Brief report

Dual roles of the C-terminal Src kinase (Csk) during developmental vascularization

Li-Juan Duan, Akira Imamoto, and Guo-Hua Fong

Here we report that C-terminal Src kinase (Csk), a tyrosine kinase that negatively regulates the activity of Src and related kinases, is important for vascular development. In Csk−/− embryos, although vascular tubules were formed and organized into capillary-like networks during the initial genesis of blood vessels, the vessels failed to engage in normal sprout formation. In chimeric embryos containing both wild-type and Csk−/− cells, the presence of wild-type cells enabled Csk−/− endothelial cells to participate in branching morphogenesis. We suggest that wild-type cells may have supplied an angiogenic factor absent in Csk−/− cells. Despite the partial rescue of vascular development in chimeric embryos, the embryos failed to form vitelline vessels and died at E9.5. These results indicate that Csk is required both for angiogenic sprouting and vascular remodeling.

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Introduction

The development of blood vessels and vascular network consists of multiple steps, including endothelial cell differentiation, de novo assembly of vascular tubules from differentiating endothelial cells, sprout formation, and the development of large vessels by the growth and merging of smaller blood vessels.1-3 Although several endothelial cell surface receptors are known to be important for vascular development,4-8 less is known about the intracellular signaling molecules that mediate these processes.

Tyrosine kinases Src, Fyn, and Yes are important regulators of cell growth, differentiation, and migration in many cell types, including endothelial cells.9-14 Surprisingly, knock-out mice for these kinases do not display developmental defects in the vascular system.15,16 One possibility is that different members of the Src family kinases have redundant functions. The C-terminal Src kinase (Csk) negatively regulates the kinase activity of multiple members of the Src family by phosphorylation of a specific tyrosine residue located at the C-termini of Src or related kinases, and it has been shown to be essential for embryonic development.9,11 However, its role in the vascular system is unclear. In this study, we investigated the role of Csk in regulating the development of the vascular system.

Study design

All mice used in this study, including Csk+/+, Flk-1+/+, and Flt-1+/+ mouse lines, were maintained in CD1 background. The “−/−” alleles of Flk-1−/− or Flt-1−/− mice carry a lacZ knock-in sequence, whose expression has been shown to specifically label endothelial cells.6,7 To facilitate the visualization of blood vessels, Csk+/− × Flk-1+/−, or Csk+/− × Flt-1+/− crosses were carried out and healthy Csk+/+, Flk-1+/+ or Csk−/−, Flt-1−/− mice were obtained. Csk−/−, Flk-1−/− or Csk+/+, Flt-1−/− embryos were obtained by the following crosses: Csk−/−, Flk-1−/− × Csk+/−, or Csk+/−, Flt-1−/− × Csk+/−.

Genotyping for Csk−/−, Flk-1−/−, or Flt-1−/− targeted alleles were previously described.7,10,17 To visualize blood vessels, X-gal staining or immunohistochemistry (IHC) was performed as previously described.17 Expression of Csk was analyzed by anti-Csk IHC (rabbit anti-Csk antibody was kindly provided by J. Cooper, Fred Hutchinson Research Institute, Seattle, WA).18 To prepare morula embryos needed for chimeric aggregation, Csk−/− females were superovulated and mated with Csk+/−, Flt-1−/− males. Morula stage embryos were collected, and each 8-cell stage morula was aggregated with 8 to 12 R1 embryonic stem (ES) cells by established methods.19

Results and discussion

To investigate the role of the C-terminal Src kinase during vascular development, we examined Csk−/− null mutants generated by homologous recombination in a previous study.10 Analysis of vascular morphogenesis at E8.2 revealed that at the gross level the genesis of the paired dorsal aorta, sinus venosus, and endocardial tubes in Csk−/− embryos was normal.

In contrast, although vessels and a capillary-like vascular network were present in the yolk sacs of E8.2 Csk−/− mice, the blood vessels appeared larger than normal. Conspicuous in Csk−/− yolk sacs was the absence of interconnecting vessels that contribute to the polygonal appearance of vascular networks in normal embryos. The absence of Csk allows wild-type cells to engage in normal sprouting and merging of smaller blood vessels.1-3 Although several vascular morphogenesis at E8.2 revealed that at the gross level the genesis of the paired dorsal aorta, sinus venosus, and endocardial tubes in Csk−/− embryos was normal.

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vessels were observed to have further increased (Figure 1C-F). In conjunction with the increased size of the yolk sac vessels, there was a marked decrease in the number of blood vessels as compared with healthy normal littermates.

In the embryo proper, the development of intersomitic vessels was significantly retarded, in contrast to the active sprouting of these vessels from the paired dorsal aortae in Csk+/+ or Csk+/− embryos (Figure 1G-H). Interestingly, branching morphogenesis of the endocardium was also affected by Csk−/− mutation (Figure 1J). These results indicate that angiogenic sprouting is a Csk-dependent process.

Consistent with a role in vascular development, Csk is expressed in endothelial cells. Figure 1E and K depict Csk expression in endothelial cells of healthy embryos (yolk sacs) immunolabeled by using anti-Csk antibody (kindly provided by J. Cooper, Fred Hutchinson Research Institute).28 As our findings indicated a decrease in the number and density of blood vessels in the yolk sac, we speculated that the basis of this phenomenon might lie in a reduction in endothelial cell numbers. Analysis of endothelial cell proliferation and apoptosis between wild-type and Csk−/− mutants (data not shown), however, revealed that there were no differences in endothelial cell proliferation or apoptosis. This finding, and our finding of increased vascular sizes that accompany diminished vascular sprouting in Csk−/− embryos, raises the possibility that endothelial proliferation, which in healthy embryos contributes to sprouting, may have contributed to the increase in vessel size instead of sprouting in Csk−/− embryos.

One possible function of Csk may be to regulate the expression of one or more secreted factors required for normal vascular morphogenesis. If that is the case, chimeric embryos containing both wild-type and mutant endothelial cells should develop with relative normalcy, as the wild-type cells in a chimera are expected to supply the factor(s) missing in Csk−/− null embryos. Further, if chimeras indeed survive longer than Csk−/− embryos, they might also reveal additional Csk functions at later developmental stages that could not be evaluated in Csk−/− null mutants, which die at E8.5. Thus, we constructed Csk+/−:Csk+/− chimeras, with Csk−/− endothelial cells marked by the lacZ allele from Flt-1−/− targeted locus. Five chimeric embryos were obtained at E9.5 that contained significant contribution by Csk−/−, Flt-1−/− cells. Examples of these embryos are shown in Figure 2B and D. The presence of X-gal–positive endothelial cells throughout the yolk sac vasculature (Figure 2B), in contrast to being limited to a small number of blood vessels, suggests that Csk−/− endothelial cells in chimeras actively participated in vascular sprouting. In the embryo proper of both wild-type and chimeric embryos, the intersomitic vessels were well formed, again indicating successful participation of Csk−/− endothelial cells in spraying activities (Figure 2D). Thus, Csk−/− endothelial cells are not intrinsically defective in spraying activities, but Csk−/− mutation may have caused the loss of one or more angiogenic factors. Unexpectedly, Csk−/− endothelial cells failed to form vitelline vessels in chimeras (Figure 2B), and embryonic growth was arrested at E9.5.
In conclusion, our data demonstrate the essentiality of Csk in vascular sprouting and strongly support a role for the Src signaling pathway in vascular development. In addition to sprouting defects, Csk<sup>−/−</sup> mutants also displayed increased vessel sizes in the yolk sac. Analyses of 2 knock-out (KO) mouse strains also exhibiting enlarged yolk sac vessels have demonstrated that impaired cardiac function results in hypoxia and a subsequent increase in vascular endothelial growth factor (VEGF) production that leads to enlarged vessels. In Csk<sup>−/−</sup> mice, however, increase in vessel size occurs between E8.2 and E8.5, before the heart begins to function in healthy embryos. This timing indicates that the formation of enlarged vessels may reflect a direct requirement for Csk. Defective vitelline vessel development in chimeras is difficult to explain in light of the rescue of intersomitic blood vessel formation by wild-type cells. The differing results likely reflect on the fact that the processes are distinct. Whereas intersomitic blood vessel formation is, for the most part, driven by sprouting, vitelline vessel formation involves a number of processes that together are embodied in the process of remodeling. During vascular remodeling, large vessels, such as vitelline vessels, are thought to form by merging of smaller vessels, and this process may require endothelial cell/cell, cell/matrix interactions, and/or endothelial migration.

Acknowledgments

We thank Ms Nancy Ryan for the preparation of histologic sections. A.I. is the co-corresponding author to whom all requests for Csk mice should be addressed.

References


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