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Relative Effects of VEGF-A and VEGF-C on Endothelial Cell Proliferation, Migration, and PAF Synthesis: Role of Neuropilin-1

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Abstract Vascular endothelial growth factor (VEGF-A) is an inducer of endothelial cell (EC) proliferation, migration, and synthesis of inflammatory agents such as platelet-activating factor (PAF). Recently, neuropilin-1 (NRP-1) has been described as a coreceptor of KDR which potentiates VEGF-A activity. However, the role of NRP-1 in numerous VEGF-A activities remains unclear. To assess the contribution of NRP-1 to VEGF-A mediated EC proliferation, migration, and PAF synthesis, we used porcine aortic EC (PAEC) recombinantly expressing Flt-1, NRP-1, KDR or KDR and NRP-1. Cells were stimulated with VEGF-A, which binds to Flt-1, KDR and NRP-1, and VEGF-C, which binds to KDR only. VEGF-A was 12.4-fold more potent than VEGF-C in inducing KDR phosphorylation in PAEC-KDR. VEGF-A and VEGF-C showed similar potency to mediate PAEC-KDR proliferation, migration, and PAF synthesis. On PAEC-KDR/NRP-1, VEGF-A was 28.6-fold more potent than VEGF-C in inducing KDR phosphorylation and PAEC-KDR/NRP-1 proliferation (1.3-fold), migration (1.7-fold), and PAF synthesis (4.6-fold). These results suggest that cooperative binding of VEGF-A to KDR and NRP-1 enhances KDR phosphorylation and its biological activities. Similar results were obtained with bovine aortic EC that endogenously express both KDR and NRP-1 receptors. In contrast, stimulation of PAEC-Flt-1 and PAEC-NRP-1 with VEGF-A or VEGF-C did not induce proliferation, migration, or PAF synthesis. In conclusion, the presence of NRP-1 on EC preferentially increases KDR activation by VEGF-A as well as KDR-mediated biological activities, and may elicit novel intracellular events. On the other hand, VEGF-A and VEGF-C have equipotent biological activities on EC in absence of NRP-1. J. Cell. Biochem. 85: 629–639, 2002.

Key words: growth factors; VEGF receptors; inflammation; angiogenesis

It is well established that angiogenesis, the sprouting of new blood vessels from the pre-existing vasculature, is a crucial requirement for several physiological conditions, such as wound healing, tissue regeneration, and uterine wall thickening [Folkman and Klagsburn, 1987; Folkman, 1991]. On the other hand, numerous reports have confirmed that angiogenesis is also involved in the pathogenesis of several disorders characterized by the uncontrolled growth of new blood vessels, such as tumor growth, atherosclerosis, proliferative retinopathies, and certain viral infections [Folkman, 1991; Moulton et al., 1999]. By virtue of its multifunctional nature, vascular endothelial growth factor (VEGF-A) is a candidate for the regulation of both physiological and pathological angiogenesis. Initially identified as a tumor-released factor that enhances vascular permeability to circulating macromolecules [Connolly et al., 1989], VEGF-A was subsequently found to promote endothelial cell (EC) morphological shape changes, migration, and proliferation [Unemori et al., 1992]. Recent studies have led to the identification of additional growth factors belonging to VEGF-A family including placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and viral

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homologue VEGF-E that share amino acids homology with VEGF-A [Park et al., 1994; Ogawa et al., 1998; Veikkola and Alitalo, 1999].

Although VEGF and other growth factors, such as acidic and basic fibroblast growth factors and epidermal growth factor, can promote EC migration and proliferation in vitro and angiogenesis in vivo, only VEGF is capable of inducing inflammation [Folkman and Klagsburn, 1987; Connolly et al., 1989; Unemori et al., 1992]. We have previously shown that VEGF-A promotes inflammation in a platelet-activating factor (PAF)-dependent manner in vivo, and that VEGF-A induces the synthesis of PAF by EC in vitro [Sirois and Edelman, 1997]. More recently, we have demonstrated by using an antisense oligonucleotide gene knockdown technique that the activation of VEGF-A tyrosine kinase receptor KDR/Flk-1 alone is sufficient to fully promote EC proliferation, migration, and PAF synthesis, whereas VEGF-A other tyrosine kinase receptor Flt-1 appears to play a very minor role in these events [Bernatchez et al., 1999].

Recent studies have shown that neuropilin-1 (NRP-1), a membrane receptor first described on axons in the developing nervous system [Fujisawa et al., 1997], also acts as a KDR/Flk-1 co-receptor [Soker et al., 1996, 1998]. In vitro, NRP-1 expression on EC was shown to enhance the binding of VEGF-A_{165} (VEGF-A) to KDR and potentiate VEGF-A-induced EC migration in an isoform-specific manner [Soker et al., 1998]. Such selectivity is attributable to the presence of VEGF-A exon 7 in VEGF-A_{165}, a domain that is lacking in VEGF-A_{121} [Soker et al., 1998]. Furthermore, conditional overexpression of NRP-1 by tumor cells in vivo was demonstrated to promote tumor angiogenesis and progression [Miao et al., 2000]. However, the role of NRP-1 in the induction of other VEGF-A-mediated events, such as PAF synthesis and EC proliferation is currently unknown.

In the present study, we used transfected porcine aortic EC (PAEC) expressing either Flt-1, NRP-1, KDR alone or KDR and NRP-1 receptors, as well as bovine aortic EC (BAEC), which express all three VEGF receptors, to investigate the contribution of NRP-1 to VEGF-A-induced EC proliferation, migration, and PAF synthesis. To determine the relative contribution of the different VEGF-A receptors, we used as control VEGF-C which binds to KDR but not to Flt-1, and according to its amino acid sequence should not bind to NRP-1. We herein report that coexpression of NRP-1 with KDR potentiates VEGF-A but not VEGF-C-induced EC proliferation, migration, and PAF synthesis. These data bring new insight on the regulation of VEGF activities in EC.

MATERIALS AND METHODS

Cell Culture

PAEC transfected with VEGF-A receptors cDNA were prepared as described previously [Soker et al., 1998] and cultured in F-12 or Dulbecco's modified eagle medium (DMEM; Life Technologies, Burlington, Canada) containing 5% fetal bovine serum (FBS; Hyclone Lab., Logan, UT), and antibiotics (Sigma Chemicals, St. Louis, MO). BAEC were isolated from freshly harvested aortas, characterized by their cobblestone monolayer morphology, Factor VIII immunohistochemistry and by diiodoindocarbocyanide acetylated LDL uptake, and cultured in DMEM + 5% FBS [Bernatchez et al., 1999].

VEGF-C Versus VEGF-A Binding to NRP-1

The radioiodination of VEGF-A (human VEGF-A_{165}, PeproTech, Rocky Hill, NJ) using IODO-BEADS as well as the binding studies were carried out as previously described [Soker et al., 1998]. Briefly, PAEC-NRP-1 were washed with PBS and supplied with binding buffer (F-12 medium, 25 mM HEPES, pH 7.5, and 0.2% gelatin). Competitive binding of VEGF-C versus ^{125}I-VEGF-A was performed by adding increasing concentrations of VEGF-C (up to 200-fold excess) to ^{125}I-VEGF-A (250 pM) at 4°C for 2 h. The cells were then washed three times with PBS and lysed with 0.2 M NaOH. Samples were collected and cell-associated radioactivity determined with a γ-counter. The experiments were done at least twice and run in triplicate. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled VEGF-A_{165}.

Western Blot Analysis of Flt-1, KDR, and NRP-1 Protein Expression and Phosphorylation

Confluent PAEC lines and BAEC (100-mm tissue culture plate) were rinsed twice with DMEM, and stimulated with PBS, VEGF-A or VEGF-C (1 nM) for various periods of time in DMEM + 1% BSA. Cells were lysed (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1.2% Triton X-100, 1 mM EGTA, 1 mM PMSF, 0.15 U/ml Aprotinin, 10 μg/ml Leupeptin, and 1 mM $NaVO_3$), the plates were scraped and the

protein concentration was determined with a protein assay kit (Bio-Rad). Total proteins (1.2 mg) were immunoprecipitated with an antibody that specifically recognizes either Flt-1, KDR or NRP-1 (Santa Cruz Biotechnologies, Santa Cruz, CA) bound to protein A-sepharose (Amersham Pharmacia Biotech). The immunoprecipitated proteins were separated by a 6% SDS–PAGE gel and transblotted overnight at 0.07 mA (25 mM Tris-Base, 190 mM Glycine, 5% MeOH) onto a Immunobilon-P PVDF membrane (Milipore, Bedford, MA). Membranes were blocked in TTBS buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100) with 3% BSA for 1 h at room temperature. Receptor phosphorylation was determined with the use of an anti-phosphotyrosine antibody (clone 4G10), whereas the presence of Flt-1, KDR or NRP-1 was detected with the use of the antibodies described for immunoprecipitation. The respective antibodies were incubated with the membranes overnight in TTBS + 0.5% BSA. Membranes were washed with TTBS and incubated with an anti-rabbit antibody coupled to horseradish peroxidase (Santa Cruz Biotechnologies). Membranes were washed with TTBS, and horseradish peroxidase was revealed by chemiluminescence. Kaleidoscope prestained standards were used for SDS–PAGE.

Mitogenic Assays

Confluent PAEC and BAEC (100 mm tissue culture plate) were washed twice with 8 ml of DMEM, and trypsinized by using trypsin-EDTA (Life Technologies). Cells were resuspended in 9 ml of DMEM containing 5% FBS and antibiotics, and a cell count was obtained by using a Coulter counter Z1 (Coulter Electronics). Cells were seeded at 10,000 cells/well of 24-well tissue culture plate, stimulated for 24 h in DMEM/5% FBS/antibiotics, and starved for 48 h in DMEM/0.25% FBS/antibiotics for G0 synchronization. The cells were stimulated for 72 h in DMEM/1% FBS/antibiotics with PBS, VEGF-A or VEGF-C (10 pM-1 nM). The cells were then harvested by trypsinization and a cell number was determined using a Coulter counter.

Chemotaxic Assays

Cell migration was evaluated using a microchamber technique (Neuroprobe, Cabin John, MD). Subconfluent PAEC and BAEC (100 mm tissue culture plate) were washed twice with 8 ml of DMEM and trypsinized using trypsin-EDTA. Cells were resuspended in 9 ml of DMEM containing 5% FBS and antibiotics, and a cell count was obtained by using a Coulter counter. The cells were resuspended in DMEM/1% FBS/antibiotics at a concentration of 1×10^6 cells/ml, and 50 μl of this solution was applied to the upper chamber, whereas the lower chamber was filled with DMEM/1% FBS/antibiotics + PBS, VEGF-A or VEGF-C (10 pM–1 nM). The two sections of the system were separated by a polycarbonate filter (5 μm pores) coated with a gelatin solution (1.5 mg/ml), and assembled. Five hours post-incubation at 37°C, the apparatus was disassembled, and the non-migrated cells were scraped with a plastic policeman, the migrated cells were stained using Quick-Diff solutions. The filter was then mounted on a glass slide using immersion oil and migrated cells were counted using a microscope adapted to a video camera in order to obtain a computer-digitized image.

Measurement of PAF Synthesis

PAF production by PAEC and BAEC was measured by incorporation of ^{3}H-acetate into lyso-PAF as described previously [Sirois and Edelman, 1997; Bernatchez et al., 1999]. Briefly, confluent PAEC and BAEC (6-well tissue culture plate) were rinsed with HBSS (Hank's balanced salt solution)/HEPES (10 mM; pH 7.4) and stimulated for 15 min in 1 ml of HBSS-HEPES (10 mM, pH 7.4) + $CaCl_2$ (10 mM) + ^{3}H-acetate (25 μCi) (New England Nuclear, Boston, MA) + PBS, VEGF-A or VEGF-C at various concentrations. The reaction was stopped by addition of acidified methanol and polar lipids were isolated by the Bligh and Dyer method [Bligh and Dyer, 1959]. Isolated lipids were evaporated under a stream of N_2 gas and purified by a silica-based normal-phase HPLC column. Fractions corresponding to ^{3}H-PAF were quantified by counting radioactivity with a β-counter. The authenticity of synthesized ^{3}H-PAF was confirmed by the similar HPLC elution pattern as standard ^{3}H-PAF (New England Nuclear) and by its ability to induce platelet aggregation as standard PAF (Avanti Polar Lipids, Alabaster, AL) [Sirois and Edelman, 1997].

RESULTS

Expression of Flt-1, NRP-1, and KDR in Transfected PAEC and Native BAEC

In order to determine the respective roles of VEGF-A receptors in the induction of VEGF-A

and VEGF-C activities, PAEC, which do not express Flt-1, KDR, or NRP-1, were transfected with either Flt-1, NRP-1, KDR, or KDR plus NRP-1 cDNA, and stable lines expressing the different receptor(s) were obtained as described previously [Soker et al., 1998]. The presence of VEGF-A receptors in native and transfected PAEC and BAEC was then confirmed by Western blot analyses. As expected, Flt-1, KDR, and NRP-1 protein expression was not detected in native PAEC (Fig. 1, lane 1). The PAEC cell line transfected with Flt-1 cDNA (PAEC-Flt-1) expressed high levels of Flt-1 (Fig. 1, lane 2), whereas the PAEC that were transfected with KDR cDNA (PAEC-KDR and PAEC-KDR/NRP-1) expressed KDR protein (Fig. 1, lanes 4 and 5). PAEC transfected with NRP-1 cDNA (PAEC-NRP-1 and PAEC-KDR/NRP-1) showed a stable protein expression of NRP-1 receptor (Fig. 1, lanes 3 and 5).

Experiments were performed on BAEC as well since they possess intracellular pathways found in typical native EC. Though we have shown that BAEC express signaling Flt-1 and KDR [Bernatchez et al., 1999], it is still unknown if BAEC express NRP-1. To address this question, we performed Western blot analyses, which revealed expression of the three VEGF-A receptors in BAEC (Fig. 1, lane 6).

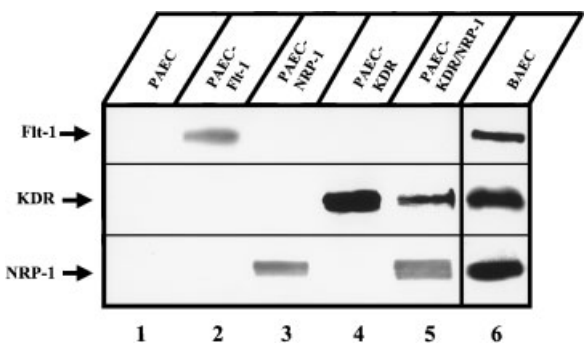

Fig. 1. Western blot analysis of the expression of VEGF receptors in transfected PAEC and native BAEC. Confluent cultures of PAEC, PAEC-Flt-1, PAEC-NRP-1, PAEC-KDR, PAEC-KDR/NRP-1, and native BAEC (lanes 1–6, respectively) were lysed, and immunoprecipitation was performed with antibodies raised against the indicated receptors. The immunoprecipitates were separated on a 7.5% SDS–PAGE gel, and proteins were transblotted overnight onto a PVDF membrane. The membrane was probed with the same antibody used for immunoprecipitation followed by incubation with a secondary antibody coupled to horseradish peroxidase, and revealed by chemiluminescence.

Characterization of VEGF-C Binding to NRP-1

Previous studies have shown that VEGF-A binds to NRP-1 through VEGF-A exon 7-encoded domain [Soker et al., 1998]. As a result, members of VEGF-A family lacking this domain such as VEGF-A_{121} do not bind to NRP-1. However, the binding properties of VEGF-C to NRP-1 have not been analyzed yet. We performed radioligand binding experiments on PAEC-NRP-1 using cold VEGF-C and ^{125}I-VEGF-A to test the binding properties of VEGF-C to NRP-1 transfected cells. As we previously demonstrated, VEGF-A binding to PAEC-NRP-1 increased in a dose-dependent manner and reached saturation at 750 pM [Soker et al., 1998]. Addition of cold VEGF-C from 5 to 50 nM did not have any significant effect on ^{125}I-VEGF-A binding to PAEC-NRP-1 (Fig. 2). This result confirms that VEGF-C is incapable of binding NRP-1.

VEGF-A and VEGF-C-Mediate Flt-1 and KDR Phosphorylation in Transfected PAEC and BAEC

We previously showed that VEGF-A could promote the phosphorylation of Flt-1 in BAEC [Bernatchez et al., 1999]. To confirm the activity of Flt-1 receptor in PAEC-Flt-1, cells were treated with VEGF-A (1 nM) and Flt-1 phosphorylation was determined by Western blot analysis (Fig. 3A). VEGF-A elicited a 3.1-fold increase in the phosphorylation of Flt-1 in PAEC-Flt-1 as compared to PBS-treated cells, which confirms the tyrosine kinase activity of Flt-1 receptor in PAEC-Flt-1. VEGF-C effect

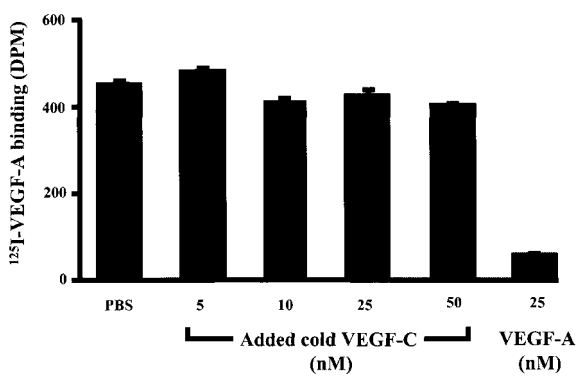

Fig. 2. Competitive binding of VEGF-C and VEGF-A to NRP-1. PAEC-NRP-1 cells were incubated with 250 pM of ^{125}I-VEGF-A_{165} and with increasing concentrations of VEGF-C (5–50 nM) or unlabeled $VEGF_{165}$ (25 nM) as described in Materials and Methods. The cells were washed, lysed, and the cell-associated radioactivity was determined using a γ counter.

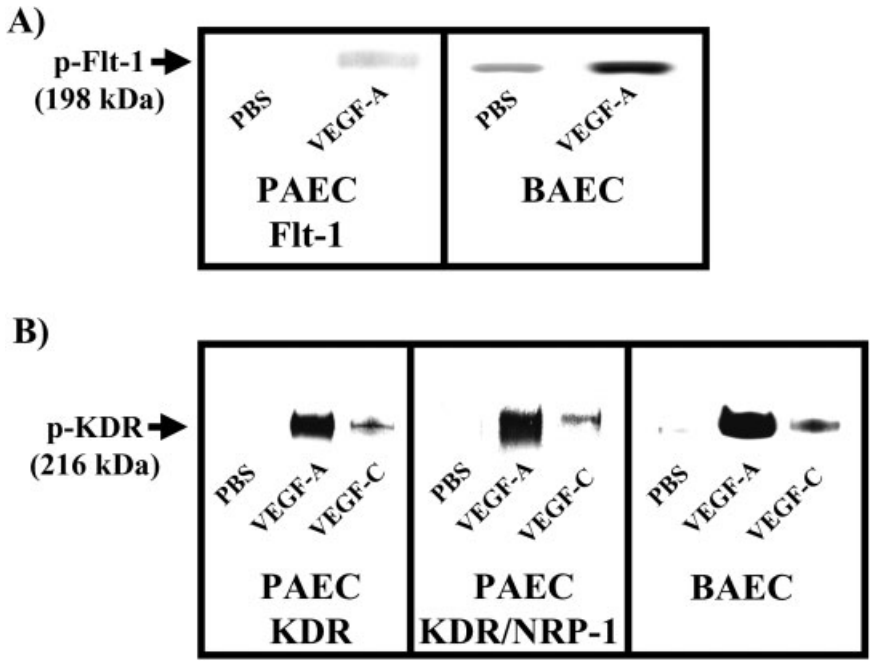

Fig. 3. Effect of VEGF-A and VEGF-C on Flt-1 and KDR phosphorylation in transfected PAEC and native BAEC. **A**: Confluent PAEC-Flt-1 or BAEC were stimulated with VEGF-A (1 nM) for 7 min and lysed. Immunoprecipitation using anti-Flt-1 antibodies was performed, and the phosphorylated form (p-Flt-1) was revealed by Western blot with an anti-phosphotyrosine antibody (clone 4G10). **B**: Confluent PAEC-KDR, PAEC-KDR/NRP-1 or BAEC were stimulated with VEGF-A and VEGF-C (1 nM) for 7 min, and then lysed. Immunoprecipitation using anti-KDR antibodies was detected as described in (A), and KDR phosphorylation (p-KDR) was determined by Western blot analysis as described in (A).

was not investigated because of its inability to bind to Flt-1 [Joukov et al., 1997; Veikkola and Alitalo, 1999].

Next, we investigated the effect of VEGF-A and VEGF-C on KDR phosphorylation. In PAEC-KDR, both VEGF-A and VEGF-C treatment increased KDR phosphorylation as compared to PBS-treated cells, though VEGF-A was 12.4-fold more potent than VEGF-C (Fig. 3B). Coexpression of NRP-1 with KDR in PAEC-KDR/NRP-1 potentiated the effect of VEGF-A, but not of VEGF-C, on KDR phosphorylation. Western blot analyses revealed that VEGF-A was 28.6-fold more potent than VEGF-C to elicit the phosphorylation of KDR in PAEC-KDR/NRP-1 (Fig. 3B). Finally, as expected, neither VEGF-A nor VEGF-C induced the autophosphorylation of NRP-1 as this receptor does not present tyrosine kinase activity (data not shown).

To confirm that coexpression of KDR and NRP-1 in native EC preferentially potentiates the effect of VEGF-A but not VEGF-C on KDR phosphorylation, we used native non-transfected BAEC that endogenously express all three VEGF-A receptors. Treatment of BAEC with VEGF-A (1 nM) increased by 31.8-fold the phosphorylation of KDR as compared to PBS-treated cells, whereas VEGF-C (1 nM) was less potent, elevating KDR phosphorylation by 5.1-fold (Fig. 3B).

VEGF-A and VEGF-C Effect on PAEC and BAEC Proliferation

As VEGF-A and VEGF-C appear to have different potency to elicit Flt-1 and KDR phosphorylation, we investigated their relative mitogenic effect on PAEC. Stimulation of quiescent PAEC with 1% FBS increased EC number of all five PAEC transfected cell lines within 72 h (Fig. 4A). The addition of VEGF-A or VEGF-C (1 nM) to PAEC, PAEC-Flt-1 and PAEC-NRP-1 at day 0 did not significantly alter the basal proliferation of these EC lines. In contrast, VEGF-A and VEGF-C (1 nM) significantly increased the proliferation of PAEC-KDR by 47 and 30%, respectively, and the proliferation of PAEC-KDR/NRP-1 by 81 and 48%, respectively, as compared to control cells treated with 1% FBS. No statistically significant differences were observed between the mitogenic effect of VEGF-A and VEGF-C on PAEC-KDR, whereas on PAEC-KDR/NRP-1, VEGF-A was significantly more potent than VEGF-C to elicit EC proliferation ($P < 0.05$) (Fig. 4A).

Next, we performed proliferation experiments on BAEC that endogenously express VEGF-A receptors including NRP-1 to compare the relative mitogenic potential of VEGF-A and VEGF-C. Stimulation of quiescent BAEC with 1% FBS increased BAEC cell count from $7{,}400 \pm 960$ to $8{,}280 \pm 820$ within 72 h. The

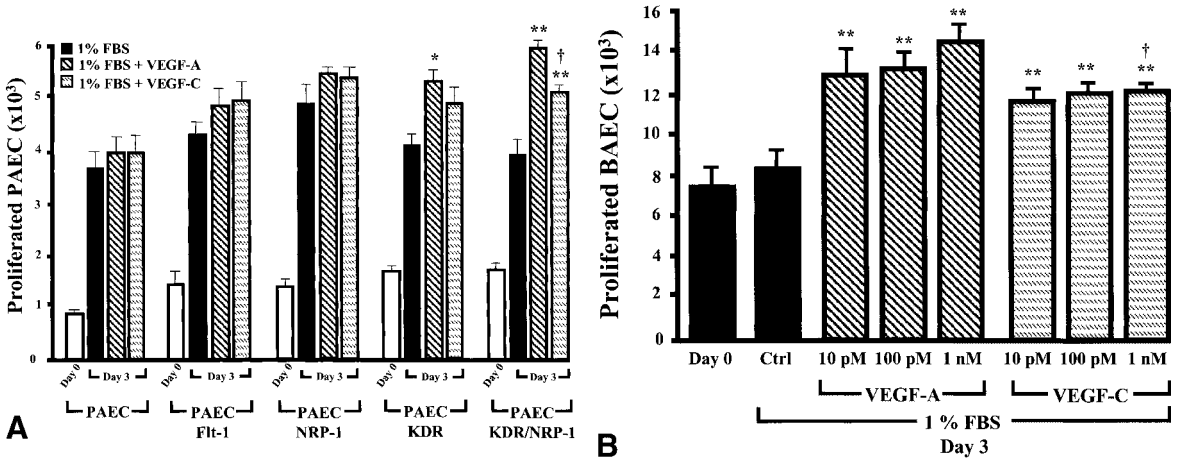

Fig. 4. VEGF-A and VEGF-C-induced proliferation of transfected PAEC and native BAEC. **A**: Quiescent PAEC, PAEC-Flt-1, PAEC-NRP-1, PAEC-KDR, and PAEC-KDR/NRP-1 (1×10^4 cells/well of a 24-well plate) were cultured in 1% FBS and stimulated with VEGF-A or VEGF-C (250 pM), and a cell count was performed 72 h post-treatment. The values are means of cell count obtained from 10 wells for each treatment. $^*P < 0.05$ and $^{**}P < 0.01$ as compared to PBS treatment, and $\dagger P < 0.05$ as compared to VEGF-A treatment. **B**: Quiescent native BAEC were stimulated with various concentrations of VEGF-A or VEGF-C (10 pM–1 nM) for 72 h as described in (A). $^*P < 0.05$ $^{**}P < 0.01$ as compared to PBS treatment, and $\dagger P < 0.05$ as compared to a treatment with a similar concentration of VEGF-A.

application of VEGF-A (10 pM, 100 pM, and 1 nM) dose-dependently increased BAEC proliferation with maximal inductions of 514, 553, and 668%, respectively. VEGF-C (10 pM, 100 pM, and 1 nM) was weaker than VEGF-A at promoting BAEC proliferation, increasing cell count by 391, 416, and 422% as compared to control (1% FBS). At the highest concentration used (1 nM), VEGF was significantly more potent than VEGF-C in mediating cell proliferation ($P < 0.05$) (Fig. 4B).

VEGF-A and VEGF-C Effect on PAEC and BAEC Migration

We also investigated the effect of VEGF-A and VEGF-C on the migration of native EC and transfected PAEC. Previously-reported checkerboard analyses have revealed that EC migration mediated through KDR activation is a result of chemotaxis and not chemokinesis [Bernatchez et al., 1999]. Stimulation of PAEC, PAEC-Flt-1, and PAEC-NRP-1 with either VEGF-A or VEGF-C (1 nM) did not increase their basal migration level. In contrast, VEGF-A and VEGF-C (1 nM) significantly increased the number of migrating PAEC-KDR cells by 40 and 42%, respectively, as compared to control levels, raising the migrated cell count from 64 ± 6.4 cells/mm^2 to 90 ± 3.2 and 91 ± 4.1 cells/mm^2, respectively (Fig. 5A). Cotransfection of NRP-1 with KDR potentiated VEGF-A, but not VEGF-C chemotactic activity. VEGF-A increased the migration of PAEC-KDR-NRP-1 cells by 77% as compared to a 45% increase mediated by VEGF-C (Fig. 5A). No statistically significant differences were observed between the chemotactic effect of VEGF-A and VEGF-C on PAEC-KDR, whereas on PAEC-KDR/NRP-1, VEGF-A was significantly more potent than VEGF-C to elicit EC migration ($P < 0.01$) (Fig. 5A).

The relative potency of VEGF-A and VEGF-C to promote the migration of BAEC was also investigated (Fig. 5B). VEGF-A (10 pM, 100 pM, and 1 nM) induced a dose-dependent increase (49, 69, and 92%) of BAEC migration as compared to PBS-treated cells, raising the migrated cell count from 208 ± 17.6 to 400 ± 12 cells/mm^2, whereas VEGF-C (10 pM, 100 pM, and 1 nM) displayed a weaker chemotactic potential, inducing a dose-dependent increase (17, 61, and 69%) in BAEC migration (Fig. 5B). At different concentrations used (10 pM and 1 nM), VEGF was significantly more potent than VEGF-C in mediating cell proliferation ($P < 0.01$ and 0.05, respectively).

VEGF-A and VEGF-C Effect on PAEC and BAEC PAF Synthesis

We have previously shown that VEGF-A elicited the synthesis of PAF from various EC

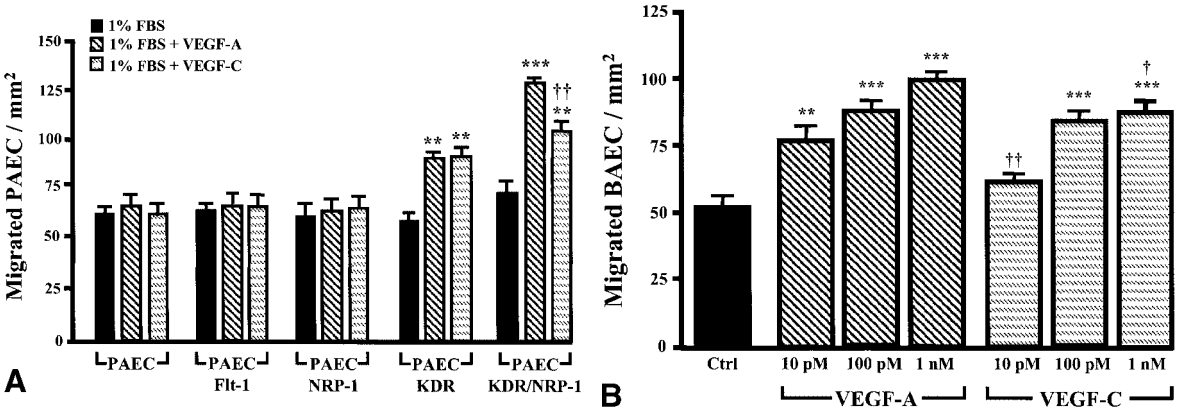

Fig. 5. Chemotactic effect of VEGF-A and VEGF-C on transfected PAEC and native BAEC: **A**: PAEC, PAEC-Flt-1, PAEC-NRP-1, PAEC-KDR, and PAEC-KDR/NRP-1 were trypsinized, resuspended in DMEM/1% FBS, and 5×10^4 cells were added in the top chamber of a modified Boyden chamber apparatus and the lower chamber was filled with DMEM/1% FBS ± VEGF-A or VEGF-C (1 nM). Five hours (5 h) post-incubation at 37°C, the migrated cells were stained and counted using a light microscope. The values are means of six cell counts per mm^2. $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to PBS treatment, and $\dagger\dagger P < 0.01$ as compared to VEGF-A treatment. **B**: BAEC were stimulated with various concentrations of VEGF-A or VEGF-C (10 pM–1 nM) as described in (A). $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to PBS treatment, and $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$ as compared to a treatment with a similar concentration of VEGF-A.

types and that KDR activation was crucial for this activity [Sirois and Edelman, 1997; Bernatchez et al., 1999]. In this study, we examined the role of NRP-1 in VEGF-mediated PAF synthesis (Fig. 6). Treatment of native PAEC, PAEC-Flt-1, and PAEC-NRP-1 with either VEGF-A or VEGF-C did not significantly induce PAF synthesis (Fig. 6A). In contrast, VEGF-A and VEGF-C were equipotent in promoting PAF synthesis in PAEC-KDR, elevating basal PAF levels by 97 and 95% (from 717 ± 298 DPM to 1,414 ± 264 and 1,401 ± 148 DPM), respectively (Fig. 6A). Interestingly, VEGF-A was 5.3-times more potent than VEGF-C to elicit PAF synthesis in PAEC-KDR/NRP-1, increasing basal PAF levels by 467% as compared to 94% ($P < 0.001$). No statistically significant differences were observed between the effect of VEGF-A and VEGF-C on PAEC-KDR PAF synthesis, whereas on PAEC-KDR/NRP-1, VEGF-A was significantly more potent than VEGF-C ($P < 0.001$) (Fig. 6A).

Similarly, we investigated the respective potency of VEGF-A and VEGF-C to elicit PAF synthesis by BAEC (Fig. 6B). We first observed a basal PAF synthesis by BAEC (548 ± 74 DPM).

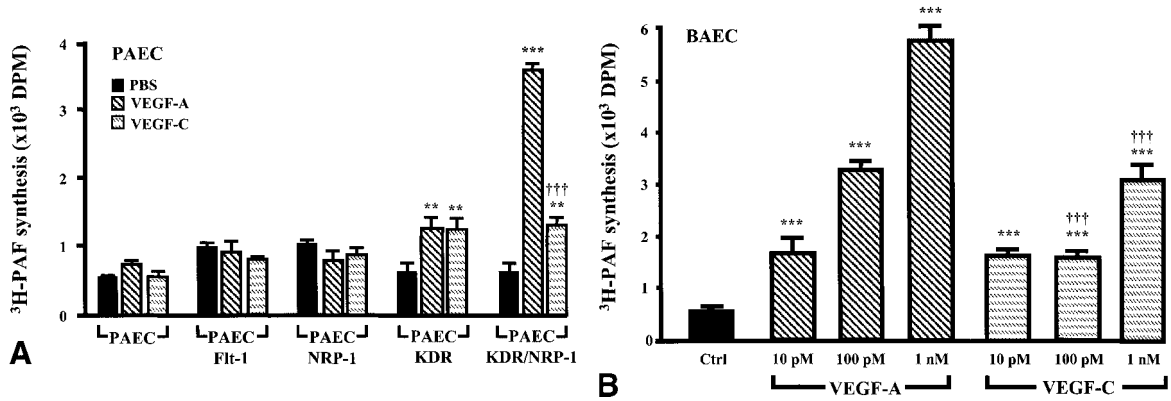

Fig. 6. PAF synthesis assay on transfected PAEC and native BAEC. **A**: Confluent PAEC, PAEC-Flt-1, PAEC-NRP-1, PAEC-KDR, and PAEC-KDR/NRP-1 (6-well tissue culture plate) were stimulated with VEGF-A or VEGF-C (1 nM) + [^{3}H]-acetate, the lipids were purified by HPLC, and the amount of [^{3}H]-PAF synthesized was determined using a β-counter. The values are means of at least four experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to PBS treatment, and $\dagger\dagger\dagger P < 0.01$ as compared to VEGF-A treatment. **B**: Native BAEC were stimulated with various concentrations of VEGF-A or VEGF-C (10 pM–1 nM) as described in (A). $^{***}P < 0.001$ as compared to PBS treatment, and $\dagger\dagger\dagger P < 0.001$ as compared to a treatment with a similar concentration of VEGF-A.

Treatment of BAEC with increasing concentrations of VEGF-A (10 pM, 100 pM, and 1 nM) elicited a dose-dependent increase in PAF synthesis, elevating basal level by up to 205, 499, and 950%, respectively. On the other hand, stimulation of BAEC with VEGF-C (10 pM, 100 pM, and 1 nM) elicited a weaker dose-dependent increase in BAEC PAF synthesis, elevating basal PAF level by 181, 192, and 467%, respectively. At the two highest concentrations used (100 pM and 1 nM), VEGF-A was significantly more potent than VEGF-C in mediating PAF synthesis ($P < 0.01$).

DISCUSSION

Vasculogenesis, the formation of new blood vessels from mesenchymal cells, as well as angiogenesis, the sprouting of blood vessels from pre-existing ones, are two highly regulated processes [Flamme et al., 1997]. There is ample data to suggest that VEGF and its two tyrosine kinase receptors Flt-1 and KDR are directly linked to both processes. For example, homozygous disruption of the KDR or Flt-1 genes leads to embryonic death due to absence of vasculogenesis and failure to assemble normal vascular channels, respectively [Fong et al., 1995; Shalaby et al., 1995]. We have shown that KDR mediates the chemotactic, mitogenic, and inflammatory effect of VEGF-A in vitro [Bernatchez et al., 1999], and that both KDR and Flt-1 participate in mediating VEGF-A angiogenic activity in vivo [Marchand et al., 2002]. However, limited data are available regarding the role of NRP-1 in VEGF-A-induced angiogenesis in vitro and in vivo. Initially shown to play a role in the development of the nervous system, NRP-1 has been shown to bind VEGF-A and serve as a co-receptor for KDR, to potentiate VEGF-A effect on EC migration and to promote tumor vascularization and development [Soker et al., 1998; Miao et al., 2000]. Herein, we report that coexpression of NRP-1 and KDR increases VEGF-induced KDR autophosphorylation, proliferation, migration, and PAF synthesis. Moreover, we present evidence that VEGF-C does not bind to NRP-1 and that it is less potent than VEGF-A to elicit EC proliferation, migration, and PAF synthesis on cells that co-express KDR and NRP-1, although its biological activities are similar to that of VEGF-A on cells that express KDR alone.

VEGF-C Does not Bind to NRP-1

VEGF-C is a secreted growth factor expressed during embryogenesis in regions of lymphatic vessel development [Joukov et al., 1997]. Based on its amino acid sequence, VEGF-C displays a limited homology to VEGF-A, which explains its ability to bind to KDR but not to Flt-1 [Joukov et al., 1997]. In addition, VEGF-C binds to VEGFR-3 (Flt-4), a tyrosine kinase receptor expressed by EC precursors during embryogenesis and is involved in the development of the lymphatic vasculature [Paavonen et al., 2000]. However, the interaction between VEGF-C and NRP-1 has not been studied. Competition experiments revealed that a 200-fold excess of unlabeled VEGF-C failed to compete with ^{125}I-$VEGF_{165}$-A binding to PAEC-NRP-1 (Fig. 2). These results clearly demonstrate that VEGF-C does not bind NRP-1, and that VEGF-C could be used as a valuable tool to assess the relative contribution of KDR and NRP-1 to EC stimulation. Previous studies have shown that VEGF-A binds to NRP-1 through its exon 7-encoded domain [Soker et al., 1998], and since the VEGF-C protein does not contain a homologous domain, this supports our results that VEGF-C is unable to bind to NRP-1.

PAEC Expressing Flt-1 do not Respond in Mitogenic, Chemotactic, and PAF Synthesis Activities When Challenged With VEGF-A or VEGF-C

We recently showed that a treatment with VEGF-A elicits Flt-1 phosphorylation in BAEC. However, Flt-1 activation in these cells did not induce proliferation or migration, and elicited a minimal increase in PAF synthesis as compared with KDR activation [Bernatchez et al., 1999]. Others have shown that Flt-1 activation by VEGF-A could mediate other activities, such as tissue factor production and cell migration [Fujisawa et al., 1997]. One possibility is that BAEC express insufficient levels of Flt-1 receptors to mediate cellular proliferation and migration, or lack crucial intracellular signaling properties required to induce a significant biological activity. To test this hypothesis, we used PAEC recombinantly expressing Flt-1 and investigated VEGF-A and VEGF-C ability to elicit proliferation, migration, and PAF synthesis. VEGF induced Flt-1 phosphorylation in these cells (Fig. 3A) but did not lead to significant biological activities under our experimental

conditions (Figs. 4 and 6). We have previously shown that under conditions where KDR expression was blocked in BAEC, the activation of Flt-1 did not lead to migration, proliferation and PAF synthesis under VEGF-A treatment [Bernatchez et al., 1999]. Together, these results suggest that Flt-1 may not have a significant role in mediating VEGF mitogenic, chemotactic, and PAF synthesis activities in EC.

Coexpression of KDR and NRP-1 Potentiates VEGF-A-Induced KDR Phosphorylation

We and others have shown that KDR undergoes autophosphorylation upon binding of VEGF [Guo et al., 1995; Bernatchez et al., 1999] and that NRP-1 increases VEGF binding to KDR [Soker et al., 1998]. Since VEGF and VEGF-C bind KDR but only VEGF binds NRP-1, we used these growth factors to investigate the effect of NRP-1 expression on KDR phosphorylation. In PAEC expressing KDR only, VEGF was found to be more potent than VEGF-C in eliciting KDR phosphorylation (Fig. 3B). Co-presence of KDR and NRP-1 in PAEC-KDR/NRP-1 potentiated VEGF-induced KDR phosphorylation but did not affect VEGF-C-induced KDR phosphorylation (Fig. 3B). These results support our conclusion that VEGF-C does not bind to NRP-1 and elicits a weaker phosphorylation of KDR in absence or presence of NRP-1 as compared to VEGF-A. In contrast to native PAEC, BAEC express all three VEGF-A receptors. Hence, this model is a valuable tool to test our current hypothesis that coexpression of KDR and NRP-1 potentiates VEGF-A activity. First, VEGF-A increased the basal phosphorylation of KDR by 31.8-fold, whereas a treatment with VEGF-C displayed a weaker 5.1-fold increase (Fig. 3B). Interestingly, these increases in KDR phosphorylation are comparable to the ones observed with PAEC-KDR/NRP-1. Taken together, these results suggest that NRP-1 significantly elevates VEGF-induced KDR phosphorylation.

Coexpression of KDR and NRP-1 Increases VEGF-A-Induced Proliferation, Migration, and PAF Synthesis

Using a gene knockdown approach, we have shown that EC proliferation, migration, and PAF synthesis induced by VEGF-A are KDR-dependent [Bernatchez et al., 1999]. To investigate the role of NRP-1 in these events, we used PAEC recombinantly expressing KDR and/or NRP-1 proteins. PAEC-KDR and PAEC-KDR/NRP-1 displayed increased proliferation, migration, and PAF synthesis when stimulated with VEGF-A or VEGF-C compared with native PAEC, confirming the crucial role of KDR (Figs. 4A and 6A). On PAEC-KDR, VEGF-A, and VEGF-C effects on proliferation, migration, and PAF synthesis did not differ significantly despite the fact that VEGF-A elicited a much greater phosphorylation of KDR than VEGF-C in these cells (Fig. 3B). This result suggests that KDR may act as "spare receptors," where maximal biological activity can be achieved without maximal KDR phosphorylation. In contrast, VEGF-A was significantly more potent than VEGF-C on PAEC-KDR/NRP-1 to elicit these cellular responses. VEGF-A was slightly more potent than VEGF-C in promoting PAEC-KDR/NRP-1 proliferation, and significantly more potent than VEGF-C in inducing their migration and PAF synthesis (Figs. 4A and 6A). Since maximal KDR-dependent activity in PAEC-KDR is observed with VEGF-C, the additive effect of VEGF-A could be attributable to co-expression of KDR and NRP-1 in PAEC-KDR/NRP-1 and suggest that VEGF-A binding to NRP-1 mediates intracellular activities independent of KDR. This hypothesis is supported by the finding that NRP-1 can interact with NRP-1 binding protein [Cai and Reed, 1999] and mediate cellular activities in absence of KDR. However, according to our data, NRP-1 alone cannot promote directly EC proliferation, migration, or PAF synthesis.

In order to confirm the capacity of NRP-1 to potentiate VEGF-A, but not VEGF-C activities, we used BAEC that express both NRP-1 and KDR. In these cells, VEGF-A was significantly more potent than VEGF-C in inducing cell migration and PAF synthesis. Taken together, these results suggest that NRP-1 potentiates KDR-mediated EC migration and PAF synthesis.

NRP-1 and PAF, Two Potent Regulators of Pathological Angiogenesis

Several studies have demonstrated that angiogenesis is regularly and perhaps invariably initiated by a local increase in vascular permeability, closely followed by the adhesion of inflammatory cells [Jackson et al., 1997, 1998].

Because of its multifunctional nature, PAF can play many crucial roles in the induction of angiogenesis. Not only is it involved in inflammatory cell rolling and adhesion [Prescott et al., 1984], in vitro studies furthermore indicate that PAF promotes the expression of potent angiogenic factors and chemokines including acid and basic fibroblast growth factor, hepatocyte growth factor, and macrophage inflammatory protein 2 [Bussolino et al., 1995; Zhixing et al., 1995], and induces the migration of cultured EC [Montrucchio et al., 2000]. As a result, the increase in PAF synthesis observed in cells co-expressing NRP-1 and KDR stimulated with VEGF-A in vitro may have broad implications in pathological angiogenesis by contributing to the development of chronic inflammatory angiogenesis, leading to increased blood flow to the angiogenic tissues.

By using a well-described PAF receptor antagonist (BN-52021, BioMol, Plymouth Meeting, PA), we have observed that PAF is critical for VEGF-induced BAEC migration [Bernatchez PN and Sirois MG, unpublished observations] but not for VEGF-induced proliferation [Sirois and Edelman, 1997]. Moreover, others have shown that PAF is involved in VEGF-mediated EC motility and angiogenesis in vivo [Montrucchio et al., 2000]. Since the presence of NRP-1 potentiates both KDR-mediated migration and PAF synthesis, NRP-1 may potentiate VEGF-A-induced EC migration indirectly through increased PAF synthesis. Moreover, as PAF appears to be involved at several stages of the angiogenic process, the pathological role of NRP-1 when coexpressed with KDR might be attributable to increased PAF synthesis by EC, resulting in sustained vascular permeability, inflammation, and EC migration. Hence, these data may suggest that PAF is central to the potentiation of VEGF-A activity by NRP-1 in the presence of KDR.

However, the intracellular signaling mechanism by which NRP-1 potentiates KDR-mediated EC stimulation is unknown. New data indicate that NRP-1 intracellular domain binds to NRP-1-binding protein [Cai and Reed, 1999], which might be involved in NRP-1-mediated activity. In addition, early events following EC stimulation with VEGF, such as activation of the phospholipase C/protein kinase C pathway [Ueno and Shibuya, 1999], might be modulated by the binding of VEGF-A to NRP-1. Future studies are aimed at determining the capacity of NRP-1 to trigger intracellular responses and to modulate KDR-mediated activation of intracellular signaling pathways. In contrast, both PAF and NRP-1 appear to play at best a minor role in VEGF-induced proliferation (Fig. 4).

In conclusion, we have presented evidence that coexpression of NRP-1 with KDR potentiated VEGF-A-induced KDR autophosphorylation, proliferation, migration, and PAF synthesis in vitro. In contrast, VEGF-C was less potent than VEGF-A at promoting these effects since it does not bind to NRP-1. Consequently, these results confirm the capacity of NRP-1 to modulate KDR-mediated biological activities.

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REFERENCES

Bernatchez P, Soker S, Sirois MG. 1999. VEGF effect on endothelial cell proliferation, migration and PAF synthesis is mediated through the activation of Flk-1 receptor. J Biol Chem 274:31047–54.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917.

Bussolino F, Arese M, Montrucchio G, Barra L, Primo L, Benelli R, Sanavio F, Aglietta M, Ghigo D, Rola-Pleszczynski M, Bosia A, Albini A, Camussi G. 1995. Platelet activating factor produced in vitro by Kaposi's sarcoma cells induces and sustains in vivo angiogenesis. J Clin Invest 96:940–952.

Cai H, Reed RR. 1999. Cloning and characterization of neuropilin-1-interacting protein: a PSD-95/Dlg/ZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. J Neurosci 19:6519–6527.

Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. 1989. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 84:1470–1478.

Flamme I, Frolich T, Risau W. 1997. Molecular mechanisms of vasculogenesis and embryonic angiogenesis. J Cell Physiol 173:206–210.

Folkman J. 1991. What is the evidence that tumors are angiogenesis-dependent. J Natl Cancer Inst 82:4–6.

Folkman J, Klagsburn M. 1987. Angiogenic factors. Science 235:442–447.

Fong GH, Rosant J, Gertenstein M, Breitman M. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating

the assembly of vascular endothelium. Nature 376:66–67.

Fujisawa H, Kitsukawa T, Kawakami A, Takagi S, Shimizu M, Hirata T. 1997. Roles of a neuronal cell-surface molecule, neuropilin, in nerve fiber fasciculation and guidance. Cell Tissue Res 290:465–470.

Guo D, Jia Q, Song HY, Warren RS, Donner DB. 1995. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with cell proliferation. J Biol Chem 270:6729–6733.

Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD. 1997. The codependence of angiogenesis and chronic inflammation. FASEB J 11:457–465.

Jackson JR, Bolognese B, Mangar CA, Hubbard WC, Marshall LA, Winkler JD. 1998. The role of platelet activating factor and other lipid mediators in inflammatory angiogenesis. Biochim Biophys Acta 1392:145–152.

Joukov V, Kaipainen A, Jeltsch M, Pajusola K, Olofsson B, Kumar V, Eriksson U, Alitalo K. 1997. Vascular endothelial growth factors VEGF-B and VEGF-C. J Cell Physiol 173:211–215.

Marchand G, Noiseux N, Sirois MG. 2002. Blockade of in vivo VEGF-mediated angiogenesis by antisense gene therapy: role of Flk-1 and Flt-1 receptors. Am J Physio 282:H194–H204.

Miao HQ, Lee P, Lin H, Soker S, Klagsbrun M. 2000. Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. FASEB J 14:2532–2539.

Montrucchio G, Alloatti G, Camussi G. 2000. Role of platelet-activating factor in cardiovascular pathophysiology. Phys Rev 80:1669–1699.

Moulton KS, Heller E, Konerding MA, Flynn E, Palinski W, Folkman J. 1999. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularisation and plaque growth in apolipoprotein E-deficient mice. Circulation 99:1726–1732.

Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M. 1998. A noval type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. J Biol Chem 273:31273–31282.

Paavonen K, Puolakkainen P, Jussila L, Jahkola T, Alitalo K. 2000. Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. Am J Pathol 156:1499–1504.

Park JE, Chen H, Winer J, Houck K, Ferrara N. 1994. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem 269:25646–25654.

Prescott SM, Zimmerman GA, McIntyre TM. 1984. Human endothelial cells in culture produce platelet-activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) when stimulated with thrombin. Proc Natl Acad Sci USA 81:3534–3538.

Shalaby F, Rossant J, Yamaguchi TP, Gertenstein M, Wu XF, Breitman ML, Schuh AC. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376:62–66.

Sirois MG, Edelman ER. 1997. VEGF effect on vascular permeability is mediated by the synthesis of platelet-activating factor. Am J Physiol 272:H2746–H2756.

Soker S, Fidder H, Neufeld G, Klagsbrun M. 1996. Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. J Biol Chem 271:5761–5767.

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. 1998. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92:735–745.

Ueno H, Shibuya M. 1999. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene 18:2221–2230.

Unemori EN, Ferrara N, Bauer EA, Amento EP. 1992. Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. J Cell Physiol 153:557–562.

Veikkola T, Alitalo K. 1999. VEGFs, receptors and angiogenesis. Semin Cancer Biol 9:211–220.

Zhixing P, Kravchenko VV, Ye RD. 1995. Platelet-activating factor stimulates transcription of the heparin-binding epidermal growth factor-like growth factor in monocytes. Correlation with an increased kappa B binding activity. J Biol Chem 270:7787–7790.