Role of vascular endothelial-cadherin in vascular morphogenesis

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SUMMARY

Vascular endothelial (VE)-cadherin is an adhesive transmembrane protein specifically expressed at interendothelial junctions. Its extracellular domain exhibits Ca²⁺-dependent homophilic reactivity, promoting cell-cell recognition. Mice deficient in VE-cadherin die at mid-gestation resulting from severe vascular defects. At the early phases of vascular development (E8.5) of VE-cadherin-deficient embryos, in situ differentiation of endothelial cells was delayed although their differentiation program appeared normal. Vascularization was defective in the anterior part of the embryo, while dorsal aortae and vitelline and umbilical arteries formed normally in the caudal part. At E9.25, organization of endothelial cells into large vessels was incomplete and angiogenesis was impaired in mutant embryos. Defects were more severe in extraembryonic vasculature. Blood islands of the yolk sac and clusters of angioblasts in allantois failed to establish a capillary plexus and remained isolated. This was not due to defective cell-cell recognition as endothelial cells formed intercellular junctions, as shown by electron microscopy. These data indicate that VE-cadherin is dispensable for endothelial homophilic adhesion but is required for vascular morphogenesis.

Key words: Vasculogenesis, Angiogenesis, Cadherin, Gene targeting, Mouse

INTRODUCTION

During embryogenesis, the cardiovascular entity is the first system to become functional and its integrity is required for embryo development and survival (Risau, 1995). Endothelial cells differentiate from mesodermic progenitors and interconnect to form a primary vascular plexus. This process is called vasculogenesis and only occurs in visceral derivatives and somites (Pardanaud et al., 1996). The primary plexus is extended by angiogenesis, which involves sprouting of new vessels and various steps of remodelling, allowing the formation of a complex vascular network with vessels of different sizes. Within the embryo and allantois, the emergence of angioblasts (i.e. endothelial progenitors) leads to the formation of the main vascular trunks: primitive endocardium, dorsal aortae, cardinal veins, vitello-embryonic and umbilical vessels (Sabin, 1917; Hirako and Hiruma, 1981). Later, other vessels such as the intersomitic vessels and the capillary sprouts of the neural epithelium derive from the primitive vasculature. In the yolk sac, endothelial cells originate from hemangioblastic progenitors differentiating into blood islands, where hematopoietic cells are surrounded by an endothelial layer (Jolly, 1940; Choi et al., 1998). Vasculogenesis further proceeds by fusion of the blood islands that form a meshwork of blood-filled capillaries. When embryo and yolk sac vasculatures interconnect, the vitelline vascular plexus develops into a highly branched vascular tree (i.e. angiogenesis) in the entire yolk sac, providing a respiratory system to the early embryo.

The description of these morphological changes has been further documented by the analysis of early endothelial markers. The sequential expression of endothelial-specific tyrosine kinase receptors defines successive differentiation steps (for a review on these receptors and their ligands, see Mustonen and Alitalo, 1995). Flk-1, one of the two receptors of vascular endothelial growth factor (VEGF), is the earliest known murine protein marking the commitment of the mesodermic cells to the endothelial lineage. It is followed by Tie-2 (the receptor of angiopoietin-1 and -2) and Flt-1 (the other VEGF receptor), and later by Tie-1 (Dumont et al., 1995). Targeted inactivation of these receptors and their ligands in transgenic mice leads to embryonic lethality due to differential defects in vasculogenesis or angiogenesis (Shalaby et al., 1995; Carmeliet et al., 1996a; Ferrara et al., 1996; Sato et al., 1995; Puri et al., 1995; Fong et al., 1995). Most of these knockouts revealed critical steps in vascular morphogenesis, especially in the establishment of vessels connections, emphasizing the role of cell-cell recognition.

In this paper, we evaluated the biological activity of vascular endothelial-cadherin (VE-cadherin) by targeted disruption in transgenic mice. VE-cadherin belongs to the cadherin family of adhesive transmembrane proteins promoting homotypic cell-cell interaction via their extracellular domain (Lampugnani et al., 1992). The cytoplasmic domain of cadherins is associated with
molecules called catenins including α-catenin, β-catenin and plakoglobin, which mediate the linkage of the cadherin cluster with the actin cytoskeleton (for a review, see Yap et al., 1997). VE-cadherin is exclusively and constitutively expressed at interendothelial junctions (Lampugnani et al., 1992). In mouse embryos, VE-cadherin transcripts have been detected as early as embryonic day 7.5 (E7.5) in the mesodermic aggregates from which blood islands originate (Breier et al., 1996). Evidence of VE-cadherin hemangioblastic expression was further substantiated by immunoreactivity of purified progenitors (Nishikawa et al., 1998). Later on, VE-cadherin was detected in all developing vessels as well as in the adult vasculature (Breier et al., 1996). Functionally, VE-cadherin is able to promote the assembly of the junctional complex and to develop homotypic adhesive activity (Lampugnani et al., 1995; Navarro et al., 1995). VE-cadherin participates in control of endothelial permeability to solutes and neutrophils (Lampugnani et al., 1992; DelMaschio et al., 1996; Allport et al., 1997). Furthermore, VE-cadherin expression has been shown to be downregulated in the disorganized cells of angiosarcomas, suggesting that this molecule may be involved in vascular morphogenesis (Martin-Padura et al., 1995). Role of catenins in cell sorting and morphogenesis had been previously reported for E-cadherin, N-cadherin and R-cadherin (Takeichi, 1995).

In previous work, we showed that VE-cadherin disruption impaired vasculogenesis in embryonic stem (ES) cells-derived embryoid bodies (Vittet et al., 1997). Although endothelial cells differentiated normally from VE-cadherin−/− ES cells, no vascular plexus formation could be observed within bodies. We report now that VE-cadherin homozygous null-mutation in transgenic mice is embryonic lethal due to severe vasculogenic defects in the yolk sac and embryo.

MATERIALS AND METHODS

Generation of mutant mice and progeny genotyping

The targeted vector was linearized by NotI, capped with hairpin-shaped oligonucleotides (Vittet et al., 1997) and electroporated into R1 ES cells, derived from 129Sv strain (Nagy et al., 1993). Transfectants were plated into R1 ES cells, derived from 129Sv hairpin-shaped oligonucleotides (Vittet et al., 1997) and mitomycin-treated neomycin-resistant primary strain (Nagy et al., 1993). Transfectants were plated on embryo.

We report now that VE-cadherin homozygous null-mutation in transgenic mice is embryonic lethal due to severe vasculogenic defects in the yolk sac and embryo.

Generation of mutant mice and progeny genotyping

The targeted vector was linearized by NotI, capped with hairpin-shaped oligonucleotides (Vittet et al., 1997) and electroporated into R1 ES cells, derived from 129Sv strain (Nagy et al., 1993). Transfectants were plated on mitomycin-treated neomycin-resistant primary embryonic fibroblasts as feeder layer and cultured in Dulbecco’s modified Eagle’s medium (GibcoBRL), supplemented with 15% fetal calf serum (Seromed) and 500 U/ml leukemia inhibitory factor (Esgro, GibcoBRL). 48 hours after transfection, 350 μg/ml G-418 (Sigma) were added to the medium and were kept until aggregation with morulas. Selection with 2 μM gancyclovir (Syntex) was performed from day 4 to day 8 post-electroporation. Resistant colonies were picked at day 10 and expanded for freezing and DNA analysis. Clones were screened for homologous recombination by Southern blot analysis using NotI digests and a 3′-external probe (Vittet et al., 1997), showing wild-type (WT) and mutated (Mut) loci. Western blot analysis of adult lung and E9.5 yolk sac extracts of offspring (Vittet et al., 1997) shows comparable protein amounts in the three yolk sacs. (C) External appearance of VE-cadherin-deficient (−/−) and wild-type (+/+) embryos at E10.5. At this age, the mutant embryo (still enveloped here by amniotic membrane) shows growth retardation, anemia, pericardial hypertrophy and incomplete folding. Scale bars, 400 μm.

Fig. 1. Targeted disruption of VE-cadherin gene. (A) Southern blot analysis of targeted ES clones (C6 and D2) and offspring using NcoI digests and a 3′-external probe (Vittet et al., 1997), showing wild-type (WT) and mutated (Mut) loci. (B) Western blot analysis of adult lung and E9.5 yolk sac extracts of offspring from heterozygous intercrosses. Presence of VE-cadherin was revealed with two different antibodies: 19E6 (50 μg of protein extracts per lane) and 11D4-1 (5 μg or 25 μg of lung or yolk sac extracts, respectively). Band at 100 kDa is a degradation product of VE-cadherin (111 kDa). Probing with a specific anti-α-tubulin antibody shows comparable protein amounts in the three yolk sacs. (C) External appearance of VE-cadherin-deficient (−/−) and wild-type (+/+) embryos at E10.5. At this age, the mutant embryo (still enveloped here by amniotic membrane) shows growth retardation, anemia, pericardial hypertrophy and incomplete folding. Scale bars, 400 μm.
Table 1. Genotype analysis of VE-cadherin<sup>+/−</sup> crosses

<table>
<thead>
<tr>
<th>Embryonic age</th>
<th>Number of liters</th>
<th>+/+</th>
<th>+/−</th>
<th>−/−</th>
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<tr>
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<td>2</td>
<td>3</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>E9.5</td>
<td>7</td>
<td>20</td>
<td>33</td>
<td>16*</td>
</tr>
<tr>
<td>E10.5</td>
<td>7</td>
<td>17</td>
<td>33</td>
<td>15*</td>
</tr>
<tr>
<td>E11.5</td>
<td>2</td>
<td>8</td>
<td>8</td>
<td>3‡</td>
</tr>
<tr>
<td>E12.5</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

*Macrosopically abnormal embryos.
‡Asystolic embryos.

GAGGCCTCAGACAG-3′ (5′) and 5′-TCTTCTTCATCGATGTCATT-3′ (3′), PCR products were electrophoresed, blotted and hybridized with 32P-labelled internal probe: 5′-GTGTTAGGGCCCTTGACTGCTGGTCCATTGAGA-3′ (3′), 5′-GTGTTAGGGCCCTTGACTGCTGGTCCATTGAGA-3′ (probe); for Tie-1, 5′-TCTTCTTCATCGATGTCATT-3′ (3′), 5′-ACACACACATCGGCTCTCAT-3′ (3′), 5′-CCACAGCACACCCACACACACAG-3′ (probe); for Tie-2, 5′-CTTCCTTACCTGCTACTTGA-3′ (5′), 5′-CCACACACACCCACACACACAG-3′ (probe); for Tie-1, 5′-CTTCCTTACCTGCTACTTGA-3′ (3′), 5′-CTTCCTTACCTGCTACTTGA-3′ (probe); for Flk-1, 5′-CTTCCTTACCTGCTACTTGA-3′ (3′), 5′-CTTCCTTACCTGCTACTTGA-3′ (probe); for Tie-1, 5′-CTTCCTTACCTGCTACTTGA-3′ (3′), 5′-CTTCCTTACCTGCTACTTGA-3′ (probe).

Western blot analysis

Lung from adult mice and E9.5 yolk sacs were lysed in 10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP40, 1% Triton X-100, containing a protease inhibitory cocktail (Boehringer Mannheim) using a micro-potter (Kontes). Protein concentration was determined with a Biorad Protein Assay. Samples were analyzed by electrophoresis on 7.5% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membrane. VE-cadherin was detected with two different rat monoclonal antibodies directed against mouse VE-cadherin (clone 19E6; a generous gift from E. Dejana and clone 11D4.1, at 1:1000 dilution of a rat monoclonal antibody (a generous gift from Dr Lafanèche).

Reverse transcriptase (RT)-PCR analysis

RT-PCR analyses were performed using exponential amplification conditions (Vittet et al., 1996). Oligonucleotides used as primers and probes for VE-cadherin, platelet endothelial cell adhesion molecule (PECAM)-1 and Flk-1 transcripts were previously described (Vittet et al., 1996). Primers for amplification of other transcripts and internal controls (Vittet et al., 1996). Primers for amplification of other transcripts and internal controls (Vittet et al., 1996). Primers for amplification of other transcripts and internal controls (Vittet et al., 1996). Primers for amplification of other transcripts and internal controls (Vittet et al., 1996).

Electron microscopy

For electron microscopy, yolk sacs were fixed 1 hour in 2.5% glutaraldehyde/0.1 M sodium cacodylate at pH 7.2, rinsed three time in 0.1 M sodium cacodylate, then postfixed in 1% osmium tetroxide/0.1 M sodium cacodylate, dehydrated in graded ethanol, embedded in Epon and sectioned at 90 nm. Examination was performed on a Jeol 1200 ExII microscope at 80 kV.

Analysis of yolk sac hematopoiesis

Yolk sacs from E9.5 embryos were dissected and treated with 0.5% collagenase/0.1% DNase I/20% fetal calf serum in Dulbecco’s modified Eagles medium for 1 hour at 37°C. After incubation, cells were dissociated by gentle flushing and counted. The total number of cells recovered per VE-cadherin<sup>+/−</sup> yolk sac was ~60% of that of the VE-cadherin<sup>+/+</sup>. Cells were plated at a density of 3x10<sup>5</sup> cells/ml in 0.9% methylcellulose/Iscover’s modified Dulbecco’s (GibcoBRL) medium supplemented with 20% fetal calf serum.

Fig. 2. RT-PCR analysis of E9.5 embryos (including allantois) and yolk sacs. Each marker RNA was co-amplified with Hprt transcript to evaluate cDNA variations between lanes. Each amplification was performed in presence (+) or in absence (−) of reverse transcriptase to detect genomic DNA contamination. One can note the loss of VE-cadherin signal in homozygous mice and the absence of βH1 mRNA within VE-cadherin<sup>+/−</sup> embryos, indicating the lack of primary erythrocytes in the embryonic compartment.

Targeted disruption of the VE-cadherin gene

paraformaldehyde/PBS at 4°C and stained with a rat monoclonal anti-mouse antibody (clone ME13.3, Vecchi et al., 1994), as described (Schlaefer et al., 1995). PECAM-1 immunoreactivity was revealed either with an alkaline phosphatase monoclonal anti-alkaline phosphatase procedure (Dako) in Fig. 6, or with an horse radish peroxidase-conjugated antibody (Jackson Laboratories) in Figs 3 and 4. For sections, embryos and yolk sacs were fixed in 1% paraformaldehyde/0.1 M phosphate buffer pH 7.4, for 16 hours at 4°C, rinsed in PBS, dehydrated through an ethanol series, cleared in xylene and paraffin embedded. Sections (7 μm) were used for hematoxylin and cosin staining or for PECAM-1 immunodetection. In this case, sections were treated with 1% trypsin (Sigma)/PBS for 30 minutes to unmask antigen. As secondary antibody, we used a cyanine 3-conjugated antibody (Jackson Laboratories) at 1:1000 dilution.

Analysis of yolk sac hematopoiesis

Yolk sacs from E9.5 embryos were dissected and treated with 0.5% collagenase/0.1% DNase I/20% fetal calf serum in Dulbecco’s modified Eagles medium for 1 hour at 37°C. After incubation, cells were dissociated by gentle flushing and counted. The total number of cells recovered per VE-cadherin<sup>+/−</sup> yolk sac was ~60% of that of the VE-cadherin<sup>+/+</sup>. Cells were plated at a density of 3x10<sup>5</sup> cells/ml in 0.9% methylcellulose/Iscover’s modified Dulbecco’s (GibcoBRL) medium supplemented with 20% fetal calf serum.
containing factors for granulomonocytic-erythroid colonies formation: 50 ng/ml stem cell factor, 25 ng/ml interleukin-3, 25 ng/ml interleukin-6, 2 U/ml erythropoietin, 10 ng/ml granulocytic-colony stimulating factor (Preprotech) (Tronik-LeRoux et al., 1995). Colonies were scored by visual inspection after 7 days in culture.

RESULTS

Generation of mice heterozygous for the VE-cadherin null-mutation

The genomic structure of the mouse VE-cadherin gene was reported previously (Huber et al., 1996). In the targeted vector (already described in Vittet et al., 1997), a neomycin phosphotransferase (neo) expression cassette replaces part of exon 2, removing the initiation codon, the signal peptide, the propeptide and the first nine amino-acids of the mature protein. R1 ES cells were transfected with this construct and subjected to positive-negative selection (see Materials and Methods). Clones targeted at VE-cadherin locus were identified by Southern blot analysis (Fig. 1A). Homologous recombination was further confirmed by hybridization with the neo probe, which failed to detect any rearrangement of the targeted locus or additional sites of integration (data not shown). Two independent clones, C6 and D2, were aggregated to morulae and contributed to the germline of chimeric males. Genotyping of progeny was performed by Southern blot analysis (Fig. 1A) or PCR.

Mice lacking VE-cadherin die at mid-gestation

Heterozygous mice appeared phenotypically normal. Viability and fecundity were similar to the wild-type animals.

No homozygous pup was detected when heterozygous animals were intercrossed. Nevertheless, ratios between heterozygotes and wild types were normal: 114 versus 57 for clone C6, and 70 versus 35 for clone D2, respectively. These results indicate that VE-cadherin mutation is recessive embryonic lethal. To determine when homozygous embryos were dying, embryos were isolated at various stages of gestation (Table 1). Up to E10.5, wild-type, heterozygous and homozygous embryos were present. From E9.5, mutant embryos were clearly abnormal and were dead at E11.5. Both transgenic lines were analyzed in parallel and developed similar phenotypes. Defects included (Fig. 1C): growth retardation, reduced number of somites (by E10.5, somites number of mutant embryos never exceeded 20, as opposed to 35 in the wild type), incomplete turning, distended pericardial cavity, pallor and fragility. The heart beat until E10.5, but contractions were weak and irregular. No blood cell was visible within VE-cadherin–/– embryos. Lack of embryonic β-globin (βH1) transcripts, as shown by RT-PCR analysis (Fig. 2), confirmed that primitive erythrocytes were absent from mutant embryos.

Loss of wild-type VE-cadherin gene expression was established by RT-PCR analysis (Fig. 2). Furthermore, using two different monoclonal antibodies against mouse VE-cadherin (Fig. 1B), no signal could be detected in mutant yolk sacs (although endothelial cells were present: see below), whereas VE-cadherin was highly expressed in lung and yolk sac extracts of wild-type and heterozygous mice (Fig. 1B). Control experiment, using anti-α-tubulin antibody, shows that similar amounts of proteins were loaded in each lane (Fig. 1B). Thus, these data indicate that the targeted VE-cadherin mutation is a null allele.

Differentiation of endothelial cells

To see whether VE-cadherin loss of function interfered with endothelial differentiation, expression of endothelial markers
Targeted disruption of the VE-cadherin gene was evaluated by RT-PCR with transcripts from E9.5 yolk sacs and embryos (including the major part of allantois). Signals obtained for Flk-1, Flt-1, Tie-2, Tie-1 and PECAM-1 (a uniform endothelial marker, although not restricted to the endothelial lineage; DeLisser et al., 1994) were similar for all three genotypes (Fig. 2). This suggests that endothelial differentiation could progress through all successive differentiation stages in intraembryonic and extraembryonic compartments of the mutants.

Vascular defects in mutant embryos

VE-cadherin−/− embryos started to show extensive cell death at E9.5; by E10.5, the entire embryo had deteriorated (data not shown). Therefore, to investigate the causes of defective development of the mutants, we performed macroscopical and histological analyses before E9.5. Embryos were first examined by whole-mount staining with a monoclonal anti-PECAM-1 antibody to show the emerging vasculature (Fig. 3A-C). At E8.5 (8-10 somites), embryonic vascularization was consistently delayed in VE-cadherin−/− embryos: (i) dorsal aortae in the caudal region were visible but appeared incompletely formed (Fig. 3B) in comparison to the wild type (Fig. 3C), (ii) sinus venosus was present but posterior cardinal veins were lacking (only exceptional vascular cords could be seen; Fig. 3A), while, in normal embryos, angioblasts amalgamated to form nearly continuous structures (Fig. 3C) and, most importantly (iii) no staining could ever be detected rostrally to the cardiac region (Fig. 3A,B), which was highly different from the wild-type or heterozygous littermates, for which a vascular network was obvious in the first aortic arch and in the head mesenchyme (Fig. 3C). On sections, endocardium was visible but endothelial cells failed to form a lumen (Fig. 3D), as opposed to the wild type (Fig. 3E). In the caudal part, no major difference could be seen between mutant and wild-type vasculatures except that mutant vessels were empty of blood cells (Fig. 3E,F). It is noteworthy that, in the mutant, vitelline artery was present on the embryonic side (Fig. 3F).

At E9.25, vascular deficiencies of VE-cadherin−/− embryos were even more pronounced (Fig. 4A) and included: the presence of a delicate plexus rather than organized large vessels (Fig. 4B), especially in the mid-trunk region and limited angiogenic extensions such as the intersomitic vessels. Along the vitello-embryonic stalk, endothelial cells assembled into vesicles (Fig. 4C) that did not form a continuous vessel, like in the heterozygous embryo (Fig. 4D). In the caudal region, both dorsal aortae and umbilical arteries started to show abnormal dilation between E9.25 and E9.5 (Fig. 4E). Development of the head microcirculation was partial (Fig. 4A) and PECAM-1 staining of sections revealed that organisation of angiogenic cords into vessels was not fully accomplished (compare Fig. 5A and B).

Extraembryonic compartments of VE-cadherin−/− mutants harboured more severe phenotypes

Angioblasts appeared in VE-cadherin−/− allantois, but instead of forming a dense vascular network, angioblasts aggregated and constituted a collection of islets (Fig. 4A). Furthermore,

### Table 2. Culture of yolk sac hematopoietic progenitors

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>BFU-E</th>
<th>GM</th>
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</tr>
<tr>
<td>−/−</td>
<td>13</td>
<td>4</td>
<td>11</td>
</tr>
</tbody>
</table>

*Yolk sacs from the same litter.

Abbreviations: BFU-E, burst-forming-erythroid; GM, granulomonocytic.

![Fig. 4](image-url) Histological analysis of E9.25 embryos. Genotypes: (A,C,E) VE-cadherin−/−; (B) VE-cadherin+/− or VE-cadherin−/−; (D) VE-cadherin+/−, (F) VE-cadherin+/+. (A,B) Whole-mount PECAM-1 staining. VE-cadherin-deficient embryos and allantois exhibited defective vascularization in both extension and organization (see text). Arrow in A shows endothelial clusters in VE-cadherin−/− allantois. (C-F) Hematoxylin and eosin stainings of embryo sections. (C) Discontinuity of the vitello-embryonic vasculature (arrow). In the caudal part, mutant embryo showed dilation of dorsal aortae and umbilical arteries (E), when compared to the wild type (F). Abbreviations: da, dorsal aorta; ua, umbilical artery; va, vitelline artery. Scale bars, (A,B,E,F) 50 μm; (C,D) 20 μm.
umbilical vessels, although visible in the embryo (Fig. 5C,E), did not penetrate the allantois. Establishment of chorioallantoic junction occurred normally in the mutant and surprisingly, vascular organisation in the placenta was similar to the normal mice (Fig. 5G,H).

Blood islands differentiated normally from yolk sac mesoderm. However, endothelial cells failed to establish a primary vascular plexus, although some blood islands surrounding the placenta fused and formed cisternae instead of the vitelline arteries (Fig. 6A-D). It is noteworthy that blood-filled islands were concentrated in the placental hemisphere whereas blood cells were very few in the embryo-linked hemisphere where endothelial clusters were empty (Fig. 6B), even at the latest stages (E10.5). This feature had been previously reported for normal embryos, before fusion of blood islands (Jolly, 1940). Our study shows that not all the yolk sac has hematopoietic potentialities.

Lack of vessel assembly in VE-cadherin−/− yolk sacs was confirmed by PECAM-1 whole-mount staining (Fig. 6F) (notwithstanding the presence of some angioblastic cords). In contrast, wild-type yolk sacs exhibited at this stage (E9.5) a honeycomb-like network in the process of remodelling (Fig. 6E). In the mutant, blood islands were constituted of blood cells densely packed by a closed endothelial layer (Fig. 5I). To see whether adherens junctions could form in the absence of VE-cadherin, blood islands were examined by electron microscopy. Mutant and wild-type yolk sacs showed similar pattern of electron-dense interendothelial junctions (Fig. 7), indicating that VE-cadherin is not required for endothelial homophilic adhesion. Interestingly, PECAM-1 was normally located at the junction, which could be better seen in intraembryonic vessels (Fig. 5E), thus confirming the establishment of large interendothelial contacts.

Altogether, these data suggest that VE-cadherin is dispensable for endothelial cells sorting and tissue cohesion but is required for vascular branching and morphogenesis.

**Vitelline hematopoiesis is normal in VE-cadherin-deficient mice**

As VE-cadherin is expressed by the hemangioblast, we reasoned that VE-cadherin gene disruption may also interfere with vitelline hematopoiesis. We thus examined the hematopoietic potential of the mutant yolk sac. E9.5 yolk sacs were dissociated by collagenase treatment and cells were cultured in semisolid medium, in the presence of factors for granulomonocytic-erythroid colonies formation (see Materials and Methods). The number of colonies of each type was similar in the mutants and in the wild type (Table 2), which indicates that primary hematopoiesis was not altered by VE-cadherin deficiency.

**DISCUSSION**

In this study, we have shown that VE-cadherin-deficient mice exhibited severe defects in both intraembryonic and extraembryonic vasculatures leading to lethal fetal wasting.

From E9.5, VE-cadherin-deficient embryos showed hypertrophy of pericardial cavity, as already described in other knockout embryos such as those lacking N-cadherin (Radice et al., 1997), Scl/Tal-1 (Shivdasani et al., 1995; Robb et al.,
Targeted disruption of the VE-cadherin gene

(1995), vinculin (Xu et al., 1998) or tissue factor (Bugge et al., 1996), which is likely attributable to yolk sac deficiency. Dilatation of distal aortae and umbilical arteries may also result from impossible flow delivery into both umbilical and vitelline vasculatures. These abnormalities, as well as extensive cell death at this gestation age, are probably due to abnormal extraembryonic vasculature and subsequent hypoxic conditions within embryo. However, at E8.5, at the time when embryonic and yolk sac circulations interconnect in the wild type, VE-cadherin−/− embryos already exhibited defective vascularization, indicating a deficiency in intrinsic embryonic vasculature.

In embryo, in situ differentiation of endothelial cells was delayed, as revealed by absence of PECAM-1 staining in the anterior region. However, the differentiation program was not altered as all the markers tested were expressed. Later, organization into a primitive meshwork occurred almost normally, but coalescence of these capillaries or direct condensation of endothelial cells into large vessels was limited in the anterior and mid-trunk region, whereas dorsal aortae, umbilical and vitelline arteries appeared normal in the caudal part. Sprouting from dorsal aortae (i.e. intersomitic vessels) was also impaired. Altogether, these data support the notion that intraembryonic anomalies are related to both vasculogenesis and angiogenesis.

Phenotype was more drastic in yolk sac and allantois, where no capillary could form whether endothelial cells were associated with hematopoietic cells (in the placental pole of the yolk sac) or appeared alone (in the rest of the yolk sac and in allantois), than within embryos. These results indicate that VE-cadherin plays a critical role in vasculogenesis and more specifically in vascular branching of extraembryonic tissues. As pointed out by Hatzopoulos et al. (1998), distinct gene expression profiles exist in embryonic and extraembryonic vasculogenesis. This diversity may be due to different environments, such as perivascular cells or the surrounding matrix (Risau and Lemmon, 1988), or to independent endothelial lineages with their own specificities.

Closer observation of blood islands showed some angiogenic sprouts, but most of them failed to interconnect and remained isolated. When blood islands fused, they formed round cisternae, rather than a capillary plexus, as opposed to VEGF+/− (Carmeliet et al., 1996a; Ferrara et al., 1996) and tissue factor−/− (Bugge et al., 1996; Carmeliet et al., 1996b) yolk sacs for instance, for which a primitive plexus was visible. This clustering suggests that VE-cadherin participates in control of cytoskeleton organization and formation of elongated structures through intracellular signalling. Cross-talk between cadherins and integrins (transmembrane proteins involved in cell-matrix adhesion) was found to regulate cell mesenchymal properties and invasiveness of epithelial cells through β-catenin signalling pathway (Novak et al., 1998). Furthermore, interaction of cytosolic β-catenin with adenomatous polyposis coli protein (for a review, see Ben-Ze’ev and Geiger, 1998) was shown to regulate epithelial tubulogenesis by promoting formation of long cell extensions, followed by assembly of cells into tubules (Pollack et al., 1997). VE-cadherin deficiency may thus alter endothelial plexus formation by modifying the β-catenin/adenomatous polyposis coli protein regulating pathway. Alternatively, a replacing molecule (see below) may promote stronger cell-cell adhesion (Huttenlocher et al., 1998), thus preventing migration and branching of endothelial cells, or may elicit other signal pathways.

In the yolk sac, blood cells were not spilled between endodermal and mesothelial layers, as would have been the case if endothelial cell-cell contacts were altered, but, on the contrary, they were densely packed by endothelial cells. Indeed, interendothelial junctions could be visualized by PECAM-1 staining and electron microscopy. Hence, other homophilic adhesive proteins may compensate for loss of VE-cadherin and provide sufficient strength to the junction for endothelial cohesion. Within mutant embryos, segregation of angioblasts from mesenchymal cells and their assembly indicate that other molecules allowed endothelial cell-to-cell recognition. Interestingly, N-cadherin, the other major cadherin expressed in human umbilical vein endothelial cells...
(although not distributed at intercellular contacts), was shown to be excluded from the junction by VE-cadherin in a model of transfected Chinese ovary cells (Navarro et al., 1998). The possibility that N-cadherin could replace VE-cadherin at the junction, in VE-cadherin-deficient mice was tested by immunostaining with anti-N-cadherin antibody (13A9; a generous gift from M. Wheelock). Embryo and yolk sac histological sections did not show any reactivity with endothelia of homozygous and wild-type embryos (whereas the hindgut and the yolk sac endoderm were highly labelled; data not shown), suggesting that N-cadherin was not the molecule supporting endothelial cell-cell adhesion. These data confirm previous observations by Radice et al. (1997).

Other homophilic adhesive molecules localized at endothelial junctions, such as PECAM-1 (Fawcett et al., 1995) or a recently identified proto-cadherin (i.e. a cadherin without catenin-binding domain) called vascular endothelial-cadherin-2 (Telo’ et al., 1998), are potential candidates. It is possible that these proteins act as docking structures and promote endothelial cell-cell assembly (Fawcett et al., 1995).

The data obtained in this study are in agreement with the phenotype of VE-cadherin-deficient embryoid bodies (Vittet et al., 1997). In this in vitro model, VE-cadherin-/- endothelial cells did not form a vascular plexus. Instead, endothelial cells remained dispersed with no elongated processes and failed to form aggregates of more than two or three cells, which parallels what we observed in mutant yolk sac and allantois. However, it is noteworthy that VE-cadherin+/- embryoid bodies also showed some alterations in vasculogenesis, which was not observed in heterozygous mice. This difference may be due to lower VE-cadherin expression in ES-derived endothelial cells than in mice or to a distinct genetic background. In addition to these data, Bach et al. (1998) reported that anti-VE-cadherin blocking antibodies inhibited capillary tube formation of human umbilical vein endothelial cells in fibrin or collagen gels, indicating that, in this model also, VE-cadherin is necessary for vascular architecture.

Lack of VE-cadherin did not significantly modify the quantity and distribution of yolk sac hematopoietic progenitors and their differentiation proceeded normally. Although it has been demonstrated that embryo (Dieterlen-Liévre et al., 1997) and possibly the allantois (Downs and Harmann, 1997; Caprioli et al., 1998) contain hematopoietic stem cells at this age (E9.5), no circulating cell could be identified within embryonic vasculature and allantois clusters.

In conclusion, mice lacking VE-cadherin exhibit one of the most severe vascular phenotypes described in gene targeting experiments, after those for Flk-1 and VEGF mutations. Furthermore, this study illustrates the morphogenetic role of cadherins, as opposed to a simply adhesive function.

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REFERENCES


Targeted disruption of the VE-cadherin gene


