

# Neuropilin-1 Mediates Collapsin-1/Semaphorin III Inhibition of Endothelial Cell Motility: Functional Competition of Collapsin-1 and Vascular Endothelial Growth Factor-165

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**Abstract.** Neuropilin-1 (NRP1) is a receptor for two unrelated ligands with disparate activities, vascular endothelial growth factor-165 (VEGF<sub>165</sub>), an angiogenesis factor, and semaphorin/collapsins, mediators of neuronal guidance. To determine whether semaphorin/collapsins could interact with NRP1 in nonneuronal cells, the effects of recombinant collapsin-1 on endothelial cells (EC) were examined. Collapsin-1 inhibited the motility of porcine aortic EC (PAEC) expressing NRP1 alone; coexpressing KDR and NRP1 (PAEC/KDR/NRP1), but not parental PAEC; or PAEC expressing KDR alone. The motility of PAEC expressing NRP1 was inhibited by 65–75% and this inhibition was abrogated by anti-NRP1 antibody. In contrast, VEGF<sub>165</sub> stimulated the motility of PAEC/KDR/NRP1. When VEGF<sub>165</sub> and collapsin-1 were added simultaneously to PAEC/KDR/NRP1, dorsal root ganglia (DRG), and

COS-7/NRP1 cells, they competed with each other in EC motility, DRG collapse, and NRP1-binding assays, respectively, suggesting that the two ligands have overlapping NRP1 binding sites. Collapsin-1 rapidly disrupted the formation of lamellipodia and induced depolymerization of F-actin in an NRP1-dependent manner. In an in vitro angiogenesis assay, collapsin-1 inhibited the capillary sprouting of EC from rat aortic ring segments. These results suggest that collapsin-1 can inhibit EC motility as well as axon motility, that these inhibitory effects on motility are mediated by NRP1, and that VEGF<sub>165</sub> and collapsin-1 compete for NRP1-binding sites.

**Key words:** angiogenesis • chemotaxis • KDR • neuronal guidance • growth cones

**V**ASCULAR endothelial growth factor (VEGF)<sup>1</sup> is a potent mitogenic and chemotactic factor for endothelial cells (EC) in vitro and an angiogenesis factor in vivo (Klagsbrun and D'Amore, 1996; Iruela-Arispe and Dvorak, 1997; Korpelainen and Alitalo, 1998; Ferrara et al., 1998; Neufeld et al., 1999). Recently, we identified neuropilin-1 (NRP1) as a receptor for VEGF,

which is expressed on the surface of EC and tumor cells (Soker et al., 1998). NRP1 is the third VEGF receptor described following KDR/Flk-1 and Flt-1, but differs from these two in that it binds VEGF<sub>165</sub> but not VEGF<sub>121</sub>. VEGF<sub>165</sub> binds to NRP1 via the VEGF exon 7-encoded domain that is lacking in VEGF<sub>121</sub> (Soker et al., 1996, 1997), whereas Flt-1 and KDR/Flk-1 bind VEGF via the exon 3- and 4-encoded domains, respectively (Keyt et al., 1996). There is some evidence that NRP1 may be a mediator of angiogenesis. Coexpression of NRP1 and KDR in porcine aortic EC (PAEC) enhanced VEGF<sub>165</sub> binding to KDR and the chemotactic activity of VEGF<sub>165</sub> for these cells, compared with PAEC expressing KDR alone (Soker et al., 1998). These results suggest that in EC, NRP1 might be acting as a coreceptor that enhances VEGF<sub>165</sub>-induced activities mediated by KDR. Overexpression of NRP1 in mice resulted in excess capillary and blood vessel formation and hemorrhaging in the embryo, contributing to embryonic lethality (Kitsukawa et al., 1995). Targeted disrupt-

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1. *Abbreviations used in this paper:* AP, alkaline phosphatase; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; DRG, dorsal root ganglia; EC, endothelial cells; NRP1, neuropilin-1; PAEC, porcine aortic EC; RAEC, rat aortic EC; VEGF, vascular endothelial growth factor.

tion of NRP1 resulted in embryonic lethality, attributable to unspecified defects in the cardiovascular system (Kitsukawa et al., 1997).

NRP1 was first described as a cell surface glycoprotein expressed on axons in the developing nervous system (Takagi et al., 1987, 1991, 1995; Fujisawa et al., 1995, 1997; Satoda et al., 1995). It was suggested that NRP1 was involved in neuronal cell recognition, axonal growth, and fasciculation in particular neuronal circuits (Kawakami et al., 1996). Targeted disruption of the NRP1 gene in mice resulted in severe abnormalities in the trajectory of the cranial and spinal efferent fibers that express NRP1 normally (Kitsukawa et al., 1997). Overexpression of NRP1 in mice resulted in sprouting and defasciculation of nerve fibers (Kitsukawa et al., 1995). Recently, it was demonstrated that NRP1 is a receptor for the semaphorins (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Mammalian semaphorins and their chick homologues, the collapsins, are a family of transmembrane or secreted glycoproteins present in neuronal and nonneuronal tissues that act as mediators of neuronal guidance (Luo et al., 1993; Tessier-Lavigne and Goodman, 1996; Kolodkin, 1996; Kolodkin and Ginty, 1997). Secreted collapsin-1 (Sema III/Sema D) acts as a localized repulsive cue that steers sensory growth cones away from the collapsin source and induces the collapse of dorsal root ganglia (DRG) neuronal growth cones (Luo et al., 1993). The collapsin/semaphorin repulsive effects on neurons are mediated by binding to NRP1 as demonstrated by the abrogation of these effects by anti-NRP1 antibodies (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Targeted disruption of Sema III resulted in inappropriate projection of sensory neurons into the spinal cord where Sema III is normally expressed (Behar et al., 1996).

These results demonstrate that NRP1 binds two ligands that appear to have opposing effects on motility. In neuronal cells, NRP1 binds semaphorin/collapsins that inhibit the motility of axons. In EC, NRP1 binds VEGF<sub>165</sub> and acts as a coreceptor for KDR by enhancing KDR-mediated chemotaxis. Since EC express NRP1, we wanted to determine whether collapsin-1 would be active in a non-neuronal cell by binding to EC and affecting EC motility via NRP1. Furthermore, since collapsin-1 and VEGF<sub>165</sub> bind to the same receptor, NRP1, we wanted to determine whether they competed with each other in functional EC and DRG assays. In this report, we demonstrate that recombinant collapsin-1 binds to EC via NRP1 and inhibits EC motility in an NRP1-dependent manner. It prevents formation of lamellipodia and depolymerizes F-actin, resulting in disorganization of the EC cytoskeleton, consistent with its effects on the growth cone cytoskeleton (Luo et al., 1997). Collapsin-1 and VEGF<sub>165</sub> compete with each other in EC motility, DRG collapse, and NRP1-binding assays, suggesting there is overlap in the NRP1 binding sites of the two ligands. In an *in vitro* angiogenesis assay, collapsin-1 inhibits EC sprouting and microvessel tube formation in aortic segments embedded in collagen gels. We conclude that collapsin-1 can inhibit not only axon motility, but EC motility as well, and that the inhibition is mediated by NRP1. Our results support the hypothesis that regulators of neuronal guidance may also be regulators of angiogenesis.

## Materials and Methods

### Materials

Human fibroblast growth factor-2 (FGF-2) and VEGF were generous gifts from Dr. Judith Abraham (Scios Inc., Sunnyvale, CA). Anti-NRP1 antibodies raised against rat NRP1 (Kolodkin et al., 1997) were kindly provided by Dr. Alex Kolodkin (Johns Hopkins University School of Medicine, Baltimore, MD). Cell culture media and lipofectamine were purchased from Life Technologies, Inc. Hygromycin B and protease inhibitors were purchased from Boehringer Mannheim. [<sup>3</sup>H]thymidine, [<sup>125</sup>I]sodium, [<sup>32</sup>P]dCTP, and GeneScreen-Plus hybridization transfer membranes were purchased from DuPont-NEN. Disuccinimidyl suberate (DSS) and IODO-BEADS were purchased from Pierce Chemical Co. RNAzol-B was purchased from TEL-TEST. DNA labeling kits were purchased from Nycomed Amersham Inc. Restriction endonucleases and ligase were purchased from New England Biolabs. X-ray films were purchased from Eastman Kodak. Recombinant human fibronectin, Falcon® CultureSlide, and other tissue culture ware were purchased from Becton Dickinson. All other chemicals were purchased from Sigma Chemical Co., unless otherwise mentioned.

### Cell Culture

Parental PAEC and PAEC expressing KDR (PAEC/KDR) were kindly provided by Dr. Lena Claesson-Welsh (University of Uppsala, Uppsala, Sweden; Waltenberger et al., 1994). The establishment of PAEC expressing NRP1 (PAEC/NRP1) and PAEC expressing KDR and NRP1 (PAEC/KDR/NRP1) were previously described by Soker et al. (1998). These cell lines were grown in F-12 medium containing 10% FCS, glutamine, penicillin, and streptomycin. Clones of rat aortic EC (RAEC) were harvested from rat aortic capillary sprouts by digesting the collagen gel with 1 mg/ml type I collagenase (Worthington Biochemical Co.) and growing the cells in complete DME, 1 g/liter glucose. FGF-2 (1 ng/ml) was added to the culture every other day. Colonies exhibiting typical EC cobblestone monolayer morphology were selected and subcultured. The identity of the cells as EC was confirmed by immunostaining for von Willebrand factor and by their mitogenic response to VEGF. Cells at passage 4–8 were used in experiments.

### Radioiodination of Collapsin-1 and Chemical Cross-linking

The radioiodination of collapsin-1 was achieved using IODO-BEADS (Soker et al., 1998) and specific activities ranging from 40,000–100,000 cpm/ng were obtained. Cross-linking of <sup>125</sup>I-collapsin-1 to PAEC was achieved using DSS as a cross-linker in the presence of 1 μg/ml heparin, as previously described (Soker et al., 1998). Cross-linked complexes were resolved by 6% SDS-PAGE and the gels were exposed to X-ray films.

### AP-VEGF<sub>165</sub> Production and Cell Binding

The preparation of alkaline phosphatase (AP)-VEGF<sub>165</sub> fusion protein and its binding to NRP1-transfected COS-7 cells was carried out using methods similar to those described previously for preparing AP-collapsins and their binding to COS-7 cells (Feiner et al., 1997). In brief, the open reading frame (ORF) of VEGF<sub>165</sub> was amplified with primers 5'-AATAATGGATCCGCACCCATGGCAGAAGGAG-3' and 5'-ATATATGCGCGCCGCTACCGCCTCGGCTTGTG-3', digested with BamHI and NotI, and cloned into a modified version of pcDNA3 (PAG-NT) that placed VEGF<sub>165</sub> downstream of AP containing a signal sequence. To prepare recombinant AP-VEGF<sub>165</sub>, 293T cells grown to ~70% confluency were transfected with 50–60 μg plasmid per 150-mm dish using calcium phosphate precipitation, and conditioned medium (CM) was collected. To quantitate AP-VEGF<sub>165</sub>, supernatants were assayed for AP activity. The amount of AP activity in CM was titrated to correspond to the activity of known amounts of AP. For binding experiments, stable COS-7 cell lines expressing NRP1 (COS-7/NRP1) were established by transfection of NRP1 cDNA in a PAG-NT vector and selection with G418. The COS-7/NRP1 cells in 48-well plates were incubated with serial dilutions of 293T cell AP-VEGF<sub>165</sub> in the absence or presence of 30 nM (~3 μg/ml) collapsin-1 or 50 nM (~1.25 μg/ml) untagged VEGF<sub>165</sub>. After binding of AP-VEGF<sub>165</sub> for 1 h at room temperature, COS-7/NRP1 cells were washed gently six times for 5 min each with PBS, fixed with 4% paraformaldehyde for 30 min, and washed extensively with PBS. Endogenous AP was heat-inactivated by incubation at 72°C for 3 h. To measure cell

bound AP-VEGF<sub>165</sub>. COS-7/NRP1 cells were incubated with a phosphatase reaction mixture consisting of 0.5 M diethanolamine, 0.25 mM MgCl<sub>2</sub>, 5 mM L-homoarginine, 0.25 mg/ml BSA, 6 mM p-nitrophenylphosphate for 3 h and OD<sub>414</sub> was measured in a MCC340 Microplate Spectrophotometer (Titertek Instruments).

### Northern Analysis

Total RNA was prepared from cells in culture using RNAzol according to the manufacturer's instructions. Samples of 15 µg RNA were separated on 1% formaldehyde-agarose gels and transferred to GeneScreen-Plus membranes. The membranes were hybridized (42°C for 18 h) using hybridization cocktails (AMRESCO) with a <sup>32</sup>P-labeled fragment of rat NRP1 cDNA corresponding to nucleotides 400–905 in the ORF. After hybridization, membranes were washed and exposed to X-ray film.

### Motility Assay

Motility assays were performed in a Boyden chamber (Neuro Probe Inc.) as described previously (Elenius et al., 1997; Soker et al., 1998). In brief, 15,000 cells in serum-free medium (F12 for PAEC, DME for RAEC) containing 0.1% BSA were added to wells in the upper chamber. Increasing concentrations of collapsin-1 were added to wells in the lower chamber, in the absence or presence of VEGF<sub>165</sub> or anti-NRP1 antibody. The upper and lower chambers were separated by a fibronectin-coated polycarbonate membrane with a pore size of 8 µm (Corning Inc.). The number of cells that had migrated through the filter after 4 h at 37°C was counted by phase microscopy. One collapsing unit (CU) is defined as the concentration required to cause 50% collapse of DRG growth cones in culture, with 1 CU being equivalent to ~3 ng/ml (Luo et al., 1993). When motility towards VEGF was measured, cells were serum-starved overnight to lower baseline migration.

### Aortic Ring Assay

The rat aortic ring assay was performed as described previously (Nicosia and Ottinetti, 1990; Miao et al., 1997). In brief, thoracic aortas were obtained from 3-mo-old Lewis rats. The fibroadipose tissue was carefully removed under a dissecting microscope, and the aortas were sliced at 1-mm intervals to obtain aortic rings. The aortic rings were placed on top of 0.1-ml collagen gels in each well of a 48-well plate and a 0.2-ml collagen solution was carefully poured on top of the ring. After the gel was formed, 0.2-ml serum-free endothelial growth medium (Life Technologies, Inc.), which favors the growth of EC but not smooth muscle cells or fibroblasts, was added and replaced every other day by fresh medium containing the indicated concentration of collapsin-1. Microvessel outgrowth was visualized by phase microscopy and the number of capillary vessels was counted throughout the course of the experiment. Microvessel structure in aortic rings previously has been characterized by light microscopy, EM, and immunohistochemical staining of von Willebrand factor (Nicosia and Ottinetti, 1990; Miao et al., 1997).

### Collapse Assay

The growth cone collapse assay using DRG isolated from E7 chick embryos was performed as described in Luo et al. (1993). Collapsin-1 dose-response curves were obtained in the presence and absence of VEGF<sub>165</sub>. VEGF<sub>165</sub> was resuspended in media to achieve a stock concentration of 1 mg/ml and was added to each culture to achieve a final concentration of 100 ng/ml. Purified recombinant collapsin-1 at increasing concentrations was added to the cultures at the same time. After a 60-min incubation at 37°C in 5% CO<sub>2</sub>, the cultures were fixed in 4% paraformaldehyde in PBS containing 10% sucrose. The tips of neurites without lamellipodia or filopodia were scored as being collapsed.

### Differential Interference Contrast (DIC) Optic Microscopy

Cells were seeded on 8-well fibronectin-coated Falcon® CultureSlides, at a density of 5,000 cells/ml in complete tissue culture medium. At 24 h after seeding, collapsin-1 (300 ng/ml) was added to the medium. The cells were incubated (30 min at 37°C), fixed and permeabilized with 0.5% glutaraldehyde and 0.2% saponin in a buffer containing 10 mM MES, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, and 5 mM glucose (2 min at 37°C). They were then further fixed (8 min at 37°C) with 1% glutaraldehyde in the same buffer. Upon completion of these treatments, the cells were washed twice with PBS,

fixed in 4% paraformaldehyde in PBS for 20 min, and further washed two times with PBS and once with PBS supplemented with 0.1% BSA. Cells were examined in a Nikon Diaphot 300 inverted microscope, using a Nikon 40× PlanFluor objective and Nomarsky differential interference contrast (DIC) optics. Digital images were captured using a Sensys KAF 1400 cooled CCD output camera (Photometrics) and controlled by an IPLab image analysis program (Scanalytics Inc.).

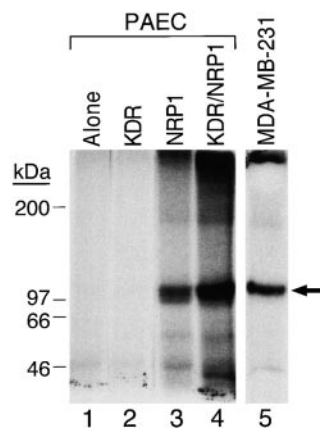
### Fluorescence Microscopy

RAEC were fixed for examining F-actin organization using conventional fluorescence microscopy. In brief, cells were washed with prewarmed serum-free DME, fixed, and permeabilized with prewarmed 0.5% glutaraldehyde, 0.2% saponin (2 min) in a cytoskeletal stabilizing (CSK) buffer containing 50 mM NaCl, 150 mM sucrose, 3 mM MgCl<sub>2</sub>, 10 mM 2-ethanesulfonic acid, pH 6.8, which maintains the integrity of the cytoskeleton. This solution was replaced and a second fixation step was carried out using 1% glutaraldehyde in CSK buffer for 10 min. Fixed specimens were washed three times with CSK buffer and exposed aldehyde groups were reduced with a freshly prepared solution of 0.5 mg/ml NaBH<sub>4</sub> in CSK buffer (40 min). After washing three times with 0.1% BSA in CSK buffer, cell actin cytoskeleton and nuclei were stained for 60 min simultaneously with fluorescein-labeled phalloidin (1:300; Molecular Probes) and DNA-binding dye, 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml; Molecular Probes). After staining, the cells were washed extensively with 0.1% BSA in CSK buffer (five times for 10 min each) and mounted in Fluoromount-G (Southern Biotechnology Associates Inc.). The samples were examined in an epifluorescence Nikon Diaphot 300 inverted microscope with a Nikon 60× PlanApo oil immersion objective. Digital images were captured using the same system.

## Results

### Collapsin-1 Binds to Endothelial Cells only if they Express Neuropilin-1

NRP1 is expressed by neuronal cells and is a receptor for members of the collapsin-1/semaphorin III family (Luo et al., 1993; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). To determine whether NRP1 can also function as a receptor for collapsin/semaphorins in nonneuronal cells, PAEC lines expressing NRP1, KDR, or both receptors (Soker et al., 1998) were incubated with <sup>125</sup>I-collapsin-1 (Fig. 1). A 100-kD protein corresponding to the size of collapsin-1, and larger size proteins possibly representing collapsin-1/NRP1 complexes, were detected in PAEC/NRP1



**Figure 1.** Cross-linking of <sup>125</sup>I-collapsin-1. <sup>125</sup>I-collapsin-1 (10 ng/ml) was bound to subconfluent cultures of PAEC (lane 1), PAEC/KDR (lane 2), PAEC/NRP1 (lane 3), PAEC/KDR/NRP1 (lane 4), and MDA-MB-231 (lane 5) cells in 6-cm dishes. The binding was carried out in the presence of 1 µg/ml of heparin. At the end of a 2-h incubation, <sup>125</sup>I-collapsin-1 was cross-linked to the cell surface. The cells were lysed and proteins were resolved by 6% SDS-PAGE. The polyacrylamide gel was dried and exposed to X-ray film. Arrow indicates the position of bound <sup>125</sup>I-collapsin-1. In this experiment, noncovalent binding, rather than covalent cross-linking of <sup>125</sup>I-collapsin-1 to NRP1, occurred.

(Fig. 1, lane 3) and PAEC/KDR/NRP1 (Fig. 1, lane 4), but not in parental PAEC (Fig. 1, lane 1) or PAEC/KDR (Fig. 1, lane 2). These results indicated that the binding of  $^{125}\text{I}$ -collapsin-1 to PAEC was totally dependent on the expression of NRP1. In addition,  $^{125}\text{I}$ -collapsin also bound to MDA-MB-231 breast carcinoma cells (Fig. 1, lane 5), previously shown to express abundant NRP1 (Soker et al., 1996, 1998).

### Collapsin-1 Inhibits Endothelial Cell Motility

Collapsin/semaphorins are inhibitors of axonal motility acting via NRP1 (Kolodkin et al., 1997). Accordingly, the ability of collapsin-1 to affect the motility of EC expressing NRP1 was tested in a Boyden chamber assay (Fig. 2). Collapsin-1 (150 ng/ml) inhibited the motility of PAEC/NRP1 and PAEC/KDR/NRP1 by ~65–70%, but did not inhibit the motility of PAEC or PAEC/KDR at all (Fig. 2 A). The specific inhibition of only those EC expressing NRP1 was consistent with the binding data. Collapsin-1 inhibition of PAEC/NRP1 and PAEC/KDR/NRP1 motility was dose-dependent with an  $\text{ID}_{50}$  (inhibitory dose) of ~3 ng/ml (Fig. 2 B). A maximal inhibition of ~65–75% occurred with ~15 ng/ml. To test whether the collapsin-1-mediated inhibition of motility was due to repulsion, collapsin-1 was placed in both upper and lower chambers. The same results were found as with the presence of collapsin-1 in the lower chamber alone (not shown), suggesting that collapsin-1 is not repelling the cells in this particular motility assay.

Semaphorin III-mediated growth cone collapse is inhibited by anti-NRP1 antibodies (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). A similar antibody preparation (Kolodkin et al., 1997) was tested on collapsin-1-treated PAEC expressing NRP1 (Fig. 2 C). In the absence of collapsin-1, the anti-NRP1 antibody had no significant effect on the motility of either PAEC/NRP1 or PAEC/KDR/NRP1 (Fig. 2 C, lane 2 compared with lane 1, for each cell type). Collapsin-1 at 150 ng/ml inhibited PAEC/NRP1 and PAEC/KDR/NRP1 motility by 55 and 65%, respectively (Fig. 2 C, lane 3 compared with lane 1, for each cell type). However, in the presence of anti-NRP1 antibody, the inhibitory effects of 150 ng/ml collapsin-1

were reduced to 18 and 14% for PAEC/NRP1 and PAEC/KDR/NRP1, respectively (Fig. 2 C, lane 4 compared with lane 3, for each cell type). Normal rabbit IgG was ineffective in inhibiting collapsin-1 activity (not shown).

While collapsin-1 inhibited NRP1-mediated motility, it did not inhibit DNA synthesis in PAEC/NRP1 or PAEC/KDR/NRP1 (not shown). Lack of any inhibitory effects in the 48-h DNA synthesis assay was taken as evidence that the inhibition of EC motility in the 4-h motility assay was not due to collapsin-1-mediated cell toxicity.

### Collapsin-1 and VEGF<sub>165</sub> Compete for PAEC/KDR/NRP1 Motility, DRG Collapse, and Binding to NRP1

Since NRP1 is a receptor for both VEGF<sub>165</sub> and collapsin-1, we wanted to test whether they would compete with each other in affecting NRP1-mediated activities and in binding to NRP1. First, we investigated the effects of collapsin-1 on VEGF<sub>165</sub> stimulatory activity and the effects of VEGF<sub>165</sub> on collapsin-1 inhibitory activity (Fig. 3). VEGF<sub>165</sub> is chemotactic for PAEC/KDR/NRP1 (Soker et al., 1998). PAEC/KDR/NRP1 were stimulated with increasing concentrations of VEGF<sub>165</sub> in the absence or presence of 150 ng/ml collapsin-1 in a Boyden chamber motility assay (Fig. 3 A). In the absence of collapsin-1, VEGF stimulated PAEC/KDR/NRP1 chemotaxis in a dose-dependent manner with a typical bell-shaped curve. However, in the presence of a constant amount of collapsin-1 (150 ng/ml), the chemotactic activity of VEGF<sub>165</sub> was reduced at each concentration of VEGF<sub>165</sub> (0.1–50 ng/ml) although never totally abrogated. About five times more VEGF<sub>165</sub> was required for half-maximal stimulation of PAEC/KDR/NRP1 chemotaxis when 150 ng/ml of collapsin-1 was present. In a reciprocal experiment, the effect of increasing concentrations of collapsin-1 (0–300 ng/ml) on the inhibition of PAEC/KDR/NRP1 motility at a constant level of VEGF<sub>165</sub> was tested (Fig. 3 B). VEGF<sub>165</sub> was added at 5 ng/ml, the optimal concentration for stimulating chemotaxis, as shown in Fig. 3 A. Collapsin-1 inhibited both basal motility and VEGF<sub>165</sub>-induced motility in a dose-dependent manner. However, in the presence of VEGF<sub>165</sub>, the motility levels were higher, about six- to sevenfold at each concentration of collapsin-1.

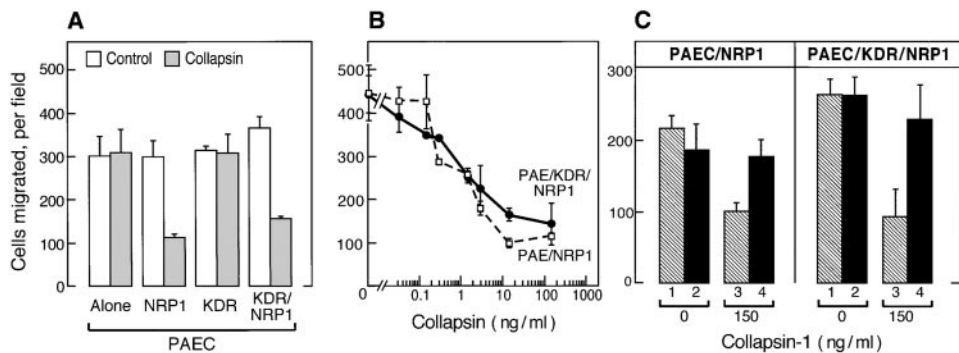
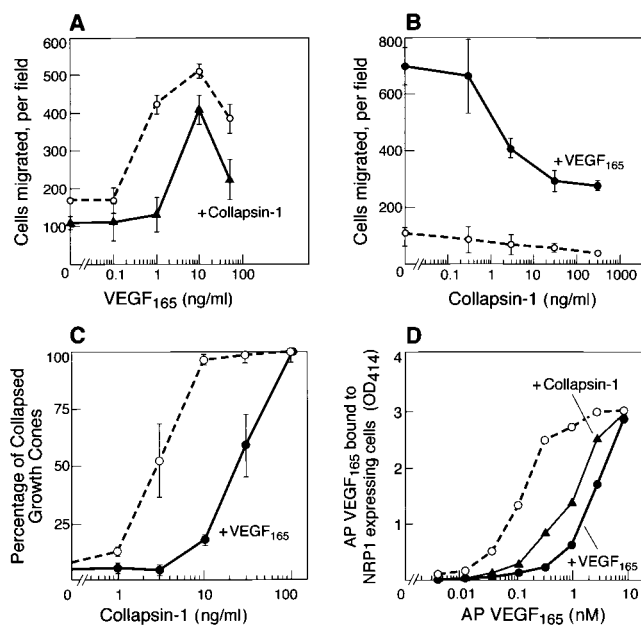


Figure 2. Collapsin-1 inhibits the motility of EC expressing NRP1. (A) PAEC, PAEC/NRP1, PAEC/KDR, and PAEC/KDR/NRP1 were seeded in the upper wells of a Boyden chamber. Collapsin-1 (150 ng/ml) was added (gray bars) or not added (white bars) to the lower wells. (B) PAEC/NRP1 and PAEC/KDR/NRP1 were seeded in the upper wells and increasing concentrations of collapsin-1 were added to the lower wells of the Boyden

chamber. (C) PAEC/NRP1 and PAEC/KDR/NRP1 were cultured in complete medium and seeded in the upper wells of a Boyden chamber. For each cell type, either 0 (lanes 1 and 2) or 150 ng/ml (lanes 3 and 4) of collapsin-1 was added in the absence (hatched bars) or presence (solid bars) of 30  $\mu\text{g/ml}$  anti-NRP1 antibodies. For each set of experiments, after a 4-h incubation, the numbers of cells that had migrated through the filter in each field were counted. Each data point represents the mean  $\pm$  SD of four independent wells.



**Figure 3.** Competition of collapsin-1 and VEGF<sub>165</sub> for PAEC/KDR/NRP1 motility, DRG collapse, and binding to NRP1 expressed by COS-7 cells. (A) PAEC/KDR/NRP1 motility at a constant concentration of collapsin-1. Serum-starved PAEC/KDR/NRP1 were seeded in the upper wells of a Boyden chamber. Increasing concentrations of VEGF<sub>165</sub> were added to the lower wells in the absence (white circles) or presence (black triangles) of 150 ng/ml collapsin-1. (B) PAEC/KDR/NRP1 motility at a constant concentration of VEGF<sub>165</sub>. Serum-starved PAEC/KDR/NRP1 cells were seeded in the upper wells of a Boyden chamber and increasing concentrations of collapsin-1 were added to the lower wells in the absence (white circles) or presence (black circles) of 5 ng/ml VEGF<sub>165</sub>. After 4 h, the numbers of migrated cells per field were counted as in Fig. 2. Each data point represents the mean  $\pm$  SD of four independent wells. (C) DRG collapse. The biological activity of collapsin-1 on DRG is attenuated by VEGF<sub>165</sub>. A growth cone collapse assay was performed in the presence (black circles) and absence (white circles) of 100 ng/ml recombinant VEGF<sub>165</sub>. The percentage of collapsed growth cones extending from explanted DRG are plotted against the concentration of recombinant collapsin-1 added to the culture. Greater concentrations of collapsin-1 are required to achieve the same level of collapse when VEGF<sub>165</sub> is present. (D) Competitive binding to COS-7/NRP1. COS-7 cells stably expressing NRP1 were incubated for 1 h with serial dilutions of CM containing AP-VEGF<sub>165</sub> in the absence (white circles) or presence of 30 nM (3  $\mu$ g/ml) collapsin-1 (black triangles) or 50 nM (1.25  $\mu$ g/ml) VEGF<sub>165</sub> (black circles). AP-VEGF<sub>165</sub> bound to COS-7/NRP1 cells was measured colorimetrically at OD<sub>414</sub>.

We also examined whether VEGF<sub>165</sub> and collapsin-1 have competing effects on growth cone motility (Fig. 3 C). Collapsin-1 induces the collapse and paralysis of specific neuronal growth cones, including those growing from explanted DRG (Luo et al., 1993). Increasing concentrations of collapsin-1 normally induce collapse with a half maximal effect at  $\sim$ 3 ng/ml. However, collapsin-1 is about sevenfold less effective in inducing growth cone collapse when 100 ng/ml VEGF<sub>165</sub> is present. Additional assays (data not shown) suggest that VEGF<sub>165</sub> is by itself neither an attractant nor a repellent for sensory growth cones.

Concentrations of VEGF<sub>165</sub> up to 200 ng/ml did not induce growth cone collapse, nor did they attract axons extending from DRG explants embedded in a collagen/matrigel matrix that contained 293T cells expressing recombinant VEGF<sub>165</sub>. Thus, the ability of VEGF<sub>165</sub> to reduce the effectiveness of collapsin-1 is unlikely to represent the activation of a parallel VEGF<sub>165</sub>-mediated attractive response, but might reflect a degree of competitive binding to NRP1.

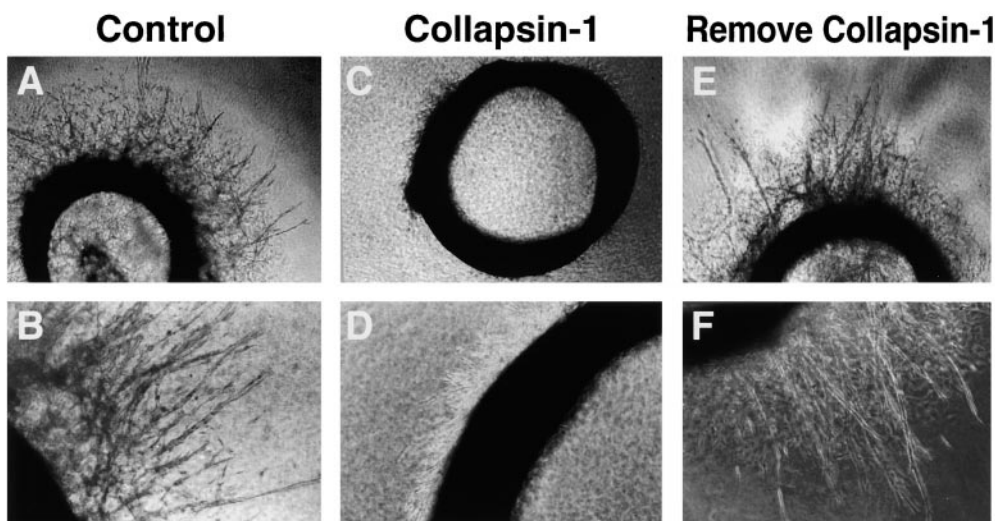
Since collapsin-1 and VEGF<sub>165</sub> compete with each other functionally, we investigated whether they would compete with each other for binding to NRP1. A stable COS-7 cell line expressing NRP1 (COS-7/NRP1) was established and incubated with increasing amounts of an AP-VEGF<sub>165</sub> fusion protein. AP-VEGF<sub>165</sub> binds to these cells in a dose-dependent manner (Fig. 3 D). No binding to parental COS-7 cells occurred (not shown). Binding of AP-VEGF<sub>165</sub> to COS-7/NRP1 cells was competed 12-fold by 50 nM untagged VEGF<sub>165</sub>. Collapsin-1 at a concentration of 30 nM inhibited AP-VEGF<sub>165</sub>-binding by  $\sim$ 10-fold. Taken together, the functional and binding results suggest that VEGF<sub>165</sub> and collapsin-1 compete with each other for binding sites on NRP1.

### ***Collapsin-1 Inhibits Microvessel Outgrowth from Aortic Rings***

The effects of collapsin-1 were tested on EC sprouting and microvessel formation in rat aortic ring segments that were embedded in collagen gels and maintained in a serum-free medium that favored microvessel outgrowth (Fig. 4). As observed by phase-contrast microscopy, branching microvessels formed a capillary network of tubes and loops with lumen-like structures at the periphery of the aortic rings, starting on day four and reaching a maximal degree of sprouting on days 12–15 (Fig. 4, A and B). The identity of the EC in the sprouts was confirmed by immunohistochemical detection of von Willebrand factor and by morphological analysis using EM (not shown). When collapsin-1 (300 ng/ml) was added on day two after embedding of the aortic rings and thereafter every second day, the outgrowth of sprouts was strongly inhibited (Fig. 4, C and D). A quantitative analysis demonstrated that collapsin-1 inhibited microvessel EC outgrowth by 80–90% (Fig. 5). When collapsin-1 was added into the culture medium on day two and washed away on day four, normal outgrowth of microvessels was observed on day 12 (Fig. 4, E and F). The reversibility of the microvessel outgrowth demonstrated that collapsin-1 inhibition of sprouting was not due to cell toxicity.

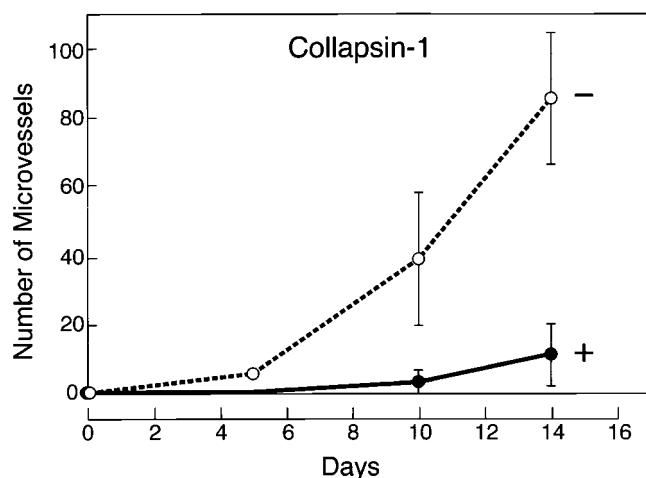
### ***Collapsin-1 Disrupts Endothelial Cell Lamellipodia Formation and Cytoskeleton Organization in an NRP1-dependent Manner***

Maintenance and physiological reorganization of the cytoskeleton play a crucial role in cell motility in response to mechanical and humoral stimuli (Hall, 1998; Mackay and Hall, 1998; Sheetz et al., 1998). RAEC sprouting from aortic rings were cultured. These cells formed a typical EC cobblestone monolayer at confluence (not shown), expressed NRP1 as demonstrated by Northern blot analysis (Fig. 6 A), and their motility was inhibited by collapsin-1 in a dose-dependent manner with an ID<sub>50</sub> of  $\sim$ 10 ng/ml



**Figure 4.** Effect of collapsin-1 on microvessel outgrowth *in vitro*. Rat aortic rings were embedded in type I collagen gels. Serum-free endothelial growth medium was added and replaced every other day with fresh medium. Microvessel structure was observed by phase microscopy on day 14 at low (A, C, and E) or high (B, D, and F) magnification. A and B, no addition; C and D, collapsin-1 (300 ng/ml) was added on day two and every second day thereafter; E and F, collapsin-1 was added on day two and the medium was replaced on day four without further addition of collapsin-1.

and a maximal inhibition of 60–65% at 30 ng/ml (Fig. 6 B). However, in the presence of anti-NRP1 antibody, the inhibitory effects of 150 ng/ml collapsin-1 were reduced to ~18% (not shown), consistent with the inhibitory effects of these antibodies on collapsin-1 inhibition of PAEC/NRP1 and PAEC/KDR/NRP1 motility that was shown in Fig. 2 C. To clarify possible mechanisms of collapsin-1 inhibition of EC, RAEC were seeded on fibronectin-coated glass chamber slides, grown for one day, treated with collapsin-1 (300 ng/ml) for 30 min, and analyzed by DIC optic microscopy (Fig. 7, A–C) and phalloidin-FITC staining (Fig. 7, D–F). RAEC typically exhibit numerous active lamellipodia, as characterized by membrane ruffling (Fig. 7, A and D). However, in the presence of collapsin-1, there was a significant retraction of the lamellipodia, as



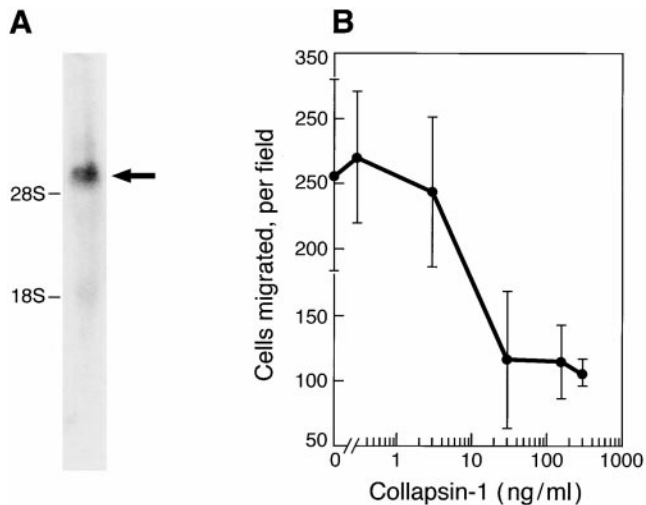
**Figure 5.** Quantification of microvessel outgrowth in the presence or absence of collapsin-1. The sprouting microvessels shown in Fig. 4, without collapsin-1 (white circles) or with 300 ng/ml collapsin-1 (black circles), were counted under a phase microscope. Each data point represents the mean  $\pm$  SD of four independent wells.

shown in Fig. 7, B and E, that occurred in 30–50% of the RAEC population. Time-lapse video microscopy showed that these alterations in lamellipodia structures began ~10 min after exposure to collapsin-1 (not shown). Within a given responsive cell, almost of all the lamellipodia were retracted. The cell membranes became thinner, ruffling was undetectable, and cell surface blebs were observed. Phalloidin-FITC staining showed that collapsin-1 treatment resulted in the loss of polymerized actin fibers (Fig. 7, E compared with D). In these cells, ~70–80% of the F-actin was depolymerized. It appeared that depolymerized actin was clustered in the retracted lamellipodia. The collapsin-1 effects on RAEC morphology were abrogated when collapsin-1 was heat-inactivated by 70°C treatment for 30 min (Fig. 7, C and F). DAPI staining of nuclei showed no DNA breakage, indicating that collapsin-1 did not induce apoptosis in the RAEC (Fig. 7, G–I).

To determine whether the collapsin-1 inhibition of lamellipodia formation observed for RAEC was mediated by NRP1, parental PAEC and PAEC expressing NRP1, KDR, or both receptors, were treated with collapsin-1 and analyzed by DIC optic microscopy (Fig. 8). Collapsin-1 did not induce morphological changes in parental PAEC (Fig. 8, A versus E) or in PAEC/KDR (Fig. 8, C versus G). On the other hand, 300 ng/ml collapsin-1 altered the morphology of PAEC/NRP1 (Fig. 8, B versus F) and PAEC/KDR/NRP1 (Fig. 8, D versus H), by inducing significant retraction of lamellipodia. Taken together, RAEC and PAEC morphology analysis suggest that collapsin-1 disorganizes EC cytoskeleton, retracts lamellipodia, and that these effects are mediated by NRP1.

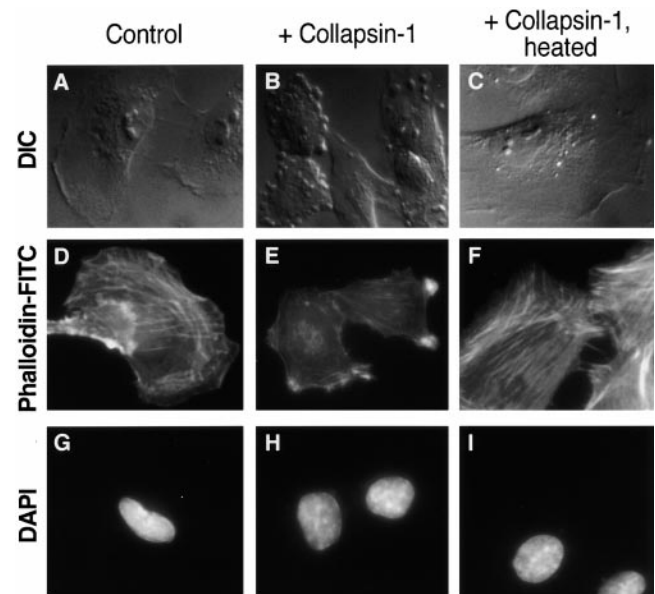
## Discussion

NRP1 is an unusual receptor in that it binds two structurally unrelated ligands that have different biological activities. In neuronal cells, NRP1 is a receptor for members of the semaphorin family (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). A subset of secreted vertebrate semaphorins repel and collapse advancing growth cones in



**Figure 6.** Collapsin-1 inhibits the motility of RAEC. RAEC were isolated from rat aortas as described in Materials and Methods and were cultured in DME containing 10% FCS and FGF-2 (1 ng/ml). (A) Northern blot analysis of total RAEC RNA with  $^{32}\text{P}$ -labeled rat NRP1 cDNA as a probe. Arrow indicates the position of rat NRP1. (B) The RAEC were seeded in the upper wells of a Boyden chamber and increasing concentrations of collapsin-1 were added to the lower wells. After 4 h, the number of migrated cells per field was counted as in Fig. 2. Each data point represents the mean  $\pm$  SD of four independent wells.

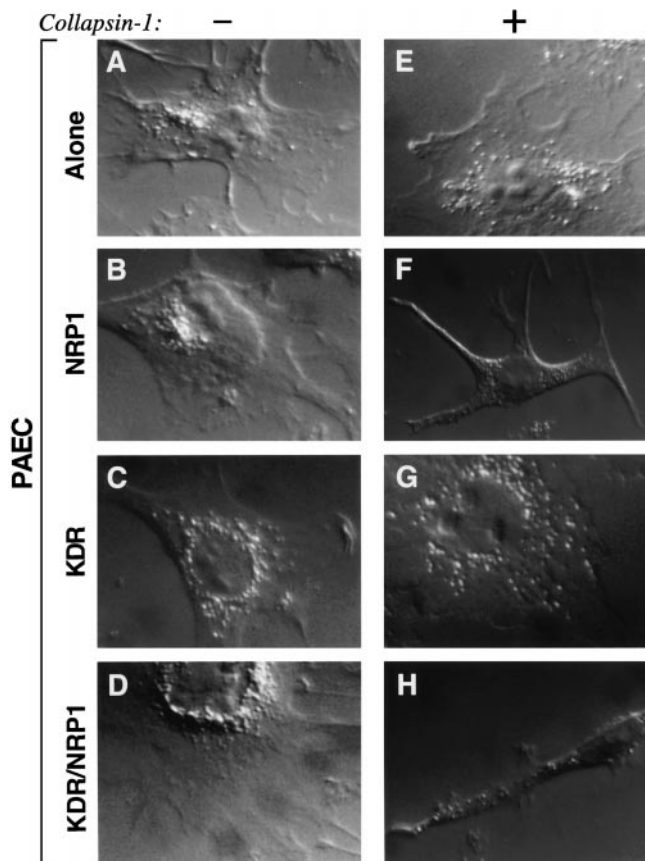
an NRP1-dependent manner. In EC, NRP1 is a receptor for VEGF<sub>165</sub>, a potent angiogenesis factor (Soker et al., 1998). Expression of NRP1 in EC enhances the ability of VEGF<sub>165</sub> to bind to KDR and to stimulate chemotaxis via KDR. Thus, depending on the ligand and cell type, interactions with NRP1 can lead to either inhibition or stimulation of cell motility. Since EC respond to VEGF<sub>165</sub> by enhanced motility, we wanted to know whether these cells would also respond to collapsin-1 (semaphorin III), the hypothesis being that collapsin-1 would inhibit EC motility in an NRP1-dependent manner. If collapsin-1 inhibited EC motility, it would be possible to determine whether or not VEGF<sub>165</sub> and collapsin-1 could compete with each other for an NRP1-dependent activity, such as motility, on the same cell type. That collapsin-1 would affect EC was not necessarily obvious, since it has been suggested that there may be additional receptor components in neurons that are required to mediate semaphorin activity (He and Tessier-Lavigne, 1997; Feiner et al., 1997; Kolodkin et al., 1997; Nakamura et al., 1998), and these putative receptors may not be present in EC. Collapsin-1 effects on EC were assayed on PAEC transfected with NRP1, primary cultures of RAEC, and EC sprouting from aortic segments embedded in collagen gels. It was found that collapsin-1 was an effective inhibitor of EC motility as follows: first, collapsin-1 bound only to PAEC expressing NRP1. Second, collapsin-1 inhibited the motility of PAEC expressing NRP1, but not the motility of parental PAEC or PAEC expressing KDR alone. The motility of PAEC/NRP1 and PAEC/KDR/NRP1, but not parental PAEC or PAEC/KDR, was inhibited by up to 60–70%, and the inhibition was abrogated by anti-NRP antibodies. The inhibition of



**Figure 7.** Collapsin-1 alters RAEC morphology. RAEC were seeded in 8-well culture slides precoated with fibronectin. The next day, medium was replaced with fresh medium (A, D, and G), medium containing 300 ng/ml collapsin-1 (B, E, and H), and medium containing 300 ng/ml collapsin-1 preheated at 70°C for 30 min (C, F, and I). After 30 min of incubation with or without collapsin-1, cells were fixed. (A–C) Analysis by DIC optic microscopy. (D–F) Staining with phalloidin-FITC and analysis by fluorescence microscopy. (G–I) The cells in D–F were stained for DNA with DAPI. The cells in A–C are not the same as those in D–F, because different fixation methods were used.

EC motility, as assayed in the Boyden chamber, did not appear to be due to collapsin-1-mediated chemorepulsion. Third, collapsin-1 inhibited the motility of primary RAEC, which express NRP1, by up to 50–60%, and this effect was abrogated by anti-NRP1 antibodies. Fourth, collapsin-1 inhibited by 80–90%, microvessel outgrowth and sprouting (in vitro angiogenesis) from rat aortic segments embedded in collagen. And fifth, collapsin-1 altered cell morphology markedly. As revealed by DIC optic microscopy, the addition of collapsin-1 resulted in the retraction of lamellipodia within minutes. Phalloidin-FITC staining demonstrated substantial loss of polymerized F-actin stress fibers in lamellipodia that are composed of a compact network of actin filaments. The changes in morphology were NRP1 dependent and did not occur in parental PAEC or PAEC/KDR. Nor did these changes occur when collapsin-1 was heat-inactivated. The collapsin-1 inhibitory effects on EC motility did not appear to be due to toxicity. For example, in the presence of collapsin-1 at relatively high concentrations, the motility of PAEC was inhibited only if they expressed NRP1. Basal DNA synthesis in PAEC was not inhibited in a 48-h assay. The inhibitory effects on EC outgrowth and sprouting from aortic segments were reversible by washing away the collapsin-1 after 48 h. DAPI staining of RAEC after collapsin-1 treatment showed intact nuclei. Taken together, these results demonstrate that collapsin-1 interacts with NRP1 in a functional manner in nonneuronal cells. Whether NRP1 alone is sufficient to





**Figure 8.** Collapsin-1 alters the morphology only of EC expressing NRP1. PAEC (A and E), PAEC/NRP1 (B and F), PAEC/KDR (C and G), and PAEC/KDR/NRP1 (D and H) were incubated for 30 min in the absence (A–D) or presence (E–H) of 300 ng/ml collapsin-1, fixed, and analyzed by DIC optic microscopy as in Fig. 7, A–C.

mediate a full inhibition of EC motility by collapsin-1 is unclear. The motility of EC was inhibited maximally by 65–80%. There may be another collapsin receptor or coreceptor present in neuronal cells, but not EC that are needed to mediate full inhibition by collapsin-1. Other semaphorin receptors do exist, for example, plexin A is a receptor for semaphorin I (Winberg et al., 1998), and there may be other novel receptors as well.

Semaphorins and VEGF<sub>165</sub> both bind to NRP1 when expressed by EC, but do they compete for the same binding sites? The extracellular domain of NRP1 consists of several subdomains (Takagi et al., 1995; Kawakami et al., 1996). These include two complement C1r/s homology-binding (CUB) domains, two domains homologous to the C1 and C2 domains of coagulation factor V and VIII, and a 170-amino acid MAM-homology domain (also known as the a, b, and c domains, respectively). In one report using full-length Sema D (Sema III), it was concluded that the CUB domain was the primary semaphorin binding site, with some contribution of the coagulation factor homology domain (Nakamura et al., 1998). Another report, using NRP1 deletion analysis, indicated that the CUB and coagulation factor domains of NRP1 were necessary and

sufficient for binding of the semaphorin III (sema) domain whereas the coagulation factor domains alone were necessary and sufficient for binding the Sema III Ig-basic domain (Giger et al., 1998). The coagulation factor homology domain alone was responsible for binding <sup>125</sup>I-VEGF<sub>165</sub>. Furthermore, the Ig-basic ligand competitively inhibited the binding of <sup>125</sup>I-VEGF<sub>165</sub> to NRP1, suggesting that the Sema III Ig-basic domain and VEGF bind to similar sites. Our own studies indicate that full-length collapsin-1 inhibits the binding of a VEGF<sub>165</sub> fusion protein to COS-7 cells expressing NRP1. However, these results are based on binding assays and might not reflect what might happen in a functional assay. In our competition assays analyzing effects on PAEC/KDR/NRP1 motility, the dose-dependent chemotactic activity of VEGF<sub>165</sub> was reduced at each concentration of collapsin-1, although not totally abrogated. About five times more VEGF<sub>165</sub> was required for half maximal stimulation of PAEC/KDR/NRP1 chemotaxis when collapsin-1 was present. In a reciprocal experiment, in which collapsin-1 inhibited basal motility in a dose-dependent manner, the motility levels were higher in the presence of constant VEGF<sub>165</sub>, about six- to sevenfold at each concentration of collapsin-1. In the DRG collapse assay, collapsin-1 was about sevenfold less effective in inducing growth cone collapse when constant VEGF<sub>165</sub> was present. In addition, the binding of a VEGF<sub>165</sub> alkaline phosphatase fusion protein to COS cells expressing NRP1 was inhibited by collapsin-1. Taken together, these results suggest that VEGF<sub>165</sub> and collapsin-1 compete with each other to some degree for binding sites on NRP1, but where the exact sites of overlap are and whether the two ligands have similar affinities for these binding sites needs to be determined.

Although both collapsin-1 and VEGF<sub>165</sub> bind to similar sites on NRP1, they appear to have different signaling mechanisms. VEGF<sub>165</sub> is a chemoattractant for PAEC expressing both NRP1 and KDR, but not for PAEC expressing NRP1 alone (Soker et al., 1998). It appears that the chemotactic activity of VEGF<sub>165</sub> is mediated primarily via KDR with NRP1 acting as a coreceptor that enhances chemotactic activity by an as yet unknown mechanism. On the other hand, collapsin-1 inhibits the migration of EC expressing NRP1 alone, and to the same extent, as EC expressing both NRP1 and KDR. Thus, it appears that collapsin-1 signals via NRP1 directly to act as an inhibitor of EC migration without the involvement of KDR. However, some other, as yet unidentified, collapsin-1 receptor may be involved.

There are some similarities in the effects of collapsin-1 on neuronal cell growth cones and EC morphology. Actin is one of the major cytoskeletal components of growth cones. F-actin is preferentially polymerized at the growth cone's leading edge. Directional growth in response to external cues is achieved by stabilizing and destabilizing the actin cytoskeleton in lamellipodia and filopodia (Luo et al., 1997). Treatment with collapsin induces a net loss of polymerized F-actin at the leading edges of the growth cones. In EC, collapsin-1 treatment results in rapid retraction of lamellipodia and substantial loss of polymerized F-actin stress fibers. Whether filopodia are affected is not clear because the EC used in our experiments have relatively few of these structures. Our results suggest that analogous



to its effects on the growth cone cytoskeleton, collapsin-1 induces the collapse of the EC cytoskeleton.

NRP1, a receptor expressed by both neuronal cells and EC that can bind both collapsin-1 and VEGF<sub>165</sub>, might provide a molecular connection between the motility events that occur in neuronal guidance and angiogenesis. In embryonic development and adults, there is a close spatial relationship between neurons and blood vessels, and it is possible that there are factors, e.g., collapsin/semaphorins, that regulate both neuronal guidance and angiogenesis. In support of this speculation, a recent report has demonstrated that another set of repulsive neuronal guidance factors, the ephrins and their receptors, the ephs, are involved in angiogenesis (Wang et al., 1998; Yancopoulos et al., 1998). During mouse development, ephrin-B2 is a marker for arterial EC and Eph-B4 is a marker for venous EC. Ephrin-B2 knockout mice display defects in angiogenesis (Wang et al., 1998), as do NRP1 knockouts (Kitsukawa et al., 1997). Future investigations will determine the extent of cross-talk between angiogenesis and neuronal guidance.

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