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Tissue Specific Expression of Alternatively Spliced Murine PECAM-1 Isoforms

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ABSTRACT **PECAM-1 (CD31) is a cell adhesion molecule that is highly expressed at the sites of endothelial cell-cell contact and at lower levels on the surface of platelets and leukocytes. It is a member of the immunoglobulin gene superfamily and undergoes alternative splicing to generate several isoforms that differ only in their cytoplasmic domains. The tissue distribution of the expression of different PECAM-1 isoforms has not been previously defined. We have examined PECAM-1 expression in various mouse tissues and endothelial cells. PECAM-1 mRNA was highly expressed in lung, heart, and kidney, and to a lower extent in brain and liver. Most endothelial cells in culture expressed high levels of PECAM-1 mRNA; however, normal mouse brain endothelial cells rapidly lost PECAM-1 expression in culture. To examine the tissue distribution of PECAM-1 isoform expression, RT/PCR was performed on the RNA isolated from various mouse tissues and mouse endothelial cells. Cloning and sequencing of the cDNA products indicated that most tissues and endothelial cells expressed several PECAM-1 isoforms at different frequencies. The PECAM-1 isoform that lacks exons 14 and 15 was most frequently detected in all cases. A novel PECAM-1 isoform that lacks exons 12 and 14 was detected in brain. An antibody to the extracellular domain of PECAM-1 reacted with two major bands, at 130 kDa and 110–120 kDa, in lysates prepared from endothelial cells or kidneys at different stages of development. An antibody prepared against PECAM-1 exon 14, which reacts only with cytoplasmic domain of PECAM-1 isoforms that contain exon 14, failed to react with the major lower molecular weight form of PECAM-1 in these lysates. Therefore, PECAM-1 isoforms that lack exon 14 are expressed in endothelial cells and tissues in developmentally regulated fashion. These results illustrate that multiple PECAM-1 isoforms are expressed in various mouse tissues and endothelial cells. Understanding the distribution of PECAM-1 isoforms, and the identity of intracellular proteins with which they may interact, will help to elucidate the role of PECAM-1 in endothelial cell–cell interactions and morphogenesis. *Dev Dyn 1999;214:44–54.***

Key words: PECAM-1; cell adhesion molecule; endothelial cells; angiogenesis; signal transduction

INTRODUCTION

Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is a member of the immunoglobulin gene superfamily. It is highly expressed at the sites of endothelial cell–cell contact, and on the surface of platelets and on hemopoietic cells at moderate levels (DeLisser et al., 1997a; Newman, 1997). PECAM-1 plays an important role during inflammatory responses by affecting leukocyte–endothelial transmigration (Muller, 1995) and activation of integrins on leukocytes and T cells (Tanaka et al., 1992; Berman et al., 1996). Its expression during early embryonic development on the surface of endothelial cells and endocardial cells suggests that PECAM-1 plays an important role in the development of the cardiovascular system (Baldwin et al., 1994). However, the role of PECAM-1 in regulation of endothelial cell proliferation and morphogenesis is not clearly understood. Culturing endothelial cells in the presence of anti-PECAM-1 antibody blocks the establishment of normal intercellular contacts and formation of an integral monolayer, while the addition of antibody to endothelial cells following formation of the monolayers does not affect cell adhesion (Albelda et al., 1990). In addition, anti-PECAM-1 antibodies block the ability of human umbilical vein endothelial cells to organize and form capillary like structures on Matrigel (Sheibani et al., 1997) and inhibit angiogenesis in mouse corneal assays (DeLisser et al., 1997b). Therefore, PECAM-1 plays an important role in initial endothelial cell–cell, and perhaps cell–-matrix, interactions that are essential during angiogenesis.

PECAM-1 participates in both homophilic and heterophilic interactions. It can bind PECAM-1 (Sun et al., 1996), proteoglycans (DeLisser et al., 1993), the $\alpha v\beta 3$ integrin (Piali et al., 1995; Buckley et al., 1996), and CD38 (Deaglio et al., 1998). These interactions are modulated, at least in part, by the cytoplasmic domain

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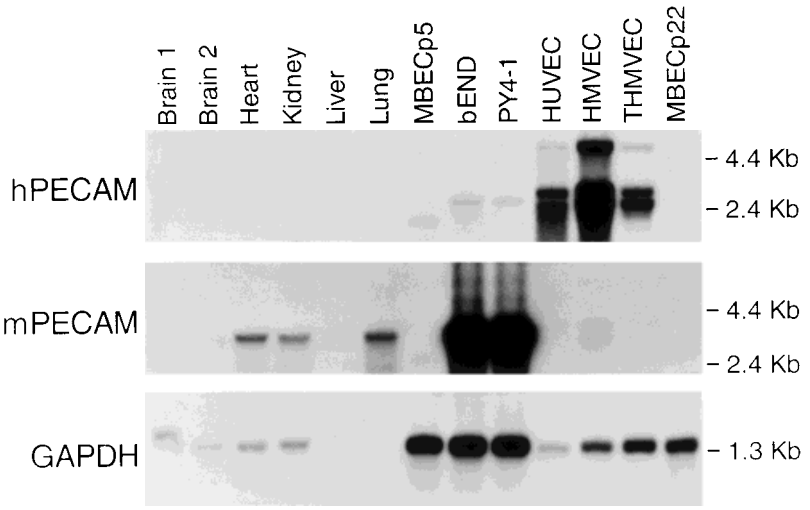

Fig. 1. Northern blot analysis of RNA isolated from various mouse tissues and endothelial cells. Approximately 30 μg of total RNA (mouse tissues) or 5 μg of poly A^+ RNA (endothelial cells) was separated on a 1.2% agarose-formaldehyde gel, transferred to zeta-probe membrane, prehybridized, and hybridized to random primer ^{32}P-labeled full-length mouse or human PECAM-1 cDNA. The blot was also probed with a cDNA for GAPDH to control for loading. Please note that the lanes corresponding to RNA from liver and lung are underloaded and only with longer exposure to GAPDH was mRNA detectable.

of PECAM-1 (Yan et al., 1995; Sun et al., 1996). PECAM-1 undergoes alternative splicing, generating several isoforms in the early mouse embryo that differ in the length of their cytoplasmic domains (Yan et al., 1995). Alterations in the cytoplasmic domain, encoded by exons 10–16, may have functional implications. The alternative splicing of exon 14 in murine PECAM-1 alters the binding characteristics of PECAM-1 when expressed in L cells (Yan et al., 1995; Sun et al., 1996). Therefore, specific interactions between PECAM-1 cytoplasmic domains and intracellular proteins may be important in modulating PECAM-1 adhesive functions. PECAM-1 undergoes tyrosine phosphorylation in response to mechanical stimulation (Osawa et al., 1997), platelet aggregation (Jackson et al, 1997a), clustering of IgE receptor (Sagawa et al., 1997a), engagement of antigen receptors on T cells (Sagawa et al., 1997b), and treatment with phosphatase inhibitors such as vanadate (Lu et al., 1996; Famiglietti et al., 1997; Jackson et al., 1997b). Phosphorylation of the tyrosine residue in exon 14 results in homophilic interaction of PECAM-1 in L cells (Famiglietti et al., 1997). In addition, tyrosine phosphorylation of PECAM-1 has been shown to promote its association with intracellular signal transducing molecules such as SHP-1 and SHP-2, tyrosine phosphatases with SH2 domains (Jackson et al., 1997a, 1997b; Masuda et al., 1997; Sagawa et al., 1997b; Cao et al., 1998). However, the physiological consequence of these phosphorylation-dependent interactions remains to be determined.

Examination of RNA prepared from early mouse embryos by RT/PCR identified seven isoforms of PECAM-1 that differed only in the length of their cytoplasmic domains (Yan et al., 1995). However, the dynamic pattern of expression and specific tissue distribution of these isoforms remain unknown. To begin understanding the role of PECAM-1 isoforms and their adhesive properties in endothelial cell–cell interactions and morphogenesis we examined the expression of PECAM-1 isoforms in various mouse tissues and endothelial cell lines. We have identified the different PECAM-1 isoforms by cloning and sequencing the products of RT/PCR performed with RNA isolated from various mouse tissues and endothelial cells. Our results indicated that all mouse tissues and endothelial cells examined express the mRNA for multiple PECAM-1 isoforms; however, the frequency at which each isoform was detected varied. The PECAM-1 isoform that lacked exons 14 and 15 (Δ14&15) was most frequently detected in all cases. We have also identified a novel PECAM-1 isoform in the brain which lacks exons 12 and 14 (Δ12&14). We prepared a polyclonal antibody that differentially reacted with PECAM-1 isoforms and provided evidence that the expression of PECAM-1 isoforms that lack exon 14 occurs in the mature kidney and endothelial cells .

RESULTS

Expression of PECAM-1 in Various Mouse Tissues and Endothelial Cells

We initially examined the expression of PECAM-1 in various postnatal day 21 (P21) mouse tissues and compared expression of PECAM-1 in several mouse and human endothelial cells in culture. Figure 1 demonstrates that all the tissues examined expressed PECAM-1 although at vastly different levels. The full-length mouse cDNA probe detected only a single mRNA band in mouse tissues and endothelial cells. PECAM-1 was highly expressed in lung as well as in heart and kidney. It was not highly expressed in brain and liver at P21. The bEND and Py4–1 mouse endothelial cells express very high levels of PECAM-1 mRNA, but no PECAM-1 mRNA was detected in normal mouse brain endothelial cells in early or late passage cells even with prolonged exposures (Fig. 1). A short exposure of the blot probed with GAPDH is shown because the poly A^+ RNA from cultured endothelial cells gave a strong signal within minutes. However, the total RNA from various mouse tissues required a much longer exposure to reveal GAPDH mRNA in lanes corresponding to liver and lung, which are underloaded compared to other

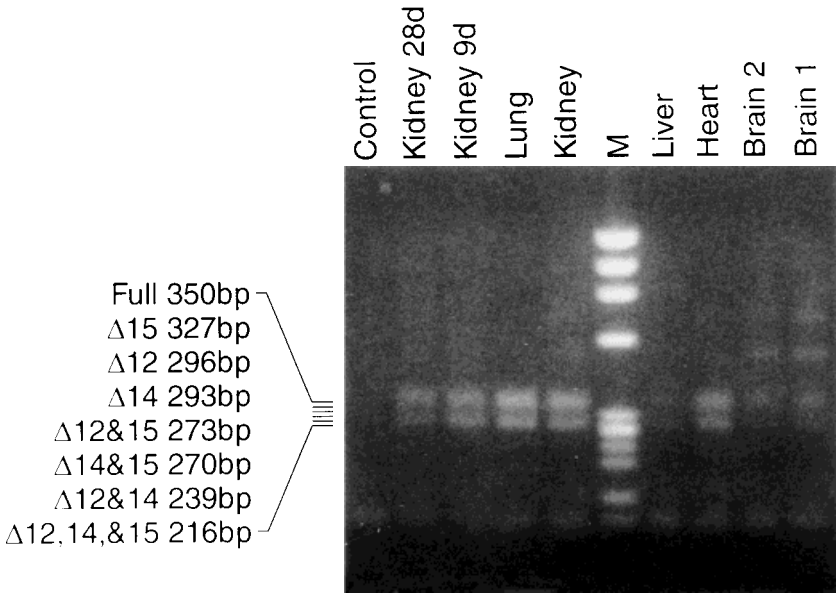

Fig. 2. The RT/PCR analysis of RNA from various mouse tissues. Total RNA was isolated from various tissues as described in the Methods section. Approximately 0.5 µg of total RNA was used for reverse transcription with random primers, and the cDNA was amplified by PCR. The PCR product was separated on a 2% agarose gel, stained with ethidium bromide, and photographed. M designates the molecular marker and the bands correspond to 1350, 1078, 872, 603, 310, 280, 234, 194, 118, and 72 bp, respectively (Molecular marker IX, Boehringer Manheim, Indianapolis, IN). The expected sizes of products derived form different PECAM-1 isoforms are indicated. The PECAM-1 isoform designations were adapted from Yan et al., 1995.

samples (data not shown). Despite the high degree of homology reported between human and mouse PECAM-1 cDNAs (Newman et al., 1990; Xie and Muller, 1993), the mouse cDNA only weakly detected human PECAM-1 mRNA or vice versa. Human endothelial cells expressed multiple RNA bands, some of which may represent alternatively spliced PECAM-1 isoforms (Kirschbaum et al., 1994).

Distribution of PECAM-1 Isoforms in Various Mouse Tissues and Endothelial Cells

It has been previously shown that several isoforms of PECAM-1, which differ only in their cytoplasmic domain, are expressed in early mouse embryos (E12) (Yan et al., 1995). However, the distribution of these isoforms in endothelial cells of various vascular beds has not been elucidated. To determine the tissue-specific expression of different PECAM-1 isoforms we performed RT-PCR using RNA isolated from various mouse tissues and several cultured mouse endothelial cells. The PCR primers encompassed the entire cytoplasmic domain and thus will amplify all PECAM-1 isoforms. Figure 2 illustrates the analysis of the RT-PCR products generated from various tissues obtained from 3-week-old mice. In the case of kidney, we also isolated RNA from 9-day and 28-day postnatal animals. Two different RNA isolations of brain were also utilized. As shown in Figure 2, all the RNA samples exhibited a similar RT-PCR pattern, although all the possible isoforms were not resolved. The expected size of the cytoplasmic domain of each isoform is indicated in Figure 2.

The presence of multiple bands suggests that multiple isoforms of PECAM-1 are expressed in various tissues. This was confirmed by cloning and sequencing the cDNAs generated by RT-PCR (see below). A similar RT-PCR product pattern was observed by using a template RNA from mouse endothelial cells (data not shown), suggesting that endothelial cells also express several different isoforms of PECAM-1 in culture.

Identification of PECAM-1 Isoforms

We next determined the identity of the cDNAs generated in RT/PCR by directly cloning these cDNAs and sequencing them. Table 1 illustrates the different isoforms of PECAM-1 and the frequency at which they were detected in various mouse tissues. Most tissues expressed several isoforms of PECAM-1, with the exception of liver, which expressed fewer isoforms. The frequency at which each isoform was detected varied. However, the PECAM-1 isoform that lacks exons 14 and 15 (Δ14&15) was most frequently detected in all tissues, except in heart where the isoform that lacks exon 15 was most frequently detected. We also detected a new isoform of PECAM-1 that lacks exons 12 and 14 (Δ12&14) in brain. The cDNA sequence of the cytoplasmic domain of Δ12&14 PECAM-1 isoform is shown in Figure 3.

Table 2 illustrates the results obtained with RT-PCR of RNA from several cultured mouse endothelial cells. The bEND cells, which are polyoma middle T-transformed mouse brain endothelial cells, and the Py4–1 cells, which are endothelial cells established from ear and tail hemangiomas of transgenic mice carrying the entire polyomavirus early region transgene, expressed several isoforms of PECAM-1. The bEND cells exhibited a very similar expression and frequency pattern of PECAM-1 isoforms to those detected in brain tissue (Tables 1 and 2). The isoform that lacked exons 14 and 15 (Δ14&15) was also most frequently detected in these cell lines (Table 2). However, a few clones representing only a single PECAM-1 isoform were detected in normal mouse brain endothelial cells in early or late passage isolates (Table 2). This is consistent with the lack of detection of PECAM-1 mRNA in Northern blots

TABLE 1. Distribution of PECAM-1 Isoforms in Mouse Tissues

PECAM-1 isoforms	Full[a]	Δ12	Δ14	Δ15	Δ12&14	Δ12&15	Δ14&15	Δ12, 14&15
Brain (18)	17	ND	11	22	6	6	33	6
Kidney (15)	27	ND	7	13	ND	7	40	7
Lung (19)	16	ND	5	11	ND	11	53	5
Heart (17)	18	6	6	29	ND	18	18	6
Liver (11)	18	ND	9	18	ND	ND	55	ND

Isoforms of PECAM-1 were identified by cloning and sequencing RT-PCR products from total RNA isolated from various mouse tissues as described in the Methods section. The numbers in parentheses indicate the total number of PECAM-1 clones examined.
ND, not detected.
[a]The number indicates the frequency in percent at which each isoform was detected.

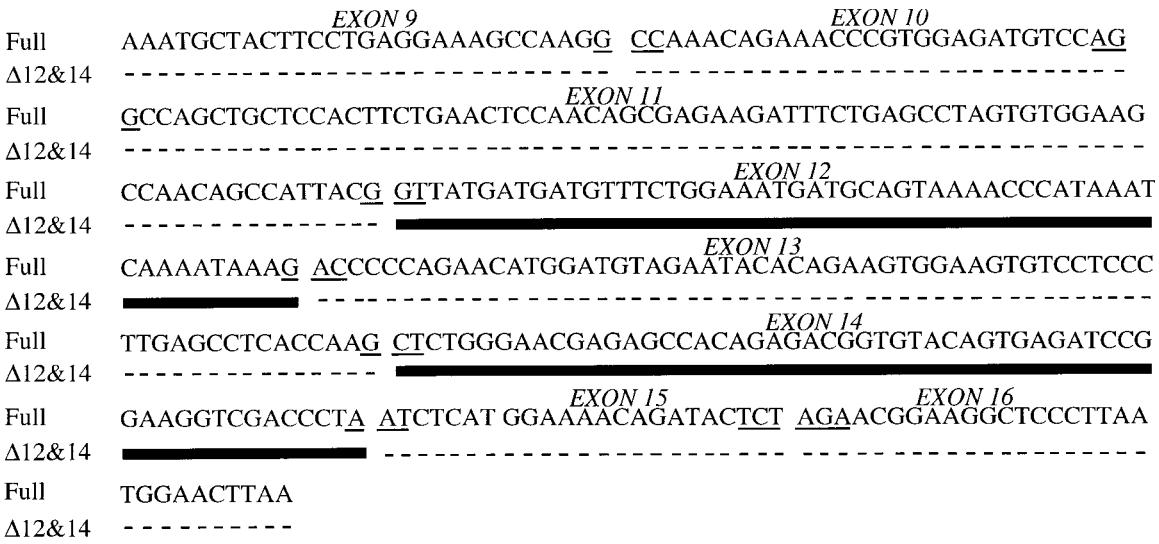

Fig. 3. The cDNA sequence of the cytoplasmic domain of the novel PECAM-1 isoform. The nucleotide sequence encoding the cytoplasmic domain of the isoform lacking exons 12 and 14 (Δ12&14) is compared to the sequence for full-length PECAM-1 isoform. Identical residues are indicated by hyphens (-) and the solid bars indicate the deleted sequences. The predicted exon sequences are separated by a space as recently indicated by Yan et al., 1995.

TABLE 2. Distribution of PECAM-1 Isoforms in Cultured Mouse Endothelial Cells

PECAM-1 isoforms	Full[a]	Δ12	Δ14	Δ15	Δ12&14	Δ12&15	Δ14&15	Δ12, 14&15
bEND (17)	18	6	18	12	ND	6	41	ND
PY4-1 (20)	5	ND	10	20	ND	10	45	10
MBECp5 (6)	ND	ND	ND	100	ND	ND	ND	ND
MBECp22 (2)	ND	ND	ND	ND	ND	ND	100	ND

Isoforms of PECAM-1 were identified by cloning and sequencing the RT/PCR products from mRNA isolated from various mouse endothelial cell lines as described in methods. The numbers in parentheses indicate the total number of PECAM-1 clones examined.
ND, not detected.
[a]The number indicates the frequency in percent at which each isoform was detected.

prepared from normal mouse brain endothelial cells, from both early and late passages (Fig. 1).

Development of Antibodies to PECAM-1 Cytoplasmic Domains and Identification of Various Isoforms

We have demonstrated that multiple PECAM-1 isoforms are present in various tissues as well as endothelial cells in culture. However, it is not known whether the mRNAs from various isoforms are translated. A major limitation to these studies is the lack of antibodies that can discriminate among these isoforms. All of the PECAM-1 antibodies available to date have been made to the extracellular domain of PECAM-1 that is shared by all isoforms. Therefore, we wished to develop antibodies to PECAM-1 cytoplasmic domains that may

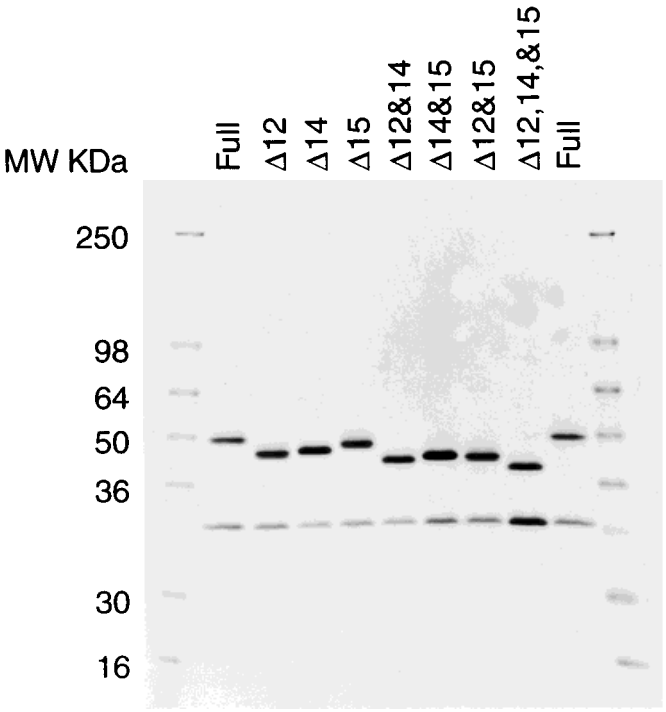

Fig. 4. Expression of cytoplasmic domains of PECAM-1 isoforms as GST-fusion. The cDNAs encoding PECAM-1 cytoplasmic domains were generated by RT/PCR and cloned in frame into the pGEX-2T expression vector. Recombinant plasmids were transformed into DH5α and the recombinant fusion proteins were purified as described in the Experimental Procedures section. Beads suspension corresponding to equal amounts of protein were boiled in SDS-sample buffer and analyzed by SDS-PAGE and Coomassie staining.

allow detection of specific isoforms. To this end, we have expressed the cytoplasmic domains of all eight PECAM-1 isoforms as GST-fusion proteins. These have provided a source of antigen for antibody production and for screening of isoform-specific antibodies. The recombinant fusion proteins were all soluble and were purified in a single step on glutathione agarose (Fig. 4). We initially used the isoforms that corresponded to the longest (full) and the shortest (Δ12, 14&15) cytoplasmic domains for antisera production. The antiserum raised against both the full length and the Δ12, 14&15 cytoplasmic domains reacted with the GST-cytoplasmic domains from all the PECAM-1 isoforms in Western blots (not shown). That is, these antibodies did not discriminate among any of the PECAM-1 isoforms. Thus, sufficient antigenic epitopes are conserved in these two isoforms. We also raised antisera to a synthetic peptide containing the amino acid sequence of murine PECAM-1 exon 14 . The peptide was synthesized with six additional histidines at its N terminus to provide an affinity tag for interaction with Ni-NTA matrix. The peptide was bound to the affinity matrix and was directly used as an immunogen for antibody production, in both chickens and rabbits, as recently described (Sheibani and Frazier, 1998a). The exon 14 peptide antibody reacted only with GST-cytoplasmic domains from PECAM-1 isoforms that contained exon 14, and not with Δ14, Δ12&14, Δ14&15, and Δ12,14&15 isoforms (Sheibani and Frazier, 1998a).

We next determined whether this antibody can differentially react with different isoforms of PECAM-1 expressed in endothelial cells. Figure 5 illustrates the Western blot of cell lysates prepared from bEND and Py4–1 cells under reduced and nonreduced conditions. The left panel was blotted with a rabbit antibody against the extracellular domain of PECAM-1 revealing a typical staining pattern for PECAM-1, that is, two major bands one at about 130 kDa and the other at about 110–120 kDa. The 130-kDa band corresponds to the full-length PECAM-1. The identity of the smaller band has been the subject of debate. However, when the same blot is probed with rabbit antibody against exon 14 (Fig.5, right panel), we only observed the staining of the 130 kDa band but not the smaller band. Thus, the smaller band contains PECAM-1 isoform(s) that lack exon 14. To our knowledge this is the first evidence for a protein product of an alternatively spliced mRNA isoform of PECAM-1. This observation is further supported by our RT-PCR data, which indicated that the PECAM-1 isoform that lacks exons 14 and 15 (Δ14&15) is the most prominent mRNA isoform in most mouse tissues and endothelial cells.

The exon 14 antibody also stained blood vessels in tissues. Figure 6 shows staining of the blood vessels in sections prepared from the rat eye. This antibody stained both the small and the large blood vessels near the optic nerve and the sclera (Fig. 6A, arrows). The blood vessels of retina are difficult to see in this section and at this level of magnification. Figure 6B shows a cross section of rat retina stained with the exon 14 antibody. A clear staining of the retinal microvessles was observed. These data suggest the coding sequence of mouse and rat PECAM-1 exon 14 must be highly conserved and share similar epitopes that cross-react with our rabbit anti-murine PECAM-1 exon 14 antibody. This antibody is therefore suitable for staining

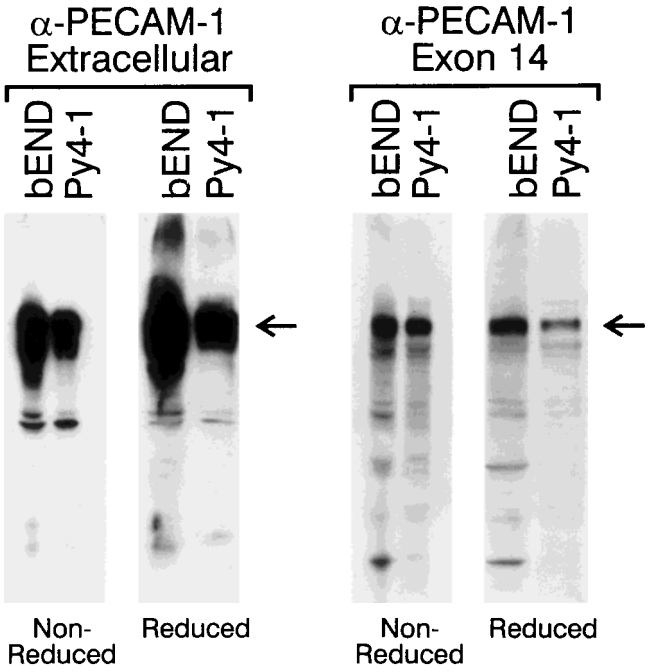

Fig. 5. Detection of PECAM-1 isoforms in mouse endothelial cells. Total cell lysates were prepared as described in Experimental procedures and analyzed by SDS-PAGE under reduced and nonreduced conditions. Proteins were transferred to nitrocellulose membrane and blotted with the rabbit polyclonal antibody to extracellular domain of PECAM-1 **(left panel)** or antibody to exon 14 peptide **(right panel)**. Please note the absence of the shorter PECAM-1 band in blot probed with antibody to exon 14 peptide, which does not recognize isoforms that lack exon 14 sequences.

frozen sections to examine the tissue distribution of some PECAM-1 isoforms in other species.

Expression of PECAM-1 Isoforms in Embryonic an Postnatal Tissues

We next determined whether different isoforms of PECAM-1 were expressed in kidneys from embryonic and postnatal mice. The kidney begins to form in the mouse on embryonic day 11 (E11), and nephrons continue to form following birth (Saxen and Sariola, 1987). By postnatal day 20 (P20) renal maturation is complete (Koseki et al., 1992). Expression of PECAM-1 isoforms was examined in lysates prepared from kidneys from E15 to P20. Figure 7A shows Western blots of these lysates incubated with an antibody to the extracellular domain (left panel) or to exon 14 peptide (right panel) of murine PECAM-1. At E15, the major 130 kDa band was detected; however, with maturation the lower molecular weight PECAM-1 became expressed and by P20 it was the predominant band detected (Fig. 7A, left panel). Thus, not only different isoforms of PECAM-1 are expressed in the kidney, but their expression is developmentally regulated. The exon 14 antibody, which reacts only with cytoplasmic domain of isoforms that contain exon 14, failed to detect the lower molecular weight band in kidney lysates (Fig. 7A, right panel). Figure 7B shows similar Western blots performed with lysates prepared from heart and liver at different stages of development. We only detected the 130-kDa band in these lysates. When these membranes were blotted with antibodies to β-catenin or α-catenin, equal amounts of proteins were observed in all lanes without any sign of degradation with maturation ruling out any general proteolysis (not shown).

DISCUSSION

PECAM-1 is an endothelial cell adhesion molecule that plays an important role in endothelial cell–cell interactions and morphogenesis (DeLisser et al., 1997b; Sheibani et al., 1997; Sheibani and Frazier 1998b). The PECAM-1 mRNA in the mouse embryo undergoes alternative splicing, generating several isoforms that differ in the length of their cytoplamic domains (Yan et al., 1995). These include the full length, Δ12, Δ14, Δ15, Δ12&15, Δ14&15, and Δ12,14&15 isoforms. As yet, the functional significance of these isoforms is poorly defined. The adhesion characteristics of PECAM-1 appear to be modulated by the presence or absence of specific cytoplasmic exons. The lack of exon 14 in PECAM-1 isoforms has been shown to favor homophilic interactions, while those containing the exon 14 participate in heterophilic interactions when expressed in L cells (Yan et al., 1995; Sun et al., 1996). In addition, phosphorylation of the tyrosine residue in exon 14 may also result in homophilic PECAM-1 interaction in L cells (Famiglietti et al., 1997). To better understand the role of PECAM-1 in endothelial cell–cell interactions and morphogenesis, we examined the expression of PECAM-1 isoforms in mouse tissue and endothelial cells. All of the tissues we examined expressed PECAM-1, although at vastly different levels, correlating in general with the vascular density. For example, PECAM-1 is highly expressed in lung, heart, and kidney with lower expression in brain and liver. The transformed mouse endothelial cell lines expressed very high levels of PECAM-1 mRNA, much higher than those reported for normal cells (Sheibani and Frazier, 1998b).

The RT-PCR analysis of RNA isolated from various mouse tissue and endothelial cells suggested expression of several PECAM-1 isoforms (Fig. 2). This was confirmed by cloning and sequencing the cDNAs generated by RT-PCR. We detected all the isoforms reported to be expressed in the early mouse embryo (E12), in postnatal mouse tissues (P21) (Yan et al., 1995). In addition, we identified a new PECAM-1 isoform (Δ12&14) expressed in the brain of postnatal mouse. The expression of this isoform was not reported in the early mouse embryo (Yan et al., 1995). Thus, most of the PECAM-1 isoforms are expressed early in the embryo and continue to be expressed postnatally. However, there may be some tissue-specific alternatively spliced isoforms, such as the Δ12&14 isoform in brain, that are expressed at later stages of development. Identification

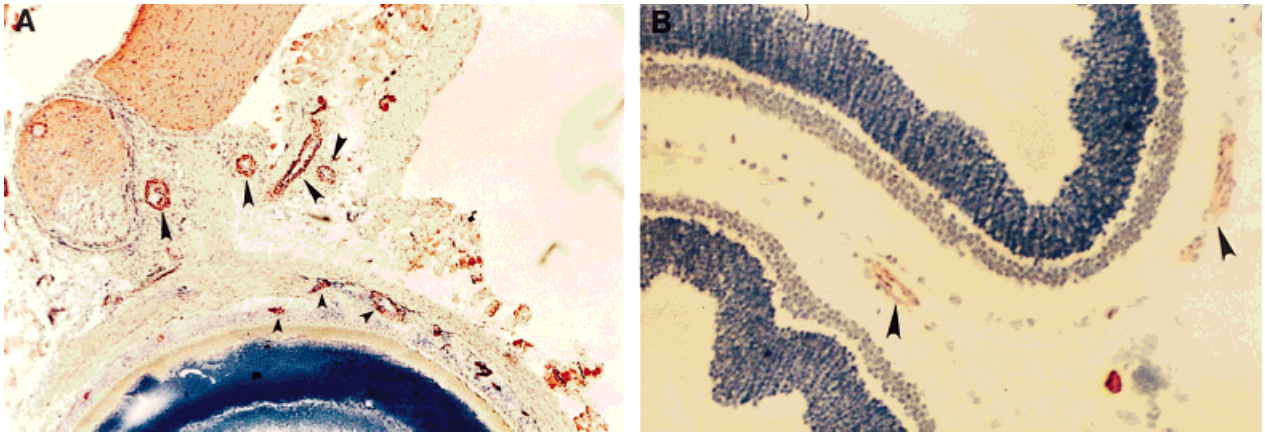

Fig. 6. Staining of the small and large blood vessels in the sections prepared from rat eye. Photomicrograph of sections originating from adult rat eye stained with anti-PECAM-1 exon 14 antibody. **A:** Cross section from the back of the rat eye (10× objective). **B:** Cross section of the rat retina (40× objective). Results are representative of more than five sets of eye. Please note staining of the large blood vessels near the optic nerve and the smaller vessels in the sclera and the retina (arrows).

of the isoforms that are translated and their tissue localization awaits development of antibodies which specifically react with these isoforms.

To begin developing such antibodies we expressed the cytoplasmic domains of all PECAM-1 isoforms as GST-fusions. We initially utilized the longest (full) and the shortest (Δ12,14&15) GST-PECAM-1 cytoplasmic domain fusion proteins, as well as the exon 14 synthetic peptide, for antibody production. The antisera prepared against both of these isoforms detected cytoplasmic domains of all the PECAM-1 isoforms. However, the antibody prepared against the exon 14 peptide only reacted with the cytoplasmic domain of isoforms that contained exon 14, thus discriminating among at least half of the known PECAM-1 isoforms (Sheibani and Frazier, 1998a). This antibody also provided the first evidence that protein isoforms of PECAM-1 other than full length are expressed in endothelial cells identifying the 110–120 kDa PECAM-1 as lacking exon 14 (Fig. 5). This finding agrees with the predominant expression of PECAM-1 isoforms that lack exon 14 in most cell lines and tissues (Table 1 and 2).

The expression of PECAM-1 isoforms was also examined in vascular beds of different tissues. We observed that different isoforms of PECAM-1 are expressed in the kidney in a developmentally regulated fashion (Fig. 7A). The isoform(s) that contain exon 14 are expressed early and are subsequently replaced by isoform(s) that lack exon 14 at later stages of development. By contrast, the isoform(s) that lack exon 14 were not detected in lysates prepared from heart and liver at either early or late stages of development (Fig. 7B). However, these data do not rule out the potential expression of other PECAM-1 isoforms that are developmentally regulated in these tissues. Therefore, differential expression of PECAM-1 isoforms may provide a mechanism by which the adhesive function of PECAM-1 can be modulated during development. Generation of isoform specific antibodies that discriminate among individual PECAM-1 isoforms will be essential in understanding the adhesive function of these isoforms in the same or different vascular beds.

Normal mouse brain endothelial cells expressed little or no PECAM-1 mRNA both in early or late passage cells (Sheibani et al., 1997) (Fig. 1). The suppression of PECAM-1 expression in normal mouse brain endothelial cells may be attributed to, or even responsible for, the rapid changes observed in the endothelial cell complex tight junctions in culture affecting the integrity of the blood-brain barrier (Risau, 1995). The role of PECAM-1 in formation and/or maintenance of complex tight junctions and its contribution to the development of the blood-brain barrier are not known. However, cytoskeletal changes which are perhaps mediated via signal transduction pathways emanating from PECAM-1 engagement may be involved (Newman et al., 1992; Risau, 1995; Ferrero et al., 1996; Kalra et al., 1996; T.T. Lu et al., 1996) and potentially modulated by expression of different PECAM-1 isoforms during development. Identification of the novel Δ12&14 PECAM-1 isoform in brain, which was not detected in whole embryo, provides further evidence that expression of PECAM-1 isoforms is developmentally regulated and may provide different adhesive properties during development.

The signaling events resulting from PECAM-1 engagement are poorly defined. The cell surface redistribution of PECAM-1 is observed concomitant with changes in the cellular cytoskeleton (Newman et al., 1992; Ferrero et al., 1996; Kalra et al., 1996). Thrombin activation of platelets results in reorganization of the cytoskeleton, serine phosphorylation of PECAM-1, and its association with the cellular cytoskeleton (Newman et al., 1992). In addition, incubation of endothelial cells with inflammatory cytokines or under hypoxic conditions results in surface redistribution of PECAM-1 and

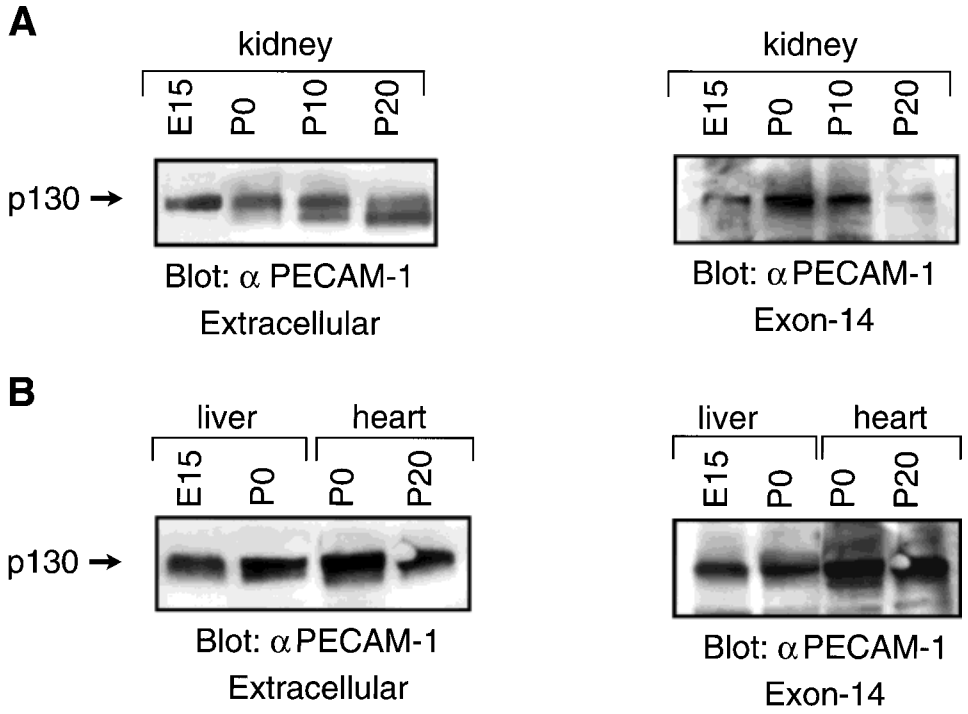

Fig. 7. Detection of PECAM-1 isoforms in embryonic and postnatal tissues. Lysates were prepared from kidney, heart, and liver at designated time points as described in experimental procedures. Using SDS-PAGE, 20 μg protein from each tissue was analyzed under reducing conditions. Proteins were transferred to nitrocellulose membrane and blotted with the rabbit polyclonal antibody to extracellular domain **(left panels)** or the antibody to exon 14 peptide **(right panels)** of PECAM-1. **A:** Blots of lysates prepared from kidneys. **B:** Blots of lysates prepared from hearts and livers. Note the presence of the major, lower molecular weight PECAM-1 only in the kidney lysates detected by the antibody to the extracellular domain but not to the exon 14 of PECAM-1.

disruption of cytoskeleton connections concomitant with activation of protein kinase C and increased phosphorylation of PECAM-1 (Ferrero et al., 1996; Kalra et al., 1996). Thus, phosphorylation of PECAM-1 on serine-threonine residues may regulate its cell surface redistribution and association with the cellular cytoskeleton. The tyrosine phosphorylation of PECAM-1 has been demonstrated in resting neutrophils (DeLisser et al., 1997a), while in endothelial cells PECAM-1 becomes phosphorylated on tyrosine residues immediately after plating the cells (Lu et al., 1996). However, the engagement of integrins, such as β1, results in cell spreading concomitant with rapid dephosphorylation of tyrosine residues in PECAM-1. Therefore, phosphorylation of the PECAM-1 cytoplasmic domain at different sites could modulate its surface distribution, cytoskeletal association, and adhesive activity. The presence of tyrosine residues in each of the cytoplasmic exons along with reports of tyrosine phosphorylation suggest potential exon-specific interactions with SH2-containing protein kinases, phosphatases, or other signaling molecules (Jackson et al., 1997a, 1997b; T.L. Lu et al., 1997; Masuda et al., 1997; Sagawa et al., 1997b; Cao et al., 1998). Characterization of alternatively spliced PECAM-1 isoforms and identification of intracellular proteins which specifically interact with these isoforms will allow us to elucidate the role of PECAM-1 in hemostasis, inflammation, and angiogenesis.

EXPERIMENTAL PROCEDURES

Cell Lines

The bEND cells are mouse brain endothelial cells (MBEC) that are transformed by polyoma middle T and cultured as described recently (Sheibani and Frazier, 1995). The Py4–1 cells (provided by Dr. V. L. Bautch, University of North Carolina, Chapel Hill, NC) are transformed endothelial cells established from ear and tail hemangiomas of transgenic mouse carrying the entire polyomavirus early region transgene and cultured as described (Dubois et al., 1991). The normal mouse brain endothelial cells were prepared and maintained as recently described (Sheibani and Frazier, 1995). Two different isolates of normal mouse brain endothelial cells were utilized, one at early passage (P5) and one at later passage (P22). HUVEC, human umbilical vein endothelial cells, and HMVEC, human dermal microvascular endothelial cells as well as the SV40 large T-transformed HMVEC (THMVEC) (provided by Dr. L. Cornelus, Washington University, St. Louis, MO) were maintained as described (Swerlik et al., 1991; Ades et al., 1992). Poly A^+ RNA was isolated from

various cell lines as described (Sheibani and Frazier, 1995).

Cloning and Isolation of Alternatively Spliced PECAM-1 Isoforms

CD-1 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and were bred and maintained at Washington University animal facility. Total RNA was isolated from various tissues using the Ultra Spec RNA isolation kit as recommended by the supplier (Biotecx Laboratories, Inc., Houston, TX). The RNA from various tissues or cell lines was utilized as template for reverse transcription in a reaction mixture of random primers and reverse transcriptase utilizing the Superscript preamplification system as described by the manufacturer (Gibco BRL, Gaithersburg, MD). To amplify the cytoplasmic domains of all possible PECAM-1 isoforms from this cDNA the following primers were used: a sense primer (5′-cgggatcc^{1941}AGGAA-AGCCAA GGCCAAA^{1958}-3′) spanning the border of exons 9 and 10 within the intracellular domain and an antisense primer (5′-cggaattc^{2288}TTGACTGTCTTAAGT-TCC^{2274}-3′) spanning the border of exon 16 and 3′-untranslated region. The primers carry a BamHI and an EcoRI recognition sequence (lowercase letters) to facilitate subsequent cloning. The polymerase chain reaction product was digested with BamHI and EcoRI, ligated into a pGEX vector (Pharmacia), and transformed into *E. coli*. Bacteria colonies were screened by BamHI and EcoRI digestion of minipreps and those with inserts were sequenced.

Northern Analysis

Approximately 5 µg of poly A^+ or 30 µg of total RNA were fractionated on a 1.2% agarose-formaldehyde gel, transferred to zeta-probe membrane (Bio-Rad), prehybridized, and hybridized to random primer ^{32}P -labeled full length cDNA probes for human PECAM-1 (Dr. P.J. Newman, The Blood Research Institute, Milwaukee, WI) or murine PECAM-1 (Dr. S.M. Albelda, University of Pennsylvania Medical School Center, Philadelphia, PA). The blots were also probed with a cDNA for GAPDH to control for loading. Please note that the exposure time varied for each probe.

Expression and Purification of GST- PECAM-1 Cytoplasmic Domain Fusion Proteins

The cytoplasmic domains corresponding to various PECAM-1 isoforms were generated by RT-PCR and directly cloned into the pGEX-2T prokaryotic expression vector (Pharmacia Biotech Inc., Piscataway, NJ). The reading frame of all constructs was confirmed by DNA sequencing. The recombinant plasmids were transformed into the *E. coli* DH5α and several ampicillin-resistant colonies were screened for expression. The overnight cultures were diluted 1:10 in LB medium with ampicillin, grown for 3 hours, and expression of fusion protein was induced by addition of 1 mM IPTG and growing for additional 2 hours. Cells were pelleted, washed with phosphate buffered saline (PBS), resuspended in PBS (1/20 of original volume), and lysed by five cycles of freezing and thawing. Triton X-100 was added to 1%, mixed, and the cell debris were pelleted by centrifugation at 14,000*g* for 10 minutes at 4°C. The supernatant was transferred to a clean tube containing glutathione-Sepharose 4B (Pharmacia Biotech Inc., Piscataway, NJ) and incubated at room temperature for 30 minutes. The contaminating proteins were removed by pelleting the beads and washing them five times with PBS.

Antibody Production

The GST-PECAM-1 fusion proteins were prepared as above and utilized as antigen for antibody production. We utilized the affinity matrix bound GST-fusion proteins as immunogens since we had shown that the matrix bound fusion protein is a better immunogen that the purified fusion protein (Sheibani and Allen-Hoffmann, 1994). Antibodies to the full length or Δ12,14,&15 isoforms were raised in chicken utilizing standard protocols (Aves Labs, Tigard, OR). We also raised an antibody to a peptide that corresponds to the exon 14 amino acid sequence. The peptide was synthesized with six histidine on its N-terminus to provide an affinity tag for binding to Ni-NTA agarose (Qiagen, Chatsworth, CA). The affinity matrix bound peptide was then utilized directly for antibody production, both in chicken and rabbit, as recently described (Sheibani and Frazier, 1998a). The titer of antibodies were monitored by ELISA and their reactivity was confirmed by Western blotting.

Western Blotting

Cell extracts prepared by lysing the cells in 20 mM Tric-HCl, 2 mM EDTA (10^6 cells/0.1 ml) and mixing with sufficient quantities of the 6X SDS sample buffer with and without β-mercaptoethanol. The GST-PECAM-1 cytoplasmic domains were purified as described above and aliquots of matrix bound fusion protein corresponding to equal amounts of proteins were boiled in the sample buffer. The protein samples were analyzed by SDS-PAGE (12% Tris-glycine gel, Novex, San Diego, CA), transferred to nitrocellulose membrane, and blotted as described previously (Sheibani and Frazier, 1995).

Immunohistochemistry

Tissues were surgically removed from rat, placed in OCT (VWR Scientific, St. Louis, MO) and rapidly frozen. Sections of 7 µm each were placed on polylysine-coated slides (Sigma, St. Louis, MO). The sections were fixed in cold acetone, washed in PBS and incubated in PBS blocking buffer (PBS containing 1% bovine albumin, 0.3% Triton X-100 and 0.2% skim milk powder) for 15 minutes. The sections were then incubated with the rabbit polyclonal antibody against PECAM-1 exon 14 (1:500) overnight at 4°C. The sections were processed

utilizing a Histostain-SP kit for rabbit primary antibodies and AEC development (Zymed, South San Francisco, CA).

Preparation of Tissue Lysates

To examine expression of PECAM-1 isoforms in tissues from embryonic and postnatal mice, FVB/N mice (Charles River, Wilmington, MA) were interbred. Embryos were removed from anesthetized pregnant female mice on day 15 of pregnancy. Metanephric kidneys, heart, and liver were surgically dissected out from embryos or postnatal mice at indicated times. Tissues were homogenized and sonicated in a modified RIPA buffer containing 142.5 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes pH 7.4, 1% NP-40, 2 mM Na_3OV_4, 2 mM NaF, and Complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) and rocked for 30 minutes at 4°C. The lysates were centrifuged for 15 minutes at 4°C to remove debris and protein concentrations were determined utilizing a Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). 20 µg of total protein lysate was electrophoresed in a 4–20% polyacrylamide gel, transferred to nitrocellulose and blotted as described above.

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