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

Takashi Nagase[1,†,*], Miki Nagase[2,†], Kotaro Yoshimura[1], Toshiro Fujita[2] and Isao Koshima[1]

[1]*Department of Plastic and Reconstructive Surgery, and* [2]*Department of Nephrology and Endocrinology, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

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 angiogenetic 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

†These authors equally contributed to this work.

Communicated by: Noriko Osumi

**Correspondence*: E-mail: tnagase@fb3.so-net.ne.jp

DOI: 10.1111/j.1365-2443.2005.00861.x

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 phenocopy 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 surrounding 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 angiogenetic 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 neurogenesis mediated by Shh.

Results

Ang-1 and VEGF are expressed within the neural tube

Although Nakao *et al.* (1988) reported that vessel sprouting 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 angiogenetic 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

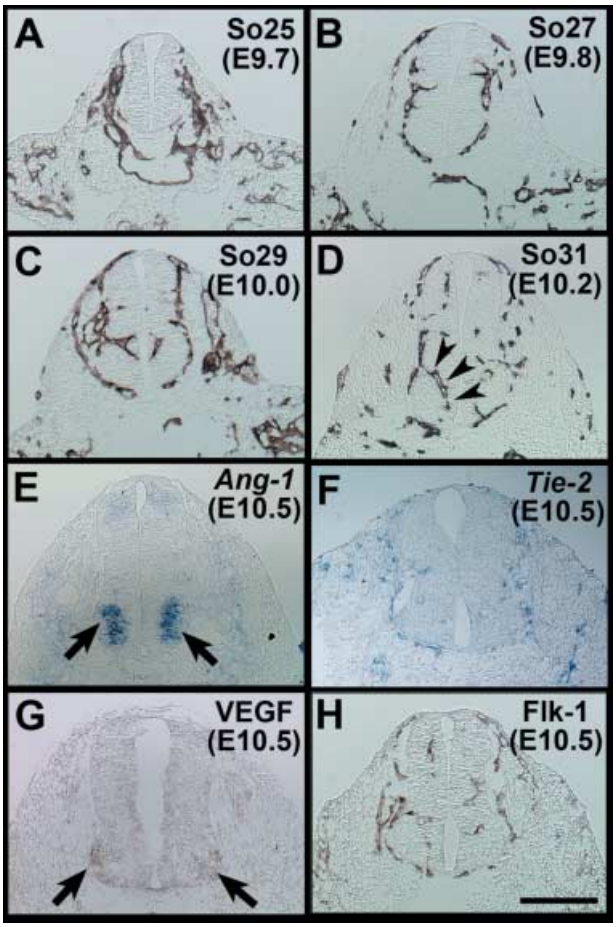

Figure 1 Vessel sprouting and angiogenetic gene expressions in the neural tube at the forelimb bud level. (A–D) Time course of vessel sprouting detected by anti PECAM-1 immunostaining. (A) At somite (So) 25 stage of E9.7, no vessel invasion can be observed. (B) At So 27 (E9.8), vessel sprouting commences. (C) At So 29 (E10.0), the sprouting is far more advanced. (D) At So 31 (E10.2), ventral arch-like vessels are observed (arrowheads). (E–H) Expressions of angiogenetic genes in E10.5 embryos. (E) *Ang-1* transcripts are detected in the ventral neural tube (arrows). (F) Expression of *Tie-2* is observed in the vascular endothelium. (G) VEGF immunostaining is detected in the neural tube, lateral to the *Ang-1* positive cells (arrows). (H) Immunostaining of Flk-1 is also found in the endothelium. Bar, 200 μm.

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

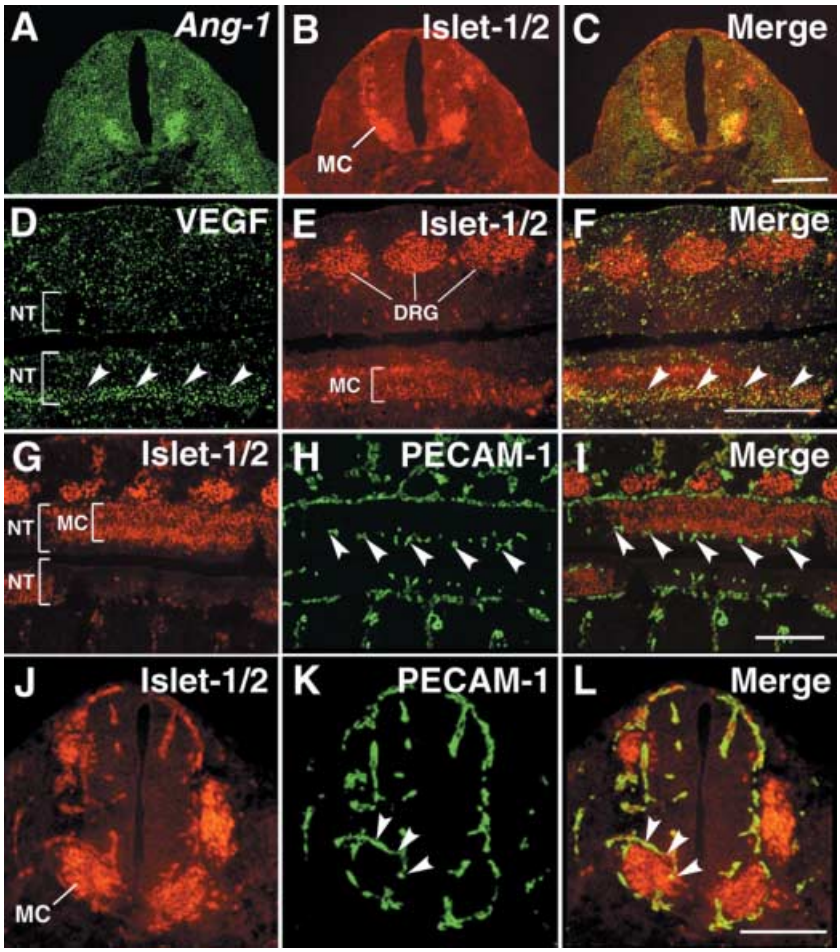

Figure 2 Localization of the motor neurons in relation to the *Ang-1*/VEGF expression and the sprouting vessels in the E10.5 mouse neural tube. (A–C) *Ang-1* is expressed in the motor neuron column (MC). (A) *Ang-1* transcripts are found in the ventral neural tube. (B) Islet-1/2 is positive in the MC. (C) *Ang-1* and Islet-1/2 are co-localized in the medial MC. (D–F) VEGF is expressed in the MC. (D) In the longitudinal section,VEGF immunostaining is observed in the lateral portion of the neural tube (NT) (arrowheads). (E) Islet-1/2 is positive in the MC and the dorsal root ganglia (DRG). (F) VEGF and Islet-1/2 are co-localized in the lateral MC (arrowheads). (G–L) The vessel sprouting is closely associated with the MCs. (G) Islet-1/2 is positive in the MC. (H) PECAM-1 is detected in the arch-like sprouting vessels (arrowheads). (I) Double staining shows that the sprouting vessels are located very close to the MC (arrowheads). (J–L) Double staining of Islet-1/2 and PECAM-1 in the cross section revealed the same results. Note that the sprouting vessels run along the surface of the MC (arrowheads in K, L). Bars, 200 μm.

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–H). 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

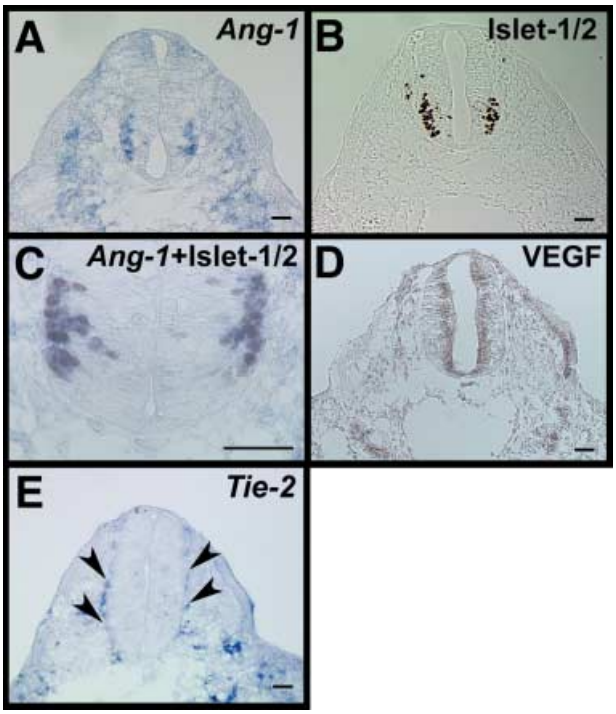

Figure 3 Expressions in *Ang-1*, VEGF and *Tie-2* in E9.5 embryos. (A) *Ang-1* is expressed in the lateral edge of the ventral neural tube (B) Islet-1/2 immunostaining is detected in the same region. (C) Double staining of *Ang-1* and Islet-1/2 shows that *Ang-1* is expressed in the motor neuron at this stage. (D) VEGF is not detected in the motor neuron, although VEGF is visible in the apical layer of the neural tube. (E) *Tie-2* transcripts are observed in the PNVP (arrowheads). Bars, 100 μm.

Table 1 Time course of expressions of Islet-1/2 and angiogenetic factors in the motor neurons, in relation to the vascular sprouting in the neural tube

	E9.0	E9.5	E9.8	E10.2
Islet-1/2	++	++	++	++
Ang-1	–	+	++	++
VEGF	–	–	+	++
Vessel Sprouting	–	–	+	++

– not detectable; + slightly detectable; ++ clearly detectable.

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 angiogenetic factors, mediate this process.

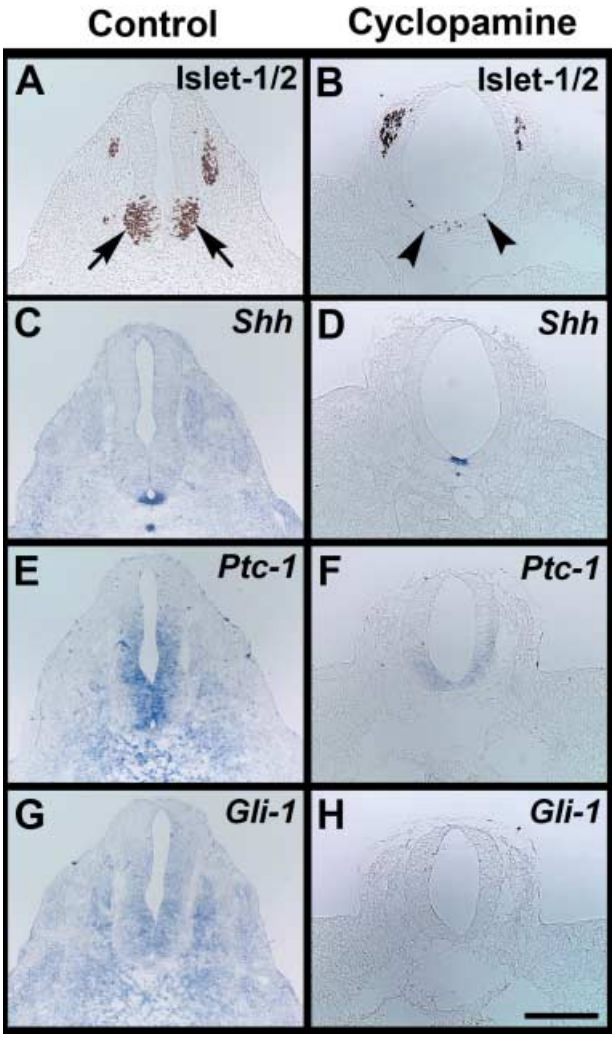

Figure 4 Cyclopamine remarkably inhibits the motor neuron induction and gene expressions of Shh signaling. (A) Islet-1/2 staining is clearly detected in the motor neurons in the control embryos (arrows). (B) Number of the Islet-1/2 positive motor neurons is reduced by cyclopamine (arrowheads). (C,D) *Shh* transcripts are detected in the notochord and the floor plate of the neural tube in both the control and the cyclopamine groups. (E) *Ptc-1* is expressed in the ventral half of the neural tube in the control embryo. (F) *Ptc-1* expression in the neural tube is weakened in the cyclopamine group. (G) *Gli-1* transcripts are diffusely detected within the neural tube and in the mesenchyme around it. (H) *Gli-1* expression is under the detection level in the cyclopamine treated embryos. Bars, 200 μm.

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

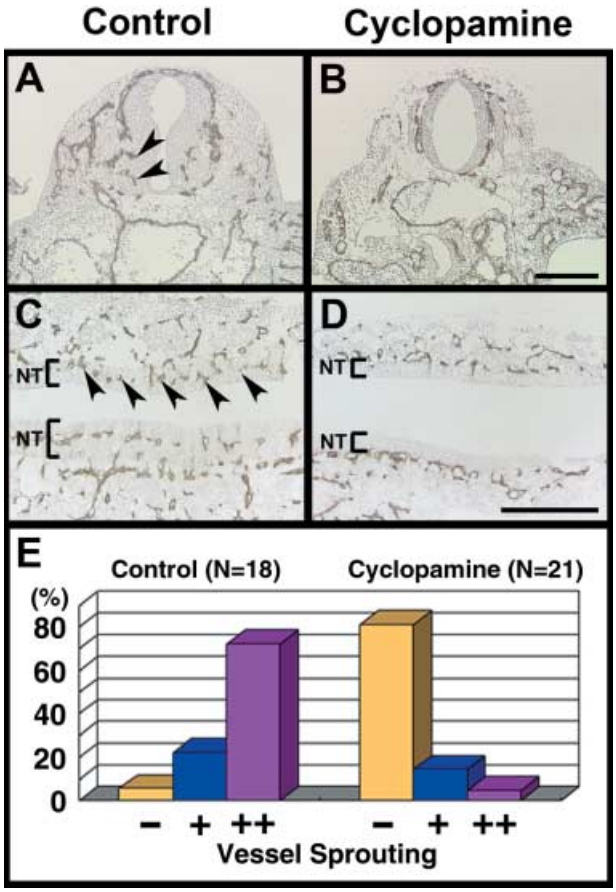

Figure 5 Absence of the motor neurons by cyclopamine causes inhibition of the vascular sprouting. (A, C) The arch-like sprouting vessels are observed in the control embryos (arrowheads). (B, D) The sprouting is remarkably inhibited by cyclopamine. Bars, 200 μm. NT: neural tube. (E) Percentages of the cultured embryos with different degrees of the vessel sprouting. Degrees of the sprouting are categorized as follows: – Sprouting is nearly completely inhibited; + Some sprouting is observed but the ventral vascular arches are not yet formed; ++ Sprouting is more advanced and the vascular arches are nearly completed.

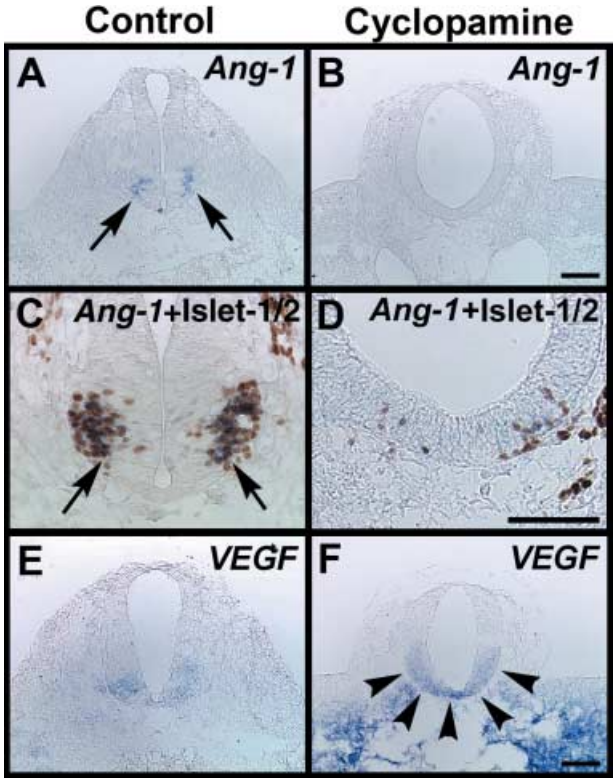

Figure 6 Cyclopamine remarkably inhibits *Ang-1* expression, but not *VEGF* expression. (A) *Ang-1* is expressed in the motor neurons in the control group (arrows). (B) *Ang-1* expression is inhibited by cyclopamine. (C) *Ang-1* and Islet-1/2 are co-localized in the control group (arrows). (D) *Ang-1* expression is not detected in the few Islet-1/2 positive cells in the cyclopamine group. (E) *VEGF* expression is found in the ventral neural tube in the control. (F) *VEGF* is up-regulated within and around the neural tube by cyclopamine (arrowheads). Bars, 100 μm.

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 trunk of Tie-2 null mouse embryos, suggesting a pivotal role of Ang-1/Tie-2 signaling. Unfortunately, there was no description of phenotype of 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 PNVP 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 immunostaining, precedent boiling with 0.01 mol/L sodium citrate was required for antigen enhancement. Biotinylated anti-rat IgG (for PECAM-1 and Flk-1) or anti-mouse IgG (for VEGF and Islet-1/2) was used as secondary antibodies at 1/200 dilution. Immunoreactivity was detected using an ABC kit (Vector Laboratories) and a metal enhanced DAB kit (Pierce, Rockford, IL, USA).

Immunofluorescence double staining was performed as follows. For PECAM-1 and Islet-1/2 double immunostaining, sections were first stained for PECAM-1 with biotinylated anti-mouse PECAM-1 (BD Pharmingen), 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.

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 Islet-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, samples were treated with hydrogen peroxide, incubated with peroxidase-conjugated anti-digoxigenin antibody, biotinyl tyramide (1 : 50), and streptavidin-FITC (1 : 500) using a biotinyl tyramide amplification kit. Thereafter, immunohistochemistry were performed with anti Islet-1/2 antibody 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 cyclopamine (TRC, Toronto, Canada) and 10 mmol/L jervine (TRC) dissolved in ethanol were stored at −30 °C as a stock solution. Two μL cyclopamine stock or 4 μL jervine stock was added to the 2 mL medium in each culture vial, yielding 20 μmol/L of cyclopamine 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-peroxidase (1 : 500) using a biotinyl tyramide amplification kit. Signals were detected with DAB.

Acknowledgements

We thank Drs Noriko Osumi, Masanori Takahashi, Tadashi Nomura, Kanae Ohsaki and Shun Nakamura for discussions and helpful comments, and Ms. Akiko Muto and Akiko Matsuura for technical supports. This work was supported by a Grant-In Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology given to T. N. (#15390537).

References

Acker, T., Beck, H. & Plate, K.H. (2001) Cell type specific expression of vascular endothelial growth factor and angiopoietin-1 and -2 suggests an important role of astrocytes in cerebellar vascularization. *Mech. Dev.* **108**, 45–57.

Adams, R.H., Diella, F., Hennig, S., Helmbacher, F., Deutsch, U. & Klein, R. (2001) The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* **104**, 57–69.

Bicknell, R. & Harris, A.L. (2004) Novel angiogenic signaling pathways and vascular targets. *Annu. Rev. Pharmacol. Toxicol.* **44**, 219–238.

Byrd, N., Becker, S., Maye, P., *et al.* (2002) Hedgehog is required for murine yolk sac angiogenesis. *Development* **129**, 361–372.

Chen, J.K., Taipale, J., Cooper, M.K. & Beachy, P.A. (2002) Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev.* **16**, 2743–2748.

Chiang, C., Litingtung, Y., Lee, E., *et al.* (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407–413.

Cohen, M.M. Jr (2003) The hedgehog signaling network. *Am. J. Med. Genet.* **123A**, 5–28.

Cooper, M.K., Porter, J.A., Young, K.E. & Beachy, P.A. (1998) Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280**, 1603–1607.

Dyer, M.A., Farrington, S.M., Mohn, D., Munday, J.R. & Baron, M.H. (2001) Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo. *Development* **128**, 1717–1730.

Emanueli, C., Schratzberger, P., Kirchmair, R. & Madeddu, P. (2003) Paracrine control of vascularization and neurogenesis by neurotrophins. *Br. J. Pharmacol.* **140**, 614–619.

Gilbert, S. (2000) *Developmental Biology*, 6th edn. Sunderland, MA: Sinauer Associates, Inc.

Gu, C., Rodriguez, E.R., Reimert, D.V., *et al.* (2003) Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev. Cell* **5**, 45–57.

Hogan, K.A., Ambler, C.A., Chapman, D.L. & Bautch, V.L. (2004) The neural tube patterns vessels developmentally using the VEGF signaling pathway. *Development* **131**, 1503–1513.

Iso, T., Hamamori, Y. & Kedes, L. (2003) Notch signaling in vascular development. *Arterioscler. Thromb. Vasc. Biol.* **23**, 543–553.

Jessell, T. (2000) Neuronal specification in the spinal cord: inductive signals and trascriptional codes. *Nature Rev. Genet.* **1**, 20–29.

Kisanuki, Y.Y., Hammer, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A. & Yanagisawa, M. (2001) Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev. Biol.* **230**, 230–242.

Koga, K., Todaka, T., Morioka, M., *et al.* (2001) Expression of angiopoietin-2 in human glioma cells and its role for angiogenesis. *Cancer Res.* **61**, 6248–6254.

Kurz, H., Gartner, T., Eggli, P.S. & Christ, B. (1996) First blood vessels in the avian neural tube are formed by a combination of dorsal angioblast immigration and ventral sprouting of endothelial cells. *Dev. Biol.* **173**, 133–147.

Kusano, K.F., Allendoerfer, K.L., Munger, W., *et al.* (2004) Sonic hedgehog induces arteriogenesis in diabetic vasa nervorum and restores function in diabetic neuropathy. *Arterioscler. Thromb. Vasc. Biol.* **24**, 2102–2107.

Lambrechts, D., Storkebaum, E., Morimoto, M., *et al.* (2003) VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nature Genet.* **34**, 383–394.

Lawson, N.D., Vogel, A.M. & Weinstein, B.M. (2002) Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev. Cell* **3**, 127–136.

Marigo, V. & Tabin, C.J. (1996) Regulation of patched by sonic hedgehog in the developing neural tube. *Proc. Natl. Acad. Sci. USA* **93**, 9346–9351.

Mukouyama, Y.S., Shin, D., Britsch, S., Taniguchi, M. & Anderson, D.J. (2002) Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* **109**, 693–705.

Nagase, T., Nagase, M., Osumi, N., *et al.* (2005) Craniofacial anomalies of the cultured mouse embryo induced by inhibition of sonic hedgehog signaling: an animal model of holoprosencephaly. *J. Craniofac. Surg.* **16**, 80–88.

Nagase, T., Sanai, Y., Nakamura, S., Asato, H., Harii, K. & Osumi, N. (2003) Roles of HNK-1 carbohydrate epitope and its synthetic glucuronyltransferase genes on migration of rat neural crest cells. *J. Anat.* **203**, 77–88.

Nakao, T., Ishizawa, A. & Ogawa, R. (1988) Observations of vascularization in the spinal cord of mouse embryos, with special reference to development of boundary membranes and perivascular spaces. *Anat. Rec.* **221**, 663–677.

Oosthuyse, B., Moons, L., Storkebaum, E., *et al.* (2001) Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nature Genet.* **28**, 131–138.

Osumi, N., Hirota, A., Ohuchi, H., *et al.* (1997) Pax-6 is involved in the specification of hindbrain motor neuron subtype. *Development* **124**, 2961–2972.

Pola, R., Ling, L.E., Aprahamian, T.R., *et al.* (2003) Postnatal recapitulation of embryonic hedgehog pathway in response to skeletal muscle ischemia. *Circulation* **108**, 479–485.

Pola, R., Ling, L.E., Silver, M., *et al.* (2001) The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nature Med.* **7**, 706–711.

Roelink, H., Augsburger, A., Heemskerk, J., *et al.* (1994) Floor

plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761–775.

Sato, T.N., Tozawa, Y., Deutsch, U., *et al.* (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70–74.

Shen, Q., Goderie, S.K., Jin, L., *et al.* (2004) Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* **304**, 1338–1340.

Suri, C., Jones, P.F., Patan, S., *et al.* (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87**, 1171–1180.

Takakura, N., Watanabe, T., Suenobu, S., *et al.* (2000) A role for hematopoietic stem cells in promoting angiogenesis. *Cell* **102**, 199–209.

Valable, S., Bellail, A., Lesne, S., *et al.* (2003) Angiopoietin-1-induced PI3-kinase activation prevents neuronal apoptosis. *FASEB J.* **17**, 443–445.

Zhang, Z. & Chopp, M. (2002) Vascular endothelial growth factor and angiopoietins in focal cerebral ischemia. *Trends Cardiovasc. Med.* **12**, 62–66.

Received: 7 January 2005
Accepted: 13 March 2005