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Oxygen Regulates Vascular Endothelial Growth Factor-Mediated Vasculogenesis and Tubulogenesis

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To determine whether low oxygen is a stimulus for endothelial cell differentiation and vascular development in the kidney, we examined the effect of low oxygen on rat metanephric organ culture, a model known to recapitulate nephrogenesis in the absence of vessels. After 6 days in culture in standard (20% O_2) or low oxygen (1–3% O_2) conditions, metanephric kidney growth and morphology were assessed by DNA measurement, and light and electron microscopy. DNA content was higher in 3% O_2-treated explants (2.5 ± 0.17 μg/kidney, $n = 9$) than in 20% O_2 explants (1.5 ± 0.09 μg/kidney, $n = 9$), $P < 0.05$. Low oxygen induced proliferation of tubular epithelial cells, resulting in enhanced number of tubules of similar size. Endothelial cells forming capillaries were localized in 3% O_2 explants by light and electron microscopy and by immunocytochemistry using endothelial cell markers. Flt-1, Flk-1, and ACE-containing cells were detected in 3% O_2-treated explants, whereas 20% O_2 explants were virtually negative. VEGF mRNA levels were 10-fold higher in 3% O_2-treated explants than in 20% O_2-treated explants. Addition of anti-VEGF antibodies to 3% O_2-treated explants prevented low oxygen-induced growth and endothelial cell differentiation and proliferation. Our data indicate that low oxygen stimulates growth by cell proliferation and induces tubulogenesis, endothelial cell differentiation, and vasculogenesis in metanephric kidneys in culture. Upregulation of VEGF expression by low oxygen and prevention of low oxygen-induced tubulogenesis and vasculogenesis by anti-VEGF antibodies indicate that these changes were mediated by VEGF. These data suggest that low oxygen is the stimulus to initiate renal vascularization.

INTRODUCTION

The origin of the kidney vasculature is unclear and the molecular mechanisms that initiate blood vessel formation in the kidney are unknown (Saxen, 1987). Renal endothelial cells could differentiate *in situ* from angioblasts by vasculogenesis, or may sprout from preexisting vessels in the surrounding mesenchyme by angiogenesis and migrate into the forming nephrons (Saxen, 1987; Sariola *et al.*, 1983; Ekblom *et al.*, 1982; Hyink *et al.*, 1996). We hypothesized that relative hypoxia may be a physiologic stimulus for the expression the angiogenic factor VEGF in the avascular mesenchyme leading to the initiation of vascular development during kidney morphogenesis.

VEGF is a secreted, direct-acting endothelial cell mitogen that stimulates angiogenesis and vascular permeability (Keck *et al.*, 1989; Leung *et al.*, 1989; Ferrara *et al.*, 1992). VEGF binding to receptor tyrosine kinases Flk-1 and Flt-1 (Quinn *et al.*, 1993; Shibuya *et al.*, 1990) expressed in angioblasts and endothelial cells is required for normal vascularization (Shalaby *et al.*, 1995; Fong *et al.*, 1995). In fact, disruption of VEGF, Flk-1, or Flt-1 genes results in embryonic lethality due to failure of vascular development (Carmellet *et al.*, 1996; Ferrara *et al.*, 1996; Shalaby *et al.*, 1995; Fong *et al.*, 1995). VEGF induces endothelial cell proliferation and paracrine upregulation of its own receptors (Kim *et al.*, 1993; Plate *et al.*, 1993). VEGF expression is upregulated by hypoxia as documented in cell lines, lung, myocardium, retina, and VEGF-producing tumors (Shweiki *et al.*, 1992; Plate *et al.*, 1993; Pe'er *et al.*, 1995; Tuder *et al.*, 1995; Banai *et al.*, 1994). VEGF mediates hypoxia-induced angiogenesis in a multitude of disease processes, including formation of collateral vessels in ischemic hearts or limbs, neovascularization of atherosclerotic plaques, retinal neo-

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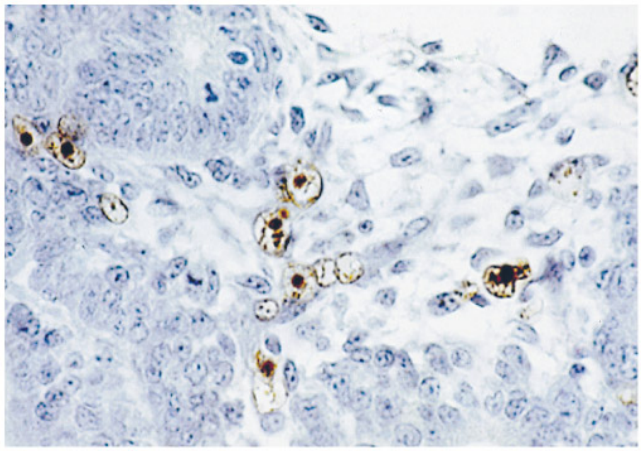

FIG. 1. Embryonic kidney prior to be placed in culture showing isolated cells containing immunoreactive Flk-1 (brown), no vascular structures are observed (original magnification, ×40).

vascularization secondary to diabetic retinopathy, or retinopathy of prematurity (Banai *et al.*, 1994; Folkman, 1995; Alon, *et al.*, 1995). Hypoxia-induced transient VEGF expression in the developing retina is tightly and sequentially followed by Flk-1 expression and development of the retinal vasculature (Stone *et al.*, 1995). In newborn kittens the rate of retinal vasculogenic cell division and vessel formation is inversely proportional to the oxygen concentration inspired, indicating that hypoxia is the physiologic stimulus for retinal vasculogenesis (Chan-Ling *et al.*, 1995).

To test the hypothesis that low oxygen stimulates VEGF-induced vasculogenesis in the developing kidney we used the metanephric organ culture, a model that recapitulates nephrogenesis in the absence of vessels, and thus is amenable to experimental induction of angiogenesis (Saxen, 1987; Sariola *et al.*, 1983). The absence of vessels in metanephric organ culture has been attributed to the putative extrametanephric origin of glomerular vessels (Saxen, 1987; Ekblom *et al.*, 1982). We postulate that the oxygen concentration used under standard culture conditions prevents VEGF expression required for endothelial cell differentiation and renal vascular development. In the present studies we examined the effect of low oxygen on metanephric organ culture to determine whether it regulates VEGF-mediated vasculogenesis.

MATERIALS AND METHODS

Metanephric Kidney Organ Culture

The culture was performed essentially as described (Sweeney and Avner, 1991). Sprague–Dawley rats were mated for 6 hr; the following morning, when the plug was observed, was considered Day 1 of gestation (E1). Intact metanephroi were microdissected from rat embryos on Day 14 of gestation (E14), were cultured in defined, serum-free medium (DMEM-F12) for 6 days at 37°C in 5% CO_2 + 20, 1, or 3% O_2. Medium was supplemented with 10 m*M* Hepes, 5 mg/ml insulin, 5 mg/ml transferrin, 2.8 n*M* selenium, 25 ng/ml PGE_1, 32 pg/ml T_3, 50 U/ml penicillin, 5 mg/ml gentamycin, 50 U/ml mycostatin. Low oxygen-treated explants were kept in airtight chambers (Billups-Rothenberg, Del Mar, CA) filled with the following gas mixes (BOC Medical Gases, Murrail Hill, NJ): 20% O_2 = 5% CO_2 + air; 1% O_2 = 5% CO_2 + 1% O_2 + 94% N_2; 3% O_2 = 5% CO_2 + 3% O_2 + 92% N_2 for 4 min at 1.2 psi. Chambers were briefly opened daily to change the medium. Explants were paired for 20% O_2 or low oxygen treatment as follows: from each embryo, one kidney was assigned to 20% O_2 and the other was assigned to 1 or 3% O_2.

In separate experiments, polyclonal anti-VEGF antibodies raised against the NH_2-terminal and COOH-terminal amino acids of human VEGF (Keck *et al.*, 1989), VEGF-ab1 (Oncogene Science Inc., Cambridge, MA) at 5 mg/ml and VEGF-ab2 (Oncogene Science Inc.) at 2.5 mg/ml were added to control and low oxygen-treated explants' medium daily for 6 days. When anti-VEGF antibodies were added, explants were paired as described above. Additional experiments were performed to prove the specificity of the antibodies' effect, explants were paired as follows: one kidney was assigned to medium + vehicle (50 m*M* $NaPO_4$ buffer + 0.77 m*M* Na azide + 1 mg% gelatin) or medium + rabbit serum and the other was assigned to medium + anti-VEGF antibodies, this scheme was repeated under 20% O_2 and low oxygen conditions.

DNA Measurements

Measurements were performed using a fluorometric method by Labarca (Labarca and Paiges, 1980). Pools of three to four explants were assessed by duplicate.

Morphologic Studies

Explants were fixed in Bouin's fixative and paraffin embedded for light microscopy examination or fixed in 2% paraformaldehyde + 2.5% glutaraldehyde and processed for electron microscopy. Briefly, explants were rinsed in 50 m*M* sodium cacodylate buffer, postfixed in 1% osmium tetroxide, and stained *en bloc* with 3% uranyl acetate in veronal acetate buffer. Then explants were dehydrated and embedded in PolyBed 812 resin (Polysciences Inc., Warrington, PA) at 60°C for 2 days. Ultrathin sections were cut on an LKB Ultratome V, counterstained with saturated uranyl acetate followed by lead citrate, examined, and photographed on a Zeiss EM 10CA transmission electron microscope at 60 kV.

Morphometric analysis. A total of 19 explants from 5 separate experiments were examined using a video-microscope system (Olympus AH-2, Sony CCD-Iris, San Jose, CA). Two nonconsecutive sections/explant were digitized. Tubular, ureteric bud and total areas were measured using an image analysis sofware (Mocha, Jaendel Inc., San Rafael, CA). Mesenchyme area was calculated as total area − (tubules + ureteric buds).

Immunocytochemistry. Flt-1, Flk-1, angiotensin-converting enzyme (ACE), and proliferating cell nuclear antigen (PCNA) were localized using specific antibodies (Shibuya *et al.*, 1990; Yamaguchi *et al.*, 1993; Caldwell *et al.*, 1976; Foley *et al.*, 1991). Sections were deparaffinized, microwaved in 10 m*M* Na–citrate, and incubated with polyclonal anti-Flt-1 (1:250) or anti-flk-1 (1:100) antibody (Santa Cruz Inc., Santa Cruz, CA) or anti-PCNA (1:150) monoclonal antibody (PC10, Novocastra, New Tyne upon Castle, UK) for 60

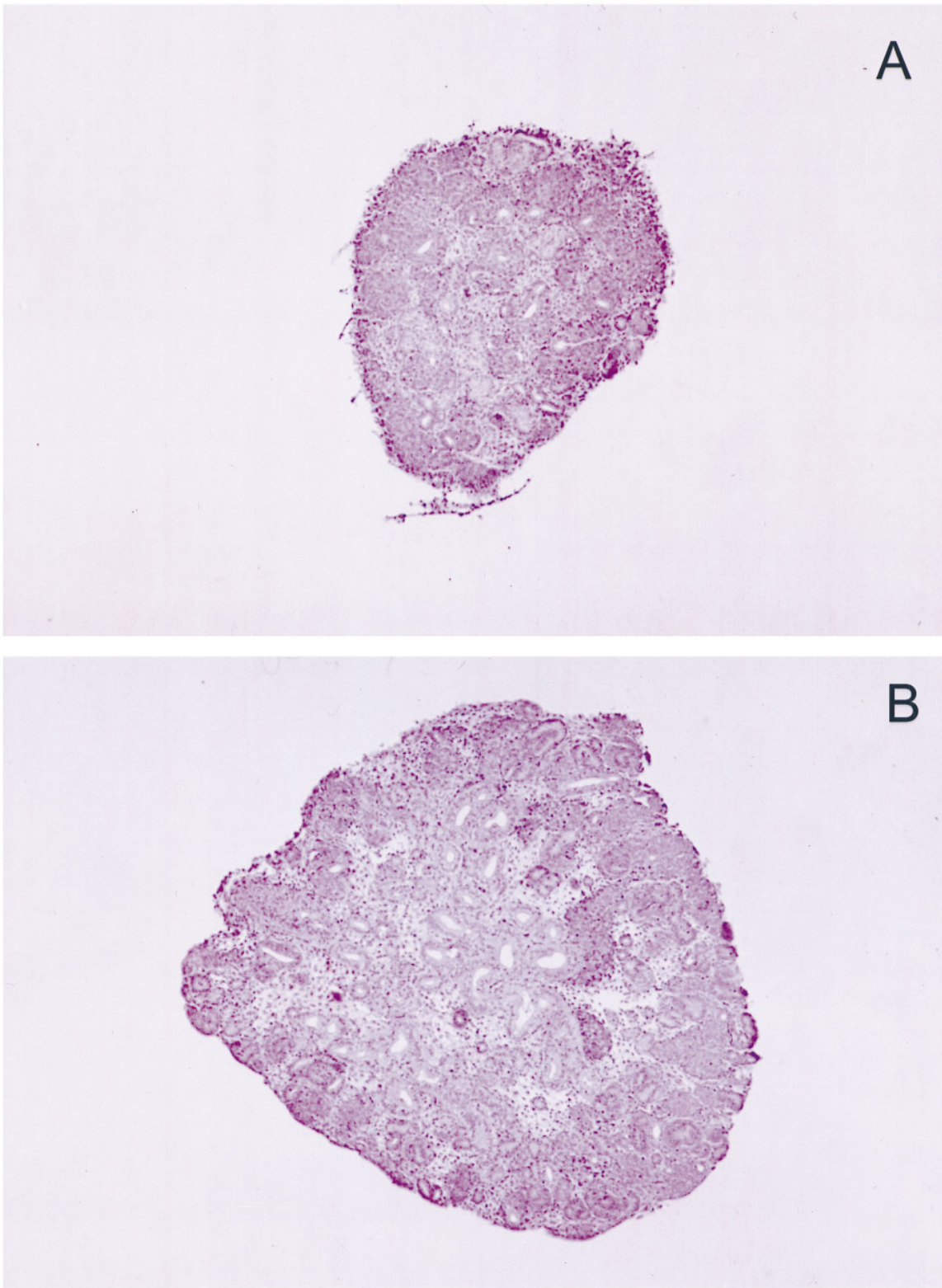

FIG. 2. (A) 20% O_2-treated explant showing ureteric bud branches, forming nephrons and mesenchyme (×13.2). (B) 3% O_2-treated explant showing intense nephrogenesis (×13.2). Please note overall size difference between A and B.

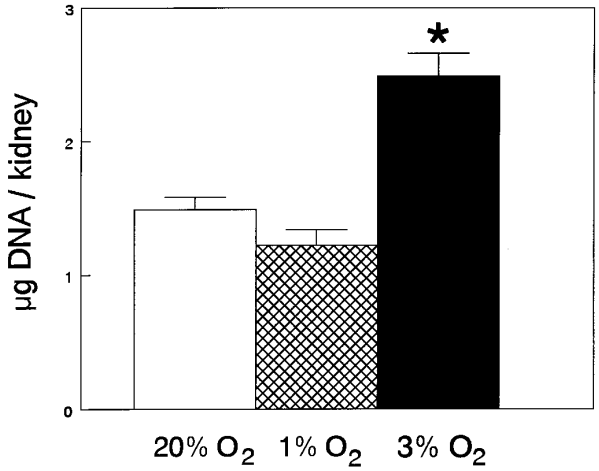

FIG. 3. DNA content (μg/kidney) from 20% O_2 (open bar, $n = 17$), 1% O_2 (hatched bar, $n = 8$), 3% O_2 (solid bar, $n = 9$) explants after 6 days in culture. * indicates $P < 0.05$.

min at room temperature or overnight at 4°C. Secondary antibody was biotinylated anti-rabbit or anti-mouse IgG, as appropriate. Reactions were detected by immunoperoxidase using the ABC technique (Hsu *et al.*, 1981). Negative controls were absence of primary or secondary antibodies, replacement of primary antibody with normal rabbit, mouse, or goat serum, and competition with specific peptide. Sections were counterstained with hematoxylin and examined by light microscopy. ACE was localized in whole mount explants (Gilbert *et al.*, 1994). Cultured explants were fixed in 4% paraformaldehyde, incubated with 0.1 U/ml neuraminidase for 60 min at 37°C, washed, and incubated with rhodamine-labeled peanut lectin (PNA, Vector, Burlingame, CA) [7.5 mg/ml] and anti-ACE polyclonal antibody (1:200) for 90 min at 37°C. Explants were washed, incubated with anti-goat fluorescein-labeled secondary antibody for 60 min, mounted, and examined in a confocal microscope (Leitz, Glenwood, NJ). Glomerular labelling with peanut lectin (PNA) provided spatial orientation for ACE-stained endothelial cells (Gilbert *et al.*, 1994).

Northern analysis. Total RNA was extracted from pools of 30–60 explants per experimental condition by standard technique (Chomczynski, 1993).

RNA samples (10-mg) were resolved in 1.2% agarose/formaldehyde gels and transferred to nylon membranes (Zetaprobe, Bio-Rad, CA). Northern blots were hybridized with a VEGF cDNA fragment (204 bp) ^{32}P-labeled by PCR. VEGF cDNA was previously sequenced to verify its identity to rat VEGF cDNA (Conn *et al.*, 1990). To verify equal loading, Northern blots were also hybridized to TGFβ1 cDNA ^{32}P-labeled by random priming (Feinberg and Vogelstein, 1983). TGFβ1 gene expression is not altered by hypoxia (Mukhopadhyay *et al.*, 1995). Blots were exposed 2–4 days to XAR film at −70°C for autoradiography.

Statistical Analysis

DNA content was expressed as mean ± SEM. Groups were compared using unpaired *t* test because several explants were

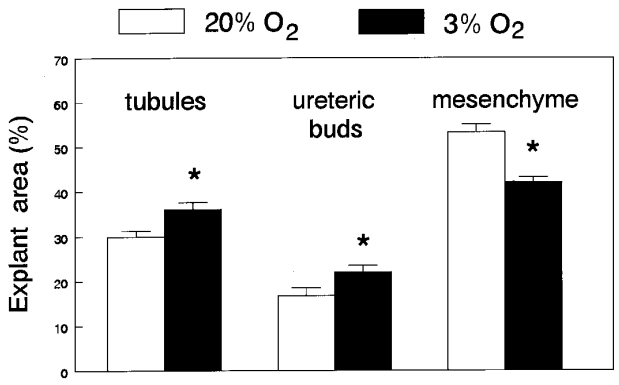

FIG. 4. Quantification of morphologic changes induced by 3% O_2 showed a relative increase in tubular and ureteric epithelia and a relative decrease in mesenchyme. Data are expressed as percentage of the total area of explants' sections. 20% O_2 (open bars, $n = 19$), 3% O_2 (solid bars, $n = 45$). * indicates $P < 0.05$.

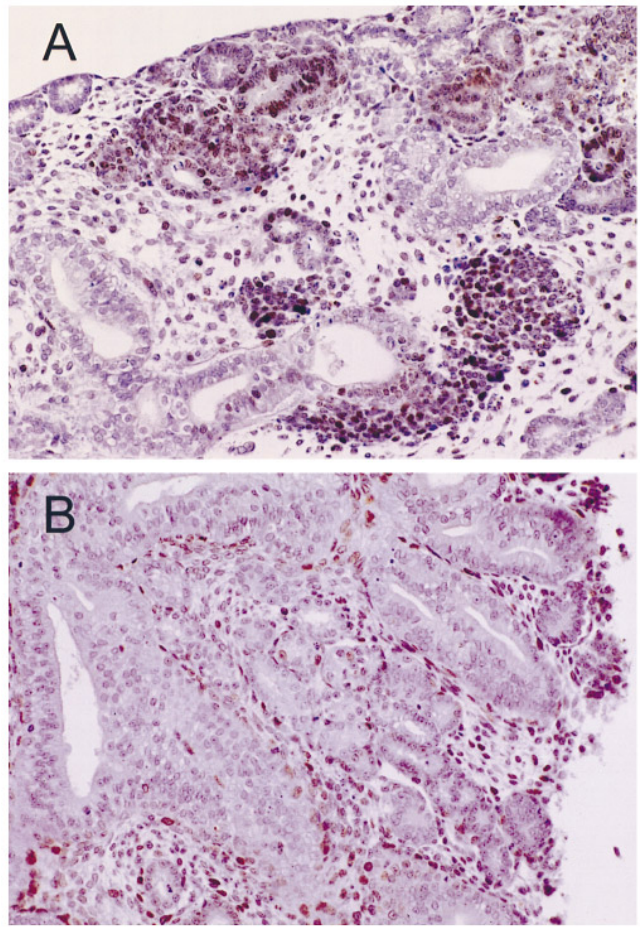

FIG. 5. (A) 3% O_2-treated explant showing numerous PCNA-immunoreactive nuclei in tubular and mesenchymal cells (original magnification, ×50). (B) 20% O_2-treated explant showing few PCNA immunoreactive nuclei (original magnification, ×80).

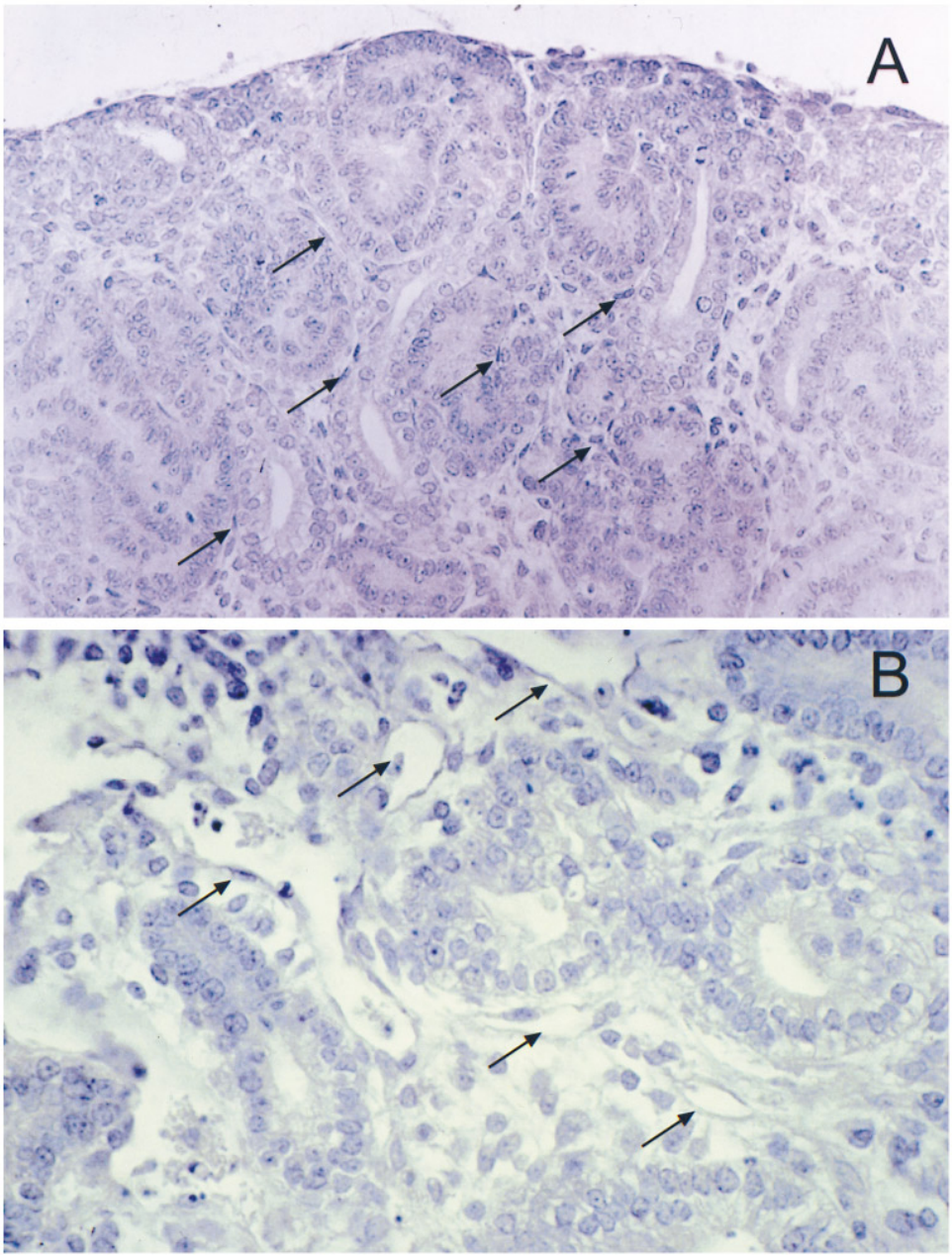

FIG. 6. (A) 3% O_2-treated explant showing endothelial cells in the vascular cleft of S-shaped body and surrounding tubular structures (arrows) (×66). (B) 3% O_2-treated explant showing forming capillary network (arrows) (×80).

pooled for DNA measurement. Statistical significance was defined as $P < 0.05$. Three nonconsecutive sections from 6–9 explants from each experimental group were examined in each immunocytochemical study.

Results of the quantitative analysis of tubular, ureteric bud, and mesenchymal areas were expressed as percentage of the examined surface area ±SEM or in mm^2; 20 and 3% O_2 groups were compared using unpaired *t* test.

RESULTS

Prior to being placed in organ culture, explants contained isolated Flk-1-expressing cells (Fig. 1), and no endothelial cells or capillaries were detected. To determine whether low oxygen is a stimulus for VEGF-mediated vasculogenesis, meta-

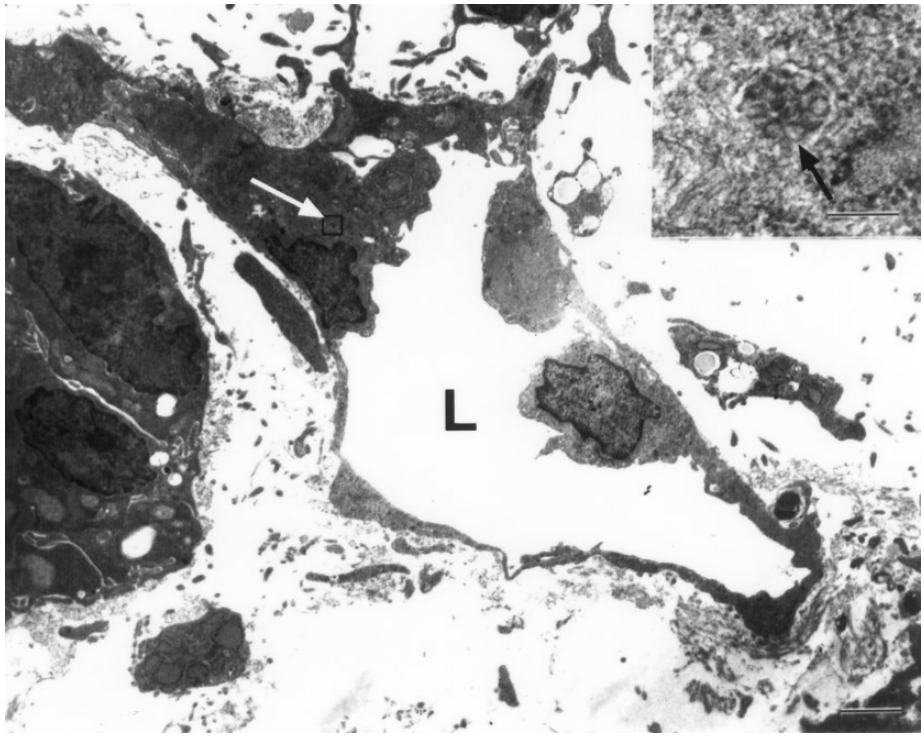

FIG. 7. 3% O_2-treated explant, electron microscopy showing two endothelial cells forming a capillary (×1250). (Inset) Higher magnification view of one of these endothelial cells showing a cross section of a Weibel–Palade body (×25,000).

nephric organs were exposed to two different levels of oxygen: 1 and 3% O_2 and compared to explants cultured in 20% O_2.

The 20% O_2 explants developed the typical metanephric organ culture epithelial differentiation; endothelial cells were not detected by light (Fig. 2A) or electron microscopy (data not shown). In addition, very few Flt-1-expressing cells and no Flk-1- or ACE-containing cells were observed in 20% O_2 explants (Figs. 8D–8F).

Low oxygen induced marked changes in explant growth and morphology. 1% O_2 reduced explant growth, as indicated by a lower DNA content than paired 20% O_2 explants (Fig. 3). Decreased growth in the 1% O_2-treated explants was associated with morphologic alterations including sparse and delayed nephrogenesis and normal ureteric bud branching (data not shown).

3% O_2 induced a striking increase in explant cell proliferation, as indicated by a higher DNA content in 3% O_2-treated than in paired 20% O_2 explants (Fig. 3). The overall size of the 3% O_2 explants (3.5 ± 0.6 mm^2) was higher than that of 20% O_2 explants (2.0 ± 0.25 mm^2), $P < 0.05$. In addition, quantification of the relative tubular, ureteric bud and mesenchymal areas showed that 3% O_2 increased tubule formation and ureteric bud growth and decreased mesenchyme expansion compared to 20% O_2 (Fig. 4). The number of tubules per explant section was higher in 3% O_2 explants (108 ± 22) than in 20% O_2 explants (56 ± 7), $P < 0.05$, whereas the area of individual tubules was not significantly different. Tubular expansion in 3% O_2 explants was due to cell proliferation, as indicated by a clear increase in PCNA immunoreactivity compared to 20% O_2 explants (Fig. 5). Immunoreactive Flt-1 was detected in tubular epithelial cells (Fig. 8A).

3% O_2 induced differentiation of endothelial cells within the explants (Figs. 6A and 6B). Endothelial cells were observed surrounding tubules and ureteric bud branches and in the vascular cleft of S-shaped bodies, often times forming capillary lumina (Figs. 6A and 6B). Electron microscopy revealed Weibel–Palade bodies within the cytoplasm of these maturing endothelial cells (Fig. 7). The developing endothelial cells expressed Flt-1 and Flk-1 (Figs. 8A and 8B), confirming their lineage. The presence of ACE-expressing cells next to and within developing glomeruli was detected by fluorescent immunolabeling (Fig. 8C).

To determine whether low oxygen upregulates VEGF expression in this model, mRNA levels were determined in 20 and 3% O_2 explants. 3% O_2 induced a 10-fold increase in steady-state VEGF mRNA level (Fig. 9).

To determine whether 3% O_2 effects on metanephric organ were mediated by VEGF, 20 and 3% O_2 explants were treated with polyclonal anti-VEGF antibodies. Anti-VEGF antibodies prevented low oxygen-induced explant growth, whereas they did not alter 20% O_2 explant growth or morphology (Fig. 10A). Rabbit serum or the antibodies' vehicle

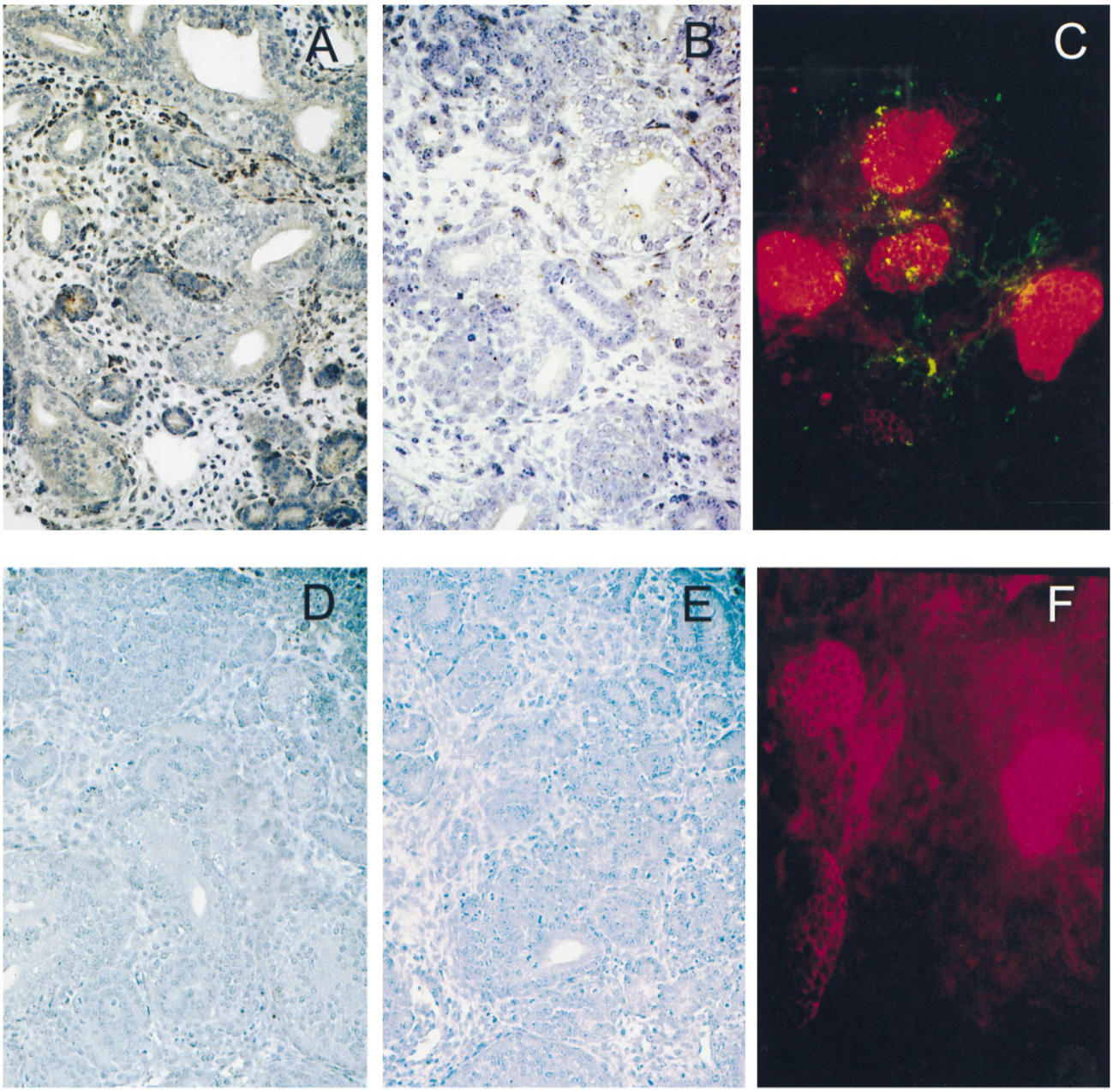

FIG. 8. (A) 3% O_2-treated explant showing immunoreactive Flt-1 localized to endothelial cells and tubular epithelial cells (original magnification, ×50). (B) 3% O_2-treated explant showing immunoreactive Flk-1 localized to endothelial cells (original magnification, ×80). (C) Whole mount 3% O_2-treated explant showing glomeruli labeled with PNA lectin (red) and FITC-labeled immunoreactive ACE (green/yellow) staining endothelial cells next and within glomeruli (original magnification, ×100). (E) 20% O_2 explant showing no detectable Flt-1 immunoreactivity (original magnification, ×80). (E) 20% O_2 explant showing no Flk-1 immunostaining (original magnification, ×80). (F) Whole mount 20% O_2 explant showing PNA-labeled glomeruli (red) and no ACE immunostaining (original magnification, ×80).

did not impair 3% O_2 explant growth (Fig. 10B), indicating that the effect of anti-VEGF antibodies was specific. Explants treated with 3% O_2 + anti-VEGF-neutralizing antibodies were morphologically indistinguishable from 20% O_2 explants, indicating that low oxygen-induced changes in explant morphology were mediated by VEGF.

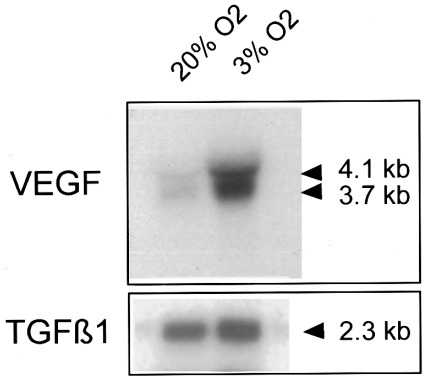

FIG. 9. 3% O_2 increases explant VEGF mRNA level. Northern analysis of 20% and 3% O_2-treated explants' total RNA (10 mg/lane) showing a 10-fold increase in VEGF mRNA. Two VEGF transcripts, 3.7 and 4.1 kb long, are detected (arrowheads). TGFβ1 mRNA level (2.3 kb) not altered by low oxygen is shown to demonstrate equal loading.

DISCUSSION

We report that low oxygen induced explant growth and differentiation of angioblasts into endothelial cells in the metanephric organ culture. Developing endothelial cell phenotype was defined by the expression of Flt-1, Flk-1, and ACE. Identification of capillary lumina and Weibel–Palade bodies by light and electron microscopy confirmed their endothelial nature (Weibel and Palade, 1964). Endothelial cell differentiation and proliferation was associated with upregulation of VEGF expression. Addition of anti-VEGF antibodies to low oxygen-treated explants prevented the described morphologic changes. Taken together, our data indicate that low oxygen induces vasculogenesis via VEGF in the metanephric organ culture.

During renal morphogenesis, Flk-1 and Flt-1 are expressed in isolated, large cells within the metanephric blastema prior to any morphologic evidence of endothelial cell differentiation. These cells are angioblasts, i.e., mesodermal cells with the potential to differentiating into endothelial cells that express the earliest markers of that lineage (Risau, 1995). As renal vascularization proceeds, Flk-1 and Flt-1 are expressed in endothelial cells, while their ligand VEGF is expressed in contiguous glomerular epithelial cells and developing tubules (Simon *et al.*, 1995; Dumont *et al.*, 1995).

These ontogenic changes in VEGF, Flt-1, and Flk-1 did not take place in the 20% O_2 metanephric organ culture. Immunoreactive Flk-1 and Flt-1 were barely detectable in 20% O_2 explants, suggesting that VEGF receptors downregulate after 6 days in culture. Flk-1 and Flt-1 downregulation was associated with a lack of endothelial cell differentiation, in agreement with previous studies showing that in metanephric organ culture glomerular and tubular epithelial differentiation occurs in the absence of vessels (Ekblom, 1987; Saxen, 1987; Bernstein *et al.*, 1981). VEGF receptor downregulation may prevent explant vascularization because Flk-1 and Flt-1 expression is required for normal endothelial cell differentiation and vascular assembly (Shalaby *et al.*, 1995; Fong *et al.*, 1995).

3% O_2 induced explant growth by cell proliferation, whereas 1% O_2 reduced explant growth and development, suggesting that the metanephric kidney has a small but precise oxygen requirement. Low oxygen has been shown to stimulate the development of blastocysts from mouse zygotes *in vitro*, whereas blastocyst formation was impaired by 20% O_2 and inhibited by 95% O_2 (Whitten, 1971). Similarly, low oxygen stimulated the proliferation of pericytes (Brighton *et al.*, 1992). 3% O_2 may be more similar to the

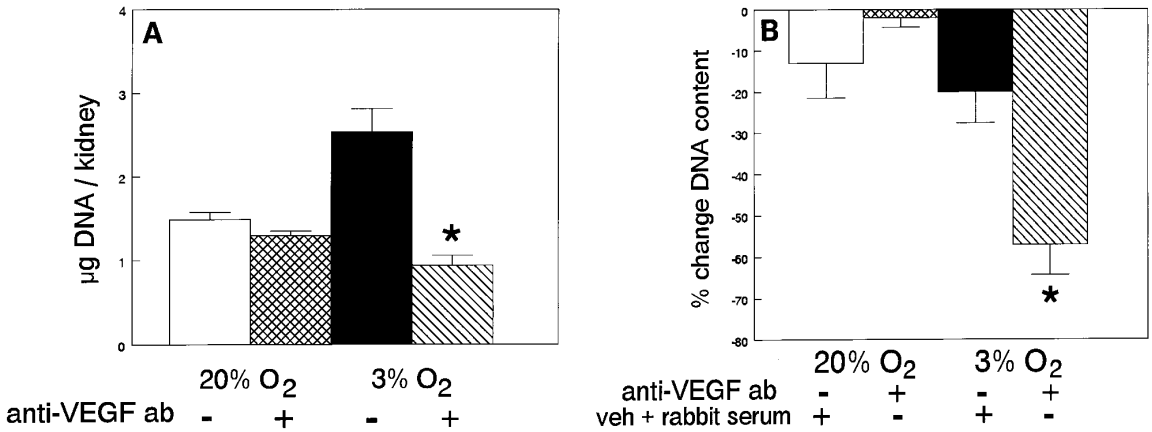

FIG. 10. Anti-VEGF antibodies prevent low oxygen-induced growth. (A) DNA content in 20% O_2 explants (open bar, $n = 12$), 20% O_2 + anti-VEGF antibodies-treated explants (cross-hatched bar, $n = 5$), 3% O_2 (solid bar, $n = 9$), 3% O_2 + anti-VEGF antibody-treated explants (hatched bar, $n = 5$). (B) Percentage change in DNA content induced by anti-VEGF, vehicle, and rabbit serum in 20 and 3% O_2-treated explants. * indicates $P < 0.05$.

naturally occurring oxygen concentration in the embryo during renal morphogenesis than the standard 20% O_2 used for organ culture. The mitogenic effect of low oxygen was not limited to endothelial cells, since the bulk of metanephric kidney cells are epithelial and mesenchymal. The increased number of tubules, expansion of ureteric buds, and reduced mesenchyme as well as the intense tubular proliferation detected by PCNA immunostaining in 3% O_2 explants suggest that low oxygen stimulated tubulogenesis. The presence of Flt-1 receptors in mesenchymal and developing tubular cells was consistent with these cell types being a target for VEGF and suggested that Flt-1 may mediate VEGF mitogenic effect.

Low oxygen-induced growth was associated with upregulation of VEGF expression, as indicated by a 10-fold increase in VEGF mRNA levels in 3% O_2 explants compared to 20% O_2 explants. VEGF upregulation by low oxygen has been shown *in vivo* and *in vitro* (Minchenko and Bauer, 1995; Mukhopadhyay *et al.*, 1995; Levy and Levy, 1995). Upregulation of Flt-1 and Flk-1 during hypoxia has been shown to be mediated by the paracrine action of VEGF (Brogi *et al.*, 1996; Kim *et al.*, 1993; Plate *et al.*, 1994). In response to low oxygen, metanephric kidneys upregulated Flt-1, Flk-1, and ACE expression in endothelial cells localized to the vascular cleft of S-shaped bodies, surrounding tubules, and ureteric bud branches. Alternatively, Flt-1-labeled cells could be mesangial cells or pericytes (Takahashi *et al.*, 1995; Decker *et al.*, 1995). This is unlikely because mesangial cells do not develop until a later stage of glomerular maturation; pericytes follow rather than precede capillary sprouting and they do not normally occur in glomerular and peritubular capillaries (Bernstein *et al.*, 1981). The observed developing endothelial cells contained Weibel–Palade bodies, cytoplasmic components considered a hallmark of endothelial cells (Weibel and Palade, 1964). Most importantly, developing endothelial cells formed capillary lumina within 3% O_2 explants. Thus our data demonstrate by several morphologic and immunocytochemical criteria that low oxygen induces vasculogenesis in the metanephric organ culture. Whether the angioblasts observed at the beginning of the experiment were ''native'' to the metanephric blastema or had previously migrated into it from the surrounding lateral mesoderm remains unclear.

In addition to its effect on VEGF expression, low oxygen upregulates several genes potentially angiogenic including PDGF, erythropoietin, nitric oxide synthase, endothelin (Kourembanas and Hannan, 1990; Kourembanas and Marsden, 1991; Porter and Goldberg, 1993). Thus, to gain insight into the mechanism of low oxygen-induced vasculogenesis in the metanephric organ culture, VEGF-neutralizing antibodies were used. This strategy has previously proved successful for inhibiting VEGF-induced endothelial cell proliferation *in vitro* and tumor angiogenesis *in vivo* (Plate *et al.*, 1993; Kim *et al.*, 1993). Indeed, addition of VEGF-neutralizing antibodies to 3% O_2 explants prevented low oxygen-induced growth and endothelial cell differentiation, indicating that the observed effects of low oxygen are mediated by VEGF. Alternatively, low oxygen-induced VEGF may regulate other genes responsible for explant growth.

To date, the lack of endothelial cell differentiation in the organ culture has been attributed to the putative extrarenal origin of kidney endothelial cells and to the angiogenic mechanism for renal vascularization (Saxen, 1987; Pinson Hyink and Abrahamson, 1995; Bernstein *et al.*, 1981; Sariola *et al.*, 1983; Ekblom *et al.*, 1982). We hypothesized that low oxygen is the physiologic stimulus that initiates vascular development by inducing VEGF expression, thus triggering a sequence of events that result in capillary formation. Elevated oxygen concentration under standard culture conditions prevents VEGF expression, resulting in Flt-1 and Flk-1 downregulation followed by the described lack of endothelial cell differentiation. The present data showing that low oxygen induces vasculogenesis in the organ culture support our hypothesis and suggest that hypoxia may be the physiologic stimulus to initiate vascularization during normal renal development. Clearly, low oxygen is not enough to support the full development of the renal vasculature *in vitro*. Vasculogenesis and angiogenesis may contribute to renal vascularization in a coordinated and orderly fashion, as it has been described in the lung and the retina (Noden, 1989; Stone *et al.*, 1995; Coffin *et al.*, 1991).

In summary, low oxygen induced VEGF-mediated vasculogenesis and tubulogenesis within metanephric organ culture by promoting differentiation and proliferation of endothelial and tubular epithelial cells. These data suggest that low oxygen is the stimulus to initiate renal vascularization.

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