Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development

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The transcription factor SCL/tal-1 is essential for blood cell development. Though it is also expressed in vascular endothelium, SCL has been considered dispensable for vessel formation. Through transgenic rescue of hematopoietic defects of SCL−/− embryos and analysis of chimeras generated with SCL−/− ES cells tagged with a transgene expressed in vascular endothelial cells, we show that SCL is essential for angiogenic remodeling of the yolk sac capillary network into complex vitelline vessels. These findings establish a role for SCL in embryonic angiogenesis and argue for critical functions in both embryonic blood and vascular cells, the descendents of the presumptive hemangioblast.

[Key Words: SCL/tal-1 gene; hematopoiesis; vascular development; tie-2 gene]

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Blood cell formation and vasculogenesis initiate at ∼E7.5 of mouse development in the yolk sac blood islands. The close temporal and spatial relationship of hematopoiesis and vascular development is consistent with the existence of a postulated common progenitor, the hemangioblast (Pardanoud et al. 1989). The SCL/tal-1 gene [hereafter termed SCL (stem cell leukemia)], which was originally identified through its translocation in acute T-cell lymphoblastic leukemia (Begley et al. 1989; Finger et al. 1989; Chen et al. 1990), encodes a basic helix–loop–helix transcription factor that is expressed specifically in hematopoietic cells (Green et al. 1991; Visvader et al. 1991), vascular endothelium (Hwang et al. 1993; Kallianpur et al. 1994), and the developing brain (Green et al. 1992). Embryos lacking SCL fail to develop past E9.5 because of the absence of yolk sac erythropoiesis (Robb et al. 1995; Shivdasani et al. 1995) and SCL−/− embryonic stem (ES) cells do not contribute to any hematopoietic lineages in chimeric mice (Porcher et al. 1996; Robb et al. 1996). Hence, SCL is believed to function either in programming commitment of ventral mesoderm to a hematopoietic fate or in the generation of hematopoietic pluripotential stem cells. From the presence of vascular endothelial cells in SCL−/− embryos it has been inferred that SCL is dispensable for vascular cell specification (Robb et al. 1995; Shivdasani et al. 1995).

To explore the potential involvement of SCL in non-hematopoietic pathways, we sought to rescue the hematopoietic defect in SCL−/− embryos by introducing an SCL cDNA–transgene specifically expressed in the hematopoietic compartment. By this approach, we uncovered an unsuspected but essential role for SCL in embryonic vessel development. By specifically tagging SCL−/− ES cells with an endothelial-expressed lacZ gene, the fate of SCL−/−-endothelial cells was tracked in chimeras. This strategy provides evidence for a primary role for SCL in angiogenesis, a process entailing the proliferation and remodeling of pre-existing endothelial cells into a mature vascular network.

Results

Transgenic rescue of hematopoiesis

To rescue the hematopoietic component of the SCL/tal-1 knockout (Robb et al. 1995; Shivdasani et al. 1995), we first generated transgenic mice harboring SCL cDNA sequences under the control of regulatory sequences of the GATA-1 gene (McDevitt et al. 1997) (Fig. 1), as its hematopoietic expression profile mimics that of SCL (Visvader et al. 1991; Green et al. 1992). Of four founders, two independent lines of transgenic mice (GATA-1/SCL-12 and GATA-1/SCL-52) expressed transgene-derived RNA at a level closely approximating that of the endogenous SCL mRNA in yolk sac (Fig. 2). SCL−/− embryos carrying the GATA-1/SCL transgene were generated by interbreeding SCL−/− Tg(GATA-1/SCL-12 or GATA-1/SCL-52) mice. Whereas SCL−/− yolk sacs are devoid of any visible blood (Fig. 1c), hematopoiesis is substantially rescued in SCL−/−Tg yolk sacs, evident as diffuse red color (Fig. 1b). SCL−/−Tg embryos, however,
remained pale and growth retarded. Like SCL−/− embryos (Robb et al. 1995; Shivdasani et al. 1995), they failed to survive beyond E9.5 because of the vascular defects described below.

Blood cells present in the yolk sacs of SCL−/− Tg embryos appeared morphologically normal (Fig. 1d,e), indicating rescue of primitive erythropoiesis. Yolk sacs contained progenitors capable of generating multiple lineages, including erythroid, myeloid, mast, and megakaryocytic cells in hematopoietic colony assays (Shivdasani et al. 1995) (Fig. 1f–i). As colonies derived from yolk sacs at this stage are of the definitive (or adult) type, we conclude that definitive hematopoiesis is also restored by SCL expression. Rescue of multiple lineages by the GATA-1/SCL transgene suggests that the transcription factors GATA-1 and SCL genes act at a similarly early point within the hematopoietic regulatory hierarchy, an inference consistent with their expression during embryogenesis (Silver and Palis 1997). Rescue, however, was quantitatively incomplete. The mean colony number per SCL−/− Tg yolk sac (based on four experiments) was 3.8-fold less for erythroid colonies/BFU-E (37 ± 8 for −/− Tg vs. 144 ± 50 for control cells, +/+ or +/+), 2.9-fold less for myeloid colonies (101 ± 50 vs. 249 ± 110) and 4.5-fold less for mixed colonies (41 ± 7 vs. 186 ± 30). The reduced potential of the SCL−/− Tg yolk sac cells to form erythroid colonies was also reflected in their relative levels of c, α, and β globin RNA (Fig. 2; data not shown). The inability to achieve full rescue may be attributable to improper timing or regulation of SCL transgene expression imposed by the GATA-1 control sequences.

SCL−/− and SCL−/− Tg yolk sacs lack vitelline vessels

The pattern of vasculature within the SCL−/− Tg yolk sacs was striking (Fig. 1b). Rather than the distinctive network of branching vitelline vessels visualized in normal embryos (Fig. 1a), a disorganized array of capillaries was observed, a finding corroborated by whole-mount staining with anti-PECAM antibodies, a specific marker of differentiated endothelial cells (Baldwin et al. 1994). The absence of vitelline vessels, but presence of an interconnecting network of smaller vessels, was also noted for SCL−/− yolk sacs (Fig. 3a–c). Individual endothelial cells of control and SCL−/− yolk sacs, however, stained similarly for PECAM (Fig. 3d–f) and CD34 (Fig. 3g,h), another marker of differentiated endothelial cells (Baumhueter et al. 1994). Both the endothelial and endodermal cell layers, as well as the smooth muscle/pericytes that constitute the lining of vessels, appeared intact by histological analysis (Fig. 3i,j) and electron microscopy (data not shown). Taken together, these data indicate that specification of vascular cells proceeds normally in the absence of SCL but that angiogenesis is defective, either as a primary or secondary consequence of the deficiency. As to be expected, this defect persists in the SCL−/− Tg embryos, as regulatory sequences of the GATA-1-based transgene are inactive in vascular cells. Histological analysis of the SCL−/− and SCL−/− Tg embryos proper suggested that the vasculature may be more disorganized than in wild-type littermates, but severe growth retardation and necrosis by E9.5 precluded a detailed analysis. We speculate that the SCL−/− Tg embryos die by E9.5 due to the absence of connecting blood vessels between the yolk sac and embryo.

Endothelial receptor tyrosine kinases are expressed in SCL−/− yolk sacs

Several genes are essential for the development and ini-
integrity of the yolk sac vascular system (Risau 1997). These include receptor tyrosine kinases and their ligands. The transcriptional status of several of these genes was evaluated by RT-PCR to search for potential SCL target genes in endothelial cells (Fig. 2; data not shown). The expression of the endothelial RTKs, Flk-1, Tie-1, and Flt-4, in SCL−/− yolk sacs was comparable to that observed in normal yolk sacs, although Tie-2 and Flt-1 RNA levels appeared to be diminished slightly (about two fold). These findings suggest that the number of endothelial cells in mutant yolk sacs is approximately the same as in wild type. The levels of TEL (Wang et al. 1997) and Ga13 (Offermanns et al. 1997) transcripts also appeared normal. Hence, none of the above genes are likely to be essential early targets of the SCL transcription factor in vascular endothelium.

Aberrant vasculature in SCL−/−/ROSA chimeras

Although it remained formally plausible that the angio-
genic defect was merely secondary to the absence of blood in SCL−/− embryos, it seemed improbable for at least two reasons. (1) The defect was evident in yolk sacs displaying substantial hematopoietic rescue; and (2) other hematopoiesis-defective embryos, such as those lacking GATA-1 or GATA-2 (Shivdasani and Orkin 1996), have normal vasculature, despite a marked decrease in blood volume. To distinguish between primary or secondary effects of SCL loss on angiogenesis, we generated two different types of chimeras. Because ES cells often display a marked bias in tissue distribution following blastocyst injection, we produced chimeras with either marked ES cells or blastocysts to follow the fate of donor SCL−/− ES cells in developing yolk sacs.

SCL−/− ES cells were first injected into blastocysts of β-galactosidase (lacZ)-expressing ROSA26 mice (Zambrowicz et al. 1997). The degree of chimerness was estimated by lacZ staining of sections through E9.5 chimeric yolk sacs (data not shown). Of particular note, we observed that the level of contribution by the SCL−/− cells correlated with the vascular, as well as the hematopoietic, phenotypes of the yolk sacs. Typical phenotypes of SCL−/−/ROSA chimeras are shown in Figure 3.
4a-c. Yolk sacs estimated to be \(~80\%\) chimeric (Fig. 4a) were pale and exhibited a patchy, disorganized network of capillaries lacking vitelline vessels, thereby recapitulating the SCL\(^{-/-}\) Tg vascular phenotype. Although a more well-developed vascular network was evident in yolk sacs exhibiting \(~20\%\) chimerism (Fig. 4b), the vessels were uniformly dilated with no clear distinction between large and small vessels. Conversely, vitelline vessels were readily visualized in low chimeric (\(<5\%\) ) yolk sacs (Fig. 4c). The analysis of SCL\(^{-/-}\)/ROSA chimeras, therefore, suggests that the presence of SCL\(^{-/-}\) cells in the developing yolk sac leads to abnormal vessel formation.

Tagged SCL\(^{-/-}\) endothelial cells do not contribute to large vessels in chimeras

To investigate a primary role for SCL in angiogenesis, we generated a second type of chimera using wild-type blastocysts and SCL\(^{-/-}\) ES cells harboring a Tie-2–lacZ reporter gene (Schlaeger et al. 1997) (Fig. 4, bottom). The Tie-2–lacZ transgene directs uniform and high-level expression to all endothelial cells. In control experiments we demonstrated that SCL\(^{-/+}\)/Tie-2–lacZ cells readily contributed to both small and large vessels of the developing yolk sac vasculature (Fig. 4d,e; note solid arrow in e). In contrast, SCL\(^{-/-}\)/Tie-2–lacZ cells contributed efficiently to small capillaries but were not observed in vitelline vessels of multiple chimeras of varying extents generated from three different marked SCL\(^{-/-}\) ES cell clones (Fig. 4f-k; note open arrow in i). In addition, the aberrant vascular architecture observed in whole yolk sacs containing a high proportion of SCL\(^{-/-}\)-derived endothelial cells paralleled the phenotypes observed for SCL\(^{-/-}\) and SCL\(^{-/-}\) Tg embryos (Fig. 4f,g), in which only small capillaries were detectable. These data provide direct evidence for an intrinsic defect in the capacity of SCL\(^{-/-}\) endothelial cells to participate in the formation of large vessels during the process of angiogenesis.

Discussion

Our goal in the studies reported here was to identify nonhematopoietic roles of SCL in development. To this end, we used cell-specific transgenic rescue to reveal functions obscured by early embryonic lethality of a knockout embryo. The relatively efficient rescue of yolk sac hematopoiesis by a transgene driven by GATA-1 regulatory sequences suggests that GATA-1 and SCL act at a similarly early point within the hematopoietic regulatory hierarchy. Furthermore, GATA-1 gene-based constructs may be useful in the rescue of hematopoietic defects of other knockout embryos. Transgenic rescue provided initial evidence for a vascular defect in the absence of SCL, a finding corroborated and extended by chimera studies, particularly employing SCL\(^{-/-}\) ES cells tagged with a Tie-2–lacZ reporter gene.
Blood vessels develop by two distinct processes, vasculogenesis and angiogenesis. Vasculogenesis involves the differentiation of endothelial cells from their mesodermal precursors and the subsequent assembly into a primary capillary plexus. During angiogenesis, these primary vessels are remodeled into a larger, more complex network. Although SCL appears to be dispensable for initial specification and proliferation of endothelial cells, the present work demonstrates that it is required for formation of branching vitelline vessels from a primary vascular plexus. Furthermore, the vascular defects associated with loss of SCL appear to be endothelial-specific, as shown by the Tie-2-lacZ transgene experiments and ultrastructure analysis indicating that the mesenchyme/smooth muscle layer is intact (data not shown).

Several genes encoding receptor tyrosine kinases (RTKs), such as Flk-1 (Shalaby et al. 1995, 1997), Flt-1 (Fong et al. 1995), Tie-1 (Puri et al. 1995; Sato et al. 1995), and Tie-2 (Dumont et al. 1994; Sato et al. 1995); and the ligands, such as vascular endothelial growth factor (VEGF) (Carmeliet et al. 1996a; Ferrara et al. 1996), angiopoietin-1 (Ang-1) (Suri et al. 1996), and tissue factor (TF) (Carmeliet et al. 1996b), are important for proper yolk sac vascular development (Risau 1997). Targeted disruption of these genes has provided insights into intracellular signaling mechanisms that govern blood vessel formation. However, none of these genes, nor the transcription factor TEL (Wang et al. 1997) or the G protein Gα13 (Offermanns et al. 1997), appear to be early targets of SCL, as shown by RT-PCR analysis.

The SCL−/− vascular defect most closely resembles that of embryos lacking Tie-2 (Dumont et al. 1994; Sato et al. 1995). Tie-2 has direct effects on the endothelium but has also been implicated in angiogenesis by virtue of an activating mutation that causes vascular dysmorphogenesis (Vikkula et al. 1996). Although targeted disruption of several other genes (Risau 1997), such as Ang-1, VEGF, TF, and 5′-hydroxycarbon receptor nuclear translocator (ARNT) (Maltepe et al. 1997), leads to similar yolk sac phenotypes, these seem to reflect aberrant interactions/signaling between the endothelium and surrounding mesenchymal cells. In contrast, SCL is required in a cell autonomous fashion for remodeling of endothelial cells into a mature vascular network.

Taken together with earlier studies, our findings establish a dual function for SCL in hematopoiesis and vascular development (Fig. 5). The close relationship between hematopoiesis and vasculogenesis in the developing yolk sac blood islands is consistent with the existence of a common progenitor, the hemangioblast (Paradanoud et al. 1989). The requirement for the receptor Flk-1 in both hematopoiesis and vasculogenesis (Shalaby et al. 1995, 1997) is compatible with this model. Although our current data do not bear directly on the existence of the hemangioblast, they do establish SCL as having a unique regulatory function, positioned at the interface between blood and vascular development. Identifying critical target genes for SCL in vascular endothelial cells should elucidate novel angiogenic pathways and their possible relationship to those already defined.

Materials and methods

Constructs and transgenic mice production

A 1.4-kb murine SCL cDNA fragment was linked to the rabbit ß-globin polyadenylation region and a 900-bp fragment spanning the SV40 early intronic region and polyadenylation signal (Lindeman et al. 1995), which also allow transgene detection, before insertion into a vector containing regulatory sequences derived from the murine GATA-1 gene (McDevitt et al. 1997). The final insert, released by SalI digestion, was introduced by pronuclear injection into fertilized eggs derived from a CD1 strain (Charles River). Southern blot analysis with the 900-bp SV40 fragment was used to identify transgenics; progeny tail DNA was subsequently typed by PCR using SV40 primers. Two independent transgenic lines were established and were crossed with SCL heterozygous mice (129 × C57BL/6N) (Shivdasani et al. 1995) to produce SCL−/−;Tg embryos. Two independent transgenic lines were established and were crossed with SCL heterozygous mice (129 × C57BL/6N) (Shivdasani et al. 1995) to produce SCL−/−;Tg embryos. A 15-kb Tie-2-lacZ insert was isolated from pT2HlaicZPA11.7 (Schlaeger et al. 1997) by Sall digestion and ligated to a fivefold molar excess of Xhol-digested PGK-puromycin cassette (2.1 kb) for 2 hr at 16°C before final purification. The DNA was electroporated into either SCL−/− or SCL−/−;Tg ES cells; the SCL−/−;Tg had also undergone two rounds of G418 selection to provide a control line (Porch et al. 1996). Puromycin-resistant clones were typed by Southern blot analysis and PCR for detection of the lacZ insert.

Yolk sac progenitor assay

These were performed essentially as described (Shivdasani et al. 1995). The yield for each collagenase-treated yolk sac (E9.5) was 1 × 10⁴ to 5 × 10⁴/ml. The disaggregated cells were plated in α-minimal essential medium supplemented with α methylcellulose, 30% FCS, 1% BSA, and 2 U/ml of erythropoietin, 50 ng/ml of recombinant c-kit ligand, 5 ng/ml of IL-11, and 10 ng/ml of IL-3.

Semiqualitative RT–PCR

RNAs were isolated from individual E9.5 yolk sacs and genomic DNA from the embryos used to confirm their genotype. RT-
PCR was performed as described (Weiss et al. 1994), using two sets of primers, one representing the specific gene and the other HPRT. In the case of SCL, 10% dimethylsulfoxide was included in the reactions. The sequence of the oligonucleotide primers has been described elsewhere (SCL (Porcher et al. 1996), HPRT and globins (Weiss et al. 1994), and Tel, Flk-1, Tie-2, and other RTKs (Carmeliet et al. 1996). Relative quantifications versus HPRT were performed using a PhosphoImager (Molecular Dynamics). Control experiments without reverse transcriptase in the cDNA synthesis reactions did not show these specific PCR products.

Immunohistochemistry and histology

Whole-mount immunohistochemistry using anti-PECAM monoclonal antibody MEC13.3 (Pharmingen) was performed essentially as described (Schlaeger et al. 1995). The stained yolk sacs were postfixed in 4% paraformaldehyde, embedded in paraffin wax, and sectioned at 8 µm. Affinity purified polyclonal anti-CD34 antibody (Baumhueter et al. 1994) was used to stain 10-µm cryosections of dissected E9.5 embryos and yolk sacs that had been previously fixed with 4% paraformaldehyde. Ten-micrometer sections were used for immunohistochemical staining: alkaline phosphatase staining was used for antibody detection.

Generation and analysis of chimeras

Chimeras were generated by blastocyst injection as described (Hogan et al. 1994). Mice carrying the ROSA26 gene trap integration (Zambrowicz et al. 1997) were obtained from Jackson Laboratories [Tg(R(RosA26)RSoR) strain]. For the ROSA chimera experiments, blastocysts were collected from matings between homozygous ROSA26 and C57BL/6N TactcBR (Tacomin) mice. A maximum of 16–18 ES cells were injected into the blastocyst cavity. Chimeric percentages were estimated by β-galactosidase staining of yolk sacs. For Tie-2-lacZ transgenic ES cell experiments, blastocysts were collected from matings between C57BL/6N TactcBRs (Taconic). To produce relatively low chimeric embryos, the number of ES cells injected into the blastocyst cavity was, on average, 8–10. Following transfer into pseudopregnant foster females, the embryos were recovered at day 9.5 or 10.5 of development. Embryos were fixed and processed for β-galactosidase as described previously (Hogan et al. 1994). After staining, embryos and their yolk sacs were postfixed with 4% paraformaldehyde and embedded in paraffin wax. Samples were sectioned at 8 µm and counterstained with neutral red.

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