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VEGFR-3/Flt-4 mediates proliferation and chemotaxis in glial precursor cells

Sandra Kranich, Kirsten Hattermann, Aljona Specht, Ralph Lucius, Rolf Mentlein *

Department of Anatomy, University of Kiel, Olshausenstraße 40, 24098 Kiel, Germany

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ABSTRACT

Neuronal and vascular cells share common chemical signals. Vascular endothelial growth factor (VEGF)-C and -D and their receptor VEGFR-3/Flt-4 mediate lymphangiogenesis, but they occur also in the brain. Quantitative RT-PCR of mouse brain tissues and cultivated cells showed that the VEGFR-3 gene is highest transcribed in postnatal brain and in glial precursor cells whereas VEGF-C and -D are variably produced by different neuronal and glial cells. In neurospheres (neural stem cells) VEGFR-3 was induced by differentiation with platelet-derived growth factor (PDGF). In functional studies with an A2B5- and nestin-positive, O4-negative murine glial precursor cell line, VEGF-C and -D stimulated phosphorylation of the kinases Erk1/2; this signal transduction was inhibited by UO126. Both peptides induced the proliferation of glial precursor cells which could be inhibited by UO126. Furthermore, VEGF-D considerably enhanced their migration into an open space in a wound-healing assay. These results show that VEGF-C/-D together with its receptor VEGFR-3 provides an auto-/paracrine growth and chemotactic system for glial precursors in the developing brain.

Vascular endothelial growth factors (VEGFs) have been originally discovered as mitogenic and chemotactic factors for the development and growth of the vascular system, but later it turned out that they target also distinct other cell types and are involved in other developmental and pathological responses (Neufeld et al., 1999; Mentlein and Held-Feindt, 2003; Ferrara, 2004). The VEGF family comprises VEGF-A (often termed only VEGF), placenta growth factor (PlGF), VEGF-B, -C, -D (also termed FiGF, c-fos induced growth factor), -E (Orf virus VEGF) and -F (from snake venom). Three signaling receptors have been identified: VEGFR-1 (Flt-1) binds VEGF-A, PlGF and VEGF-B; VEGFR-2 (KDR or Flk-1) binds VEGF-A, -C, -D and -E; and VEGFR-3 (Flt-4) is targeted by VEGF-C and -D (Takahashi and Shibuya, 2005; Otrock et al., 2007). Processed mouse VEGF-D is reported to target selectively murine VEGFR-3 (Baldwin et al., 2001). In addition, co-receptors as the neuropilins are known. VEGFRs have similar structures, belong to the receptor tyrosine kinases (RTK), in particular to the PDGF (platelet-derived growth factor) receptor family. VEGFRs are 180–230 kDa integral membrane glycoproteins that dimerize and undergo autophosphorylation at tyrosine residues upon ligand binding. Their signal transduction involves phosphorylation of further intracellular proteins, e.g. phosphoinositide-3-kinase (PI3-kinase) or mitogen-activated protein kinases (MAPKs).

In the brain, VEGF-A, VEGFR-1 and -2 are best investigated. VEGF-A is expressed by neurons and astrocytes and exerts pleiotropic effects on neural cells as well as microglial cells (Breier et al., 1992; Sondell et al., 1999, 2000; Jin et al., 2002; Forstreuter et al., 2002; Schänzer et al., 2004; Greenberg and Jin, 2005). In contrast, little is known about the occurrence and functions of VEGF-C and -D and its selective receptor VEGFR-3. In the vascular system, VEGF-C and -D function as specific regulators of lymphangiogenesis (Joukov et al., 1996; Lee et al., 1996; Jeltsch et al., 1997); their effects are mediated via the VEGFR-3 that is mainly expressed on lymphatic endothelial cells (Kaipainen et al., 1995). However, recent evidence has shown that VEGF-C and VEGFR-3 are also expressed in the developing *Xenopus* and mouse brains and act on neural progenitors (Le Bras et al., 2006). Furthermore, VEGF-C, -D and VEGFR-3 are expressed in some glioblastomas despite their lack of lymphatic vessels (Debinski et al., 2001; Jenny et al., 2006; Ma et al., 2008).

Therefore, we wanted to elucidate in more detail the role of VEGFR-3 and its ligands VEGF-C and -D in glial function, their regulation, signal transduction and biological effects. We investigated their expression in murine brain, various murine brain cell types and chose an immortalized A2B5-positive glial precursor cell

Abbreviations: BMP-2, bone morphogenetic protein-2; CNTF, ciliary neurotrophic factor; ERK, extracellular-signal related kinase; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; LIF, leukemia inhibitory factor; MEK, mitogen-activated protein kinase kinase; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription polymerase chain reaction; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

* Corresponding author. Tel.: +49 431 2460; fax: +49 431 1557.
E-mail address: rment@anat.uni-kiel.de (R. Mentlein).

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line that expresses high levels of VEGFR-3 for functional studies. We show that VEGFR-3 signals primarily via the Erk1/2 pathway and induces proliferation as well as chemotaxis of these glial precursor cells.

1. Experimental procedures

1.1. Peptides and inhibitors

Recombinant mouse VEGF-C and VEGF-C_{152S} were from RELIATech (Braunschweig, Germany), VEGF-D from R&D Systems (Minneapolis, MN, USA), VEGF-A_{165} from PeproTech (Rocky Hill, NJ, USA); other cytokines and growth factors (recombinant human forms) were from Immunotools (Friesoythe, Germany). UO126 was purchased from Biomol (Hamburg, Germany) and applied in DMSO solution.

1.2. Tissue samples and cell cultures

All murine tissue samples and cell cultures were obtained from E18, P6 and adult (3-month old) C57B6 J mice (Charles River, Les Oncins, France). Primary murine astroglial and neuronal cells were prepared and analyzed for purity with cell type-specific antibodies as described (Lucius and Mentlein, 1991; Mentlein et al., 1998); b.End3 mouse endothelioma cells, TR6BC1 murine Schwannoma cells and Neuro 2a mouse brain neuronal tumor cells were from the European Collection of Cell Cultures ECACC (Salisbury, UK); immortalized BV-2 murine microglial cells were kindly provided by Elisabetti Blasi, University of Perugia, Italy; Olig-*neu* cells (Jung et al., 1995) were a kind gift from J. Trotter, Heidelberg, Germany. Primary cells were at least 95% immunocytochemically pure for cell type-specific markers except neurons (about 50%).

Immortalized glial precursor cells (GPCs) were obtained from dissociated cells of brains of P2-mice that were transfected (calcium phosphate method) with a temperature-sensitive SV40-construct (pSVLTtsA58 is friendly gift from Dr. P. Rouget, Paris; compare Thi et al., 1998). Stable transfected cells were obtained by addition of G418-sulfate (400 μg/ml, added first after 2 days) over 4 weeks. Stable transfection was verified by immunostaining for SV40-T-large antigen (antibody from Dr. P. Rouget). From stable transfected cells, a clone was selected by panning for the glial marker A2B5: polystyrol culture dishes (for cultivation of bacteria, Sarstedt; Nürnbrecht, Germany) were coated with anti-mouse IgG (from goat, Dako, Glostrup, Denmark; 10 μg/ml in phosphate-buffered saline for 1 h at room temperature, then washed and blocked with bovine serum albumin), then incubated for 1 h at 4 °C with cells pre-labeled with anti-A2B5 (mouse monoclonal, Chemicon; 1:40 in phosphate-buffered saline for 45 min at 4 °C, then washed), and non-adherent cells repeatedly removed by gentle shaking; the finally adherent cells were detached by trypsin treatment and further cultivated in Dulbecco's modified Eagle medium (DMEM; #42430 from Invitrogen, Karlsruhe, Germany) plus 10% fetal calf serum (FCS; PAA Laboratories, Pasching, Austria), 6 mM L-glutamine (Sigma–Aldrich) and each 100 IU/l penicillin and streptomycin at 35 °C.

Murine neural stem cells (NSC) were prepared from the subventricular zone of P6-mice and cultivated in a defined NSC medium (DMEM/F12, Invitrogen) with 2 mM glutamine, 0.6% glucose, 9.6 ng/ml putrescine dihydrochloride, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 25 μg/ml insulin, 2 μg/ml heparin and 4 mg/ml bovine serum albumin (all from Sigma–Aldrich) plus 10 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF). Within the first week of culture they formed free floating neurospheres. Differentiation of murine NSC was initialized by replacing the above medium with fresh NSC medium without EGF and bFGF, but plus additives (none, 2% FCS for 3 days, or growth factors at 10 ng/ml every second day for total of 10 days). Free floating neurospheres got adherent during this time and cells changed morphology.

1.3. Quantitative RT-PCR

RNA was isolated with the TRIZOL reagent, digested by DNase (Promega, Mannheim, Germany), and cDNA synthesized with RevertAid^{TM} H Minus M-muLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). Real time PCR was performed (Held-Feindt et al., 2006) in three replicates of each sample using TaqMan primer probes (Assays on demand; Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as positive control and normalizer. Cycle of threshold (C_T) of each sample was determined, and ΔC_T values = C_TGene of interest − C_TGAPDH calculated and relative expression averaged. For each gene, linear dependence of C_T values from the numbers of copies on a logarithmic scale was verified by using different amounts of cDNA; one magnitude yielded a ΔC_T of 3.33 ($2^{3.33} = 10$). Relative gene expression was calculated with $2^{(\text{normalized } C_T \text{ non-stimulated} - \text{normalized } C_T \text{ stimulated})} = n$-fold of control. TaqMan assays had the following identification numbers and reporter sequences:

- mVEGF-A, Mm00437304_m1, ACCATGCCAAGTGGTCCCAGGCTGC;
- mVEGF-C, Mm00437313_m1, AGCAACATTACCACAGTGTCAGGCA;
- mVEGF-D, Mm00438965_m1, GCCTGGGACAGAAGACCACTCTTAC;
- mVEGFR-1, Mm00438980_m1, TCACCGTGCAAGGAACCTCAGACAA;
- mVEGFR-2, Mm00440099_m1, AATAGAAGGTGCCCAGGAAAAGACC;
- mVEGFR-3, Mm00433337_m1, CCCAGCGCAGCCTCCGGAGGCGGCA;
- mGAPDH, Mm99999915_g1, CGGTGTGAACGGATTTGGCCGTATT;
- mGFAP, Mm00546086_m1, ACCTCCAGATCCGAGAAACCAGCCT;
- mNestin, Mm00450205_m1, GCTACATACAGGACTCTGCTGGAGG;
- mCD133 (prominin 1), Mm00477115_m1, GACTCGTACTGGTGGCTGGGTGGCT.

1.4. Immunocytochemistry

Adherent cells were seeded onto poly-D-lysine (PDL)-coated cover slips and grown to sub-confluence, were either fixed 4% paraformaldehyde in Hepes-buffered saline, pH 7.4 (all VEGFRs) or fixed with an ice-cold acetone/methanol mixture (1:1), washed and immunostained as described (Hattermann et al., 2008). Primary antibodies used in 1:100 dilutions were: anti-VEGFR-1 (anti-mouse VEGFR-1, goat IgG; R&D Systems, Wiesbaden, Germany; AF471), anti-VEGFR-2 (anti-mouse VEGFR-2, rat monoclonal IgG_1; R&D Systems; MAB443), anti-mouse VEGFR-3 (anti-Flt-4; goat IgG, R&D Systems; AF743); anti-nestin (mouse monoclonal 1:100; R&D Systems; MAB1259), anti-O4 (mouse monoclonal 1:20; Roche, Mannheim, Germany; 1518925), anti-A2B5 (mouse monoclonal 1:100; Chemicon; MAB312). Secondary antibodies were goat anti-rabbit or anti-mouse or donkey anti-rat or anti-goat polyclonal antibodies labeled with Alexa Fluor 488 (1:1000; Invitrogen). Nuclei were counterstained with diamidino-2-phenylindole (DAPI; Molecular Probes).

1.5. Stimulation of cells and analysis of kinase phosphorylation

Confluent cells were washed twice with serum free medium containing 0.1% BSA (each 1 h) before stimulation that was done in fresh serum free medium with 0.1% BSA. Inhibitors were pre-incubated for 1 h before adding the stimulators and their concentration maintained during the experiments. Stimulated cells were washed with ice-cold phosphate-buffered saline (2× PBS), lysed with Triton-lysis-buffer (50 mM Tris–HCl, pH 7.8, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM Na_3VO_4), and lysates with equal amounts of proteins (5 μg) separated by SDS-PAGE (10% gel), and Western blots with chemiluminescence detection (ECL Advance^{TM} Western Blotting Detection Kit, Amersham/GE Healthcare, Buckinghamshire, UK) generated using anti-pAkt (Ser473; 1:250, Cell Signaling Technology, Danvers, MA, USA; #9271S), anti-pErk1/2 (Thr202/Tyr204; 1:500; #9101S). Blots were re-probed (Mentlein et al., 2004) after stripping with methanol and 0.1 mol/l glycine/HCl buffer, pH 2.5, with anti-Akt or anti-Erk2 (1:500, Cell Signaling Technology #9272; Santa Cruz sc-1647).

1.6. Proliferation assays

Proliferation assays were performed with sub-confluent cells which had been pre-cultivated for 2 days in medium with 10% FCS. Subsequently, cells were washed and then incubated with medium containing 0.2% FCS with or without stimulators added. After 24 h, DNA was measured fluorimetrically using the CyQuant-method (Mentlein et al., 2001) and proliferation was expressed as % of the non-stimulated control (4 individual dishes for each stimulus).

1.7. Scratch assays

For scratch (wound-healing) assays, cells were cultured to optical confluence in 35 mm dishes (TPP, Pasching, Austria). The cell layer was scratched with a pipette tip, the medium was replaced with fresh one (containing 10% FCS) with or without stimulators. The wound healing was continuously monitored by video camera under a microscope equipped with an object chamber heated to 35 °C and with CO_2 (7%) ventilation. For quantitative evaluation, randomly selected areas of the scratch were analyzed at the beginning and at different times for areas with invaded cells and normalized for the original open area according to: normalized area = (area at 0 h − area 10 h)/area at 0 h × 100.

1.8. Statistical analysis

Values are given as means ± standard deviations (SD). Values below detection limit after 40 PCR cycles were assumed as $\Delta C_T = 21$ for statistical analysis as GAPDH signals of 10 ng RNA input are supposed to appear at cycle 19 (C_T value of 19). Statistical significance was analyzed by a two-tailed unpaired Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

2. Results

2.1. VEGFR-3 is highly expressed in murine glial precursor cells (GPCs)

Murine brain tissues as well as various brain cell types (primary cultures, immortalized cells and cell lines) were analyzed for transcription of VEGF-A, -C, -D, VEGFR-1, -2 and -3 mRNA by quantitative RT-PCR (Fig. 1). In total brain of different developmental stages, all factors and receptors were detected, but with