Conditional inactivation of VEGF-A in areas of collagen2a1 expression results in embryonic lethality in the heterozygous state

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SUMMARY

VEGF-A has been implicated in regulating the initial angiogenic invasion events that are essential for endochondral bone formation. VEGF-A mRNA expression was indeed found in the sclerotome of the developing somite and in the limb-bud mesenchyme at E10.5 in mouse development but declined during chondrogenesis and became upregulated in hypertrophic chondrocytes prior to angiogenic invasion. To determine the functional importance of VEGF-A expression in the developing chondrogenic tissues, VEGF-A was conditionally inactivated during early embryonic development using Collagen2a1-Cre transgenic lines. Deletion of a single VEGF-A allele in Collagen2a1-Cre-expressing cells results in embryonic lethality around E10.5. This lethality is characterized by aberrant development of the dorsal aorta and intersomitic blood vessels, along with defects in the developing endocardial and myocardial layers of the heart.

A small percentage of VEGFFllox/+, Collagen2a1-Cre fetuses survive until E17.5, show aberrant endochondral bone formation and develop a heart phenotype resembling a dilated form of ischemic cardiomyopathy. These results provide insights into the function of VEGF-A in heart and endochondral bone formation and underscore the importance of tightly controlled levels of VEGF-A during development.

Key words: Vascular endothelial growth factor (VEGF), Angiogenesis, Cartilage, Collagen2a1, Cre/loxP, Conditional gene targeting, Heart development, Cardiomyopathy

INTRODUCTION

The VEGF-Flk/Flt1 signaling system has been demonstrated to be essential for the proper development of the vascular system (Carmeliet et al., 1996; Ferrara et al., 1996; Fong et al., 1995; Shalaby et al., 1995). There are currently four known members of the VEGF family (VEGF-A through VEGF-D), which have different expression patterns and vary in their affinity and specificities in binding to the Flk1 family of receptor tyrosine kinases (Flt1, Flk1, Flt4) (for reviews see Carmeliet and Collen, 1999; Eriksson and Alitalo, 1999; Ferrara, 1999). Murine VEGF-A is transcribed from a single gene that is alternatively spliced to produce three different mRNA species (VEGF-A120, VEGF-A164, VEGF-A188; Neufeld et al., 1996). VEGF-A120 diffuses freely in the surrounding extracellular matrix whereas the other isoforms show increased binding to the heparin-rich extracellular matrix. The three isoforms also differ in their mitogenic potency; VEGF-A120 is approximately 100-fold less mitogenic than the VEGF-A164 and VEGF-A188 isoforms (Carmeliet and Collen, 1999).

The importance of maintaining threshold levels of VEGF-A during development was first demonstrated using conventional gene targeting. Loss of a single VEGF-A allele was shown to be embryonic lethal with heterozygous mice dying between E11 and E12 due to severe defects in angiogenesis and blood island formation (Carmeliet et al., 1996; Ferrara et al., 1996). Similarly, VEGF receptor-2+/− (Flk1+/−) mice die in utero even earlier than the VEGF-A heterozygotes between E8.5 and E9.5, as a result of an early defect in the development of hematopoietic and endothelial cells (Shalaby et al., 1995). In the Flk1−/− fetuses, yolk-sac blood islands were completely absent as early as E7.5. Organized blood vessels could not be observed in the Flk1−/− fetuses or yolk sacs at any developmental stage, and hematopoietic progenitors were severely reduced (Shalaby et al., 1995). Finally, VEGF receptor-1−/− (Flt1−/−) fetuses, although possessing endothelial cells in both intraembryonic and extraembryonic sites, failed to organize the endothelium, exhibited abnormal vascular channels and died in utero at mid-somite stages (Fong et al., 1995).

As a result of these early embryonic lethals, it has been impossible to investigate the role of VEGF-A signaling in later developmental stages of murine organogenesis. Gerber et al. (1999a) have recently used two independent approaches to inactivate VEGF-A in neonatal mice. One approach used the Cre/LoxP recombination system whereas the other approach involved the administration of a soluble mFlt (1-3)-IgG chimeric receptor protein that is capable of sequestering VEGF-A protein. This study demonstrated that VEGF-A is
required for endothelial cell survival in vivo in the neonate mouse (Gerber et al., 1999a). The Cre/LoxP system has also been recently utilized by Carmeliet et al. (1999) to determine the developmental consequences of expression of only the VEGF-A120 isoform during development. Interestingly, VEGF-A120 homozygous mice die shortly after birth because of ischemic cardiomyopathy (Carmeliet et al., 1999).

With the advent of the Cre/LoxP recombination system and the existence of mice carrying a conditional VEGF-A allele, it is possible to specifically inactivate VEGF-A in a cellular and organ-specific manner during development. The cartilage anlage is a unique tissue in that it initially undergoes much of its early development as an avascular tissue. It is thought that the cartilage is kept avascular because of a tightly controlled balance between the release of anti-angiogenic and angiogenic molecules (Dascalzi Cancedda et al., 1995). During the vascular invasion of this cartilage anlage, it is believed that anti-angiogenic molecules such as Chondromodulin-I are downregulated and angiogenic molecules such as VEGF-A and FGFs are upregulated (Baron et al., 1994; Gerber et al., 1999b; Suzuki, 1996). As a result of this vascular invasion process, bone-forming cells (osteoblasts) and cartilage/bone-resorbing cells (chondroclasts/osteoclasts) are brought into the cartilaginous matrix and remodel the cartilage template into bone (Caplan, 1990).

In order to investigate whether VEGF-A has an important role in this initial vascular invasion process, a developmental expression analysis was conducted. In addition, loss-of-function experiments were performed to determine if VEGF-A is necessary in determining the timing and extent of vascular invasion into the developing cartilage. Several transgenic lines were established that express the Cre recombinase under the control of human Collagen2a-1 promoter and enhancer sequences (Cheah et al., 1995). Cre activity was not limited to chondrogenic tissues but instead recapitulated the reported expression pattern of the endogenous Collagen2a-1 gene including expression in the developing eye, epidermis, heart myocardium and the endoderm of the developing yolk sac in addition to chondrogenic tissues (Cheah et al., 1991). Mice that delete VEGF-A in collagen2a-1-expressing cells died around day E10.5 in the heterozygous state due to aberrant development of the dorsal aorta and intersomitic blood vessels, along with defects in the developing endocardial and myocardial layers of the heart. In contrast, fetuses that develop till E17.5 exhibit aberrant endochondral bone formation and display a heart phenotype resembling a dilated form of ischemic cardiomyopathy (Carmeliet et al., 1999).

MATERIALS AND METHODS

Generation of Collagen2a1-Cre transgenic mice

The bacterial Cre recombinase sequences were excised from the PTZ19R plasmid (gift from Klaus Rajewski) as a SalI fragment and subcloned into the pSP72 vector (Promega). Bovine polyadenylation sequences (pA) (Pfarr et al., 1986) were subsequently cloned 3' of the bacterial Cre sequences. The CreA fragment was excised from the pSP72 plasmid and cloned as a HindIII fragment into the PAA2 plasmid replacing the bacterial lacZ gene (gift from Kathryn Cheah; Cheah et al., 1995). The Coll2a1-CrepA+ sequences were excised from the plasmid backbone as a NotI fragment and used in DNA microinjection experiments using B6CBAF1 embryos as previously described (Hogan, 1994). Five high-copy-number founder animals were obtained and analyzed by Southern analysis. These founder animals were bred with wild-type B6CBAF1 animals. F1 offspring were subsequently bred with animals that were homozygous for the ‘floxed’ VEGF-A allele (VEGFfloxFloxp) (Gerber et al., 1999a) and with lacZ-reporter mice (Akagi et al., 1997). The offspring from these breedings were genotyped as previously described (Akagi et al., 1997; Gerber et al., 1999a).

RESULTS

VEGF-A expression in chondrogenic tissues

To determine the expression pattern of VEGF-A mRNA in developing chondrogenic tissues, a developmental expression analysis was conducted on wild-type (WT) embryos at embryonic day E10.5, 13.5, 15.5 and 17.5. VEGF-A was detected in the limb-bud mesenchyme and in the developing somites (Fig. 1A,B). As a control, hybridization experiments were performed with a flk1 antisense (AS) probe that showed...
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specific expression in the endothelium of the developing intersomitic blood vessels (Fig. 1C). At E13.5, no significant expression of VEGF-A mRNA was detected in the developing cartilage compared with sense control transcripts (data not shown). However, at E15.5, VEGF-A mRNA could be detected in proliferating chondrocytes in certain cartilaginous structures such as the developing rib cartilage prior to active angiogenic invasion (Fig. 1D,E). VEGF-A mRNA was continuously expressed in the hypertrophic chondrocytes during active angiogenic invasion of the developing cartilage primordia (Fig. 1F).

Generation and analysis of Collagen2a1-Cre transgenics

To conditionally inactivate the ‘floxed’ VEGF-A allele in developing chondrogenic tissues, five high copy number Cre lines were generated in which Cre recombinase was under the transcriptional control of human Collagen2a1 promoter and enhancer sequences (Cheah et al., 1995). The ability of these elements to direct cartilage-specific transgene expression was confirmed by generating Collagen2a1-lacZ transgenics (Fig. 2A). Essentially all transgenic fetuses examined at E11.5 and E12.5 exhibited cartilage-specific lacZ expression regardless of copy number and integration site (Fig. 2A and data not shown). Whole-mount mRNA ISH analysis conducted on E12.5 Collagen2a1-Cre transgenic fetuses demonstrated cre mRNA expression specifically in developing chondrogenic tissues (Fig. 2B). Coll2a1-Cre transgenic lines were crossed with lacZ-reporter animals (Akagi et al., 1997) to examine sites of Cre activity (Fig. 3A). Whole-mount X-gal staining analysis conducted on E12.5 double transgenic fetuses revealed a much broader staining pattern than the expected cartilage-specific staining pattern observed in the Collagen2a1-lacZ fetuses described in Fig. 2A. These results also conflicted with the cartilage-specific expression of cre mRNA found by whole-mount ISH (Fig. 2B). Histological analysis of sectioned material revealed X-gal staining in the developing eye (Fig. 3B), in the neuroepithelium (Fig. 3C), in the epidermis (Fig. 3D) and in cranial mesenchymal cells (Fig. 3F). The expected expression in the developing proliferating chondrocytes in the head region (Fig. 3E) was not observed.

Fig. 1. Expression of VEGF-A during chondrogenic differentiation. (A) Whole-mount mRNA in situ hybridization (ISH) analysis was conducted on E10.5 WT embryos using a VEGF-A anti-sense (AS) probe. Arrows indicate high levels of VEGF-A expression in limb-bud mesenchyme in both hindlimb and forelimb. (B) Higher power magnification of embryo in A. Asterisks indicate VEGF-A expression in developing somites. The arrow indicates VEGF-A expression in the posterior limb-bud. (C) Whole-mount mRNA in situ analysis was conducted on E10.5 wild-type embryo using a flk1 AS probe. Arrows indicate vascular endothelial staining of the intersomitic blood vessels. (D,E) VEGF-A expression in proliferating chondrocytes. (D) Dark-field image of radioactive in situ hybridization conducted on section from wild-type E15.5 rib cartilage using a VEGF-A AS probe. Arrows indicate increased silver grain density surrounding proliferating chondrocytes compared with background signal obtained using a VEGF sense probe on adjacent section (E). (F) Non-radioactive in situ hybridization conducted on growth plate cartilage from E17.5 developing rib cartilage using a VEGF-A AS probe. Arrows indicate VEGF-A expression in hypertrophic chondrocytes. Invading vasculature (V) Magnification in D-F: ×400.

Fig. 2. Generation of and expression analysis in Collagen2a1-transgenic mice. (A) β-gal staining shows lacZ transgene expression limited to chondrogenic tissues in the head, limb primordia and vertebral column in an E11.5 Collagen2a1-lacZ fetus. ISH analysis detected cre mRNA transcripts predominantly in chondrogenic tissues in a Collagen2A1-Cre fetus compared with background staining observed in a control WT fetus (B). Whole-mount mRNA in situ analysis was conducted on E12.5 transgenic and WT fetuses using a cre AS probe. Arrows indicate cre expression in the developing limb cartilage. Asterisks indicate cre expression in developing vertebrac.
transgenic and was heterozygous for the floxed allele \((\text{coll}2a1-\text{Cre}, \text{VEGF}^{\text{Flx}+/+})\). These results implied that there was lethality associated with Cre-mediated deletion of a single floxed VEGF-A allele in \(\text{collagen}2a1-1\)-expressing cells although these fetuses still possessed one wild-type allele. In order to determine the point of lethality, fetuses were isolated starting at E10.5.

Greater than 95% of mutant fetuses \((\text{coll}2a1-\text{Cre}, \text{VEGF}^{\text{Flx}+/+})\) showed altered development at E10.5. The mutants appeared from initial gross morphological assessment to be necrotic and were 30-40% smaller than their control littermates \((\text{VEGF}^{\text{Flx}+};\) data not shown). The mutant fetuses displayed poorly developed branchial arches and had underdeveloped or absent forelimb buds (asterisks in Fig. 4A,B). The forebrain regions of the mutants also appeared malformed (Fig. 4A,B). The vasculature along the developing axial regions of the mutants appeared to be aberrant and there was a clear lack of proper intersomitic vessel development (arrows in Fig. 4B,M). The dorsal aorta was incompletely formed and showed signs of vascular hemorrhaging (arrows in Fig. 4B,C,I). There was also a significant decrease in the number and complexity of vessels in the mutant yolk sacs (Fig. 4D,E). This aberrant vascular development in the yolk sac was indicative of Cre activity present in the developing endoderm. This was confirmed through whole-mount mRNA in situ analysis conducted on mutant yolk sacs which showed the presence of \(\text{cre}\) transcripts (data not shown).

In the heart, the common atrium and primitive ventricle were developmentally delayed and the pericardial cavity was distended and filled with fluid (Fig. 4F,G). The vascular defects found in the initial morphological assessment were confirmed through whole-mount mRNA in situ analysis (Fig. 4H,I). The number of flk1-positive cells was decreased and the mutant endothelium appeared disorganized compared with controls (Fig. 4H,I). Closer inspection of the caudal regions of the mutant embryos showed decreased organization of flk1-positive cells and altered formation of intersomitic blood vessels (arrows in Fig. 4L,M). Whole-mount ISH analysis also demonstrated a reduced complexity in the degree of endothelial branching in the flk1-positive mutant endocardium (arrows in Fig. 4J,K). Histological analysis revealed that the endocardium was less intricately branched and appeared to be detached from the underlying myocardium (Fig. 5D,F). There was variance in the severity of the heart phenotype observed. The differences in myocardial thickness in the mutant hearts correlated with the degree of endocardial degeneration (Fig. 5D,E). Another consequence of the endocardial degeneration observed in the mutants was the improper formation and/or maintenance of the endocardial cushions (EC in Fig. 5D,F). There appeared to be greater amounts of cardiac jelly in the mutant hearts (asterisks in Fig. 5D,F). As a result, the endocardium was greatly constricted resulting in significant decreases in the lumen size of the developing ventricle (V in Fig. 5C) and/or atria (A in Fig. 5E).

The vascular phenotypes observed in the mutant fetuses were consistent with the localized deletion of a single VEGF-A allele in regions of \(\text{cre}\) expression reported in Fig. 3. These results implied that VEGF-A expression in \(\text{collagen}2a1-1\)-expressing cells is essential for the establishment of the cardiovascular system of the mouse.

**Fig. 3.** Cre activity detected in Collagen2a1-expressing cells at E12.5. (A) Schematic representation of Cre-mediated recombination of the reporter locus in double transgenic Collagen2A1-Cre/lacZ reporter mice. Cre activity/X-gal staining was detected in (B) the developing eye, (C) the neuroepithelial lining of the developing ventricle and (D) the epidermis. Cre activity/X-gal staining was detected in (E) the mesenchymal condensations of the head, (F) sites of the developing cranial mesenchyme near the developing brain vesicles and (G) the chondrocytes of the developing vertebrae and spinal chord. Cre activity was detected in the myocardial layer of the developing (H) atria and (I) ventricles of the heart. (J) Higher power magnification \((\times 400)\) of the developing ventricle showed Cre activity/X-gal staining in the cardiomyocytes of the trabecular of the myocardial layer. A, atrium; C, cranial mesenchymal condensations; CM, cranial mesenchyme; Ep, epidermis; M, myocardium; NE, neuroepithelium; S, spinal chord; V, ventricle; VC, vertebral chondrocytes. Magnification in B-I, \(\times 100; J, \times 400\).

**Early lethality of Collagen2a1-Cre, VEGF\(^{\text{Flx}+/+}\) heterozygous fetuses**

Several Collagen2a1-Cre founder mice were crossed with mice that were homozygous for the floxed VEGF-A allele \((\text{VEGF}^{\text{Flx}/\text{Flx}};\) Gerber et al., 1999a). Out of over 100 offspring obtained from five different transgenic lines only 1 stillborn mouse was delivered that carried the Collagen2a1-Cre reporter locus in double transgenic Collagen2A1-Cre/+; data not shown). The mutant fetuses displayed poorly developed branchial arches and had underdeveloped or absent forelimb buds (asterisks in Fig. 4A,B). The forebrain regions of the mutants also appeared malformed (Fig. 4A,B). The vasculature along the developing axial regions of the mutants appeared to be aberrant and there was a clear lack of proper intersomitic vessel development (arrows in Fig. 4B,M). The dorsal aorta was incompletely formed and showed signs of vascular hemorrhaging (arrows in Fig. 4B,C,I). There was also a significant decrease in the number and complexity of vessels in the mutant yolk sacs (Fig. 4D,E). This aberrant vascular development in the yolk sac was indicative of Cre activity present in the developing endoderm. This was confirmed through whole-mount mRNA in situ analysis conducted on mutant yolk sacs which showed the presence of \(\text{cre}\) transcripts (data not shown).

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Fig. 4. Early lethality in coll2a1-Cre, VEGF<sup>Flox/+</sup> heterozygotes at E10.5. Gross morphological assessment of a heterozygous coll2a1-Cre, VEGF<sup>Flox/+</sup> fetus (B) demonstrates a dramatically altered appearance compared to a VEGF<sup>Flox/+</sup> littermate (A). Mutant fetuses show altered branchial arch development and severe cranial defects. The heterozygous mutant fetus (B) lacks the forelimb bud (asterisks indicate limb buds). The arrows in B show impaired vascular development in the dorsal aorta and intersomitic blood vessels. Mutant fetuses show incomplete dorsal aorta formation and signs of vascular hemorrhaging (arrow in C). Yolk sacs from mutants (E) demonstrate dramatic decreases in major blood vessels compared with yolk sacs from control littermates (arrows in D). Mutant hearts (G) showed a distended, fluid-filled cardiac cavity and a pronounced delay in cardiac development of the atria and ventricles compared with a control heart (F). Whole-mount mRNA in situ analysis was conducted on mutant (I) and control fetuses (H) using a flk1 AS probe, which demonstrates the dramatic disorganization of the developing endothelium in mutant fetuses. flk1-positive cells in a mutant fetus (I) appear discontinuous and show regions of vascular hemorrhaging, (arrow in I). The intersomitic blood vessels often appear to be discontinuous in mutants particularly in the caudal region (M). This is in contrast to the organized intersomitic blood vessels observed in a control littermate (L). The endocardium in a mutant (K) also appears less developed and collapsed, and lacks the characteristic branching architecture observed in a control heart (J).

Fig. 5. Histological analysis of coll2a1-Cre, VEGF<sup>Flox/+</sup> hearts at E10.5. Mutant hearts show a dramatic decrease in the thickness of the myocardium and extent of trabeculae formation (lines in C, E). Control hearts showed a thicker myocardium and more elaborate trabeculae formation (line in A). Higher power magnification (×400) of control hearts (B) show that the endocardium is in close contact with the underlying myocardium and that there are properly formed endocardial cushions separating the atria and ventricle. High-power magnification (×400) of the mutant hearts shows that there is variability in the degree of degeneration. The mutant heart in F is more severely affected than the heart seen in D. The mutant endocardium appears detached from the underlying myocardium and is much thinner with less-developed trabeculae (D,F). There is an increased distance between the endocardium and myocardium implying greater amounts of cardiac jelly present in between the two heart layers compared with controls (asterisks in B,D,F). The detachment of the endocardium results in a decrease in lumen size of the ventricle (C) and/or atrium (E). The endocardial cushions in the mutant hearts show aberrant formation and/or increased degeneration. A, atrium; EC, endocardial cushion; E, endocardium; Ep, epicardium; M, myocardium; V, ventricle. Asterisks indicate cardiac jelly. Magnification of A,C,E, ×100; B,D,F, ×400.
Altered heart and bone formation in E17.5 mutants

Histological analysis of three E17.5 mutant fetuses demonstrated a marked decrease in the thickness of the myocardium in the developing ventricle compared to controls (Fig. 6A,E). Higher power magnification of the mutant ventricles showed an increased disorganization and a decreased density in the developing cardiac myofibers (Fig. 6B,F). The cardiomyocytes were much more fibrous and spindle shaped, lacking the characteristic morphology of the control cardiomyocytes (Fig. 6C,G). Vessel density counts showed a significant decrease in the number of microvascular endothelial cells in the affected hearts (arrows in Fig. 6C,G). Tunnel analysis revealed a massive increase in the number of apoptotic endothelial cells lining major blood vessels. There was also a significant increase in the number of Tunnel-positive cardiomyocytes present in the mutant hearts compared to control littermates (Fig. 6D,H).

E17.5 mutant fetuses were also smaller than control littermates (data not shown). Histological inspection of the developing long bones from four E17.5 mutants revealed an increased hypertrophic chondrocyte zone of the growth plates compared with controls (dashed lines in Fig. 7A-D). This increase was present in several long bones including the humerus and tibia and the distance between the proximal and distal growth plates was reduced (data not shown). Serial sections from E17.5 mutant fetuses were examined and the length of the hypertrophic chondrocyte zone of the cartilage from several different long bones was measured. The hypertrophic zone was on average approximately twice as long as the hypertrophic zone from control littermates (Fig. 7A-D). The average length of the hypertrophic zone in mutant long bones was 213±39 μm whereas the length of the hypertrophic zone in control littermates was 98±33 μm. In addition to this increased length of the hypertrophic zone, the vasculature in the mutants did not invade the hypertrophic cartilage to the same extent as in control littermates (red dashed lines in Fig. 7E,F). The vasculature in the mutant fetuses also showed decreased sprouting at the growth plate/trabecular bone interface (Fig. 7E,F).

DISCUSSION

To specifically address the role of VEGF-A expression in chondrogenic tissues during development a conditional inactivation approach using the Cre/LoxP recombination system was chosen. The human Collagen2a-1 promoter and enhancer sequences that were used in this analysis to direct cre expression have previously been used to direct transgene expression specifically to developing chondrogenic tissues (Cheah et al., 1995). The present analysis has revealed that these elements are also capable of directing Cre expression in non-cartilagenous cell types at early time points in embryonic development including the developing eye, epidermis, heart myocardium and endoderm of the developing yolk sac, in addition to chondrogenic tissues. Interestingly, these sites of non-cartilagenous expression correlate with the previously reported expression pattern of the endogenous murine collagen2a-1 gene (Cheah et al., 1991). Deletion of VEGF-A in collagen2a-1-expressing cells has resulted in a heterozygous lethality that recapitulates many of the phenotypic alterations that were present in the original VEGF-A+/− fetuses (Carmeliet et al., 1996; Ferrara et al., 1996). These results allow for a more complete understanding of the role of VEGF-A in the development of essential embryonic structures and organ systems.

Role of VEGF expression in developing chondrogenic tissues

VEGF-A mRNA is expressed in the developing sclerotome and limb bud mesenchyme at E10.5. After this point, VEGF-A expression declines in chondrogenic tissues and gradually becomes upregulated around E17.5 in hypertrophic chondrocytes in bones undergoing active angiogenic invasion. These results are consistent with previous reports of VEGF-A expression in the axial somites in zebrafish and Xenopus.

Fig. 6. Histological analysis of coll2a1-Cre, VEGFFlx/+ hearts at E17.5. Mutant hearts at E17.5 show a dramatic decrease in the thickness of the myocardium of the left ventricle (E) compared with a control heart (A). Higher power magnification (×100) of the mutant myocardium (F) shows that the cardiac myofibers of the trabeculae were much more disorganized than in control hearts (B). The cardiomyocytes of the mutant hearts (G) show a more fibrous and spindle-shaped architecture compared with the normal cytoarchitecture of the control cardiomyocytes (C). It appeared from histological inspection that there is significantly more microvasculature (white arrows) in the myocardium of the control (C) than in the mutant myocardium (G). Tunnel analysis reveals increased apoptosis in the mutant vascular endothelial cells lining major blood vessels and in the cardiomyocytes (H) compared with no apoptosis seen in the control (D). Magnification of A,E, ×40; B,F, ×100; C,G,D, ×400.
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(Cleaver and Krieg, 1998; Liang et al., 1998) and in hypertrophic chondrocytes in mice and humans (Gerber et al., 1999b; Horner et al., 1999).

In both the zebrafish and *Xenopus* systems, VEGF-A expression in axial structures has been implicated in playing a role in angioblast migration and the formation of the dorsal aorta (Cleaver and Krieg, 1998; Fouquet et al., 1997). Characterization of zebrafish mutants (floating-head and no-tail) along with cloning and characterization experiments have determined that VEGF-A is expressed under notochordal control in the ventromedial portion of each somite in cells adjacent to the notochord (Fouquet et al., 1997; Liang et al., 1998). This site of VEGF-A expression corresponds to the developing sclerotome of the mouse. Cre-mediated deletion of a single floxed VEGF-A allele in the developing sclerotomal mesenchyme of the somite of *coll2a1-Cre, VEGF flox/+* fetuses has resulted in defects in the development of the dorsal aorta and intersomitic blood vessels in mutant fetuses. These results underscore the importance of VEGF-A expression in the developing sclerotome of the mouse to ensure proper dorsal aorta formation.

Another structure that showed altered development in mutant fetuses was the developing limb-bud, which was either developmentally retarded or absent. VEGF-A expression was detected in the developing limb-bud mesenchyme. This site of VEGF-A expression may serve to recruit blood vessels from the dorsal aorta. Decreasing the levels of VEGF-A in the developing limb-bud mesenchyme resulted in decreased vasculature invasion in the mutants, which may, in turn, have resulted in a delay or absence of differentiation stimuli that are essential for limb-bud development. These results are consistent with previous observations that the vasculature plays a major role in the morphogenesis and pattern formation of the developing limb (Caplan, 1985).

Mutant fetuses at E17.5, which were only obtained at low frequencies (4/150), exhibited an increased hypertrophic chondrocyte zone in the developing growth plate. These results are similar to the phenotypic alterations recently observed in *MMP-9/Gelatinase-B−/−* mice and 24-day-old neonates treated with a VEGF-A sequestering protein (Gerber et al., 1999b; Vu et al., 1998). Decreasing the levels of VEGF-A protein through sequestration (Gerber et al., 1999b) or through genetic means has resulted in decreased blood vessel invasion, reduced bone lengthening, and increases in the hypertrophic chondrocyte zone. Further support for the importance of VEGF-A in endochondral bone development has come from a recent report on the expression of VEGF-A in hypertrophic chondrocytes and the existence of autocrine-paracrine signalling events present at the growth plate of developing long bones (Carlevaro et al., 2000).

**Role of VEGF in heart development**

Recent gene-targeting and expression analysis in the mouse system has demonstrated the importance of VEGF-A signaling in the initial stages of heart development. *Flk1−/−* fetuses in addition to having a complete lack of endothelial and hematopoietic development also showed a lack of endocardial cell development (Shalaby et al., 1995). VEGF-A has previously been detected in the underlying endoderm in precardiac regions and in the developing myocardium (Dumont et al., 1995). Recently, the developmental expression of VEGF-A in the heart has been investigated more thoroughly using a lacZ-tagged VEGF-A allele (Miquerol et al., 1999). At around E9.5 there is a major change in lacZ/VEGF-A expression such that the compact trabecular layers of the myocardium become the major source of VEGF-A expression in the heart.
Deletion of a single floxed VEGF-A allele in the myocardium in mutant fetuses resulted in several phenotypic alterations in the heart that demonstrate the importance of threshold levels of VEGF-A expression in myocardial-endocardial interactions. Mutant hearts displayed a less intricately folded endocardium, which was detached from the myocardial layer. The myocardial wall appeared to be less developed and thinner with a more collapsed appearance. The endocardial cushions were also abnormally formed in mutant fetuses. These phenotypes are similar to alterations observed in several mouse mutants recently reported, including homozygous null mutations in the angiopoietin-1/Tie2 system and the neuregulin/ErbB2-4 system (Gassmann et al., 1995; Kramer et al., 1996; Sato et al., 1995; Suri et al., 1996). Similar phenotypic alterations have also been reported in mice that are homozygous null for the MEF2C transcription factor (Bi et al., 1999; Lin et al., 1998). The heart defects exhibited in MEF2C−/− fetuses was shown to be related to decreased expression levels of VEGF-A and Angiopoietin-1 in the myocardium that resulted in endocardial defects due to decreased signaling via Flk1/Tie2 receptors present on the endocardium (Bi et al., 1999). As a result of these endocardial defects, there was a loss of reciprocal paracrine signals to the myocardium, which prevented cardiomyocyte maturation and trabeculae formation. These reciprocal signals provided by the endocardium may involve molecules such as neuregulins, which have been implicated in signaling through ErbB-2/4 receptors present on cardiomyocytes (Gassmann et al., 1995; Kramer et al., 1996; Lee et al., 1995).

The small proportion of mutant E17.5 fetuses that survived the initial embryonic lethality at around E10.5 demonstrated a decreased vascular density in the heart and displayed an increased apoptotic index in both the vascular endothelium and cardiomyocytes compared with control littermates. VEGF-A has been shown to act as an endothelial survival factor both in vitro and in vivo (Gerber et al., 1998, 1999a). Cre-mediated deletion of a single VEGF-A allele in the myocardium resulted in increased endothelial apoptosis and a decreased nutritional and oxygen supply to the myocardium. As a result, the cardiomyocytes were exposed to continued ischemic conditions. During myocardial ischemia, it is known that cardiomyocytes may either undergo apoptotic cell death or they may survive chronic ischemia through a process termed ‘hibernation’ (Elsasser and Schaper, 1995; Vanoverschelde et al., 1997). Through this process cardiomyocytes try to adapt to low oxygen levels through reversibly downregulating contractile function to re-equilibrate energy expenditure and supply. However, as may be the case with the surviving collagenα1-Cre, VEGF Flox/+ fetuses, continued ischemia led to progressive degeneration, atrophy and interstitial fibrosis of the myocardium. Given the above histological criteria, it appeared that the hearts of these animals displayed a severe form of dilated cardiomyopathy (Towbin, 1998; Towbin et al., 1999). The heart phenotype seen in the mutant fetuses at E10.5 and E17.5 is much more severe than the cardiomyopathy recently reported (Carmeliet et al., 1999). These authors have shown that overexpression of the VEGF-A120 isoform was sufficient to rescue the early lethality exhibited in the VEGF-A−/− fetuses, but was insufficient to ensure proper development of the heart myocardium throughout neonatal life. These findings implied that there are specific functions of the VEGF-A164 and VEGF-A188 isoforms that VEGF-A120 overexpression cannot compensate for. The results obtained with the collagenα1-Cre, VEGF Flox/+ fetuses, in which there is decreased amounts of all three VEGF-A isoforms in the developing myocardium, establish the specific and essential functions for the maintenance of threshold levels of the longer VEGF-A isoforms to ensure proper heart formation.

In summary, this is the first report of a conditional inactivation of VEGF-A during mouse development. These results provide definitive proof that threshold levels of VEGF-A are extremely important during embryonic development. This study has demonstrated the essential role of sclerotomal sources of VEGF-A in dorsal aorta and intersomitic blood vessel formation in the mouse. Specific deletion of a single VEGF-A allele in the myocardium has provided conclusive evidence for the necessity of threshold levels of VEGF-A for proper endocardial/myocardial development. The present conditional approach used to inactivate VEGF-A expression in developing chondrogenic tissues has provided cell-specific evidence for the importance of VEGF-A in endochondral bone growth. In addition, these results imply that there is an important developmental link between VEGF-A expression in Collagen2a1-expressing cells and the establishment of a functional vascular network and cardiovascular system.

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