VEGF Spatially Directs Angiogenesis during Metanephric Development *in Vitro*

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Vascular endothelial growth factor (VEGF) is required for endothelial cell differentiation, vasculogenesis, and normal glomerular vascularization. To examine whether VEGF plays a role as a chemoattractant for the developing kidney vasculature, avascular metanephric kidneys from rat embryos (E14) were cocultured with endothelial cells. To determine whether VEGF directly provides chemoattractive guidance for migration, we examined migration of endothelial cells toward VEGF-coated beads. Mouse glomerular endothelial cells expressing β-galactosidase (MGEC) were isolated from Flk-1(+/−) heterozygous mice and passaged 4–12 times. Upon 24 h culture on collagen I gels MGEC formed a lattice or capillary-like network. Embryonic metanephroi were cocultured with MGEC on collagen I gels for 1–6 days in defined media, stained for β-galactosidase, and examined by light microscopy. Metanephric organs induced a rearrangement of the endothelial cell lattice and attracted MGEC. MGEC invaded the metanephric organs forming capillary-like structures within and surrounding the forming nephrons. This process was accelerated and amplified by low oxygen (3% O_2) and was prevented by anti-VEGF neutralizing antibodies. MGECs migrated toward VEGF-coated beads, whereas PBS-coated beads did not alter MGEC networks. We conclude that VEGF produced by the differentiating nephrons acts as a chemoattractant providing spatial direction to developing capillaries toward forming nephrons during metanephric development *in vitro*.   © 2000 Academic Press

Key Words: VEGF; angiogenesis; directional migration; chemoattraction; metanephros; organogenesis; vascular development.

INTRODUCTION

The mechanisms guiding the complex organization of the renal vasculature are unknown and the origin of renal endothelial cells remains uncertain. Metanephric endothelial cells may differentiate from angioblasts by vasculogenesis or may sprout from extrarenal mesenchymal vessels by angiogenesis (Potter, 1965; Ekblom *et al.,* 1982; Sariola, 1991). The angiogenic origin is supported by data from interspecies transplantation experiments (Sariola, 1991; Sariola *et al.,* 1983). Several lines of evidence support a vasculogenic origin. First, we and others have identified angioblasts in the avascular metanephric mesenchyme (Robert *et al.,* 1996; Tufro-McReddie *et al.,* 1997; Loughna *et al.,* 1998). Second, we showed that when exposed to increased levels of endogenous or exogenous VEGF, avascular embryonic kidneys develop capillaries (Tufro-McReddie *et al.,* 1997; Tufro *et al.,* 1999). Finally, recent transplantation experiments indicate that embryonic kidneys' endo-

thelial cells can derive from the blastema or from extrarenal sources depending on the environment (Robert *et al.,* 1998; Abrahamson *et al.,* 1998; Sequeira Lopez *et al.,* 1999). Taken together, the available information suggests that renal vascularization occurs by both vasculogenic and angiogenic mechanisms.

Vascular endothelial growth factor (VEGF) and its receptors Flk-1 and Flt-1 are major regulators of vascular morphogenesis. The VEGF system is responsible for endothelial cell differentiation, migration, proliferation, tube formation, and vessel assembly (Fong *et al.,* 1995; Shalaby *et al.,* 1995). Moreover, threshold levels of VEGF are required for maintaining the differentiation, correct assembly, and maturation of endothelial cells during development, as indicated by lethal vascular abnormalities resulting from partial ablation of VEGF expression in VEGF(+/−) and inducible VEGF(−/−) mice (Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996; Nagy and Rossant, 1996; Gerber *et al.,* 1999). In embryonic human, mouse, and rat kidneys VEGF localizes

to S-shaped bodies and later on to glomerular visceral and parietal epithelial cells and tubular cells (Breier *et al.,* 1992; Brown *et al.,* 1992; Simon *et al.,* 1995; Tufro *et al.,* 1999). Blockade of VEGF with a neutralizing antibody and partial ablation of VEGF expression in newborn mice resulted in edema formation and disturbed vessel and glomerular capillary tuft development, suggesting that VEGF plays an active role in postnatal renal vascularization (Kitamoto *et al.,* 1997; Gerber *et al.,* 1999). We hypothesized that developing podocytes and renal tubular epithelial cells expressing VEGF provide chemoattractive guidance for angioblasts and endothelial cells to migrate toward the appropriate sites within the developing nephron, i.e., vascular cleft and surrounding tubules. The present studies examined whether exogenous endothelial cells can contribute to metanephric vascularization *in vitro* and whether VEGF mediates endothelial cell migration in this model. These experiments showed that VEGF produced by metanephric embryonic kidneys in culture is a chemoattractant for exogenous endothelial cells that spatially directs their migration toward developing nephrons.

METHODS

Endothelial cell culture on collagen I gels. Glomerular endothelial cells were isolated from Flk-1(+/−) mice that harbor the LacZ coding region following the Flk-1 promoter and thus express the LacZ gene in endothelial cells and their precursors. Glomeruli were isolated by sieving in Hanks' solution as described (Margolin *et al.,* 1995), with few modifications (Ballermann, 1989; Uchida and Ballermann, 1992), and were plated in fibronectin-coated dishes. Endothelial cells outgrown from glomeruli were cloned and were used between passages 4 and 12, during which time consistent β-galactosidase expression was confirmed. Confluent Flk-1(+/−) glomerular endothelial cells expressing β-Gal were trypsinized and counted and 1.5×10^5 cells were plated on collagen I gels (2 mg/ml). Endothelial cells plated on gels were cultured at 37°C in a 5% CO_2/air mix (Control) for 24 h in MCDB 131 medium with heparin-Na 50 μg/ml, endomitogen 150 μg/ml, bFGF 1 ng/ml, pen/strep 50 U/ml, and 15% FBS.

Endothelial cell–metanephric organ coculture. Flk-1(+/−) glomerular endothelial cells expressing β-Gal (MGEC) were cocultured with embryonic kidney explants from E14 rats for 1, 2, 3, or 6 days. Since Sprague–Dawley rat embryonic kidneys do not express β-galactosidase, exogenous endothelial cells were detected upon β-Gal staining. For staging of the embryos, animals were bred 6 h, and the following day was considered day 1 of gestation. Intact metanephroi were microdissected from rat embryos at E14. The metanephric organ culture was performed as described (Tufro-McReddie *et al.,* 1997). Metanephroi were placed on top of the collagen gels with the endothelial cell network in organ culture inserts within six-well culture dishes, partially immersed in defined, serum-free medium (DMEM-F12) at 37°C in a 95% humidity incubator. Medium was supplemented with Hepes 10 mM, insulin 5 mg/ml, transferrin 5 mg/ml, selenium 2.8 nM, PGE_1 25 ng/ml, T3 32 pg/ml, penicillin 50 U/ml, mycostatin 50 U/ml and changed daily. Explant medium was used for the cocultures, since preliminary studies showed that endothelial cells preserve their morphology and growth pattern in it during the study period. Cocultures were grown at 37°C in a 5% CO_2/air mix (Control) or in low oxygen (3% O_2). Low-oxygen explants were kept in air-tight chambers (Billups-Rothenberg, Del Mar, CA) filled for 4 min at 1.2 psi with the gas mix 3% O_2/5% CO_2/92% N_2 for 1, 2, 3, or 6 days except for the few minutes required for daily medium changes. Five coculture experiments were performed using four to six paired explants under each condition and at each time point.

In additional experiments ($n = 3$), polyclonal anti-VEGF neutralizing antibodies (Oncogene, Cambridge, MA) VEGF-ab1 (5 mg/ml) and VEGF-ab2 (2.5 mg/ml) were added to control ($n = 11$ explants) and low-oxygen-treated ($n = 12$ explants) cocultures' medium daily. The specificity of these antibodies' effect on metanephric kidneys in culture has been previously confirmed (Tufro-McReddie *et al.,* 1997). Medium + rabbit serum was used as control for the antibodies' effect ($n = 4$ explants). At the end of the study periods collagen gels with the cocultures were harvested, fixed, and stained for β-Gal as described below, counterstained with nuclear fast red and examined by light microscopy, or immunostained and examined by confocal microscopy as whole-mount preparations.

β-Gal staining. Flk-1(+/−) glomerular endothelial cell–explant cocultures were washed in PBS and fixed in 0.2% glutaraldehyde for 30 min. Then, they were washed and stained with 0.5% X-Gal/DMSF in phosphate/ferrocyanide buffer for 2 h, washed, and postfixed in 10% buffered formalin for 12 h at 4°C (Shalaby *et al.,* 1995).

Immunocytochemistry. Cocultures were postfixed, washed, permeabilized with 0.5% Triton X, blocked with 1% rat serum, and incubated with anti-mouse CD31 (1:100) (Mec13.3 clone; Pharmingen, San Diego, CA) antibody for 60 min (Vecchi *et al.,* 1994). Secondary antibody was a biotinylated, fluorescein-labeled anti-rat IgG (Vector Laboratories, Burlingame, AL). Negative controls were absence of primary or secondary antibody and replacement of primary antibody by preimmune rat serum. Fluorescent immunolabeling was detected by confocal microscopy (Leitz, Glenwood, NJ).

FIG. 1. Flk-1(+/−) glomerular endothelial cells form a capillary-like network and express β-galactosidase when grown for 24 h on collagen I gels. (A) Inverted microscope view; (B) β-Gal staining (blue) (original magnification ×100).

FIG. 2. Embryonic kidneys induce a rearrangement of the endothelial cell lattice. Coculture of embryonic kidneys and Flk-1(+/−) glomerular endothelial cells on collagen I gels for 6 h seen under inverted microscope. (A) Control; (B) low oxygen. Note endothelial cells beginning to align toward the explants. (original magnification ×40).

FIG. 3. The endothelial cell network completely rearranged and endothelial cells aligned toward the explants. Coculture of paired embryonic kidneys and Flk-1(+/−) glomerular endothelial cells for 48 h. (A) Control; (B) low oxygen showing a larger explant, numerous cells forming an interface around it, and aligned MGEC (original magnification ×20).
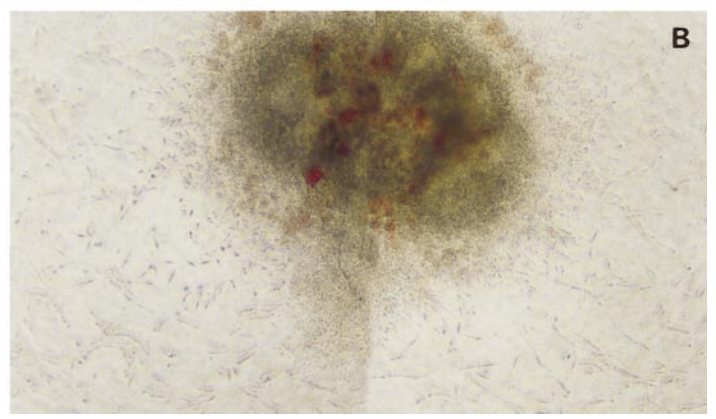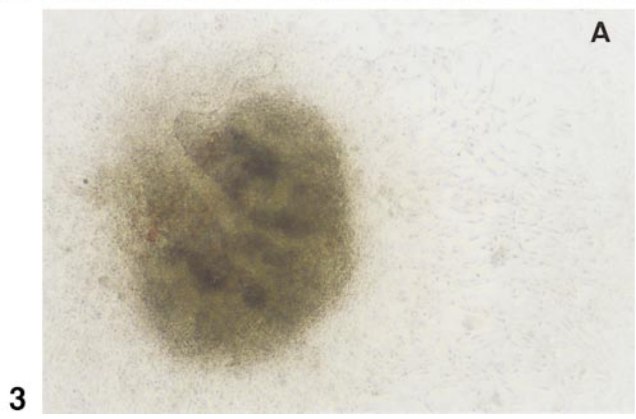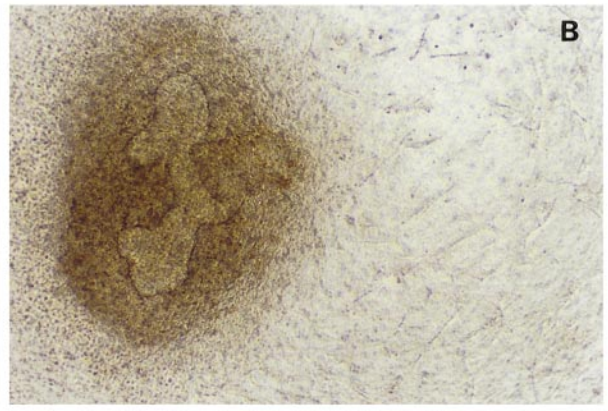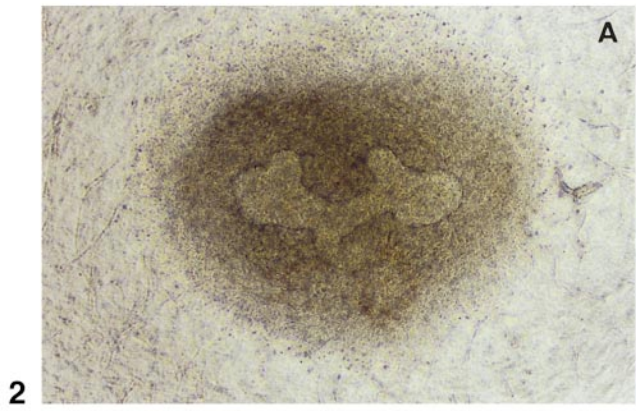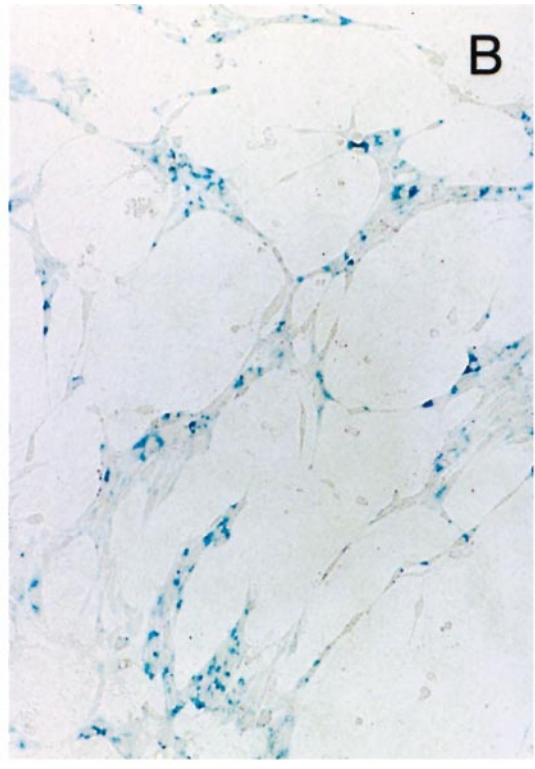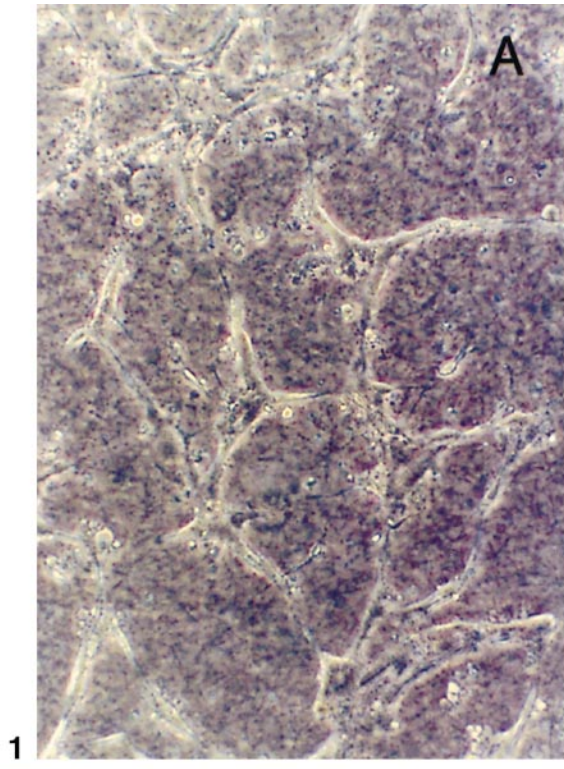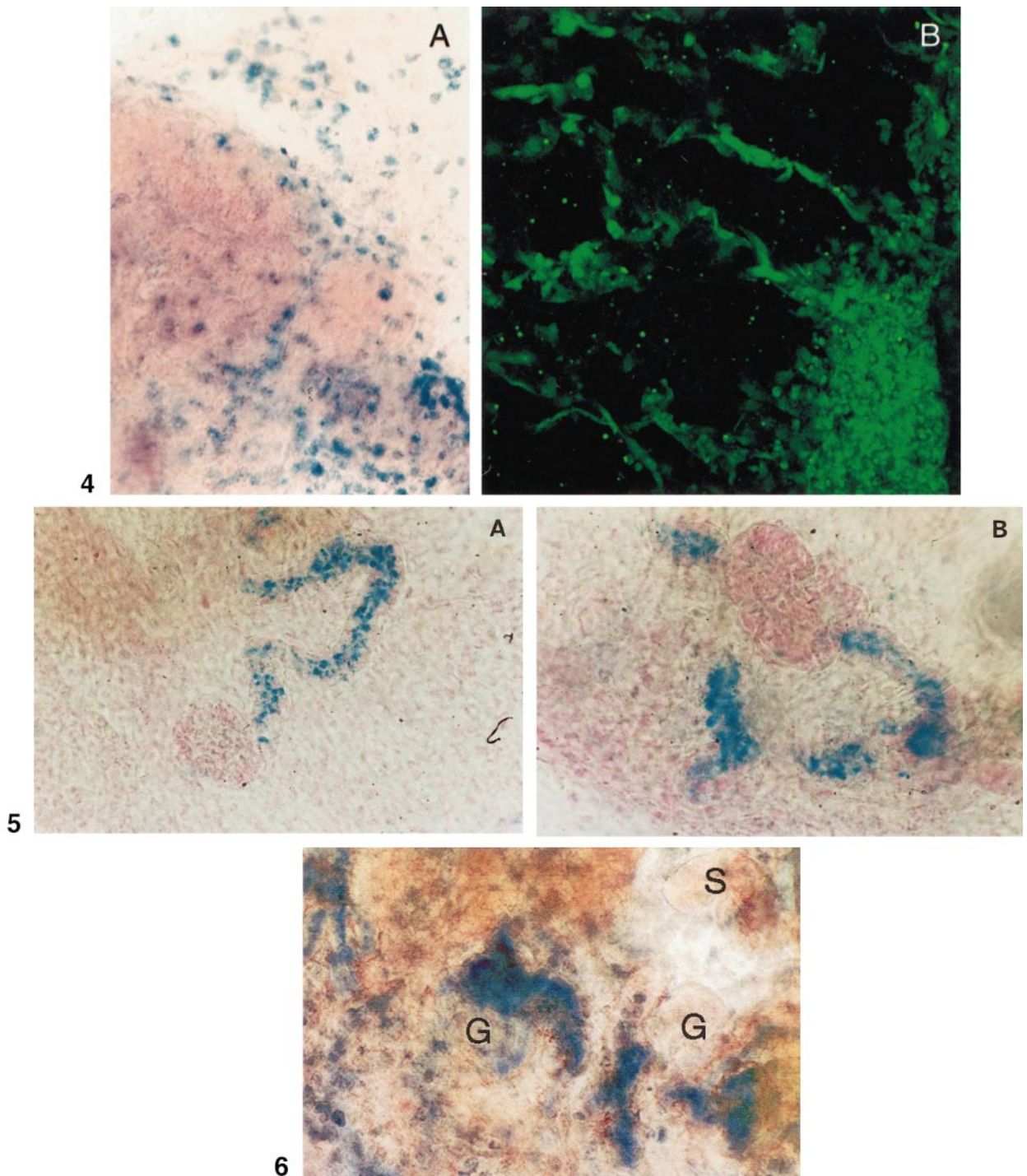
FIG. 4. MGEC migrate into the embryonic kidneys *in vitro.* Coculture of embryonic and Flk-1(+/−) glomerular endothelial cells for 72 h under control conditions. (A) β-Gal staining showing endothelial cells (blue) invading the explant. (B) CD31 immunostaining showing endothelial cells (green) invading the explant.

FIG. 5. Migrating MGEC form capillaries that reach the developing glomeruli. Coculture of embryonic kidneys and Flk-1(+/−) glomerular endothelial cells for 6 days under control conditions. Whole-mount β-Gal staining showing endothelial cells (blue) that migrated toward developing glomeruli and formed capillary-like structures. (A) Developing glomerulus with afferent-appearing capillary; (B) glomerulus with afferent- and efferent-appearing capillaries. Note the paucity of β-Gal+ cells inside the glomeruli.

FIG. 6. Migrating MGEC form numerous capillaries in and around developing nephrons under low-oxygen conditions. Coculture of embryonic and Flk-1(+/−) glomerular endothelial cells for 6 days in low oxygen. Whole-mount β-Gal staining showing innumerable β-Gal+ endothelial cells forming capillaries in and around forming nephrons. G, glomerulus; S, S-shaped body.

VEGF-coated beads. Affi-Gel blue agarose beads (Bio-Rad) were soaked in rhVEGF (1 µg/ml) + 0.1% BSA for 1 h at 37°C, washed in PBS three times, and then placed on top of MGEC networks (1.5 × 10⁵ cells plated on collagen I gels 24 h before) (Vaahtokari *et al.,* 1996). Control beads were soaked in PBS + 0.1% BSA for 1 h at 37°C and washed in PBS three times. MGEC networks with VEGF-coated or PBS-coated beads were also exposed to polyclonal anti-VEGF neutralizing antibodies (Oncogene, Cambridge, MA) VEGF-ab1 (5 µg/ml) and VEGF-ab2 (2.5 µg/ml). Migration of MGEC toward the beads was examined 6, 12, 24, and 48 h afterward by inverted microscopy. Two collagen I gels with four to six beads each were analyzed under each experimental condition. The experiment was repeated three times.

Morphometric analysis and statistics. Cocultures were examined using a videomicroscope system (Olympus AH-2, Sony CCD-Iris, San Jose, CA). To assess the extension of the MGEC rearrangement after 48 h of coculture, we measured the distance between the periphery of the explant and the farthest MGEC aligned toward it in 11 explants. To quantitate MGEC migration into the explants we examined a total of 37 explants from three separate experiments. We counted the number of β-Gal⁺ cells per standard explant area (0.0625 mm²) at high magnification using a grid. β-Gal⁺ cells from eight standard areas per explant were counted in 6–17 explants under each experimental condition. An average β-Gal⁺ cell number was calculated for each explant, then data were pooled for each experimental condition. In addition, the area of the explants containing β-Gal⁺ cells was measured and expressed as a percentage of the total explant area. Data are expressed as means ± SEM. Results from control, low-oxygen, and low-oxygen + neutralizing antibodies were compared using one-way ANOVA. Statistical significance was defined $P < 0.05$.

RESULTS

To examine whether exogenous endothelial cells can contribute to metanephric vascularization *in vitro,* Flk-1(+/−) MGEC expressing β-galactosidase were isolated and cocultured with avascular rat embryonic kidneys that do not express the LacZ gene. MGEC plated on collagen I gels for 24 h formed capillary-like structures in random directions that resembled a primitive vascular plexus, readily detected by direct visualization and β-Gal staining (Figs. 1A and 1B). Coculture of avascular rat metanephric organs with MGEC induced a spatial rearrangement of the endothelial cell lattice, such that the endothelial cells were directed toward the explants. This process started at 6 h of coculture (Figs. 2A and 2B) and became clearly evident by 48 h of coculture, suggesting that endothelial cells were migrating toward the explants (Figs. 3A and 3B). At this time an interface developed between the explants and the endothelial cell network (Fig. 3B) and the spatial reorganization of the endothelial cell network extended 1373 ± 44.6 µm ($n = 11$) radially surrounding each explant. At 72 h of coculture β-galactosidase staining and immunofluorescent staining with anti-CD31 antibody showed that aligned mouse endothelial cells migrated into the explants and that the cell interface consisted mostly of endothelial cells (Figs.

4A and 4B). Rat endothelial cells do not express β-Gal and are not recognized by monoclonal anti-CD31 antibodies (Vecchi *et al.,* 1994), indicating that β-Gal-positive cells were not endogenous to the explants or growing out from them. Taken together, these data strongly suggested that metanephroi provided a chemoattractive signal to the mouse endothelial cells.

After 6 days of coculture migrating MGEC were located in specific sites within the explants, forming capillary-like structures that reached the developing glomeruli resembling afferent and efferent vessels (Figs. 5A and 5B), suggesting that guidance for the migrating endothelial cells toward the forming nephrons was provided. Surprisingly, few intraglomerular β-Gal⁺ endothelial cells were detected.

Since low oxygen upregulates VEGF in this model (Tufro-McReddie *et al.,* 1997), cocultures were subjected to low oxygen to examine whether VEGF was involved in the directed endothelial cell migration. Low-oxygen exposure accelerated and accentuated the spatial reorganization and migration of the endothelial cells into the explants (Figs. 2B and 3B). After 6 days of coculture numerous β-Gal-expressing endothelial cells were identified within and surrounding the developing nephrons (Fig. 6), strongly suggesting that VEGF produced by the explants is involved in MGEC directed migration.

To determine whether VEGF mediates MGEC directional migration toward the explants, cocultures under control and low-oxygen conditions were exposed to anti-VEGF neutralizing antibodies or rabbit serum. Anti-VEGF neutralizing antibodies minimized migration of β-Gal⁺ endothelial cells into the explants (Fig. 7).

Quantitation of the changes induced by low oxygen and VEGF neutralizing antibodies is summarized in Fig. 8. The number of β-Gal⁺ endothelial cells in the explants was at least four times higher under low-oxygen than under control conditions (28 + 1 vs 6 + 0.1 β-Gal⁺ cells/std area). The explant area containing β-Gal⁺ endothelial cells was two to three times larger under low-oxygen than under control conditions (54 + 3% vs 20 + 1%). Anti-VEGF neutralizing antibodies decreased the number of β-Gal⁺ endothelial cells in the explants to half of control and 1/10 of low-oxygen conditions (2.6 + 0.3 vs 6 + 0.1 vs 28 + 1 β-Gal⁺ cells/std area). The percentage explant area containing β-Gal⁺ cells decreased similarly. These data indicate that VEGF mediates directional endothelial cell migration into the metanephric kidney *in vitro.*

To examine whether VEGF directly provides chemoattractive guidance for MGEC migration, we assessed MGEC migration toward VEGF-coated beads, a source of VEGF fixed to an inert surface. As shown in Fig. 9 MGEC migrated toward the VEGF-coated beads (Fig. 9B) but did not migrate toward the PBS-coated beads (Fig. 9A). Anti-VEGF neutralizing antibody (Fig. 9C) inhibited MGEC migration toward VEGF-coated beads. This experiment demonstrates that

VEGF provides a direct chemoattractive cue to MGEC networks.

DISCUSSION

The present studies report that metanephric embryonic kidneys in culture chemoattract exogenous endothelial cells and spatially direct their migration toward developing nephrons. Hypoxia accelerated and amplified endothelial cell migration into the metanephric organs and anti-VEGF neutralizing antibodies prevented it, indicating that this process is mediated by VEGF produced by the metanephroi. Using VEGF-coated beads we showed that VEGF provides a direct chemoattractive signal to glomerular endothelial cells.

Mouse glomerular endothelial cells isolated from Flk-1(+/−) mice form a network of capillary-like tubes growing in random directions when plated on collagen I gels in a fashion similar to that described for endothelial cells from other organs and species (Folkman and Haudenschild Christian, 1980; Montesano *et al.,* 1983; Nicosia and Villaschi, 1995). It appears that endothelial cells receive yet undefined morphogenetic signals from extracellular matrix components that are responsible for their cell–cell interactions and behavior, such as monolayer, tube, or network forming. The similar behavior described in many endothelial cell types cultured in ECM gels suggests that the machinery for tube formation is inherent to the endothelial cell and that various ECM components provide signals specific to endothelial cell subtypes (Montesano *et al.,* 1983; Kubota *et al.,* 1988; Madri and Williams, 1983).

Upon 6–24 h of coculture with metanephric organs chemoattraction was detected by a rearrangement of endothelial cells surrounding the explants, such that they aligned toward the metanephroi. This rearrangement was followed by migration of mouse endothelial cells into the rat metaphroi, as indicated by two independent methods: β-Gal expression and CD31 immunostaining. β-Gal-positive endothelial cells formed an interface immediately surrounding the explants and a fraction of them actually invaded the metanephroi. Clearly, this fraction was larger under low-oxygen conditions. Since hypoxia stimulates VEGF expression in the explants (Tufro-McReddie *et al.,* 1997) as well as in endothelial cells (Liu *et al.,* 1995; Ferrara, 1999), and VEGF induces endothelial cell proliferation and migration (Ferrara, 1999; Noiri *et al.,* 1998), we were unable to discriminate between increased chemoattraction and increased endothelial cell activity (proliferation/migration) under low-oxygen conditions. It is also possible that both VEGF actions were stimulated simultaneously. Irrespective of the mechanism, quantitation of the β-Gal-positive cells in the explants confirmed that low oxygen stimulated MGEC migration into the explants.

Mouse endothelial cells migrated into the metanephroi in a nonrandom fashion, establishing capillary-like structures that reached the forming glomeruli and surrounded developing tubules. These data indicate that the metanephric kidney in culture elicits the signals required to direct the migration of endothelial cells to the proper places within the developing nephrons. Our data are consistent with classic transplantation experiments showing that signals from the local mesenchyme determine the pattern of vessel development, rather than the origin of the endothelial cells (Noden, 1989, 1991). The nature of the signal(s) remains unclear so far. Although some β-Gal-positive endothelial cells entered the glomeruli, capillary loop formation was not fully completed under control or low-oxygen conditions. The lack of complete capillarization of glomeruli suggests that signals from mesangial cells (not present in this model) may be required for the capillary loop terminal differentiation. Analysis of PDGF-R knockout mice and recently from Pod1 null mice showing failure of mesangial and podocyte cell development and glomerular capillarization are congruent with this possibility (Lindahl *et al.,* 1998; Quaggin *et al.,* 1999).

We postulated that VEGF, produced by the metanephric blastema and the differentiating nephrons, provides a chemoattracting signal that drives endothelial cell migration within the metanephroi. We have previously demonstrated that low oxygen dramatically upregulates VEGF expression and induces vasculogenesis in this model (Tufro-McReddie *et al.,* 1997). The present experiments demonstrate that when exogenous endothelial cells are available they are capable of invading the explants in an organized manner, similar to that occurring during normal development. These data support the notion that vasculogenesis and angiogenesis contribute to renal vascularization in a coordinated fashion (Tufro-McReddie *et al.,* 1997). The fact that hypoxia enhances and accelerates migration of exogenous endothelial cells into the metanephroi strongly suggests that VEGF is involved. Using anti-VEGF neutralizing antibodies that prevented capillary formation in the metanephroi, we showed that VEGF or a gene regulated by VEGF mediates the organized migration of endothelial cells in this model. We further showed that VEGF bound to agarose beads attracts MGEC, indicating that VEGF chemoattractive signal is sufficient to elicit MGEC migration. Consistent with these data, VEGF induced vectorial movement of endothelial cells, mediated by nitric oxide in cell migration assays (Noiri *et al.,* 1998). It is possible that a concentration gradient of VEGF established by the generation of membrane-bound, ECM-bound, and soluble VEGF isoforms may drive endothelial cell migration. The unavailability of antibodies specific for the different VEGF isoforms precluded testing this hypothesis directly. Analysis of isoform-specific VEGF mutant mice may allow the clarification of this issue in the near future (Carmeliet *et al.,* 1999).

In summary, VEGF produced by the differentiating
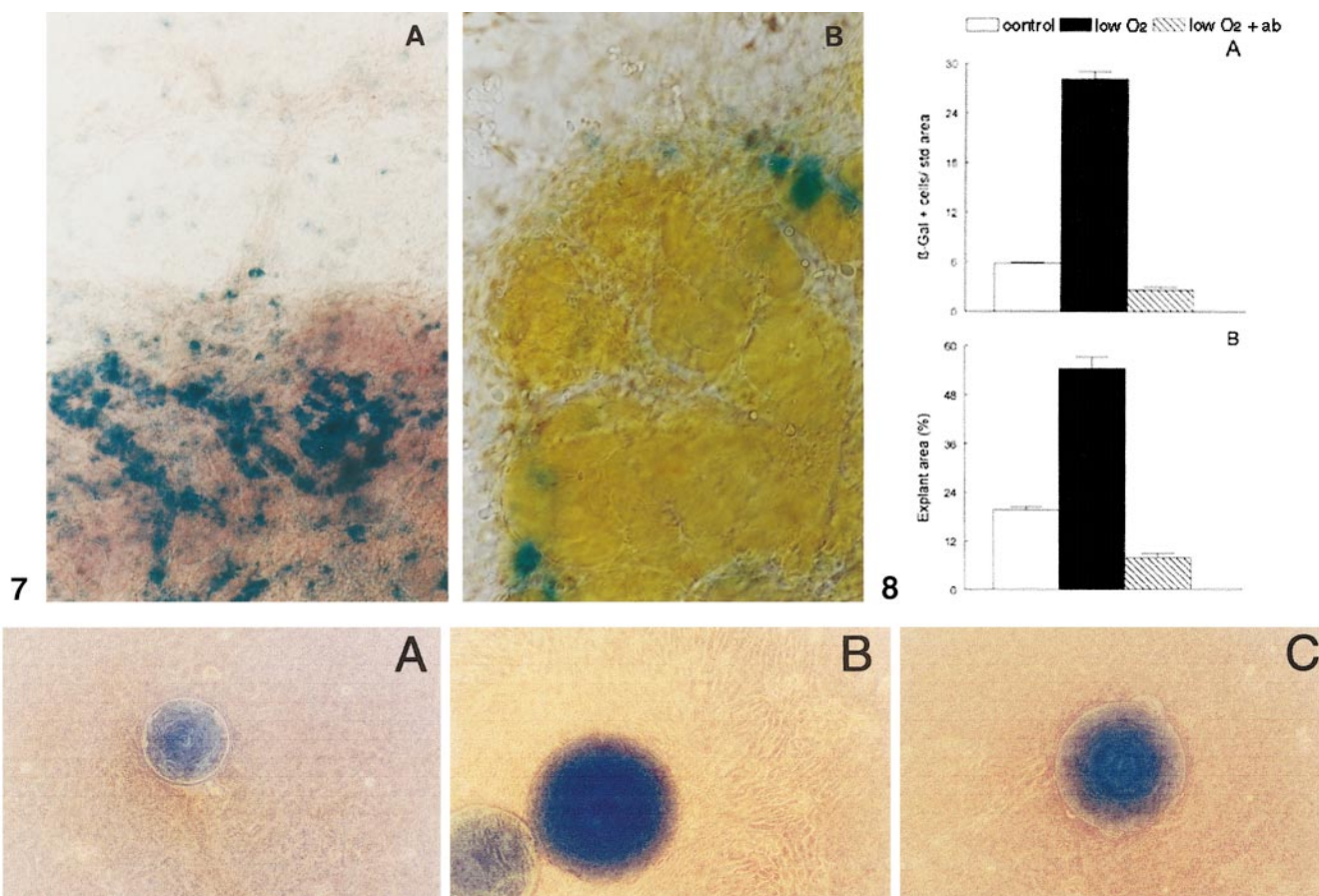
FIG. 7. Neutralizing anti-VEGF antibodies prevent MGEC migration into the explants. Coculture of embryonic and Flk-1(+/−) glomerular endothelial cells for 6 days. (A) Low oxygen showing β-Gal$^+$ endothelial cells forming capillaries; (B) low oxygen and anti-VEGF antibodies showing very few β-Gal$^+$ endothelial cells in the explant.

FIG. 8. Quantitation of β-Gal$^+$ MGEC in the explants. (A) Bar graph showing the number of β-Gal$^+$ MGEC per standard area (0.0625 mm^2) in control, low oxygen, and low oxygen + anti-VEGF neutralizing antibodies explants. (B) Bar graph showing the percentage explant area containing β-Gal$^+$ MGEC under the same experimental conditions.

FIG. 9. VEGF-coated beads attract MGEC. (A) MGEC network with PBS-coated bead showing no migration toward the bead; (B) MGEC network with VEGF-coated bead showing numerous MGEC next to the beads; (C) MGEC network with VEGF-coated bead + anti-VEGF neutralizing antibodies added to the medium showing that MGEC migration is inhibited.

nephrons is a chemoattractant for exogenous endothelial cells that spatially directs their migration toward developing nephrons *in vitro.* These data suggest that under naturally occurring low-oxygen conditions vasculogenesis and angiogenesis contribute to renal vascularization.

ACKNOWLEDGMENTS

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