far the most strongly expressed in HEV mRNA and is designated hevin.

mRNA abundance in the MECA-79$^+$ and MECA-79$^-$ tonsillar stromal cell fractions was compared by hybridization of the respective cDNA probes with Southern blots prepared with cDNA encoding hevin, CD34, and IgA (Figures 3b–3d). The MECA-79$^+$ (HEV) cDNA probe intensely hybridized with the hevin cDNA (Figure 3d, lane 3), whereas the MECA-79$^-$ (HEV-depleted tonsillar stromal cell) cDNA probe was negative with hevin cDNA (Figure 3c, lane 3). The probes were also hybridized with a CD34 cDNA, because CD34 is a major sulfo- and sialoglycoprotein of HEV that bears carbohydrate ligands for L-selectin (Baumhueter et al., 1993), and because CD34 is highly expressed on HEV in human tonsils (Girard and Springer, 1994). The MECA-79$^+$ but not the MECA-79$^-$ tonsillar stromal cell cDNA probe hybridized with CD34 (Figures 3c and 3d, lanes 2). Interestingly, the intensity of hybridization of the MECA-79$^+$ (HEV-enriched) cDNA probe was at least 20-fold greater with hevin cDNA than CD34 cDNA, suggesting that hevin is abundantly expressed in human HEV (Figure 3d, compare lanes 2 and 3). By contrast with the above results, the MECA-79$^-$ (HEV-depleted) but not the MECA-79$^+$ (HEV-enriched) cDNA probe strongly hybridized with IgA cDNA, suggesting that the MECA-79$^-$ tonsillar stromal cell preparation contained immunoglobulin A (IgA)-secreting plasma cells.

In Situ Hybridization Analysis of Hevin mRNA Distribution in Human Tonsils

To confirm the HEV-specific expression of hevin mRNA within human tonsils, a digoxigenin-labeled cDNA probe was hybridized in situ with cryostat tissue sections from

Table 1. HEV Cell Purification

Fraction	Total cells	HEV cells	HEV cells (percent)	Enrichment
Tonsil suspension			<0.025	1
Stromal cell suspension	4×10^8	3.8×10^6	1	40
Magnetic cell fraction	6×10^6	3.7×10^6	60	2400

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 4a and 4e). An IgA probe was used as a control. It hybridized intensely with single scattered cells that were numerous near the crypt epithelium areas of the tonsil and are likely to be IgA-secreting plasma cells (Figure 4f). The IgA signal was detected after chromogen development for 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 tonsils, 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 (DDDGDDDGDD, 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 (Kretsinger, 1980). The presence of a putative signal peptide and the lack of an internal hydrophobic membrane-spanning 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 SPARC-Like 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-

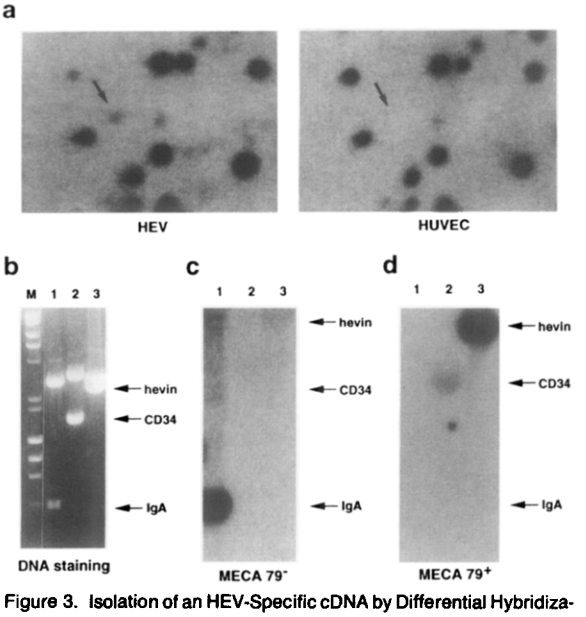


Figure 3. Isolation of an HEV-Specific cDNA by Differential Hybridization Screening

(a) Primary screening. Duplicate filters from the HEV cDNA library were hybridized with HEV and HUVEC cDNA probes. The arrow indicates the position of a clone containing a cDNA (hevin) specifically detected with the HEV cDNA probe.

(b–d) Secondary screening. cDNAs encoding IgA (lane 1), CD34 (lane 2), and hevin (lane 3) were released from plasmid DNA by digestion with EcoRI and XhoI, separated on a 1% agarose gel, stained with EtBr (b), and transferred onto nitrocellulose filters. Duplicate filters were then hybridized with a MECA-79⁻ tonsillar stromal cell cDNA probe (MECA-79⁻), which is depleted of HEV cDNAs but contains many cDNAs from IgA-secreting cells (c), and a MECA-79⁺ cDNA probe (MECA-79⁺), which is enriched in cDNAs from HEV endothelial cells (d). The positions of the IgA, CD34, and hevin cDNAs are indicated by arrows.

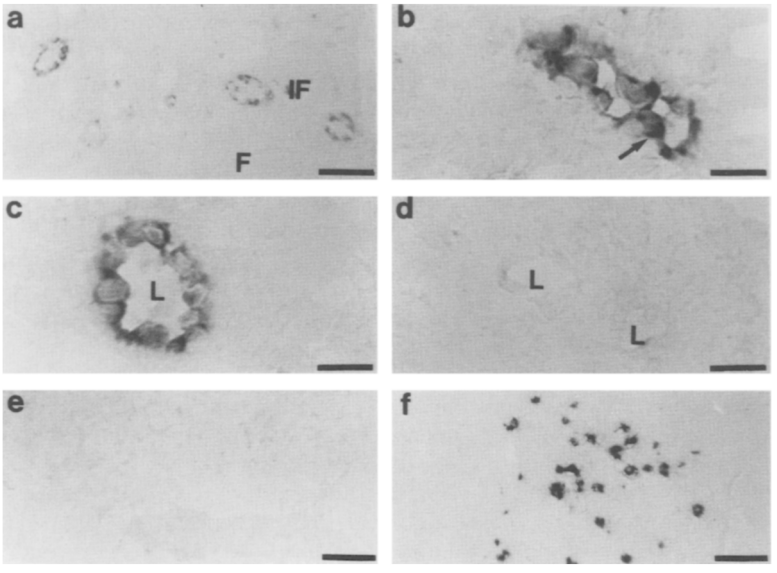


Figure 4. In Situ Hybridization to Hevin mRNA in Human Tonsil

Cryostat sections (8 μm) of human tonsils were hybridized with digoxigenin-labeled cDNA probes. (a–e) Hevin mRNA detection (4 hr of chromogen reaction).

(a) Hybridization signals are present in all HEV. Positive HEV are found in the interfollicular areas (IF) while follicles are negative (F). Scale bar 120 μm.

(b) A longitudinally sectioned HEV.

(c) A cross-sectioned HEV. Signals are located predominantly in the cytoplasm of the plump high endothelial cells (arrow). L, lumen of the HEV. Scale bar, 30 μm.

(d) Lack of expression of hevin mRNA in flat-walled vessels of the tonsils. L, lumen of negative thin-walled vessels. Scale bar, 30 μm.

(e) No detectable signals are seen in the crypt epithelium area of the tonsils. Scale bar, 120 μm.

(f) IgA mRNA detection (15 min of chromogen reaction). Strong hybridization signals are observed in IgA-producing cells infiltrating the crypt epithelium area of the tonsils. Scale bar, 120 μm.

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 116/120 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 neuroretina (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 62% 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

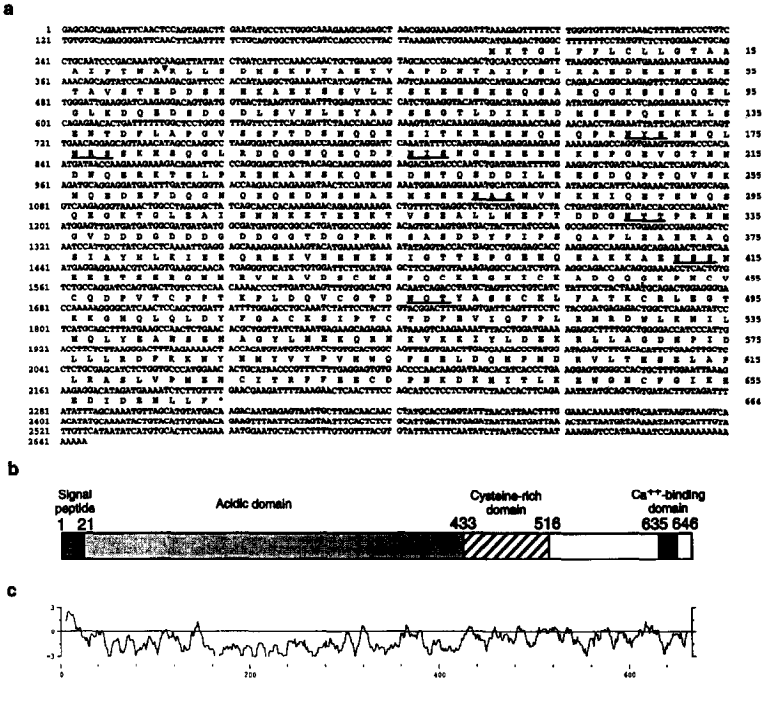


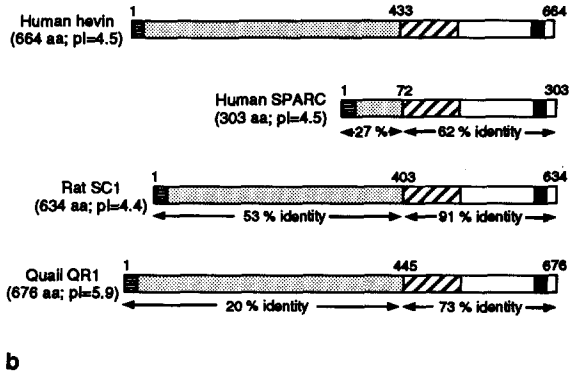
Figure 5. Sequence of the cDNA Encoding Hevin

(a) Nucleotide and deduced amino acid sequence of the 2645 bp hevin cDNA. The numbers on the right indicate amino acid positions in the unique long open reading frame. The putative signal sequence cleavage site is marked with an arrow. The polyadenylation signal AATAAA is located 16 bp upstream of the poly(A) tail. Putative N-linked glycosylation sites are underlined.

(b) Schematic representation of the hevin protein. Horizontal hatched lines, putative signal peptide; shaded box, acidic domain; diagonal hatched lines, cysteine-rich domain; closed box, EF-hand calcium-binding domain.

(c) Hydropathic profile determined by the Kyte and Doolittle algorithm (Kyte and Doolittle, 1982). Hydrophobic regions are assigned positive values. These sequence data are available from EMBL/GenBank/DDBJ under accession number X82157.

a



b

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Hevin CMSFQCKRGH ICKADQQGKP HCVCQDPVTC P.PTKPLDQV CGTDNQTYAS 481
 SC1  --n------- t--t--h--- -------e-- -.-a-i---a ---------- 451
 QR1  -rn-h----k v-q--k---- s-i----aa- -.s--dykr- -----k--dg 493
SPARC -qnhh--h-k v-el-ennt- m------ts- -a-igefek- -sn--k-fd- 121

Hevin SCHLPATKCR LEGTKKGHQL QLDYFGACKS IPTCTDFEVI QFPLRMRDWL 531
 SC1  ---------m ---------- ---------- --a------a ---------- 501
 QR1  t-q--g---q -----m-r-- h---m----h --h---y--n ---------- 543
SPARC ---f-----t --------k- h---i-p--y --p-l-s-lt e--------- 171

Hevin KNILMQLYEA NSEHAGYLNE KQRNKVKKIY LDEKRLLAGD HPIDLLLRDF 581
 SC1  ---------p -pk-g----- ---s------ ---------- ---e------ 551
 QR1  ------y--r dqdtsaf-t- ---------- -n----vs-e --ve---h-- 593
SPARC --v-vt---r dedn.nl-t- --klr----h en----e--- --ve--a--- 220

Hevin KKNYHMYVYP VHWQFSELDQ HPMDRVLTHS ELAPLRASLV PMEHCITRFF 631
 SC1  ---------- -----n---- --a--i---- ---------- ---------- 601
 QR1  e------l-- -----yq--- --v--s---- ---------- ---------- 643
SPARC e---n--if- -----gq--- --i-gy-s-t -------p-i -----t---- 270

Hevin EECDPNKDKH ITLKEWGHCF GIKEEDIDEN LLF 664
 SC1  ---------- ---------- ---------- --- 634
 QR1  q---gdq--l ------c--- a------n-- --- 676
SPARC -t--ldn--y -a-d--ag-- ---qk---kd -vi 303
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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, SC1, 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 SC1, quail QR1, and human SPARC. Dashed lines indicate amino acid identities with hevin. Dots represent gaps introduced to align sequences.

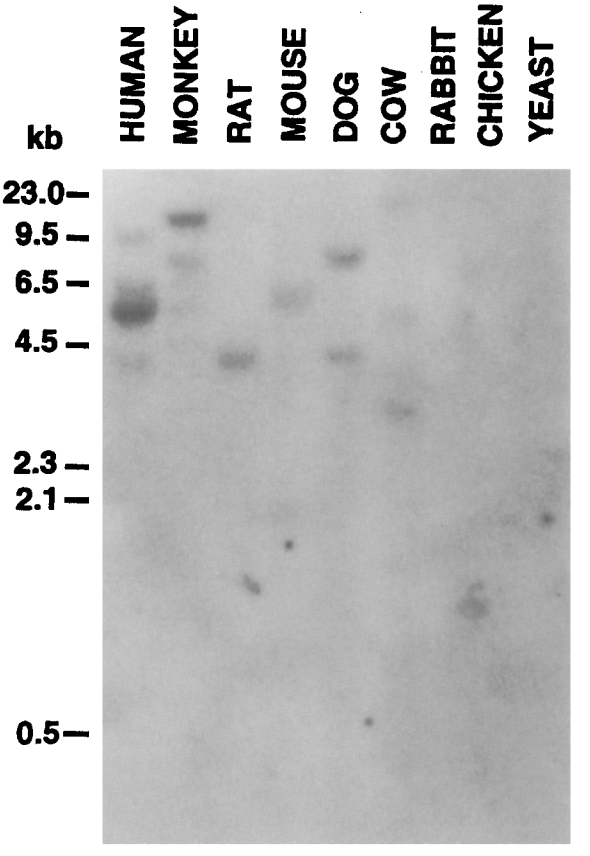

Figure 7. Cross-Species Hybridization of Hevin cDNA with Genomic DNAs

A Southern blot containing 4 μg EcoRI-digested genomic DNA from nine different eukaryotic species (zoo blot, Clontech) was hybridized with a ^{32}P-labeled full-length hevin cDNA probe. After a moderately stringent wash (2× SSC, 0.1% SDS at 65°C), the blot was exposed to X-ray film at −80°C for 48 hr with intensifying screens.

that is similar to the long N-terminal domain of hevin, SC1, and QR1, in the sense that all contain clusters of acidic residues that impart an overall acidic pI to the proteins. Interestingly, the 14 cysteine residues found in SPARC are all present at homologous positions in hevin, SC1, and QR1. 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 SC1 and quail QR1, 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 SC1 cDNA probe (data not shown); close comparison of rat and human hybridizing fragments was hindered because only a short rat SC1 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 SC1.

Distribution of Hevin mRNA Transcripts in Human Tissues

Since SC1 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-

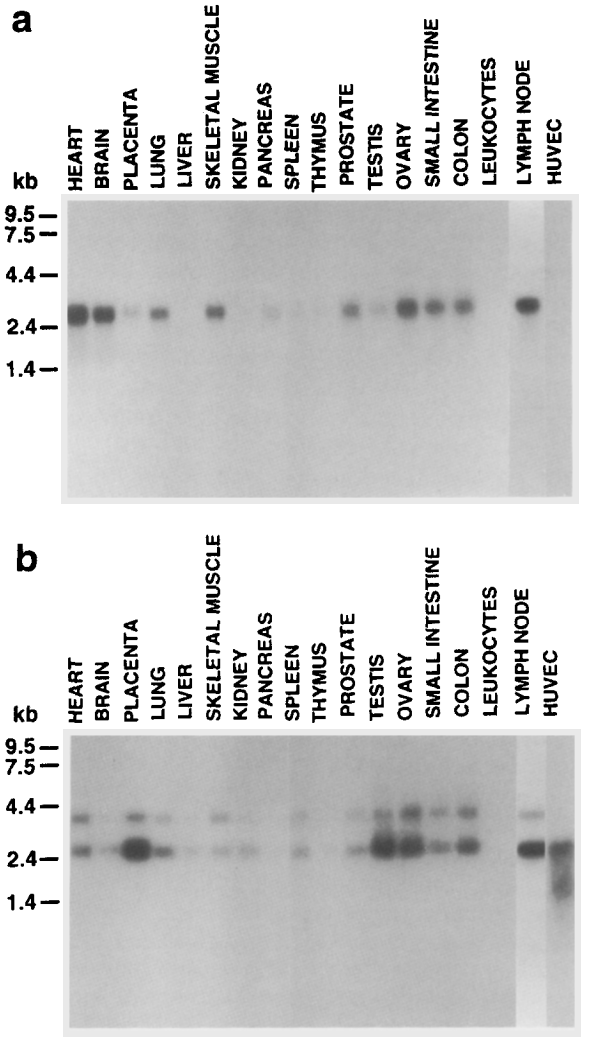

Figure 8. Northern Blot of Hevin cDNA with mRNA from Different Human Tissues

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 ^{32}P-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 ^{32}P-labeled human SPARC cDNA fragment.

ing to the size of the hevin cDNA (2645 bp), is detected at high levels in lymph node, brain, heart, lung, skeletal muscle, ovary, small intestine, and colon. This 2.7 kb mRNA is also present at lower levels in placenta, pancreas, testis, spleen, and thymus, but is absent in kidney, liver, and peripheral blood leukocytes. Furthermore, hevin mRNA was absent in HUVEC. Since high levels of hevin mRNA are expressed in many tissues that do not contain HEV, we conclude that expression of hevin is restricted to HEV in tonsils, but can occur on other cell types in other tissues.

To investigate further the relationship between hevin and SPARC, the Northern blots were reprobed with a human SPARC cDNA probe (Villarreal et al., 1989). Hevin and SPARC showed no cross-hybridization under the high stringency hybridization conditions used. Two mRNA species of 2.4 and 3.8 kb, distinct from the 2.7 kb hevin message, are detected with the SPARC probe (Figure 8b). Interestingly, the tissue distribution of SPARC message is clearly different from that of hevin. For example, SPARC is expressed to high levels in placenta and testis, and lower levels in brain, while hevin has an opposite expression pattern in these tissues. Moreover, while hevin mRNA is not present in HUVEC, SPARC mRNA is clearly detected in these cells.

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 (Ager 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 × 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 fifths 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, 1993) 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., 1987). 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 6). 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 to 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 (Mendis 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 inferred from the cloning of the hevin cDNA by differential screening, a method that allows only the detection of abundant mRNAs 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 cytokines 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 (Patthy 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, confirmation 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 removal 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 DNAse I (Sigma Chemical Company, St. Louis, Missouri). After this mild collagenase treatment, the stromal elements suspension is placed on the steel screen and subjected to flushing to remove lymphocytes that were adherent to or trapped between stromal elements. The stromal elements that remain after this step are resuspended in RPMI 1640 containing 0.5% collagenase and 2 μg/ml DNAse I and incubated 60 min at 37°C with shaking. At the end of this strong collagenase digestion, remaining stromal cell aggregates are eliminated by retention on the steel screen, and the cells that pass through the screen are collected. The stromal cell suspension from four tonsils contains approximately 4×10^8 total cells and 4×10^6 high endothelial cells (see Table 1; Figure 2b).

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 tissue culture (rat IgMκ), (Streeter et al., 1988) for 15 min at 4°C (4×10^8 cells in 4 ml MECA-79 tissue culture supernatant), washed with phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), incubated for 15 min at 4°C with a mouse MAb anti-rat κ light chain (mouse IgG1, AMAC, Westbrook, Maine) diluted to 10 μg/ml in PBS, 1% BSA, and washed again with PBS, 1% BSA. The cells (in a final volume of 4 ml) are then incubated for 15 min at 10°C with a rat anti-mouse IgG1 MAb conjugated to superparamagnetic microbeads (Miltenyi Biotech, Sunnyvale, California) diluted one fifth in PBS, 1% BSA, washed and, in a fourth step, incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG1 antibodies (Southern Biotechnology Associates, Birmingham, Alabama). After a final wash in PBS, 1% BSA, the cells are resuspended in 20 ml PBS, 1% BSA and applied to the top of a type A2 separation column (Miltenyi Biotech, Sunnyvale, California) in the Magnetic Cell Sorting magnetic separator with flow rate regulated by a 21 gauge needle. The column is washed with 20 ml PBS, 1% BSA at a higher flow speed regulated with a 18 gauge needle and magnetic cells are then eluted by removing the column from the magnetic field and washing with 10 ml PBS, 1% BSA.

Immunofluorescence Microscopy and Flow Cytometry

For immunohistochemistry, 8 μm acetone-fixed frozen sections of human tonsils were stained with the MAb MECA-79 in culture supernatant diluted to one half with PBS, 1% BSA, for 1 hr in a moist chamber at room temperature, washed with PBS (three times for 5 min), incubated with an FITC-labeled mouse MAb anti-rat κ light chain (AMAC, Westbrook, Maine), washed again with PBS (three times for 5 min), then coverslipped using fluoromount-G media (Fisher Scientific, Pittsburgh, Pennsylvania). Fluorescence microscopy of tissue sections or small aliquots of cell suspensions was with a Zeiss microscope (Carl Zeiss, Thornwood, New York). Photographs were taken using a Nikon microflex UFX-IIA (Nikon, Garden City, New York) and Kodak T-Max 400 film. For flow cytometry, with an EPICS V (Coulter, Hialeah, Florida) a small fraction (10^4 cells) of the FITC-labeled stromal cell and magnetic cell populations was resuspended in 100 μl of PBS, 1% paraformaldehyde.

cDNA Library Construction and Screening

Magnetic cell fractions (total of 4.1×10^7 cells) containing purified high endothelial cells were pooled from seven separate preparations from a total of 28 tonsils. Poly(A) RNA was isolated with the mRNA isolation kit (Stratagene, La Jolla, California). A unidirectional cDNA library was prepared from this RNA using the λZAPII vector and cDNA cloning reagents from Stratagene (La Jolla, California). The original library contained 6×10^6 recombinants before amplification. Primary screening of this library was performed by differential hybridization using as the plus probe HEV cDNA prepared during cDNA library construction (60% MECA-79$^+$ HEV cells) and, as the minus probe, a mixture of 80% HUVEC cDNA and 20% tonsillar leukocyte cDNA. Probes were ^{32}P-labeled by random priming (GIBCO BRL, Grand Island, New York). Duplicate filter lifts (Amersham, Arlington Heights, Illinois) from five plates, each containing 3000 plaques, were hybridized at 42°C overnight in 50% formamide, 5× Denhardt's solution, 0.5% SDS, 5× SSC, 50 μg/ml tRNA, and 50 μg/ml herring sperm DNA. The membranes were then washed with 1× SSC, 0.1% SDS at room temperature (two times for 15 min) and 0.1× SSC, 0.1% SDS at 55°C (two times for 15 min). Plaques from differentially hybridizing clones were picked and the corresponding pBluescript II SK(−) phagemids rescued by in vivo excision using Exassist helper phage (Stratagene, La Jolla, California). For the secondary screening, inserts were released from the plasmid DNA by digestion with restriction enzymes EcoRI and XhoI, separated on a 1% agarose gel, stained with ethidium bromide, and transferred to nitrocellulose filters. Duplicate filters were then screened by differential hybridization with ^{32}P-labeled cDNA probes from MECA-79$^+$ (purified HEV) and MECA-79$^-$ (HEV-depleted) tonsillar stromal cells, using the same hybridization conditions as for the primary screening. The MECA-79$^+$ magnetic stromal cell fraction used for probe generation contained 3×10^6 high endothelial cells (90% purity determined by fluorescence microscopy). mRNA was isolated from these cells, and first-strand cDNA synthesized with the superscript preamplification system (GIBCO BRL, Grand Island, New York) was ^{32}P-labeled by random priming (GIBCO BRL, Grand Island,

New York). The MECA-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 sequences was carried out on a VAX computer using the Genetics Computing Group program of the Wisconsin Genetics Group (Devereux et al., 1984). The program BLASTP (Altschul et al., 1990) was used to compare the hevin-deduced amino acid sequence with all sequences present in the National Center for Biotechnology Information nonredundant 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 prepared 4% paraformaldehyde in PBS at room temperature for 15 min, washed in 3× PBS, then twice in 1× PBS, dehydrated in 30%, 60%, 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× SSC, 10× Denhardt, 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× SSC, 0.05% SDS (two times for 20 min). To remove unspecifically bound probe, slides were incubated at 50°C in 0.1× 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 (nucleic acid detection system Genius 3, Boehringer-Mannheim Corporation, Indianapolis, Indiana).

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, Indiana) 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 65°C for 18 hr in 5× SSC, 10× Denhardt's solution, 2% SDS, 100 μg/ml herring sperm DNA, with a full-length hevin cDNA (2.6 kb) probe ^{32}P-labeled by random priming (GIBCO BRL, Grand Island, New York). The blot was then washed sequentially with 2× SSC, 0.1% SDS at room temperature (two times for 30 min) and 2× 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 nitrocellulose filter (Hybond N, Amersham, Arlington Heights, Illinois). Filters were hybridized at 42°C overnight in 50% formamide, 5× SSC, 10× Denhardt's solution, 2% SDS, 100 μg/ml herring sperm DNA, washed with 2× SSC, 0.05% SDS at room temperature (two times for 20 min), and with 0.1× SSC, 0.1% SDS at 50°C (two times for 30 min), and exposed, with two intensifying screens, to Kodak XAR-5 film at −80°C for 3 hr. A 1.1 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 ^{32}P-labeled by random priming (GIBCO BRL, Grand Island, New York) and used as probes in the Northern blots.

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