Cell, Vol. 102, 199–209, July 21, 2000, Copyright ©2000 by Cell Press

A Role for Hematopoietic Stem Cells in Promoting Angiogenesis

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Summary

Angiogenesis is an important event for embryonic organogenesis as well as for tissue repair in the adult. Here, we show that hematopoietic stem cells (HSCs) play important roles for angiogenesis during embryogenesis. To investigate the role of HSCs in endothelial cell (EC) development, we analyzed AML1-deficient embryos, which lack definitive hematopoiesis. These embryos showed defective angiogenesis in the head and pericardium. Para-aortic splanchnopleural (P-Sp) explant cultures on stromal cells (P-Sp culture) did not generate definitive hematopoietic cells and showed defective angiogenesis in the *AML1* null embryo. Disrupted angiogenesis in P-Sp cultures from *AML1* null embryos was rescued by addition of HSCs or angiopoietin-1 (Ang1). HSCs, which express Ang1, directly promoted migration of ECs in vivo and in vitro. These results indicate that HSCs are critical for angiogenesis.

Introduction

Vascular development consists of two different processes, vasculogenesis and angiogenesis. In both processes, bidirectional signaling between endothelial cells (ECs) and the surrounding mesenchymal cells is critical (Folkman and D'Amore 1996). Many molecules have been isolated that regulate the processes of vasculoangiogenesis and are involved in maintaining the integrity of vessels by recruitment and formation of the periendothelial layer or by mediating interactions between arteries and veins (Folkman and D'Amore 1996; Hanahan 1997; Gale and Yancopoulos 1999). Among them, two receptor tyrosine kinase subfamilies are characterized by their largely endothelial specific expression. One family includes Flt-1/VEGFR1, Flk-1/KDR/VEGFR2, and Flt-4/VEGFR3, all of which are members of the vascular endothelial growth factor (VEGF) receptor family. Critical roles for Flt-1, Flk-1, and Flt-4, as well as that of VEGF, have been demonstrated by analysis of genetically engineered mice mutant in these proteins (Fong et al., 1995; Shalaby et al., 1995; Ferarra et al., 1996; Dumont et al., 1998). The other family includes TIE1/TIE and TIE2/TEK. Targeted mutation of TIE1 or TIE2 demonstrates that these receptors, like VEGFRs, play a critical role in embryonic vascular formation (Dumont et al., 1994; Puri et al., 1995; Sato et al., 1995). Embryos deficient in TIE2 or TIE1 fail to establish structural integrity of vasculature, resulting in hemorrhage at E9.5 and E13.5, respectively. Compared with the early defect in vasculogenesis seen in *VEGF* or *VEGFR* mutant embryos, mice lacking *TIE1* or *TIE2* exhibit later defects in angiogenesis and vascular remodeling as well as in vascular integrity.

A first step in angiogenesis in the adult vessel is dissociation of periendothelial cells (pericytes) tightly adhering to ECs. The balance between adhesion and dissociation of ECs and pericytes depends on angiopoietins, the ligands for TIE2. Angiopoietin-1 (Ang1) deficient embryos show severe vascular abnormalities, as observed in TIE2 deficient embryos. These abnormalities include insufficient branching of the cardinal vein or capillaries in the pericardium, lack of remodeling of vessels in the yolk sac, and insufficient heart development (Suri et al., 1996). Moreover, ultrastructural analysis reveals that close adhesion between ECs and pericytes is inhibited in the absence of TIE2 or Ang1. A naturally occurring antagonist of Ang1, designated Ang2, blocks the ability of Ang1 to activate TIE2 (Maisonpierre et al., 1997). Transgenic mice expressing Ang2 exhibit phenotypes similar to those observed in TIE2 or Ang1 deficient embryos (Maisonpierre et al., 1997). Thus, while Ang1 is expressed in mesenchyme surrounding large vessels in the embryo and is widely expressed in the adult, Ang2 is expressed only at sites of vascular remodeling (Maisonpierre et al., 1997). This observation suggests that Ang2 blocks an otherwise constitutive stabilizing signal by Ang1 and promotes localized vessel destabilization. Destabilization of vessels by Ang2 may contribute to either vessel regression by blocking factors required for survival of ECs produced by pericytes or new vessel sprouting. Prolonged overexpression of Ang2 without VEGF promotes vessel regression, although vessel sprouting occurs when Ang2 and VEGF are coexpressed (Asahara et al., 1998; Holash et al., 1999).

Recently, we found that Ang1 stimulated integrin-dependent adhesion of TIE2 expressing cells to fibro-

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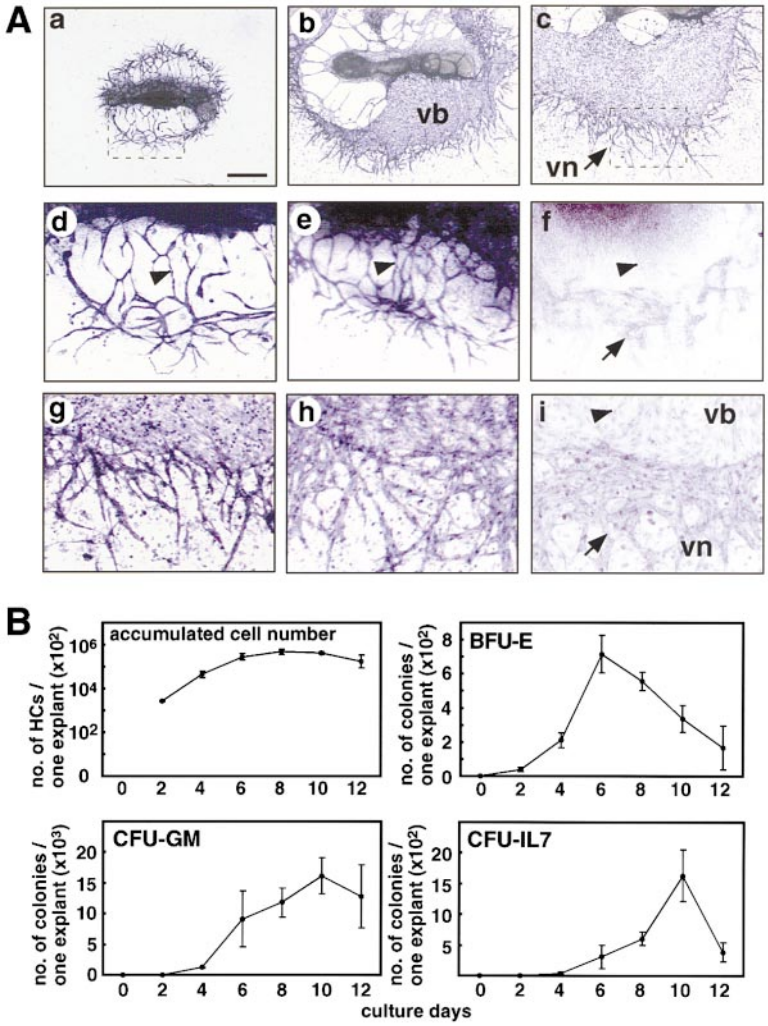

Figure 1. P-Sp Culture System Supports Vasculo-angiogensis and Hematopoiesis

(A) The development of ECs in P-Sp cultures from E9.5 mouse embryos. Culture plates were fixed after 2 (a,d,e, and f), 4 (b), and 8 (c,g,h, and i) days of culture, and stained with anti-PECAM-1 (a–d and g), -Flk-1 (e and h), or -TIE2 (f and i) mAbs, respectively. (d) and (g) are the higher maginification of areas of (a) and (c) indicated by the dashed box, respectively. Migrating ECs from the explant highly expressed PECAM-1 (arrowhead in d) or Flk-1 (arrowhead in e). TIE2 expression was faint in migrating ECs (arrowhead in f) or vb (arrowhead in i), although it was strong in sprouting ECs (arrows in f and i). Isotype matched control IgG showed no staining in the cells of this P-Sp (data not shown). Abbreviations: vb, vascular bed; vn, vascular network. Scale bars: (a–c) 400 μm; (d–j) 133 μm. (B) Hematopoiesis in P-Sp cultures. Cells from P-Sp cultures on OP9 cells were harvested at indicated days, and the number of HCs was counted after removal of adherent cells by Sephadex G10. Subsequently, cells were transferred to methylcellulose semisolid culture. The number of progenitor cells forming a colony was counted after 7 days of culture.

nectin or collagen (Takakura et al., 1998; Sato et al., 1998). This role is consistent with the close adhesion between ECs and pericytes promoted by Ang1. However, it has been reported that Ang1 promotes vessel sprouting by acting as a chemoattractant. (Witzenbichler et al., 1998). Vessel sprouting begins with dissociation of pericytes from ECs, a process mediated by inactivation of Ang1 signaling. Subsequently, ECs may sprout toward Ang1-producing tissues. As recruitment of pericytes is followed by migration of ECs, Ang1-producing cells may promote vessel sprouting at a distance. To examine the angiogenesis mediated by TIE2–angiopoietin system, we established a new culture system supporting angiogenesis and hematopoiesis using the para-aortic splanchnopleural (P-Sp) region (pre-AGM region; aorta-gonad-mesonephros region), a site where definitive HSCs are committed from hemangioblasts (Cumano et al., 1996; Medvinsky and Dzierzak, 1996). Here, we show that Ang1 derived from HSCs promotes vessel sprouting into avascular areas and that this stimulation is critical in tissues where Ang1-producing mesenchymal cells are absent. Moreover, we demonstrate that HSCs are required for angiogenesis by presenting a detailed histological analysis of capillary formation in AML1 deficient mice, which lack HSCs completely.

Results

Vasculo-angiogenesis in the P-Sp Culture System

To characterize the interaction between ECs and hematopoietic cells (HCs), an in vitro culture system for vasculo-angiogenesis and hematopoiesis was developed. P-Sp explants from the E9.5 embryos were cultured on OP9 stromal cells. In this culture, PECAM-1$^+$ ECs form a sheet-like structure (vascular bed, vb) and subsequently form a network (vascular network, vn) in the periphery of the endothelial sheet. To analyze vasculo-angiogenesis in this culture system, EC growth was observed using the markers PECAM-1, Flk-1, and TIE2. At the early stages of culture, PECAM-1- and Flk-1-positive ECs were generated from the P-Sp explant. These cells migrated and formed a circle around the explant on OP9 cells (Figure 1A, a,d, and e). TIE2 expression was weak in migrating ECs, although sprouting ECs in the circle expressed TIE2 strongly (Figure 1A, f). Subsequently, PECAM-1-, Flk-1-positive ECs surrounding the explant proliferated and formed a vascular bed, and a large number of ECs sprouted from the vascular bed and formed network-like structure (Figure 1A, b, c, g, and h). Compared with the weak expression of TIE2 in ECs in the vascular bed, sprouting ECs highly expressed TIE2

(Figure 1A, i). Inhibition of Flk-1 signaling by addition of soluble Flk-1 receptors to the culture disrupted formation of both the vascular bed and network of ECs, and addition of soluble TIE2 inhibited network formation of ECs (Takakura et al., 1998). In this culture system, we define vasculogenesis as the process in which PECAM-1^+Flk-1^+TIE2^{low} immature ECs migrate from the explant and form a vascular bed. Angiogenesis is defined as the process of sprouting of PECAM-1^+Flk-1^+TIE2^+ ECs from such a bed and subsequent network formation.

The development of HCs was observed in this culture system. HCs, which were initially observed in the periphery of the vascular bed, migrated into the vascular network area and proliferated. As the number of HCs increased, erythroid, granulocyte-macrophage, and lymphoid progenitor cells increased on days 6–10 of culture (Figure 1B).

Lack of Hematopoiesis and Angiogenesis in P-Sp Cultures from AML1 Deficient Mice

AML1 deficient mice provide a tool to analyze the interaction between hematopoiesis and angiogenesis. Disruption of *AML1* leads to failure in the development of definitive hematopoiesis and lethality at E12.5 (Okuda et al., 1996; Wang et al., 1996). Mutant embryos exhibit hemorrhages in the ventricles of the central nervous system and in the vertebral canal and within the pericardial space and peritoneal cavity. We analyzed the vascular development of AML1 deficient mice (Okada et al. 1998) in vivo and in vitro. Whole mounts of E11.5 embryos were stained with the PECAM-1 monoclonal antibody (mAb) to visualize all ECs. Mutant embryos were the same size as wild-type (wt) embryos at E11.5 (Figure 2A). The analysis showed that extensive vascular branching and remodeling into large and small vessels occurred normally in the head region of both wt and mutant embryos by E11.5. However, the number of small capillaries in mutant embryos (Figure 2C) was less in the hindbrain than that observed in wt (Figure 2B); moreover, large vessels in the mutant embryo (Figure 2C) contained fewer branches than those seen in wt embryos (Figure 2B). In mutant embryos, less branching of capillaries was observed in vessels of the pericardium (Figures 2D and 2E) and in the vitelline artery of the yolk sac (Figures 2F and 2G). Consistent with defective angiogenesis in the head, massive hemorrhages and aneurysms were observed in mutant embryos (Figure 2I). Such abnormalities were not seen in wt embryos (Figure 2H).

To analyze the interaction between HSCs and ECs, we observed the development of ECs in P-Sp cultures from *AML1* mutant embryos. As expected, HCs were not generated from P-Sp explants of *AML1* mutant embryos (Figure 3A, b and d). In contrast, explants from wt embryos developed many round cells adhering to the presumptive vascular network area (Figure 3A, a and c). Cells harvested from cultures derived from wt embryos formed several hematopoietic colonies in a semisolid medium; however, *AML1* mutant embryos did not generate hematopoietic progenitor cells in these conditions (Table 1). After fixation of the culture plate, we examined the development of ECs. The wt explant generated vascular beds and networks (Figure 3A, e); on the other hand, poor vascular network formation was observed in cultures of *AML1* mutant explants (Figure 3A, f).

To evaluate further the effect of HCs on angiogenesis, we examined explants from *c-Myb* mutant embryos (Mucenski et al., 1991). *c-Myb* mutant embryos generate a small number of HCs and thereby provide a model in which the effect of an intermediate number of HCs on angiogenesis can be tested. As expected, *c-Myb* mutant explants contained smaller populations of HCs than did explants derived from wt embryos. *c-Myb* mutant-derived P-Sp cultures contained megakaryocytes and monocyte/macrophages, but no erythroid cells (data not shown). Moreover, the number of hematopoietic progenitor cells in *c-Myb* mutant cultures was a hundred-fold less than that observed in wt explants (Table 1). In proportion to the number of HCs, the area of the vascular network was reduced in cultures of *c-Myb* mutant explants (Figure 3B).

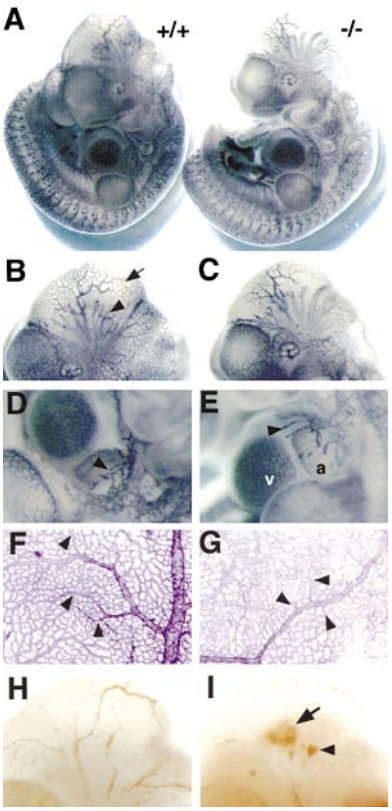

Figure 2. Defective Angiogenesis in *AML1* Mutant Embryos

(A) Whole-mount PECAM-1-stained wt and *AML1* mutant embryos at E11.5. (B–E) High power views of (A).

(B and D) Wild-type and (C and E) mutant embryos. Arrowhead or arrow in (B) indicates highly branched small capillary in the hindbrain or network forming cardinal vein, respectively. Counterparts in mutant (C) show less branching or less caliber change of vessels. A network of capillaries surrounded the pericardium (arrowheads) in wt heart (D), while comparable network formation was absent in mutants (E). Abbreviations: v, ventricles; a, atrium.

(F and G) Yolk sac vascularization. Note less branching of the vitelline artery in mutants (G, arrowheads) compared to wt embryos (F, arrowheads). Gross appearance of the wt (H) and mutant (I) heads of E11.75 embryos. Hemorrhage (arrow) and aneurysm (arrowhead) was observed in the mutants.

HCs Promote Angiogenesis in P-Sp Cultures

To test the hypothesis that HCs promote angiogenesis, HSC-enriched population from the bone marrow of transgenic mice harboring green fluorescent protein (GFP) under the transcriptional control of the β actin

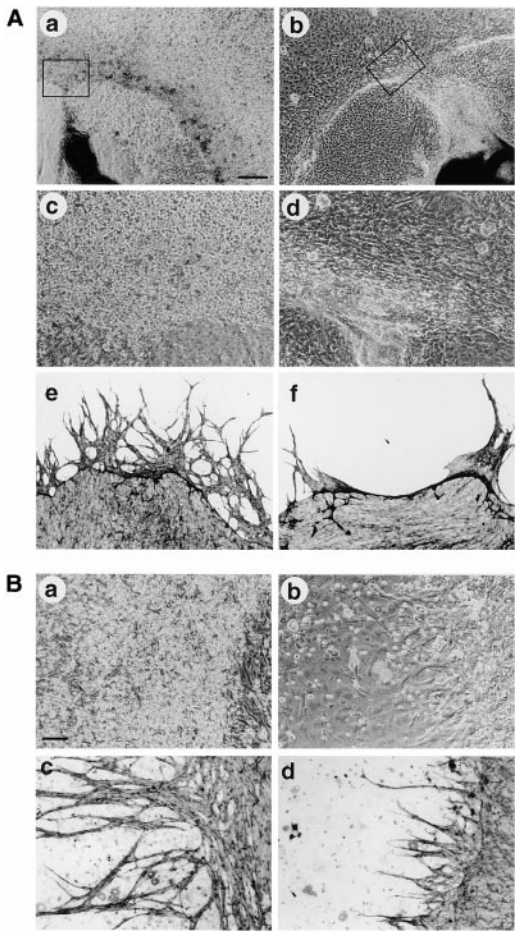

Figure 3. Requirement of HSC Development for Angiogenesis In Vitro

P-Sp explants from (A) wt (a, c, and e) and *AML1* mutants (b, d, and f) or (B) wt (a and c) and *c-Myb* mutants (b and d) were dissected at E9.5, and cultured on OP9 cells. (A) Phase contrast microscopy (a–d). (c) and (d) are the higher magnification of areas of (a) and (b) indicated by the box, respectively. (e and f) P-Sp cultures stained with anti-PECAM-1 mAb. Scale bars: (a, b, e, and f) 200 μm; (c and d) 50 μm. (B) Phase contrast microscopy demonstrates that the number of round cells in *c-Myb* mutant cultures (b) was less than that observed in wt culture (a). (c and d) P-Sp cultures stained with anti-PECAM-1 mAb. Scale bar: 50 μm.

promoter (Okabe et al., 1997) was sorted by flow-cytometry and added to P-Sp cultures of *AML1* mutant embryos. In these transgenic mice, all tissues except erythrocytes and hair fluoresce, allowing us to visualize precisely the morphology of nonmutant cells. As expected, addition of HSCs rescued defective angiogenesis in *AML1* mutant embryos (Figure 4A, b and c). The fine vascular network coincides with the region where HCs formed a colony (Figure 4A, a). Their round morphology confirmed that exogenous HCs did not differentiate into elongated ECs (Figure 4A, d).

Ang1 Expression in HSCs

Since results from the P-Sp culture system suggested that extrinsic signals from HCs promote angiogenesis, we searched for factors that could mediate this process. As shown in Figure 5A, Ang1 and VEGF were expressed in bone marrow HSC-enriched population ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$) but not in mature HCs ($CD45^+Lin^+$). During embryogenesis, $CD45^+c\text{-}Kit^+CD34^+$ cells are defined as HSCs (Yoder et al., 1997). As observed in the bone marrow, $CD45^+c\text{-}Kit^+CD34^+$ cells highly expressed Ang1 but not VEGF. Other angiogenesis related genes such as Ang2 and ephrinB2 were not expressed in either mature or immature HCs (data not shown). To clarify whether an HSC-enriched population produces Ang1 as a protein, we sorted HSC-enriched population from E12.5 fetal liver and adult bone marrow of mice heterozygous for Ang1-targeted allele in which β-galactosidase (LacZ) expression is under control of Ang1 promoter (Suri et al., 1996) and examined the expression of Ang1 and LacZ (Figures 5B and 5C). We detected Ang1 protein in HSC-enriched population (Figure 5B, a and c) by anti-Ang1 antibody but not in mature HCs (Figure 5B, b and d). The frequency of Ang1 positive cells was 90% in $CD45^+c\text{-}Kit^+CD34^+$ cells of the fetal liver, and 50% in $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells of adult bone marrow. Moreover, we confirmed that LacZ positive cells in HSC-enriched population were detected at a similar frequency to that observed in Ang1 expression (data not shown). Finally, the LacZ gene was expressed in HSC-enriched population abundantly in an mRNA level by RT-PCR analysis (Figure 5C). To determine whether HSCs participate in angiogenesis, we examined whether HSCs exist in the head region where severe angiogenic defects are observed in the *AML1* mutants. At E10.5, the primary sites of hematopoiesis have been reported to be in the yolk sac and AGM region (Medvinsky and Dzierzak, 1996; Yoder et al. 1997). To examine the localization of HSCs, embryos at E10.5 were dissected into three parts: the yolk sac, AGM region, and head. The surface phenotype of dissociated cells was analyzed by FACS (Figure 5D). $CD45^+$ HCs were divided into $CD34^+c\text{-}Kit^+$ cells and $CD34^{dull/-}c\text{-}Kit^{dull/-}$ cells. The frequency of $CD34^+c\text{-}Kit^+$ cells in the head was low compared with that observed in the yolk sac and AGM region. Limiting dilution of $CD34^+c\text{-}Kit^+$ cells or $CD34^-c\text{-}Kit^-$ cells cocultured with OP9 cells revealed that multipotential hematopoietic progenitors were included in the $CD34^+c\text{-}Kit^+$ fraction but not in the $CD34^-c\text{-}Kit^-$ fraction. Moreover, the incidence of hematopoietic progenitors in $CD34^+c\text{-}Kit^+$ cells was not different in the yolk sac, AGM region, and head (Figure 5D). These findings indicate that HSCs migrated to the head after circulation was established.

Ang1 on HSCs Plays an Important Role in Vascular Network Formation

These findings suggested that Ang1 expressed on HCs, especially HSC-enriched populations, promotes angiogenesis. To test this hypothesis, we added $CD45^+c\text{-}Kit^+CD34^+$ cells from E10.5 *Ang1* mutants and wt embryos to P-Sp cultures of *AML1* mutant embryos. As observed in bone marrow HSCs (Figure 4A), $CD45^+c\text{-}Kit^+CD34^+$ cells from wt embryos rescued defective network formation of ECs (Figure 4B, a); however, the same number of HSC-enriched cells from *Ang1* mutant embryos could not (Figure 4B, b). Moreover, when soluble TIE2 receptors or Ang2 were added simultaneously with HSCs enriched from wt embryo to inactivate Ang1 signaling via TIE2 in P-Sp cultures, HSC-mediated rescue of defective angiogenesis of *AML1* mutants was completely abolished (data not shown). To test whether Ang1 alone rescues the angiogenic defects observed in *AML1*

Table 1. Hematopoietic Progenitors in Cultured P-Sp Explants

AML1	Colonies per explant					
	Wild type		Heterozygote		Homozygote	
	Erythroid	GM	Erythroid	GM	Erythroid	GM
	118 ± 4	1580 ± 90	100 ± 4	1292 ± 132	0 ± 0	0 ± 0
c-Myb	Colonies per explant					
	Wild type		Heterozygote		Homozygote	
	Erythroid	GM	Erythroid	GM	Erythroid	GM
	108 ± 17	1034 ± 55	109 ± 13	1006 ± 76	0 ± 0	14 ± 8

P-Sp from AML-1 or c-Myb +/+, +/−, and −/− E9.5 embryos were cultured on OP9 cells for 10 days. Non adherent cells were then assayed for hematopoietic colonies in semisolid media using methylcellulose supplemented with IL-3, SCF, and Epo. The number of hematopoietic colonies was scored after 7 days in culture. The total number of colonies (>40 cells) per explant was obtained from three independent experiments and expressed as the mean ± SEM (n = 3). "Erythroid" indicates a colony containing definitive erythrocytes. "GM" indicates a colony containing granulocytes and/or macrophages.

mutants, Ang1* (see Experimental Procedures) was added to P-Sp cultures derived from *AML1* mutant embryos. 100 ng/ml Ang1* did not rescue the angiogenic phenotype effectively (data not shown); however, 300 ng/ml Ang1* promoted angiogenesis to a level equivalent to wt (Figure 4B, d). Interestingly, addition of the same amount of Ang1* to P-Sp cultures of wt embryos altered the morphology of the vascular bed to a lattice-like network structure (Figure 4B, c, arrow). It is assumed that *AML1* or *cMyb* mutant embryos expressed Ang2 at higher levels than did wt embryos; however, there was no upregulation of Ang2 mRNA expression in P-Sp regions and whole embryos from *AML1* or *cMyb* mutants.

Ang1 Expression in Pericytes and HCs

It has been reported that periendothelial cells such as pericytes and smooth muscle cells constitutively secrete Ang1 (Davis et al., 1996). We analyzed the expression of Ang1 in the P-Sp culture system and in vivo. First, to determine whether pericytes exist in P-Sp cultures, we looked for α smooth muscle actin (αSMA)-positive cells. We observed two types of vascular beds marked by the presence or absence of αSMA^+ cells. One was a lattice-like vascular bed that was αSMA positive (Figure 6A, a; box by dashed line and b). The other was a smooth monolayer of ECs that was αSMA negative. Based on distribution of $PECAM\text{-}1^+$ cells, the sprouting of ECs did not appear to depend on αSMA^+ cells (Figure 6A, c). Ang1 expression was detected in two cell types. One type was umbrella shaped (Figure 6A, d), and these cells also expressed αSMA (data not shown). The other type was round HCs (Figure 6A, e) and hematopoietic clusters showing a cobble stone appearance (Figure 6A, f). Consistent with the area in which HCs express Ang1, $PECAM\text{-}1^+$ ECs migrated from the vascular bed (Figure 6A, g) and formed a network.

As observed by in situ hybridization analysis (Suri et al., 1996), Ang1 expression was detected in myocardial cells (Figure 6B, a–d), periendothelial cells surrounding large vessels (Figure 6B, e–h), and mesenchymal cells in the intersomite region (data not shown) of E10.5 embryos. Consistent with a lack of αSMA^+ periendothelial cells, we did not detect Ang1 expression in mesenchymal cells surrounding small vessels at this stage; however, some mesenchymal cells not adhering to vessels expressed Ang1 (data not shown). Although overall, there is little difference in Ang1 expression between *AML1* mutant (Figure 6B, d and h) and wt (Figure 6B, c and g) embryos, round cells, which were lacking in the *AML1* mutants (Figure 6B, d and l), expressed Ang1 highly (Figure 6B, c and k) in wt embryos. Those round cells were located outside the lumen of the vessel and expressed CD45 (Figure 6B, i). $CD45^+$ cells were not seen in *AML1* mutants (Figure 6B, j), indicating that round cells expressing Ang1 and CD45 are HCs.

HSCs Regulate Vascular Network Formation

To examine the effect of HSCs in angiogenesis, we compared capillary formation of wt and *AML1* mutant embryos in the E10.5 brain ectoderm. In wt embryos, initially, ECs seemed to migrate toward $CD45^+$ HCs in the neuronal layer (Figure 7A, a). These $CD45^+$ cells migrating in front of ECs also expressed c-Kit or CD34 (data not shown), indicating that $CD45^+$ cells are in the HSC population. After ECs formed a network, HSCs remained near ECs (Figure 7A, b), as observed in the P-Sp culture (Figure 6A, g). In E10.5 *AML1* mutants, ECs migrating into the ectodermal layer showed disorganized structures. For example, many pods sprouted from one capillary (Figure 7A, c), and ECs did not form tube-like structures, leading to hemorrhage in the ectoderm (Figure 7A, d).

Next, to test whether HSCs promote angiogenesis in vivo, we used a method for assessing angiogenesis requiring reconstituted basement membrane (Passaniti et al., 1992). An HSC-enriched population sorted from adult bone marrow was prestained with PKH26 red fluorescence and mixed with matrigel in the presence or absence of soluble TIE2 receptors. Gels were injected subcutaneously into the abdominal region of adult mice. Matrigel mixed with HSCs without soluble TIE2 receptors (Figure 7B, a) appeared yellow, suggesting a large number of erythrocytes within the gel. On the other hand, matrigel mixed with HSCs with soluble TIE2 receptors appeared white (Figure 7B, c). In the section, PECAM-1 labeled ECs were localized near PKH26-labeled HSCs and formed a capillary network as observed in the neuronal layer (Figure 7A, b). Soluble TIE2 receptors inhibited this network of ECs (Figure 7B, d). Gels without HSCs or with Lin^+ mature HCs could not promote capillary formation (data not shown). Taken together, the data suggests that HSCs promote migration of ECs through TIE2 and contribute to EC network formation in vivo.

Finally, we investigated the migration of ECs sorted from E10.5 embryos (Figure 7C, a) induced by Ang1

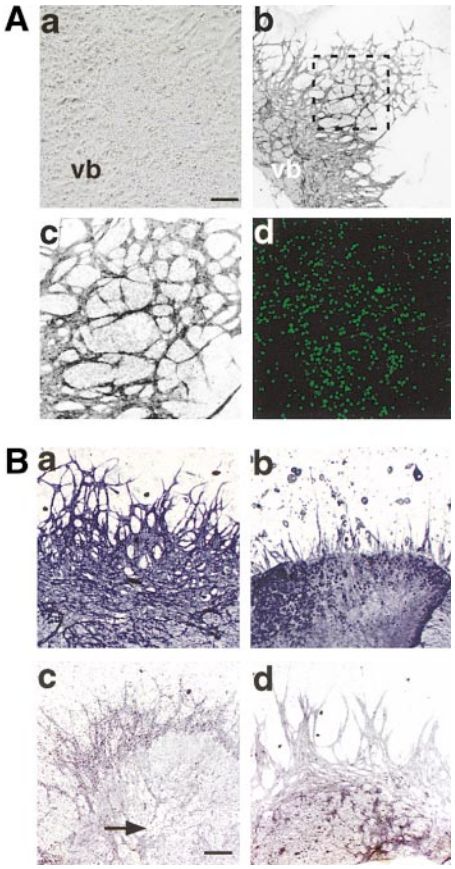

Figure 4. Rescue of Defective Angiogenesis in *AML1* Mutant P-Sp Cultures

(A) Rescue of defective angiogenesis in *AML1* mutants by HSCs enriched from adult bone marrow. (a) Phase contrast microscopy shows that exogenous HSCs form hematopoietic colonies beside the vascular bed (vb) of *AML1* mutant P-Sp cultures. (b) PECAM-1 staining in (a). (c) A high power view of the area indicated by the dashed box in (b). (d) Detection of GFP-positive cells by fluorescence microscopy in (c). Scale bars: (a and b) 250 μm; (c and d) 67 μm.

(B) Rescue of defective angiogenesis in *AML1* mutants by HSCs from embryos (a and b) or Ang1 (c and d). HSC-enriched populations from E10.5 wt (a) or *Ang1* mutant (b) embryos from the same litter were added to the *AML1* mutant P-Sp culture. Three hundred nanograms per milliliter of Ang1* was added to the P-Sp culture of wt (c) and *AML1* mutant (d) embryos. A lattice-like structure (arrow in c). Scale bars: (a and b) 125 μm; (c and d) 250 μm.

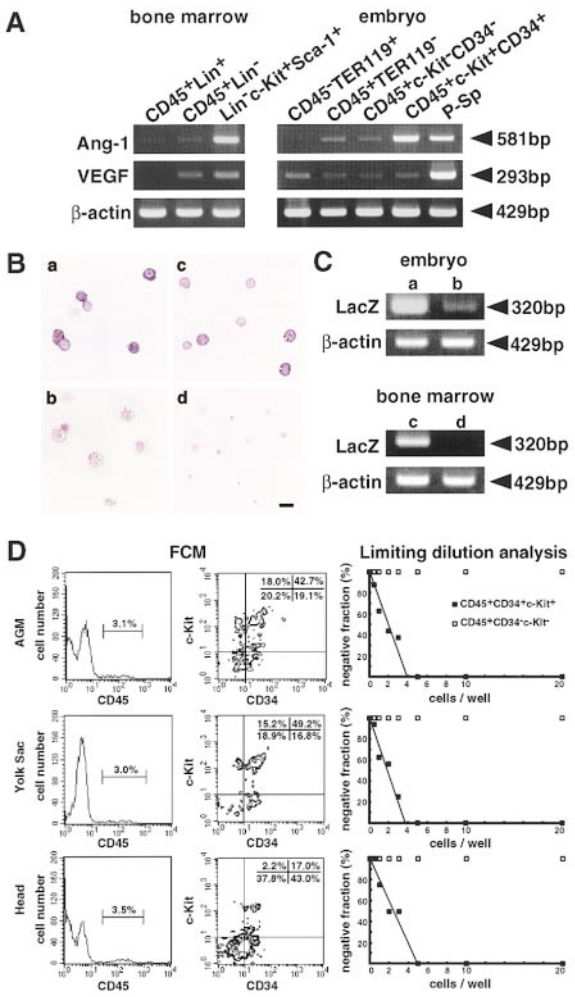

Figure 5. Expression of Ang1 on HC and Existence of HSCs in the Head of E10.5 Embryos

(A) Analysis of growth factor mRNA expression in HCs from adult bone marrow or E10.5 embryos by reverse transcriptase-PCR. $CD45^+Lin^+$; mature HCs, $CD45^+Lin^-$; immature HCs, $Lin^-c\text{-}Kit^+Sca\text{-}1^+$; HSC-enriched population, $CD45^-TER119^+$; primitive erythrocytes, $CD45^+TER119^-$; HCs other than primitive erythrocytes, $CD45^+c\text{-}Kit^-CD34^-$; mature HCs, $CD45^+c\text{-}Kit^+CD34^+$; and HSC-enriched population. β actin mRNA served as a loading control.

(B) Production of Ang1 on HSC-enriched population. $CD45^+c\text{-}Kit^+CD34^+$ cells (a) and $CD45^+c\text{-}Kit^-CD34^-$ cells (b) sorted from E12.5 fetal liver of mice heterozygous for Ang1-targeted allele ($Ang1^{+/-}$) in which β galactosidase (LacZ) expression is under control of Ang1 promoter, and $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells (c) and Lin^+ cells (d) from adult bone marrow of $Ang1^{+/-}$ mouse were stained by anti-Ang1 antibody. Ang1 expression is visualized as a dark blue reaction product. Nuclei were stained with nuclear fast red. Scale bar: 20μm.

(C) mRNA expression of LacZ on sorted HCs. Lanes (a)–(d) correlate with that described in Figure 5B.

(D) Incidence of HSCs in E10.5 embryos. Cells from the AGM region, yolk sac, or head region from E10.5 embryos were stained with anti-CD45, CD34, and c-Kit mAbs and analyzed by flow cytometry. $CD45^+$ cells were gated (left histogram, percentages of $CD45^+$ cells are indicated in the panel), and examined for expression of CD34 and c-Kit (middle panel, percentages of each fraction are indicated in the upper right quadrant). Sorted $CD45^+c\text{-}Kit^+CD34^+$ cells or $CD45^+c\text{-}Kit^-CD34^-$ cells were evaluated by a limiting dilution assay for their ability to differentiate into multiple hematopoietic lineages. The frequency of multipotential hematopoietic progenitors in $CD45^+c\text{-}Kit^+CD34^+$ cells was 1/2.6 (AGM), 1/2.4 (yolk sac), or 1/3.2 (head) according to a Poisson analysis.

or HSCs. Ang1* led to a dose-dependent increase in directed migration of $TIE2^+$ ECs but did not promote migration of control $CD45^-PECAM\text{-}1^-TIE2^-$ cells (Figure 7C, b). Ang2 did not stimulate chemotactic activity (data not shown). $CD45^+c\text{-}Kit^+CD34^+$ HSCs from E10.5 embryos also promoted migration of $TIE2^+$ ECs in a dose-dependent manner. This migration was completely suppressed by soluble TIE2 receptors (TIE2-Fc) but not by CD4-Fc control protein or by soluble Flk-1 receptors (Flk-1-Fc). $CD45^+c\text{-}Kit^-CD34^-$ mature HCs also promoted migration of $TIE2^+$ ECs, but less efficiently than did HSCs. Moreover, migration of ECs stimulated by mature HCs was also blocked by soluble TIE2 receptors (Figure 7C, c). These findings indicate that HSC-induced migration of $TIE2^+$ECs depends on Ang1.

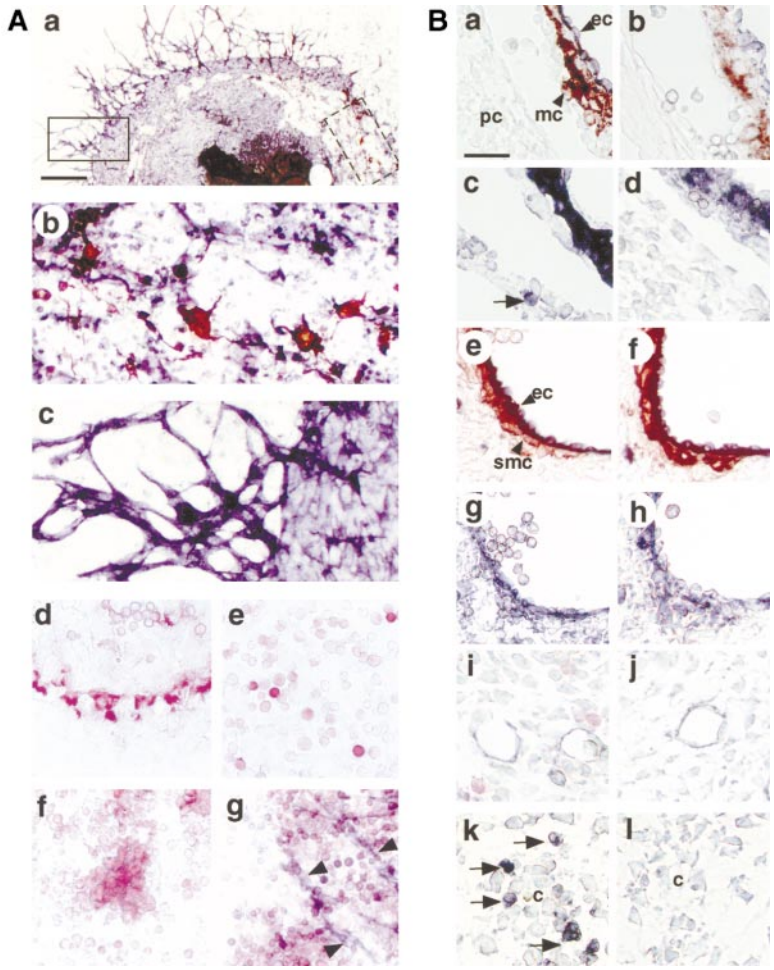

Figure 6. Localization of Smooth Muscle Cells and Expression of Ang1 in P-Sp Cultures and Embryonic Tissues

(A) (a–c) P-Sp explants cultured on OP9 cells for 10 days were stained with anti-PECAM-1 mAb (blue) and anti-α smooth muscle actin (αSMA) mAb (brown). (b and c) High-power view of the dashed (b) or solid (c) box in (a), respectively. (d–f) P-Sp explants cultured as above were stained with anti-Ang1 antibody. (d) Umbrella-shaped pericytes in the vascular bed were positive for Ang1. Round HCs (e) and hematopoietic clusters (f) on the vascular network were also Ang1-positive. (g) PECAM-1 (blue) and Ang1 (red) expression. Arrowheads indicate PECAM-1$^+$ ECs. Scale bars: (a) 500 μm; (c and d) 125μm; (d, f, and g) 40 μm; (e) 25 μm.

(B) Ang1 expression in tissues of E10.5 embryos. (a–d) the heart, (e–h) dorsal aorta, and (i–l) head from wt (a, c, e, g, i, and k) or *AML1* mutants (b, d, f, h, j, and l). (a, b, e, and f) Double staining with anti-PECAM-1 (blue) and anti-αSMA (brown) mAbs. abbreviations: c, capillary; ec; endothelial cells, mc; myocardial cells, pc; pericardial cells, smc; smooth muscle cells. (c, d, g, h, k, and l) Ang1 expression. Ang1 expression was detected in mc and smc from both *AML1* mutant and wt embryos. Round HCs express Ang1 (arrows in c and k). (i and j) Double staining with anti-CD45 (red) and PECAM-1 (blue) mAbs. Scale bar: 40 μm.

Discussion

In this paper, we develop the P-Sp culture system to analyze HSC function and present a model that HSCs promote angiogenesis through Ang1. Stem cells may have a capacity to contribute to tissue organization through specific cytokines. To clarify the interaction between HSCs and ECs, we used *AML1* mutant embryos, which do not generate definitive HSCs and exhibit defective vasculature in vivo. We demonstrate defective angiogenesis in P-Sp explants cultured from these embryos and show that this defect is secondary to hematopoietic failure.

In P-Sp cultures from wild-type embryos, two types of vascular bed formation are observed. One is a lattice-like bed in which αSMA$^+$ cells adhering to ECs express Ang1; the other is a smooth vascular bed that lacks αSMA$^+$ cells. Since Ang1 expressed on pericytes promotes vessel remodeling (Suri et al., 1996), the presence of pericytes may promote formation of fine networks in the vascular bed in an Ang1-dependent manner, as observed in vivo. Moreover, we do not observe αSMA$^+$ cells in regions of the vascular network where extensive sprouting of ECs occurs. During early embryogenesis, capillary formation proceeds without pericytes. Expression of Ang1 is not always observed in mesenchymal cells around capillaries. Sprouting of new vessels in the head and pericardium is analogous to sprouting of ECs in the P-Sp culture system. In *AML1* mutant embryo, many vessels are present at locations where Ang1 expression is normally observed in mesenchymal cell compartment. Histological analysis revealed that capillary formation is dependent on Ang1 from HSCs in an area where Ang1-producing mesenchymal cells are absent. Based on observations of capillary formation in neuronal layers, HSCs appear to determine the direction of vessel sprouting, since migration of ECs in *AML1* mutant embryos is severely disorganized. In addition, vascular phenotypes in *AML1* mutants, such as poor branching of the cardinal vein in the head and poor network formation in the pericardium, are quite similar to those observed in *Ang1* mutant embryos (Suri et al., 1996). HSCs derived from wt embryos or adult bone marrow rescue angiogenesis defects seen in *AML1* mutants in the P-Sp culture, but HSCs derived from *Ang1* mutant embryos can not, indicating that HSC-derived Ang1 is critical for angiogenesis.

Role of AML1 in Vasculo-angiogenesis

Mutations in several genes, such as *Scl* (Robb et al., 1995; Shivdasani et al., 1995a), *flk-1* (Shalaby et al., 1995), and *TIE2* (Dumont et al., 1994; Sato et al., 1995, Takakura et al. 1998), affect both endothelial and hematopoietic cell development. These genes are expressed in both lineages. It is important to determine whether the effects of mutations in these genes on endothelial

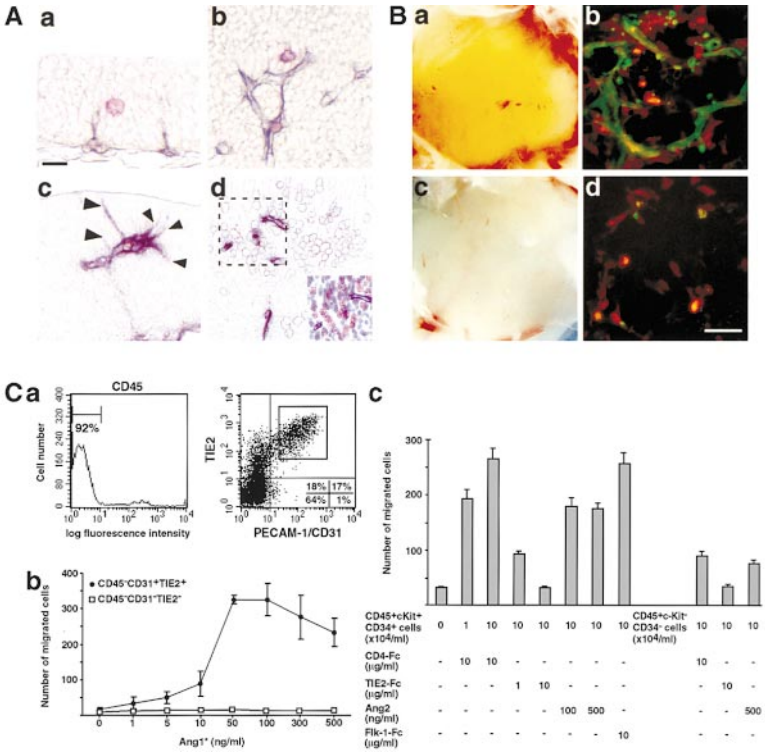

Figure 7. Vessel Sprouting by HSCs

(A) Sections from E10.5–E11.5 embryos double stained with anti-CD45 (red) and anti-PECAM-1 (blue) mAbs. Vessels in the brain ectodermal layer of wt (a and b) or *AML1* mutant (c and d) embryos. Note that ECs in the *AML1* mutant embryo sprouted in many directions (arrowheads) from one capillary (c). (d) Disorganized capillaries lead to hemorrhage in *AML1* mutants. Inset shows hematoxylin-eosin staining in the box. Scale bar: 25 μm.

(B) HSCs promoted capillary sprouting in adult mice. Matrigels containing HSCs and CD4-Fc chimeric protein (a and b) or HSCs and soluble TIE2 receptors (c and d) were injected subcutaneously near the abdominal midline. (a and c) Appearance of matrigels on day 4. (b and d) Fluorescence microscopic analysis of sections from matrigels. HSCs were labeled with PKH26 (red) before mixing with gels. ECs were visualized as green following staining with FITC-conjugated PECAM-1 mAb. Scale bar: 50 μm.

(C) Migration of ECs induced by Ang1 or HSCs. (a) Cells from total embryos at E10.5 stained with anti-CD45, -PECAM-1, and -TIE2 mAbs were analyzed by flow cytometry. After gating the $CD45^-$ fraction (left panel), $CD31^+TIE2^+$ cells (indicated by box in right panel) or $CD31^-TIE2^-$ cells used as negative controls were sorted. (b) Diluted Ang1* (0–500 ng/ml) was applied to the lower chamber. A total of 4×10^2 cells/well were seeded into the upper chamber, and cells were allowed to migrate for 4 hr. (c) $CD45^+$c-Kit^+CD34^+ cells or $CD45^+$c-Kit^-CD34^- cells were sorted as above (Fig. 5C) and seeded into the lower chamber in the presence or absence of the factors indicated. A total of 4×10^2 $CD45^-CD31^+TIE2^+$ ECs were applied to upper chamber and allowed to migrate for 4 hr.

and hematopoietic cells are primary or secondary. Recently, an analysis of *Scl* gene function was reported (Visvander et al., 1998). Through transgenic rescue of hematopoietic defects of *Scl* mutant embryos and analysis of chimeras generated with *Scl* null ES cells tagged with a transgene expressed in vascular ECs, SCL was shown to affect EC development primarily.

AML1 encodes the DNA binding subunit of a transcription factor of the core binding factor family. Members of this family are thought to regulate expression of several genes, such as the T cell antigen receptor, myeloperoxidase, granulocyte-macrophage colony stimulating factor, interleukin-3, and colony stimulating factor receptor 1 (Tenen et al., 1997). Consistent with these observations, homozygous disruption of the *AML1* gene severely impairs definitive hematopoiesis but does not affect primitive erythropoiesis (Okuda et al., 1996; Wang et al., 1996; Okada et al., 1998). The reason for the lethal hemorrhage observed in *AML1* mutant embryos is not understood. Initially, platelet deficiency was thought to lead to hemorrhage; however, mice mutant in the *NF-E2* gene exhibit severe defects in platelet development but do not hemorrhage at early embryonic stages (Shivdasani et al., 1995b). Recently, it has been reported that AML1 is expressed in ECs in sites where early HSCs are thought to emerge, such as the yolk sac, vitelline and umbilical arteries, and the dorsal aorta in the AGM region (North et al., 1999). An autonomous effect in ECs by *AML1* gene disruption has been suggested to cause hemorrhage; however, AML1 expression in ECs is not observed in the brain and heart where severe abnormalities in angiogenesis are observed in *AML1* deficient embryos. Impaired angiogenesis in *AML1* mutants was clearly rescued by HSCs in the P-Sp culture system. This finding suggests that disorganized EC development and hemorrhage seen in *AML1* mutant embryos are secondary effects due to a lack of definitive HSCs.

HSCs Promote Vessel Sprouting

An important question in vasculo-angiogenesis, especially in vessel sprouting, is how ECs migrate to sites where tissues require nutrients or oxygen. Although findings shown here indicate that ECs migrate toward Ang1 producing HSCs, the basic mechanism of how HSCs migrate from the intraluminal cavity into parenchymal cells at a restricted point of a vessel is unclear. A report that peripheral $CD34^+$ hematopoietic progenitors express high levels of matrix metallo-proteinases (MMP)-2 and -9 (Janowska-Wieczorek et al., 1999) may shed light on this mechanism. Our preliminary data also show that embryonic HSCs ($CD45^+$c-Kit^+CD34^+ cells) express MMP-9 strongly (data not shown). Moreover, these HSCs express TIE2 and adhere to fibronectin (FN) following stimulation by Ang1 (Takakura et al., 1998). Taken together, these results suggest that HSCs adhere to FN on ECs near the ischemic region, digest the matrix, and transmigrate through the basement membrane of capillary ECs into parenchymal cells. Therefore, we hypothesize that FN production on the intraluminal surface of ECs is the initial step in migration of HSCs and ECs.

Ang1 and VEGF may have important clinical applications in treatment of ischemia because of their strong angiogenic effects observed in cornea assays and transgenic mice. (Asahara et al., 1998; Suri et al., 1998). Soluble Ang1 rescues network formation in *AML1* mutant

mice in vitro; however, HSCs from embryos and adult mice could promote vascular network formation more effectively than soluble Ang1. In transgenic mice expressing Ang1 in the skin under control of the K14 keratinocyte specific promoter, hypervascularity was observed in the dermis but not in other organs, indicating a localized effect of Ang1 (Suri et al., 1998). Administration of Ang1 results in widespread stimulation of $TIE2^+$ ECs, while HSCs may promote localized angiogenesis. Therefore, findings presented here may have important clinical applications.

Experimental Procedures

Animals

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). AML1, c-Myb, or Ang1 mutant mice were generated as described (Mucenski et al., 1991, Suri et al., 1996; Okada et al., 1998). Transgenic mice with GFP (gift from Dr. M. Okabe, Osaka University, Osaka, Japan) were generated as described (Okabe et al., 1997). Animal care in our laboratory was in accordance with the guidance of Kumamoto University for animal and recombinant DNA experiments.

In Vitro Culture of P-Sp and Hematopoietic Progenitors

P-Sp culture conditions were as described (Takakura et al., 1998). In brief, P-Sp explants of E9.5 embryos were cultured on OP9 stromal cells (Nakano et al., 1994) in RPMI1640 (GIBCO BRL, Gaithersburg, MD) with 10% fetal calf serum (FCS; JRH Bioscience, Lenexa, KS) and 10^{-5} M 2ME (Sigma, St. Louis, MO) supplemented with IL-6 (20 ng/ml), IL-7 (20 ng/ml) (gifts from Dr. T. Sudo, Toray Industries Inc., Kamakura, Japan), SCF (50 ng/ml) (a gift from Chemo-Sero-Therapeutic Co., Ltd., Kumamoto, Japan), and Epo (2 U/ml) (a gift from Snow-Brand Milk Product Co, Tochigi, Japan) at 37°C in humidified 5% CO_2 air. $3x10^2$ sorted HSCs were added to P-Sp cultures 4 days after initiation of the culture. 300 ng/ml Ang1* (a gift from Dr. G. D. Yancopoulos, Regeneron Pharmaceuticals, Inc., Tarrytown, NY) was added to P-Sp cultures of wt and *AML1* mutant embryos. Ang1 used in in vitro studies was a recombinant version of Ang1 with a modified NH_2 terminus and mutated Cys^{245} and has been designated Ang1* (Maisonpierre et al., 1997).

The in vitro colony assay for hematopoietic progenitors was performed as described previously (Takakura et al., 1996).

Immunohistochemistry

An anti-PECAM-1 antibody (MEC13.3, Pharmingen, San Diego, CA), anti-Flk-1 antibody (AVAS12α1,Pharmingen), anti-TIE2 antibody (TEK4) (Yano et al., 1997), horseradish peroxidase (HRP)-conjugated anti-α smooth muscle actin antibody (1A4, DAKO, Glustrup, Denmark), anti-Ang1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-CD45 or biotinylated anti-CD45 antibody (30-F11, Pharmingen), were used for staining of tissues. First antibodies were developed with HRP-conjugated anti-rat or anti-goat Ig antibody (Biosource, Camarillo, CA), respectively. Biotinylated antibodies were developed with alkaline phosphatase (ALP) conjugated streptavidin (DAKO). Diaminobenzidine (DAB; Dojin Chem., Kumamoto, Japan) or 3-amino-9-ethyl carbazole (AEC; DAKO) was used for the HRP color reaction. New fuchsin (DAKO) or 5-bromo-4-chloro-3-indoxyl phosphate/ nitro blue tetrazolium chloride (BCIP/NBT; Boehringer Mannheim, Mannheim, Germany) was used for the ALP color reaction. Procedures for immunohistochemistry have been described elsewhere (Takakura et al., 1998).

Cell Preparation and Flow Cytometry

Embryos were staged by somite counting. AGM or P-Sp was isolated using the methods described (Godin et al., 1995). Methods for cell preparation were as described previously (Takakura et al., 1998). The cell-staining procedure for flow cytometry was as described previously (Takakura et al., 1996). The mAbs used in immunofluorescence staining were anti-CD45, -CD34, -c-Kit (2B8), -PECAM-1, -Sca-1 (E13–161.7), Mac-1 (M1/70), Gr-1 (RB6–8C5), B220 (RA3–6B2), anti-CD4 (L3T4), -CD8 (53–6.72), and Ly-76 (TER-119), all of which were purchased from Pharmingen. A mixture of Mac-1, Gr-1, B220, anti-CD4, -CD8, and Ly-76 was used as a lineage marker (Lin). Also used was anti-TIE2 (TEK4). All mAbs were purified and conjugated with FITC, PE, or biotin. Biotinylated antibodies were visualized with PE- (GIBCO BRL), FITC- (Sigma), or APC- (Caltag, Burlingame, CA) conjugated streptavidin. The stained cells were analyzed and sorted by FACSvantage (Becton Dickinson, San Jose, CA).

Limiting Dilution Assay

Sorted cells were cultured on OP9 cells in the media as described in P-Sp culture. Cultures were scaled down to 20, 10, 5, 3, 2, 1, and 0.5 in 0.1 ml for a limiting dilution assay. When the recovered cells are composed of at least three lineages, such as erythroid, myeloid, and lymphoid cells, we score these as positive wells containing multipotential hematopoietic progenitors.

RT-PCR Analysis

Procedures for RT-PCR analysis have been described elsewhere (Takakura et al., 1998). Sequences of specific primers used in RT-PCR of mAng1 and mβ actin were as described (Takakura et al., 1998).

To amplify mVEGF and LacZ, forward primer was 5′CTTCCT ACAGCACAGCAGATGTGAA3′ and the reverse was 5′TGGTGACA TGGTTAATCGGTCTTTC3′ for VEGF, and forward primer was 5′GCGTTACCCAACTTAATCG3′, and the reverse was 5′ TGTGAGC GAGTAACAACC3′ for LacZ, respectively. Each cycle consisted of a 30 s denaturation at 94°C, 2 min of annealing at 64°C, and a 2 min extension at 72°C.

In Vivo Neovascularization Using Matrigel

Preparation, injection, and processing of matrigel (Becton Dickinson, Bedford, MA) were as described (Passaniti et al., 1992) with some modifications. Briefly, 8-week-old C57BL mice were injected subcutaneously with 0.5 ml matrigel and 37 units heparin/ml (Sigma), 20 ng VEGF/ml (Pepro Tec, London, UK), 50 ng SCF /ml, and $2x10^4$ $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells from the bone marrow of 8-week-old C57BL mice in the presence or absence of soluble TIE2 receptors (20 μg/ml), or CD4-Fc chimeric protein as a control (20 μg/ml) near the abdominal midline using a 25-guage needle. HSCs were labeled with PKH26 (PKH26 red fluorescent cell linker mini kit, Sigma) before mixing with matrigel. Matrigels were dissected on day 4 from mice, and gel sections were stained with FITC-conjugated anti-PECAM-1 mAb (Pharmingen).

Cell Migration Assay

EC migration assays were performed using a 48-well microchemotaxis chamber (Neuroprobe). Tweny-five microliters of Ang1* diluted in serum free media (SA, a gift from Chemo-Sero-Therapeutic Co., Ltd) or sorted HCs suspended in SA (10^4/ml or 10^5/ml) in the presence or absence of CD4-Fc, TIE2-Fc, Flk-1-Fc (Takakura et al., 1998), or Ang2 (gift from Dr. G. D. Yancopoulos) were placed in the lower chamber of a Boyden chamber. After plating polycarbonate filter with a pore size of 5 μm (Nucleopore; Costar, Cambridge, MA) between lower and upper chamber, $4x10^2$ sorted ECs suspended in 50 μl of SA medium containing 0.5% BSA were seeded in the upper compartment. The migrated cells were counted under microscope after staining with Giemsa solution.

Acknowledgments

We thank Drs. Michael L. Mucenski, Masaru Okabe, and G. D. Yanchopoulos for providing us with c-Myb mutant mice, GFP transgenic mice, and Ang1 mutant mice, respectively. This work was supported in part by grants-in-aid for scientific research (06277104 and 10470211) from the Ministry of Education, Science, and Culture of Japan.

Received November 17, 1999; revised June 1, 2000.

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