Semaphorin 3A–Vascular Endothelial Growth Factor-165 Balance Mediates Migration and Apoptosis of Neural Progenitor Cells by the Recruitment of Shared Receptor

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The dynamic and coordinated interaction between cells and their microenvironment controls cell migration, proliferation, and apoptosis, mediated by different cell surface molecules. We have studied the response of a neuroectodermal progenitor cell line, Dev, to a guidance molecule, semaphorin 3A (Sema3A), described previously as a repellent–collapsing signal for axons, and we have shown that Sema3A acts as a repellent guidance cue for migrating progenitor cells and, on prolonged application, induces apoptosis. Both repulsion and induction of cell death are mediated by neuropilin-1, the ligand-binding component of the Sema3A receptor. The vascular endothelial growth factor, VEGF165, antagonizes Sema3A-induced apo-

ptosis and promotes cell survival, migration, and proliferation. Surprisingly, repulsion by Sema3A also depends on expression of VEGFR1, a VEGF165 receptor, expressed in Dev cells. Moreover, we found that these repulsive effects of Sema3A require tyrosine kinase activity, which can be attributed to VEGFR1. These results indicate that the balance between guidance molecules and angiogenic factors can modulate the migration, apoptosis (or survival), and proliferation of neural progenitor cells through shared receptors.

Key words: apoptosis; semaphorin; VEGF; migration; neuropilin; VEGFR1

During development, cell–cell and cell–matrix interactions provide essential information for controlling cell fate in terms of migration, growth, death, and differentiation (Bissell et al., 1982; Reichardt and Tomasselli, 1991). Cell surface receptors are instrumental in coordinating these interactions between cells and their microenvironment, which includes growth factors, hormones, and extracellular matrix components (Adams and Watt, 1993; Basbaum and Werb, 1996; Juliano, 1996; Thomasset et al., 1998; Bissell et al., 1999). During the development of the CNS, proliferation and cell migration are two of the most striking morphogenetic processes that require both the spatially and temporally coordinated control of pathway choice and cell survival to ensure that progenitor cells differentiate in appropriate locations (Graham et al., 1996). Primitive neuroectodermal tumors (PNETs) display similar properties to CNS progenitors (Trojanowski et al., 1994; Rorke et al., 1997). We have described previously an undifferentiated cell line, Dev, derived from a

cerebellar PNET (a medulloblastoma) that behaves as a pluripotent neural progenitor (Giraudon et al., 1993; Dufay et al., 1994; Derrington et al., 1998). In the present study, we used this cell line as a CNS model system to characterize the molecular and cellular mechanisms involved in cell migration, proliferation, and apoptosis in response to specific guidance and proliferative–angiogenic factors expressed in the local cellular environment.

Short- and long-range guidance factors acting in the developing CNS have been characterized extensively (Bolz et al., 1993; Tessier-Lavigne and Goodman, 1996). Both positive (attractive–permissive) and negative (repulsive–inhibitory) molecules are essential for axonal guidance and in providing local signals regulating the accessibility of regions to growing axons (Tessier-Lavigne and Goodman, 1996). These guidance molecules are bifunctional signals that can have both attractant and repellent effects. For example, chemorepellent semaphorins are chemoattractant for several types of neurons (Bagnard et al., 1998; De Castro et al., 1999; Püschel, 1999; Wong et al., 1999). Moreover, Sema3A (collapsin-1/semaphorin III/semaphorin D) (Semaphorin Nomenclature Committee, 1998) is implicated in the patterning of neural crest cell migration in the developing chick (Eickholt et al., 1999). The effects of Sema3A are mediated through neuropilin-1 (NRP1), a major component of the Sema3A receptor (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). NRP1 is also a receptor for a specific isoform of vascular endothelial growth factor (VEGF), VEGF165 (Soker et al., 1998). VEGF, for which there are two different receptors, VEGFR1 and VEGFR2, is an important factor in the early development of the vascular system (Fong et al., 1995; Hanahan, 1997) and also plays an essential role in tumor cell survival and proliferation (Plate et

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al., 1992) and in angiogenesis during embryogenesis (Millauer et al., 1993; Peters et al., 1993). This convergence of two different factors, semaphorin and VEGF, with quite different functions (axonal guidance and angiogenesis), on the same receptor, NRP1, prompted us to investigate their roles in the migration and proliferation of primitive neuroectodermal cells. Our results demonstrate that the balance between Sema3A and VEGF165 can result in alternative cellular responses, such as migration, apoptosis, or proliferation, which involve NRP1 and/or VEGFR1.

MATERIALS AND METHODS

Cell lines. The established Dev cell line was derived from a human cerebellar PNET tumor (medulloblastoma) (Giraudon et al., 1993; Dufay et al., 1994; Derrington et al., 1998). The cells were grown in DMEM supplemented with 10% fetal calf serum (FCS) and 10 μ g/ml gentamycin (all from Life Technologies, Gercy Pontoise, France).

Human embryonic kidney 293 cells (HEK293 cells) (CRL 1573; American Type Culture Collection, Manassas, VA) stably transfected with an expression vector containing cDNA coding for Flag-His-Sema3A (Adams et al., 1997) (cell line 602.108), used as a source of Sema3A, were cultured in minimal essential medium containing 5000 U/ml penicillin, 5 mg/ml streptomycin, 200 mM L-glutamine, 10% FCS, and 1 mg/ml G418 (Life Technologies). Sema3A was purified using an anti-Flag M2 affinity gel (Sigma, St. Quentin Fallavier, France), and its protein concentration was determined using the Bradford method. The membrane preparations used in the stripe assay were obtained as described previously (Götz et al., 1992; Bagnard et al., 1998), and membrane stripes were prepared according to the technique of Walter et al. (1987); cells were grown for 24 hr on lanes of alternating substrates and then fixed in 4% paraformaldehyde, and the number of cells in each type of lane was determined using phase-contrast optics (Zeiss, Jena, Germany).

Human umbilical vein endothelial cells (Huvec) were kindly provided by Dr. Macovschi (Institut National de la Santé et de la Recherche Médicale U.352, Lyon, France) and used at the first passage.

Receptor affinity probes. An alkaline phosphatase (AP)-Sema3A-expressing cell line was produced as described previously (Adams et al., 1997). AP-Sema3A binding sites were detected as described previously (Bagnard et al., 1998). Competition experiments with unlabeled Sema3A confirmed the specificity of AP-Sema3A binding (data not shown).

In situ hybridization. The neuropilin-specific antisense oligodeoxynucleotide probe CAGACATGTGATACCAGAAGGTCATGCAGT was 3'-labeled using a digoxigenin oligonucleotide tailing kit (Roche Molecular Biochemicals, Mannheim, Germany). The slides were washed twice for 5 min in PBS, fixed in 4% paraformaldehyde-PBS for 5 min at room temperature (RT), and rinsed three times in PBS. Endogenous peroxidase activity was quenched by incubating the slides for 10 min at RT in PBS containing 6% H₂O₂. The slides were then rinsed three times in PBS, dehydrated in a graded alcohol series, washed three times for 5 min in PBS, and then prehybridized for 1.5 hr at 40°C with 100 μ l of hybridization buffer containing 50% formamide, 2 $\times$ SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.0), 1 $\times$ Denhardt's solution, 500 μ g/ml salmon sperm DNA, 250 μ g/ml tRNA, 100 μ g/ml polyadenyl, 5 μ g/ml polydesoxyadenyl, and 10% dextran sulfate. One hundred microliters of hybridization buffer was mixed with 1 ng of the oligoprobe, and the mixture was applied to the slides, which were then incubated overnight in a humid chamber at 40°C before being sequentially washed twice for 10 min at RT in 2 $\times$ SSC, twice for 15 min at RT in 1 $\times$ SSC, once for 30 min at 45°C in 0.5 $\times$ SSC, once for 15 min at RT in 0.25 $\times$ SSC, and once for 5 min at RT in PBS. They were then incubated for either 1 hr at RT or overnight at 4°C with AP-conjugated sheep anti-digoxigenin antibody (Roche Molecular Biochemicals), diluted 1:500 in 10% FCS-PBS, and then washed four times for 10 min at RT in PBS. Bound label was detected by incubating the slides for 3–5 min at RT in a developing buffer containing nitroblue-tetrazolium-chloride and 5-bromo-4-chlorindolyl-phosphate (Roche Molecular Biochemicals).

Reverse transcription-PCR and Southern hybridization. Total RNA was resuspended in diethylpyrocarbonate-treated water and reverse transcribed for 1 hr at 42°C using 10 U/ μ l Moloney murine leukemia virus reverse transcriptase (Life Technologies) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 2 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dTTP, 0.5 mM dGTP, and 100 ng of oligo-dT 12–18 (Amersham Pharmacia Biotech, Orsay, France). PCR amplification was performed using 2 ng/ml reverse-transcribed RNA, 0.025 U/ml Taq DNA

polymerase (Life Technologies), 0.4 mM 5' primer, 0.4 mM 3' primer, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM MgCl₂, and 0.2 mM each dATP, dCTP, dGTP, and dTTP. Templates were first denatured at 95°C for 5 min. A typical PCR cycle consisted of denaturation (45 sec at 95°C), annealing (45 sec at 58°C), and extension (1 min at 72°C). Thirty-two cycles were used for NRP1, 22 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 40 for VEGFR2; in the last two cases, annealing was at 52°C. For VEGFR1, the mixture was first incubated at 94°C for 5 min, followed by 40 cycles of 1 min at 94°C, 2 min at 58°C, and 2 min at 72°C. cDNAs (40 ng) were amplified using the following primers designed from the DNA sequences: for human NRP1 (GenBank accession number AF 018956) (He and Tessier-Lavigne, 1997), CTG GTG AGC CCT GTG GTT TAT TCC as the 5' primer and ACT AAT GTC ATC CAC AGC AAT CCC as the 3' primer; for human GAPDH, GGA GAT TCA GTG TGG TGG as the 5' primer and GGC TCT CCA GAA CAT CAT CC as the 3' primer; for human VEGFR1 (GenBank accession number AFO 63657), ATT CTG ACG GTT TCT ACA AGG AG as the 5' primer and TCC TGT CAG TAT GGC ATT GAT TG as the 3' primer; and for human VEGFR2 (Möhle et al., 1997), CTG GCA TGG TCT TCT GTG AAG CA as the 5' primer and AAT ACC AGT GGA TGT GAT GCG G as the 3' primer. The PCR amplification products were resolved on 2% agarose gels and photographed as ethidium bromide fluorescent bands. To verify the identity of the amplified sequences, Southern hybridization was performed using [³²P]ATP 5'-end-labeled internal oligonucleotides complementary to the mRNA sequence of the studied gene. The oligonucleotides used were GAC ATC AAG AAG GTG GTG AAG CAG G for GAPDH, ACT GCA TGA CCT TCT GGT ATC ACA TGT CTG for NRP1, TTC CTG TCA GTA TGG CAT TGA TTG G for VEGFR1, and AAT CTC TGG TGG AAG CCA CG for VEGFR2. An autoradiographic film was then exposed to the labeled membranes. All samples analyzed for NRP1, VEGFR1, and VEGFR2 expression by reverse transcription-PCR were also tested for GAPDH expression to confirm the integrity and quantity of the RNA.

Western blots. Huvec and Dev cells were trypsinized and washed three times with PBS. The cell pellets were resuspended in PBS plus complete protease inhibitor (Roche Molecular Biochemicals), sonicated, and centrifuged for 10 min at 100 $\times$ g at 4°C, the supernatants were centrifuged for 30 min at 100,000 $\times$ g at 4°C, and the final pellets were suspended in lysis buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA, and 1% sodium deoxycholate); after sonication, the samples were left on ice for 1 hr and then centrifuged for 30 min at 14,000 rpm at 4°C.

For the detection of NRP1, the supernatants were subjected to SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), which was then blocked for 1 hr in Tris buffer containing 0.1% Tween 20 and 5% bovine serum albumin and incubated overnight with the anti-NRP1 antibody described below, rinsed three times for 5 min in PBS, and then incubated for 2 hr at RT with peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). Bound antibody was detected using an ECL kit (Covalab, Oullins, France).

For the detection of VEGFR1 and VEGFR2, the solubilized membrane proteins were immunoprecipitated overnight at 4°C by shaking with either polyclonal rabbit anti-VEGFR1 antibodies (C-17; Santa Cruz Biotechnology, Santa Cruz, CA) or a monoclonal mouse anti-VEGFR2 antibody (C-1158; Santa Cruz Biotechnology), 100 μ l of protein A-Sepharose was added, and incubation was continued for 2 hr at 4°C. The protein A-Sepharose was then washed three times with lysis buffer, and the immunoprecipitated proteins were electrophoresed as described above. The secondary antibodies used for VEGFR1 and VEGFR2 were, respectively, peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) and peroxidase-conjugated goat anti-mouse antibody (Bioss, Compiègne, France).

The anti-NRP1 antibodies were raised using a synthetic 14 amino acid peptide (NH₂-CEHDSHAQLRWVL-CONH₂) from the MAM domain of NRP1 (Chen et al., 1998). A rabbit was immunized intradermally with 50 μ g of peptide in complete Freund's adjuvant and boosted twice with the same amount of peptide in incomplete Freund's adjuvant, and then the antibodies formed 74 d after immunization were purified on an NRP1 peptide-Sepharose column, with the bound antibodies being eluted with 0.1 M glycine, pH 2, and the eluant neutralized with 0.1 M Tris, pH 8. Preimmune serum served as the control. The anti-NRP1 antibodies blocked the repulsive activity of Sema3A on dorsal root ganglia neurons cocultured with Sema3A-expressing HEK293 cells (data not shown).

Antisense targeting. Phosphorothionate-modified VEGFR1 oligonucleotides were synthesized and purified by Biognostik (Göttingen, Germany). The control, provided by Biognostik, was a GC-matched randomized-sequence oligonucleotide (missense). Cells were grown on glass coverslips for 2 d in the presence of 2 μ M VEGFR1 antisense or missense oligonucleotides and then examined for downregulation of VEGFR1 expression by immunocytochemistry. Serum-free medium containing 5% BSA and 1 μ g/ml polyclonal rabbit anti-VEGFR1 antibodies (C-17; Santa Cruz Biotechnology) was added to the living cells for 3 hr at 37°C, and then the cells were gently washed and fixed for 10 min at –20°C in acetone. The slides were air-dried and washed in PBS, and then goat anti-rabbit antibodies (Molecular Probes, Eugene, OR), diluted 1:100 in PBS, were added. After 2 hr of incubation, the slides were washed and mounted in PBS-glycerol for fluorescence microscopy.

Time-lapse video microscopy. Cells were grown on glass coverslips coated with laminin (1 μ g/ml; Sigma) and then transferred to Petriperm dishes (Heraeus). Time-lapse video microscopy was performed as described previously (Hübener et al., 1995). Images were taken every 5 min for up to 48 hr (Metamorph time-lapse software; Imaging Technology). The average migration speed, expressed as micrometers per hour, was determined for individual cells by measuring the distance between the initial and final positions of the center of the cell. Collapse assays were performed by injection of 1 ml of conditioned medium from Sema3A-expressing HEK293 cells after 4 hr of migration in the absence of Sema3A, with cellular collapse being determined by the transient loss or total retraction of cell processes 20 min after injection. Control experiments were performed using medium from untransfected HEK293 cells. Because collapse was reversible and the whole process could be repeated, only the first retraction was taken into account. Collapse assays were also performed using 50 ng/ml purified Sema3A and 1 μ g/ml anti-NRP1 or anti-VEGFR1 antibodies. In these experiments, cellular morphological changes were analyzed after fixation in 4% paraformaldehyde after 4 hr incubation with the test agents.

Detection of apoptosis. DNA fragmentation was visualized by staining DNA with propidium iodide (PI). Cells grown on glass coverslips and preincubated with Sema3A for 24 hr were fixed for 1 hr at –20°C in 70% ethanol and washed with PBS. A solution of 25 μ g/ml PI and 50 U/ml RNase (Sigma) was added for 15 min at RT, and then, after several washes in PBS, the coverslips were mounted in Moviol for fluorescence microscopy analysis.

The DeadEnd colorimetric apoptosis detection system [terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL); Promega, Charbonnières, France] was used to visualize apoptotic cells. After fixation for 1 hr at –20°C in 70% ethanol, Dev cells (5×10^{-5} cells in suspension) were stained with PI to detect and quantify apoptotic cells (sub-G₀/G₁ DNA fraction) using flow cytometry. DNA fragments were extracted for 1 hr at 4°C in 5 mM Na₂PO₄–2.5 mM citric acid–0.01% Tween 20 and removed by washing in PBS and centrifugation. The pelleted cells were resuspended in PBS, and the nonfragmented DNA was stained for 15 min in PBS containing 25 μ g/ml PI and 50 U/ml RNase. Fluorescence was detected using a Counter XL flow cytometer (Beckman Coulter, Miami, FL) with an argon laser (488 nm wavelength) and an A615 nm optical filter (FL3). In blocking experiments, 1 μ g/ml antibodies against NRP1, VEGFR1, or VEGFR2 was added to the culture. A general caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp (Z-VAD) and interleukin-1 β converting enzyme (ICE) inhibitor, were used at a concentration of 25 μ M.

Motility assay. The motility assay was performed in a Boyden chamber. [³⁵S]Methionine-labeled cells (0.1×10^{-6} per well) were plated in serum free-medium containing 0.1% BSA. The cells were grown in the upper chamber, which was separated from the lower chamber by a poly-L-lysine (PL)-coated polycarbonate membrane with a pore size of 8 μ m (Falcon, Pont de Claix, France). After 12 hr of culture, the nonmigrating cells were removed using a plastic policeman, and the labeled migrating cells in the membrane were counted using a 1600 TR liquid scintillation counter (Packard, Meriden, CT). In some experiments, different concentrations of VEGF165 were added to the lower compartment of the Boyden chamber.

Proliferation assay. Dev cells were seeded at 1.7×10^{-5} cells per well in six-well culture dishes, stimulated for 24 hr in DMEM containing 10% FCS, and then starved for 1 d in DMEM containing 0.2% FCS, before being treated with various concentrations of VEGF165 (0–100 ng/ml), antibodies against VEGFR1, VEGFR2, or NRP1 (50 ng/ml), genistein (5 μ M), or antisense VEGFR1 oligomers (2 μ M, added at the beginning of the culture). The cells were then trypsinized, and the number of living

Figure 1. Semaphorin binding sites and neuropilin-1 expression. *A*, Semaphorin binding sites were detected using AP-Sema3A. The *left panel* shows control staining after addition of conditioned medium without AP-Sema3A, and the *right panel* shows that Dev cells were strongly labeled after addition of AP-Sema3A-containing medium. *B*, *In situ* hybridization demonstrating expression of neuropilin-1 by Dev cells (*left*, control cells; *right*, cells treated with antisense probe). *C*, NRP1 mRNA levels determined by reverse transcription-PCR after 24 hr of culture; a molecular weight ladder is shown on the *left*. *D*, Western blot of Dev cells showing a single band at the expected molecular weight for NRP1 (110 kDa). *E*, Immunocytochemical labeling of NRP1 on Dev cells after 24 hr of culture. The *left panel* shows control staining with preimmune serum, and the *right panel* shows staining with anti-NRP1 antibodies.

cells was assessed by Trypan blue dye exclusion using a hemocytometer. The data are expressed as the mean $\pm$ SEM for three independent experiments.

RESULTS

Detection of specific Sema3A binding sites associated with neuropilin-1 expression

An AP-Sema3A fusion protein (Bagnard et al., 1998) was used to detect the presence of Sema3A binding sites on Dev cells (Fig. 1*A*). All cells in Dev cultures bound AP-Sema3A, the binding sites being especially dense on cellular processes. Binding could be blocked by an excess of untagged Sema3A, demonstrating the specificity of AP-Sema3A binding (data not shown).

Figure 2. Dev cells express VEGFR1. *A*, Expression of VEGFR1 and VEGFR2 mRNA in Dev cells and Huvec. Equal amounts of RNA were reverse-transcribed to generate cDNA. The cDNA was subjected to VEGFR1- and VEGFR2-specific PCR amplification (580 and 790 bp products, respectively) using paired primers. cDNA from all samples was also subjected to GAPDH-specific PCR amplification (500 bp product). The *left lane* on each micrograph represents molecular weight markers. *B*, Southern analysis with specific internal probes for VEGFR1, VEGFR2, or GAPDH. *C*, Western blot analysis of VEGFR1 and VEGFR2 expression in Huvec and Dev cells, showing that, whereas VEGFR1 is equally expressed in Dev cells and Huvec, VEGFR2 is mainly restricted to Huvec. *D*, Immunohistochemical staining of VEGFR1 on Dev cells treated for 48 hr with missense (control) or VEGFR1 antisense oligonucleotides. Note the decrease in VEGFR1 immunolabeling in the VEGFR1 antisense-treated cells compared with the missense-treated cells.

Expression of NRP1, a major component of the Sema3A receptor (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), could be detected in Dev cells by both *in situ* hybridization (Fig. 1*B*) and reverse transcription-PCR (Fig. 1*C*). Western blots (Fig. 1*D*) and immunochemical analysis (Fig. 1*E*) using our anti-NRP1 antibody confirmed the presence of the protein at the cell surface.

VEGFR1 is expressed by Dev cells

Because VEGF165 binds to NRP1, we investigated whether other VEGF165 receptors were expressed by Dev cells. Compared with Huvec, Dev cells expressed high levels of VEGFR1 but almost no VEGFR2, as shown by reverse transcription-PCR (Fig. 2*A*), Southern blotting (Fig. 2*B*), and Western blotting (Fig. 2*C*), using specific probes or anti-VEGFR1 or anti-VEGFR2 antibodies. VEGFR1 expression on Dev cells, as assessed by immunohistochemical analysis, was dramatically reduced after 48 hr incubation with a VEGFR1 antisense probe (Fig. 2*D*).

Sema3A mediates repulsion of migrating Dev cells by a process involving NRP1 and VEGFR1

To explore the role of Sema3A during Dev cell migration, we used a monostripe assay used previously for analysis of axonal guidance (Bagnard et al., 1998). During the test, the Dev cells, which initially were equally distributed on the alternating lanes coated with PL alone or with PL coated with membranes, prepared from either Sema3A-expressing cells or untransfected

Figure 3. Sema3A is a repellent signal for migrating Dev cells. *A*, Micrographs of Dev cells grown on alternating lanes composed of poly-L-lysine alone or poly-L-lysine covered with cell membranes prepared from a cell line stably expressing Sema3A or from untransfected HEK293 cells (*Cont*). Dev cells avoid the stripes containing Sema3A. *B*, Quantification of repulsion after 24 hr of culture by counting the percentage of cells in each lane containing either control membranes or Sema3A-containing membranes, with or without treatment with 1 μ g/ml antibodies against NRP1, VEGFR1, or VEGFR2, 20 μ M genistein, or 2 μ M VEGFR1 antisense or missense oligonucleotide. (χ^2 analysis; ** p < 0.01; ns, not significant compared with *No treatment*).

HEK293 cells, moved to Sema3A-negative regions (Fig. 3*A*). Quantification of this effect showed that, after 24 hr of culture, 88.3% of the cells were found on lanes not containing Sema3A (p < 0.05 compared with control experiment; χ^2 analysis) (Fig. 3*B*). Double-stripe assays using alternating lanes coated with Sema3A-containing or control membranes were performed to verify that the absence of Dev cells on Sema3A-containing mem-

branes was not attributable to better cell adhesion on membrane-free stripes (9.9% cells on Sema3A-containing membrane stripes; 90.1% cells on control membrane stripes). Time-lapse analysis also confirmed that the cells adhered equally well to PL stripes or membrane-containing stripes 2 hr after deposition of the cells on the alternating substrates and that the cells then gradually moved away from the Sema3A-containing membranes. Interestingly, we did not observe significant cell death during these stripe assays, regardless of the type of membrane used (data not shown). Thus, cells exposed to Sema3A-enriched local territories avoided such regions in preference for a Sema3A-free environment, as seen with axon guidance.

As shown in Figure 3*B*, this repulsive effect of Sema3A could be significantly blocked ($p < 0.01$; χ^2 analysis) by anti-NRP1 antibodies, VEGF165, or VEGF121 (37.6, 35.5, or 32.1%, respectively, of the cells remaining on Sema3A-containing membrane stripes compared with 11.7% without treatment). Surprisingly, addition of anti-VEGFR1 antibody in the absence of VEGF165 or VEGF121 was also able to block the repulsive effect of Sema3A (38.1% of cells remaining on Sema3A-containing membranes compared with 11.7% without treatment; $p < 0.05$; χ^2 analysis). No such significant effect was seen using anti-VEGFR2 antibodies (21.3% of cells remaining on Sema3A-containing membranes; nonsignificant by χ^2 analysis). Together, these results suggested that VEGFR1 was implicated in the repulsive effect of Sema3A on migrating Dev cells. Pharmacological inhibition of VEGFR1 potential activity by genistein, a general tyrosine kinase inhibitor, confirmed the involvement of VEGFR1 in the transduction of the Sema3A repulsive effect. This was further supported by antisense targeting experiments; after 24 hr of culture, cells treated with a VEGFR1 antisense probe, resulting in dramatic blocking of VEGFR1 expression (Fig. 2*D*), were almost equally distributed on Sema3A-containing and control membrane stripes, whereas missense probe-treated cells continued to avoid Sema3A-containing lanes (40.1% of cells on Sema3A-containing membrane stripes after antisense treatment compared with 17.2% after missense treatment) (Fig. 3*B*). Because inhibition of tyrosine kinase can result in nonspecific inhibition of cell migration, we performed time-lapse video microscopy to quantify Dev cell migration with and without treatment with genistein or anti-VEGFR1 antibody and found a significant reduction in the speed of migration of cells treated with 20 μ M genistein (29.9 ± 11.9 μ m/hr; $p < 0.001$) or with 1 μ g/ml anti-VEGFR1 antibody (26.5 ± 11.7 μ m/hr; $p < 0.001$) compared with control cultures (50.7 ± 21.1 μ m/hr); however, the general mobility of the cells was unaffected by either treatment.

In a second set of experiments, we used time-lapse video microscopy to study the migration of Dev cells exposed to soluble Sema3A. Dev cells were monitored for up to 48 hr while migrating on laminin-coated glass coverslips. Under control conditions, the cells extended long processes and migrated with an average velocity of 45.9 ± 25.1 μ m/hr ($n = 16$ cells). However, 10–20 min after addition of soluble recombinant Sema3A, they showed a striking change in behavior and morphology, with the cells stopping migrating and their processes collapsing (Fig. 4*A*, seen in all 48 cells analyzed). In 11 of the 48 cells examined, the entire morphology was transiently affected, with the cell body rounding up and all extensions being retracted (Fig. 4*B*). The collapse of cell processes was reversible, with the cells extending new processes after a few minutes until a new round of retraction occurred. After 2 d in culture with Sema3A, cell motility was arrested and the cells died, whereas, under control conditions, the

Figure 4. Time-lapse recording of Dev cell migration. Images were taken every 5 min for 48 hr; the state of the cell after 15, 30, or 45 min of recording is shown from left to right. *A*, High magnification of Dev cell processes. Scale bar, 6 μ m. Ten to 15 min after addition of Sema3A to the culture medium, the cells stopped migration and their processes collapsed (* indicates time of injection). *B*, In some cases, the whole cell transiently collapsed for several minutes before new processes were extended. Scale bar, 20 μ m. * indicates time of injection. *C*, Collapse assay on Dev cells using 50 ng/ml Sema3A alone or together with 1 μ g/ml anti-NRP1 or anti-VEGFR1 antibodies (* $p < 0.05$, χ^2 analysis compared with control; † $p < 0.05$, χ^2 analysis compared with Sema3A alone).

cells survived and continued to migrate (data not shown). To further characterize the Sema3A-induced cellular collapse, we performed the collapse assay after addition of soluble recombinant Sema3A, in the absence and presence of blocking antibodies, and found that addition of anti-NRP1 or anti-VEGFR1 antibodies blocked the induction of collapse, suggesting a direct role of NRP1 and VEGFR1 in Sema3A-induced cellular collapse (Fig. 4*C*).

The Sema3A–NRP1 interaction mediates apoptosis in the Dev cell line

To study the consequences of prolonged exposure to Sema3A, induction of cell death was analyzed after 24 hr of culture in the presence of Sema3A, with cell nuclei being stained with PI to detect DNA fragmentation as an indicator of apoptosis. Under these conditions, the majority of cells showed DNA fragmentation, whereas those grown in the absence of Sema3A contained nuclei with a normal morphology (Fig. 5*A*). To confirm that cells died by an apoptotic process, we also used the TUNEL method and found that only 3–5% of cells treated for 24 hr with medium from untransfected cells contained apoptotic bodies, whereas 70–80% of cells treated with Sema3A-containing medium (concentrations ranging from 30 to 60 ng/ml) were stained (Fig. 5*B*). To quantify this effect, cells were incubated with various concentrations of purified Sema3A, and their DNA content was measured by flow cytometry (Fig. 5*C*). As shown in Figure 5*D*, induction of apoptosis by Sema3A was concentration-dependent,

Figure 5. Sema3A induces apoptosis. *A*, Nuclei of Dev cells stained with propidium iodide to visualize DNA fragmentation associated with apoptosis. Whereas cultures treated with control medium showed intact nuclei (*top*), DNA fragmentation was seen in numerous cells cultured in the presence of Sema3A (*bottom*). *B*, TUNEL assay confirming that only 3–5% of controls (*top*) died by apoptosis compared with 70–80% of cells treated with Sema3A (*bottom*). *C*, Apoptotic cells were quantified by measuring the sub-G₀/G₁ DNA fraction using flow cytometry analysis after propidium iodide staining (*a*, 80% apoptosis; *b*, 3% apoptosis). *D*, The Sema3A effects were dose-dependent with a maximal effect (100% apoptosis) at 350 ng/ml Sema3A and a EC₅₀ of 0.5 nM.

with the maximal effect (100% apoptosis) being seen at 350 ng/ml Sema3A, with an EC₅₀ of 0.5 nM. Sema3A-mediated apoptosis could not be detected at incubation periods of <24 hr, and all cells had died by 72 hr (data not shown).

As shown in Figure 6*A*, anti-NRP1 antibody suppressed Sema3A-induced apoptosis in a dose-dependent manner, whereas preimmune serum was ineffective. Addition of anti-VEGFR1

Figure 6. Reversal of Sema3A-induced apoptosis. *A*, Anti-NRP1 antibodies, but not preimmune serum, blocked Sema3A-induced apoptosis. A partial block of apoptosis was seen with 1 μg/ml antibody, and 2 μg/ml had a greater effect. Preimmune serum had no effect. (χ^2 analysis; $**p < 0.01$; ns, not significant). Anti-VEGFR1 antibody (1 μg/ml) had no effect on Sema3A-induced apoptosis, in contrast to its blocking effect on Sema3A-mediated repulsion. *B*, The number of apoptotic Dev cells was determined after culture in the presence of 50 ng/ml Sema3A and VEGF165 (50, 100, or 200 ng/ml; left 4 gray bars), 50 ng/ml VEGF165 plus 1 μg/ml anti-VEGFR1 antibody (first black bar), 50 ng/ml VEGF121 (last gray bar), or 50 ng/ml VEGF121 plus 1 μg/ml anti-VEGFR1 antibody (last black bar). *C*, Sema3A-induced cell death in the presence of a tyrosine kinase inhibitor (20 μM genistein) or caspase inhibitors (25 μM Z-VAD or ICE).

antibodies (1 μg/ml) did not block Sema3A-induced apoptosis, suggesting that VEGFR1 was not required for Sema3A apoptotic signaling.

VEGF165 antagonizes Sema3A-induced apoptosis and increases Dev cell survival

Because VEGF165 has been shown recently to compete with Sema3A for binding to NRP1 (Soker et al., 1998), we examined whether competition between Sema3A and VEGF165 in Dev cells had an effect on apoptosis. As shown in Figure 6*B*, addition of 50 ng/ml VEGF165 partially blocked the apoptosis induced by 50 ng/ml Sema3A (70 and 18% apoptosis in the absence or

presence, respectively, of VEGF165; $p < 0.05$; χ^2 analysis). Higher concentrations of VEGF165 (100–200 ng/ml) completely blocked the effect of Sema3A; at 200 ng/ml, VEGF165 even reduced apoptosis significantly below the basal level of control cultures, suggesting an additional effect on cell survival ($0.6 \pm 1.1\%$ with 200 ng/ml VEGF165 compared with $3.5 \pm 0.3\%$ under basal conditions; $p < 0.05$; χ^2 analysis). Surprisingly, VEGF121 was also able to block Sema3A-induced apoptosis ($18.3 \pm 3.5\%$ apoptotic cells; $p < 0.001$), a similar action to its effect in reversing the repulsive effect of Sema3A during migration assays. Addition of anti-VEGFR1 antibody to block VEGFR1 showed that VEGF121 had an absolute requirement for VEGFR1 to exert its effect in blocking Sema3A-induced apoptosis, whereas VEGFR1 partially interfered with the block of Sema3A-mediated apoptosis by VEGF165 (Fig. 6*B*, dark bars).

Finally, as shown in Figure 6*C*, 20 μ M genistein did not block Sema3A-induced apoptosis, and caspase inhibitors only partially blocked cell death (30% block with ICE and 40% block with Z-VAD; $p < 0.001$), suggesting the involvement of other, caspase-independent, intracellular pathways during this apoptotic cascade that must be primarily independent of tyrosine kinase activity.

VEGF165 promotes Dev cell migration and proliferation

To further investigate the role of VEGF165 in primitive neuroectodermal cells, we tested the effects of increasing concentrations of VEGF165 on Dev cell migration and proliferation. The effect of VEGF165 on Dev cell motility was assessed using a Boyden chamber assay. As shown in Figure 7*A*, maximal stimulation of migration was seen after treatment with 1 ng/ml VEGF165 (+53% migration compared with control conditions). Cell counting revealed a significant increase in cell proliferation (+30%) in the presence of 50 ng/ml VEGF165 ($1.24 \pm 0.15 \times 10^{-6}$ cells compared with $0.95 \pm 0.09 \times 10^{-6}$ cells under control conditions; $p < 0.01$); this effect was dose-dependent, with a maximal effect at 50 ng/ml (Fig. 7*B*). Similar results were obtained using [3 H]thymidine DNA incorporation to quantify effects of VEGF165 on Dev cells proliferation (data not shown). Moreover, as shown in Figure 7*C*, the proliferative effect of VEGF165 could be blocked by 1 μ g/ml anti-VEGFR1 antibody ($0.94 \pm 0.10 \times 10^{-6}$ cells; $p < 0.01$), treatment with 2 μ M VEGFR1 antisense oligonucleotide ($1.11 \pm 0.05 \times 10^{-6}$ cells; $p < 0.01$), or 5 μ M genistein ($0.92 \pm 0.02 \times 10^{-6}$ cells; $p < 0.01$), whereas addition of 1 μ g/ml anti-VEGFR2 antibody ($1.30 \pm 0.08 \times 10^{-6}$ cells; NS), anti-NRP1 antibody ($1.43 \pm 0.09 \times 10^{-6}$ cells; NS), or treatment with 2 μ M VEGFR1 missense oligonucleotide ($1.37 \pm 0.05 \times 10^{-6}$ cells; NS) had no effect.

DISCUSSION

Sema3A acts as a repellent molecule for the neural progenitor cells Dev, causes cell processes to collapse, and, after prolonged exposure, leads to apoptosis. Recently, one VEGF splice variant (VEGF165), which is structurally different from Sema3A, has been shown to bind to NRP1 with a similar affinity to Sema3A (Soker et al., 1998). Moreover, it has been shown that competitive inhibition by VEGF165 suppresses the endothelial cell motility mediated by the Sema3A–NRP1 interaction (Miao et al., 1999). We have now demonstrated that, in neural progenitor cells exposed to Sema3A, competition between these two ligands modulates not only cellular motility but also the apoptotic process. This effect is mediated by NRP1 and blocked by VEGF165, which also stimulates cell survival and proliferation. Strikingly,

Figure 7. Effect of VEGF165 on Dev cell migration and cell proliferation. *A*, Increasing concentrations of VEGF165 were added to the lower chamber of a Boyden chamber, and Dev cells migration was measured after 12 hr at 37°C. The rate of migration was assessed by counting [3 S]methionine-labeled cells in the polycarbonate membrane after removing cells in the upper well. Results are presented as counts per minute (mean $\pm$ SEM for 3 independent experiments). *B*, Proliferation of Dev cells determined by cell counting after culture for 72 hr in the presence of increasing concentrations of VEGF165. The maximal proliferative effect was seen using 50 ng/ml VEGF165. *C*, Effect of the combination of 50 ng/ml VEGF165 and various treatments on Dev cell proliferation. Results were compared with the effects obtained with 50 ng/ml VEGF165 alone (No treatment). Student's *t* test; * $p < 0.01$; ** $p < 0.001$; ns, not significant.

the repulsive effect of Sema3A during migration requires VEGFR1 activity.

Sema3A induced dose-dependent apoptosis of Dev cells. Time-lapse imaging on a permissive substrate (laminin) revealed that

Dev cell migration was rapidly abolished in Sema3A-containing medium. In this case, cell processes collapsed and cell death occurred after 24 hr. Moreover, when offered a choice between alternating substrates with or without Sema3A, Dev cells avoided lanes containing membrane-bound Sema3A. Thus, Sema3A acts as a repellent cue, delineating territories nonpermissive for PNET cells, as described for neural crest cells (Eickholt et al., 1999). As seen in the case of axonal repulsion in the developing brain (Messersmith et al., 1995; Püschel et al., 1995; Wright et al., 1995; Bagnard et al., 1998), Sema3A might induce progenitor cells to migrate away from the territory containing the repellent signal and then restrict the random migration of precursor cells to specific pathways. When cells are not able to avoid such an inhibitory region, prolonged exposure to Sema3A may trigger an apoptotic signal. This is further supported by the fact that apoptotic Dev cells were seen only after continuous exposure to Sema3A for up to 24 hr. Interestingly, during neural crest development, alterations in growth factor availability change the fate and migration pattern of neural crest-derived precursors (Wehrle-Haller and Weston, 1995; Wehrle-Haller et al., 1996). Moreover, in addition to their growth- and differentiation-enhancing effects, some neurotrophins (NGF, BDNF, and neurotrophin-3) can induce cell death if present at inappropriate levels or times (Von Bartheld et al., 1994; Casaccia-Bonnel et al., 1996). The effects of Sema3A on both motility and apoptosis show that it can exert two different effects, and which effect is produced in a given situation may depend on its spatiotemporal distribution, as described for axonal repulsion (Bagnard et al., 2000). It remains unclear whether Sema3A-induced apoptosis is a direct consequence of repetitive cellular collapse or reflects the activation of an independent signal transduction pathway. However, total or partial collapse (Fan and Raper, 1995) requires tight control of actin cytoskeleton rearrangement, which is known to be essential during cell adhesion, migration, and survival (Aspenstrom, 1999). Activation of an independent signal transduction pathway is suggested by the fact that Sema3A–NRP1-induced apoptosis in Dev cells was only partially dependent on caspases, which are often involved in apoptotic pathways (for review, see Green, 1999), because this process was not completely blocked by a general caspase inhibitor, Z-VAD. Thus, caspase-independent pathways must be involved, as suggested in the Sema3A-induced apoptosis of sensory neurons (Gagliardini and Fankhauser, 1999).

Interestingly, a recent study showed upregulation of Sema3A and collapsin response mediator protein, which preceded dopamine-induced apoptosis in dopaminergic neurons and resulted in cell death with direct exposure to Sema3A (Shirvan et al., 1999). Moreover, the DCC (deleted in colorectal cancer) gene product, a receptor for the guidance molecule, netrin-1, induces apoptosis in the absence of ligand binding (Mehlen et al., 1998). In addition, nerve growth factor, involved in the guidance of embryonic sensory neurons (Gundersen and Barret, 1980), induces apoptosis in human PNETs expressing TrkA receptors (Muragaki et al., 1997). Thus, our data provide additional evidence that guidance molecules for axons or migrating cells can also function as death signals and that induction of apoptosis is mediated by the same receptors involved in guidance. In this report, we showed that Dev cells express NRP1 and that an antibody directed against the MAM domain of NRP1 prevented Sema3A-induced apoptosis and inhibition of migration. Strikingly, the addition of either VEGF165 or VEGF121 abolished the effects of Sema3A. It appears that the block of apoptosis by VEGF165 is attributable to direct competition with Sema3A for

Figure 8. Model showing how the Sema3A–VEGF balance modulates cell proliferation, migration, repulsion, and apoptosis. After binding to NRP1 and recruitment of VEGFR1, Sema3A induces cell repulsion. Prolonged exposure to Sema3A leads to apoptosis. VEGF165 binds to NRP1 and VEGFR1. This VEGF isoform is able to block Sema3A-mediated repulsion and apoptosis by directly competing with Sema3A for binding to NRP1, thus preventing the formation of the coreceptor NRP1–VEGFR1 and/or to activate a survival pathway through its binding to VEGFR1. VEGF121 only binds to VEGFR1 and can block Sema3A effects by preventing the formation of the NRP1–VEGFR1 coreceptor and/or by activation of a survival pathway involving VEGFR1 or other cell surface molecules.

binding to NRP1. Thus, as in the competitive signaling between TrkA and p75 NGF receptors that determines cell survival (Yoon et al., 1998), the balance between the expression of different NRP1 ligands and their receptors probably determines whether cell migration or apoptosis occurs. It remains unclear, however, how VEGF121, which does not bind to NRP1 (Soker et al., 1998), can block both migration inhibition and apoptosis. Because addition of anti-VEGFR1 antibody was able to prevent VEGF121 from blocking Sema3A-induced apoptosis, the effects mediated by VEGF121 after its binding to VEGFR1 may result from the stimulation of a parallel intracellular pathway, which counteracts apoptosis. As illustrated in the model shown in Figure 8, VEGF165 can exert its blocking activity via NRP1 competitive inhibition and/or VEGFR1 activation.

VEGF binds with high affinity to the tyrosine kinase receptors VEGFR1 (Flt1) and VEGFR2 (KDR/Flk1) (Neufeld et al., 1999). Generally, cells express both VEGF receptors. VEGFR2 is a positive regulator for the commitment of endothelial cells (Shalaby et al., 1995) and stimulates their proliferation and migration (Bernatchez et al., 1999). In monocytes–macrophages, VEGFR1 mediates tissue factor induction and chemotaxis (Barleon et al., 1996; Clauss et al., 1996) and has transforming and morphogenic potential (Maru et al., 1998). Interestingly, VEGFR2 is expressed by retinal progenitor cells, and it is first expressed at the onset of neuronal differentiation at which time VEGF is present, suggesting a physiological function for VEGF and its receptor in the developing retina (Yang and Cepko, 1996). Dev cells were shown to express VEGFR1, which appears to mediate their proliferation in response to VEGF stimulation, as demonstrated by antisense targeting, tyrosine kinase pharmacological inhibition, and a specific antibody directed against VEGFR1, consistent with results obtained in sinusoidal endothelial cells expressing high levels of VEGFR1 (Yamane et al., 1994).

The relatively weak stimulation of Dev cell migration and proliferation by VEGF165 may be attributed to the lack of the VEGFR2 receptor, which is considered to intensify functions in cells expressing both receptors (Kanno et al., 2000). Strikingly, we found that the inhibitory effect of Semaphorin 3A on migration was abrogated by an anti-VEGFR1 antibody or treatment with a VEGFR1 antisense oligonucleotide. Moreover, genistein, a tyrosine kinase inhibitor, inhibited the Semaphorin 3A-dependent repulsion. These data suggest that tyrosine kinase activity is required during Semaphorin 3A signaling. The lack of a repulsive effect after genistein treatment was not attributable to nonspecific alteration of Dev cell mobility, because migration was only partially reduced (by 45%). Thus, the repulsive effect of Semaphorin 3A requires the activity of a tyrosine kinase, such as VEGFR1, because anti-VEGFR1 antibody also suppressed the inhibitory effect of Semaphorin 3A. A recent study demonstrated that NRP1 binds with high affinity to VEGFR1 and that this interaction inhibits the binding of VEGF165 to NRP1 (Fuh et al., 2000). Thus, VEGFR1 might serve as a coreceptor for NRP1 in the modulation of Semaphorin 3A signaling. This is supported by the fact that NRP1 is considered to be an essential component of the semaphorin 3A receptor but requires a partner to form a functional receptor (Renzi et al., 1999). It has been shown that the plexin–NRP1 complex acts as a receptor for Semaphorin 3A (Takahashi et al., 1999; Tamagnone et al., 1999; Rohm et al., 2000). Our results strongly support the idea that VEGFR1, which has tyrosine kinase activity, may have a similar function during Semaphorin 3A-mediated inhibition of cell migration. This effect must be exerted during the initial steps of Semaphorin 3A signaling, because blocking of VEGFR1 has no effect on Semaphorin 3A-induced apoptosis once the cells have been exposed for some time to Semaphorin 3A. Thus, recruitment of VEGFR1 during Semaphorin 3A-mediated cell repulsion may allow cells to migrate away from the repulsive territory and, consequently, may promote cell survival. Because cell migration is completely abolished after prolonged exposure to Semaphorin 3A, persistent inhibition of cell migration may trigger an apoptotic process.

Our data provide evidence for a novel regulatory mechanism that determines the migration, apoptosis, and proliferation of neural progenitor cells and involves a balance between the repellent signal Semaphorin 3A and the growth factor VEGF165. Cells adapt their response (migration, apoptosis, or proliferation) to the signal, depending on both ligand availability and the recruitment of receptor components, such as NRP1 and VEGFR1, on neuroectodermal progenitor cells. The interplay between ligand–receptor recruitment and selective signaling pathways for migration or apoptosis is under investigation. Because Semaphorin 3A is expressed during embryonic development in regions of cell proliferation, such as the ventricular zone of the neocortex (Bagnard et al., 1998), a region in which apoptotic waves occur, this mechanism may also be involved in the early morphogenetic events associated with the migration and apoptotic elimination of neuronal progenitor cells during cortex development.

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