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Exogenous vascular endothelial growth factor induces malformed and hyperfused vessels during embryonic neovascularization

(vasculogenesis/angiogenesis/blood vessel development/cardiac morphogenesis)

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ABSTRACT **Vascular endothelial growth factor (VEGF) is a potent and specific endothelial mitogen that is able to induce angiogenesis *in vivo* [Leung, D. W., Cachianes, G., Kuang, W.-J., Goeddel, D. V. & Ferrara, N. (1989) *Science* 246 1306–1309]. To determine if VEGF also influences the behavior of primordial endothelial cells, we used an *in vivo* vascular assay based on the *de novo* formation of vessels. Japanese quail embryos injected with nanomolar quantities of the 165-residue form of VEGF at the onset of vasculogenesis exhibited profoundly altered vessel development. In fact, the overall patterning of the vascular network was abnormal in all VEGF-injected embryos. The malformations were attributable to two specific endothelial cell activities: (*i*) inappropriate neovascularization in normally avascular areas and (*ii*) the unregulated, excessive fusion of vessels. In the first instance, supernumerary vessels directly linked the inflow channel of the heart to the aortic outflow channel. The second aberrant activity led to the formation of vessels with abnormally large lumens. Ultimately, unregulated vessel fusion generated massive vascular sacs that obliterated the identity of individual vessels. These observations show that exogenous VEGF has an impact on the behavior of primordial endothelial cells engaged in vasculogenesis, and they strongly suggest that endogenous VEGF is important in vascular patterning and regulation of vessel size (lumen formation).**

It is generally accepted that new blood vessels can be established by either vasculogenesis or angiogenesis (1, 2). Vasculogenesis is the *de novo* establishment of blood vessels and vascular networks from mesoderm-derived endothelial cell precursors (angioblasts). In contrast, the expansion of the vasculature by angiogenesis is dependent on the generation of additional endothelial cells from preexisting vascular beds. Thus, it is the source of the newly generated endothelial cells that best distinguishes vasculogenesis from angiogenesis. Despite this difference, it is likely that many of the mechanisms and regulators that control new vessel formation are common to the two processes. For instance, endothelial cells engaged in either vasculogenesis or angiogenesis appear to use similar extracellular matrix adhesive mechanisms (3, 4).

Considerable work has demonstrated that polypeptide growth factors, such as acidic and basic fibroblast growth factors, platelet-derived endothelial cell growth factor, and vascular endothelial growth factor, exert a wide range of effects on endothelial cells and are able to induce angiogenesis both *in vivo* and *in vitro* (for reviews, see refs. 5 and 6). However, the effects of such factors on vasculogenesis have only recently been examined. Using an *in vitro* approach, Flamme and coworkers (7, 8) showed that basic fibroblast growth factor can induce pluripotent cells of the quail blastodisc to undergo vasculogenesis. There is also reason to believe that vascular endothelial growth factor (VEGF) may influence the process of vasculogenesis. Data supporting this possibility are that VEGF acts as an endothelial cell-specific mitogen (9), that VEGF mRNA has a widespread distribution in fetal tissue of mice and rats (10, 11), and that VEGF receptor mRNA is present in embryonic murine and avian endothelial cells (12–14).

VEGF exists in at least four forms that are generated by alternative splicing from a single gene: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, with the subscript indicating the number of amino acid residues. The $VEGF_{165}$ form is the most abundant gene product in a number of tissues (15). In this study recombinant human $VEGF_{165}$ ($rhVEGF_{165}$) was tested for its ability to influence vasculogenesis in an *in vivo* vascular assay based on the *de novo* formation of blood vessels in the quail embryo (16). We report that microinjected $rhVEGF_{165}$ profoundly alters the behavior of primordial endothelial cells. To the best of our knowledge, this report provides the first direct experimental evidence that links VEGF to *de novo* blood vessel formation, vasculogenesis.

MATERIALS AND METHODS

Embryo Microinjection and Microsurgery. Methods for the microinjection of early stage Japanese quail embryos (*Coturnix coturnix japonica*) have been described (16, 17). To deliver reagent to the interstitial space between the endoderm and the splanchnic mesoderm, a site of active vasculogenesis, embryos were microinjected through the endoderm. All embryos in this study were injected at a site caudolateral to the last formed somite. The micropipette was positioned by using a Leitz micromanipulator coupled to a Narishige hydraulic drive manipulator. Approximately 25 nl of reagent was delivered by utilizing the pneumatically driven Pico-Injector (Medical Systems, Greenvale, NY). Volumes were set by regulation of pressure and time and were calibrated by collecting 10 ejections in a calibrated 1-μl Microcaps pipette (Drummond Scientific, Broomall, PA).

All embryos in this study received a single injection on one side of the midline and were then placed ventral side down on a nutrient agar culturing medium (see below). To examine the development of the intersomitic arteries, embryos were injected on one side, transferred to culture dishes, and then immediately bisected along the midline, using a microsurgical scalpel. This procedure allows a comparison of vessel development in specimens of identical somitic stage.

Injected Reagents. $rhVEGF_{165}$ was expressed in the insect cell line sf21; the level of endotoxin was <0.1 ng/μg of cytokine (obtained from R&D Systems). In addition, equivalent VEGF effects were more recently obtained with recombinant cytokine supplied by N. Ferrera (Genentech). Lyophilized VEGF and the carrier protein human serum albumin (HSA) were dissolved in water to obtain a final concentration of 0.5 ng/nl and 25 ng/nl, respectively. The estimated con-

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Abbreviations: VEGF, vascular endothelial growth factor; rh-, recombinant human; HSA, human serum albumin; LSCM, laser scanning confocal microscope.

centration of rhVEGF$_{165}$ in an injected embryo is 56 nM. This value is based on the following assumptions: (*i*) the embryonic volume at five somites is 5 mm^3 or 5 μl; (*ii*) 12.5 ng of recombinant protein was injected per embryo; and (*iii*) the molecular mass of dimeric rhVEGF$_{165}$ is 45,000 Da. The VEGF carrier protein HSA was used in control injections (USP grade, Baxter Healthcare, Glendale, CA). The sterile HSA, supplied as a 25% solution, was diluted with phosphate-buffered saline to a final concentration of 25 ng/nl.

Embryo Culture. Embryo/ring assemblies of VEGF-injected (n = 32), control HSA-injected (n = 16), and control culture embryos (n = 21) were placed ventral side down in 35-mm plastic Petri dishes (Falcon) containing an egg supernatant/agar mix (18). These dishes were then placed in a 150-mm dish containing water-saturated paper towels and were incubated for 7 hr at 37°C, in a humidified CO_2/air mixture (10%/90%).

Fixation and Immunolabeling of Whole-Mounted Embryos. At the end of the culture period, the embryo/ring assemblies were removed from the culture dishes, rinsed in phosphate-buffered saline, and fixed in 3% paraformaldehyde for 45 min. Examination of these embryos as whole mounts revealed no differences in morphology that would distinguish them from similarly staged normal embryos. Immunolabeling the vasculature of these whole-mounted embryos was performed as previously described (16), using the supernatant from the QH1 hybridoma (Developmental Studies Hybridoma Bank, University of Iowa) and fluorochrome-conjugated affinity-purified goat anti-mouse secondary antibodies (Jackson ImmunoResearch). After immunolabeling, the embryo/ring assemblies were mounted ventral side up under a no. 1 coverslip, using an anti-photobleaching mounting medium (19). After analysis using the laser scanning confocal microscope, a subset of embryos were embedded in paraffin and sectioned by conventional methods.

Laser Scanning Confocal Microscopy. Embryos were analyzed by using a Bio-Rad MRC-1000 laser scanning confocal microscope (LSCM). The LSCM allowed multiple focal planes of the intact whole-mounted embryo to be compressed into one image. The primordial endothelial cells and blood vessels of the early embryo were detected by using the monoclonal antibody QH1, as described above.

Image Processing. Whole-mounted embryos were scanned in a plane parallel to the embryonic plate, using 5× and 10× objective lenses. Sequential optical planes were acquired along the vertical (z) axis through the full thickness of a stage 8–9 quail embryo (approximately 80 μm). The stored graphics files were then collapsed to a single virtual image by using the manufacturer's proprietary software (Bio-Rad). This processing resulted in a QH1-derived fluorescence map of all the blood vessels and angioblasts in the quail embryo. The vascular map graphics file was imported into Adobe Photoshop for image processing. The cross section in Fig. 3 *c* and *d* was acquired as an LSCM immunofluorescent image, then rendered as a surface relief image in Photoshop.

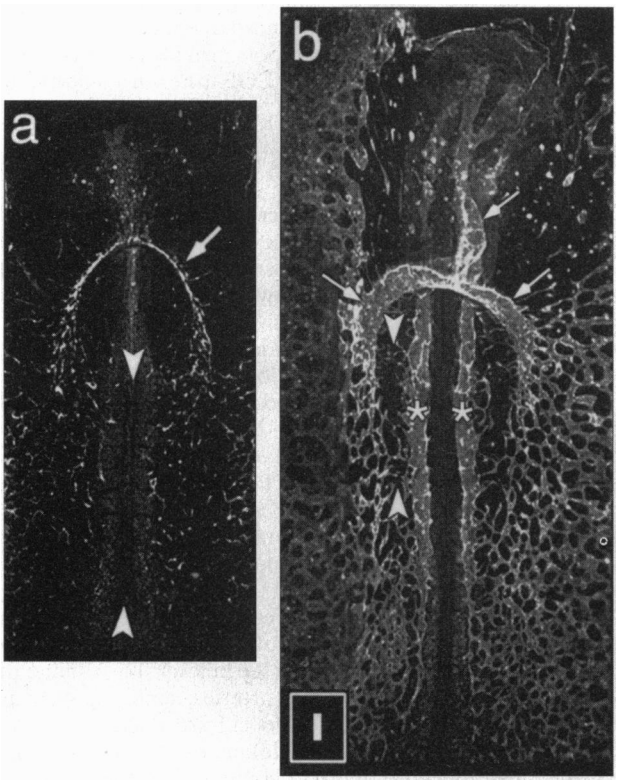

FIG. 1. Whole-mounted normal quail embryos were examined with the LSCM. The images depict a road map-like view of all blood vessels as viewed from above. In *a* the midline of the five-somite embryo is marked by the two arrowheads. Lateral to the arrowheads, small clusters or cords of endothelial cells (angioblasts) are observed as bright foci. Cranially, the presumptive heart endothelium appears as an arc of immunopositive cells (arrow). *b* illustrates the extensive changes to the vasculature that occur during the experimental incubation period. Well-defined vessels, which form during the 7-hr interval, distinguish the two stages (*a* and *b*). For example, the dorsal aortae (asterisks) and the primordial heart endothelial tubes (arrows) are prominent in the nine-somite embryo (*b*). The arrowheads in *b* designate an avascular zone; the vertical bar is the size of a nine-somite embryo, 3.5 mm. (×25.)

RESULTS

Normal Vasculogenesis. The vascular pattern of a normal five-somite embryo, shown in Fig. 1*a*, is representative of the state of vessel development at the time VEGF was injected into experimental embryos. Primordial endothelial cells and vessels were visualized with the monoclonal antibody marker QH1 (20). Although organized vessels and vascular networks are not yet apparent, QH1-positive immunofluorescent primordial endothelial cells are evident as bright cords and foci. The time frame over which the experiments were conducted was chosen to coincide with an active period of vasculogenic activity. The

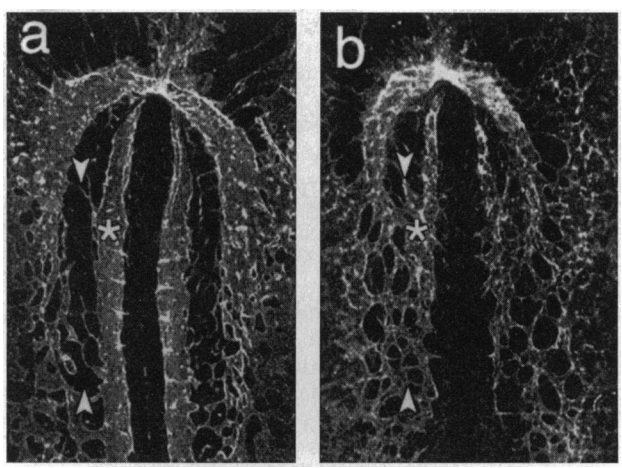

FIG. 2. The normally avascular zone, between the arrowheads in *a*, has been invaded by an inappropriate vasculature in VEGF-injected embryos (arrowheads in *b*). Treated embryos also exhibit aortae that are clearly disorganized. A hallmark of normal aortic development is the organization of clear medial and lateral boundaries (asterisk in *a*); however, in the case of VEGF-treated embryos, well-defined boundaries are totally lacking (asterisk in *b*). Both control and VEGF-treated embryos were injected with human serum albumin (625 ng in 25 nl), while the latter group also received 125 ng of rhVEGF$_{165}$. Embryos were injected at the five-somite stage, similar to Fig. 1*a*. (×35.)

extent of vascular development during this period (7 hr) is demonstrated in the normal nine-somite embryo shown in Fig. 1*b*. At the nine-somite stage well-defined vessels and an easily recognizable vascular pattern are evident. The largest vessels depicted in Fig. 1*b* are the heart endothelium (arrows) and the dorsal aortae (asterisks); also evident is an extensive network of smaller lateral vessels. It is important to point out that avascular zones, which appear as unlabeled black regions throughout the embryo, are a critical component of the embryonic vascular pattern. Note, in particular, the two avascular regions that separate the dorsal aortae from the developing heart (Fig. 1*b*, region between the arrowheads).

VEGF Alters Vasculogenesis *in Vivo*. The rhVEGF$_{165}$-injected embryos (five somites) reveal obvious and profound malformations in blood vessel patterning (Fig. 2*b*) compared with control-injected embryos (Fig. 2*a*). Most abnormal was the elaboration of an inappropriate vascular network in the area between the dorsal aortae and the developing heart tubes (arrowheads, Fig. 2*b*). These supernumerary vascular networks join the dorsal aortae (asterisk) directly to the inflow regions of the heart (presumptive atrial region). In contrast, normal embryos (Fig. 1*b*) and control-injected embryos (Fig. 2*a*) maintain a separation (avascular zone) between the future arterial and venous trunks.

Developmental Age Is a Determinant of Induced Abnormalities. When embryos were injected with rhVEGF$_{165}$ 2 hr later in development (six-somite stage) the severity of the vascular abnormalities was significantly increased compared with five-somite embryos (compare Figs. 2*b* and 3*b*). In fact, embryos injected at six somites were virtually devoid of avascular regions. A continuous vascular structure directly connects the dorsal aortae to the inflow area of the endocardium (between the arrows in Fig. 3*b*).

The increase in the level of vascular fusion observed in embryos injected at six somites leads to the total loss of individual vessel identity (compare Fig. 3 *a* and *b*). Indeed, the dorsal aortae, the heart endothelial tubes, and the lateral vascular networks are no longer discernible as separate vessels. These data show that the stage of vascular development at which VEGF was injected directly influences the severity of the abnormalities observed.

VEGF Induces Vascular Hyperfusion. To determine whether the enlarged endothelial structures observed in the VEGF-injected embryos (Fig. 3*b*) contained lumens, cross sections of this, and other, embryos were prepared. Comparison of the images in Fig. 3 *c* and *d* strongly confirms the *en face* observation (above) that VEGF injection causes the loss of individual vessel identity. The images illustrate that individual vessels present in controls (Fig. 3*c*) merged to form large sinuses in VEGF-injected embryos (Fig. 3*d*). Thus, dorsal aortae (da), normally avascular spaces (bracket), and lateral vessels (arrowheads), which are easily identified in normal and control embryos (Fig. 3*c*), are fused in the VEGF-treated embryos (bracket, Fig. 3*d*). The transverse sections depicted in Fig. 3 *c* and *d* were cut from the embryos shown in *a* and *b* at the level indicated by the double-headed arrow.

Intersomitic and Vertebral Arteries. The alterations to normal vessel development that resulted from injection of VEGF also influenced the formation of the intersomitic and vertebral arteries. The latter vessels are reported to be the first that form in the embryo by the process of angiogenesis (1). In all treated embryos, the formation of the intersomitic and

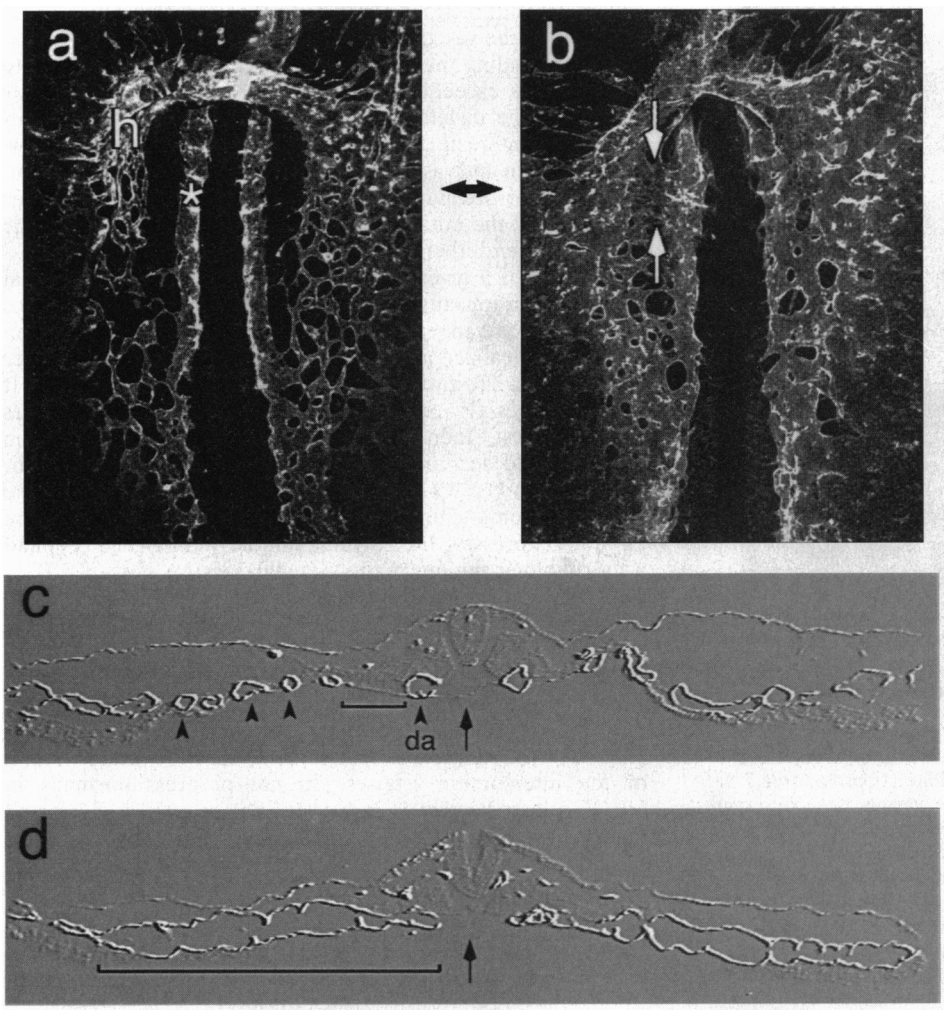

FIG. 3. Embryos injected with VEGF at six somites (*b*) induced distinctly different anomalies compared with embryos that were injected at the five-somite stage (see Fig. 2*b*). In fact, when injected at six somites the QH1-positive cells formed two continuous structures which virtually eliminated avascular zones on each side of the midline. Compare the control vascular pattern in *a* with that of the VEGF-injected embryo in *b*. Most obvious is the absence of the critically important avascular space between the heart (h) and the aorta (asterisk) in the VEGF-treated embryos (arrows). (*a* and *b*, ×35.) The transverse section in *c* was prepared from the control-injected embryo shown in *a*, while the section shown in *d* is from the corresponding VEGF-injected embryo in *b*. The arrow marks the midline in both images. Lateral to the midline in the control are the dorsal aortae (da), followed in sequence by the avascular zone (small bracket), and a series of small vessels (arrowheads). In the VEGF-injected embryo, no distinct vessels or avascular spaces are present. Instead, large sinuses (bracket) have obliterated normal vessel patterning. The sections shown were taken from the level of the second somite indicated by the double-headed arrow between *a* and *b*. (*c* and *d*, ×50.)

vertebral arteries appeared to be retarded. To investigate specifically the effects of VEGF on this process, embryos were injected, then immediately bisected (see *Materials and Methods*). Comparison of the injected and control halves revealed marked alterations in the development of these vessels (Fig. 4). Conspicuous by their absence on the VEGF-injected side were the intersomitic arteries associated with the newest somites (arrowheads). In addition, the primitive vertebral arteries are missing (brackets, VEGF). The latter vessels appear as longitudinal branches of intersomitic arteries 1–3 (brackets, Control).

DISCUSSION

VEGF Stimulates Angioblasts to Change Behavior. Prior to this study, the various properties of VEGF have been investigated in the context of functionally mature endothelial cells, especially during angiogenesis. Here we tested the effects of VEGF on primordial endothelial cells. The results establish that the introduction of exogenous VEGF into developing embryos causes profound vascular malformations. To our knowledge, this is the first evidence that VEGF affects the behavior of endothelial cells engaged in *de novo* vessel formation—vasculogenesis. Together with VEGF receptor studies, the data reported here strongly suggest that this growth factor is a regulator of both angiogenesis and vasculogenesis. However, it is important to note that the responses rhVEGF$_{165}$ elicited during vasculogenesis differed markedly from the rhVEGF$_{165}$ response observed by others during angiogenesis. For example, when rhVEGF$_{165}$ was used to induce angiogenesis in functionally mature vessels of the day-13 chick chorioallantoic membrane (CAM), the response was characterized by formation of arterially derived vessel with a "brush-like appearance" (21). In contrast, the supernumerary vessels that formed in rhVEGF$_{165}$-microinjected embryos (Fig. 2*b*) did not exhibit this brush-like angiogenic morphology.

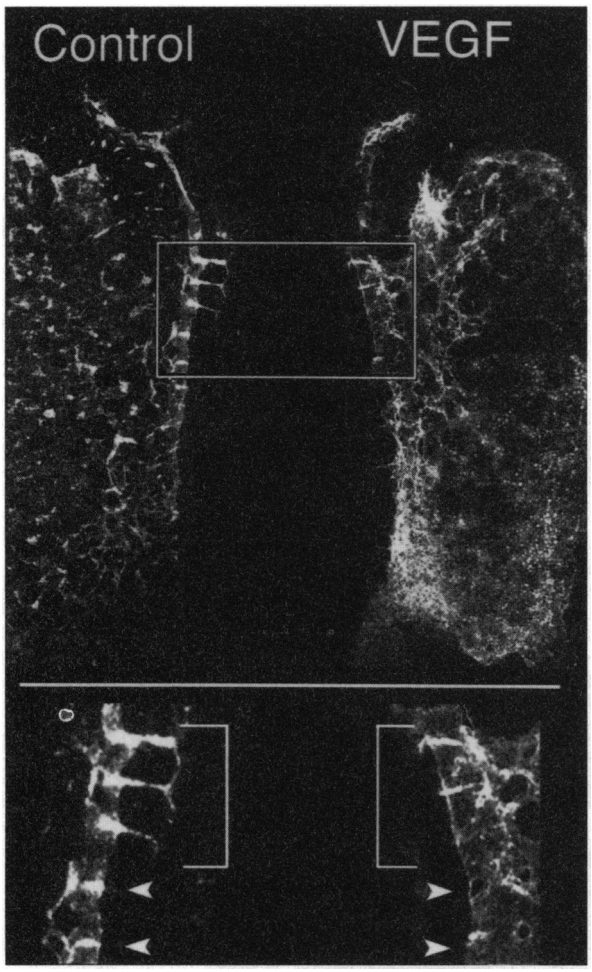

FIG. 4. Intersomitic and vertebral artery development was perturbed by VEGF injection. Five-somite embryos were injected, as before, then immediately cut along the midline (*Upper*). After 7 hr, clear alterations in the formation of these vessels were observed (boxed area). This region is depicted at higher magnification in *Lower*. The intersomitic arteries in the VEGF-injected halves were either missing (arrowheads) or stunted (brackets), whereas, in the uninjected halves, intersomitic and vertebral vessel development was normal (Control). Thus, alterations in vasculogenesis induced by injection of VEGF lead to malformed intersomitic vessels and aborted development of the vertebral arteries (the fine longitudinal branches of the intersomitic arteries). (*Upper*, ×30; *Lower*, ×60.)

VEGF Induces Alterations in Vessel Lumens and Vessel Patterns. The appearance of supernumerary vessels in VEGF-treated embryos demonstrated that proper vascular development is dependent not only on new vessel formation, but also on the strict maintenance of avascular regions. The importance of avascular regions was demonstrated when the normally avascular areas, such as those lying between the developing heart and the dorsal aortae (Fig. 1*b*), were invaded by vessels (Figs. 2*b* and 3*b*). This result raises the interesting possibility that inhibition of local VEGF activity is responsible for maintenance of avascular zones.

Perhaps the most far-reaching finding of this study was that injection of exogenous rhVEGF$_{165}$ was able to induce the formation of vessels with grossly enlarged lumens. Currently little is known about the mechanisms that regulate this fundamental aspect of vascular development. Extensive studies *in vitro* (22–24) and more recently *in vivo* (4, 16) have shown that cell–matrix interactions play a critical role in the initial establishment of the vascular lumen.

Understanding the fundamental mechanisms that regulate vessel size is exceedingly important, because size is directly related to the developmental fate of a given vessel. In this regard it is interesting to note that at nine somites, blood flow has not begun and vascular smooth muscle cells are not present (J. E. Hungerford and C.D.L., unpublished observations). This suggests that the initial regulation of vessel size is an intrinsic property of endothelial cells and nearby epigenic factors.

The size of a given vessel is determined in part by initial lumen formation and by vascular fusion. There is evidence that, in the avian embryo, small pleiomorphic vessels can undergo a localized and controlled fusion that leads to larger vessels (refs. 25 and 26 and unpublished observations). If embryonic vessels are actively engaged in fusion, as the studies above suggest, then relatively modest increases in fusion activity could account for the abnormally large "vessels" or sacs observed in VEGF-injected embryos. Significantly, the cavernous hemangiomas formed by yolk sac vessels in murine embryos expressing the polyoma middle-T oncogene (27) had a morphology similar to the vascular sacs we observed (Fig. 3*d*). It is also noteworthy that Wilting *et al.* (21) observed venous-derived sinusoidal or lacunar vessels in chorioallantoic membrane after stimulation with VEGF$_{165}$.

Although the focus of this study centered on vasculogenesis, we were also able to observe the effects of exogenous rhVEGF$_{165}$ on the formation of intersomitic vessels (1). Our data show that the vascular processes, necessary for the formation of the intersomitic arteries, do not progress normally in VEGF-injected embryos. In fact, the aortae of VEGF-injected embryos do not produce normal numbers of intersomitic arteries, and those that do form fail to branch properly (Fig. 4). This observation suggests that proper formation of the dorsal aortae is required for development of intersomitic vessels. We do not mean to imply, however, that VEGF is preventing angiogenesis, but rather that abnormal vasculogenesis has an impact on later vessel patterning. This result was

surprising. In fact, we had expected exogenous rhVEGF$_{165}$ to enhance the formation of vascular sprouts, structures that are the hallmark of angiogenesis.

How Does VEGF Mediate Its Effect? Since VEGF is a known endothelial mitogen it is possible this activity could account for vascular malformations observed. Considering all the data, however, it is highly unlikely that VEGF mediated its effects through mitogenic activity. The most compelling argument supporting this contention is that endothelial cells in injected embryos engaged in exceedingly abnormal cell movements and behavior. For instance, the colonization of avascular areas by these cells implies alterations in motility, spreading/elongation, extracellular matrix adhesion, and cell–cell contact. Moreover, the time interval from injection to altered morphology is short. VEGF-mediated effects were noticeable as early as 5 h after injection (not shown). Therefore, it is very questionable whether 5–7 h is sufficient time to accumulate inordinate numbers of extraneous angioblasts.

The results showed that VEGF induced a more robust effect in six-somite, compared with five-somite, embryos. Since we know very little about the early events in the angiogenic lineage, it is not possible to determine why the additional time made such a marked difference. However, we noted that injection at the four-somite stage (data not shown) yielded vessel patterns very similar to the five-somite injection shown in Fig. 2*b*. This observation suggests that some fundamental change occurs during the five- to six-somite time interval. It may be that at six somites there are simply more angioblasts, and/or the angioblasts that are present are more actively engaged in fusion and extension of processes. Thus, injection at this time yields a more vigorous response.

VEGF is also known as vascular permeability factor (28, 29). It is conceivable that some of the angioblastic responses we observed are due to the permeability-increasing properties of this cytokine. For instance, the angioblasts may engage in hyperfusion due to a secondary effect caused by an increase in primordial vessel permeability. However, since there is no circulation at these early stages it is difficult to test changes in vascular permeability.

VEGF and VEGF Receptors in the Early Embryo. The fact that exogenous VEGF stimulated the formation of supernumerary vessels suggests that, if endogenous VEGF is present, its activity must be tightly regulated. One method of possible regulation involves interaction with the extracellular matrix (ECM). Indeed, recent studies have demonstrated that heparin-like molecules can regulate the binding of VEGF$_{165}$ to its receptors (30, 31). In this regard, there is evidence that the ECM regulates VEGF by sequestering the factor in latent form, which can later be activated by the serine protease plasmin (32). Regardless of the mechanism, injection of exogenous VEGF into quail embryos must have circumvented normal regulatory control and thus led to excessive stimulation of endogenous VEGF receptors. Two members of the receptor tyrosine kinase family (Flt-1 and Flk-1) have been identified as high-affinity VEGF receptors (13, 33). Exogenous VEGF binding to these or similar receptor tyrosine kinases could influence multiple signaling pathways, leading to the abnormal behavior we observed.

Future experimental manipulation of vasculogenic cells may shed light on one of the most interesting questions regarding blood vessel formation: What prevents the early embryo from overproducing blood vessels and becoming, in effect, an angioma? Why, in areas with competent mesoderm and presumably all the necessary requirements, are there areas where nascent vessels do not form?

We dedicate this paper to the memory of our close friend and colleague Michael Solursh. Michael was a constant source of inspiration and good cheer. His enthusiasm for developmental biology will be sorely missed. We thank Dr. Brenda Rongish and Dr. Scott Argraves for helpful suggestions with the manuscript. This work was supported by National Institutes of Health Grants R01 HL37709, R01 HL45348, and P01 HL52813 to C.D.L.

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