Angiogenesis within the developing mouse neural tube is dependent on sonic hedgehog signaling: possible roles of motor neurons

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Embryonic morphogenesis of vascular and nervous systems is tightly coordinated, and recent studies revealed that some neurogenetic factors such as Sonic hedgehog (Shh) also exhibit angiogenic potential. Vascularization within the developing mouse neural tube depends on vessel sprouting from the surrounding vascular plexus. Previous studies implicated possible roles of VEGF/Flk-1 and Angiopoietin-1(Ang-1)/Tie-2 signaling as candidate molecules functioning in this process. Examining gene expressions of these factors at embryonic day (E) 9.5 and 10.5, we unexpectedly found that both VEGF and Ang-1 were expressed in the motor neurons in the ventral neural tube. The motor neurons were indeed located in the close vicinity of the infiltrating vessels, suggesting involvement of motor neurons in the sprouting. To substantiate this possibility, we inhibited induction of the motor neurons in the cultured mouse embryos by cyclopamine, a Shh signaling blocker. The vessel sprouting was dramatically impaired by inhibition of Shh signaling, together with nearly complete loss of the motor neurons. Expression of Ang-1, but not VEGF, within the neural tube was remarkably reduced in the cyclopamine treated embryos. These results suggest that the neural tube angiogenesis is dependent on Shh signaling, and mediated, at least in part, by the Ang-1 positive motor neurons.

Introduction

Embryonic morphogenesis is mostly accompanied by the development of vascular system which nourishes organs. Vascular formation in the developing nervous system is exquisitely regulated, considering that vessels are of mesodermal and neurons are of ectodermal origins. Molecular basis of this meticulous coordination has become an intriguing topic of recent researches (Emanueli et al. 2003). For example, Mukouyama et al. (2002) reported that association of arteriogenesis and neurogenesis in the skin is mediated by the vasculotropic action of VEGF released from the neurons. Shen et al. (2004) reported that neurogenesis from the neural stem cells are stimulated by endothelial cells. In addition, several molecular cues are shared by vasculo-angiogenesis and neurogenesis (Bicknell & Harris 2004), such as VEGF-neuropilin system (Gu et al. 2003), Eph-ephrin system (Adams et al. 2001), and Notch-Delta system (Iso et al. 2003), harmonizing these two distinct processes.

Sonic hedgehog (Shh) is a representative morphogen of various tissues and organs (Cohen 2003), and recent studies indicated that Shh has pivotal roles for both neurogenesis and angiogenesis. As for neurogenesis, morphological patterning within the neural tube is finely regulated through balancing effects between dorsalizing signal of TGF-β superfamily (such as BMP4) and ventralizing signal of Shh (Gilbert 2000). Induction of the motor neurons in the ventral neural tube requires Shh signaling released from the notochord and the floor plate (Roelink et al. 1994; Chiang et al. 1996; Jessell 2000). Angiogenetic potential of hedgehog family has also been enthusiastically investigated in a recent few years. Shh promotes aortogenesis via VEGF in zebrafish.
embryos (Lawson et al. 2002), and stimulates angiogenesis in ischaemic limbs or muscles in adult mice (Pola et al. 2001, 2003). Furthermore, Kusano et al. (2004) recently reported induction of nerve vessels and restoration of nerve functions by exogenous Shh in peripheral neuropathy in diabetic rats, suggesting that Shh is one of the main coordinators between angiogenesis and neurogenesis. Shh signaling can be blocked by steroidal alkaloids cyclopamine and jervine (Cooper et al. 1998; Chen et al. 2002). Their blocking effects can be monitored by down-regulation of a receptor Patched-1 (Ptc-1) and a downstream transcription factor Gli-1, since Ptc-1 and Gli-1 are direct targets of Shh signaling itself (Marigo & Tabin 1996). We previously reported that cyclopamine caused holoprosencephalic craniofacial anomalies, a pheno-
copy of the Shh null embryos (Chiang et al. 1996), along with down-regulation of Ptc-1 and Gli-1, in the mouse whole embryo culture system (Nagase et al. 2005). In the present study, we focused on vascular phenotype 
within the neural tube in the cyclopamine treated embryos.

Vessel formation in the neural tube may be a suitable model for investigating the coordination of angiogenesis and neurogenesis, and depends on two mechanisms in the chick embryo: direct recruitment of angioblasts into the neural tube and angiogenetic sprouting from the sur-
rrounding perineural vascular plexus (PNVP) (Kurz et al. 1996). In the mouse embryo, the vascularization in the 
neural tube depends solely on the sprouting from the PNVP, which occurs at embryonic day (E) 9–10 (Nakao et al. 1988). Molecular mechanisms of the neural tube vascularization have been partly unraveled. Hogan et al. (2004) reported that VEGF expressed in the E9.0–9.5 neural tube recruits angioblasts around the neural tube to 
form the PNVP. Sato et al. (1995) reported impaired sprouting from the PNVP into the trunk neural tube in the E9.5 Tie-2 null embryos. These reports suggest that VEGF/Flk-1 or Angiopoietin-1(Ang-1)/Tie-2 signaling may be candidate molecular players regulating the vessel invasion into the neural tube. However, the paper by Hogan et al. (2004) did not describe VEGF involvement in the sprouting stage from the PNVP. Also, there was no 
description of angiopoietin expression in the paper by Sato et al. (1995), because angiopoietins were not identified when this paper was published. Therefore, we consider that roles of these angiogenic factors have not been fully 
proven, because of insufficient investigations regarding precise expressions and functions of these factors.

In this study, we examined expression patterns of genes encoding Ang-1, VEGF and their receptors in mouse embryos of E9.5–10.5. Unexpectedly, we found that Ang-1 and VEGF were both expressed in the motor 
neurons. The vessel sprouts from the PNVP were indeed observed in the close vicinity of the motor neurons. Furthermore, the sprouting was remarkably reduced when the motor neurons were eliminated by blockade 
of Shh signaling with cyclopamine in the whole embryo culture. The Ang-1 expression was also undetectable, but the VEGF expression was up-regulated, in the cyclopamine treated embryos. Our results suggest a 
pivotal role of the Ang-1 positive motor neurons in the angiogenesis within the neural tube. This study also 
implicates a novel, previously unrecognized mechanism of harmonization between angiogenesis and neuro-
genesis mediated by Shh. 

Results
Ang-1 and VEGF are expressed within the neural tube

Although Nakao et al. (1988) reported that vessel sprout-
ing from the PNVP occurs at around E9–10, precise 
timing of the vascularization is still unclear. Thus, we first 
determined the timing of sprouting from the PNVP by 
immunostaining against PECAM-1, an endothelial marker 
(Fig. 1A–D). At E9.7, PNVP was already formed but no 
sprouting was detected at the forelimb bud level (Fig. 1A). 
Vessel sprouting into the neural tube commenced at E9.8 
at the lateral border of the neural tube (Fig. 1B). The 
sprouting vessels grew in the medio-ventral direction 
thereafter (Fig. 1C), and finally we observed formation of 
arch-like vascular connections between the lateral edge and the floor plate by E10.2 (Fig. 1D). We focused on 
the ventral arch-like vessels in the following analysis.

As stated above, Ang-1/Tie-2 signaling or VEGF/Flk-
1 signaling may be molecular candidates mediating this 
process. We thus explored gene expression patterns of 
these angiogenic factors in the E10.5 neural tube at the 
forelimb bud level. Abundant Ang-1 expression was 
detected in the ventral neural tube (Fig. 1E). VEGF 
immunostaining was also found in the neural tube, just 
lateral to the Ang-1 positive cells (Fig. 1G). Expressions of 
their receptors, Tie-2 and Flk-1, were observed in 
endothelial cells within and around the neural tube 
(Fig. 1F,H). Expression of Ang-2, another ligand of 
Tie-2, was under the detection level (data not shown).

Ang-1 and VEGF are expressed in the motor neurons 
near the sprouting vessels

Location of the Ang-1 and VEGF positive cells suggests 
that these are the motor neurons, which are induced in the 
ventral neural tube at around the same developmental
stage (Jessell 2000). To test this possibility, we next performed double staining with anti Islet-1/2 antibody, a marker of the motor neurons and the dorsal root ganglia (Osumi et al. 1997). Ang-1 positive cells in the ventral neural tube were, indeed, included within medial subpopulation of the Islet-1/2 positive motor neuron columns (Fig. 2A–C). VEGF immunolocalization was also clearly observed, in the longitudinal section, in the lateral portion of the motor neuron columns (Fig. 2D–F).

These findings indicated Ang-1 and VEGF expressions in the subpopulation of the motor neurons.

Furthermore, we examined localization of the motor neurons and the sprouting vessels by double immunostaining with anti Islet-1/2 and anti PECAM-1 antibodies. The sprouting vessels, which were arranged in a row in the longitudinal section, were detected just medial to the motor neuron columns (Fig. 2G–I). In the transverse section, the arch-like vessels ran along the medial surface of the motor neuron columns (Fig. 2J–L). These data suggest close association of the vessel sprouting with the motor neurons.

**Ang-1 and VEGF have already been expressed in the motor neurons at the time of the sprouting**

We further investigated expression patterns of Ang-1 and VEGF in the earlier embryos, to examine whether expressions of these molecules are in parallel to the timing of the vessel sprouting as described above. At E9.5, just precedent to sprouting, Ang-1 transcripts were clearly observed in the lateral surface of the neural tube, near the entry point of the sprouting vessels (Fig. 3A). These cells were double positive for Ang-1 and Islet-1/2 (Fig. 3B,C), indicating that Ang-1 is expressed in the motor neurons as early as E9.5. VEGF expression was visible in the apical layer of the neural tube at E9.5, but not observed in the Islet-1/2 positive motor neurons (Fig. 3D). Its expression in the motor neurons was commenced at E9.75 (data not shown), when the sprouting was started. Tie-2 expression could be observed in the PNVP at E9.5 (Fig. 3E) as previously reported (Kisanuki et al. 2001), suggesting possible interaction with Ang-1 expressed in the motor neuron.

Detailed analysis of time course of Ang-1 and VEGF expressions revealed that these expressions were already found at least when or just before the sprouting commenced (Table 1). These data further suggest the possibility that Ang-1 and VEGF expressed in the motor neurons provide molecular cues for sprouting vessel path-finding.

**Blockade of Shh signaling inhibits motor neuron induction in the cultured embryos**

To obtain more direct evidence supporting possible involvement of the motor neurons in the neural tube angiogenesis, we planned to examine whether the vascular sprouting is altered if the motor neurons are deleted. As stated earlier, Shh signaling from the notochord and the floor plate is essential for induction of the motor neurons. First, we examined the effect of Shh
signaling inhibitor cyclopamine on the motor neuron induction in the cultured mouse embryos. We cultured E8.5 (somite 8–12) embryos till E10.2 (somite 31–34), with or without 20 μmol/L cyclopamine in the culture medium.

Islet-1/2 positive motor neurons were clearly detected in the control group cultured without cyclopamine (Fig. 4A). In contrast, the neural tube became round and number of the Islet-1/2 positive cells in the neural tube was remarkably reduced in the cyclopamine treated embryos (Fig. 4B), showing that blockade of Shh signaling results in inhibition of the motor neuron induction. The dorsal root ganglia were detected by Islet-1/2 immunostaining, indicating that effect of Shh signaling blockade was found only in the ventral portion of the neural tube and the surrounding mesenchyme. Down-regulation of Ptc-1 and Gli-1 was obviously noted in and around the neural tube in the section at the forelimb bud level of the cyclopamine treated embryos (Fig. 4C–F). Because Ptc-1 and Gli-1 themselves are targets of Shh signaling as stated earlier, these findings can be regarded as a confirmation of Shh signaling blockade in this protocol.

**Blockade of Shh signaling remarkably impairs vascular sprouting into the neural tube**

Next, we investigated whether the vessel sprouting was inhibited when the motor neurons were eliminated by cyclopamine. The arch-like vessels were observed in the control group as seen in the normal embryos (Fig. 5A,C). On the other hand, vascularization of the neural tube was dramatically impaired in the cyclopamine treated group (Fig. 5B,D), in which even immature sprouts were very scarcely observed.

We estimated the neural tube vascularization semi-quantitatively by categorizing them into three levels, which was described in detail in the legends (Fig. 5E). Thirteen embryos out of 18 (72.2%) in the control group were rated as ++, showing grossly normal arch-like vessels. In contrast, 17 embryos out of 21 (81.0%) in the
cyclopamine group exhibited almost complete inhibition of vessel sprouting (Fig. 5E), categorized as –. Taken together, these data suggest a possibility that the neural tube angiogenesis is dependent on Shh signaling, and that the motor neurons, which express angiogenic factors, mediate this process.

Expression of Ang-1, but not VEGF, is reduced in the neural tube of the cyclopamine treated embryos

Although we have confirmed expressions of both Ang-1 and VEGF in the motor neurons, we do not know which
is more responsible for the neural tube angiogenesis. To obtain a clue to answer this question, we further investigated expression changes of the Ang-1 and VEGF in the cyclopamine treated embryos. Ang-1 expression within the neural tube was remarkably reduced by cyclopamine, compared with the control group (Fig. 6A, B). Double staining of Ang-1 in situ hybridization and Islet-1/2 immunohistochemistry revealed that Ang-1 expression, which was detected in the motor neurons in the control group (Fig. 6C), was not detectable within a few Islet-1/2 positive motor neuron remnants in the cyclopamine treated embryos (Fig. 6D). These observations support a possibility that Ang-1 mediates the sprouting into the neural tube. On the other hand, VEGF expression was up-regulated within and around the neural tube in the cyclopamine treated embryos (Fig. 6E, F). Up-regulated VEGF expression was not accompanied with augmented vascularization, suggesting that VEGF is not a main contributor for the sprouting, at least in this situation.

Administration of 20 µmol/L jervine, another inhibitor of Shh signaling, resulted in the similar morphological change of the neural tube, reduction of the Islet-1/2 positive motor neurons, and the inhibition of the vessel sprouting (data not shown). Together with the findings by Sato et al. (1995) in Tie-2 null embryos, these data suggest that the Shh dependent angiogenesis within the neural tube may be mediated by Ang-1 expressed in the motor neurons.
Discussion

We presented here that Ang-1 and VEGF were expressed in the motor neurons. This is the first paper, as far as we know, describing Ang-1 expression in the embryonic or adult motor neurons. VEGF expression in the motor neurons was previously reported in the adult animals and humans, especially in reference to pathogenesis and prevention of ischaemic cell death of the motor neurons in amyotrophic lateral sclerosis (Oosthuyse et al. 2001; Lambrechts et al. 2003). Several authors indicated that Ang-1 is expressed in normal and abnormal postnatal brain tissue and prevents neuronal apoptosis (Acker et al. 2001; Koga et al. 2001; Zhang & Chopp 2002; Valable et al. 2003). Interestingly, Takakura et al. (2000) reported that Ang-1 positive hematopoietic stem cells are located in the E10.5–11.5 cranial neuroepithelium, mediating vascular sprouting. However, there is no previous report describing Ang-1 expression in neurons in the embryonic central nervous system.

We consider that Ang-1, rather than VEGF, is responsible for the sprouting process by the following three reasons. First, expressions of Ang-1 and its receptor Tie-2 were observed at E9.5 sufficiently before the sprouting. Second, reduction of Ang-1 expression by cyclopamine was clearly accompanied by the impaired sprouting into the neural tube. Third, a report by Sato et al. (1995) indicated that vessel sprouting into the neuroepithelium is inhibited in the Ang-1 knockout mice regarding the neural tube vascularization (Suri et al. 1996). It is yet to be elucidated from the present study whether VEGF is dispensable in the sprouting process, since a possibility that both Shh and VEGF pathways are involved has not been fully ruled out. However, earliest expression of VEGF was only observed at E9.7–9.8, slightly later than Ang-1 expression and just when the sprouting commences (Table 1). If VEGF is a primary cue for the sprouting, its expression should be sufficiently earlier than E9.7. Also, VEGF up-regulation in the cyclopamine treated embryos was not associated with vessel sprouting. These findings suggest a less important role of VEGF than that of Ang-1 in the sprouting, at least in our experimental model. There may be two possibilities for explaining the VEGF up-regulation by cyclopamine: VEGF expression is negatively regulated by Shh signaling. Alternatively, reduced blood supply and local hypoxia cause compensatory up-regulation of VEGF. If the latter is the case, VEGF may have some additional roles in the sprouting: adjusting angiogenesis according to oxygen demand. In any case, further experiments such as blockade of VEGF/Flk-1 signaling should be required addressing roles of VEGF in the sprouting. Notably, VEGF is necessary for PNVP formation in E9.0–9.5 (Hogan et al. 2004), and it should be borne in mind that results of the VEGF blocking experiment can be easily obscured by the defects in PNVP formation itself.

We also demonstrated in this paper that the sprouting into the neural tube was dramatically inhibited by the cyclopamine treatment in the cultured embryos. Shh controls embryonic morphogenesis of various tissues and organs, such as the face, limb buds, gastrointestinal tract, skin appendages and the central nervous system (Cohen 2003). Shh also attracts interests of recent researchers as a potent angiogenetic factor (Pola et al. 2001, 2003; Lawson et al. 2002; Kusano et al. 2004). Another member of hedgehog family, Indian hedgehog, also mediates vasculo-angiogenesis of the mouse yolk sacs (Dyer et al. 2001; Byrd et al. 2002). The inhibitory effect of cyclopamine on the Hedgehog signaling was confirmed in our experimental protocol, because Ptc-1 and Gli-1 were down-regulated by cyclopamine, and another inhibitor jervine produced the same phenotypes as cyclopamine. Inhibitory actions of these agents may be common among Hedgehog families, not specific to Shh. However, indispensable roles of Shh signaling in the motor neuron induction have been generally accepted (Roelink et al. 1994; Chiang et al. 1996; Jessell 2000). We thus consider that the vessel sprouting into the neural tube examined in this study can be regarded as a unique, unexpected example of Shh dependent angiogenesis.

Mode of actions of Shh signaling in angiogenesis may be either direct or indirect. For example, Shh dependent arteriogenesis in the zebrafish embryos is indirect and mediated by up-regulation of VEGF in the somites (Lawson et al. 2002). Several possibilities should be taken into consideration for describing our present data. A possibility of direct regulation of angiogenesis by Shh, irrelevant of Ang-1 expression in the motor neuron, is not plausible, because angiogenetic defects in Tie-2 null mice (Sato et al. 1995) may not be associated with change of Shh signaling. It may be natural to consider that the effect of Shh signaling is indirect: Shh signaling induces the motor neurons, and Ang-1 expression is probably associated with the presence or absence of the motor neurons. The Ptc-1 and Gli-1 positive area (where Shh signaling is functioning, see Fig. 4E,G) includes, but is not specific to, localization of the Ang-1 positive motor neurons, and this finding may further support an idea of the indirect action of Shh signaling. However, a possibility that Shh directly regulates Ang-1 expression in the motor neurons has not been fully ruled out, since
a few Islet-1/2 positive cells were devoid of Ang-1 transcripts in the cyclopamine treated group (Fig. 6D). Further studies will be required to determine whether this is due to just a problem of sensitivity in our Ang-1 in situ hybridization protocol, or really due to the direct regulation of Ang-1 expression by Shh.

In summary, we found that Ang-1 and VEGF were expressed in the motor neurons, in the close vicinity to the sprouting vessels. The sprouting was dramatically reduced in the absence of the motor neurons when Shh signaling was inhibited by cyclopamine. Expression of Ang-1, not VEGF, was reduced in this condition. Our results suggest that the vascular sprouting from the sprouting vessels is Shh signaling dependent, and that Ang-1 expressed in the motor neurons may be a molecular cue for the sprouting vessels.

**Experimental procedures**

**Animals**

E9.5–10.5 mouse embryos were dissected out from pregnant ICR mice (Clea Japan, Tokyo, Japan), which were anesthetized with ether and sacrificed by cervical dislocation. Embryos were fixed overnight at 4 °C in 4% paraformaldehyde, cryosectioned in 14 µm thickness and were thaw-mounted on to VECTABOND (Vector Laboratories, Burlingame, CA, USA) coated glass slides.

All the procedures using the animals were approved by the ethical committee of University of Tokyo Graduate School of Medicine.

**Immunohistochemistry**

Immunohistochemistry on sections was carried out as previously described (Nagase et al. 2003, 2005) with slight modifications. For diaminobenzidine (DAB) staining, the cryosections were incubated overnight at 4 °C with primary antibodies in the following dilutions: rat anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM-1) (MEC13.3; BD Pharmingen, San Diego, CA, USA), 1/500; mouse anti-human VEGF (BD Pharmingen), 1/300; rat anti-mouse Flk-1 (BD Pharmingen), 1/100; and mouse anti-mouse Islet-1/2 (40.2D6. Developmental Studies Hybridoma Bank), 1/100. For VEGF and Islet-1/2 double staining, sections were first boiled for antigen retrieval, samples were immunolabeled with anti-Iselet-1/2 antibody and Cy3-conjugated anti-mouse IgG. Signals were detected with DAB.

**In situ hybridization**

cDNA probes for mouse Ang-1 (U83509; bases 338–848), Ang-2 (AF004326; bases 251–748), Tie-2 (X71426; bases 1583–2181), and VEGF (S38083; bases 139–588) were cloned by RT-PCR. The obtained cDNA fragments were subcloned into pBluescript II SK- and sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) to confirm their authenticity. cDNA probes for mouse Shh, Ptc-1, and Gli-1 were kind gifts from Dr J. Motoyama.

In situ hybridization with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Wako, Osaka, Japan) was performed as previously described (Nagase et al. 2003, 2005). Some sections were subsequently immunolabeled with anti-Iselet-1/2 antibody and visualized with DAB.

Fluorescence double staining for Ang-1 mRNA and Islet-1/2 protein was carried out as follows. First, sections were hybridized with Ang-1 anti-sense probe. After washing, sections were incubated with hydrogen peroxide, incubated with peroxidase-conjugated antibody, biotinylated anti-goat IgG, streptavidin-peroxidase, biotinyl tyramide, and streptavidin-FITC, which was followed by reaction with mouse anti-mouse Islet-1/2 and Cy3-conjugated anti-mouse IgG.

**Mouse whole embryo culture**

Mouse whole embryo culture was performed as previously described (Nagase et al. 2003, 2005). 20 mmol/L cycloamine (TRC, Toronto, Canada) and 10 mmol/L jervine (TRC) dissolved in ethanol were stored at −30 °C as a stock solution. Two µL cycloamine stock or 4 µL jervine stock was added to the 2 mL medium in each culture vial, yielding 20 µmol/L of cycloamine or jervine. Two µL or 4 µL of ethanol was added to the 2 mL medium in the control group. Using the whole embryo incubator (Ikemoto Rika, Tokyo, Japan), E8.5 ICR mouse embryos (somite 8–12 stage) were cultured until the stage corresponding to E10.2 (somite 31–34 stages).

For PECAM-1 immunostaining using the cultured embryos, the samples were incubated with 1 : 100 dilution of biotinylated anti-PECAM-1 antibody, and subsequently reacted with streptavidin-peroxidase (1 : 500), biotinyl tyramide (1 : 50), and streptavidin-FITC (1 : 500) using a biotinyl tyramide amplification kit (PerkinElmer Life Sciences, Boston, MA, USA). Subsequently, after boiling for antigen retrieval, samples were immunolabeled with mouse anti-mouse Islet-1/2 and Cy3-conjugated anti-mouse IgG (Jackson Immunolaboratories Inc., West Grove, PA, USA). For VEGF and Islet-1/2 double staining, sections were first boiled for antigen enhancement, incubated with goat anti-mouse VEGF at dilution of 1/300 (Santa Cruz Biotechnology Inc., California, CA, USA), biotinylated anti-goat IgG, streptavidin-peroxidase, biotinyl tyramide, and streptavidin-FITC, which was followed by reaction with mouse anti-mouse Islet-1/2 and Cy3-conjugated anti-mouse IgG.
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