Tissue-specific distributions of alternatively spliced human PECAM-1 isoforms

YONGJI WANG,1 XIAOJING SU,1 CHRISTINE M. SORENSON,2 AND NADER SHEIBANI1,3

1Departments of Ophthalmology and Visual Sciences, 2Pharmacology, and 3Pediatrics, University of Wisconsin, Madison, Wisconsin 53792

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Wang, Yongji, Xiaojing Su, Christine M. Sorensen, and Nader Sheibani. Tissue-specific distributions of alternatively spliced human PECAM-1 isoforms. Am J Physiol Heart Circ Physiol 284: H1008–H1017, 2003.—Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a cell adhesion molecule that is highly expressed on the surface of endothelial cells and some hematopoietic cells. Its cytoplasmic domain is encoded by multiple exons, which undergo alternative splicing. Here, we demonstrate that the human PECAM-1 cytoplasmic domain undergoes alternative splicing, generating six different isoforms. RT-PCR cloning and DNA sequence analysis indicated that human tissue and endothelial cells express multiple isoforms of PECAM-1, including the full-length PECAM-1 and five other isoforms, which lack exon 12, 13, 14, or 15 or exons 14 and 15. The full-length PECAM-1 is the predominant isoform detected in human tissue and endothelial cells. This is in contrast to murine endothelium, in which the PECAM-1 isoform lacking exons 14 and 15 is the predominant isoform. The PECAM-1 isoform lacking exon 13 detected in human tissue and endothelial cells is absent in murine endothelium. The expression pattern of PECAM-1 isoforms changes during tube formation of endothelial cells on Matrigel, which may indicate specialized roles for specific isoforms of PECAM-1 during angiogenesis. The data presented here demonstrate that human PECAM-1 undergoes alternative splicing, generating multiple isoforms in vascular beds of various tissues. Therefore, the regulated expression of these isoforms may influence endothelial cell adhesive properties during angiogenesis and/or vasculogenesis.

CD31; alternative splicing; cell adhesion; angiogenesis

ALTERNATIVE mRNA splicing can impact development and many biological functions. Alternative splicing of mRNAs greatly expands the number of gene products produced from a single coding sequence. Recently, expressed sequence tag analysis indicated a much higher rate of alternative splicing in human genes than was previously believed to occur (3). Therefore, identification of the function of the alternatively spliced products should play a crucial role in understanding developmental processes and biological functions. We previously demonstrated that multiple isoforms of platelet endothelial cell adhesion molecule-1 (PECAM-1), generated by alternative splicing, are expressed in the vascular beds of various mouse tissues in a developmentally regulated fashion (24). Furthermore, we showed that cultured murine endothelial cells express multiple isoforms of PECAM-1 in a pattern similar to that observed in vivo. However, the identification and distribution of PECAM-1 isoforms in vascular beds of human tissue and endothelial cells require further delineation.

PECAM-1 (CD31) is a member of the Ig gene superfamily that is expressed by endothelial cells, platelets, monocytes, neutrophils, and specific subsets of T cells. PECAM-1 is a relatively large gene (110 kbp) containing 16 exons encoding the 6 Ig-like extracellular domains (exons 3–8), a transmembrane domain (exon 9), and a relatively long cytoplasmic domain (exons 10–16) (18, 31). Exons 1 and 2 encode the 5′-untranslated region and the signal peptide. The predicted size of PECAM-1 (711 amino acids) is ~80 kDa. However, fully processed PECAM-1 is ~130 kDa, with carboxydrate residues (9 asparagine-linked glycosylation sites) accounting for ~40% of its molecular mass (6, 18). The cytoplasmic domain of PECAM-1 (exons 10–16) undergoes alternative splicing, generating multiple isoforms with different adhesive properties (24, 32).

PECAM-1 is believed to participate in homophilic (1, 16, 27) and heterophilic (7, 15) interactions influencing cell adhesive mechanisms. It can bind PECAM-1 (27), proteoglycans (7, 15), α5β3-integrin (4, 19, 30), and CD38 (5). Interaction of PECAM-1 with integrins may modulate their adhesive properties, as recently demonstrated for α5β3-integrin. Intracellular dimerization and oligomerization of PECAM-1 expressed in human kidney epithelial cells enhance their adhesion to fibronectin through α5β3-integrin (33). The PECAM-1 cytoplasmic domain plays an active role in modulation of cellular adhesive properties. PECAM-1 exon 14 is an important modulator of PECAM-1 adhesive properties. PECAM-1 isoforms that contain exon 14 participate in heterophilic aggregation, whereas those that lack exon 14 participate in homophilic aggregation when expressed in L cells (32). In addition, phosphorylation or
loss of the tyrosine residue in exon 14 promotes homophilic aggregation in L cells (8). Therefore, tyrosine phosphorylation or exclusion of exon 14 by alternative splicing can modulate PECAM-1 adhesive properties.

We recently demonstrated that expression of different murine PECAM-1 isoforms in Madin-Darby canine kidney cells differentially modulates the ability of these cells to form cadherin-mediated cell-cell adhesion (25). This is mediated by the differential ability of the PECAM-1 isoforms to activate the mitogen-activated protein kinase-extracellular signal-regulated kinase pathway. These cells also exhibit alterations in their integrin expression pattern and adhesion to vitronectin through αβ5-integrin (unpublished data). Therefore, PECAM-1 isoforms play an active role in regulation of endothelial cell adhesive properties through modulation of intracellular signaling pathways. Identification of the isoforms involved and the signaling pathways affected is critical for understanding the development of PECAM-1 in angiogenesis and vascular development.

Here, we demonstrate that multiple isoforms of PECAM-1 are expressed in vascular beds of various human tissues and endothelial cells. The alternative splicing is less frequent in human than in murine PECAM-1 and generates fewer isoforms. Full-length PECAM-1 is the predominant isoform detected in vascular beds of human tissue and endothelial cells. In contrast, in murine endothelium, the predominant isoform lacks exons 14 and 15. We also show that expression of PECAM-1 isoforms is regulated during capillary formation of endothelial cells on Matrigel. Thus developmental and species-specific regulation of PECAM-1 isoform expression may play an important role during angiogenesis by influencing cell adhesive mechanisms.

MATERIALS AND METHODS

Cell lines. Human umbilical vein endothelial cells (HUVEC), human dermal microvessel endothelial cells (HMVEC), and simian virus 40 large T-transformed HMVEC (TMVEC; provided by Dr. L. Cornelus, Washington University, St. Louis, MO) were maintained as described previously (24, 28). Briefly, HUVEC (passages 2–8) were grown in medium 199 with Earle’s salts containing 25 mM HEPES, 100 µg/ml endothelial cell mitogen (BioMedical Technology, Stoughton, MA), 100 µg/ml heparin (Sigma), and 20% fetal bovine serum (BioWhittaker). HMVEC (passages 2–8) were maintained in EBM medium (Clonetics) containing 10% heat-inactivated fetal bovine serum, 0.2 µM dibutyryl cAMP (Sigma), 1 µg/ml hydrocortisone (Sigma), and 10 ng/ml epidermal growth factor (Intergen). TMVEC were grown in MCDB131 medium containing 10% human serum, 0.2 mM l-glutamine, 2 µg/ml hydrocortisone (Sigma), and 100 µg/ml endothelial cell mitogen (BioMedical Technology). All endothelial cells were grown on gelatin-coated plates. Human leukemia HEL, Jurkat, and U937 cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum.

Analysis of PECAM-1 mRNA expression. Total RNA from various human tissues (human total RNA panel I) was obtained from Clontech (BD Biosciences, Palo Alto, CA). Poly(A)⁺ RNA was isolated from different human cell lines as described previously (23). The RNAs, 5 µg of poly(A)⁺ or 10 µg of total RNA, were size fractionated on a 1.2% agarose-formaldehyde gel, transferred to the membrane (Zeta-probe, Bio-Rad), prehybridized, and hybridized to the random-primed [32P]-labeled full-length human PECAM-1 cDNA (a gift of Dr. Peter Newman, Southeastern Wisconsin Blood Center). The blot was also probed with a cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control for loading. Exposure time varied for each probe.

RT-PCR analysis and DNA sequencing. Total RNA or mRNA was utilized as template for RT-PCR (Superscript One-Step RT-PCR, Gibco BRL, Gaithersburg, MD) to amplify the cytoplasmic domain of all possible PECAM-1 isoforms. The sense primer was designed as 5’-agtggacct CGAGCTTCAATGTTTCTCTTT-3’, which spans the border of exons 9 and 10 within the intracellular domain. The antisense primer was designed as 5’-ggagagctc CTTGCTGCTGATGTTGTTT-3’, which amplifies the 5’-untranslated region. The primers carry a BanHI and an EcoRI recognition sequence (lowercase letters) to facilitate subsequent cloning. PCR products were examined on 2.5% agarose gels to assess their integrity and expected size. For cloning, PCR products were directly purified using the QIAquick PCR purification kit (Qiagen), digested with BanHI and EcoRI, and cleaned using the same kit. The digested PCR products were then ligated into the pGEX-2T vector (Pharmacia), cut with the same enzymes, and transformed into Escherichia coli DH5α. Bacterial colonies were screened by BanHI and EcoRI digestion of plasmid DNA minipreps, and those with inserts were sequenced using the Big Dye reagent (Perkin-Elmer) as described previously (21). All PCR were performed in a gradient cycler (Eppendorf). For DNA sequencing, the following PCR parameters were used: 96°C for 5 min followed by 25 cycles of 96°C for 30 s, 50°C for 15 s, and 72°C for 1 min. The DNA samples were ethanol precipitated and prepared for analysis by the DNA sequencing facility at the University of Wisconsin Biotechnology Center.

Identification of PECAM-1 isoforms. The exonic mutation sites of human PECAM-1 cDNA molecules were identified by comparison of the mutant sequences with that of the wild type (12, 18). The isoform with a new junction in the cDNA sequence that lacks exon 12 (Δ12) was identified at G²¹₃⁰, A²¹³⁰ (loss of 55 bp), the isoform that lacks exon 13 (Δ13) at G²¹₃⁰, A²¹₃₅ (loss of 64 bp), the isoform that lacks exon 14 (Δ14) at G²₂₅₂, A²₂₃₁₀ (loss of 58 bp), the isoform that lacks exon 15 (Δ15) at G²₂₅₂, A²₂₃₁₃ (loss of 24 bp), and the isoform that lacks exons 14 and 15 (Δ14&15) at G²₂₅₂, A²₂₃₁₃ (loss of 81 bp). The expected size for the wild-type form (full-length PECAM-1 cytoplasmic domain) is 351 bp according to the human PECAM-1 cDNA sequence. However, the alternatively spliced isoforms have variable sizes, smaller than the wild type. Absence of exon 15 in the Δ15 and Δ14&15 isoforms changes the reading frame terminating upstream of the commonly utilized termination codon (see Figs. 3 and 4).

Three-dimensional culture of endothelial cells. Matrigel (10 mg/ml; Collaborative Research) was applied at 0.5 ml/35-mm tissue culture dish and incubated at 37°C for 30 min to allow it to harden. HUVEC (macroversal) or HMVEC (microvascular) were prepared by trypsinization, washed with growth medium once, and resuspended at 1.5 × 10⁵ cells/ml. Cells (2 ml) were gently added to the Matrigel-coated plates, incubated at 37°C, and monitored for 6–24 h. During the incubation, endothelial cells initially organize to islands of cells that elongate and migrate to form a capillary-like network within 18–24 h. Endothelial cells were recovered from Matrigel at different times after they
were plated using the MatriSperse cell release solution (BD Labware, Bedford, MA) as recommended by the supplier. This method has been successfully utilized for differential cloning of endothelial cell adhesion molecules during tube formation (11). Briefly, the medium was removed, the adherent cells on the Matrigel were rinsed three times with cold phosphate-buffered saline (PBS), MatriSperse solution was added at 2 ml/35-mm dish, and cells or Matrigel was scraped into a 50-ml conical tube. Each dish was rinsed with an additional 2 ml of MatriSperse solution, added to the collecting tube, and incubated on ice for 1 h to release the adherent cells from Matrigel. The cells were recovered by centrifugation, washed with cold PBS three times, and used for isolation of mRNA with the ExpressDirect mRNA Capture and RT System for RT-PCR (Pierce, Rockford, IL). After the last PBS wash, the cell pellet was resuspended and lysed on ice to release poly(A)⁺ RNA, which is captured in tubes containing immobilized oligo(dT). The captured poly(A)⁺ RNA was washed with a high-salt and then a low-salt wash buffer to remove contaminating DNA or poly(A)⁻ RNA. The captured poly(A)⁺ RNA was then directly used for RT-PCR. Therefore, the mRNA capture, cDNA synthesis, and RT-PCR are performed in the same tube for each sample. The RT-PCR was performed with Superscript One-Step RT-PCR ( Gibco BRL). The RT-PCR products were then cloned and sequenced as described above.

Western blot analysis of PECAM-1 isoforms. The hematopoietic HEL cells express moderate levels of PECAM-1 on their surface. The PECAM-1 expression in these cells is modulated by incubation with phorbol myristate acetate (PMA) (9, 10). PMA induces the differentiation of these cells toward a megakaryocytic lineage. These cells express multiple isoforms of PECAM-1 at very similar frequencies, unlike human tissue and endothelial cells, where full-length PECAM-1 is expressed at the highest frequency, thus making HEL cells more feasible for analysis of PECAM-1 isoform products. In addition, the expression pattern of the PECAM-1 isoforms changes on incubation of these cells with PMA (unpublished observations). To demonstrate that the product of the different PECAM-1 isoform is translated, we examined the cell lysates prepared from PMA- or DMSO (control)-treated HEL cells by Western blotting. HEL cells were incubated with 20 nM PMA (Calbiochem, San Diego, CA) or DMSO (solvent control) for different times. After PMA treatment, cells were pelleted, washed with cold Tris-buffered saline (20 mM Tris and 150 mM NaCl, pH 7.4), and lysed in 0.5 ml of lysis buffer [20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Roche Biochemicals)] with a brief sonication. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce). Equal amounts of protein (25 μg) were analyzed by SDS-PAGE (4–20% Tris-glycine gel; Invitrogen), transferred to nitrocellulose, and blotted with an antibody that recognizes the extracellular domain of human PECAM-1 (reacts with all PECAM-1 isoforms; SEW 16, a gift of Dr. Peter Newman) or an antibody that recognizes exon 14 of murine PECAM-1 (reacts with isoforms that contain exon 14) (24). After incubation with appropriate secondary antibody, the blot was washed and developed using enhanced chemiluminescence (Amersham).

RESULTS

Expression of PECAM-1 in various human tissues and cells. PECAM-1 plays an important role in endothelial cell-cell and cell-matrix interactions, which are essential during vasculogenesis and/or angiogenesis (17, 22). Here, we examined expression of PECAM-1 mRNA in vascular beds of various human tissues and compared it with expression of PECAM-1 in human endothelial and hematopoietic cells. Figure 1 is a Northern blot demonstrating that PECAM-1 is expressed in all the tissues and cell lines examined, although at vastly different levels. PECAM-1 was highly expressed in kidney, lung, and trachea and at lower levels in brain, heart, and liver. PECAM-1 was highly expressed in HUVEC and at moderate levels in the hematopoietic cells. A short exposure of the blot probed with GAPDH is shown, because poly(A)⁺ RNA from the cell lines gives a strong signal within several hours compared with the total RNA from human tissue. Therefore, total RNA from various tissues required a much longer exposure to reveal GAPDH mRNA. Human tissue and cell lines expressed multiple RNA bands for PECAM-1, which may represent alternatively spliced PECAM-1 isoforms, the identity of which required further analysis.

Tissue-specific distribution of human PECAM-1 isoforms. The tissue-specific distribution of PECAM-1 isoform(s) has not been defined in human endothelium. To determine the expression pattern of different PECAM-1 isoforms in vascular beds of various human tissues and endothelial cells, RT-PCR was performed on RNA isolated from various human tissues and endothelial cells (see MATERIALS AND METHODS). The primers encompassed the entire cytoplasmic domain, thus having the potential to amplify all PECAM-1 isoforms. All the RNA samples from human tissue and endothelial cells exhibited a similar RT-PCR pattern, with a predominant band corresponding to the expected size of the full-length PECAM-1 cytoplasmic domain (Fig. 2). We did not observe the multiple band patterns we had previously seen in mouse tissue and endothelial cells, suggesting fewer PECAM-1 isoforms (24). However, it is difficult to resolve all the potential isoforms in this
manner because of similarity in size and differences in their abundance. To confirm that these results were due to the presence of fewer PECAM-1 isoforms in human tissue and endothelial cells, we next cloned and sequenced the cDNA products generated by RT-PCR.

Identification of PECAM-1 isoforms in vascular beds of various human tissues and endothelial cells. The identity of the cDNAs generated by RT-PCR was determined by direct cloning and sequencing (see MATERIALS AND METHODS). Tables 1 and 2 show the isoforms of PECAM-1 and the frequency at which they were detected in vascular beds of various human tissues and endothelial cells. Multiple isoforms of human PECAM-1 were detected in vascular beds of human tissue (Table 1) and human endothelial cells (Table 2). We detected full-length PECAM-1 as well as isoforms lacking exon 12, 13, 14, or 15 or exons 14 and 15. The PECAM-1 isoform that lacks exon 13 has not been previously detected in murine endothelium. The full-length PECAM-1 isoform is the predominant isoform detected in vascular beds of human tissue and endothelial cells. The PECAM-1 isoform that lacks exon 14 was detected in all human tissues and endothelial cells, whereas the isoforms that lack exon 12, 13, or 15 or exons 14 and 15 were detected in vascular beds of some tissues and endothelial cells. Human heart, lung, trachea, HUVEC, and THMVEC express a higher number of different isoforms. The cDNA and amino acid sequences of all the PECAM-1 isoforms detected in this study are shown in Figs. 3 and 4, respectively. The absence of exon 15 changes the reading frame, resulting in utilization of an upstream termination codon shortening the cDNA by three amino acids and incorporation of six different amino acids upstream of the termination codon (Figs. 3 and 4). Our sequence data are in strong agreement with the published sequence of human PECAM-1 (18). The putative splice sites of human PECAM-1 mRNA molecules were derived from Newman et al. (18) and Kirschbaum et al. (12). This is the first systematic investigation of the alternatively spliced PECAM-1 isoforms in human endothelium. Therefore, our results provide further support for the presence of alternatively spliced human PECAM-1 isoforms with the potential for different adhesive properties.

**Distribution of PECAM-1 isoforms during endothelial cell tube formation.** To gain a better understanding of the adhesive function of different PECAM-1 isoforms in capillary formation, we examined the pattern of PECAM-1 isoforms during endothelial cell tube formation on Matrigel. Most endothelial cells rapidly organize and form capillary-like structures when plated on Matrigel. This assay recapitulates the later stages of angiogenesis and has been utilized to assess the effects of angiogenic stimulators and/or inhibitors on endothelial cell sprouting. Figure 5 shows the ability of HUVEC and HMVEC to organize and form cordlike structures. After 6 h on Matrigel, both cell types started to reorganize to form visible capillary-like networks with large islands of cells. After 24 h, cells further reorganized to form capillary-like structures, with HUVEC exhibiting the most uniform and largest capillary-like structures.

We examined changes in the pattern of PECAM-1 isoforms during this process by RT-PCR analysis of

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Table 1. Distribution of PECAM-1 isoforms in human tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>Full-length</th>
<th>Δ12</th>
<th>Δ13</th>
<th>Δ14</th>
<th>Δ15</th>
<th>Δ14&amp;15</th>
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<tbody>
<tr>
<td>Brain</td>
<td>32</td>
<td>94</td>
<td>ND</td>
<td>ND</td>
<td>3</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>33</td>
<td>97</td>
<td>ND</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Heart</td>
<td>29</td>
<td>83</td>
<td>ND</td>
<td>7</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Liver</td>
<td>34</td>
<td>94</td>
<td>ND</td>
<td>ND</td>
<td>6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lung</td>
<td>27</td>
<td>74</td>
<td>ND</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Trachea</td>
<td>32</td>
<td>88</td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values represent frequency (percentage) at which each isoform was detected; n, total number of platelet endothelial cell adhesion molecule (PECAM)-1 clones examined. Isoforms of PECAM-1 were identified by cloning and sequencing RT-PCR products from total RNA isolated from various human tissues. ND, not detected.

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RNA isolated from proliferating cells and cells plated on Matrigel for 6 or 24 h. The RT-PCR patterns of RNAs prepared from HUVEC and HMVEC growing in two-dimensional culture or on Matrigel after different times were similar. The RT-PCR analysis demonstrated a predominant band of the expected size for full-length PECAM-1 (Fig. 2 and results not shown). To identify the PECAM-1 isoform patterns during tube formation, the RT-PCR products were directly cloned and sequenced. Tables 3 and 4 demonstrate the isoforms detected in HUVEC and HMVEC during tube formation, respectively. Full-length PECAM-1 was the predominant isoform detected in both cell types, consistent with that observed in most tissues. However, changes in the isoforms expressed did occur. Thus alternative splicing may provide a mechanism for modulation of PECAM-1 adhesive properties during angiogenesis.

Table 2. Distribution of PECAM-1 isoforms in cultured human endothelial cells

<table>
<thead>
<tr>
<th></th>
<th>Full-length</th>
<th>Δ12</th>
<th>Δ13</th>
<th>Δ14</th>
<th>Δ15</th>
<th>Δ14&amp;15</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>32</td>
<td>90</td>
<td>ND</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>HMVEC</td>
<td>21</td>
<td>85</td>
<td>5</td>
<td>ND</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>TMHVEC</td>
<td>27</td>
<td>78</td>
<td>7</td>
<td>ND</td>
<td>7</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values represent frequency (percentage) at which each isoform was detected; n, total number of PECAM-1 clones examined. Isoforms of PECAM-1 were identified by cloning and sequencing RT-PCR products from mRNA isolated from various human endothelial cells. HUVEC, human umbilical vein endothelial cells; HMVEC, human dermal microvessel endothelial cells; TMHVEC, Simian virus 40 large T-transformed HMVEC.

Fig. 3. cDNA sequence of cytoplasmic domain of human PECAM-1 isoforms. Nucleotide sequence encoding cytoplasmic domain of isoforms lacking exon 12, 13, 14, or 15 or exons 14 and 15 is compared with sequence for full-length PECAM-1 isoform. Deleted sequences are indicated by dashes. Predicated exon sequences of human PECAM-1 were adapted from Newman et al. (18).
Expression of the alternatively spliced PECAM-1 isoforms. To determine whether alternatively spliced isoforms of PECAM-1 are translated, we examined the expression pattern of PECAM-1 isoforms in the hematopoietic HEL cells during their megakaryocytic differentiation. HEL cells express moderate levels of PECAM-1, which is modulated during their PMA-induced differentiation. The frequencies at which different PECAM-1 isoforms are detected in these cells, particularly after PMA treatment, are very similar. This is in contrast to human endothelial cells, where the full-length isoform occurs at the highest frequency. Therefore, HEL cells provide a suitable system to determine whether the product of different PECAM-1 isoforms can be translated. The RT-PCR analysis and DNA sequencing demonstrated that HEL cells express full-length isoforms and five additional isoforms of PECAM-1, including Δ13, Δ14, Δ15, Δ13&14, and Δ14&15 (29). This pattern dramatically changes on PMA treatment and includes a number of isoforms that lack exon 12, including Δ12&14 and Δ12,13&14 (unpublished observations), which are the predominant isoforms detected. Figure 6 shows the Western blot analysis of cell lysates prepared from HEL cells after incubation with PMA or DMSO (control). The same blot was incubated with an antibody to human PECAM-1 (SEW 16), which reacts with the extracellular domain of PECAM-1 (recognizes all PECAM-1 isoforms; Fig. 6, left) or an antibody to murine exon 14 [recognizes PECAM-1 isoforms containing exon 14 (24); Fig. 6, right]. The human PECAM-1 antibody recognized a band corresponding to the full-length PECAM-1 (130 kDa) in DMSO-treated

Fig. 4. Amino acid sequence of cytoplasmic domain of human PECAM-1 isoforms lacking exon 12, 13, 14, or 15 or exons 14 and 15 is compared with amino acid sequence for full-length PECAM-1 isoform. Deleted sequences are indicated by dashes. Putative exon sequences of human PECAM-1 were indicated according to cDNA sequence data (see Fig. 3 legend). Underscored sequences indicate changes in reading frame of amino acid sequence. * Different reading frames utilized.

Fig. 5. Formation of capillary networks by HUVEC and HMVEC on Matrigel. Phase micrographs show HUVEC and HMVEC incubated on Matrigel for 0, 6, and 24 h and photographed (×4 objective). Experiment was repeated at least twice with identical results.
cells. However, two additional lower-molecular-weight bands (~110 and 120 kDa) were detected in PMA-treated cells (Fig. 6, left). These bands were not detected when the same blot was probed with the murine exon 14 antibody (Fig. 6, right), even after longer exposure. The murine exon 14 antibody only detected a band corresponding to the full-length PECAM-1 in PMA- or DMSO-treated cells. Thus the lower-molecular-weight bands are the products of PECAM-1 isoforms lacking exon 14. This is consistent with the pattern of PECAM-1 isoforms detected in HEL cells after PMA treatment.

**DISCUSSION**

Alternative splicing of pre-mRNA and protein phosphorylation are fundamental mechanisms for regulating protein structure and function (2, 26). Alternative splicing of pre-mRNA increases protein diversity from a single gene (3), with many proteins having multiple functional isoforms. More than one-third of human genes display variably spliced mature mRNAs, including N-cadherin, Down syndrome cell adhesion molecule (20), and PECAM-1 (12, 22). Therefore, the estimated 35,000–80,000 genes in the human genome could easily produce several hundred thousand different proteins (2). Determining how these sometimes subtle changes in sequence affect protein function is a crucial question in developmental and cell biology impacting apoptosis, tumor progression, neuronal connectivity, tuning of cell excitation and cell contraction, and angiogenesis. PECAM-1 (CD31) is a cell adhesion molecule that is highly expressed on the surface of endothelial cells and localizes to the sites of cell-cell contact in monolayer culture of endothelial cells and in the vasculature. It plays an important role in endothelial cell-cell interactions during angiogenesis (22) and leukocyte-endothelial cell interaction during transendothelial migration (14).

We recently demonstrated that alternatively spliced PECAM-1 isoforms are expressed in vascular beds of various mouse tissues, and their expression is developmentally regulated (24). Here we demonstrate that 1) PECAM-1 is expressed in various human tissues at vastly different levels, 2) PECAM-1 undergoes alternative splicing, generating multiple isoforms in vascular beds of human tissue and endothelial cells, 3) the full-length PECAM-1 isoform is the predominant isoform detected in human endothelium, whereas the Δ14&15 PECAM-1 is the predominant isoform in mouse endothelium, 4) the PECAM-1 isoform that lacks exon 13 is detected in human endothelium but is absent in murine endothelium, 5) the expression pattern of PECAM-1 isoforms changes during tube formation on Matrigel, with the full-length PECAM-1 as the predominant isoform, and 6) different human PECAM-1 isoforms can be translated.

Understanding the adhesive function of PECAM-1 isoforms is essential for elucidating the role of PECAM-1 in endothelial cell-cell and cell-matrix interactions during vasculogenesis and/or angiogenesis. Although the Δ13 and Δ14 PECAM-1, as well as a soluble form of PECAM-1, have been identified in HUVEC and U937 cells (10, 12, 18), the presence of other PECAM-1 isoforms in human endothelium requires further characterization. Here, we show that the full-length and Δ12, Δ13, Δ14, Δ15, and Δ14&15 isoforms are detected in vascular beds of human tissue and endothelial cells (Tables 1 and 2). In human tissue and endothelial cells, the full-length PECAM-1 is the predominant isoform detected. In contrast, our data in murine tissue and endothelial cells demonstrated that Δ14&15 is the predominant isoform (24), whereas the isoform lacking exon 13 is not present. The Δ14&15 isoform was detected in human lung but at a lower frequency than in mouse tissue and endothelial cells. Human heart, lung, and trachea, HUVEC, and THMVEC expressed higher

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**Table 3. Distribution of PECAM-1 isoforms in HUVEC during tube formation on Matrigel**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>n</th>
<th>Full-length</th>
<th>Δ12</th>
<th>Δ13</th>
<th>Δ14</th>
<th>Δ15</th>
<th>Δ14&amp;15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32</td>
<td>90</td>
<td>ND</td>
<td>3</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>28</td>
<td>82</td>
<td>ND</td>
<td>3</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>24</td>
<td>29</td>
<td>97</td>
<td>ND</td>
<td>3</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values represent frequency (percentage) at which each isoform was detected; n, total number of PECAM-1 clones examined. Isoforms of PECAM-1 were identified by cloning and sequencing RT-PCR products from mRNA isolated from HUVEC at 0, 6, and 24 h during tube formation.

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**Table 4. Distribution of PECAM-1 isoforms in HMVEC during tube formation on Matrigel**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>n</th>
<th>Full-length</th>
<th>Δ12</th>
<th>Δ13</th>
<th>Δ14</th>
<th>Δ15</th>
<th>Δ14&amp;15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21</td>
<td>85</td>
<td>5</td>
<td>ND</td>
<td>10</td>
<td>ND</td>
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</tr>
<tr>
<td>6</td>
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<td>90</td>
<td>ND</td>
<td>5</td>
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</tr>
<tr>
<td>24</td>
<td>23</td>
<td>82</td>
<td>9</td>
<td>ND</td>
<td>ND</td>
<td>9</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values represent frequency (percentage) at which each isoform was detected; n, total number of PECAM-1 clones examined. Isoforms of PECAM-1 were identified by cloning and sequencing RT-PCR products from mRNA isolated from HMVEC at 0, 6, and 24 h during tube formation.
numbers of different isoforms through alternative splicing of PECAM-1. Human lung shows more diversity of PECAM-1 isoforms than other human tissues, endothelial cells, and primary hematopoietic cells, including platelets and T lymphocytes (Table 1) (29). Thus these data strongly argue against the possibility that the presence of different PECAM-1 isoforms in human tissue is the result of contaminating blood cells. Therefore, multiple isoforms of PECAM-1 are expressed by human endothelium in a tissue-specific pattern (Table 1).

The PECAM-1 isoforms are generated by alternative splicing of the exons that encode the PECAM-1 cytoplasmic domain. Our results indicate that the frequency at which human PECAM-1 mRNA undergoes alternative splicing is much lower than that detected in murine PECAM-1 mRNA (24). Indeed, in human endothelium, the full-length PECAM-1 is the predominant isoform detected, whereas the Δ14&15 isoform is the predominant isoform in murine endothelium. In addition, murine tissue and endothelial cells express a greater number of different isoforms. We previously showed that not only is the product of the murine PECAM-1 isoform(s) present in the endothelium, but its expression is developmentally regulated (24). This was accomplished by development of an antibody to exon 14 that recognizes only isoforms containing exon 14 but not the isoforms that lack exon 14 (24). However, all these isoforms are recognized by an antibody that reacts with the extracellular domain of PECAM-1. The murine PECAM-1 exon 14 sequence is very similar to human PECAM-1 exon 14, and the antibody to murine exon 14 recognizes the human PECAM-1 (Fig. 6, right). We recently demonstrated that HEL cells express mRNAs for multiple PECAM-1 isoforms at very similar frequencies (29). In contrast, human tissue and endothelial cells express full-length PECAM-1 at the highest frequency. Thus HEL cells, which express several isoforms of PECAM-1 at similar frequencies, are well suited for detection of various isoform products by Western blot analysis. Using HEL cell protein lysates, we demonstrate that the product of several human PECAM-1 isoforms is translated during their PMA-mediated differentiation. The pattern of products detected by Western blot is consistent with the pattern of isoforms detected by RT-PCR cloning and sequencing (see RESULTS). The major glycosylation sites of PECAM-1 are extracellular (6), and exon 14 antibody (made to the cytoplasmic domain) fails to detect these isoforms. Therefore, we believe that the presence of the different PECAM-1 products in PMA-treated HEL cells is mainly due to alternative splicing and is not the result of alterations in PECAM-1 glycosylation or the presence of other contaminating proteins. In addition, the presence of multiple PECAM-1 isoform mRNAs in polysome preparations provides further support for their ability to be translated (29).

The regulation of PECAM-1 alternative splicing is poorly understood. Despite a 67% sequence homology between murine and human PECAM-1 cDNAs, they fail to significantly cross hybridize in Northern blots (24). Thus there are significant differences in the primary structure of mouse and human PECAM-1 mRNA molecules. A single-nucleotide difference reduces the intrinsic strength of the 3′-splicing site of the survival motor neuron gene exon 7 by twofold, whereas the strength of the 5′-splicing site of the exon 7 is not affected (13). Therefore, the differences in the DNA sequences of human and murine PECAM-1 may contribute, at least in part, to the differences observed in the expression pattern of the alternatively spliced PECAM-1 isoforms. In addition, these differences may indicate utilization of different mechanisms for modulation of PECAM-1 adhesive properties in human endothelium compared with mouse endothelium. However, the specific function of the alternatively spliced isoforms in modulation of cell adhesive properties during human vascular development and angiogenesis requires further delineation.

To determine whether isoform changes occur during angiogenesis, we examined the distribution of PECAM-1 isoforms during tube formation of human endothelial cells on Matrigel. This assay recapitulates only the later stages of angiogenesis. Cultured human endothelial HUVEC (large vessel) and HMVEC (small vessel) were allowed to form capillary-like networks on Matrigel. Tables 3 and 4 show that there are changes in the PECAM-1 isoforms expressed during tube for-

![Fig. 6. Western blot analysis of PECAM-1 isoforms in HEL cells. Lysates were prepared from control (DMSO) or phorbol myristate acetate (PMA)-treated HEL cells at 0, 1, 2, and 4 days. Equal amounts of protein (25 μg) from each sample were analyzed under reducing conditions utilizing SDS-PAGE. Proteins were transferred to nitrocellulose membrane and blotted with a rabbit polyclonal antibody that reacts with the extracellular domain (left) or exon 14 (right) of PECAM-1. Note the presence of the lower-molecular-weight PECAM-1 bands in cells treated with PMA for 2 and 4 days detected by antibody that reacts with extracellular domain of PECAM-1 but not exon 14.](http://ajpheart.org/Downloadedfromhttp://ajpheart.org/)
mation, but the predominant isoform remains the full-length PECAM-1. The significance of these isoform changes during angiogenesis requires further characterization of the adhesive properties of these isoforms. Perhaps the different PECAM-1 isoforms have specific adhesive properties, the expression of which is regulated during angiogenesis/vasculogenesis.

We recently demonstrated that expression of murine PECAM-1 with and without exon 14 (Δ15 or Δ14&15) can differentially modulate cadherin-mediated cell-cell interactions when expressed in Madin-Darby canine kidney cells (25). This is mediated through the differential ability of these isoforms to activate the mitogen-activated protein kinase-extracellular signal-regulated kinase pathway. Therefore, PECAM-1 can play an active role in modulation of cell-cell interactions, and its functionalization is dependent on formation of adherens junction (25). Isoform switching provides a mechanism by which PECAM-1 can modulate cell-cell interactions. Strong cadherin-mediated cell-cell adhesions are counterintuitive at early stages of angiogenesis when cells are highly migratory, whereas later these interactions are essential for physiological integrity of the mature vessels. This is consistent with our observation during kidney vascular development, where isoforms that contain exon 14 (e.g., Δ15) are expressed early and later replaced with isoforms that lack exon 14 (e.g., Δ14&15) (24, 25). Therefore, characterization of the adhesive properties of the alternatively spliced PECAM-1 isoforms and identification of the intracellular proteins that specifically interact with these isoforms will allow us to further elucidate the role of PECAM-1 in hemostasis, inflammation, and angiogenesis.

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