Vascular Endothelial Growth Factor Receptor-2-Mediated Mitogenesis Is Negatively Regulated by Vascular Endothelial Growth Factor Receptor-1 in Tumor Epithelial Cells

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Vascular endothelial growth factor (VEGF) receptors are present on nonendothelial cells suggesting that VEGF may mediate nonendothelial effects during organogenesis and tumorigenesis. Here we show that VEGF receptor-1 (VEGFR-1) negatively regulates VEGFR-2-mediated proliferation via nitric oxide (NO) in an epithelial cancer cell line ECV304. Cell proliferation was assessed by [³H]thymidine incorporation, fluorescent-activated cell-sorting analysis, and cell number using a Coulter Counter. Total NO generated by the action of nitric oxide synthase was measured by Seivers NOA 280 Nitric Oxide Chemiluminescence Analyser. VEGF (1 ng/ml) stimulated DNA synthesis and increased ECV304 cell number in a manner that was inhibited by a neutralizing anti-VEGFR-2 antibody. In contrast, VEGF (50 ng/ml) stimulated NO release in a manner that was inhibited by functionally neutralizing anti-VEGFR-1 antibody. Blockage of the VEGFR-1 receptor signal with anti-VEGFR-1 stimulated DNA synthesis and increased cell number. Cell-cycle analysis showed that inhibition of VEGFR-1 increased the transition from G₁ to S phase whereas inhibition of VEGFR-2 blocked the VEGF-mediated transition from G₁ to S phase. Finally, the addition of NO donors suppressed both VEGF-mediated proliferation and the increase in growth after blockade of VEGFR-1. Conversely, inhibition of VEGF mediated NO release by nitric oxide synthase inhibitor, L-monomethyl-L-arginine, restored the mitogenic effect of VEGF. These findings identify a dose-dependent reciprocal regulatory mechanism for VEGF via its two receptors. It shows that VEGFR-1 induces cell cytostasis via NO and as such is a suitable target for molecular strategies suppressing tumorigenesis. (*Am J Pathol* 2001, 158:265–273)

Vascular endothelial growth factor (VEGF) stimulates proliferation and migration of endothelial cells and mediates *in vitro* and *in vivo* angiogenesis.¹ It is generally accepted that the vascular endothelium is the specific target of VEGF action. VEGF mediates its effects by binding with high affinity to two tyrosine kinase receptors VEGF receptor-1 (VEGFR-1/Flt, 1 kd, 16 to 114 pmol/L)² and VEGFR-2 (KDR kd, 75 to 125 pmol/L).³

VEGF is critical for solid tumor growth.^{4,5} Many studies demonstrate a marked increase in VEGF mRNA levels in human tumors, where VEGF is thought to promote tumor driven neovascularization in a paracrine manner.¹ Withdrawal of VEGF from xenografted c6 gliomas resulted in blood vessel regression and endothelial cell death, whereas overexpression of VEGF resulted in the formation of metastatic neoplasms,⁶ suggesting that VEGF is a good target for therapeutic intervention against tumor driven angiogenesis. However, a recent article demonstrating the ability of aggressive uveal melanoma cells to form vascular channels independent from endothelium has suggested an additional mechanism of tumor perfusion.⁷ These authors suggest that aggressive melanomas may facilitate tumor perfusion by forming blood-carrying vessels independent from tumor angiogenesis and therefore anti-tumor therapies targeting endothelial cells alone would not be fully effective.⁷

Numerous studies have demonstrated that cells of nonendothelial origin also express functional VEGF receptors. VEGF was reported to increase DNA synthesis in dendritic antigen-presenting cells⁸ and promoted the growth of uterine smooth muscle cells.⁹ Moreover, on the addition of exogenous VEGF, VEGFR-1 was shown to mediate monocyte migration,¹⁰ to induce nitric oxide (NO) production in trophoblasts,¹¹ and to stimulate matrix metalloproteinase expression in vascular smooth muscle cells.¹² Recently, VEGF was shown to play a dual role in

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kidney development. It promoted both vasculogenesis and tubulogenesis in rat embryos by stimulating both endothelial and tubular epithelial cell proliferation.¹³ Furthermore, VEGF was also identified to be a specific survival factor for the tubular epithelial cell line NRK52-E.¹⁴ More importantly, both VEGF and its receptors are expressed on primary and metastatic melanoma cell lines,¹⁵ as well as on both epithelial and endothelial cells from breast,¹⁶ and ovarian carcinomas.¹⁷

Recently, pancreatic cancer Capan-1 cells were shown to express VEGFR-1 and VEGFR-2 mRNA, and to proliferate in response to VEGF stimulation.¹⁸ These data suggest an additional autocrine manner of tumor cell growth by VEGF. We previously demonstrated that VEGF stimulated trophoblast cell growth via VEGFR-2¹⁹ and NO release via VEGFR-1. It was suggested that VEGFR-1 negatively regulated proliferation.¹¹ In support of this hypothesis, Herold-Mende and co-workers²⁰ recently demonstrated that stimulation with exogenous VEGF resulted in inhibition of cell proliferation and migration in VEGFR-1-expressing tumor cells. These observations support the notion that VEGF may exert similar functional roles in tumor epithelial cells as in endothelial cells.

In this study we investigated the functional significance of epithelial VEGF receptors using selective blockade of VEGFR-1 and VEGFR-2 in an epithelial carcinoma cell line ECV304²¹ that undergoes tube formation, like endothelial cells, in an *in vitro* assay.²² The interaction between VEGFR-1 and VEGFR-2 was further elucidated to determine whether a negative regulatory mechanism mediated by VEGFR-1 and NO occurs in epithelial cancer cells to regulate VEGFR-2-mediated mitogenesis.

Materials and Methods

Reagents

All cell culture reagents were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). Recombinant VEGF₁₆₅ was purchased from Strathmann Biotech GmbH (Hanover, Germany). All chemical reagents for NO research; sodium nitroprusside (SNP) or *N*-(b-D-glucopyranosyl)-*N*₂-acetyl-S-nitroso-D,L-penicillaminamide (glyco-SNAP-1) and ¹⁴G-monomethyl-L-arginine (L-NNA) and tyrosine kinase inhibitors were purchased from Calbiochem Novabiochem Corporation (Nottingham, UK). The polyclonal VEGF receptor anti-VEGFR-1 and anti-VEGFR-2 antibodies used in this study have been demonstrated to be highly selective and show no cross-reactivity.¹¹ The anti-VEGFR-1 antibody has been demonstrated to neutralize VEGF-mediated monocyte migration, known to be mediated by VEGFR-1¹⁰ whereas the anti-VEGFR-2 antibody had no effect.²³ Conversely, in human umbilical vein endothelial cells and VEGFR-2-overexpressing porcine aortic endothelial cells the anti-VEGFR-2 antibody neutralized VEGF-mediated migration whereas the anti-VEGFR-1 antibody had no effect.²³

Cell Culture

An epithelial carcinoma cell line, ECV304, was purchased from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). The ECV304 cells have recently been shown to be genetically identical to T24/83 a human bladder cancer epithelial cell line.²¹ Cells were grown in medium 199 (ICN) with Hanks' salts and supplemented with 10% fetal bovine serum, 5,000 IU/ml penicillin, 5,000 µg/ml streptomycin, and L-glutamine (200 mmol/L), pH 7.4, in a humidified incubator (37°C, 5% CO₂). Cells were routinely passaged at 90% confluence.

[³H]Thymidine Incorporation

ECV304 cells were plated in 24-well plates in growth medium (H199, 10% fetal calf serum) at a density of 10,000 cells per well. After the cells had grown to 70 to 80% confluence they were rendered quiescent by incubation for 48 hours in serum-free H199, containing 0.2% bovine serum albumin (BSA). Water-soluble VEGF₁₆₅, SNP, or L-NNA were added to the cells at the indicated concentrations and combinations and incubated for 30 hours at 37°C. For the inhibitor studies anti-VEGFR-1 or anti-VEGFR-2 antibodies were added at 30 ng/ml (previously determined as the IC₅₀ value; see Maniotis et al⁷) for 30 minutes before to stimulation with 10 ng/ml VEGF₁₆₅. During the last 6 hours of the 30-hour incubation, cells were labeled with 0.2 µCi/ml [methyl-³H]thymidine (Amersham, Bucks., UK). After incubations, cells were washed with phosphate-buffered saline (PBS), fixed in 5% ice-cold trichloroacetic acid, and washed with 100% ethanol. Cells were lysed in PBS, 0.2% BSA, 1% Triton X-100, and 1 mmol/L NH₄ OH, and incorporated [³H]thymidine measured in a liquid scintillation β counter. Data are expressed as a mean ± SEM of three independent experiments.

Cell Count Assay

ECV304 cells were seeded in 6-well plates at 200,000 cells/well, in growth medium, grown for 24 hours, and serum-starved as above for a further 48 hours. Stimulation's were initiated by addition of VEGF₁₆₅ (1 to 50 ng/ml) or the anti-VEGFR-1 or anti-VEGFR-2 (30 ng/ml) antibodies, and the cells then incubated for 48 hours at 37°C. After incubation the cells were washed with PBS before addition of 1 ml Hepes (1.19 g/L), MgCl₂ (0.153 g/L) solution plus Zapoglobin (Coulter Electronics Ltd., Harpenden, Herts., UK), and a 10-minute incubation at 37°C. Cells were collected in solution and added to 9 ml 0.9% NaCl, 0.05% formalin solution in optically clear pots, and stored at 4°C until counted in a standardized Coulter Counter (Coulter Electronics Ltd.). Three 100-µl aliquots of cell suspension per sample were used to determine the average cell number per well. Results were corrected for the dilution factor and are expressed as a mean ± SEM of three independent experiments performed in triplicate determinations.

Fluorescent-Activated Cell-Sorting (FACS) Analysis

ECV304 cells were seeded in T₂₅ flasks in growth medium. After cells had grown to 70 to 80% confluence, they were rendered quiescent in 0.2% BSA H-199 throughout 24 hours. Cells were incubated with VEGF₁₆₅ at 1 and 10 ng/ml, or the anti-VEGFR-1 or -VEGFR-2 antibodies (30 ng/ml), both alone and in the presence of VEGF₁₆₅ for 24 hours. After incubation, cells were collected and washed with PBS, fixed in 70% ethanol in PBS (4°C, 30 minutes), washed with PBS, and treated with RNase A (200 µg/ml, 37°C, 30 minutes) before staining with propidium iodide (100 µg/ml) and FACS analysis. Data are expressed as a representative of three independent experiments displaying similar trends. Statistical differences are calculated from a mean ± SEM of three independent experiments.

Measurement of NO

ECV304 cells were seeded at 100,000 cells/well in 24-well plates, grown to confluence and rendered quiescent throughout 24 hours. Cells were stimulated with increasing concentrations of VEGF₁₆₅ (1 to 50 ng/ml) in serum-free H199 containing 0.2% BSA. Incubations were performed for 1 hour in a final volume of 0.5 ml at 37°C. For the inhibitor studies, cells were pre-incubated with either 100 µmol/L genestein or increasing concentrations of the anti-VEGFR-1 or anti-VEGFR-2 antibodies for 30 minutes before the addition of VEGF₁₆₅ (50 ng/ml) and incubated further for 60 minutes. Reactions were terminated by removal of the supernatant that was subsequently centrifuged and stored at -80°C for NO analysis. Levels of total NO were measured in the gas phase using a standardized Sievers NOA 280 chemiluminescence analyzer (Analytix, Durham, UK) as previously described.¹¹ Results are corrected for background levels of NO present in culture medium alone, and are expressed as a nmol/L NO/ml and as a mean ± SEM of three independent experiments performed in triplicate determinations.

Results

VEGF Stimulates Dose-Dependent ECV304 Cell Proliferation

We first examined the effect of increasing concentrations of VEGF₁₆₅ on ECV304 cell DNA synthesis by [³H]thymidine incorporation and cell proliferation by cell counting as described in Materials and Methods. Stimulation with VEGF₁₆₅ (1 to 25 ng/ml) caused a significant increase in [³H]thymidine incorporation as compared to control in quiescent ECV304 cells ($P < 0.001$, $n = 3$) (Figure 1A). Maximal stimulation was observed with 2 ng/ml VEGF₁₆₅ that caused a $191.69 \pm 8.7\%$ increase in DNA synthesis. Above this concentration levels of DNA synthesis reached a plateau until the addition of 50 ng/ml VEGF₁₆₅, where VEGF-mediated [³H]thymidine incorporation decreased and showed no significant difference from basal

Figure 1. Effect of VEGF₁₆₅ on ECV304 cell growth. Subconfluent ECV304 cells were serum-starved for 24 hours and stimulations were initiated by the addition of 1 to 50 ng/ml VEGF₁₆₅ followed by a 30-hour (A) or 48-hour (B) incubation at 37°C. DNA synthesis and cell number were assessed as described in Materials and Methods. A: Stimulatory effect of VEGF₁₆₅ on ECV304 DNA synthesis. B: Effect of VEGF₁₆₅ on ECV304 cell number. VEGF induces epithelial ECV304 cell proliferation with an EC₅₀ of 1 ng/ml. Data are expressed as a mean ± SEM of three independent experiments performed in triplicate determinations per experiment. Statistical analysis was performed using the Student's unpaired *t*-test. *, Significant difference as compared to control. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

values. Similarly, addition of low VEGF₁₆₅ concentrations (1 to 10 ng/ml) significantly increased epithelial cell numbers. VEGF stimulated a maximal increase in cell number at 1 ng/ml VEGF₁₆₅ ($182.57 \pm 4.12\%$, $P < 0.0001$, $n = 3$) whereas higher concentrations of VEGF (25 to 50 ng/ml) demonstrated no significant difference from control (Figure 1B). These results demonstrate that VEGF acts as a potent mitogen in the epithelial cell line ECV304 with an EC₅₀ value of 1 ng/ml.

Effect of Blockade of the VEGF Receptors on Basal ECV304 Cell Proliferation

Neutralization of VEGFR-1 by the anti-VEGFR-1 antibody (hatched bars) stimulated a significant increase ($198.97 \pm 23.5\%$ above basal) in [³H]thymidine incorporation in ECV304 cells ($P < 0.01$, versus control, $n = 3$) (Figure 2A). Confirmation that blockade of VEGFR-1 stimulated cell proliferation was assessed by cell counting and showed a $123.74 \pm 2.67\%$ increase above control

Figure 2. Blockade of the VEGFR-1 receptor stimulates ECV304 cell proliferation. Subconfluent quiescent ECV304 cells were incubated with 20 or 30 ng/ml anti-VEGFR-1 (hatched bars) or anti-VEGFR-2 (solid bars), for 30 hours (A) or 48 hours (B) and DNA synthesis and cell number assessed. **A:** Effect of neutralization of VEGFR-1 and VEGFR-2 on ECV304 DNA synthesis. **B:** Effect of neutralization of VEGFR-1 and VEGFR-2 on ECV304 cell number. Addition of anti-VEGFR-1 antibody caused a significant increase above basal levels (white bar) in [³H]thymidine incorporation and cell number. Data are expressed as the mean $\pm$ SEM of three independent experiments assayed in triplicate. Statistical analysis was performed using the Student's unpaired *t*-test. *, Significant difference as compared to control. **, $P < 0.01$; ***, $P < 0.0001$.

($P < 0.0001$, $n = 3$) (Figure 2B). In contrast, blockade of VEGFR-2 (solid bars) had no significant effect on either [³H]thymidine incorporation or cell numbers as compared to control (Figure 2).

Blockade of VEGFR-2 Inhibits VEGF-Mediated ECV304 Cell DNA Synthesis

To further investigate the relative roles of the VEGF receptors on epithelial cell growth, VEGF-induced DNA synthesis was studied in the absence or presence of the neutralizing anti-VEGFR-1 or anti-VEGFR-2 antibodies. Subconfluent quiescent ECV304 cells were pre-incubated for 30 minutes with 30 ng/ml of anti-VEGFR-1 (hatched bars) or anti-VEGFR-2 (gray bars) antibody before a 30-hour incubation with 1 ng/ml VEGF₁₆₅ (Figure 3). Addition of VEGF₁₆₅ (1 ng/ml) stimulated a highly significant increase ($204.98 \pm 15.56\%$) in incorporated [³H]thymidine in ECV304 cells (black bar, $P < 0.001$ versus control, $n = 3$; Figure 3). As before, under basal conditions in the absence of exogenous VEGF, neutralization of the VEGFR-1 receptor by the anti-VEGFR-1

Figure 3. Effect of blockade of VEGFR-1 or VEGFR-2 on VEGF-mediated ECV304 DNA synthesis. Subconfluent quiescent ECV304 cells were preincubated for 30 minutes with neutralizing anti-VEGFR-1 or anti-VEGFR-2 (30 ng/ml) antibodies before stimulation with 1 ng/ml VEGF₁₆₅ and a 30-hour incubation. DNA synthesis was assessed as before. Neutralization of VEGFR-2 inhibited VEGF-mediated DNA synthesis whereas the anti-VEGFR-1 antibody had no inhibitory effect. Data are expressed as the mean $\pm$ SEM of three independent experiments assayed in triplicate. Statistical analysis was performed using the Student's unpaired *t*-test. In the presence of anti-VEGFR-1 an asterisk indicates a significant difference as compared to control, in all other cases comparisons were made with VEGF stimulation. ***, $P < 0.0001$.

antibody stimulated a highly significant increase ($366.67 \pm 31.49\%$) in DNA synthesis ($P < 0.0001$ versus control, $n = 3$) whereas the anti-VEGFR-2 antibody had no effect (Figure 3). In contrast in the presence of VEGF₁₆₅ (1 ng/ml), the anti-VEGFR-1 antibody had no effect on VEGF-mediated [³H]thymidine incorporation, whereas the anti-VEGFR-2 antibody completely attenuated the VEGF-mediated increase in DNA synthesis ($P < 0.0001$ versus VEGF 1 ng/ml, $n = 3$; Figure 3). These results conclusively demonstrate that VEGF stimulates epithelial ECV304 cell proliferation via VEGFR-2. Moreover it is suggested that neutralization of the VEGFR-1 results in a removal of a signaling pathway that negatively regulates cell proliferation.

Blockade of VEGFR-1 Potentiates VEGF-Mediated Cell-Cycle Progression

FACS analysis was performed to further investigate the effect of VEGF receptor subtype blockade on cell-cycle progression. Quiescent cells were incubated with VEGF₁₆₅, or with anti-VEGFR-1 or anti-VEGFR-2 (30 ng/ml) antibodies in the absence or presence of VEGF₁₆₅ for 24 hours (Figure 4). VEGF₁₆₅ stimulated a significant increase in cell number and caused a mean increase in S-phase cells of $180 \pm 4.5\%$ as compared to the control ($P < 0.001$, $n = 3$). Likewise, blockade of VEGFR-1 increased the proportion of cells in S-phase cells by $135.75 \pm 1.03\%$ ($P < 0.001$ versus control, $n = 3$). Moreover, the anti-VEGFR-1 antibody in the presence of VEGF₁₆₅ induced a further shift in cell-cycle progression from S to G₂ phase. The blockade of VEGFR-1 pathway by anti-VEGF-1 antibody resulted in the potentiation of VEGF-mediated increase in G₂ phase cells (1 ng/ml VEGF $31.28 \pm 11.49\%$ and 10 ng/ml VEGF $39.78 \pm 17.13\%$) in comparison to equivalent concentrations of VEGF₁₆₅ alone. In contrast, neutralization of VEGFR-2 inhibited cell-cycle progression from G₁- to S-phase cells

Figure 4. VEGF and neutralization of VEGFR-1 promote cell-cycle progression. Representative cell-cycle analysis of DNA content, measured by propidium iodide staining and FACS analysis as described in Materials and Methods. Quiescent ECV304 cells were incubated with VEGF₁₆₅ at 1 or 10 ng/ml or the anti-VEGFR-1 or anti-VEGFR-2 antibodies 30 ng/ml both alone and in the presence of VEGF₁₆₅ for 24 hours. Data are expressed as a representative of three independent experiments demonstrating similar results and as a percentage of G₁ (white bars), S (hatched bars), or G₂ (solid bars) phase cells over the total cell number of each sample.

leading to an accumulation of cells in G₁ phase ($74.36 \pm 9.35\%$, $P < 0.001$, versus control G₁, $n = 3$) (Figure 4). These results show that the anti-VEGFR-2 antibody inhibits VEGF-mediated cell-cycle progression from G₁ to S phase whereas conversely the anti-VEGFR-1 antibody promotes this transition. This effect may be caused by potentiation of VEGF-VEGFR-2 interaction or it may be that neutralization of VEGFR-1 results in removal of a pathway governing S-phase entry and cell proliferation.

VEGF Stimulates NO Release via VEGFR-1 in ECV304 Cells

We have previously demonstrated that VEGF stimulates NO release via VEGFR-1 in a human trophoblast cell line.⁷ To investigate if this mechanism and result of receptor activation was the same in epithelial cells confluent quiescent ECV304 cells were first incubated with 1 to 50 ng/ml VEGF₁₆₅ for 1 hour and the media collected for NO analysis. Stimulation of the ECV304 cell line with 1 to 25 ng/ml VEGF₁₆₅ had little or no effect on NO release as compared to control levels (Figure 5A). In contrast a

Figure 5. VEGF stimulates dose-dependent NO release via VEGFR-1 in ECV304 cells. **A:** Dose-dependent VEGF-mediated NO release in ECV 304 cells. Confluent quiescent ECV304 cells were incubated with increasing concentrations (1 to 50 ng/ml) of VEGF₁₆₅ for 1 hour. The media was collected and levels of total NO assessed by chemiluminescent analysis as described in Materials and Methods. **B:** Inhibition of VEGF-mediated NO release by genistein. For the inhibitor studies cells were pre-incubated for 30 minutes with a tyrosine kinase inhibitor 100 μmol/L genistein (B, gray bars) or with increasing concentrations of the anti-VEGFR-1 (C, gray and hatched bars) or anti-VEGFR-2 (D, spotted and cross-hatched bars) antibodies before the addition of 50 ng/ml VEGF₁₆₅ (hatched bars) for a further 1 hour. **C:** Inhibition of VEGF-stimulated NO release by neutralization of VEGFR-1. **D:** Potentiation of VEGF-stimulated NO release by neutralization of VEGFR-2. The results are expressed as nmol/L NO per well, corrected for background levels of NO and as a mean SEM of three independent experiments performed in triplicate determinations. Statistical analysis was performed using the unpaired Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

further increase in VEGF₁₆₅ concentration to 50 ng/ml mediated a significant $231 \pm 32.4\%$ increase in release of NO ($P < 0.001$ versus control, $n = 3$) (Figure 5A). For the inhibitor studies ECV304 were pre-incubated with genestein or the neutralizing anti-VEGFR-1 or anti-VEGFR-2 antibodies before stimulation with 50 ng/ml VEGF as detailed in Materials and Methods. Pre-incubation with 100 $\mu\text{mol/L}$ genestein inhibited VEGF-stimulated NO release by $71.95 \pm 6.6\%$ ($P < 0.001$, versus VEGF, $n = 3$) (Figure 5B). More interestingly, pre-incubation of cells with the anti-VEGFR-1 antibody resulted in complete inhibition of VEGF-mediated NO release ($P < 0.0001$ versus VEGF, $n = 3$), (anti-VEGFR-1: 50 ng/ml, $103.78 \pm 1.47\%$; 100 ng/ml, $96.0 \pm 2.19\%$; 250 ng/ml, 99.02 ± 1.25) (Figure 5C). The anti-VEGFR-1 antibody alone had no stimulatory effect on basal levels of NO in the culture medium. In contrast, pre-incubation with the anti-VEGFR-2 antibody alone stimulated a significant increase above control levels comparable to levels observed on stimulation with VEGF₁₆₅ alone ($289 \pm 25\%$, $P < 0.0001$ versus control, $n = 3$) (Figure 5D). Moreover, blockade of VEGFR-2 had no inhibitory effect on VEGF₁₆₅-mediated NO release and at higher anti-VEGFR-2 antibody concentrations (100 to 250 ng/ml), there was a 37% potentiation of VEGF-mediated NO release (Figure 5D). These results demonstrate that VEGF stimulates NO release via tyrosine kinase-dependent activation of VEGFR-1 in the epithelial ECV304 cell line.

NO Suppresses VEGF-Stimulated ECV304 Cell Proliferation

To investigate whether the growth-suppressive property of VEGFR-1 could be attributable to release of NO via VEGFR-1, DNA synthesis was first assessed in ECV304 cells incubated with the anti-VEGFR-1 antibody (30 ng/ml) alone (solid bar) and in the presence of NO donors. Neutralization of the VEGFR-1 receptor stimulated a significant increase in epithelial cell DNA synthesis ($P < 0.001$, $n = 3$) as compared to control (Figure 6A). Addition of SNP (hatched bars) in the presence of the anti-VEGFR-1 antibody significantly attenuated the observed increase in [³H]thymidine incorporation by 83.34% for 10^{-6} mol/L ($P = 0.0055$, versus anti-VEGFR-1, $n = 3$) and by 93.1% for 10^{-4} mol/L ($P = 0.002$, $n = 3$). Similarly, the addition of glyco-SNAP-1 (cross hatched bars) (10^{-6} mol/L) caused a 64.3% reduction in [³H]thymidine incorporation as compared to anti-VEGFR-1 antibody alone ($P = 0.0116$, $n = 3$) and a higher concentration (10^{-4} mol/L) inhibited DNA synthesis to control levels demonstrating a 109.8% reduction ($P = 0.0003$, $n = 3$; Figure 6A). These results show that addition of exogenous NO suppresses epithelial cell proliferation induced by neutralization of VEGFR-1.

Finally to investigate whether VEGF-mediated NO release was down-regulating VEGF-mediated ECV304 cell proliferation, cells were either stimulated with 1 ng/ml VEGF₁₆₅ alone, and in the presence of increasing concentrations of SNP (hatched bars) (Figure 6B), or with 50 ng/ml VEGF alone, and in presence of increasing con-

Figure 6. Effect of NO donors and NO inhibitors on VEGF-mediated epithelial DNA synthesis. **A:** Exogenous NO suppresses epithelial cell proliferation stimulated by neutralization of VEGFR-1. Subconfluent quiescent ECV304 cells were incubated for 30 hours with anti-VEGFR-1 antibody (30 ng/ml) alone (solid bar), and in the presence of the NO donors, SNP (hatched bars), or Glyco-SNAP-1 (cross hatched bars) (10^{-6} and 10^{-4} mol/L) and DNA synthesis assessed as before. **B:** Inhibitory effect of exogenous NO on VEGF-mediated epithelial cell proliferation. Quiescent ECV304 cells were incubated with increasing concentrations of SNP (hatched bars) in the presence of VEGF₁₆₅ (1 ng/ml). Solid bar, VEGF (1 ng/ml) alone. **C:** Inhibition of VEGF-mediated NO release by L-NNA stimulates ECV304 DNA synthesis. Quiescent ECV304 cells were incubated with increasing concentrations of L-NNA (cross-hatched bars) in the presence of VEGF₁₆₅ (50 ng/ml). The solid bar in this case shows VEGF (50 ng/ml) alone. DNA synthesis was assessed by addition of 0.2 $\mu\text{Ci/ml}$ [³H]thymidine for the last 6 hours of a 30-hour incubation. Data are expressed as the mean $\pm$ SEM of three independent experiments assayed in triplicate. Statistical analysis was performed using the Student's unpaired *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

centrations of L-NNA (Figure 6C). VEGF₁₆₅ 1 ng/ml caused a doubling in [³H]thymidine incorporation (solid bar) whereas 10 nmol/L SNP alone (gray bar) had no effect as compared to control (white bar) (Figure 6B). However, in the presence of 1 ng/ml VEGF, increasing SNP concentrations attenuated VEGF-mediated ECV304 DNA synthesis to control levels (Figure 6B). Low picomolar SNP concentration demonstrated no effect on VEGF-mediated [³H]thymidine incorporation although an increase in nanomolar and micromolar SNP concentration significantly attenuated VEGF-mediated DNA synthesis by $61.65 \pm 8.3\%$ and $97.89 \pm 7.98\%$, respectively ($P < 0.001$, versus VEGF 1 ng/ml, $n = 3$) (Figure 6B).

In contrast, incubation of the ECV304 cells with 50 ng/ml VEGF₁₆₅ (the concentration that stimulated maximal NO release), or 1 μ mol/L L-NNA had no proliferative effect as compared to control (solid bar) (Figure 6C). In contrast, in the presence of 50 ng/ml increasing concentrations of L-NNA VEGF₁₆₅ (cross hatched bars) stimulated a highly significant increase in ECV304 DNA synthesis (10 nmol/L L-NNA: $158.06 \pm 7.96\%$; 1 μ mol/L L-NNA: $184.94 \pm 17.41\%$) as compared to VEGF stimulation alone ($P < 0.0001$, $n = 3$) (Figure 6C). Cell viability was also assessed by trypan blue exclusion and the concentrations of SNP, glyco-SNAP-1, and L-NNA used in this study shown to not to affect cell viability. These findings identify a dose-dependent reciprocal mechanism for VEGF acting via VEGFR-2 and VEGFR-1 to regulate epithelial cell proliferation.

Discussion

This study demonstrates through selective blockade of the VEGFR-1 and VEGFR-2 receptors that VEGF stimulates ECV304 cell proliferation via VEGFR-2, and that VEGFR-1 negatively regulated this effect via the production of NO. Hence, VEGF may fulfil a fundamental role in facilitating tumor proliferation and tissue organogenesis through dual epithelial and endothelial effects. Moreover, the addition of exogenous NO suppressed VEGF-mediated ECV304 cell proliferation, whereas inhibition of VEGF-mediated NO restored the proliferative activity of VEGF. We conclude that the VEGFR-1 receptor plays an important role in regulating VEGF-mediated epithelial growth via NO, leading to cell-cycle arrest and as such is a potential target of therapeutic intervention targeting cell growth.

VEGF has previously been reported to stimulate proliferation of dendritic antigen-presenting cells⁸ uterine smooth muscle cells,⁹ tubular epithelial cells,¹³ and Capan-1 cells.¹⁸ In this study, we conclusively demonstrate that VEGF stimulates epithelial ECV304 cell proliferation. Moreover, using a neutralizing anti-VEGFR-2 antibody we show that VEGFR-2 mediates the proliferative effect of VEGF. A further study has shown that VEGF mediates an increase in phosphorylated extracellular signal regulated kinase (ERK) associated with VEGFR-2 immunoprecipitates in the ECV304 cell line and inhibition of ERK activity resulted in the attenuation of VEGF-mediated DNA synthesis.²⁴ It is well known that VEGF promotes endothelial

cell proliferation via the VEGFR-2.²⁵ VEGF stimulated DNA synthesis in VEGFR-2-transfected porcine aortic endothelial cells but had no biological effect on VEGFR-1-transfected cells.^{26,27} To our knowledge this is the first demonstration of a specific role for the VEGFR-2 receptor in promoting epithelial cell proliferation.

In the present study, we show that blockade of the VEGFR-1 signal using a neutralizing anti-VEGFR-1 antibody potentiated both basal and VEGF-mediated epithelial cell growth. This indicates that the VEGFR-1 mediates a negative regulatory pathway controlling VEGFR-2-mediated cell proliferation. We reported a similar role for the VEGFR-1 in a trophoblast cell line of placental origin where the addition of exogenous VEGF stimulated NO release through VEGFR-1 whereas inhibition of VEGFR-1 promoted DNA synthesis.¹¹ VEGF was also reported to stimulate NO release in cultured human umbilical vein endothelial cells¹¹ and intact arterial strips,²⁸ and we have recently shown that this NO release was mediated by the specific activation of VEGFR-1 in endothelial cells.²³ In this study, addition of 50 ng/ml of VEGF resulted in maximal release of NO from ECV304 cells. Furthermore, the neutralization of VEGFR-1 by the anti-VEGFR-1 antibody completely attenuated the observed VEGF-mediated NO release in these epithelial cells. In contrast, neutralization of the VEGFR-2 by the anti-VEGFR-2 antibody potentiated both endogenous and exogenous VEGF-mediated NO release. These data conclusively demonstrate a specific biological role of the VEGFR-1 in mediating VEGF-stimulated NO release in epithelial ECV304 cells.

A recent study showed that transfection of metastatic murine K-1735 cells with inducible NOS was associated with cytosclerosis or cytolysis via apoptosis depending on NO output.²⁹ Moreover the NO-generating anti-anginal vasodilators, isorbide mononitrate and dinitrate, inhibited *in vivo* angiogenesis in the chick chorioallantoic membrane assay³⁰ and inhibited tumor growth and metastasis in mice implanted with lung Lewis carcinoma cells.³¹ In the present study, the dose-dependent VEGF-mediated NO release displayed an inverse correlation with the observed concentration-dependent effect of VEGF on ECV304 proliferation. This suggests that VEGFR-1-mediated

Dose Dependant Activation of the VEGF receptors

Figure 7. A schematic representation of the proposed reciprocal regulatory signaling mechanisms mediated by VEGF and its two receptors VEGFR-1 and VEGFR-2.

ated NO was acting as a negative regulator of cell growth. Addition of NO donors in the presence of 1 ng/ml VEGF suppressed VEGF-mediated DNA synthesis in the ECV304 cell line. In addition, NO donors antagonized DNA synthesis induced by the inhibition of VEGFR-1 by the anti-VEGFR-1 antibody demonstrating that NO acts as a negative regulator of ECV304 cell growth. NO donors have similarly been reported to inhibit mRNA encoding both VEGF and its receptors,³² and to inhibit cell proliferation in endothelial,³³ trophoblast,¹¹ and vascular smooth muscle cells.³⁴ Interestingly the concentration of NO donors used in this study has been reported to induce apoptosis in endothelial cells,³⁵ however the ECV304 cell line did not display any signs of cell death as assessed by trypan blue exclusion assay. This may be explained by the observation that ECV304 cells are tumor cell line and express a relatively high basal activity of NOS,²¹ and therefore may possess a higher tolerance for NO levels than primary endothelial cells.

Studies using NO synthase inhibitors further support the role of NO as an endogenous inhibitor of the angiogenic process.³⁶ The inhibitors N^G-monomethyl-L-arginine and nitro-L-arginine methyl ester caused a significant stimulation in angiogenesis whereas the NO donors SNP and superoxide dismutase inhibited thrombin-induced angiogenesis in the chick chorioallantoic membrane assay.³⁶ In this study, inhibition of maximal VEGF-mediated NO release (50 ng/ml) by the addition of L-NNA restored the proliferative effect of VEGF in the ECV304 cell line. We conclude that increasing levels of NO released via VEGFR-1 by relatively high VEGF concentration acts to down-regulate VEGFR-2-mediated epithelial cell proliferation. This data supports the hypothesis that low concentrations of NO can be pro-angiogenic and protumor growth, whereas high concentrations can have the opposite effect.³⁷ Although NO was also demonstrated to positively contribute to the angiogenic properties of VEGF^{38,39} these studies did not use a range of VEGF concentrations and therefore were unable to dissect the significance of NO levels to cell growth. Interestingly, gene-targeting studies in embryonic mice revealed that *flt-1*^{-/-} homozygotes showed a disorganization of blood vessels displaying an overgrowth of endothelial cells within the vessel lumens, and died at E8.5 to E9.0.⁴⁰ This genetic study first suggested that VEGFR-1 acts a negative regulator for endothelial cell proliferation, although it was thought that the VEGFR-1 functioned as a trapping molecule sequestering VEGF away from VEGFR-2.⁴¹ In this study we show that a NO-dependent signaling mechanism mediated by the specific activation of VEGFR-1 is responsible for the regulation of VEGF-mediated epithelial cell proliferation.

In conclusion, the VEGFR-1 receptor plays an important role in regulating VEGFR-2-mediated epithelial growth via NO. A model is proposed for VEGF action during tissue development through reciprocal regulatory pathways that are mediated by VEGFR-1 and VEGFR-2 (Figure 7). Perturbation of these pathways in tumor growth may facilitate tumor proliferation through dual epithelial and endothelial effects. It is postulated that overexpression of VEGFR-1 in tumors will inhibit their growth

and as such, VEGFR-1 may provide an additional target for therapeutic intervention in the control of tumor progression.

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