Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression

Lucile Miquerol1,*, B. Lowell Langille2 and Andras Nagy1,3,‡

1Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5, Canada
2University Health Network/Toronto General Hospital, 200 Elizabeth St, Toronto, ON, M5G 2C4, Canada
3Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, M5S 1A8, Canada
*Present address: LGPD-IBDM, campus de Luminy, case 907, 13288 Marseille cedex 9, France
‡Author for correspondence (e-mail: nagy@mshri.on.ca)

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SUMMARY

Previous work has shown that heterozygocity for a null mutation of the VEGF-A gene, resulting in a 50% reduction in VEGF-A expression, is embryonic lethal at embryonic day (E) 9.5 in mice. We now show that two- to threefold overexpression of VEGF-A from its endogenous locus results in severe abnormalities in heart development and embryonic lethality at E12.5-E14. The mutant embryos displayed an attenuated compact layer of myocardium, overproduction of trabeculae, defective ventricular septation and abnormalities in remodeling of the outflow track of the heart. In addition, aberrant coronary development was characterized by formation of oversized epicardial vessels, apparently through vasculogenesis. We infer that embryonic survival requires a narrow window of VEGF-A expression.

Key words: Vascular endothelial growth factor, Gene regulation, Cardiogenesis, Coronary development, Septation, Mouse.

INTRODUCTION

Vascular endothelial growth factor (VEGF-A; Vegf – Mouse Genome Informatics) is a potent stimulator of both embryonic vascular development and pathological angiogenesis. It induces differentiation, proliferation and migration of endothelial cells that contribute to vessel formation through both vasculogenesis and angiogenesis (Ferrara et al., 1987; Leung et al., 1989). It is also an endothelial-cell survival factor (Benjamin and Keshet, 1997; Benjamin et al., 1999; Gerber et al., 1999). VEGF-A exerts its biological activities by binding two tyrosine kinase receptors VEGFR1 (Flt1 – Mouse Genome Informatics; Shibuya et al., 1990; De Vries et al., 1992) and VEGFR2 (Kdr – Mouse Genome Informatics; Millauer et al., 1993; Mustonen and Alitalo, 1995), both of which are expressed by endothelial cells. The deletion of either of these receptors by gene targeting leads to embryonic lethality by E8.5/E9.5, because of vascular defects (Fong et al., 1995, 1999; Shalaby et al., 1995). The absence of VEGFR2 blocks the differentiation of mesodermal cells to angioblasts, while the absence of VEGFR1 induces an overproduction of endothelial cells and vessel malformation. These data indicate the complex and multiple functions of this growth factor and its receptors. During embryonic development, VEGF-A is present in many tissues from the late blastocyst stage (Miquerol et al., 1999). The largely complementary localization of the endothelial cell specific VEGF-A receptors on endothelium and VEGF-A in the surrounding tissues indicates paracrine regulation by this factor.

An absolute requirement for VEGF-A during embryonic development has been demonstrated by gene targeting. Deletion of one of the VEGF-A alleles is sufficient to disturb normal embryonic development of the mouse and cause midgestational lethality (Carmeliet et al., 1996; Ferrara et al., 1996). This heterozygous (50%) expression of the gene for VEGF-A leads to compromised endothelial proliferation and/or differentiation that prevents the establishment of a normal circulatory system. This haploinsufficiency suggests a requirement for precise VEGF-A regulation during development.

Heterozygous lethality obscured the production of an F2 generation and the study of embryos homozygous for the null allele. This limitation was bypassed when completely embryonic stem (ES) cell-derived embryos were made by aggregating homozygous VEGF-A-deficient ES cells with tetraploid embryos (Carmeliet et al., 1996; Nagy and Rossant, 1999). The resulting homozygote embryos showed even more severe vascular defects. Homozygous embryos, like heterozygotes, died at midgestation (E9.5) owing to cardiovascular dysfunction.

Expression of the gene for VEGF-A is tightly controlled and isstimulated under hypoxic conditions through both transcriptional and post-transcriptional mechanisms (Ikeda et al., 1995; Levy et al., 1996). In the promoter region, a 47 bp HIF-1 (hypoxia-inducible factor 1)-binding element at 985-939 bp 5’ of the start codon mediates, in part, sensitivity to hypoxia (Levy et al., 1995; Liu et al., 1995; Forsythe et al., 1996). Hypoxia also induces factors that regulate VEGF-A mRNA
stability by interacting with the 3' untranslated region (3'UTR) (Levy et al., 1996; Claffey et al., 1998; Dibbens et al., 1999), thereby greatly increasing the half-life of the normally short-lived VEGF-A transcripts. To address whether both regulatory elements are essential for normal embryonic development, we disconnected the 3' regulation of VEGF-A from the 5' regulatory and coding regions. Using gene targeting, we inserted a lacZ gene followed by an SV40 polyadenylation signal (Jang et al., 1988; Takeuchi et al., 1995) between the stop codon and the 3'UTR of the gene. We show that this alteration of the gene for VEGF-A induces modest elevation of VEGF-A level that leads to embryonic lethality by day E12.5, owing to cardiac failure. These results demonstrate the importance of tightly regulating VEGF-A expression during cardiac development.

MATERIALS AND METHODS

RNA analyses
Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out as previously described (Carmeliet et al., 1996), using oligomers 1 in exon 3 (5'-GCCCTGGGTGCTGCCCACGGCAGAGCA-3'), and 3 in VEGF-A exon 8 3'UTR (5'-TTGGCGATTTAGCAGCATTA-3') for amplification of the wild-type (WT) allele and oligomers 1 and 4 in the IRES sequence (5'-GATACTTGGCTGCTAG-3') for amplification of the targeted allele.

Northern blots were prepared using 10 μg of total RNA extracted from E12.5 tissues or E9.5 whole embryo by TRIzol reagent (GibcoBRL, Life Technology). VEGF-A cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as probes.

Protein analysis
Enzyme-linked immunosorbant assays (ELISAs) were performed on total proteins extracts in 250 mM Tris-HCl (pH 7.5) from embryonic tissues and embryos using mouse VEGF-A QuantikineM kit (R&D systems). Values given in the results are means of protein concentration and are expressed in pg/ml. Statistical analysis was performed by t-test. Comparison of VEGF-A concentration between WT and VEGFlacZ-KI/VEGFlacZ-KI mutants revealed P values <0.05.

Histology, immunohistochemistry and β-galactosidase staining
Embryos were dissected, fixed in cold 4% paraformaldehyde in PBS, paraffin embedded, sectioned and stained with haematoxylin-eosin. For immunohistochemistry and β-galactosidase (β-gal) staining, E12.5 embryos were fixed in cold 4% paraformaldehyde for 1 hour, then embedded and frozen in OCT compond after passage through overnight sucrose gradients (15% and 30% sucrose in PBS). Serial cryosections were produced from these embryos and used for both reactions. PECAM immunostaining, was performed using the rat monoclonal anti-CD31 antibody (R&D systems) as primary antibody and following the manufacturer’s protocol. Staining of sections for β-gal was done as described previously (Miquerol et al., 1999).

RESULTS

Disruption of the 3'UTR regulation induces overproduction of VEGF-A
To study the role of the VEGF-A 3'UTR regulatory element during embryonic development, we inserted an IRES (internal ribosome entry site)-lacZ cassette in the last exon of the gene after the stop codon, and placed a SV40 polyadenylation signal after the lacZ coding sequence (Fig. 1A). The IRES sequence allows independent production of VEGF-A and β-galactosidase (β-gal), from the same bicistronic mRNA. This allele allowed heterozygous survival.

Gene targeting was performed on R1 ES cells (Nagy et al., 1993) and the loxP-flanked selectable marker was removed from the targeted allele by crossing heterozygous animals with a general deleter Cre transgenic line (Lobe and Nagy 1998; Lobe et al., 1999). Mice heterozygous for this allele (designated as VEGFlacZ-KI) were viable and fertile, and provided an excellent tool with which to characterize the expression pattern of VEGF-A during development and adulthood (Miquerol et al., 1999), including normal and pathological situations. The level of VEGF-A expression was studied in the F2 generation embryos by examining VEGF+/+, +/VEGFlacZ-KI and VEGFlacZ-KI/VEGFlacZ-KI genotypes. Northern-blot analyses performed on E9.5 and E12.5 embryos showed increased levels of mRNA production of the targeted allele compared with the wild type (Fig. 1C). To test whether the increased mRNA level yielded increased protein, we measured levels of VEGF-A in embryos by ELISA. The levels of VEGF-A protein correlated well with the VEGFlacZ-KI allele dosage (Fig. 1D). The relative increase over wild-type levels varied among the different tissues of the E12.5 embryos. The heart showed the highest (threelfold) increase, whereas other organs in homozygous VEGFlacZ-KI/VEGFlacZ-KI embryos (lung, liver and brain) exhibited increased expression of twofold or less.

We have previously reported that the pattern of expression of VEGF-A during embryonic development (Miquerol et al., 1999) is unaffected by insertion of lacZ into the 3'UTR. In addition, the VEGFflacZ-KI allele correctly expressed the three isoforms of VEGF-A (VEGF120, VEGF164 and VEGF188) as in wild-type embryos (Fig. 1B). These results strongly suggest that the 3'UTR insertion of the IRES-lacZ-pA sequence increases the stability of the VEGF-A transcripts, which results in increased mRNA levels and increased VEGF-A production.

Threefold VEGF-A levels induce embryonic cardiac failure
The increased VEGF-A expression in the VEGFflacZ-KI homozygous embryos did not result in any obvious cardiovascular phenotype before E12.5. Whole-mount immunostaining for platelet endothelial adhesion molecule (PECAM), which is expressed by vascular endothelial cells, did not reveal any change in vessel density or caliber in the embryo proper. Yolk sac vascular development followed a normal timecourse, and yielded a normal capillary density and formation of larger vessels. Thus, early vessel development occurred normally. In addition, there was no obvious alteration in heart development up to E11.5 (data not shown).

Despite normal early vascular development, embryos homozygous for the VEGFflacZ-KI allele died between E12.5 and E14.5. These embryos were edematous (Fig. 2A,B) and displayed dilatation of the developing jugular lymph sacs (Fig. 2LJ), features that are consistent with death from congestive heart failure and/or to increased permeability of vessels as a consequence of the elevated VEGF-A levels.

The hearts of the homozygous VEGFflacZ-KI embryos were significantly larger than those of wild-type and heterozygous littermates (Fig. 2C,D) and histological sections revealed that
VEGF-A overexpression induces embryonic cardiopathy

the ventricular wall of the mutants was abnormally thin (Fig. 2E,F). The compact layers of both ventricles were reduced and the trabeculation of the inner wall was substantially more elaborate than in wild-type mice (Fig. 3A,B). The extensive trabeculation was frequently accompanied by disruption (internal trabeculation) of the interventricular septum. The enlargement of the trabecular layer was associated with an increase in endocardial tissue (Fig. 3E,F), visualized by PECAM immunostaining (Fig. 3G,H). The coronary vessels were also significantly enlarged and very large vessels were frequently observed on the epicardial surface of the heart (Fig. 3A-D). When these vessels were followed in serial sections, they frequently opened into the ventricular cavities. However, vessels that were blind-ended at one or both ends were occasionally observed; a finding that indicates these vessels may have originated through vasculogenesis, then enlarged substantially without linking to other vessels. Increased PECAM immunostaining suggests the presence of an increased number of endothelial cells in this region (arrows in Fig. 3H).

Outflow tract remodeling was also defective in these embryos, as demonstrated by incomplete or delayed turning of the outflow tracts and delayed interventricular septation (Fig. 2G,H).

The VEGF<sup>KI/KI</sup> cardiac phenotype reflects VEGF-A involvement during heart development

The VEGF<sup>lacZ-KI</sup> allele strongly expressed the lacZ reporter in the trabeculae, the interventricular septum and the outflow tract of the heart (Fig. 4). Interestingly, the outflow tract was the only site where endothelial cells of heterozygous embryos expressed lacZ, indicating VEGF-A expression in these cells (Miquerol et al., 1999). The pattern of lacZ expression correlated with the cardiac defects observed in the mutants; therefore, we infer that VEGF-A overexpression was directly responsible for the developmental defects of the heart.

DISCUSSION

In this report, we have studied the consequences of disturbing VEGF-A 3'UTR on embryonic development. Replacement of this region by an IRES-lacZ-SV40pA sequence induced
modest overproduction of VEGF-A that caused cardiac failure in homozygous mutant embryos.

Hypoxia stabilizes VEGF-A mRNA by promoting the binding of proteins to several AU-rich sequences in the 3'UTR (Levy et al., 1997; Dibbens et al., 1999). In this paper, we show that the replacement of the VEGF-A 3'UTR by SV40pA sequence increased the level of VEGF-A mRNA. We believe that the observed increase in mRNA produced from our altered allele was due to increased stability of the mutant transcripts, compared with that of the wild type. We have also shown that this elevated VEGF-A mRNA generated an increased protein level that was somewhat tissue specific. This finding is consistent with the observation that the extent of VEGF-A hypoxia-induced mRNA stability is distinctly tissue or cell-type dependent (Claffey et al., 1998). Differences in tissue responsiveness may be attributed to the nature of the hypoxia-induced proteins that bind the 3'UTR.

We have demonstrated that two- to threefold overexpression of VEGF-A is deleterious to embryonic heart development. This observation, together with the earlier finding that haploinsufficiency for VEGF-A resulted in a lethal phenotype,
indicates that VEGF-A must be precisely controlled for cardiovascular development to proceed normally. The lethal heart phenotype associated with VEGF-A overexpression demonstrates that this factor is crucial in defining positional and differentiation clues for remodeling of this developing organ. Because VEGF-A has complex functions and there is little tolerance for variations in its expression level, we anticipate that peripheral vascular development is also sensitive to overexpression of the gene. Elucidating this sensitivity awaits studies that exploit conditional rescue of the heart to allow examination of later effects of VEGF-A overexpression.

It has been shown previously that overexpression of VEGF-A can be deleterious for embryonic development. Retrovirally induced overexpression of VEGF-A in chick limb-induced hypervascularization (Flamme et al., 1995) and direct injection of VEGF-A into the midbrain or the forelimb of a quail embryo caused severe embryonic malformations of the heart and the venous system (Feucht et al., 1997). These data confirmed the direct effect of VEGF-A on the development of the cardiovascular system. The absence of vascular defects in our model probably reflects a VEGF-A dosage effect; consequently, the heart may be more sensitive than the vasculature, or sensitive earlier, to elevation of VEGF-A expression.

The phenotype observed in the homozygous VEGFloxZ:KI/VEGFloxZ:KI embryos was characterized by heart malformations that affected septation, the development of the myocardium and the development of the coronary vasculature. Our results indicate that VEGF-A regulates the coordinated development of the compact myocardium and the trabeculae, probably via an interaction between myocardial and endocardial cells. Increased levels of VEGF-A in the myocardium appear to enhance trabeculation at the expense of the compact layer. This interpretation is consistent with findings that crosstalk between myocardium and endocardium is essential for a normal trabeculation. For instance, trabeculation is absent in embryos with null mutation of the ErbB2, ErbB3 and ErbB4 receptors, which are expressed in myocytes, and after mutation of the ligand for these receptors, neuregulin, which is expressed by the endocardium (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Erickson et al., 1997). In our model, overexpression of VEGF-A induces endocardial development as shown by PECAM staining and overexpression of VEGFR2 (not shown).

The coronary vasculature proceeds by vasculogenesis and angiogenesis (Mikawa and Fischman, 1992; Rakusan et al., 1994). Endothelial precursors reach the heart from the proepicardial organ, with the epicardium, and form tubes by vasculogenesis. These primary vessels subsequently develop by branching and sprouting angiogenesis. VEGF-A expression coincides with coronary vasculogenesis and angiogenesis (Tomane et al., 1999). Recently, it has been shown that hypoxia is an important inducer of coronary vasculogenesis and angiogenesis, and that VEGF-A signaling plays a major role in this induction (Tomane et al., 1999). In homozygous VEGFloxZ:KIF/VEGFloxZ:KI embryos, overexpression of VEGF-A impairs the development of the epicardium in association with an overgrowth of the coronary vasculature. Together, these data suggest that normal coronary vasculogenesis depends on an appropriate level of VEGF-A expression under the control of a hypoxia-sensitive element in the 3'UTR sequence.

The profound effect of a modest overexpression of VEGF-A is a strong testament to the importance of this growth factor during cardiovascular development. Our findings fit well with the previously observed angiogenic effects of VEGF-A in ischemic limb tissue or myocardium (Tsurumi et al., 1997; Baumgartner et al., 1998; Losordo et al., 1998; Rosengart et al., 1999), when presumably low levels of expression were obtained after plasmid injection or adenovirus transfection. Nonetheless, the very tight physiological regulation that is normally exercised over the expression of VEGF-A, and the serious consequences resulting from mild deviations from normal levels, underscore the need for caution when manipulating VEGF-A level for therapeutic purposes.
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