Microvessel formation from mouse embryonic aortic explants is oxygen and VEGF dependent

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Received 26 November 2001; accepted in final form 11 April 2002

During embryogenesis, the early vascular plexus derives from mesoderm by differentiation of vascular endothelial cells that have not yet formed a lumen (angioblasts) into primitive blood vessels. This process is termed vasculogenesis. After the primary vascular plexus is formed, more endothelial cells are generated that can form capillaries by sprouting or by intussusception from their vessel of origin in a process designated angiogenesis (11, 21).

Concurrent sprouting and nonsprouting angiogenesis are involved in the vascularization of organs such as the lung, brain, and kidney. Sprouting angiogenesis from the embryonic aorta predominates in the developing kidney (19–21).

Vascular endothelial growth factor (VEGF), produced in vascular or extravascular tissues during blood vessel formation, serves as a key endothelial growth and chemotactic agent (4, 6, 10, 11). A decrease in O₂ concentration, i.e., hypoxia, activates VEGF gene and protein expression (5, 10, 11). In addition to VEGF, hypoxia induces the expression of agents that regulate VEGF gene and VEGF receptor expression, such as hypoxia-inducible factor (HIF)-1 (1, 4, 21, 26).

The finding of abnormal vascular development in the HIF-1α-deficient mouse is strong, albeit indirect, evidence of a key role for hypoxia in embryonic blood vessel formation (25). Supporting evidence is provided by the observations that HIF-1α and VEGF are spatially and temporally colocalized in regions within developing mouse embryos in which the hypoxia marker pimonidazole can also be detected (14).

One widely studied organ culture model of aortic-based angiogenesis employs adult mouse aortic rings from which microvessels develop in serum-free matrix culture under normoxic (17, 18), but not hypoxic, conditions (3). In contrast to what is known about the growth requirements for capillary formation from adult aortic rings, little is known about the growth of aortic rings from developing embryos (2).

To define more precisely in a model of embryonic aortic angiogenesis the roles that O₂ and VEGF might play in organ vascularization, we developed a model in which formation of capillary-like structures from mouse embryonic thoracic aorta explants can be demonstrated in a three-dimensional collagen gel matrix. Low O₂ and exogenous VEGF are required for angiogenesis in our explant system. Low O₂ enhances the expression of the VEGF receptor Flk-1.

We propose that hypoxia and VEGF play important roles in stimulating angiogenesis from the developing thoracic aorta and that Flk-1 upregulation is one mechanism by which hypoxia renders embryonic aortic tissue more responsive to VEGF.

MATERIALS AND METHODS

Preparation of three-dimensional collagen gels. Collagen gels were prepared according to the method described by Nicosia and Ottinetti (17) with some modification. Briefly, eight volumes of purified rat tail type I collagen (Sigma

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Chemical, St. Louis, MO) solubilized in 0.02 N acetic acid (2 mg/ml) were neutralized with one volume of premixed solution containing 200 mM HEPES, 200 mM NaHCO3, 0.05 N NaOH, and one volume of 10× MCDB-131 (Sigma Chemical). The mixture was kept on ice to preclude gelation before it was dispensed into 24-well tissue culture plates.

Embryonic aortic explant culture. Pregnant C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized and killed by cervical dislocation on embryonic day 14 unless otherwise specified, and the embryos were retrieved by cesarean section. Mouse embryonic dorsal aortas (thoracic level to vessels supplying the mesonephros and metanephros) were visualized using an American Optical dissecting microscope, surgically dissected from embryos, and washed twice in serum-free MCDB-131 supplemented with penicillin-streptomycin. Aortic explants (~500 μm long) were sectioned and washed twice in serum-free medium. Immediately after collagen solution (300 μl) was dispensed in 24-well culture plates, the aortic explants were embedded in the collagen solution, and the plate was incubated at 37°C for 15 min to promote gelation. Some of the dissected aortas were fixed directly with zinc fixative (Pharmingen, San Diego, CA), embedded in paraffin, sectioned, and analyzed for histology.

After gelation, MCDB-131 (300 μl) supplemented with 5% heat-inactivated fetal bovine serum (GIBCO BRL, Gaithersburg, MD) was applied, and the culture was maintained at 37°C in 5% CO2-95% room air or under low (5%) O2 conditions. For low O2 conditions, explants were kept in an air-tight chamber (Billups-Rothenberg, Del Mar, CA) that had been filled for 5 min at 20 l/min with 5% O2-5% CO2-90% N2. The chamber was regassed daily. Repeated measurement with a Fyrite O2 indicator (Bacharach, Pittsburgh, PA) confirmed 5% O2. The media were changed every other day. Therefore, for brief periods every 2 days, explants cultured under 5% O2 were exposed to room air.

The following additions were made to cultures as indicated: recombinant human VEGF (rhVEGF), anti-human VEGF-neutralizing antibody (α-hVEGF Ab), and soluble Flt-1 (sFlt-1) fusion protein (R & D Systems, Minneapolis, MN). Each culturing condition included at least four cultures. Experiments were repeated at least three times.

Antibodies and histological staining reagents. Rat anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31), monoclonal antibody Mec13.3 biotin-conjugated anti-mouse antibody (1:1000; Santa Cruz Biotechnology) at 4°C, monoclonal antibody 5C7-FK1 (Flk-1) and Flt-1 from Santa Cruz Biotechnology (Santa Cruz, CA); and Alexa Fluor 488-conjugated antibody were purchased from Pharmingen; anti-mouse anti-rat antibody, and FITC-conjugated anti-rat antibody were purchased from ImmunoChem, St. Louis, MO) solubilized in 0.02 N acetic acid (2 mg/ml) were neutralized with one volume of premixed solution containing 200 mM HEPES, 200 mM NaHCO3, 0.05 N NaOH, and one volume of 10× MCDB-131 (Sigma Chemical). The mixture was kept on ice to preclude gelation before it was dispensed into 24-well tissue culture plates.

Western blotting analysis. Western blotting analysis was performed as described previously (23) with modifications. Briefly, aortic explants were removed from gels after 18 h of culture, rinsed with ice-cold phosphate-buffered saline (PBS), and resuspended in 80 μl of lysis buffer (1% Nonidet P-40, 20 mM Tris·HCl, pH 7.5, 10% glycerol, 1 mM leupeptin, 1 mM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate). After incubation for 30 min at 4°C, insoluble materials were removed by centrifugation of cell extracts at 16,000 g for 20 min. Cell lysates normalized by protein content were placed in 5× Laemmli sample buffer, heated at 95°C for 10 min, separated by SDS-10% polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Identical protein loading was confirmed by staining membranes using Ponceau S dye (Sigma Chemical). The membranes were incubated for blocking by a 60-min treatment at room temperature in 10 mM Tris·HCl, pH 7.4, 100 mM NaCl, and 0.1% Tween 20 with nonfat dry milk and then immunoblotted with mouse anti-Flk-1 antibody (1:1,000) or rabbit anti-Flt-1 antibody (1:1,000; Santa Cruz Biotechnology) at 4°C. The membranes were further probed with secondary antibody, and the enhanced chemiluminescence immunoblotting detection system (Amersham Pharmacia Biotech) was used according to the manufacturer’s instructions. Immunoblots were quantified using NIH Image 1.56 software. The ratio of protein expression was expressed as relative density divided by control values.
Morphological and quantitative analysis. Aortic explants, along with the collagen gels, were rinsed with ice-cold PBS twice and fixed with Bouin’s solution (Sigma Chemical) for 30 min at room temperature. Samples were washed twice with PBS and treated with 1% Triton X-100 in PBS for 60 min and then incubated with 3% bovine serum albumin in PBS overnight at 4°C. After they were washed with PBS three times, samples were incubated with Gs-IB4 (20 μg/ml) or indicated antibody (primary and secondary antibody) according to the manufacturer’s instructions. Gels were then sandwiched between glass slides and coverslips using GVA mounting medium (Zymed, South San Francisco, CA) and subjected to fluorescence microscopy analysis. Some samples were subjected to immunohistochemical analysis using an avidin-biotin immunoperoxidase technique (24).

Outgrowth of capillary structures was quantified by calculating the number of newly formed capillary sprouts within the cultures on a daily basis according to the method described by Nicosia and Ottinetti (17) with some modification as delineated below. Cultures were examined under bright-field microscopy using a Zeiss phase-contrast inverted microscope. The criteria used for analysis were as follows: 1) Capillary sprouts were distinguished from fibroblastic mesenchymal cells by their unique morphology (greater thickness and uniformly cohesive growth pattern). 2) The dichotomous branching of one sprout generated two new sprouts. 3) Because anastomosis between two converging vessels usually forms loop structures, each loop was counted as two sprouts (17). We confirmed that these structures were positive for Gs-IB4 staining and an endothelium-specific marker molecule PECAM-1 (7) and that there was no statistical difference between the number of capillary outgrowths determined using bright-field and fluorescence imaging.

Fluorescent microscope images were captured using a Nikon Eclipse 800 microscope with a Spot 2 cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI) using Spot software version 2.1. Images were imported into Adobe PhotoShop 5.0 for final processing and layout.

Electron microscopy. Electron microscopy was carried out as described previously (22). Briefly, tissues were fixed in situ with 3% (wt/vol) glutaraldehyde buffer and then postfixed in OsO4. Samples were dehydrated in ethanol and embedded in Polybed 812 resin. Thin sections were stained with lead acetate and lead citrate and examined through a transmission electron microscope.

Statistics. Values are means ± SE. Data were analyzed by an analysis of variance combined with Fisher’s protected least significant difference. Differences with $P < 0.05$ were considered to be significant.

RESULTS

Identification of endothelial cells within embryonic aorta. Figure 1A is a bright-field phase-contrast photomicrograph of a mouse aorta freshly dissected on embryonic day 14 shown in a longitudinal view. Endothelial cells are present at the intimal surfaces of the
aortic explants, as demonstrated by positive staining for each of the two endothelial markers as shown in cross sections: Gs-IB4 in a non-paraffin-embedded section shown amidst generalized nuclear PI staining (Fig. 1B) and PECAM-1 in a paraffin-embedded section (Fig. 1C).

Effect of hypoxia on embryonic aortic explants. Figure 2A is a phase-contrast photomicrograph of an embryonic day 14 mouse aortic explant immediately after it was embedded in a collagen gel shown in longitudinal view. If cultured under 5% O2 for 8 days, the appearance of the explant changes in a way that suggests migration of cells into the gel (Fig. 2B). To determine whether this is the case, explants cultured for 2 (Fig. 2, C and F), 4 (Fig. 2, D and G), or 8 days (Fig. 2, E and H) were stained for Gs-IB4 without PI (Fig. 2, C–E) or concurrently with PI (Fig. 2, F–H). A few Gs-IB4-positive structures are detected around the aortic explants after 2 days (Fig. 2C). As delineated by arrows, these structures stain also with PI (green + red = yellow), consistent with a cellular identity (Fig. 2F). The number of cells and the degree of migration increase after 6 days (Fig. 2, D and G) and increase further after 8 days (Fig. 2, G and H).

When explants are cultured for 8 days under room air (Fig. 3), a less robust outgrowth of cells is observed (cf. Fig. 2, B, E, and H with Fig. 3).

Effect of exogenous VEGF on embryonic aortic explants. To ascertain whether the addition of a known mediator of angiogenesis to explants affects their growth in vitro, aortas were cultured under 5% O2 in the presence of rhVEGF. A phase-contrast photomicrograph of an aortic explant grown for 8 days under 5% O2 in the presence of rhVEGF (50 ng/ml) is shown in Fig. 4A. Structures with a capillary-like morphology (Fig. 4A) that stain positively for PECAM-1 (Fig. 4B) and Flk-1 (Fig. 4C) can be delineated.

Gs-IB4 and PI staining of explants grown under 5% O2 in the presence of rhVEGF (50 ng/ml) revealed that the capillary-like structures stain for Gs-IB4 (Fig. 4C) and PI (Fig. 4D) by 4 days of culture and that their formation is increased on day 6 (Fig. 4, E and F) and increased further on day 8 (Fig. 4, G and H).

Capillary-like structure (microvessel) growth is quantified in Fig. 5. After 8 days of culture, some microvessels are detected when explants are grown without VEGF. However, the number is only ~10% of that in the presence of 10 ng/ml rhVEGF. rhVEGF (10–50 ng/ml) stimulates capillary formation in a concentration-dependent manner.

Electron microscopy of a capillary-like structure demonstrates cells characteristic of mouse vascular endothelium (15) surrounding an open lumen (Fig. 6). Cells are connected to adjacent cells by nondesmosomal junctions. Nuclei of these cells are slightly invaginated. The cytoplasm contains numerous organelles, including mitochondria, bundles of thin filaments, membrane-bound granules, dense bodies, and plentiful pinocytotic vesicles of variable size. The luminal surface of the cells is ruffled with slender cell processes. Pinched-off fragments of plasma membrane-bound cytoplasm contain multivesicular organelles.

In contrast to the case for explants grown under 5% O2, addition of rhVEGF (50 ng/ml) to explants grown under room air does not result in formation of capillary-like structures (Fig. 7).

Effect of VEGF sequestering on capillary formation from aortic explants. Addition of sFlt-1 to endothelial cells is thought to sequester available VEGF and, thereby, obviate its effect (8). Anti-VEGF-neutralizing antibody has a similar action. To ascertain whether
sequestration of VEGF affects the growth of capillary-like structures in explants grown under 5% O$_2$, aortas were cultured for 8 days in the absence or presence of various concentrations of sFlt-1 or α-hVEGF Ab with or without rhVEGF (50 ng/ml).

In the absence of exogenous VEGF, addition of sFlt-1 (400 ng/ml) has no effect on the pattern of Gs-IB$_4$ or PI staining of explants (cf. Fig. 8, A and B, with Fig. 2, E and H). In contrast, in the presence of rhVEGF, addition of sFlt-1 reduces in a concentration-dependent manner the number of capillary-like structures.

The effects of sFlt-1 on microvessel growth illustrated in Fig. 8 are quantitated in Fig. 9A. In the absence of VEGF, the number of microvessels is very small (control) and is not affected by sFlt-1 (Fig. 9A, left). In the presence of VEGF (50 ng/ml), the number of microvessels is decreased progressively by increasing concentrations of sFlt-1 (Fig. 9A, right).

Similarly, α-hVEGF Ab has no effect on the low level of microvessels observed in the absence of exogenous VEGF (control; Fig. 9B, left).

Fig. 4. A: photomicrograph of aortic explant after 8 days of growth under 5% O$_2$ in the presence of recombinant human vascular endothelial growth factor (rhVEGF, 50 ng/ml). Arrows delineate capillary-like structures. B and C: immunohistochemistry of capillary-like structures arising from aortic explants after 8 days of growth. B: PECAM-1-positive cells organized into capillary-like structures (arrow). C: Flk-1-positive cells organized into capillary-like structures. D–H: cells were visualized after 2 (C and D), 4 (E and F), or 8 (G and H) days of culture by Gs-IB$_4$ staining. Some samples were stained also with PI (D, F, and H). Arrows delineate capillary-like structures. Scale bar is shown in A.

Fig. 5. Number of microvessels in explant cultures grown under 5% O$_2$ in the presence of rhVEGF. rhVEGF stimulated capillary formation in a dose-dependent manner. **P < 0.01 vs. 0 ng/ml VEGF (day 8). ††P < 0.01 vs. day 0 of the same group. Values are means ± SE of 4 independent experiments.
DISCUSSION

The process of blood vessel formation during development is VEGF dependent. VEGF is thought to exert actions on endothelial cells via binding to two main endothelial receptors: VEGF receptor-1 [also known as fms-like tyrosine kinase (Flt-1)] and VEGF receptor-2 [kinase insert domain-containing receptor/fetal liver kinase (Flk-1)] (1, 9). Homozygous deletions of Flt-1 or Flk-1 impair vascular development in embryos.

Hypoxia is known to be a potent stimulator of endogenous VEGF production (14, 21). Hypoxia-induced VEGF production can provide a major stimulus for the angiogenesis that accompanies organ formation. For

As is the case for sFlt-1, addition of rhVEGF-neutralizing antibody to explants grown in the presence of VEGF reduces the number of microvessels to the control levels seen in the absence of rhVEGF (Fig. 9B, right).

Effect of hypoxia on VEGF receptor expression in aortic explants. To determine whether the action of VEGF to enhance formation of microvessels in cultures of embryonic aorta might be mediated via up-regulation of one or more VEGF receptors, we performed Western analysis of Flk-1 and Flt-1 protein in homogenates of freshly isolated explants (control) and explants cultured for 18 h under room air (normoxia) or 5% O2 (hypoxia). As illustrated in Fig. 10, levels of Flk-1, but not Flt-1, are upregulated compared with baseline when explants are grown under low O2 conditions.
example, in the developing retina, astrocytes that migrate together with neurons away from existing blood vessels secrete VEGF in response to the resulting hypoxia. Angiogenesis is stimulated toward the VEGF-producing astrocytes and the neurons that have accompanied them (16).

Hypoxia can also serve as a stimulus to upregulate Flt-1 and Flk-1, independent of its actions to enhance VEGF expression (16). In the case of Flk-1, the process of hypoxia-induced upregulation was demonstrated in human umbilical vein endothelial cells and in transfected porcine aortic endothelial cells. Flk-1 upregulation was reversible, maximal after 24 h of hypoxia, and regulated at a posttranscriptional level (27).

In the present studies, we use embryonic aortic explants cultured in a three-dimensional type I collagen gel matrix to show that formation of capillary-like structures is dependent on 5% O2 and addition of exogenous VEGF. Flk-1 receptors in explants grown under 5% O2 are upregulated relative to Flk-1 receptors in explants grown under room air. This observation is consistent with the basis for enhanced sensitivity to VEGF in explants grown under 5% O2 being an increase in VEGF binding to Flk-1.

In our aortic explant system, the number of microvessels formed from aortic explants is low in the absence of added VEGF, even under 5% O2. This is shown by direct visual comparison of Fig. 2A (no added VEGF) with Fig. 4A (VEGF added). In the absence of VEGF, the low microvessel number is not affected by sFlt-1 or α-hVEGF Ab (Figs. 8 and 9). Our interpretation of these data is that whatever VEGF may be produced in mouse embryonic aortic cultures or may be present in serum supplementing culture media is insufficient to act as an effector of the angiogenic response in vitro.

It is possible that in vivo, as is the case in developing retina (16), the angiogenic stimulus for the embryonic aorta, at least the thoracic section we used for experiments, is provided by VEGF produced by nonvascular tissues outside the aorta itself. One possible source is the developing kidney (12) (see Perspectives).

Alternatively, the microvessels produced under 5% O2 could reflect a component VEGF-independent microvessel formation or an inability of our methodology to detect a reduction by sFlt-1 or α-hVEGF Ab in the setting of low levels of VEGF-stimulated microvessel formation.
Perspectives

To our knowledge, ours is the first description of a model of angiogenesis from embryonic mouse aorta. Because we utilized thoracic aortas obtained at the level from which lateral vessels supplying the mesonephros and metanephros arise, it is possible that our model will prove useful in delineating the mechanism by which vascularization occurs in a developing organ for which angiogenesis from this part of the aorta plays a major role (the kidney).

Fig. 9. Effect of VEGF sequestering on number of microvessels formed in aortic explants cultured for 8 days under 5% O₂ in the presence or absence of rhVEGF (50 ng/ml). A: effect of sFlt-1. ††P < 0.01 vs. control. **P < 0.01 vs. 0 ng/ml sFlt-1 and 50 ng/ml VEGF. B: effect of VEGF-neutralizing antibody (α-hVEGF Ab). ††P < 0.01 vs. control. **P < 0.01 vs. 50 ng/ml VEGF. Values are means ± SE of 4 independent experiments.

Fig. 10. Western analysis of Flk-1 and Flt-1 in freshly isolated mouse embryonic aortas (control) and 18 h after culture under room air (normoxia) or 5% O₂ (hypoxia). Values are means ± SE of 4 experiments. **P < 0.05 vs. control.
REFERENCES


