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VEGF directs newly gastrulated mesoderm to the endothelial lineage ☆

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Abstract

Herein, we investigated the role of VEGF signaling in the earliest events in vasculogenesis and found that it exerts critical effects shortly after mesodermal cells form by gastrulation. We showed that VEGF treatment of embryos caused an increase in the population of newly gastrulated mesodermal (NGM) cells that express the transcription factor TAL1. This increase in TAL1-positive cells was attributed to VEGF induction of VEGF receptor-2 (Flk1)-positive NGM cells that would normally not have been induced due to the limited availability of VEGF in the NGM. Evidence that VEGF-mediated induction of NGM cells is relevant to the endothelial lineage is the finding that induced TAL1-positive cells in the NGM formed ectopic structures whose cells exhibited characteristics of endothelial cells, including the ability to integrate into the vascular network and express the QH1 antigen. Finally, we showed that VEGF-induced TAL1 expression in the NGM which resulted in the formation of ectopic structures was mediated by Flk1 but not Flt1 signaling. In summary, we have established that VEGF signaling is critical to allocation of NGM to the endothelial lineage.

Keywords: VEGF-A; TAL1/SCL; Flk1; Vasculogenesis; Angioblast; Hematopoiesis

Introduction

Vasculogenesis, the de novo formation of blood vessels, has several key components beginning with lineage formation from undifferentiated mesodermal cells followed by successive morphogenic events that culminate in the formation of a vascular epithelium (i.e., a blood vessel). Although vascular endothelial growth factor (VEGF) has been linked to all aspects of the morphogenic events associated with blood vessel formation, its precise role in endothelial lineage formation is uncertain. Genetic ablation of either VEGF or VEGF receptor-2 (VEGFR-2/Flk1) in mice leads to defective endothelial and hematopoietic lineage formation (Carmeliet et al., 1996; Ferrara et al., 1996). These defects highlight a lineage interrelationship that has long been a subject of investigation (Choi, 2002; de Bruijn et al., 2002; Jaffredo et al., 1998, 2000; Jordan, 1916; Munoz-Chapuli et al., 1999; Sabin, 1920). Indeed, endothelial cells and hematopoietic cells share expression of numerous proteins including CD31/PECAM (Newman and Albelda, 1992), CD34 (Fina et al., 1990), Tie1 and 2 (Dumont et al., 1995), and the bHLH transcription factor TAL1 (Kallianpur et al., 1994).

Along with Flk1, TAL1 is one of the earliest described markers of the endothelial lineage (Drake et al., 1997; Kallianpur et al., 1994). This and other findings implicate TAL1 in the regulation of the endothelial lineage. For example, ectopic expression of TAL1 in zebrafish and mice promotes the generation of the endothelial lineage (Gering et al., 1998, 2003). Additionally, a genetic mutation (cloche) that leads to Flk1 deficiency and a consequential failure in vasculogenesis could be rescued by forced expression of TAL1 (Liao et al., 1997, 1998). Experiments described herein further evaluated the relationship between VEGF

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signaling and TAL1 expression in the origins of the endothelial lineage.

Materials and methods

Quail microinjection, microsurgery, and ex ovo culture

Methods for microinjection, microsurgery, and ex ovo culture have been previously described (Drake and Little, 1995, Drake et al., 1992, 1997). Briefly, Japanese quail (*Coturnix coturnix japonica*) embryos at the 4–6 somite stage were removed from the yolk using paper rings, rinsed in phosphate-buffered saline (PBS) and placed ventral side up in 35-mm Petri dishes containing a bed of 5% agar. Approximately 25 nl of recombinant human vascular endothelial growth factor 165 ($rhVEGF_{165}$) (R&D Systems, Minneapolis, MN) at 0.5 mg/ml was microinjected into the interstitial space between the endoderm and splanchnic mesoderm using glass micropipettes. Stage-matched control embryos were cultured alongside experimental, microinjected embryos. To allow the contralateral side of an embryo to act as a control, microsurgery was performed on the VEGF-injected embryos. After injection on one side, as described above, embryos were washed in PBS to eliminate residual VEGF and then bisected along the midline. All embryos were cultured for either 7 or 14 h (37°C, 5% CO_2) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL/Life Technologies, Baltimore, MD) containing 10% chick serum (Gibco BRL/Life Technologies), 1% Penn/Strep, and 1% Glutamine (Gibco BRL/Life Technologies).

Explant culture

In vitro studies were conducted to discriminate between induction and migration as a mechanism for the increased number of TAL1-positive cells observed as a response to VEGF. Using a double-edged, carbon steel blade (Electron Microscopy Sciences, Ft. Washington, PA), small pieces of tissue from either the region containing NGM or more cranial regions lateral to the second somite were isolated. Tissue from the NGM region lacking endothelial cells was dissected so as to avoid any contaminating lateral tissues that contain blood vessels. Isolated tissues were cultured in the presence or absence of VEGF (1 μg/ml) for 7 h (37°C, 5% CO_2) in DMEM (Gibco BRL/Life Technologies) containing 10% chick serum (Gibco BRL/Life Technologies), 1% Penn Strep, and 1% Glutamine (Gibco BRL/Life Technologies).

Fixation, immunolabeling, preparation of sections, and their immunolabeling

Fixation, immunolabeling, laser scanning confocal microscopy (LSCM), and image processing of early stage avian embryos have been described previously (Drake et al., 1992, 1997). Following the culture period, embryos or cultured explants were rinsed in PBS and fixed in 4% paraformaldehyde (45 min, 25°C; Sigma, St. Louis, MO). After washing in PBS plus 0.01% azide (PBSA), the vitelline membrane was removed from embryos and both embryos and explants were permeabilized in absolute methanol (1 h, −20°C), rehydrated through a series of ethanol/water solutions (100%, 80%, 50%, 30%) and then blocked in 3% bovine serum albumin/DPBSA (8–12 h, 4°C). The embryos were washed and immunolabeled using QH1 hybridoma supernatant and rabbit anti-recombinant TAL1 antibody. TAL1 antibody was obtained from Stephen J. Brandt (Vanderbilt University and VA Medical Center, Nashville, TN). The QH1 hybridoma developed by F. Dieterlen was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. After three 30-min washes in PBSA, embryos and explants were immunolabeled with appropriate fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch Labs, Inc., West Grove, PA). Embryos were then mounted on glass slides under no. 0 cover slips (Fisher Scientific, Sewanee, GA) using an anti-photobleaching mounting medium (Giloh and Sedat, 1982).

To evaluate the morphology of VEGF-induced QH1/TAL1-expressing structures, quail embryos previously treated with VEGF for 14 h and evaluated by whole-mount immunofluorescence microscopy were further processed for cross-sectional analysis of the structures. Briefly, embryos were dehydrated through a series of graded ethanol: water solutions (30%, 50%, 80%, and 100%) for 30 min each. The embryos were infiltrated with 100% Histo-Clear (National Diagnostics, Atlanta, GA) for 2 h and then processed through a graded series of Histo-Clear/paraffin solutions (3:1, 1:1, 1:3) for 15 min each. The embryos were finally infiltrated with 100% paraffin two times for 1 h each, embedded in paraffin, cut into 3-μm transverse and saggital sections using a Spencer microtome, floated onto glass slides, and allowed to dry over low heat. Sections were then cleared using Histo-Clear (3 × 10 mins), rehydrated with a graded series of ethanol: water solutions (100% and 95%) and transferred into PBSA. Sections were mounted under no. 0 cover slips using an anti-photobleaching mounting medium (Giloh and Sedat, 1982).

Microscopy and imaging

Whole-mounted embryos or sections of embryos were imaged using either a Bio-Rad MRC-1024 laser scanning confocal microscope (BioRad, Microscopy Division, Cambridge, MA) or a Leica DMR light microscope equipped with epifluorescence and differential interference contrast (DIC) optics (Vashaw Scientific, Raleigh, NC). Images were processed using Image J 1.31v (National Institutes of Health) and Adobe© Photoshop© 7.0 software (Adobe Systems, San Jose, CA).