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VEGF mediates angioblast migration during development of the dorsal aorta in *Xenopus*

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SUMMARY

Angioblasts are precursor cells of the vascular endothelium which organize into the primitive blood vessels during embryogenesis. The molecular mechanisms underlying patterning of the embryonic vasculature remain unclear. Mutational analyses of the receptor tyrosine kinase flk-1 and its ligand vascular endothelial growth factor, VEGF, indicate that these molecules are critical for vascular development. Targeted ablation of the *flk-1* gene results in complete failure of blood and vascular development (F. Shalaby et al. (1995) *Nature* 376, 62-66), while targeted ablation of the *VEGF* gene results in gross abnormalities in vascular patterning (P. Carmeliet et al. (1996) *Nature* 380, 435-439; N. Ferrara et al. (1996) *Nature* 380, 439-442). Here we report a role for *VEGF* in patterning the dorsal aorta of the *Xenopus* embryo. We show that the diffusible form of VEGF is expressed by the hypochord, which lies at the embryonic midline immediately dorsal to the location of the future dorsal aorta. We find that, initially, no *flk-1*-expressing angioblasts are present at this location, but that during subsequent development, angioblasts migrate from the lateral plate mesoderm to the midline where they form a single dorsal aorta. We have demonstrated that VEGF can act as a chemoattractant for angioblasts by ectopic expression of VEGF in the embryo. These results strongly suggest that localized sources of VEGF play a role in patterning the embryonic vasculature.

Key words: Vascular endothelial growth factor (VEGF), *flk-1*, *VEGFR-2*, Vasculogenesis, Endothelial cell, Angioblast, Posterior cardinal vein

INTRODUCTION

The first major blood vessels in the embryo, the dorsal aortae, posterior cardinal veins, vitelline veins and also the endocardial tube, are formed through the coalescence of endothelial precursor cells (angioblasts). This process is called vasculogenesis (Poole and Coffin, 1991; Hirakow and Hiruma, 1981; Risau and Flamme, 1995). The locations where these endothelial cells initially assemble outline the architecture of the embryonic vascular system. Subsequent elaboration of the vascular system occurs primarily through the modification of extensive capillary networks (Evans 1909), or in the case of smaller vessels, by sprouting from pre-existing vessels via a process termed angiogenesis (Clark and Clark, 1939; Klosovskii, 1963; Wagner, 1980). Vascular precursor cells that contribute to the primary vascular plexus are initially scattered throughout the mesoderm and can assemble either at the location where they arise, or following migration to the location of the developing vessel (Noden, 1989, 1990; Poole and Coffin, 1988, 1991). Indeed, the ability of endothelial cells to migrate is critical for the formation of the primary blood vessels of the embryo (Noden, 1988; Christ et al., 1991; Wilting et al., 1992; Pardanaud and Dieterlen-Lievre, 1993). At present many questions about the establishment of the embryonic vascular network remain unanswered, including the origins of the vascular precursor cells and the nature of the signals responsible for directing their migration to the positions where the primary vascular structures will form.

Peptide growth factors and their transmembrane receptors play essential roles in the early events of vasculogenesis (Gospodarowicz et al., 1989; Klagsbrun and D'Amore, 1991; Dumont et al., 1992; Yamaguchi et al., 1993; Risau and Flamme, 1995). Examples include members of the tyrosine kinase family of growth factor receptors, especially the vascular endothelial growth factor (VEGF) receptors flk-1 and flt-1 (Shibuya et al., 1990; Eichmann et al., 1993; Yamaguchi et al., 1993; Millauer et al., 1993; Flamme et al., 1995a), the angiopoietin receptor tie-2 (tek) (Dumont et al., 1992; Schnurch and Risau, 1993; Sato et al., 1993; Iwama et al., 1993; Maisonpierre et al., 1997) and the receptor tie-1 (tie) for which a ligand has not yet been identified (Korhonen et al., 1994; Partanen et al., 1992; Iwama et al., 1993; Hatva et al., 1995). The expression of the genes for these four receptors is almost exclusively restricted to the endothelial cell lineage. Expression of *flk-1* is amongst the earliest markers of embryonic angioblasts, however transcript levels decline at about the time that angioblasts differentiate into mature blood vessels (Dumont et al., 1995). The other receptor genes, *flt-1*, *tie* and *tek*, are expressed slightly later than *flk-1* and transcript levels remain high after vessel differentiation. The importance

of these receptor tyrosine kinases have recently been revealed by mutational analysis. Targeted mutation of *flk-1* in mice results in embryos that lack all vasculature and blood (Shalaby et al., 1995), whereas *flt-1* mutation results in abnormalities in vessel organization (Fong et al., 1995). *tie* and *tek* appear to be required for endothelial survival and angiogenesis respectively (Puri et al., 1995; Sato et al., 1995). These studies point to the primary importance of *flk-1* for early vascular development, though the very early lethality of the *flk-1* null mouse embryos makes study of the embryonic function more difficult. Of particular significance to this current study, in vitro experiments using *flk-1*$^{-/-}$ embryonic stem cells indicate that cell autonomous expression of *flk-1* is essential for vascular precursor cell migration (Shalaby et al., 1997).

The flk-1 receptor and its high affinity ligand VEGF show a generally coordinated pattern of developmental expression in mouse (Dumont et al., 1995), quail (Flamme et al., 1995a) and frog (Cleaver et al., 1997). VEGF was originally identified as a powerful mitogen for endothelial cells, and as an inducer and regulator of angiogenesis (Ferrara and Henzel, 1989; Risau, 1991). While the possibility that VEGF acts as a chemoattractant for endothelial cells has been suggested in previous reports (Dumont et al., 1995; Beck and D'Amore, 1997; Waltenberger et al., 1996), no experiments have been carried out to address this possibility. Overexpression of *VEGF* in several different embryonic systems causes dramatic perturbations in blood vessel development (Flamme et al., 1995b; Drake and Little, 1995; Cleaver et al., 1997) and targeted ablation of *VEGF* results in gross abnormalities in vascular development and patterning (Carmeliet et al., 1996; Ferrara et al., 1996). Homozygous mutant mice die at 10.5 d.p.c showing delayed endothelial cell development, vascular defects and tissue necrosis. Significantly for this study, VEGF mutant embryos show a complete failure of dorsal aorta development.

Although the molecular mechanisms underlying vascular patterning are unknown, axial structures have been suggested as a source of patterning signals. Studies in chick demonstrate that migrating endothelial cells never cross the midline of the embryo (Hahn, 1908) and that the midline barrier is related to signals from the notochord (Klessinger and Christ, 1996). Work using zebrafish mutants demonstrates that axial signals, perhaps emanating from the notochord, play a role in organizing axial blood vessel formation (Sumoy et al., 1997; Fouquet et al., 1997). In floating head (*flh*) and no tail (*ntl*) mutant embryos, both of which lack a notochord, the dorsal aorta does not develop, while the axial vein is less affected. This vascular defect is most likely due to the failure of *flk-1*-expressing vascular precursor cells to migrate from the intermediate cell mass (ICM) towards the midline of the embryo as described by Al-Adhami and Kunz (1977). These mutant studies do not indicate whether the role of the notochord in the formation of the dorsal aorta is direct, through the action of a molecule secreted by the notochord itself, or indirect, through changes induced by the notochord in adjacent tissues.

Using the *Xenopus* embryo, we have investigated the role of the Flk-1/VEGF signaling pathway in development of the dorsal aorta. Using in situ hybridization analysis, we show that the Flk-1 ligand, VEGF, is strongly expressed in the hypochord, a transient tissue located immediately beneath the notochord. Further experiments demonstrate that, during the mid-tailbud stages, vascular precursor cells migrate from the lateral plate mesoderm, below the somites and over the underlying endoderm, toward the midline of the embryo where they organize into the dorsal aorta. We suggest that the hypochord functions as a concentrated source of diffusible VEGF which acts as a chemoattractant for *flk-1*-expressing vascular precursor cells, directing their migration to the dorsal midline. In support of this hypothesis, we demonstrate that localized expression of VEGF in the embryo can induce the ectopic migration of angioblasts. We propose that VEGF is the axial signal that regulates development of the dorsal aorta in the *Xenopus* embryo.

MATERIALS AND METHODS

Whole-mount in situ hybridization

Digoxigenin-labeled RNA probes containing *X-msr*, *flk-1* and *VEGF* sequences were generated as described previously (Cleaver et al., 1997). Whole-mount in situ hybridization and sectioning of whole-mount embryos was performed with modifications as described by Cleaver et al. (1997).

Embryo manipulation and dissection

Embryos were staged according to Nieuwkoop and Faber (1994). Vitelline membranes were removed manually in NAM. Operations to remove portions of the lateral plate mesoderm were accomplished using a tungsten knife and plastic Petri dishes coated with 1% agarose. Embryos were allowed to heal under glass bridges, in NAM/2 overnight at 13˚C. The embryos were then incubated in 20% Steinberg's solution until stage 35, when they were fixed in MEMPFA prior to whole-mount in situ hybridization. Embryos lacking visible angioblast marker expression in the posterior cardinal vein regions were then assayed for the presence of dorsal aorta angioblasts by sectioning. Embryos dissected to isolate somite tissue and the hypochord, were placed in 95% ethanol containing 5% acetic acid. Dissections were carried out in this solution using a tungsten knife. The hypochord and notochord tissues were isolated in a single fraction because of their tight association. RNA was subsequently isolated from the small tissue fragments as previously described (Melton and Cortese, 1979).

DiI labeling and cryostat sections

A 0.25% stock solution of the lipophilic dye 1,1-dioctadecyl-3,3,3,3 tetramethylindocarbo-cyanine perchlorate (DiI, Molecular Probes) in 100% ethanol was diluted immediately before injection in 0.3 M sucrose and injected at a concentration of 0.05%. Injections were achieved by inserting a micropipet under the epidermis into the mesenchyme of the lateral plate mesoderm and expelling a small volume of dye (4.6 nl) using a Nanoject system (Drummond). DiI labeling was also carried out by contacting cells with a crystal of DiI. Labeling was performed in the lateral plate mesoderm at three locations along the trunk of the embryo: rostral near the pronephric anlage, middle and caudal near the future rectal diverticulum. The location of labeling in injected embryos was determined using a Zeiss compound microscope. Approximately 20-50 cells were labeled per injection. Embryos were allowed to develop to stage 34-36 and were then fixed in 4% paraformaldehyde/0.25% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) overnight at 4˚C. Embryos were then washed in 0.1 M PBS and left in 20% sucrose overnight at 4˚C. Embryos were frozen in methylbutanol at –80˚C and embedded in Tissue-Tek OCT compound (Miles Inc.). Sectioning was accomplished using a cryostat (Model OTF, Bright Instr. Co. Ltd) at a thickness of 25 μm.

RT-PCR

RNA from somite or hypochord tissues, from individual embryos, was copied into cDNA. One tenth of the reverse transcription reaction served as a template for PCR. Oligonucleotides recognizing the extreme 5′ and 3′ end of the VEGF coding region were used to amplify all three alternatively spliced transcripts. In order to estimate the relative amounts of RNA in the hypochord/notochord fraction versus the somite fraction, semiquantitative PCR was carried out using primers for the ubiquitous EF1α sequence (Krieg et al, 1989). PCR amplification was performed using standard conditions (55˚C annealing), with 20 cycles for the EF1α amplification and 25 cycles for the *VEGF* amplification. Oligos recognizing *VEGF* were 5′-ATCATGAACTTTCTG-3′ and 5′-TCACCGTCGTGGCTT-3′. Oligos recognizing EF1α were 5′-CAGATTGGTGCTGGATATGC-3′ and 5′-ACTGCCTTGATGACTCCTAG-3′.

Microinjection of embryos and transplantations

Pigmented donor embryos overexpressing VEGF were prepared by microinjection of 200 pg of *VEGF* DNA in the expression vector pXeX (Cleaver et al., 1997) plus lineage tracer at the 2-cell stage. Injected embryos were cultured at 13°C in 100% Steinberg's solution, 3% Ficoll, overnight and then at 13°C in 20% Steinberg's solution until they reached stage 22-24. Embryos were then sorted under fluorescence microscopy to identify embryos that received the injection in the posterior gut region. At stage 22-24, small pieces of VEGF-expressing mesodermal and ectodermal tissue were extirpated from the posterior gut region in NAM buffer and transplanted under the epidermis of an albino host embryo. Embryos were allowed to heal in 50% NAM buffer for 5-24 hours under glass bridges. Once healed, the embryos were either labeled with DiI in the region of the posterior cardinal vein precursors or fixed for in situ hybridization. Identical VEGF-expressing tissue samples were also cultured as explants in 50% NAM for the same period as the embryos. The presence of angioblasts in the embryos or explants was assayed by whole-mount in situ hybridization using *X-msr* probe (Devic et al., 1996).

RESULTS

VEGF expression in axial tissues

Using whole-mount in situ hybridization, we have examined *flk-1* and *VEGF* expression during early development of the *Xenopus* embryo (Cleaver et al., 1997). As previously reported for mouse and avian embryos (Dumont et al., 1995; Flamme et al., 1995a), cells expressing *flk-1* receptor transcripts are usually closely juxtaposed to cells expressing *VEGF*. One exception, however, is in the axial tissues of the early tailbud *Xenopus* embryo, where *VEGF* is expressed at high levels at the midline of the embryo, while the closest angioblasts (marked by *flk-1* expression) are located several hundred microns away in the lateral plate mesoderm, ventral to the somites. We have examined the expression of *VEGF* and *flk-1* in axial tissues in more detail. Sections through the posterior trunk of an early tailbud embryo (stage 28) show *VEGF* expression in the somites (Fig. 1A), and also very prominently in the hypochord (Fig. 1B). The hypochord is a transient structure consisting of a single row of cells, which is located just ventral to the notochord of amphibian and fish embryos, and immediately dorsal to the endoderm (Gibson, 1910; Lofberg and Collazo, 1997). The dorsal aorta develops immediately ventral to the hypochord, but the mechanisms driving its formation are unknown.

The pattern of *VEGF* expression in the somites and the hypochord remains relatively constant until the mid-tailbud stage (stage 32-34), after which expression declines in both tissues. This decrease in expression is first noticeable in anterior tissues, but then extends posteriorly (data not shown). By the late tailbud (stage 37) *VEGF* transcripts can no longer be detected in the hypochord. The hypochord itself persists after the formation of the dorsal aorta (which occurs at about stage 35) until about stage 42 when the cells flatten and shrink. The ultimate fate of the hypochord cells remains unknown.

Migration of angioblasts to the midline precedes dorsal aorta formation

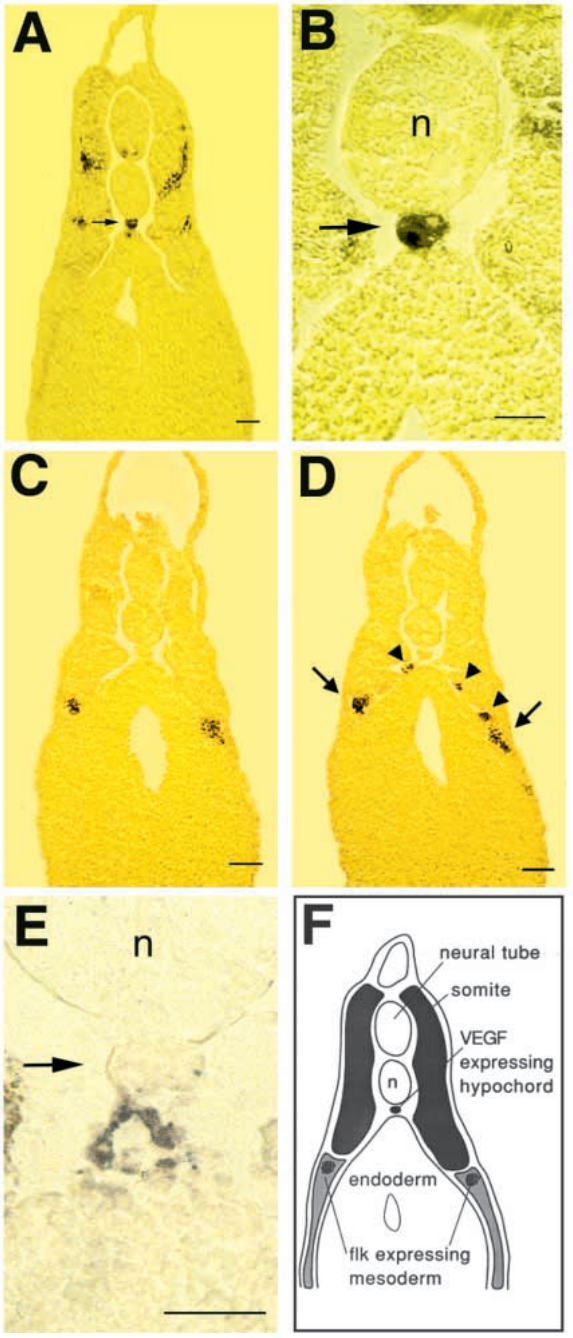
During the tailbud stages (stage 22-32), developmental analysis of *flk-1* expression indicates that a population of angioblasts is present in the mesenchyme of the lateral plate mesoderm, ventral to the somites, and some distance from the *VEGF*-expressing hypochord (Fig. 1C). This is the position where the posterior cardinal veins will later differentiate. However, examination of sections from mid-tailbud stage embryos (stage 32-34) reveals that a subset of *flk-1*-expressing angioblasts appear to migrate over the dorsal-most endoderm, underneath the somites, towards the midline of the embryo (Fig. 1D). In each 10 μm section, approximately 2-6 cells can be observed at intermediate positions between the lateral plate and the hypochord. Migration is first detected in the more anterior tissues of the stage 32 embryo, then in progressively more posterior tissues down the length of the embryo. The wave of migration is apparently complete by stage 34. Expression of *flk-1* in the vascular endothelial cells declines significantly once they reach the midline.

Commencing at about stage 34, formation of a single dorsal aorta in the trunk of the embryo takes place as the endothelial precursor cells organize into a vessel immediately ventral to the hypochord. Although expression decreases rapidly as development proceeds, *flk-1* transcripts can be detected at very low levels in the cells of the differentiated dorsal aorta, shortly before the commencement of blood circulation (Fig. 1E). Concomitant formation of the posterior cardinal veins occurs laterally, at the location of the *flk-1*-expressing cells ventral to the somites. Once the dorsal aorta has formed, the levels of *VEGF* mRNA detected in the hypochord also decline significantly. A schematic representation of the axial tissue layers and the *VEGF* and *flk-1*-expression patterns is presented in Fig. 1F. Note the relative positions of the *VEGF*-expressing hypochord and the lateral plate mesodermal tissues where *flk-1*-expressing angioblasts are located.

Lateral plate mesoderm cells migrate to the position of the dorsal aorta

To demonstrate that migration of cells from the lateral mesoderm to the midline of the embryo does indeed occur, we have carried out labeling experiments using DiI. Labeling of mesenchymal cells of the lateral plate mesoderm of early tailbud embryos (stage 24) was accomplished by injection of a DiI solution. An embryo soon after DiI labeling is shown in Fig. 2A. The embryos were then allowed to develop for approximately 36 hours, until the early tadpole stage (stage 34-36) when they were fixed and sectioned. DiI-labeled cells were visualized in tissue sections using fluorescence microscopy. In the DiI-labeled embryos, individual labeled cells can be observed at some distance from the lateral plate mesoderm where they were labeled (Fig. 2B). Even later during

development, individual labeled cells can be observed within the endothelial layer of the differentiated dorsal aorta (Fig. 2C). Significant medial migration of labeled cells was observed in 85% of experimental embryos (23/27). In the small number of negative cases, the DiI injection probably missed the position of the vascular precursors. Typically, the cells have migrated 200 to 300 μm from the original position of labeling. Lineage labeling of cells after stage 34, fails to reveal any cell movement towards the dorsal midline, indicating that the period of migration has passed. Conversely, lineage labeling of cells earlier during development (stage 22), and assaying prior to stage 32, reveals that no migration has yet occurred (data not shown). Overall, these experiments demonstrate that at least some angioblasts from the lateral plate region migrate to the dorsal midline and contribute to the formation of the dorsal aorta. Due to the uncertainties of DiI labeling, however, it is difficult to determine whether lateral plate angioblasts contribute to the dorsal aorta rather rarely, or whether they represent a major proportion of the dorsal aorta cells.



Removal of lateral plate mesoderm prevents dorsal aorta formation

Additional experiments have been performed to determine whether a dorsal aorta can form in the absence of lateral plate mesoderm tissue. As diagrammed in Fig. 3A, lateral plate mesoderm tissue was dissected from both sides of early tailbud stage embryos (stage 22-24). Care was taken to minimize damage to the overlying epidermis, underlying endoderm and somites. The manipulated embryos were allowed to develop to the late tailbud stage (stage 35) when they were assayed for the presence of vascular structures. In these experiments, the vascular marker, *X-msr* (Devic et al., 1996) was used in preference to *flk-1* because it is expressed at much higher levels in differentiated vascular tissue. Sections through the unmanipulated region of an experimental embryo show the presence of endothelial cells in the posterior cardinal veins, laterally, and in the single dorsal aorta, located immediately ventral to the hypochord (Fig. 3B). In more posterior sections through the microdissected region of the same embryo, there is a complete absence of vascular precursor cells, both at the position of the posterior cardinal veins, (as expected) and at the position of the dorsal aorta (Fig. 3C). Complete elimination of the dorsal aorta, not merely a reduction in size, was observed in 100% of manipulated embryos (22/22). If dissection is performed on only one side of the embryo, dorsal aorta development is hindered on the experimental side, while the unmanipulated side shows the presence of endothelial cells (Fig. 3D). We conclude from these experiments that a subpopulation of angioblasts, originally located in the lateral plate mesoderm, migrates to the dorsal midline to form the dorsal aorta. Removal of this population of migratory angioblasts results in the complete elimination of dorsal aorta precursor cells. Angioblasts that remain in the lateral plate, ventral to the somites, differentiate to form the posterior cardinal veins.

Fig. 1. In situ hybridization reveals expression of *VEGF* in the hypochord and *flk-1* in migrating endothelial precursor cells. (A) Section through the posterior trunk of an early tailbud embryo (stage 30) showing *VEGF* expression in the somites and in the hypochord (arrow). (B) Higher magnification of the hypochord (arrow) stained for *VEGF* expression. The notochord is indicated (n). (C) Section through the trunk of an early tailbud embryo (stage 28) showing expression of *flk-1* in groups of endothelial precursor cells in the mesenchyme of the lateral plate mesoderm. *Flk-1* transcripts are located at the position of the developing cardinal veins, prior to blood vessel formation. (D) Section positionally equivalent to C through the trunk of a mid-tailbud embryo (stage 33) showing migrating angioblasts (arrowheads) that express *flk-1*. Position of the posterior cardinal veins is indicated (arrows). (E) High magnification view of the dorsal aorta (stage 35) stained for *flk-1* expression. Hypochord is indicated (arrow). (F) Schematic representation of tissue layers and expression domains of *VEGF* and *flk-1*. Note that the hypochord lies immediately dorsal to endodermal tissue and at some distance from lateral plate mesoderm tissue. Bar, 40 μm (A,C,D) and 20 μm (B,E).

The diffusible form of VEGF is preferentially expressed in the hypochord

Studies of VEGF in vitro demonstrate that only homodimers of the lowest molecular weight form of VEGF ($VEGF_{122}$ in *Xenopus*) are free to diffuse, while homo- or heterodimers containing the larger forms of the VEGF protein are effectively restricted to the immediate vicinity of the cell of origin (Ferrara et al., 1992). If VEGF emanating from the hypochord is to play a role as a chemoattractant during dorsal aorta development, we hypothesize that the hypochord expresses a significant proportion of the diffusible form of VEGF. To test this hypothesis, we have used RT-PCR to examine the *VEGF* transcripts expressed by the hypochord. A schematic representation of the dissection separating the hypochord from the somites is presented in Fig. 4A. Note that the hypochord consists of a single row of cells beneath the notochord and, for technical reasons, cannot be separated from the notochord in this dissection. This is not likely to be a problem, however, since *VEGF* expression has not been detected in the notochord in any species and so RT-PCR results from the notochord/hypochord tissue fraction will reflect only hypochord VEGF transcript levels. Analysis of the alternatively spliced forms of *VEGF* mRNA expressed in the hypochord/notochord fraction and in the somites is presented in Fig. 4B. This result indicates that the hypochord expresses, almost exclusively, transcripts encoding the small, diffusible form of VEGF. The somites, in contrast, express significant proportions of the mRNAs encoding the intermediate and large forms, in addition to diffusible $VEGF_{122}$. These results indicate that the hypochord expresses a form of VEGF consistent with chemotactic signaling.

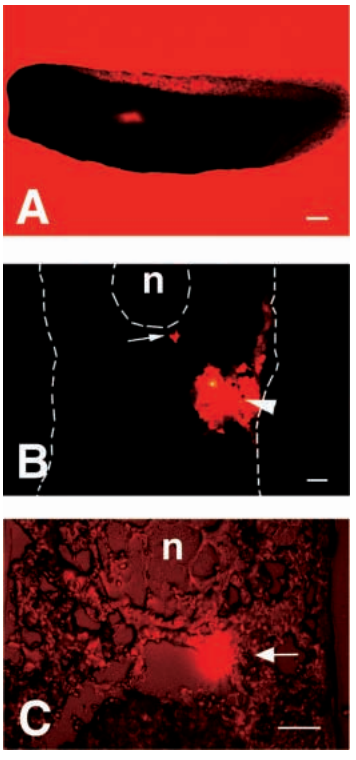


Fig. 2. Lineage tracing of lateral plate mesoderm cells showing migration to the position of the dorsal aorta. (A) Embryo labeled with DiI at the position of the posterior cardinal vein (stage 28). (B) Cryostat section, viewed under fluorescence microscopy, of a tailbud embryo (stage 33) which was labeled with DiI in the lateral plate mesoderm at stage 25. The arrowhead marks the position of DiI labeling and the arrow indicates a cell that has migrated towards the midline near the notochord (n). (C) Cryostat section showing a high magnification view of the dorsal aorta of a later tailbud embryo (stage 35) in which a DiI-labeled cell has become incorporated into the endothelial layer (arrow). Notochord is indicated (n). Bar, 150 μm (A), 20 μm (B,C).

Ectopic VEGF causes ectopic angioblast migration

To directly determine whether VEGF is capable of mediating angioblast migration, we have used a tissue transplantation approach to ectopically express $VEGF_{122}$ in ventral lateral plate mesoderm. In these experiments, VEGF-expressing tissue is generated by microinjection of *Xenopus* embryos with *VEGF* DNA (Fig. 5A). At stage 22-24, VEGF-expressing mesoderm, devoid of endogenous angioblasts, is transplanted into a host embryo at a location ventral to the posterior cardinal vein region. The donor tissue is pigmented while host embryos are albino, facilitating identification of the *VEGF*-expressing tissue. As shown in Fig. 5B, angioblasts migrate ventrally and accumulate within the VEGF-expressing implant. This migration often results in a depletion of the pool of angioblasts at the position of the future posterior cardinal veins. No accumulation of angioblasts is ever observed in implants that do not express VEGF. Migration of angioblasts towards implants was observed in 24% of embryos (15/63) implanted with VEGF-expressing tissue. The fact that migration does not occur in all cases can be attributed to the mosaic expression of injected plasmid DNA, which lowers the actual number of implants that strongly express VEGF. In addition, some implants may have been located too far from the posterior cardinal vein position, placing the angioblasts out of range of the VEGF gradient. As a control to ensure that angioblasts are not induced within the transplanted tissue itself, explants of VEGF-expressing tissue have been cultured separately and angioblasts are never observed in these tissues (Fig. 5C).

To demonstrate that angioblasts originating in the lateral plate mesoderm are indeed migrating towards the implant, DiI labeling has been used to mark cells at the position of the posterior cardinal veins. At a time consistent with the migration of angioblasts to the dorsal aorta (stage 33), embryos were assayed by fluorescent microscopy. Labeled cells from the region of the lateral plate mesoderm can be observed in the process of migrating towards the *VEGF*-expressing implant (Fig. 5D). Angioblasts have reproducibly been observed to migrate several hundred micrometers from the position of DiI-labeling, towards the ectopic source of $VEGF_{122}$. Migration of DiI-labeled cells is never observed towards implants not expressing VEGF. Overall, these experiments demonstrate that localized sources of $VEGF_{122}$ are capable of inducing angioblast migration in vivo.

DISCUSSION

In this report, we describe the formation of the dorsal aorta in the posterior trunk of the *Xenopus* embryo. This process involves the migration of *flk-1*-expressing endothelial

precursor cells to the location of the future single dorsal aorta at the embryonic midline. Supporting this model, we find that the hypochord, which lies at the embryonic midline, immediately dorsal to the position where the dorsal aorta will differentiate, is a highly concentrated source of the diffusible form of VEGF and we have demonstrated that VEGF can act as a chemoattractant for angioblasts in the embryo. We propose that a chemotactic gradient of VEGF is responsible for guiding migration of angioblasts during development of the dorsal aorta in *Xenopus*.

Expression of *VEGF* in the hypochord

In addition to the somites, the major domain of *VEGF* expression in the axial region of the *Xenopus* embryo is the hypochord. The hypochord, first described in Selachian embryos – sharks and dogfishes – (Leydig, 1852), is a transient, endodermally derived, rod-like structure found immediately ventral to the notochord in the embryos of fish and Amphibia (Reinhart, 1904; Gibson, 1910). Although its function is completely unknown, the location of the hypochord ventral to the notochord has led to the suggestion that it plays a role in positioning the dorsal aorta (Gibson, 1910; Lofberg and Collazo, 1997). Two additional pieces of information support this possibility. First, *VEGF* is expressed at high levels in the hypochord in *Xenopus* (Cleaver et al., 1997 and Fig. 1A,B). Second, the existence of the hypochord in the embryo is temporally correlated with the assembly of angioblasts to form the dorsal aorta. It seems plausible that a gradient of VEGF is established between the hypochord and the lateral regions of the embryo and this gradient mediates the directed migration of angioblasts.

Angioblasts migrate towards the dorsal midline

In tailbud stage embryos, a subset of *flk-1*-expressing cells can be observed at intermediate positions between the lateral plate mesoderm and the hypochord (Fig. 1D). When these cells reach the dorsal midline, immediately ventral to the hypochord, they differentiate to form a single dorsal aorta (Fig. 1E). We have demonstrated in two ways that these cells are indeed migrating angioblasts that arise exclusively in the lateral plate mesoderm. First, lineage tracing indicates that cells labeled in the lateral plate mesoderm can later contribute to the endothelial cell layer of the dorsal aorta (Fig. 2C). This shows that at least some lateral plate mesoderm cells can migrate towards the dorsal midline and become part of the dorsal aorta. However, it is not clear from these experiments whether this is an occasional event and the dorsal aorta can form in the absence of these lateral plate mesoderm angioblasts, or whether the dorsal aorta develops exclusively from lateral plate angioblasts. To address this question, we have carried out dissection experiments in which lateral plate mesoderm cells, including the population of angioblasts, are removed from both sides of the developing embryo. In the manipulated region of the trunk, angioblasts are completely absent and neither a dorsal aorta nor posterior cardinal veins form (Fig. 3C). The complete absence of dorsal aorta formation strongly suggests that angioblasts originating in the lateral plate mesoderm represent a major proportion of cells contributing to the dorsal aorta. Indeed it seems likely that the dorsal aorta is composed entirely of migrating angioblasts from the lateral plate mesoderm and their descendants. This experiment also eliminates the possibility that a significant number of dorsal aorta angioblasts arise either from the underlying endoderm or from the adjacent medial somitic tissues, since the dissection only involves superficial tissues in the lateral plate. However, the possibility that angioblasts arising from the ventrolateral portion of the somites (Wilting et al., 1995; Dieterlen-Lievre, 1997) may contribute to the dorsal aorta cannot be ruled out, since the dissection may slightly damage these tissues. We conclude from these experiments that a pool of endothelial precursor cells exists in the lateral plate mesoderm of the neurula stage embryo and that these cells contribute to the formation of both the posterior cardinal veins and the dorsal aorta.

The role of VEGF in angioblast migration

The results presented above demonstrate that a population of *flk-1*-expressing angioblasts migrate towards the embryonic midline. In addition, we have shown that a localized source of

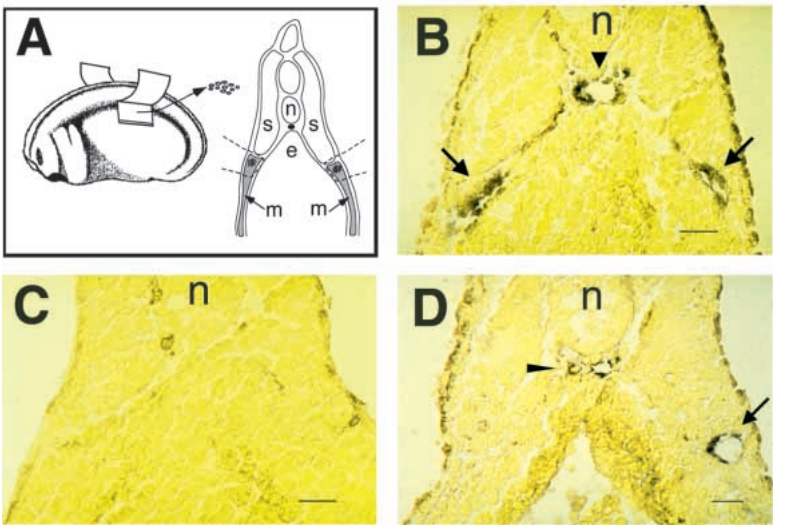


Fig. 3. Removal of lateral plate angioblasts eliminates dorsal aorta formation. (A) Schematic representation of microdissection experiment. Lateral plate mesoderm at the position of the posterior cardinal veins is removed, without damaging underlying endoderm or midline structures. Notochord (n), somite (s), endoderm (e) and mesoderm (m) are indicated. (B) Section through the unmanipulated region of the trunk of a stage 35 embryo, showing *X-msr* expression in the differentiated blood vessels. The posterior cardinal veins are evident laterally on each side of the embryo (arrows), and the single dorsal aorta (arrowhead) is located immediately under the hypochord. The position of the notochord is indicated (n). (C) Section through the dissected region of the trunk (more posterior) of the embryo shown in B. Posterior cardinal veins and dorsal aorta are not detectable by *X-msr* staining. Note the absence of damage to the midline structures as the hypochord, notochord and underlying endoderm are undisturbed. (D) Section through embryo where the lateral plate mesoderm on one side only was dissected. Note the absence of the posterior cardinal vein on the manipulated side of the embryo and the abnormality in the dorsal aorta at the midline (narrow arrowhead). The posterior cardinal vein on the opposite side is undisturbed (arrow). Bars, 25 μm (B-D).

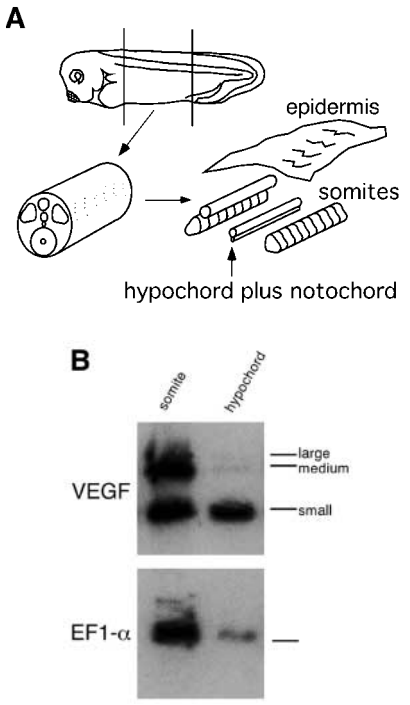


Fig. 4. The hypochord expresses transcripts for the small, diffusible form of the VEGF protein. (A) Schematic depiction of the dissection of a stage 32 embryo to separate the hypochord from the somites. (B) RT-PCR analysis demonstrates that transcripts encoding the small, diffusible form of VEGF are preferentially expressed in the hypochord relative to the intermediate or large forms. Positions of the PCR products representing the large, medium and small transcripts are indicated to the right of the figure. Total amount of RNA in the hypochord and somite fractions was estimated using the ubiquitous EF1-α sequence.

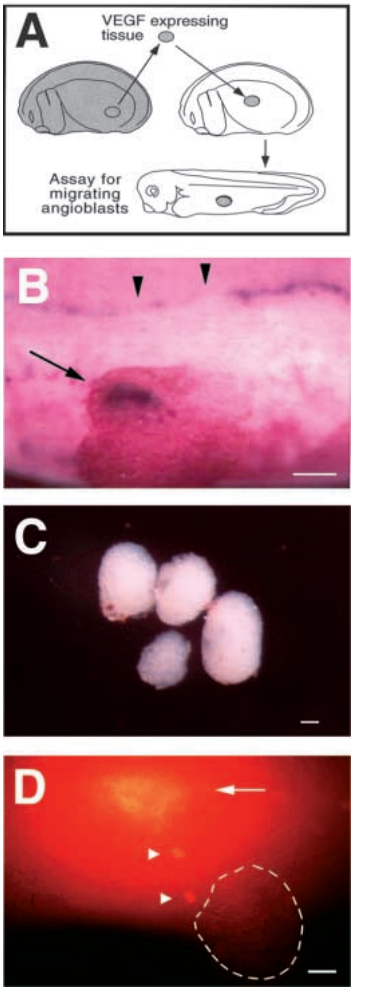


Fig. 5. VEGF can mediate ectopic migration of angioblasts. (A) Diagram illustrating experiment to generate localized sources of VEGF in the embryo. (B) In situ hybridization detection of angioblasts using *X-msr* probe. Angioblasts have migrated into the implant (arrow). Notice the depletion of angioblasts from the position of the posterior cardinal vein (arrowheads). (C) In situ hybridization analysis of *VEGF*-expressing tissue explants after culture in isolation. No *X-msr*-expressing cells are detected in these explants. (D). DiI labeling of lateral plate mesoderm, showing migration of angioblasts towards the VEGF-expressing implant (indicated by dashed outline). Position of the original DiI labeling is indicated by the arrow, and migrating cells are indicated by arrowheads. Bar, 200 μm (B) and 100 μm (C,D).

VEGF exists at the embryonic midline (Fig. 1B) and that $VEGF_{122}$ is sufficient to mediate angioblast migration (Fig. 5). Although we cannot conclusively demonstrate that angioblast migration to the dorsal midline is mediated by VEGF signaling, this proposal is plausible and is supported by additional evidence. First, it has previously been demonstrated that different forms of VEGF protein exhibit different biochemical properties, and that only homodimers of the lowest molecular weight form of the protein are free to diffuse (Ferrara et al., 1992). Our experiments show that the great majority of the *VEGF* transcripts expressed by the hypochord encode the diffusible form of the protein (Fig. 4B) and, therefore, establishment of a concentration gradient is possible. To the best of our knowledge, this is the first demonstration of tissue-specific alternative splicing of the *VEGF* primary transcript. Second, mice that are homozygous mutant for the *VEGF* gene show a complete absence of dorsal aorta formation (Carmeliet et al., 1996). This result strongly implies a direct role for VEGF in dorsal aorta development in the mammalian embryo. At present however, it is not clear which midline structures in the mouse embryo strongly express VEGF, or how VEGF expression might be involved in patterning development of the paired dorsal aortae which later fuse into a single vessel.

The specific roles of the different VEGF forms expressed in the somites and in the hypochord remain an open question. Studies of the different human VEGF proteins indicate that the intermediate form of VEGF, $VEGF_{165}$, exhibits greater biological activity than $VEGF_{121}$ in most assays (Keyt et al., 1996). For example, in mitogenic potency assays, $VEGF_{165}$ is 50- to 100-fold more potent than $VEGF_{121}$, and the carboxyl terminal domain of $VEGF_{165}$ has been shown to be critical for this activity. No experiments however, have determined whether one of the VEGF proteins preferentially induces directed cell movement. Therefore, it is possible that the different forms of VEGF mediate very different responses in the embryo, such as proliferation or chemotaxis.

Why do a proportion of the lateral plate *flk-1*-expressing

angioblasts migrate to form the dorsal aorta, while others remain in place to form the posterior cardinal veins? The molecular cues that determine which endothelial precursor cells will migrate are completely unknown, but there are several possibilities. First, some cells may be inherently competent to respond to chemotactic VEGF signaling while others are not. Second, it is possible that only those cells directly in contact with the VEGF-expressing somites are signaled to initiate migration. For example, we have shown that both diffusible and non-diffusible forms of *VEGF* are expressed by the somites, and it is therefore possible that the lower surface of the somites provides a signal for adjacent angioblasts to begin migration. The migration of angioblasts towards *VEGF*-expressing implanted tissue would argue against this possibility, however. Third, the surface properties of the endoderm or of the extracellular matrix between the endoderm and the ventral surface of the somite may allow only a subset of the angioblasts to migrate. Cells not in contact with the appropriate surface may remain in the lateral plate mesoderm. Lastly, it is possible that the somites simply provide a physical barrier to the migration of a portion of the angioblast population, allowing some to move towards the midline while blocking others. Additional experiments will be carried out to address this question.

Patterning of the dorsal aorta

Axial signals from the notochord are known to be involved in patterning the midline and paraxial tissues. The notochord has been shown to induce cell fate changes that affect the differentiation of the floor plate (Placzek et al., 1990), somites (Brand-Saberi et al., 1993; Halpern et al., 1993) and motor neurons (Yamada et al., 1993). Recent experiments examining zebrafish mutants have suggested that the notochord plays a role in organizing axial blood vessel formation (Sumoy et al., 1997; Fouquet et al., 1997). Using *flk-1* expression as a marker for angioblasts, these experiments show that the floating head (*flh*) and no tail (*ntl*) mutant embryos, which lack a notochord, also fail to form a dorsal aorta. Mosaic analysis reveals that transplanted wild-type cells can form notochord tissue in *flh* mutants and that a dorsal aorta primordium assembles in these embryos (Fouquet et al., 1997). Based on our observations in *Xenopus*, we suggest that the notochord is required for induction of the hypochord in zebrafish, and that the hypochord is subsequently responsible for patterning of the dorsal aorta by directing migration of angioblasts to a position below the notochord. Consistent with this model, previous observations of zebrafish embryos have reported medial migration of angioblasts towards the dorsal midline (Stockard, 1915; Al-Adhami and Kunz, 1977). The expression pattern of *VEGF* has not been reported in zebrafish, but we predict that the hypochord will also express *VEGF* in this organism.

It is not clear whether a structure equivalent to the hypochord exists in the embryos of other vertebrates. If not, which structure or what signals are responsible for directing the formation of the dorsal aorta in these organisms? It has been suggested that in Amphioxus, which does not have a hypochord, the epibranchial groove may play an analogous role in directing dorsal aorta location (Gibson, 1910). In amniote embryos, a vestigial structure reminiscent of the epibranchial groove exists at the midline and this could conceivably play an analogous role (Prenant, 1898; de Meuron, 1886; Wheeler, 1899). However, it is possible that in many organisms, completely different embryonic structures express VEGF and have taken on a role in vascular patterning. As mentioned previously, one specific defect observed in the homozygous VEGF mutant mice is the complete absence of the dorsal aorta over its entire length (Carmeliet et al., 1996). In mice, *VEGF* does not appear to be expressed in midline tissues, such as in the notochord, although it is expressed at high levels in the underlying endoderm (Dumont et al, 1995). Thus, the endoderm may have taken over an inductive role. *Xenopus* embryos express VEGF in the somites and it is possible therefore, that expression of VEGF by the somites is important for dorsal aorta patterning in other organisms. We note that the paired dorsal aortae in the avian embryo initially assemble immediately ventral to the somites (Flamme et al., 1995a) and that the somites are now reported to be a source of VEGF expression in the avian embryo (Aitkenhead et al., 1998).

Our observations indicate that the series of cellular events leading to development of the dorsal aorta varies between organisms. In *Xenopus,* the posterior cardinal veins develop from angioblasts that appear to arise in situ in the lateral plate mesoderm, at the location where they will differentiate. In contrast, the single dorsal aorta results from an aggregation of angioblasts that have migrated a significant distance under the somites towards the midline. These major vessels then differentiate almost simultaneously. In quail, however, the paired dorsal aortae develop significantly earlier than the posterior cardinal veins. In addition, QH-1 staining reveals that endothelial cells of the posterior cardinal veins migrate from the periphery of the embryo before they coalesce and differentiate (Poole and Coffin, 1991). The differences in vascular patterning between *Xenopus* and quail may reflect a difference in the location of endothelial precursor cells or may be the result of differences in the pattern of *VEGF* expression. Recent work, however, indicates that the formation of the dorsal aorta in avians also involves the medial movement of angioblasts (C. J. Drake and C. D. Little, personal communication). Considering the differences in morphology of the avian and amphibian embryos, the moderate movement of angioblasts observed in birds may be analogous to the more extensive movements observed in frog embryos . Further examination of angioblast migration and VEGF expression patterns in different species will contribute to our understanding of these fundamental patterning events.

In conclusion, our results indicate that a previously undescribed mechanism underlies formation of the dorsal aorta in *Xenopus*. These results show that a single dorsal aorta forms in the posterior trunk following migration of angioblasts from lateral tissues and coalescence into a vessel at the midline. The single dorsal aorta in posterior regions of the *Xenopus* embryo does not form by fusion of paired dorsal aortae as described in the classical literature (Nieuwkoop and Faber, 1994). In addition, our results suggest that VEGF expression in the hypochord is responsible for mediating migration of angioblasts to the dorsal midline. It seems reasonable to predict that localized sources of VEGF will play a general role in patterning of other major vessels during vertebrate vascular development.

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