Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant

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Abstract
Angiogenesis is a fundamental vertebrate developmental process that requires signalling by the secreted protein vascular endothelial growth factor-A (VEGF-A). VEGF-A functions in the development of embryonic structures, during tissue remodelling and for the growth of tumour-induced vasculature. The study of the role of VEGF-A during normal development has been significantly complicated by the dominant, haplo-insufficient nature of VEGF-A-targeted mutations in mice. We have used morpholino-based targeted gene knock-down technology to generate a zebrafish VEGF-A morphant loss of function model. Zebrafish VEGF-A morphant embryos develop with an enlarged pericardium and with major blood vessel deficiencies. Morphological assessment at 2 days of development indicates a nearly complete absence of both axial and intersegmental vasculature, with no or reduced numbers of circulating red blood cells. Molecular analysis using the endothelial markers fli-1 and flk-1 at 1 day of development demonstrates a fundamental distinction between VEGF-A requirements for axial and intersegmental vascular structure specification. VEGF-A is not required for the initial establishment of axial vasculature patterning, whereas all development of intersegmental vasculature is dependent on VEGF-A signalling. The zebrafish thus serves as a quality model for the study of conserved vertebrate angiogenesis processes during embryonic development. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: zebrafish; VEGF-A; fli-1; flk-1; angiogenesis; vasculature; morpholino; morphant

Introduction
Signalling by members of the vascular endothelial growth factor (VEGF) gene family is implicated in the formation of vasculature during embryogenesis, during wound healing and for the growth of tumour-induced vasculature (for reviews, see Carmeliet and Collen, 1997; Ferrara, 1999). Pioneering work in mice with VEGF-A demonstrates the extreme dose responsiveness of the mouse embryo to VEGF-A signalling during development. Loss of a single copy of the VEGF-A gene induces haplo-insufficient lethality by day 9.5 pc (Ferrara et al., 1996; Carmeliet et al., 1996). This biological hurdle to the genetic investigation of VEGF-A requirements during later development has resulted in a series of experiments using conditional knock-out strategies (Gerber et al., 1999; Haigh et al., 2000) or dominant negative proteins (Gerber et al., 1999). A more recent approach to address this problem used intravenous injection of antisense oligonucleotides in pregnant mice to reveal loss of function requirements of VEGF-A function during murine embryogenesis (Driver et al., 1999).
Zebrafish embryos develop externally and have only limited requirements for a functioning circulatory system during early development. For example, embryos with no circulating red blood cells due to porphyria live through the first 3 days of development (Ransom et al., 1996), a time period which includes all of segmentation and organogenesis in the fish embryo. Multiple mutations in cardiovascular development were isolated in the initial large-scale chemical mutagenesis screens (Stainier et al., 1996; Chen et al., 1996; Weinstein et al., 1995). The zebrafish has the potential to rapidly assess the biological role of angiogenic factors required for this essential vertebrate process.

We used morpholino-based oligonucleotides (morpholinos, MO; Summerton, 1999) to create a VEGF-A loss of function developmental model to circumvent potential haplo-insufficient genetic complications in zebrafish. These VEGF-A morphant embryos display an enlarged pericardium and a modest body size decrease and have severe deficiencies in vascular development. We show that the establishment of the axial and intersegmental vasculature has distinct requirements for VEGF-A signalling, as revealed by analysis of the expression of two endothelial markers, the tyrosine kinase VEGF receptor, flk-1 (Sumoy et al., 1997; Fouquet et al., 1997; Thompson et al., 1998) and the early vascular marker, the transcription factor fli-1 (Thompson et al., 1998; Brown et al., 2000). Initial axial expression of these markers is not altered in VEGF-A morphant embryos, while no intersegmental expression of these markers is detected. Both axial and intersegmental vasculature fail to function in VEGF-A morphant embryos, however, indicating a role for VEGF-A beyond the establishment of flk-1 and fli-1 expression in blood vessel formation. Similar phenotypes were observed in some mutations found in chemical mutagenesis screens, suggesting a possible role for these genes in VEGF-A signalling in the zebrafish embryo.

Materials and methods

Morpholino injections

Morpholino antisense oligonucleotides were purchased from Gene-Tools, LLC (Corvallis, OR). Solutions were prepared and injected as described in Nasevicius and Ekker (2000): VEGF-A-1 (predicted start codon is underlined); 5′-GTATCAAATAACAAACCCAGTTCAT-3′; VEGF-A-1D4 (four-base mismatch), 5′-GTAaCAAATAAACAACCAtGTTgAT-3′; VEGF-A-3, 5′-TAAGAAAAGCGAAGCTGCTGGGTATG-3′.

FITC–Dextran injections

Microangiography was performed similarly to the method described in Weinstein et al. (1995). Fluorescein isothiocyanate–Dextran (FITC–Dextran) with a molecular weight of 200000 Da (SIGMA, catalogue #FD-2000S) was used for these studies. The Dextran was solubilized in 1× Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6) at 2 mg/ml concentration. 10 μl of the prepared solution was injected into the sinus venosus/cardinal vein of the anaesthetized 48 h embryo. The visualization and photography was performed on a ZEISS Axioskop 2 microscope using a standard FITC filter set.

Red blood cell (RBC) fluorescence visualization in urod morpholino-injected embryos

The fluorescence of red blood cells was observed and photographed as described in Nasevicius and Ekker (2000).

RNA localization

Whole mount in situ hybridization was performed as described in Jowett (1999). Hybridization was performed at 65°C. Riboprobes for fli-1 and flk-1 were synthesized using plasmids zffli-1 and zfflk-1 (Thompson et al., 1998), digested with EcoRI and SmaI, respectively. T7 polymerase was used for riboprobe synthesis.

Tissue sectioning and visualization

Embryos with FITC–Dextran visualized vasculature were fixed overnight, embedded into paraffin using standard procedures, and sectioned. FITC–Dextran fluorescence outlining blood vessels in unprocessed tissue sections was visualized using ZEISS Axioskop 2 microscope with a FITC filter set. Histological haematoxylin–eosin staining of the sections was subsequently carried out using a standard protocol.
Digital photography

Bright-field and in situ photography was performed on a ZEISS Axioplan 2 microscope using Nikon CoolPix 990 (bright-field) or Kodak DCS 420 (in situ) digital cameras. For fluorescent photography, a ZEISS AxioCam or a Nikon CoolPix 990 digital camera was used.

Results and discussion

Zebrafish VEGF-A is expressed during embryogenesis in the anterior nervous system, in mesoderm flanking the prospective heart fields, and in somitic mesoderm that flanks the developing endoderm (Liang et al., 1998). We generated morpholino (Summerton, 1999) antisense oligonucleotides against VEGF-A to analyse the requirements of this gene during embryonic development. Morpholinos have been recently shown to be effective at gene inactivation during the first 2 days of zebrafish development (Nasevicius and Ekker, 2000). We term the loss of function effects due to morpholinos a ‘morphant’ phenotype to distinguish this assessment of gene function from that of classical mutant analyses.

VEGF-A morphant embryos develop with no overt phenotype during the first day of development. The VEGF-A morphant phenotype at 2 days of embryogenesis, however, consists of an enlarged pericardium, no circulating red blood cells, a slight reduction in neural tube and overall body size, and little or no functioning vasculature (Figure 1B). In a subset of embryos, red blood cell accumulation can be noted in the ventral tail (Figure 1C).

We used two separate fluorescent assays to assess vascular function in detail. First, we generated fluorescent-labelled red blood cells through the inactivation of the uroporphyrinogen decarboxylase (urop; Wang et al., 1998) gene using morpholinos (Nasevicius and Ekker, 2000). Red blood cells quantitatively accumulate in the anterior hypochord (Figure 2B). To analyse the vasculature directly, we injected fluorescein isothiocyanate–Dextran (FITC–Dextran) into the sinus venosa/cardinal vein of an anaesthetized 48 h embryo (Figure 2C). This microangiography assay labels the entire vasculature of the zebrafish embryo, including the yolk sac, heart, head, axial and intersegmental blood vessels (Weinstein et al., 1995). These structures are differentially sensitive to VEGF-A signalling. At high dose injections of VEGF-A morpholino, the only vasculature detectable in these animals using this method is found in the heart and yolk (Figure 2D). The vasculature either fails to form at all or contains no functioning connections to the heart in these embryos. To distinguish between these possibilities we performed histological analyses on these most severely effected embryos (Figure 2G–J). Neither dorsal aorta nor axial vein were noted in the injected embryos (Figure 2I, J). We also observed a frequent but less severe phenotypic class of embryos with heart, yolk and head blood vessels (Figure 2E); no axial or intersegmental vasculature was observed in these embryos, however. The least severe phenotypic classification was represented by embryos with reduced intersegmental vasculature and normal heart, yolk, head and axial blood vessels (Figure 2F). The penetrance of these phenotypic classes is very dose-dependent (Table 1), consistent with the strong dose dependence of VEGF-A function in mouse embryos (Ferrara et al., 1996; Carmeliet et al., 1996). Heterozygous mouse VEGF-A mutants showed a reduced dorsal aorta detected by histological analysis. Fewer intersegmental blood vessels were also detected by a tissue-specific lacZ expression (Carmeliet et al., 1996). Lack of the dorsal aorta was indicated by histological analysis in homozygous mouse VEGF-A mutants (Carmeliet et al., 1996), suggesting that the most severe zebrafish morphant classes represent a nearly complete loss of function phenotype. However, while mouse VEGF-A mutants also display hearts with underdeveloped myoblasts (Ferrara et al., 1996; Haigh et al., 2000), the heart in zebrafish VEGF-A morphants has an essentially normal appearance with a slightly enlarged atrium and ventricle, possibly due to higher cardiac pressure (histological analysis not shown).

We assessed the phenotypic effects of two VEGF-A morpholinos of non-overlapping sequence and of a four-base mismatch sequence (see Materials and methods) to confirm the specificity of targeting (Table 2). The four-base mismatch morpholino VEGF-A-D4 demonstrates the sequence-specific nature of the noted effects of the VEGF-A-1 morpholino (Table 2). To test independently for the specificity of targeting to the endogenous VEGF-A gene, we used a second morpholino of completely independent sequence (VEGF-A-3). This very potent morpholino caused the same
phenotypic effects on development, including a dose-dependent reduction of vascular function (Table 2), pericardial oedema and blood accumulation in the tail (data not shown). The observed differential efficacy might be due to the different secondary structure of the morpholinos or the targeted mRNA region. Alternatively, the effect might be caused by the higher VEGF-A-3 predicted melting temperature due to higher G/C content (48%) as compared to VEGF-A-1 (28%). We conclude that the observed effects are due to morpholino-based inactivation of VEGF-A gene function through the specific inhibition of VEGF-A transcript translation.

A number of genes whose mutation results in cardiovascular defects were observed in the chemical mutagenesis screens (Stainier et al., 1996; Chen et al., 1996). None of these mutations strongly resemble the VEGF-A morphant effects, although mutant embryos with overlapping phenotypes were

Table 1. Microangiography analysis of VEGF-A-1 morpholino injection effects at 48 h

<table>
<thead>
<tr>
<th>Observed phenotypes (frequency, %)</th>
<th>Injected VEGF-A-1 morpholino dose (ng)</th>
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</thead>
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<tr>
<td></td>
<td>3</td>
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<tr>
<td>Heart and yolk vasculature only</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>No axial or intersegmental vasculature</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>No/reduced intersegmental vasculature</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>Normal vasculature</td>
<td>23 ± 10</td>
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<tr>
<td>Pericardial oedema</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Blood accumulation in anterior hypochord</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Blood accumulation in tail</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Total embryo number</td>
<td>110</td>
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</table>

Four experiments were performed with each morpholino dose. The phenotype frequencies in every experiment were averaged and entered into the table as average frequency. The differences between the average frequency and the individual experiment frequencies were averaged and entered into the table as a standard error.
Table 2. Microangiography analysis of VEGF-A-D4 and VEGF-A-3 morpholino injected embryos at 48 h

<table>
<thead>
<tr>
<th>VEGF-A-D4 morpholino</th>
<th>Injected dose (ng)</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>18</th>
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<tbody>
<tr>
<td>Heart and yolk vasculature only</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>2 ± 3</td>
<td>3 ± 0</td>
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<td></td>
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<tr>
<td>No axial or intersegmental vasculature</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>No/reduced intersegmental vasculature</td>
<td>7 ± 0</td>
<td>6 ± 3</td>
<td>5 ± 2</td>
<td>22 ± 1</td>
<td>14 ± 3</td>
<td></td>
</tr>
<tr>
<td>Normal vasculature</td>
<td>92 ± 1</td>
<td>94 ± 3</td>
<td>93 ± 3</td>
<td>75 ± 2</td>
<td>84 ± 3</td>
<td></td>
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<td>Total embryo number</td>
<td>163</td>
<td>116</td>
<td>119</td>
<td>76</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>VEGF-A-3 morpholino</th>
<th>0.5</th>
<th>1.5</th>
<th>3</th>
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<tr>
<td>Heart and yolk vasculature only</td>
<td>0 ± 0</td>
<td>13 ± 13</td>
<td>27 ± 11</td>
</tr>
<tr>
<td>No axial or intersegmental vasculature</td>
<td>0 ± 0</td>
<td>24 ± 24</td>
<td>40 ± 13</td>
</tr>
<tr>
<td>No/reduced intersegmental vasculature</td>
<td>18 ± 8</td>
<td>37 ± 11</td>
<td>24 ± 14</td>
</tr>
<tr>
<td>Normal vasculature</td>
<td>81 ± 2</td>
<td>26 ± 26</td>
<td>10 ± 10</td>
</tr>
<tr>
<td>Total embryo number</td>
<td>32</td>
<td>71</td>
<td>69</td>
</tr>
</tbody>
</table>

Four (VEGF-A-D4 morpholino) or two (VEGF-A-3 morpholino) experiments were performed with each morpholino dose. The phenotype noted. Multiple loci result in embryos with cardiac oedema, and a similar accumulation of blood in the ventral tail fin was noted due to disorganized endothelia in the scotch tape (sco) mutation (Chen et al., 1996). Several mutations with altered circulation were noted (Stainier et al., 1996; Chen et al., 1996), including gridlock, which encodes for a bHLH protein that is required only for arterial development (Weinstein et al., 1995; Stainier et al., 1996; Zhong et al., 2000). The role of these genes in VEGF signalling awaits molecular genetic characterization of the remaining loci.

We analysed the expression of two endodermal vascular markers in VEGF-A morphant embryos. The transcription factor fli-1 is a very early marker of vascular cell fate specification (Thompson et al., 1998; Brown et al., 2000). In wild-type 26 h embryos, fli-1 is expressed in the forming dorsal aorta and axial vein (axial vessels; arrowhead in Figure 3A) and in the intersegmental vasculature in overlying somites (Figure 3A, arrows). In embryos that fail to complete vascular development, only a subset of the vascular expression pattern of these genes is altered, contrary to all vasculature sensitivity to VEGF-A knock-down. No detectable intersegmental expression is noted (Figure 3B).

**Figure 2.** Visualization of vasculature defects in VEGF-A-1 morphants using microangiography (Weinstein et al., 1995). (A, C, G, H) Wild-type embryos. (B, D–F, I, J) Embryos injected with 9 ng VEGF-A-1 morpholino. (A, B) 36 h embryos, with red blood cells (RBC) visualized by the injection of 9 ng uroD morpholino. (A) Fluorescing RBC highlight axial vasculature (white arrowhead), head vasculature (white arrows), yolk sac (yellow arrowhead) and heart (red arrowhead). (B) In the VEGF-A-1 morphant, RBC are localized only to anterior aorta (white arrowheads). (C–F) 48 h embryos with vasculature visualized by FITC-Dextran injection. (C) Wild-type embryo injected with FITC-Dextran. Axial vasculature (white arrowhead), intersegmental blood vessels (yellow arrows), head vasculature, yolk sac (yellow arrowhead) and heart (red arrowhead) are visible. (D) The strongest effect observed upon VEGF-A-1 morpholino injection. The injected FITC-Dextran spreads throughout the yolk sac and heart only. Head, axial and intersegmental blood vessels are not visible. (E) Moderate VEGF-A-1 morpholino injection effect. The injected FITC-Dextran highlights yolk sac, heart, and head blood vessels only. (F) Weak VEGF-A-1 morpholino injection effect. No/few intersegmental blood vessels are observed (yellow arrows). Yolk sac, heart, head vasculature and axial blood vessels appear to be normal. In panels A–F, axial vessels are indicated by white arrowhead, intersegmental blood vessels are indicated by yellow arrows, head vasculature is indicated by white arrow, heart is indicated by red arrowhead, and yolk sac is indicated by yellow arrowhead. (G–I) Mid-trunk cross-sections of strongly effected 48 h VEGF morphant embryos. (G, I) Fluorescence images of sectioned embryos with FITC-Dextran visualized vasculature. Dorsal aorta (white arrow) and axial vein (white arrowhead) are indicated by FITC-Dextran fluorescence in the wild-type embryo (G), while the vasculature is absent in the injected embryo (I; white arrow and arrowhead indicate where dorsal aorta and axial vein would normally be). (H, J) Haematoxylin–eosin-stained sections shown in (G) and (I), respectively. White arrows indicate present (H) or absent (J) dorsal aorta. White arrowheads indicate present (H) or absent (J) axial vein. Bars = 0.2 mm.
coinciding with the exquisite intersegmental vascular endoderm sensitivity to VEGF signalling (Table 1). The cells are either not properly specified or fail to migrate during formation of the intersegmental vessels. Similar results were obtained upon analysis of expression of the VEGF receptor, flk-1. flk-1 transcript distribution is very similar to that of fli-1 in the trunk and tail of wild-type embryos (Figure 3C). In VEGF-A morphant embryos, intersegmental but not axial expression is absent (Figure 3D). A significant reduction in flk-1 gene expression was noted in mouse embryos with no VEGF-A activity (Carmeliet et al., 1996). A less extreme lack of flk-1-expressing cells in the intersegmental vasculature was also observed in the mouse with the partial and conditional VEGF-A knock-out (Haigh et al., 2000). The results in Figure 3 represent work with 9 ng VEGF-A-1 injection-dose embryos; 18 ng injection-dose embryos display the same specific loss of expression only in the intersegmental regions for both fli-1 and flk-1 (data not shown).

The distinct responsiveness of the expression of the endothelial marker fli-1 in intersegmental vessels to VEGF-A signalling demonstrates a dual role for VEGF during vascular development. First, VEGF-A is required for proper axial vessel formation but not for initial axial vessel patterning. Second, VEGF-A is required for intersegmental vessel cell specification or migration and, presumably, for subsequent vascular formation. The lack of a requirement for VEGF signalling for flk-1 expression is consistent with previous observations of paracrine modes of VEGF signalling (reviewed in Ferrara, 1999). The expression of the VEGF receptor flk-1 is, however, VEGF-dependent during intersegmental vascularization. This latter observation suggests a possible autoregulatory loop, functioning during vasculogenesis of the intersegmental vessels.

The strong conservation of VEGF function from fish to mammals implicates this as a fundamental vertebrate biological pathway. The use of morpholino-based gene targeting represents a new tool in the genetic repertoire of vertebrate biologists and,
combined with the excellent embryology of the zebrafish, will be extremely powerful in the elaboration of gene function for similarly conserved developmental processes. This method will help further elaborate the role of VEGF during embryogenesis through the targeted knockdown of other players in this signalling pathway in this outstanding model system.

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