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The effect of VEGF on blood vessels and blood cells during *Xenopus* development

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Abstract

Vascular endothelial growth factor (VEGF) is known to play an essential role in vascular development. We have overexpressed $VEGF_{122}$ or $VEGF_{170}$, which are equivalent to mouse $VEGF_{120}$ and $VEGF_{164}$, in developing *Xenopus* embryos. Overexpression of $VEGF_{170}$ but not $VEGF_{122}$ demonstrated an absence of expression of hematopoietic markers α-globin and GATA-1 but only in the posterior portion of the blood island. Interestingly, strong signals of endothelial markers, msr, fli-1, and tie-2, were detectable in those regions, instead of hematopoietic markers. These results suggested both that injection of $VEGF_{170}$ resulted in disturbance of vasculogenesis in the posterior portion of the blood island, with excessive production of endothelial cells at the expense of blood cells, and that the anterior and posterior portions of the VBI may have distinct characteristics.

Keywords: VEGF; Hematopoiesis; Vasculogenesis; Angiogenesis

A close relationship between the blood and endothelial cells exists during development. Hematopoiesis is known to occur in two distinct phases, termed primitive and definitive hematopoiesis, based on the time of initiation, site of development, cell morphology, globin content, and potential to differentiate [1]. Primitive red blood cells are produced exclusively in the ventral blood island (VBI, a functional equivalent of the extraembryonic yolk sac blood islands of mammals) of *Xenopus*. In the VBI, both hematopoietic and endothelial cells simultaneously arise, forming structures in which primitive hematopoietic cells are surrounded by endothelial cells, giving rise to the vitelline vein [2]. Definitive blood cells arise predominantly from the dorsolateral plate (DLP, equivalent to the intraembryonic aorta-gonads-mesonephros region). Definitive hematopoiesis is closely related to the posterior cardial vein [2,3]. These close spatial and temporal relationships between blood and endothelial cells have led to the hypothesis that they have a bipotential precursor, the hemangioblast [4,5]. Consistent with this concept, it was reported that cultured ES-derived cells can give rise to the two lineages [6–8]. Mice lacking Flk-1, its VEGF receptor, die due to lack of endothelial and hematopoietic cells [9]. Flk-1 null embryonic stem cells also fail to contribute to either endothelium or hematopoiesis in chimeric mice [10]. These studies are all consistent with a close relationship between blood and endothelial cells.

Vascular endothelial growth factor (VEGF) is encoded by a single VEGF gene, and several VEGF isoforms are produced from this gene by alternative splicing [11]. In *Xenopus*, the VEGF gene is alternatively spliced to produce at least three isoforms; $VEGF_{122}$, $VEGF_{170}$, and $VEGF_{190}$ (equivalent to mouse $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$, respectively) [12]. VEGF is known to play an essential role in vascular formation. Mice lacking VEGF die due to

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insufficient formation of endothelium [13,14]. Furthermore, it was shown that addition of VEGF to the culture medium enhanced endothelial differentiation of Flk-1-positive cells from the avian yolk sac [15]. In addition, in adult mice, administration of VEGF results in enhancement of bone marrow-derived endothelial progenitor cells, which are involved in neovascularization [16]. Furthermore, overexpressed $VEGF_{122}$ results in disorganized vascular structures in *Xenopus* embryos [12]. Although all these findings indicate that VEGF plays an essential role in vascular formation, the distinct function of VEGF in hematopoietic/angioblast differentiation remains unknown. In the present study, we employed *Xenopus* embryo, as the function of specific genes can be directly assayed by mRNA micro-injection. In addition, *Xenopus* embryos can be assessed without a functional circulatory system during development. For example, circulating blood is not critical to early embryonic development [17]. Thus to examine the functional roles of $VEGF_{122}$ and $VEGF_{170}$ during development, we have overexpressed these sequences in developing *Xenopus* embryos.

Materials and methods

Embryo generation and RNA microinjection. *Xenopus laevis* embryos were generated using standard techniques and staged, according to Nieuwkoop and Faber [18]. For blastomere injections, regularly cleaving embryos were selected, and 250 or 500 pg synthetic mRNA encoding $VEGF_{122}$, $VEGF_{170}$ or LacZ was microinjected into each blastomere at the 8-cell stage. For lineage tracing, RNA encoding LacZ was coinjected at 1000 pg per blastomere. Injected embryos were cultured in 3% ficoll/100% Steinberg's solution for 12 h and then in 80% Steinberg's solution, until sacrifice.

In vitro transcription. For mRNA production, $VEGF_{122}$, $VEGF_{170}$, and LacZ cDNAs were cloned into pCS2+. In vitro transcribed RNA was prepared from linearized plasmid DNA using a Megascript Kit (Ambion). Concentration and integrity of the RNA were checked by measuring OD_{260} and agarose gel electrophoresis in the presence of formaldehyde.

Whole embryo in situ hybridization and LacZ staining. Whole embryo in situ hybridization was performed as described previously [19]. LacZ staining was performed as previously described [3].

Results

Edema induced by overexpression of $VEGF_{122}$ or $VEGF_{170}$

We injected 500 pg mRNA coding either LacZ, $VEGF_{122}$ or $VEGF_{170}$ mRNA into dorsal-vegetal and ventral-vegetal blastomeres at the 8-cell stage. Embryos injected with LacZ, $VEGF_{122}$ or $VEGF_{170}$ mRNA were indistinguishable by visual inspection during the first day of development. The first alteration in the embryos injected with $VEGF_{122}$ or $VEGF_{170}$ mRNA became visible after the onset of heartbeat as compared to injection of LacZ. The embryos began to show edema in the ventral region, which became more severe as development proceeded (Figs. 1C and E). These embryos were allowed to further develop to the stage when circulating red blood cells can be observed easily in the heart and blood vessels in control embryos injected with LacZ mRNA (Fig. 1B, yellow arrowhead). Although the beating heart was visible in the dorsal portion of the pericardial cavity in embryos injected with $VEGF_{122}$ (Fig. 1D, yellow arrowhead) or $VEGF_{170}$ mRNA (Fig. 1F, yellow arrowhead), only few circulating blood cells were seen in the heart and blood vessels; instead, the blood cells pooled around the developing ventral gut, where primitive hematopoiesis had occurred (Figs. 1D and F, blue arrowheads).

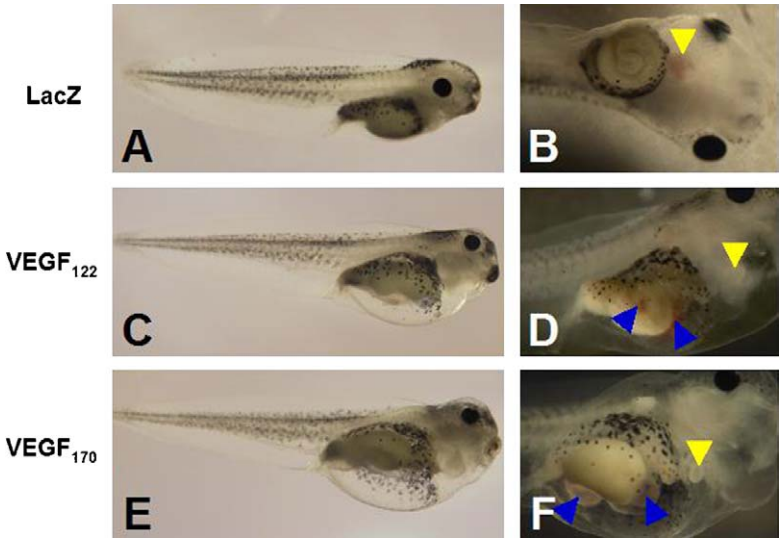

Fig. 1. Edema induced by overexpression of $VEGF_{122}$ or $VEGF_{170}$. Tadpoles injected with (A,B) LacZ mRNA, (C,D) $VEGF_{122}$ mRNA or (E,F) $VEGF_{170}$ mRNA. (A, C, and E) Lateral views of stage 42 embryos. (B, D, and F) Ventral views of stage 46 embryos. Yellow arrowheads indicate beating heart. Red blood cells are visible in the heart of a control tadpole injected with LacZ mRNA (B), but are rare in tadpoles injected with $VEGF_{122}$ or $VEGF_{170}$ mRNA (D,F). Blue arrowheads indicate pooled blood cells.

Analysis of formation of endothelial cells at stage 32/33

The above observations revealed that injection of $VEGF_{122}$ or $VEGF_{170}$ mRNA led to obstruction of the circulation of red blood cells. Thus, these results raise the possibility that vascular development might be abnormal. To examine this further, we analyzed the embryonic vasculature by whole-mount in situ hybridization with endothelial markers, tie-2 [20] and msr [21], prior to the onset of circulation, when no sign of edema was detectable in the embryos injected with $VEGF_{122}$ or $VEGF_{170}$ mRNA. Inspection of stained embryos failed to reveal obvious defects in blood vessels of the head and posterior cardial vein, albeit that the posterior cardial vein was slightly faint compared with the control. Furthermore, although intersomic veins tended to be underdeveloped, the intersomic veins sprouting from the posterior cardinal veins formed normally, suggesting that angiogenesis sprouting from cardinal veins was undisturbed. In contrast, a specific defect was observed in the vitelline vascular network. While a highly structured plexus of vitelline vessels was observed in control embryos (Figs. 2A and G), vascular hyperfusion was detectable in the vitelline vascular network of embryos injected with $VEGF_{122}$ (Figs. 2B and H, yellow arrowheads) or $VEGF_{170}$ mRNA (Figs. 2C and I, yellow arrowheads). These vascular networks were no longer discernible as separate vessels.

Analysis of formation of blood cells in VBI at stage 32/33

Since the vitelline veins are closely related to primitive hematopoiesis, one might assume that hematopoietic development in the VBI might also be disrupted by overexpression of $VEGF_{122}$ or $VEGF_{170}$. Thus, we studied the expression pattern of hematopoietic markers, α-globin and GATA-1 [22], and endothelial markers, tie-2 [20] and msr [21], in the VBI by double whole-mount in situ hybridization. Embryos injected with LacZ (Figs. 2D and J) or $VEGF_{122}$ mRNA (Figs. 2E and K) showed a marked difference in the expression patterns of endothelial cells and hematopoietic cells in the VBI region. While α-globin-positive cells formed a Y-shaped structure in the VBI region, tie-2- or msr-positive cells were less detectable in the region. In contrast, in embryos injected with $VEGF_{170}$ mRNA, α-globin- (Figs. 2F and L, red arrowheads) and GATA-1-positive cells (Fig. 2O, black arrowheads) were undetectable in the posterior portion of the VBI. Irrespective of whether $VEGF_{170}$ mRNA was injected into the dorsal- or ventral-vegetal blastomere, hematopoietic marker expression was absent only in the posterior portion of the VBI. However, injection into the dorsal-vegetal blastomere appeared to be more effective than that into the ventral-vegetal blastomere (Table 1). Strong signals of endothelial markers, but not hematopoietic markers, were detectable in the posterior portion of the VBI (Figs. 2F and L, red arrowheads). Interestingly, co-injection of $VEGF_{122}$ (250 pg) with $VEGF_{170}$ (250 pg) attenuated the effects of the injection of $VEGF_{170}$ (250 pg) mRNA alone (Table 2).

Lineage analysis of hematopoietic cells

Flk-1, the VEGF receptor, was reported to be involved in hematopoietic and endothelial cell migration,

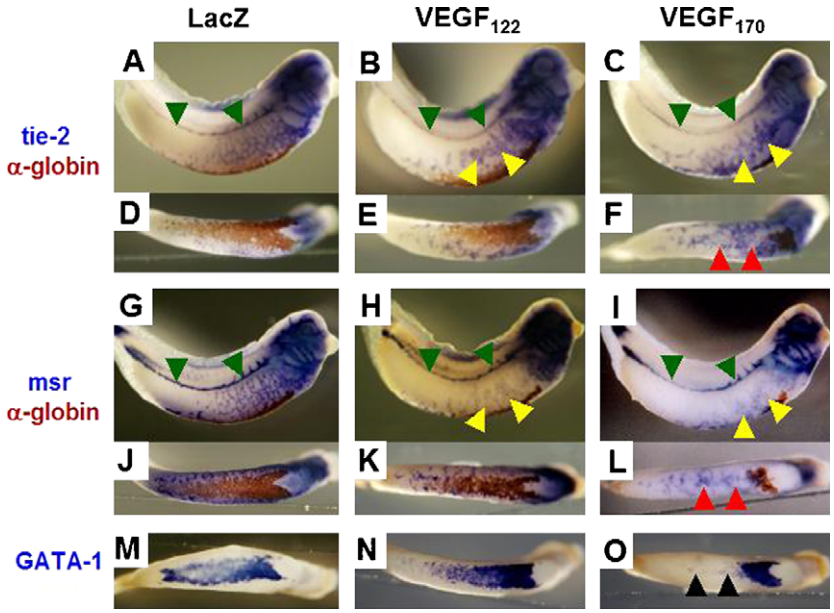

Fig. 2. Expression pattern of hematopoietic markers (α-globin and GATA-1) and endothelial markers (tie-2 and msr) shown by in situ hybridization. Embryos injected with (A, D, G, J, and M) LacZ, (B, E, H, K, and N) $VEGF_{122}$ or (C, F, I, L, and O) $VEGF_{170}$ mRNA into dorsal-vegetal blastomeres. (A–C and G–I) Lateral views of embryos. (D–F, J–L, and M–O) Ventral views of embryos. (A–F) Expression pattern of α-globin and tie-2. (G–L) Expression pattern of α-globin and msr. Yellow arrowheads indicate vascular hyperperfusion in vitelline venous network. Green arrowheads indicate intersomic veins. Red arrowheads indicate ectopic expression of endothelial markers, but not hematopoietic marker. (M–O) Expression pattern of GATA-1. Black arrowheads indicate absence of expression of GATA-1. Anterior is right for all embryos.

Table 1
Gene expression pattern in VBI in response to injected $VEGF_{122}$ or $VEGF_{170}$ mRNA

RNA	Blastomere	α-Globin expression (%)				GATA-1 expression (%)			
		n	Normal	Decrease or absent		*n*	Normal	Decrease or absent	
				Posterior VBI	Throughout VBI			Posterior VBI	Throughout VBI
$VEGF_{122}$	DV	31	61	29	10	30	53	30	17
$VEGF_{170}$	DV	31	32	68	0	27	26	67	7
LacZ	DV	40	77	10	13	35	89	11	0
$VEGF_{122}$	VV	23	83	13	4	23	70	30	0
$VEGF_{170}$	VV	30	60	40	0	27	52	48	0
LacZ	VV	22	95	5	0	22	95	5	0
$VEGF_{122}$	DV and VV	25	76	4	20				
$VEGF_{170}$	DV and VV	21	29	52	19				
LacZ	DV and VV	18	83	17	0				

Summary of gene expression pattern obtained in injection experiments. Frequency of gene expression pattern is given as percentages. *n* represents the number of embryos scored from independent experiments. Embryos were scored for absent staining throughout VBI or the posterior portion of VBI in embryos injected with mRNAs (500 pg) to dorsal-vegetal blastomere (DV), or ventral-vegetal blastomere (VV) or dorsal-vegetal and ventral-vegetal blastomeres (DV and VV).

Table 2
α-Globin expression in VBI in response to co-injected $VEGF_{122}$ and $VEGF_{170}$ mRNA

RNA	Blastomere	α-Globulin expression (%)			
		n	Normal	Decrease or absent	
				Posterior VBI	Throughout VBI
$VEGF_{122}$(250 pg)	DV	32	69	6	25
$VEGF_{170}$(250 pg)	DV	34	29	50	21
$VEGF_{122}$(250 pg) + $VEGF_{170}$(250pg)	DV	32	56	25	19

Frequency of gene expression pattern is given as percentages. *n* represents the number of embryos scored from independent experiments. Embryos were scored for absent staining throughout VBI or the posterior portion of VBI in embryos injected with mRNAs to dorsal-vegetal balstomere (DV).

as Flk-1 null cells are unable to colonize the developing mouse yolk sac region [10]. It is possible that absence of hematopoietic cells in the posterior portion of the VBI is due to abnormal cell migration. To test this hypothesis, we performed lineage-tracing experiments by co-injection of LacZ and $VEGF_{170}$ mRNA into two dorsal- or ventral-vegetal blastomeres of 8-cell embryos. Consistent with the previous reports [3,23,24], the anterior and posterior portions of the VBI were derived from dorsal-vegetal and ventral-vegetal blastomeres of 8-cell embryos, respectively (Figs. 3A and B). Although α-globin expression was absent only in the posterior portion of the VBI of embryos injected with $VEGF_{170}$ mRNA, no marked difference in the localization of LacZ activity was observed between control (Figs. 3A and B) and experimental embryos (Figs. 3C and D). Importantly, no LacZ-positive cells migrated into the dorsal portion of the VBI of embryos co-injected with LacZ and $VEGF_{170}$ mRNA into two ventral-vegetal blastomeres (Figs. 3D). These results excluded the possibility that overexpression of VEGF leads to abnormal hematopoietic cell migration.

Analysis of endothelial and blood cell formation in VBI at early stage

As described above, ectopic expression of endothelial markers was observed in the posterior portion of the VBI of embryos injected with $VEGF_{170}$ mRNA. Endothelial cells of the vitelline vein emerge in the ventral region, called vasculogenesis, and migrate to form the vitelline venous system, called angiogenesis [2,12,20,21]. To determine whether ectopic endothelial expression is due to disturbance of early (vasculogenesis) or the late steps (angiogenesis) in vascular formation, we examined the expression patterns of endothelial markers, tie-2 [20], msr [21], and fli-1 [25], at the early stage, when vasculogenesis occurs. While these endothelial markers formed a ring around the prospective VBI in control embryos (Figs. 4A, C, and E), strong signals of endothelial markers were detectable in the posterior portion of the prospective VBI in embryos injected with $VEGF_{170}$ mRNA (Figs. 4B, D, and F). These results suggest that injection of $VEGF_{170}$ resulted in disturbance of vasculogenesis, with excessive production of endothelial cells at the expense of blood cells.

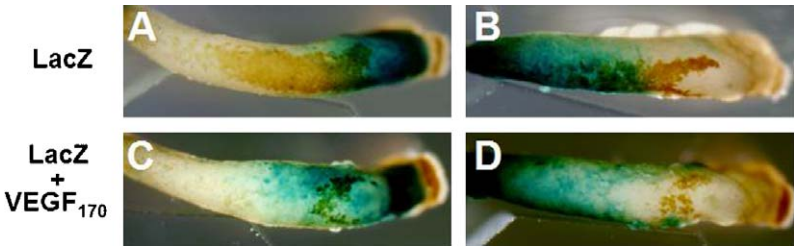

Fig. 3. Expression pattern of α-globin in embryo lineages traced with LacZ mRNA. Embryos were injected with LacZ mRNA into (A) two dorsal-vegetal or (B) ventral-vegetal blastomeres at the 8-cell stage. Embryos were co-injected with LacZ and $VEGF_{170}$ mRNA into (C) two dorsal-vegetal or (D) ventral-vegetal blastomeres at the 8-cell stage.

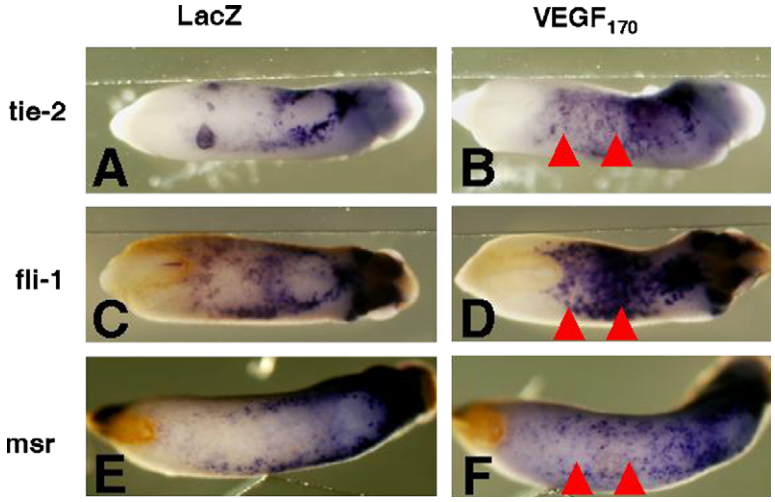

Fig. 4. Expression pattern of tie-2, fli-1, and msr in stage 26/28 embryos shown by in situ hybridization. Embryos were injected with either (A, C, and E) LacZ mRNA or (B, D, and F) $VEGF_{170}$ mRNA into dorsal-vegetal blastomeres. Expression pattern of (A,B) tie-2, (C,D) fli-1, and (E,F) msr. Red arrowheads indicate ectopic expression in the posterior portion of the prospective VBI.

Discussion

Differences in the endothelial cells between vitelline veins and posterior cardinal veins

In the present paper, it was shown that exogenous $VEGF_{122}$ or $VEGF_{170}$ in *Xenopus* embryos resulted in hyperperfusion in the vitelline vasculature, but not the intersomic veins sprouting from the posterior cardinal veins. The present results support the previous reports in *Xenopus* [12] and in chick embryos [26]. There might be differences between the vitelline veins and intersomic veins during developmental angiogenesis under VEGF signaling. Recently, programming differences between the vitelline vein and posterior cardinal veins were demonstrated in terms of the dependence of fli-1 expression on BMP signaling [2]. In addition, although EphB4, member of Eph family of receptor tyrosine kinases, was expressed in vascular vitelline network and posterior cardinal veins, blockade of EphB4 signaling by the dominant receptor in *Xenopus* embryos disrupted angiogenesis of only the intersomic veins, but not the vitelline vascular network [27]. In addition to the close association between primitive hematopoiesis and the vitelline vein and between definitive hematopoiesis and the posterior cardinal veins, the present and previous studies revealed that the endothelial cells of the vitelline vein may be different from those of the posterior cardinal veins, as primitive hematopoiesis is different from definitive hematopoiesis.

Differences in putative blood cells between anterior and posterior portion of VBI

The present study showed that overexpression of $VEGF_{170}$ resulted in the absence of hematopoietic cells only in the posterior portion of the VBI, without obviously abnormal hematopoietic cell migration. In addition, the present study demonstrated that injection of $VEGF_{170}$ mRNA resulted in the detection of ectopic expression of endothelial cell markers in the posterior portion of the prospective VBI. Furthermore, overexpression of $VEGF_{170}$ increased the region of tie-2, fli-1, and msr-expressing cells in vasculogenesis. These results suggest that injection of $VEGF_{170}$ resulted in disturbance of vasculogenesis, with excessive production of endothelial cells at the expense of blood cells. These results were consistent with previous reports. As VEGF induced the emergence of an endothelial lineage from Flk-1-positive

cells from the avian yolk sac, with a reduction of hematopoietic differentiation [15], proper levels of VEGF may be needed to maintain hematopoietic/angioblastic differentiation. These results were confirmed by the observation that mice lacking one copy of the VEGF gene died in utero due to aberrant blood vessel formation, although they showed hematopoietic differentiation [13,14]. There are two possible mechanisms for the disturbance of vasculogenesis by $VEGF_{170}$. First, hematopoietic precursors in the posterior portion of the VBI might be diverted to an angioblastic fate under the influence of ectopic $VEGF_{170}$. Second, the angioblastic precursors may be positively affected by overexpression of $VEGF_{170}$ while the blood cells are affected negatively. Importantly, injection of $VEGF_{170}$ resulted in the disturbance of vasculogenesis only in the posterior portion of VBI. Thus, our present results suggest that the anterior and posterior portions of the VBI have distinct characteristics. Since co-injection of $VEGF_{122}$ with $VEGF_{170}$ attenuated the abnormality induced by $VEGF_{170}$, $VEGF_{122}$ might play the pivotal role in the disturbance of vasculogenesis. The previous study demonstrated that various VEGF isoforms exert different biological functions during retinal angiogenesis [28]. Furthermore, it is shown that neuropilin-1 is a $VEGF_{170}$-specific receptor, but is not specific for $VEGF_{122}$ [29]. Specific VEGF isoforms might be important for vascular development.

Acknowledgments

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