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$VEGF_{165}$ Mediates Formation of Complexes Containing VEGFR-2 and Neuropilin-1 That Enhance $VEGF_{165}$-Receptor Binding

Shay Soker,[1]* Hua-Quan Miao,[2] Masashi Nomi,[1] Seiji Takashima,[2] and Michael Klagsbrun[2,3]

[1]Department of Urology, Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115
[2]Department of Surgical Research, Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115
[3]Department of Pathology, Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Abstract Co-expression of NRP1 and (VEGFR-2) KDR on the surface of endothelial cells (EC) enhances $VEGF_{165}$ binding to KDR and EC chemotaxis in response to $VEGF_{165}$. Overexpression of NRP1 by prostate tumor cells in vivo results in increased tumor angiogenesis and growth. We investigated the molecular mechanisms underlying NRP1-mediated angiogenesis by analyzing the association of NRP1 and KDR. An intracellular complex containing NRP1 and KDR was immunoprecipitated from EC by anti-NRP1 antibodies only in the presence of $VEGF_{165}$. In contrast, $VEGF_{121}$, which does not bind to NRP1, did not support complex formation. Complexes containing $VEGF_{165}$, NRP1, and KDR were also formed in an intercellular fashion by co-culture of EC expressing KDR only, with cells expressing NRP1 only, for example, breast carcinoma cells. $VEGF_{165}$ also mediated the binding of a soluble NRP1 dimer to cells expressing KDR only, confirming the formation of such complexes. Furthermore, the formation of complexes containing KDR and NRP1 markedly increased ^{125}I-$VEGF_{165}$ binding to KDR. Our results suggest that formation of a ternary complex of $VEGF_{165}$, KDR, and NRP1 potentiates $VEGF_{165}$ binding to KDR. These complexes are formed on the surface of EC and in a juxtacrine manner via association of tumor cell NRP1 and EC KDR. J. Cell. Biochem. 85: 357–368, 2002.

Key words: angiogenesis; VEGF; KDR; neuropilin-1

The role of VEGF in promoting physiological and pathological angiogenesis is well established. Most tumor cells produce high levels of VEGF [Dvorak et al., 1991] and VEGF antagonists such as anti-VEGF antibodies and soluble VEGF receptors inhibit tumor vascularization and significantly repress tumor growth [Kim et al., 1993; Kendall et al., 1996]. VEGF activities are mediated by high-affinity receptor tyrosine kinases (RTKs) associated primarily with endothelial cells (EC). These RTKs are Flt-1 (VEGFR-1) and KDR (VEGFR-2 and the mouse homologue Flk-1) [de Vries et al., 1992; Terman et al., 1992]. Recently, we identified another VEGFR, neuropilin-1 (NRP1) [Soker et al., 1996, 1998], which was first described as a cell surface glycoprotein expressed on axons and independently shown to be a receptor for the semaphorin/collapsin family of neuronal guidance mediators [Fujisawa and Kitsukawa, 1998]. NRP1 binds $VEGF_{165}$ but not $VEGF_{121}$ since the NRP1-binding site in $VEGF_{165}$ is encoded by VEGF exon 7, a domain that is lacking in $VEGF_{121}$ [Soker et al., 1997, 1998]. In addition to neurons and EC, tumor cells also express NRP1. Some tumor cell lines express abundant NRP1; for example, prostate and breast carcinoma cell lines possess $1–2 \times 10^5$ NRP1 copies/cell. The K_d of $VEGF_{165}$ binding to NRP1 in tumor cells is 2.8×10^{-10} M, approxi-

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Hua-Quan Miao's present address is ImClone Systems, New York, NY 10014, USA.

Seiji Takashima's present address is Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita Osaka 565-0871, Japan.

*Correspondence to: Dr. Shay Soker, Children's Hospital, Department of Urology, 300 Longwood Avenue, Boston, MA 02115. E-mail: shay.soker@tch.harvard.edu

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mately the same as for $VEGF_{165}$ binding to EC NRP1 [Soker et al., 1996]. Unlike EC, which express KDR and NRP1, tumor cell lines that we have examined express NRP1 but neither KDR nor Flt-1 [Soker et al., 1996, 1998].

There is ample evidence that directly links NRP1 to angiogenesis. In cell culture studies, we established that co-expression of NRP1 and KDR in porcine aortic EC (PAE cells) enhanced $VEGF_{165}$ binding to KDR and the KDR-mediated chemotactic activity of $VEGF_{165}$ [Soker et al., 1998]. Overexpression of NRP1 in mice resulted in excess capillary and blood vessel formation and hemorrhaging in embryos, contributing to embryonic lethality [Fujisawa and Kitsukawa, 1998]. Disruption of NRP1 was embryonic lethal and exhibited various types of vascular defects, including impairment of neural vascularization, transposition of large vessels, and insufficient development of vascular networks in the yolk sac [Fujisawa and Kitsukawa, 1998]. Mice engineered to express only $VEGF_{121}$, the VEGF isoform that doesn't bind to NRP1, have fewer coronary vessels and a fourfold reduction in capillary density in the heart. One possible explanation is that $VEGF_{121}$ alone is insufficient for normal angiogenesis because it cannot bind to NRP1 [Carmeliet et al., 1999]. Finally, conditional overexpression of NRP1 in prostate carcinoma cells resulted in a significant increase in tumor angiogenesis and growth, characterized by high microvessel density, dilated blood vessels, increased proliferating EC, and notably less tumor cell and EC apoptosis, compared to non-induced controls [Miao et al., 2000]. The mechanism underlying the enhanced $VEGF_{165}$ binding and activity, is unknown. Unlike other VEGF receptors, the NRP1 cytoplasmic portion does not contain a consensus tyrosine kinase domain [Soker et al., 1998]. Several studies have shown that deletion of the cytoplasmic portion of NRP1 does not interfere with its activity as a mediator of growth cone collapse [Nakamura et al., 1998]. Thus, one possible mechanism for an NRP1 role in angiogenesis that is emerging is that it potentiates the interactions between $VEGF_{165}$ and KDR resulting in increased VEGF activity.

In order to understand the molecular mechanisms that enable NRP1 to promote angiogenesis we have analyzed the association of NRP1 and KDR. We found that in the presence of $VEGF_{165}$, anti-NRP1 antibodies can immunoprecipitate KDR from EC expressing both KDR and NRP1, suggesting that a ternary complex containing $VEGF_{165}$, NRP1, and KDR is formed. In addition, when cells expressing NRP1 only were co-cultured with cells expressing KDR only, anti-NRP1 antibodies immunoprecipitated KDR, suggesting that NRP1 on one cell could associate with KDR on a different cell, via $VEGF_{165}$. These results were confirmed by the binding of soluble NRP1 dimers to cells expressing KDR only in the presence of $VEGF_{165}$. Furthermore, formation of complexes containing NRP1 and KDR enhanced $VEGF_{165}$ binding to KDR. These results indicate that NRP1 plays a role in promoting intracellular and juxtacrine associations with KDR in order to potentiate VEGF activity.

MATERIALS AND METHODS

Human recombinant $VEGF_{165}$ and $VEGF_{121}$ were produced in Sf-21 insect cells infected with recombinant baculovirus vectors encoding either human $VEGF_{165}$ or $VEGF_{121}$ as previously described [Soker et al., 1996]. Fc-sNRP1 cDNA was constructed by fusing the extracellular portion of human NRP1 (containing domains a, b, and c) to the murine IgG2α Fc fragment, as previously described for Fc-Flk-1 cDNA [Kuo et al., 2001]. The chimeric cDNA was subcloned into a mammalian expression vector, pCDNA3.1 (Invitrogen, Carlsbad, CA), that was used to transfect CHO cells. A stable clone expressing high levels of Fc-sNRP1 was selected and Fc-sNRP1 protein was purified from the conditioned medium by Protein G affinity chromatography. Anti-Flk-1 (C-1158) and anti-NRP1 (C-19 and H-286) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibodies (4G-10) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). "Re-Blot" Western blot striping kits were purchased from Chemicon (Temecula, CA). Reagents and antibodies for immunohistochemical analyses were purchased from Vector laboratories, Inc. (Burlingame, CA). Cell culture media were purchased from Life Technologies (Grand Island, NY). Anti-KDR antibodies, DAPI, heparin, hygromycin B, protease and phosphatase inhibitors were purchased from Sigma (St. Louis, MO). ^{125}I-Sodium and enhanced luminol reagent "Renaissance" were purchased from DuPont NEN (Boston, MA). Disuccinimidyl suberate (DSS) and IODO-BEADS were purchased

from Pierce Chemical Co. (Rockford, IL). X-ray films, Con A, and Protein G Sepharose were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). Immobilon-P PVDF membranes were purchased from Millipore (Bedford, MA).

Cell Culture

Human umbilical vein EC (HUVEC) were obtained from American Type Culture Collection (ATCC, Rockville, MD), and grown on gelatin coated dishes in M-199 medium containing 20% fetal calf serum (FCS) and a mixture of glutamine, penicillin, and streptomycin (GPS). Basic fibroblast growth factor (2 ng/ml) was added to the culture medium every other day. Parental porcine aortic endothelial (PAE) cells and PAE cells expressing KDR (PAE/KDR), NRP1 (PAE/NRP1) and both receptors (PAE/KDR/NRP1), were previously described [Soker et al., 1998] and were grown in F12 medium containing 10% FCS and GPS. MDA-MB-231 (231) cells were obtained from ATCC, and grown in DMEM containing 10% FCS and GPS. CHO/Fc-sNRP1 cells were grown in MEM-α containing 10% FCS and GPS.

VEGF Receptor Tyrosine Phosphorylation and Immunoprecipitation

Cells were grown in 10 cm dishes, starved for 18 h in the presence of 0.5% FBS, washed, and the medium was replaced with 2.5 ml binding buffer containing DMEM, 1 mg/ml BSA, and 1 μg/ml heparin. Dishes were incubated with or without 20 ng/ml VEGF for 30 min on ice and then at 37°C for 7 min. The cells were washed extensively with ice cold phosphate buffer saline (PBS) and lysed on ice with 1 ml of 20 mM Tris pH 7.0, 0.1 M NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 5 mM NaF, 10 mM $Na_4P_2O_7$ and protease inhibitors. Cell lysates were incubated with anti-NRP1 antibodies (C-19, 1–2 μg/ml) for 18 h at 4°C and immune complexes were precipitated with protein G. Alternatively, cell lysates were incubated with Con A Sepharose.

Western Blot

Immunoprecipitated and ConA-associated proteins were resuspended in SDS sample buffer, heated at 95°C for 5 min and proteins were resolved by 7.5% SDS–PAGE and transferred to PVDF membrane using semi-dry blotter (Amersham-Pharmacia Biotech). The membranes were blocked with 3% BSA in TBS/T (20 mM Tris 7.4, 140 mM NaCl, and 0.1% Tween 20), incubated overnight with primary antibody and subsequently probed with peroxidase-labeled secondary antibody. For Western blot, the first probe used were anti-Flk-1 antibodies (C-1158). These antibodies were stripped with "Re-Blot" and the membrane was probed with anti-phosphotyrosine antibodies (4G-10), stripped again and probed with anti-NRP1 antibodies (H-286). The membranes were developed with enhanced luminol reagent "Renaissance" and exposed to X-ray film, according to the manufacturer's instructions.

Binding of Fc-sNRP1 to Cells and Immunohistochemistry

Fc-sNRP1 was purified from conditioned media of CHO cells overexpressing the fusion protein using Protein G affinity chromatography. The identity of Fc-sNRP1 protein in the eluted fractions was determined using anti-NRP1 (H286) and anti-mouse IgG antibodies. PAE and PAE/KDR cells grown in 24 well dishes were washed with PBS and incubated with 5 μl ($\sim$ 50 ng) of Fc-sNRP1 in 200 μl DMEM containing 2 mg/ml BSA and 1 μg/ml heparin, in the presence or the absence of 20 ng/ml $VEGF_{165}$. After 1 h incubation on ice, cells were washed three times with cold PBS and fixed with 4% paraformaldehyde for 10 min at 37°C. The cells were washed three times with PBS and incubated with biotinylated anti-mouse IgG antibodies and probed with fluorescein-avidin conjugate. Alternatively, cells were incubated with anti-KDR antibodies and probed in the same manner. Cell nuclei were subsequently stained with DAPI. Cells were visualized using fluorescence microscopy and blue (DAPI) and green (fluorescein) images were acquired and overlaid using PhotoShop®.

Radio-Iodination of VEGF, Binding and Cross-Linking

Radio-iodination of $VEGF_{165}$ using IODOBEADS was carried out as previously described [Soker et al., 1996]. The specific activity ranged from 40,000–100,000 cpm/ng protein. Binding and cross-linking experiments using ^{125}I-$VEGF_{165}$ were performed as previously described [Soker et al., 1996]. ^{125}I-$VEGF_{165}$ cross-linked complexes were resolved by 6% SDS-PAGE and the gels were exposed to X-ray film.

RESULTS

Formation of Complexes Containing NRP1 and KDR on the Surface of EC is $VEGF_{165}$ Dependent

Co-expression of NRP1 and KDR in EC increases the binding of $VEGF_{165}$ to KDR and as a consequence its chemotactic activity for EC [Soker et al., 1998]. In order to understand the molecular mechanisms that underlie this activation, interactions between NRP1 and KDR were analyzed. Porcine aortic EC (PAE) engineered to express KDR (PAE/KDR) or both KDR and NRP1 (PAE/KDR/NRP1) [Soker et al., 1998] and HUVEC were incubated transiently in the presence or the absence of $VEGF_{165}$ (Fig. 1). Cells were lysed and anti-NRP1 antibodies (Fig. 1, lanes 1–6) were used to immunoprecipitate NRP1 and any proteins associated with NRP1, followed by Western blot with anti-Flk-1 antibody (Fig. 1, lanes 1–6, top), anti-phosphotyrosine antibody (Fig. 1, lanes 1–6, center), and anti-NRP1 antibody (Fig. 1, lanes 1–6, bottom). In the presence of $VEGF_{165}$, a single band of 220 kDa KDR protein (Fig. 1, lanes 4 and 6, top) that was tyrosine phosporylated (Fig. 1, lanes 4 and 6, center), was immunoprecipitated by anti-NRP1 antibodies from PAE/KDR/NRP1 and HUVEC lysates. In contrast, KDR was not immunoprecipitated from PAE/KDR lysates (Fig. 1, lane 2, top and center). These results suggest that the anti-NRP1 antibodies immunoprecipitate an NRP1/KDR complex via NRP1 and do not interact directly with KDR. Anti-NRP1 antibodies immunoprecipitated similar amounts of NRP1, in the presence and the absence of $VEGF_{165}$, indicating equal sample loading (Fig. 1, lanes 3–6, bottom).

In order to demonstrate that the cell lines used for these experiments expressed similar amounts of KDR and that KDR levels were not changed due to the presence and the absence of $VEGF_{165}$, cell lysates were incubated with Concanavalin (Con) A-Sepharose which binds glycoproteins such as NRP1 and KDR (Fig. 1, lanes 7–12). Examination of Con A-associated proteins demonstrated that similar amounts of KDR could be detected, in the presence and the absence of $VEGF_{165}$, in PAE/KDR cells (Fig. 1, lanes 7 and 8, top), PAE/KDR/NRP1 cells (Fig. 1, lanes 9 and 10, top) and HUVEC (Fig. 1, lanes 11 and 12, top). Furthermore, KDR was tyrosine phosphorylated only in the presence of $VEGF_{165}$ (Fig. 1, lanes 8, 10, and 12, center). Con A precipitation yielded similar amounts of NRP1, in the presence and the absence of $VEGF_{165}$, indicating equal sample loading (Fig. 1, lanes 9–12, bottom). Together, these results suggest that $VEGF_{165}$ mediates the formation of a stable complex containing NRP1 and KDR in EC.

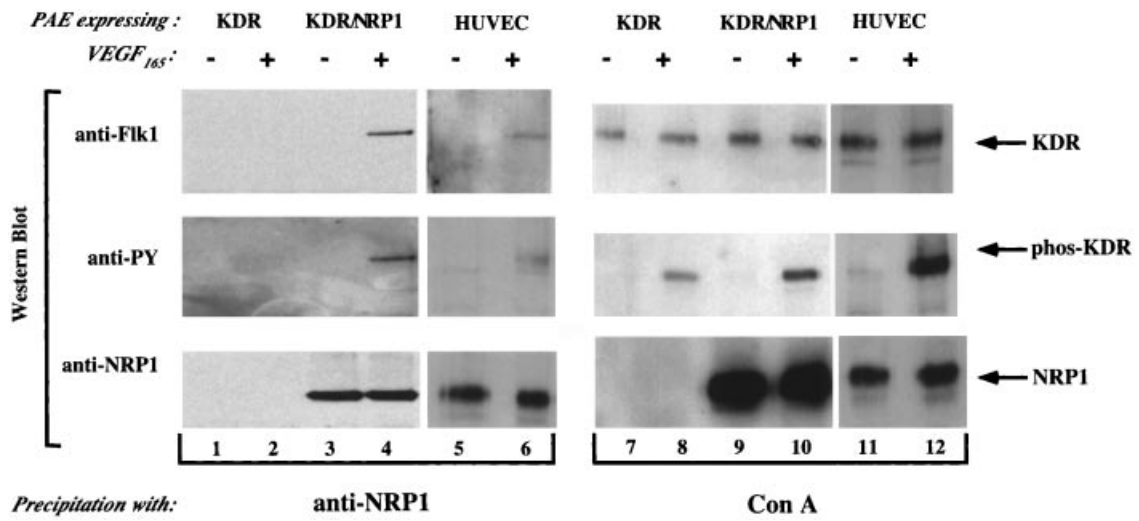

Fig. 1. VEGF mediates the formation of complexes containing NRP1 and KDR on EC. Serum-starved PAE/KDR (**lanes 1**, **2**, **7**, and **8**), PAE/KDR/NRP1 (**lanes 3**, **4**, **9**, and **10**) and HUVEC (**lanes 5**, **6**, **11** and **12**) were incubated in the presence (lanes 2, 4, and 6) or the absence (lanes 1, 3, and 5) of $VEGF_{165}$ for 30 min on ice and then for 7 min at 37°C. Cell lysates were prepared and immunoprecipitated with anti-NRP1 antibodies (lanes 1–6) or precipitated with Con A beads (lanes 7–12), as described in Materials and Methods. The precipitates were analyzed by Western blot with anti-Flk-1 antibodies (top), anti-phosphotyrosine antibodies (center), and anti-NRP1 antibodies (bottom). Arrows indicate the position of KDR, tyrosine phosphorylated KDR (phos-KDR), and NRP1.

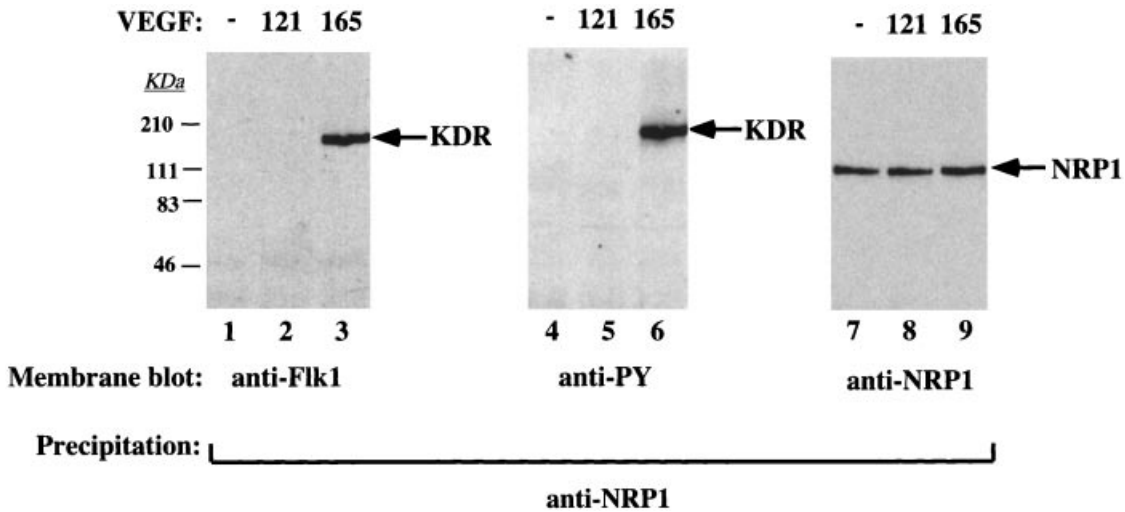

Fig. 2. Isoform-specific requirement for $VEGF_{165}$ to generate complexes containing NRP1 and KDR. Serum-starved PAE/KDR/NRP1 were incubated with $VEGF_{121}$ (**lanes 2**, **5**, and **8**), $VEGF_{165}$ (**lanes 3**, **6**, and **9**), or no VEGF (**lanes 1**, **4**, and **7**) for 30 min on ice and then at 37°C for 7 min. Cell lysates were prepared and immunoprecipitated with anti-NRP1 antibodies as in Figure 1. The precipitates were analyzed by Western blot with anti-Flk-1 antibodies (left), anti-phosphotyrosine antibodies (center), and anti-NRP1 antibodies (right). Arrows indicate the position of KDR and NRP1.

$VEGF_{165}$ Isoform-Specific Promotion of NRP1/KDR Complex Formation

The formation of an NRP1/KDR complex in PAE/KDR/NRP cells, as indicated by immunoprecipitation of tyrosine phosphorylated KDR with anti-NRP1 antibodies, was dependent on the presence of $VEGF_{165}$ (Fig. 2, lanes 3 and 6, respectively). No KDR signal could be detected in the absence of VEGF (Fig. 2, lanes 1 and 4). In contrast, anti-NRP1 antibodies failed to immunoprecipitate KDR from PAE/KDR/NRP1 cells that were incubated with $VEGF_{121}$ (Fig. 2, lanes 2 and 5). The latter result is consistent with the finding that $VEGF_{121}$ does not bind to NRP1 [Soker et al., 1996, 1998]. Anti-NRP1 antibodies immunoprecipitated similar amounts of NRP1 in the presence and the absence of $VEGF_{165}$ and $VEGF_{121}$ (Fig. 2, lanes 7–9), indicating equal sample loading. These results suggest that the binding of $VEGF_{165}$ to NRP1 is essential for the association between NRP1 and KDR.

Juxtacrine Interactions Between NRP1 and KDR are Mediated via $VEGF_{165}$

Formation of NRP1/KDR complexes, in the presence of $VEGF_{165}$, can be demonstrated not only when NRP1 and KDR are expressed by the same cell type but also in co-culture of two different cell types, one expressing NRP1 only and the other expressing KDR only. Co-cultures of PAE/KDR and PAE cells (Fig. 3, lanes 1 and 2) and of PAE/KDR and PAE/NRP1 cells (Fig. 3, lanes 3 and 4) were incubated in the presence or the absence of $VEGF_{165}$ and cell lysates were immunoprecipitated with anti-NRP1 antibodies followed by Western blot with anti-KDR antibody (Fig. 3, top), anti-phosphotyrosine antibody (Fig. 3, center), and anti-NRP1 antibody (Fig. 3, bottom). A single band of 220 kDa KDR protein that was tyrosine phosporylated, was immunoprecipitated from a co-culture of PAE/KDR and PAE/NRP1 cells in the presence (Fig. 3, lane 4, top and center) but not in the absence (Fig. 3, lanes 1 and 3, top and center) of $VEGF_{165}$. No KDR signal could be detected in the co-culture of PAE/KDR and PAE cells (Fig. 3, lane 2, top and center). These results suggest that an intercellular complex between KDR and NRP1 is formed in the presence of $VEGF_{165}$. To test this hypothesis further, PAE/KDR cells were co-cultured with MDA-MB-231 (231) breast carcinoma cells (Fig. 3, lanes 5 and 6), which express NRP1 as their only VEGF receptor [Soker et al., 1996, 1998]. Tyrosine phosphorylated KDR was immunoprecipitated by anti-NRP1 antibodies from the co-culture of PAE/KDR and 231 cells in the presence of $VEGF_{165}$ (Fig. 3, lane 6, top and center, respectively). A small amount of phosphorylated KDR was detected in the PAE/KDR-231 cell co-cultures that had not been treated with

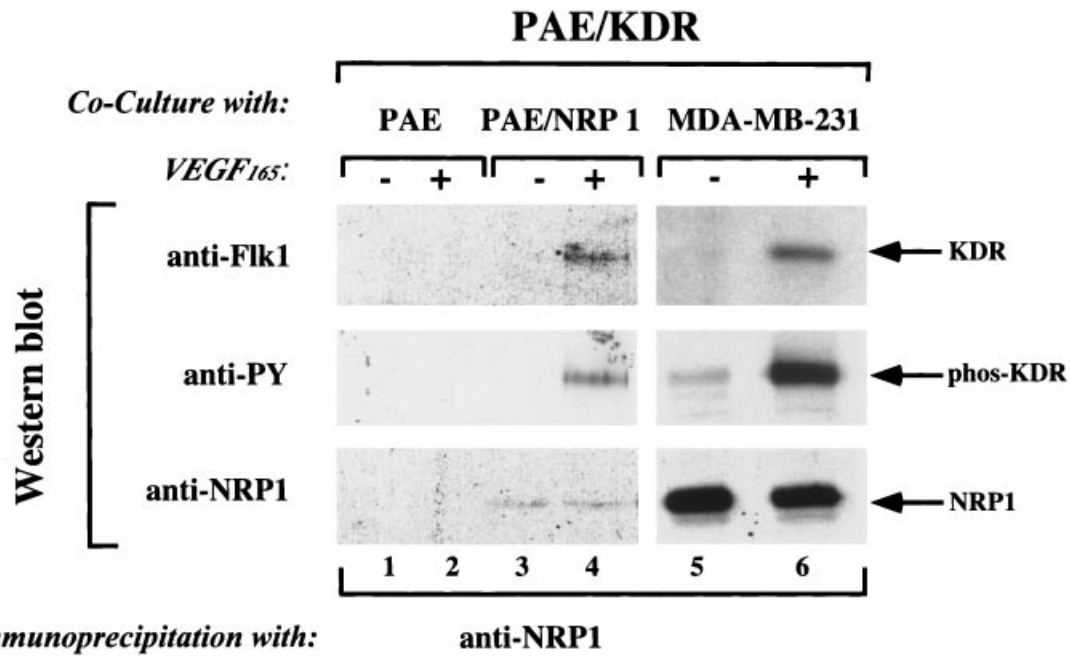

Fig. 3. $VEGF_{165}$ mediates formation of intercellular complexes between cells expressing only NRP1 or only KDR. Approximately 2.5×10^6 PAE/KDR cells were co-cultured with the same number of PAE cells (**lanes 1** and **2**), PAE/NRP1 cells (**lanes 3** and **4**) or MDA-MB-231 breast carcinoma cells (**lanes 5** and **6**) in 10 cm dishes. Co-cultures were incubated in the presence (lanes 2, 4, 6 and 8) or the absence (lanes 1, 3, 5, and 7) of 20 ng/ml $VEGF_{165}$. Immunoprecipitation and Western blot with anti-KDR antibodies (top), anti-phosphotyrosine antibodies (center), and anti-NRP1 antibodies (bottom) were as in Figure 1. Arrows indicate the position of KDR, tyrosine phosphorylated KDR (phos-KDR), and NRP1.

$VEGF_{165}$ (Fig. 3, lane 5, center), probably due to endogenous secretion of $VEGF_{165}$ from 231 cells (data not shown).

In the presence of $VEGF_{165}$ the amount of phosphorylated KDR that was immunoprecipitated from the PAE/KDR-231 cell co-culture was higher than the amount precipitated from PAE/KDR-PAE/NRP1 co-culture (Fig. 3, lane 6 vs. 4, center), and was correlated with the higher amount of endogenous NRP1 expression by 231 cells compared with recombinant expression of NRP1 in PAE/NRP1 cells (Fig. 3, lanes 5 and 6 vs. 3 and 4, bottom). In the various co-cultures, the anti-NRP1 antibodies immunoprecipitated the same levels of NRP1 in the absence or the presence of $VEGF_{165}$, demonstrating equal loading (Fig. 3, lane 3 vs. 4 and lane 5 vs. 6, bottom). Together, these results suggest that $VEGF_{165}$ promotes formation of an intercellular juxtacrine complex containing tumor cell-associated NRP1 and KDR from an adjacent endothelial cell.

$VEGF_{165}$ Mediates the Binding of Soluble NRP1 Dimer to PAE/KDR Cells

To demonstrate that KDR can associate with exogenous NRP1 in the presence of $VEGF_{165}$, we tested the ability of soluble NRP1 to bind KDR on PAE/KDR cells. It has recently been shown that soluble NRP1 (sNRP1) dimers could substitute for cell-associated NRP1 and promote $VEGF_{165}$-induced KDR phosphorylation in EC derived from NRP1-deficient mice [Yamada et al., 2001]. We produced chimeric proteins containing the extracellular portion of NRP1, with a, b, and c domains [Soker et al., 1998], fused upstream to the Fc domain of murine IgG [Kuo et al., 2001]. The chimeric Fc-sNRP1 proteins were secreted as dimers and bound ^{125}I-$VEGF_{165}$ but not ^{125}I-$VEGF_{121}$ (not shown). Fc-sNRP1 was incubated with PAE and PAE/KDR cells in the presence or the absence of $VEGF_{165}$ (Fig. 4A). Bound Fc-sNRP1 was detected by anti-mouse IgG antibodies coupled with fluorescein (green), and cell nuclei were detected by staining with DAPI (blue). Approximately 20% of PAE/KDR cells bound Fc-sNRP1 on their membranes but only in the presence of $VEGF_{165}$ (Fig. 4A, panel 1 vs. 2). In contrast, no bound Fc-sNRP1 was detected on PAE cells incubated with Fc-sNRP1 in the presence or the absence of $VEGF_{165}$ (Fig. 4A, panels 3 and 4, respectively). Anti-KDR antibodies immunostained all PAE/KDR cells, suggesting homogenous KDR expression by these cells, whereas no KDR

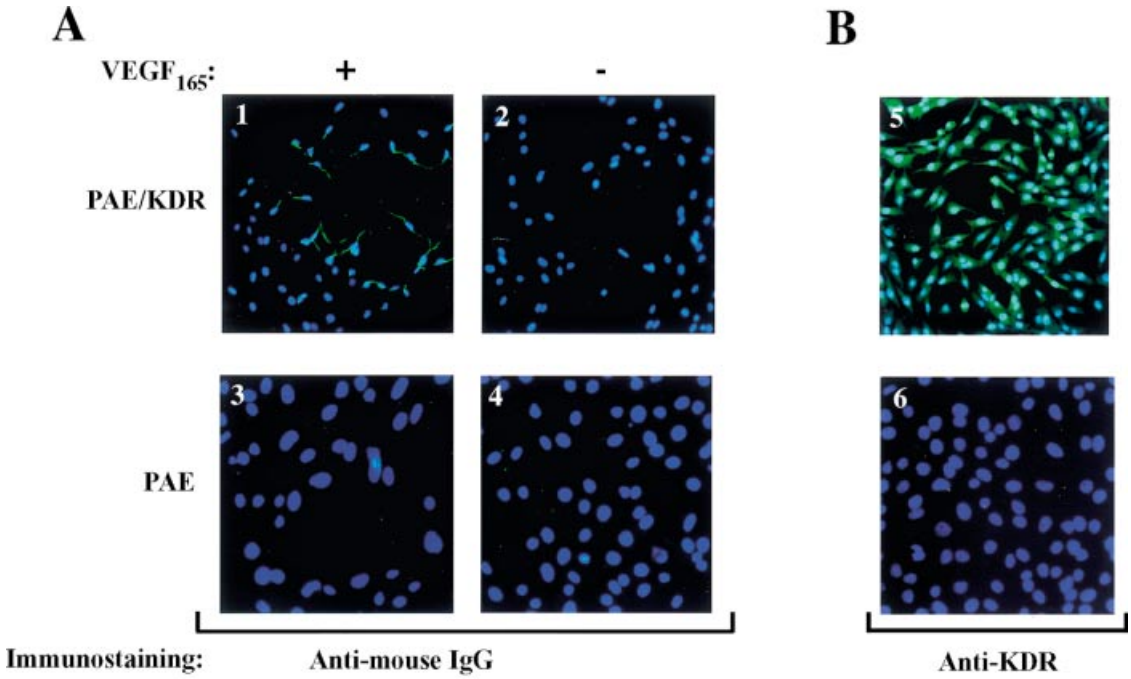

Fig. 4. The binding of soluble NRP1 dimers to PAE/KDR cells is $VEGF_{165}$-dependent. **A**: PAE/KDR (**panels 1** and **2**) and PAE (**panels 3** and **4**) cells, grown in 24 well dishes were incubated for 1 h on ice with Fc-sNRP1 (~250 ng/ml) in the presence (panels 1 and 3) or the absence (panels 2 and 4) of 20 ng/ml $VEGF_{165}$. **B**: PAE/KDR (**panel 5**) and PAE (**panel 6**) cells were grown as described in A and incubated with mouse anti-KDR antibodies for 1 h at room temperature. Cells were washed with PBS, fixed and incubated with biotinylated anti-mouse IgG antibodies. Antibodies were detected using fluorescein-avidin cojugate (green) and cell nuclei were further stained with DAPI (blue). Cells were visualized using fluorescence microscopy and green and blue images were acquired and overlaid using PhotoShop.

staining was detected in PAE cells (Fig. 4B, panels 5 and 6, respectively). These results indicate that some but not all KDR molecules on the surface of PAE/KDR cells are occupied by sNRP1, even though saturating amounts of $VEGF_{165}$ (20 ng/ml) were used. Together, these results support our hypothesis that, in the presence of $VEGF_{165}$, NRP1 associates with KDR on the surface of EC in a juxtacrine manner.

$VEGF_{165}$ Binding to KDR is Enhanced in the Presence of NRP1

We next analyzed ^{125}I-$VEGF_{165}$ binding and cross-linking to receptors in co-cultures that enable KDR and NRP1 to be present in close proximity. First, we cross-linked ^{125}I-$VEGF_{165}$ to PAE cells expressing NRP1 alone, KDR alone, or to both receptors (Fig. 5A). Cross-linking of ^{125}I-$VEGF_{165}$ to PAE/NRP1 cells resulted in a 175 kDa ^{125}I-$VEGF_{165}$-NRP1 doublet (Fig. 5A, lane 1) and cross-linking to PAE/KDR cells resulted in a single 240 kDa ^{125}I-$VEGF_{165}$-KDR complex (Fig. 5A, lane 2). Cross-linking of ^{125}I-$VEGF_{165}$ to PAE/KDR/NRP1 cells resulted in the formation of a 175 kDa labeled doublet containing NRP1 and a 240 kDa labeled complex that contained KDR (Fig. 5A, lane 3). The binding of ^{125}I-$VEGF_{165}$ to KDR when NRP1 was co-expressed with KDR in PAE/KDR/NRP1 cells was increased approximately threefold compared to PAE/KDR cells (Fig. 5A, lane 3 vs. 2), confirming previous results [Soker et al., 1998]. The binding of ^{125}I-$VEGF_{165}$ to NRP1 in PAE/KDR/NRP1 cells (Fig. 5A, lane 3) was slightly increased by less than twofold compared to PAE/NRP1 cells (Fig. 5A, lane 1).

Cross-linking of ^{125}I-$VEGF_{165}$ to PAE/KDR cells co-cultured with PAE/NRP1 cells (Fig. 5B, lane 3) resulted in 175 kDa labeled complex containing NRP1 and a 240 kDa labeled complex containing KDR. As expected, only a 240 kDa complex was formed in co-culture of PAE/KDR cells with PAE cells (Fig. 5B, lane 2) or when PAE/KDR cells were cultured alone (Fig. 5B, lane 1). The binding of ^{125}I-$VEGF_{165}$ to KDR in PAE/KDR cells was 2.5-fold higher in co-cultures of PAE/KDR with PAE/NRP1 cells than in co-cultures of PAE/KDR with PAE cells (Fig. 5B, lane 3 vs. 2).

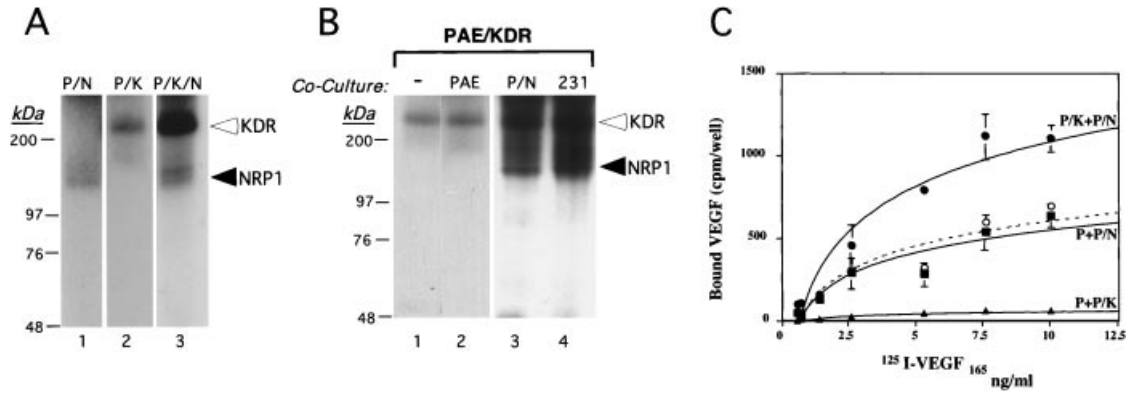

Fig. 5. Co-expression of NRP1 and KDR results in increased ^{125}I-VEGF$_{165}$ binding. **A**: ^{125}I-VEGF$_{165}$ (5 ng/ml) was bound and cross-linked to PAE/NRP1 (**lane 1**) PAE/KDR (**lane 2**) and to PAE/KDR/NRP1 (**lane 3**) cells. The ^{125}I-VEGF$_{165}$ cross-linked complexes were resolved by 6% SDS/PAGE and the gels were exposed to X-ray film. Arrows denote radiolabled complexes containing KDR (240 kDa) and NRP1 (175 kDa). **B**: PAE/KDR cells (lane 1) or co-cultures of PAE/KDR with PAE (lane 2), PAE/NRP1 (lane 3) and 231 (**lane 4**) cells were grown as described in Figure 3. ^{125}I-VEGF$_{165}$ was bound and cross-linked to co-cultures and cross-linked complexes were analyzed as in panel B. **C**: Similar cell numbers (5×10^4) of PAE and PAE/NRP1 cells (closed squares, P + P/N), of PAE and PAE/KDR cells (closed triangles, P + P/K) and of PAE/NRP1 and PAE/KDR cells (closed circles, P/N + P/K) were seeded together in wells of a 48 well dish. Cells were washed with PBS and incubated with increasing amounts (0.5–10 ng/ml) of ^{125}I-VEGF$_{165}$ for 2 h on ice. Unbound ^{125}I-VEGF$_{165}$ was washed and cell-associated radioactivity was determined. The sum of radioactivity in P + P/N mix and P + P/K mixture was calculated for each ^{125}I-VEGF$_{165}$ concentration (open circles, broken line).

To test if similar effects could be produced by tumor cells expressing NRP1 only, ^{125}I-VEGF$_{165}$ was cross-linked to co-cultures of 231 and PAE/KDR cells (Fig. 5B, lane 4). Complexes containing NRP1 (175 kDa) and KDR (240 kDa) were observed in these co-cultures. However, in the co-cultures of PAE/KDR with 231 cells, the binding of ^{125}I-VEGF$_{165}$ to KDR in PAE/KDR cells was threefold higher than in co-cultures of PAE/KDR with PAE cells (Fig. 5B, lane 4 vs. 2). These results suggest that the binding of VEGF$_{165}$ to tumor cell NRP1 enhances its binding to KDR on adjacent EC.

In order to quantify the increase in VEGF$_{165}$ binding to KDR in these co-cultures, the levels of bound VEGF$_{165}$ were analyzed. ^{125}I-VEGF$_{165}$ was bound to co-cultures of PAE and PAE/KDR (P + P/K) cells, PAE and PAE/NRP1 (P + P/N) cells, or PAE/NRP1 and PAE/KDR (P/K + P/N) cells (Fig. 5C). Co-cultures of P + P/K cells bound very little ^{125}I-VEGF$_{165}$ ($\sim$50 cpm/well) even at high ^{125}I-VEGF$_{165}$ concentrations (10 ng/ml). In contrast, co-cultures of P + P/N cells showed a logarithmic ^{125}I-VEGF$_{165}$ binding curve that was saturated at approximately 7.5–10 ng/ml with maximal binding of approximately 600 cpm/well. Subsequently, the sum of ^{125}I-VEGF$_{165}$-binding to P + P/K and to P + P/N co-cultures was calculated (Fig. 5C, broken line). ^{125}I-VEGF$_{165}$ binding to co-cultures of P/K + P/N cells showed a logarithmic curve that was saturated at approximately 7.5 ng/ml. Interestingly, the amount of ^{125}I-VEGF$_{165}$ bound to P/K + P/N co-cultures at this concentration was approximately twofold higher than the sum of ^{125}I-VEGF$_{165}$-binding to P + P/K and to P + P/N co-cultures (1,100 vs. 650 cpm/well, at 7.5 ng/ml ^{125}I-VEGF$_{165}$). These observations suggest that there may be a synergism that results in higher VEGF$_{165}$ binding when KDR and NRP1 from different cells are brought together as a result of juxtacrine activation.

DISCUSSION

NRP1 is a high affinity receptor for semaphorins and VEGF$_{165}$ and is expressed by neuronal, endothelial, and tumor cells [Soker, 2001]. However, NRP1 does not seem to induce intracellular signaling on its own [Nakamura et al., 1998], probably because its cytoplasmic domain lacks a consensus kinase domain [Fujisawa and Kitsukawa, 1998]. One possibility for EC that express both NRP1 and KDR is that NRP1 mediates KDR activity by serving as a co-receptor. In this study, we analyzed receptor interactions and found that complexes containing KDR and NRP1 are formed on the surface of EC. These results were obtained by demonstrating that anti-NRP1 antibodies immunoprecipitated KDR from EC expressing both NRP1 and KDR. Furthermore, KDR could only be immunopre-

cipitated by anti-NRP1 antibodies in the presence of $VEGF_{165}$, suggesting that a ternary complex containing NRP1, $VEGF_{165}$, and KDR was formed. No complex formation occurred in the presence of $VEGF_{121}$, which is consistent with our earlier observations that NRP1 is an isoform specific receptor for $VEGF_{165}$ [Soker et al., 1996, 1998]. These results suggest that anti-NRP1 antibodies do not interact directly with KDR and that NRP1 does not interact directly with KDR. Within the ternary complex, KDR is tyrosine phosphorylated and is thus activated, indicating that simultaneous binding of $VEGF_{165}$ to NRP1 and KDR does not interfere with KDR activation. $VEGF_{165}$ is capable of binding simultaneously to KDR and NRP1, since it uses the exon 4-encoded domain to bind KDR [Keyt et al., 1996] and the exon 7-encoded domain to bind NRP1 [Soker et al., 1996]. Taken together, the results suggest that $VEGF_{165}$ bridges KDR and NRP1, bringing them into close proximity, but does not result in direct receptor to receptor contact (Fig. 6). Interestingly, it has been reported that unlike KDR, NRP1 interacts directly with Flt-1 with a high affinity. However, in contrast to KDR, Flt-1 competed with NRP1 for the binding to $VEGF_{165}$ [Fuh et al., 2000].

When NRP1 is expressed in EC, the binding of $VEGF_{165}$ to KDR in these cells is enhanced considerably confirming our previous results [Soker et al., 1998]. Consistent with these results, a recent report has demonstrated, using a BIAcore system for binding analysis, that the immobilized NRP1 extracellular domain binds $VEGF_{165}$, and increases the affinity of $VEGF_{165}$ for the extracellular domain of KDR [Fuh et al., 2000]. The ^{125}I-$VEGF_{165}$ binding experiments in the present and in previous studies [Soker et al., 1996, 1998] did not predict an increase in the affinity of $VEGF_{165}$ to KDR. It is possible that NRP1 enables more KDR on the cell surface to bind $VEGF_{165}$ without changing the affinity between the ligand and the receptor, as was

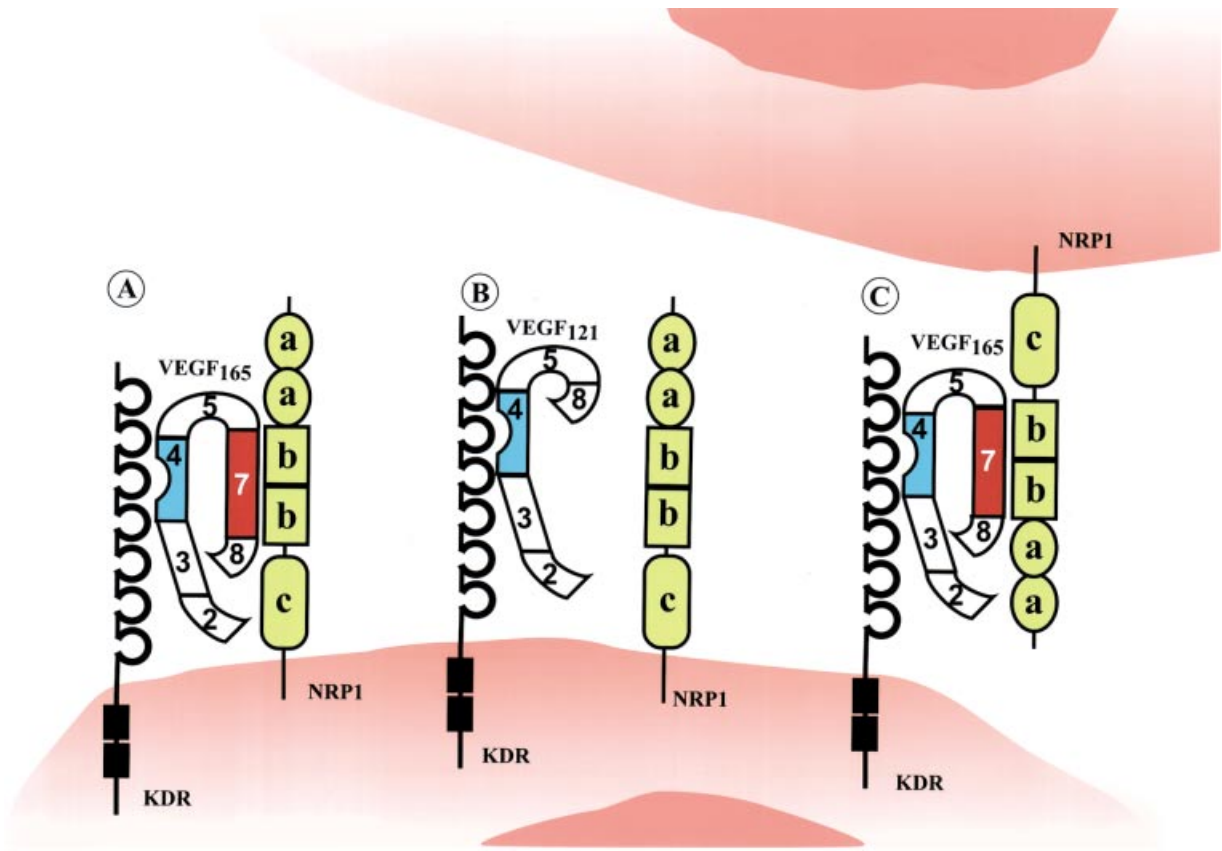

Fig. 6. Model of the formation of intracellular and intercellular complexes containing NRP1, VEGF, and KDR. **A**: $VEGF_{165}$ binds to NRP1 via the exon 7-encoded domain and to KDR via its exon 4-encoded domain to form a ternary complex in which $VEGF_{165}$ bridges the two receptors in the same cell, for example, EC. **B**: $VEGF_{121}$ binds to KDR as does $VEGF_{165}$ but since it lacks exon 7 $VEGF_{121}$ does not bind to NRP1 and no ternary complex is formed. **C**: A juxtacrine ternary complex is formed by interactions of $VEGF_{165}$ with KDR and NRP1 on two different cells, for example, EC and tumor cells.

suggested for the role of heparin in promoting $VEGF_{165}$ binding to receptors on EC [Gitay-Goren et al., 1992].

A novel finding is that complexes containing KDR and NRP1 can be formed between two different cells, such as two neighboring EC or an EC and an adjacent tumor cell. When cells expressing KDR only were co-cultured in the presence of $VEGF_{165}$ with cells expressing NRP1 only, anti-NRP1 antibodies immunoprecipitated tyrosine phosphorylated KDR. This result could be possible only if the two cell types were bridged by $VEGF_{165}$ via the two receptors, suggesting a $VEGF_{165}$-mediated juxtacrine mechanism that brings two cell types in proximity (Fig. 6). In these co-cultures, ^{125}I-$VEGF_{165}$ binding was approximately twofold higher than the sum of binding to each cell type, suggesting a synergism between NRP1 and KDR in $VEGF_{165}$ binding. Furthermore, a soluble NRP1 dimer bound to PAE/KDR cells in situ but only in the presence of $VEGF_{165}$. This result is consistent with a previous report that soluble NRP1 dimers were able to partially restore sprouting activity in explants from embryonic tissue of $NRP1^{-/-}$ mice [Yamada et al., 2001]. The authors suggested that addition of exogenous soluble NRP1 resulted in enhanced KDR activity in EC. Our results indicate that exogenous soluble NRP1 forms a complex together with KDR and $VEGF_{165}$, and provide a molecular mechanism for their hypothesis.

An intriguing possibility is that $VEGF_{165}$ mediates juxtacrine interactions between tumor cells and EC via the two receptors. Many tumor cells express abundant NRP1, but do not express KDR. For example, we have shown that breast carcinoma MDA-MB 231 cells express NRP1, but not KDR and bind $VEGF_{165}$ to NRP1 [Soker et al., 1996]. The traditional model is that tumor cells express $VEGF_{165}$, which is a paracrine growth factor for EC. However, the data presented here suggest that tumor cells can also interact with EC in a juxtacrine manner, enhancing localized tumor-EC interactions. Furthermore, the binding of ^{125}I-$VEGF_{165}$ to cells is enhanced markedly when PAE/KDR cells are co-cultured with tumor cells expressing high levels of NRP1. Tumor cell-EC juxtacrine interactions might explain in part our finding that conditional overexpression of NRP1 in tumor cells in vivo results in increased tumor angiogenesis [Miao et al., 2000]. Thus, tumor cells are not only a source of angiogenic $VEGF_{165}$ but are also a source of NRP1, which acts to enhance $VEGF_{165}$-mediated KDR activity.

A recent study [Whitaker et al., 2001] using COS cells expressing KDR and NRP1 showed that anti-NRP1 antibodies precipitated KDR protein even in the absence of $VEGF_{165}$. One difference in these two studies may be the ability of COS cells to express endogenous $VEGF_{165}$ unlike PAE cells which do not. This explanation would be consistent with our finding that in 231 tumor cell-PAE/KDR co-cultures, anti-NRP1 antibodies immunoprecipitated KDR in the absence of exogenous $VEGF_{165}$ due to endogenous 231 tumor cell $VEGF_{165}$ levels. The amount of immunoprecipitated KDR was significantly increased in the presence of 20 ng/ml $VEGF_{165}$, suggesting that the association between NRP1 and KDR is enhanced in the presence of $VEGF_{165}$.

NRP1 forms other complexes, for example with plexins. NRP1 is a receptor for neuronal guidance receptors [Soker, 2001]. Sema 3A repulses axons and this activity is mediated by binding to NRP1 [Fujisawa and Kitsukawa, 1998]. However, while NRP1 is necessary, it is not sufficient to mediate axon repulsion since it is incapable of transmitting a Sema 3A signal to the growth cone interior. Rather, NRP1 binds plexin 1, which is a transmembrane receptor that is capable of stimulating signal transduction [Nakamura et al., 2000; Rohm et al., 2000]. Plexin 1 alone does not bind Sema 3A but a NRP1/plexin 1 complex has a higher affinity for Sema 3A than NRP1 alone. Complex formation between NRP1 and plexin 1 results in enhanced binding of the ligand to NRP1. However, in the case of $VEGF_{165}$, KDR/$VEGF_{165}$/NRP1 complex formation results in increased ligand binding to KDR. Recently, it has been shown that NRP1 forms a complex with L1 CAM, a cell adhesion molecule, via their extracellular domains [Castellani et al., 2000]. In the absence of L1 CAM, Sema 3A does not repel cortical axons. Together, these results suggest that NRP1 forms complexes with a number of different receptors, thereby mediating ligand activity. The NRP1 homologue, NRP2, forms complexes with Flt-1 but the biological consequence of this interaction is not clear as yet [Gluzman-Poltorak et al., 2001]. Interestingly, ^{125}I-$VEGF_{121}$ was associated with the Flt-1/NRP-2 complexes, probably through independent binding to Flt-1. The association of non-signaling receptors and signaling tyrosine kinase receptors,

creating high affinity binding sites might be a general phenomenon. For example, betaglycan (TGF beta type III receptor) and p75 are non-signaling co-receptors for TGF-β [Massague, 1992] and NGF [Chao and Hempstead, 1995], respectively.

The proactive role of NRP1 in enhancing KDR activity and tumor cell-EC cell–cell interactions via the two receptors suggests that antagonizing NRP1 may be a feasible anti-VEGF and anti-tumor strategy. We have previously identified NRP1-specific antagonist, a fusion protein containing the 44 amino acid exon 7-encoded domain of VEGF, which mediates $VEGF_{165}$ binding to NRP1 [Soker et al., 1997]. Exon 7-GST protein inhibited the binding of $VEGF_{165}$ to HUVEC and 231 cells and inhibited $VEGF_{165}$-induced HUVEC proliferation. Using a similar approach, 12 amino acid peptides derived from exon-6 of VEGF were shown to specifically inhibit ^{125}I-$VEGF_{165}$ binding to HUVEC and subsequently prostacyclin production [Jia et al., 2001]. The inhibitory effect was greater for ^{125}I-$VEGF_{165}$ binding to NRP1 than binding to KDR. These results suggest that interfering with $VEGF_{165}$ binding to NRP1 decreased KDR activity.

In summary, we have demonstrated that NRP1, KDR, and $VEGF_{165}$ form a ternary complex, both on the surface of EC or between tumor cells and EC. Formation of this ternary complex may possibly explain how the expression of NRP1 enhances $VEGF_{165}$ interactions with KDR. Tumor cell NRP1 interaction with EC KDR via $VEGF_{165}$ may provide a novel mechanism for juxtacrine activation of tumor angiogenesis.

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