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Developmental Biology 289 (2006) 329 – 335
DEVELOPMENTAL BIOLOGY
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Vascular endothelial growth factor-B (VEGFB) stimulates neurogenesis: Evidence from knockout mice and growth factor administration

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Received for publication 5 January 2005, revised 7 October 2005, accepted 14 October 2005
Available online 7 December 2005

Abstract

Vascular endothelial growth factor-B (VEGFB) is an angiogenic and neuroprotective protein that reduces hypoxic and ischemic neuronal injury. To determine if VEGFB also regulates neurogenesis in the adult brain, we studied the effects of VEGFB administration in vitro and in vivo, as well as the effect of VEGFB gene knockout (KO) in mice, on bromodeoxyuridine (BrdU) incorporation and expression of immature neuronal markers in the subgranular zone (SGZ) of the hippocampal dentate gyrus and the forebrain subventricular zone (SVZ). Intracerebroventricular VEGFB administration increased BrdU incorporation into cells of neuronal lineage both in vitro and in vivo, and VEGFB-KO mice showed impaired neurogenesis, consistent with a neurogenesis-promoting effect of VEGFB. In addition, intraventricular administration of VEGFB restored neurogenesis to wild-type levels in VEGFB-KO mice. These results suggest a role for VEGFB in the regulation of adult neurogenesis, which could have therapeutic implications for diseases associated with central neuronal loss.

Keywords: Vascular endothelial growth factor-B; Neurogenesis; Angiogenesis

Introduction

Members of the vascular endothelial growth factor (VEGF) family of proteins were identified based on their angiogenic and vessel permeability-inducing properties (Keck et al., 1989; Leung et al., 1989) and were subsequently found to couple hypoxia to angiogenesis in a wide range of tissues (Shweiki et al., 1992). The prototypical VEGF, VEGFA, also exerts direct, survival-promoting effects on nonvascular cells, including hematopoietic cells (Gerber et al., 2002), cardiomyocytes (Giordano et al., 2001), renal tubular epithelial cells (Kanellis et al., 2000), and neurons (Jin et al., 2000b). For example, VEGFA administration has neuroprotective effects in animal models of stroke (Hayashi et al., 1998; Sun et al., 2003; Zhang et al., 2000) and motor neuron disease (Azzouz et al., 2004; Lambrechts et al., 2003; Sopher et al., 2004). VEGFA also stimulates neurogenesis, or the production of new neurons, in the adult brain (Jin et al., 2002b; Louissaint et al., 2002), and this may be a key feature of the vascular niche (Palmer et al., 2000; Shen et al., 2004) that fosters neurogenesis in select brain regions. VEGFA acts through two receptor tyrosine kinases, VEGFR1 and VEGFR2, and also interacts with co-receptor neuropilins that may function in ligand presentation, but most known effects of VEGFA have been ascribed to VEGFR2 (Neufeld et al., 1999).

VEGFB is an angiogenic growth factor (Silvestre et al., 2003) with 88% amino acid sequence homology to VEGFA (Olofsson et al., 1996) and selective affinity for VEGFR1 (Olofsson et al., 1998). VEGFB-knockout (KO) mice show abnormalities of cardiac development and function (Aase et al., 2001; Bellomo et al., 2000) and hypoxia-induced remodeling of the pulmonary vasculature (Wanstall et al., 2002), but no change in the expression of VEGFA or VEGF receptors, or in VEGFA-induced angiogenesis (Aase et al., 2001). VEGFB is also expressed in the brain, where it is induced by brain injury (Nag et al., 2002). We reported recently that VEGFB-KO mice exhibit increased infarct size and more severe neurological deficits following stroke induced by occlusion of the middle cerebral artery (Sun et al., 2004), consistent with a neuroprotective function of VEGFB. VEGFB also reduced hypoxic death of cultured cerebral cortical neurons in vitro (Sun et al., 2004), suggesting that its protective effect may involve a direct

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doi:10.1016/j.ydbio.2005.10.016

interaction with neurons. In view of the dual neuroprotective and neurogenesis-promoting effects identified previously for VEGFA, we investigated the possibility that VEGFB might also regulate neurogenesis, using the VEGFB-KO mice referred to above.

Methods

Cell culture

Primary cultures enriched in cells of neuronal lineage (75% βIII-tubulin and 82% Hu-immunopositive) were prepared from wild-type (WT) 15-day Charles River CD1 mouse embryos. Cell suspensions were plated at 2.0×10^5 cells/cm^2 on 24- or 96-well poly-D-lysine-coated plastic tissue culture dishes in defined medium Neurobasal/B27 (Life Technologies, Inc., Rockville, MD) containing 2 mM glutamine, 25 U/ml penicillin, and 25 μg/ml streptomycin. Cultures were kept in a humidified, 5% CO_2 incubator at 37°C for 4 days, and then maintained for an additional 2 days in the absence or presence of VEGFB (10–100 ng/ml; R&D Systems Inc., Minneapolis, MN). This duration of exposure to VEGFB was chosen to achieve a maximal, time-dependent effect. BrdU (50 μg/ml; Sigma, St. Louis, MO) was added for the final 24 hr, after which BrdU incorporation was measured using a commercially available ELISA kit (Roche, Indianapolis, IN) and cell proliferation was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Jin et al., 2002a).

BrdU and cell-type marker proteins in neuronal cultures were localized by dual-label immunocytochemistry using mouse monoclonal anti-BrdU (Roche, Indianapolis, IN; 1:200), mouse monoclonal anti-nestin (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100), and mouse monoclonal anti-NeuN (Chemicon; 1:200) as primary antibodies and FITC- and rhodamine-conjugated secondary antibodies (Jackson Immuno-Research Laboratories, Inc.; 1:200). Controls included preabsorbing the primary antibody and omitting the secondary antibody. A Nikon PCM-2000 laser-scanning confocal microscope and Simple PCI imaging software (Compix, Cranberry Township, PA) were used for visualization.

VEGFB-knockout mice

Animal experiments were approved by local committee review and conducted according to NIH guidelines. VEGFB-KO mice (Bellomo et al., 2000) were obtained from Jackson Laboratories (Bar Harbor, ME), bred in house, and genotyped by polymerase chain reaction using tail-tip genomic DNA (Sun et al., 2004). Mice were used at 10–12 weeks of age.

RNA and protein measurements

To compare VEGFA, VEGFR1, and VEGFR2 mRNA expression in WT and VEGFB-KO mice, total RNA was isolated and RT-PCR was performed using a standard protocol and the following primers: VEGF-A sense, CTGACATGAAGGAAGAGGAG; VEGF-A antisense, GTGTCTACAGGAATCCCAGA; VEGFR1 sense, AAGATTACATCCCCCTCAAT; VEGFR1 antisense, GAGCTTCCAGAATGAAAATG; VEGFR2 sense, GTAAAAGCAGGGAGTCTGTG; VEGFR2 antisense, GTGGTGGAAAGAACAACACT. PCR products (VEGFA, 189bp; VEGFR1, 124bp; VEGFR2, 275bp) were detected on 2% agarose gels, and their optical density was measured using Scion imaging software.

For Western blotting, protein was extracted from mouse brain and 100 μg per lane was electrophoresed on 12% SDS-PAGE and transferred to polyvinyldifluoridine membranes. Membranes were blocked with 5% nonfat dried milk in PBS/0.1% Tween 20 and incubated overnight with goat polyclonal anti-VEGFB (R&D, Minneapolis, MN; 1:100), rabbit polyclonal anti-VEGFR1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500), or mouse monoclonal anti-VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500). Blots were washed with PBS/0.1% Tween-20 and incubated at room temperature for 60 min with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology; 1:2000). Peroxidase activity was visualized with a chemoluminescence substrate (NEN, Boston, MA) and quantified by computerized densitometry.

BrdU administration

BrdU (50 mg/kg in saline i.p.) was given twice daily for 3 days and WT and VEGFB-KO mice were killed 3 days or 2 weeks later. Mice were perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate-buffered saline (pH 7.4).

Immunohistochemistry

For single-label immunohistochemistry, 50 μm sections were cut with a cryostat and stored at −80°C. Sections for BrdU immunohistochemistry were treated with 2 M HCl at 37°C for 30 min and rinsed in 0.1 M boric acid (pH 8.5) at room temperature for 10 min. Sections were incubated in blocking solution (2% goat serum/0.3% Triton X-100/0.1% bovine serum albumin in phosphate-buffered saline) for 30 min at room temperature, and with mouse monoclonal anti-BrdU (Roche Applied Science, 1:200) or goat polyclonal anti-DCX (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100) at 4°C overnight. Sections were washed with phosphate-buffered saline, incubated with biotinylated goat-anti-mouse secondary antibody (Vector Laboratories, 1:200) for 1 h at 25°C, washed, and placed in avidin–peroxidase conjugate (Vector Laboratories) solution for 1 h. The horseradish peroxidase reaction was detected with 0.05% diaminobenzidine and 0.03% H_2O_2. Processing was stopped with H_2O and sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped in permanent mounting medium (Vector Laboratories). Sections were examined with a Nikon E300 epifluorescence microscope. For double-label immunohistochemistry, paraffin-embedded, 6-μm sections were deparaffinized with xylene and rehydrated with ethanol. The same BrdU and DCX primary antibodies mentioned above were used, as well as FITC- and rhodamine-conjugated secondary antibodies (Jackson Immuno-Research Laboratories, Inc.; 1:200). Fluorescence signals were detected by Nikon PCM-2000 laser-scanning confocal microscopy. Controls included omitting primary or secondary antibody.

Cell counting

BrdU- or DCX-immunopositive cells were counted blindly in six diaminobenzidine-stained, 50 μm coronal sections per animal, spaced 200 μm apart. Cells were counted under high-power (200×) on a Nikon E300 microscope with a Magnifire digital camera, and the image was displayed on a computer monitor. Results were expressed as the average number of BrdU-positive cells per section.

Klenow staining

To evaluate the possibility that VEGFB increases the number of BrdU-labeled cells by reducing cell death rather than increasing proliferation, damaged cells containing DNA strand breaks were detected using the Klenow fragment of DNA polymerase I, as described (Jin et al., 1999).

Intracerebroventricular administration of VEGFB in vivo

Wild-type or VEGFB-KO mice and adult male Sprague–Dawley rats were anesthetized with 4% isoflurane in 70% N_2O/30% O_2 and implanted with osmotic minipumps (Alzet 1003D; Alza Corporation, Mountain View, California, USA). The cannula was placed in the right lateral ventricle, 2.0 (mice) or 3.8 mm (rats) deep to the pial surface, −0.82 (mice) or −0.6 mm (rats) anteroposterior relative to bregma, and 1.5 (mice) or 1.3 mm (rats) lateral to the midline. VEGFB (10 μg/ml) or artificial cerebrospinal fluid (aCSF) vehicle (128 mM NaCl, 2.5 mM KCl, 0.95 mM $CaCl_2$, and 1.99 mM $MgCl_2$) was infused by the intracerebroventricular route at 1 μl/h for 3 days. BrdU (50 mg/kg i.p. in saline) was given twice daily for 3 days and animals were killed 3 days to 2 weeks later. Immunohistochemistry and cell counting were performed as described above.

Statistics

The statistical significance of differences between means was evaluated by Student's *t* test for single comparisons and by ANOVA followed by post hoc *t* tests for multiple comparisons, with $P < 0.05$ considered significant.

Results

Addition of VEGFB to cerebral cortical cultures produced concentration-dependent enhancement of BrdU incorporation, which increased by ~30% in the presence of 100 ng/ml VEGFB (Fig. 1A). Immunocytochemistry showed that BrdU labeling was associated with cells that expressed markers of immature (e.g., nestin) but not mature (e.g., NeuN) cells of neuronal lineage (Fig. 1B). This is consistent with our prior finding that only ~2% of cells in these cultures are of nonneuronal (glial or endothelial) lineage (Jin et al., 2002a). Metabolic activity in cultures, measured by the reduction of MTT, increased by ~25% over the same range of VEGFB concentrations (Fig. 1C), consistent with an increase in cell number.

As reported previously (Sun et al., 2004), VEGFB-KO mice lacked VEGFB immunoreactivity in cerebral neurons and vasculature, but were phenotypically indistinguishable from WT mice under normal conditions during life. VEGFB-KO mice showed no secondary decreases in VEGFA or VEGF receptor RNA or protein expression that might lead one to misattribute the effects of such changes to deletion of VEGFB (Figs. 2A–B; note that VEGFR1 expression in brain is undetectable by Western blot under basal conditions, but becomes apparent after injury; Jin et al., 2000c).

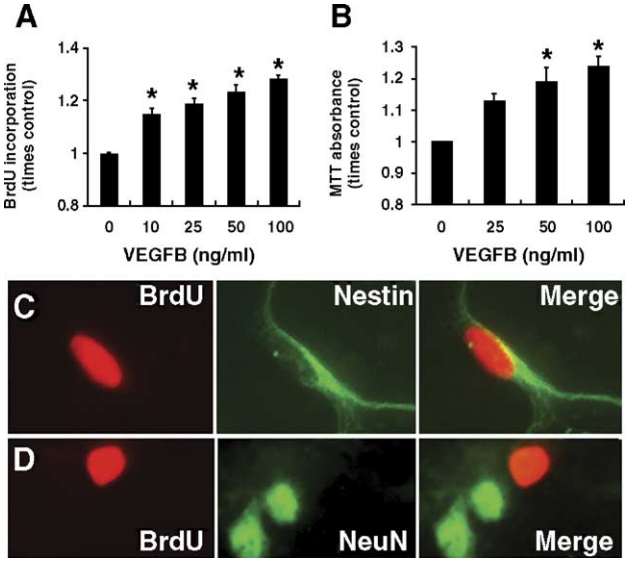


Fig. 1. VEGFB stimulates BrdU incorporation in murine cortical cultures in vitro. Cultures enriched in neuronal precursors were treated with the indicated concentrations of VEGFB for 48 h. BrdU (50 μg/ml) was added, where indicated, for the last 24 h of VEGF exposure. BrdU incorporation (A) and MTT absorbance, an index of cell number, (B) were measured and expressed in relation to control (0 ng/ml VEGFB) values. BrdU immunoreactivity was co-localized with expression of the neuroepithelial precursor cell marker nestin (C), but not with the mature neuronal marker NeuN (D). Data are mean values ± SEM from at least 3 cell cultures per concentration of VEGFB (A, B) or representative fields (C, D). *$P < 0.05$ compared to 0 ng/ml VEGFB.

After administration of BrdU, VEGFB-KO mice showed reduced BrdU immunolabeling in the two major neuroproliferative brain regions—the subgranular zone (SGZ) of the hippocampal dentate gyrus and the forebrain subventricular zone (SVZ) (Fig. 2C). Counting of BrdU-immunopositive cells in brain sections showed significantly reduced cell counts in SGZ and SVZ of VEGFB-KO mice (Fig. 2D). Intraventricular administration of VEGFB to VEGFB-KO mice restored BrdU labeling to wild-type levels in both SGZ and SVZ, which argues that the absence of VEGFB is sufficient to explain impaired neurogenesis in VEGF-KO mice.

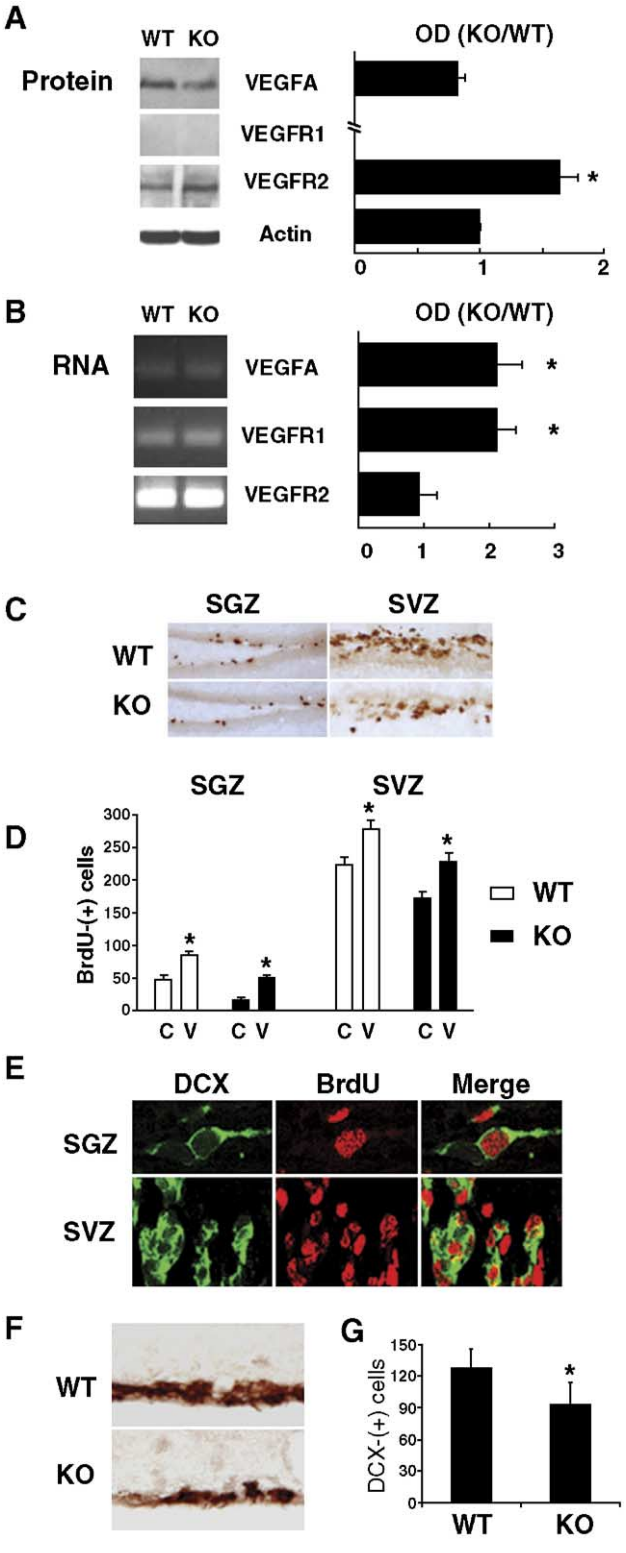
BrdU immunoreactivity in SGZ and SVZ was associated for the most part with expression of the immature neuronal marker DCX (Fig. 2E), indicative of a neuronal lineage for BrdU-labeled cells in SGZ and SVZ. In line with this observation, DCX immunolabeling in SVZ of VEGFB-KO mice was decreased to the same (~30%) extent as was BrdU labeling at 3 days after BrdU administration (Figs. 2F–G). Reduced BrdU-immunopositive cell counts in VEGFB-KO mice could, in principle, result not only from impaired proliferation, but also from enhanced cell death. To evaluate this possibility, we used Klenow labeling to detect CA1 cells with DNA strand breaks in SGZ from WT and VEGFB-KO mice (Jin et al., 1999). Fig. 3 shows that whereas Klenow labeling was prominent in CA1 from mice subjected to global cerebral ischemia (positive control), it was rare in SGZ from both WT and VEGFB-KO mice. Consequently, it is difficult to ascribe decreased BrdU labeling in VEGFB-KO mice to increased cell damage leading to death.

If neurogenesis is decreased in the absence of VEGFB expression, then the administration of exogenous VEGFB to normal animals might stimulate neurogenesis. To test this possibility, VEGFB or aCSF vehicle was infused into the cerebral ventricles of rats given BrdU over the same 3-day period, and brain sections were stained for BrdU and for DCX at 3 days and 2 weeks after completing these treatments. Figs. 4A–B shows that VEGFB infusion increased BrdU labeling in SGZ at 2 weeks and in SVZ at both 3 days and 2 weeks after BrdU administration, reproducing in reverse the effects of VEGFB gene knockout in mice. Again, BrdU labeling in these regions was associated primarily with DCX-expressing, neuronal cells (Figs. 4C–E).

Discussion

The principal finding we report here is that VEGFB stimulates neurogenesis in the adult brain. The evidence for this is that neurogenesis, demonstrated by BrdU labeling and confirmed by immunohistochemical detection of co-expressed neuronal lineage markers, is reduced in VEGFB-knockout mice and enhanced in VEGB-treated neuronal cultures and in rats given VEGFB by intraventricular injection. VEGFB, therefore, joins a growing list of growth factors that can regulate the production of new neurons in the brain, including the better-characterized VEGF homolog VEGFA (Jin et al., 2002b; Louissaint et al., 2002; Sun et al., 2003).

What is the relationship between VEGFA- and VEGFB-induced neurogenesis? VEGFA and VEGFB act through overlapping but nonidentical signaling systems—VEGFA activates both VEGFR1 and VEGFR2 receptor tyrosine kinases, whereas the effects of VEGFB are mediated by VEGFR1 alone; both VEGFA and VEGFB can also bind to neuropilin co-receptors (Matsumoto and Claesson-Welsh, 2001). VEGFA binds to VEGFR1 with higher affinity than to VEGFR2, but the interaction with VEGFR2 elicits a greater increase in protein kinase activity, and the cytoproliferative activity of VEGFA appears to be attributable primarily to its effect on VEGFR2 (Ferrara et al., 2003). The extent to which VEGFA and VEGFB increase neurogenesis also differs. Thus, whereas both VEGFA (Jin et al., 2002b) and VEGFB (Fig. 1) increased BrdU labeling and cell number by 20–30% in vitro, VEGFA increased in vivo BrdU labeling in DG by ~600% and in SVZ by ~270% (Sun et al., 2003), while VEGFB-induced increases were only ~50% in both regions (Fig. 4). Consequently, the manner in which VEGFA and VEGFB promote proliferation and survival of neuronal precursors is likely to differ. The observation that neurogenesis is decreased in VEGFB-KO mice provides further evidence for nonidentical effects of the two VEGF isoforms.



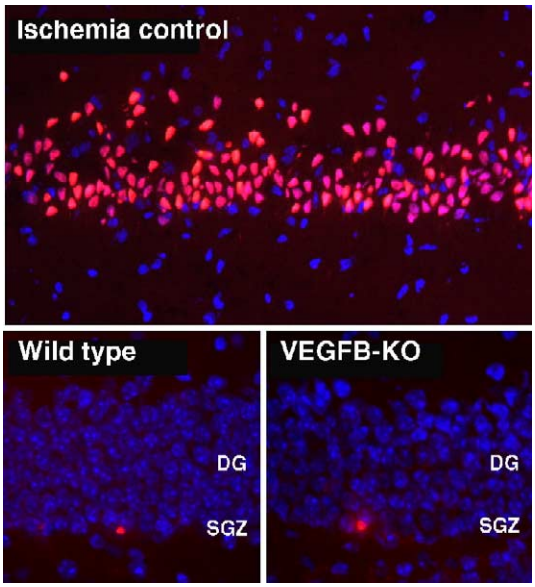


Fig. 3. VEGFB deletion is not associated with increased cell death in SGZ. Klenow labeling of cells with DNA strand breaks (pink), which was prominent in the rat hippocampal CA1 region 72 h after transient (15 min) global cerebral ischemia (top; positive control), was detected only rarely in SGZ (lower cell layer of dentate gyrus, DG) of WT mice (bottom left) and was not increased in VEGFB-KO mice (bottom right). Nuclei are stained with DAPI (blue). Data are representative fields from 3 rats or 6 mice per condition.

Fig. 2. BrdU incorporation into SGZ and SVZ is reduced in VEGFB-KO mice. BrdU was given i.p. for 3 days and then localized by immunohistochemistry in SGZ and SVZ. (A) Western blots of VEGFA, VEGFR1, and VEGFR2 protein expression in brain of wild-type (WT) and VEGFB-KO mice, and densitometry from three blots per protein, showing the ratio of optical densities in VEGFB-KO versus WT mice (mean ± SEM, $*P < 0.05$ compared to WT). (B) PCR of VEGFA, VEGFR1, and VEGFR2 mRNA expression in brain of wild-type (WT) and VEGFB-KO mice, and densitometry from three blots per mRNA, showing the ratio of optical densities in VEGFB-KO versus WT mice (mean ± SEM, $*P < 0.05$ compared to WT). (C) BrdU-immunopositive cell counts showed reduced labeling (D) in SGZ and SVZ of VEGFB-KO mice (c), and restoration of WT levels in KO mice given intracerebroventricular VEGFB (v). BrdU immunoreactivity was co-localized with expression of the immature neuronal marker DCX in SGZ and SVZ of VEGFB-KO mice (E). SVZ of VEGFB-KO mice also showed reduced immunohistochemical expression of DCX (F, G). Data are representative fields (C, E, F) or mean values ± SEM (D, G) from 6 mice per condition. $*P < 0.05$ compared to control.

That VEGFB should have the capacity to trigger the production of neurons is not surprising, in that VEGFB has mitogenic effects on hematopoietic cells (Casella et al., 2003), and some hematopoietic growth factors, including stem cell factor (Casella et al., 2003) and erythropoietin (Shingo et al., 2001), also promote neurogenesis. Other (e.g., angiogenic) effects of VEGFB involve activation of Akt (Silvestre et al., 2003), which has been implicated in neuronal effects of VEGFA (Jin et al., 2000a). VEGFB-induced angiogenesis is associated with increased production of NO by endothelial NO synthase (Silvestre et al., 2003), and alterations in NO synthesis have been implicated in neurogenesis (Zhang et al., 2001). VEGFA-induced neurogenesis in vitro is inhibited by the VEGFR2 inhibitors SU1498 and oxindole I, the phospholipase C inhibitor U73122, the protein kinase C inhibitor GF102390X, the phosphatidylinositol 3-kinase inhibitor wortmannin, and the extracellular signal-related kinase kinase inhibitor PD98059 (Jin et al., 2002b; Zhu et al., 2003). The signaling pathways through which the interaction of VEGFB with VEGFR-1 also promotes neurogenesis will be important to investigate in future studies.

Adult neurogenesis may represent a mechanism for responding to and mitigating brain injury. One respect in which VEGFA and VEGFB differ is that only the former appears to be inducible by hypoxia (Enholm et al., 1997), and hypoxia-inducible VEGFA production could help couple brain injuries, such as ischemia, to enhanced neurogenesis. Whether cerebral ischemia induces VEGFB is uncertain, but VEGFB expression was increased by traumatic thermal injury (Nag et al., 2002), and could therefore be affected by other pathological processes as well.

Two questions that are raised but cannot be answered by this study are why there appears to be so much redundancy in the ability of growth factors to stimulate neurogenesis and what functional significance, if any, to attach to this phenomenon. The apparent redundancy may reflect incomplete understanding of the complexity of neurogenesis signaling. For example, different growth factors may be induced by different physiological or pathological events (as in the disparate effects of hypoxia on VEGFA and VEGFB), act on different subpopulations of neuronal precursor cells, or promote different stages of neurogenesis (e.g., proliferation, survival, differentiation, or migration). The ability to demonstrate defects on neurogenesis resulting from growth factor gene-knockout, as reported for fibroblast growth factor-2 (Yoshimura et al., 2001), leukemia inhibitory factor (Bauer et al., 2003), and now VEGFB, indicates that what redundancy may exist in the regulation of neurogenesis is incomplete. The functional significance of VEGFB-induced or other forms of

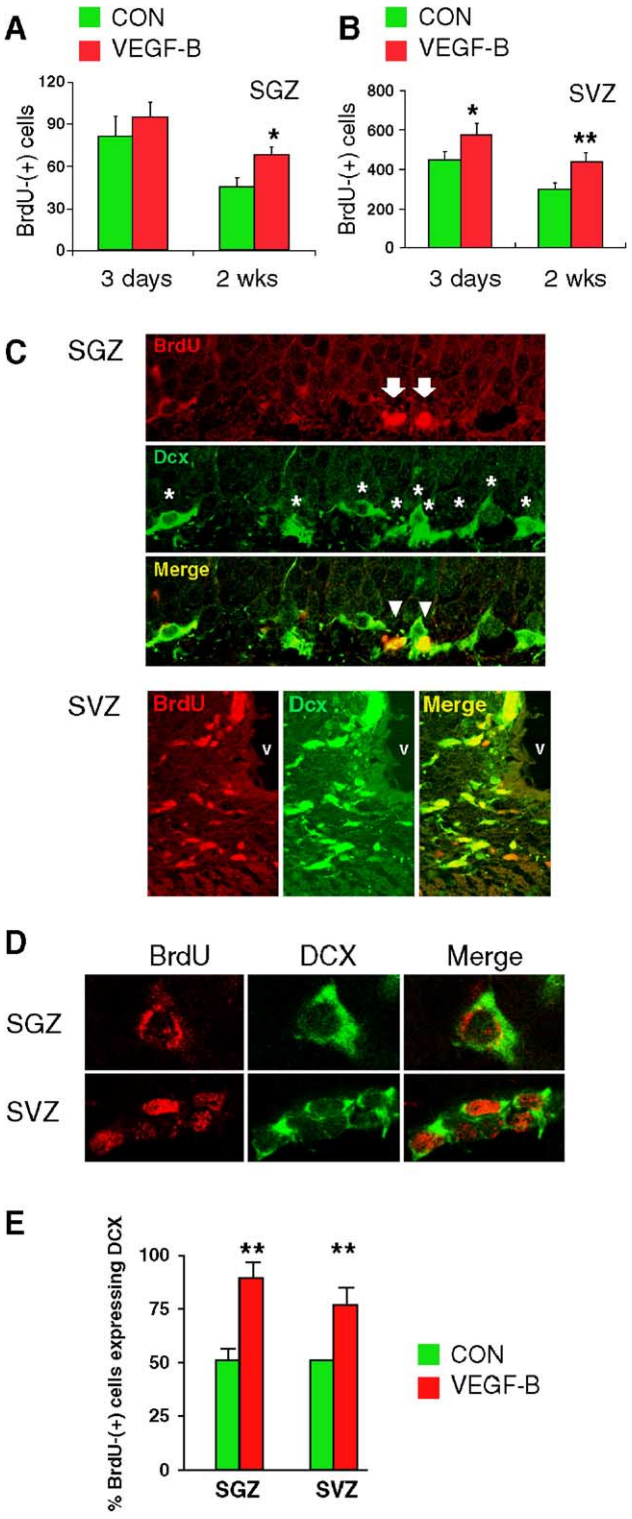


Fig. 4. VEGFB administration increases BrdU incorporation into SGZ and SVZ of adult rats. VEGFB was given by the intraventricular and BrdU by the intraperitoneal route for 3 days. VEGFB increased BrdU labeling in SGZ (A) and SVZ (B). BrdU labeling co-localized with DCX expression in some but not all BrdU-labeled cells, as seen at low magnification in both SGZ and SVZ of control rats (C); in upper panel, arrows indicate BrdU-positive nuclei, asterisks Dcx-positive cell bodies, and arrowheads dual-labeled cells; in lower panel, v indicates lateral ventricle. Co-localization of BrdU and Dcx in SGZ and SVZ of VEGFB-treated rats is seen at higher magnification in panel D. VEGFB treatment also increased the percentage of BrdU-labeled cells that expressed Dcx (E), which is consistent with, but not necessarily indicative of, a stimulatory effect on neuronal differentiation, as well as on cell proliferation. Data are mean values ± SEM (A, B, E) or representative fields (C, D) from 6 rats per condition. The percentage of BrdU-positive cells that expressed Dcx was increased after VEGFB administration, from ~50% to ~90% in DG, and from ~50% to ~75% in SVZ. *$P < 0.05$, **$P < 0.01$ compared to no VEGFB administration (CON).

adult neurogenesis is difficult to establish. Nevertheless, current data indicate that neurons born in the adult brain have the capacity to become functional (Carleton et al., 2003; Jessberger and Kempermann, 2003; Song et al., 2002; van Praag et al., 2002), and that inhibiting neurogenesis has functional consequences as well (Raber et al., 2004). It is, therefore, reasonable to hypothesize that VEGFB may exercise distinctive effects on adult neurogenesis, and that these effects could have therapeutic application in efforts to replace cerebral neurons that die as a result of brain injury or disease.

Acknowledgments

Supported by National Institutes of Health grants NS35965 and NS44921.

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