VEGF-B inhibits apoptosis via VEGFR-1–mediated suppression of the expression of BH3-only protein genes in mice and rats

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Despite its early discovery and high sequence homology to the other VEGF family members, the biological functions of VEGF-B remain poorly understood. We revealed here a novel function for VEGF-B as a potent inhibitor of apoptosis. Using gene expression profiling of mouse primary aortic smooth muscle cells, and confirming the results by real-time PCR using mouse and rat cell lines, we showed that VEGF-B inhibited the expression of genes encoding the proapoptotic BH3-only proteins and other apoptosis- and cell death–related proteins, including p53 and members of the caspase family, via activation of VEGFR-1. Consistent with this, VEGF-B treatment rescued neurons from apoptosis in the retina and brain in mouse models of ocular neurodegenerative disorders and stroke, respectively. Interestingly, VEGF-B treatment at the dose effective for neuronal survival did not cause retinal neovascularization, suggesting that VEGF-B is the first member of the VEGF family that has a potent antiapoptotic effect while lacking a general angiogenic activity. These findings indicate that VEGF-B may potentially offer a new therapeutic option for the treatment of neurodegenerative disorders.

Introduction

The VEGF family incorporates 5 structurally related ligands that bind differentially to 3 receptor tyrosine kinases (VEGFR-1, -2, and -3) and the semaphorin receptors neuropilin 1 (NP-1) and NP-2. Among the 5 VEGF family members, VEGF is the prototypical angiogenic factor, with a potent and universal angiogenic effect in most physiological and pathological conditions (1–3). The placenta growth factor (PlGF) is believed to play important roles in pathophysiological angiogenesis (4), while VEGF-C and VEGF-D are mainly involved in lymphangiogenesis (5). VEGF has recently been shown to be a potent neuroprotective factor (6, 7). However, the therapeutic potential of its neuroprotective effect is limited by its potent angiogenic activity (4), while VEGF-C and VEGF-D are mainly involved in lymphangiogenesis (5). VEGF has recently been shown to be a potent neuroprotective factor (6, 7). However, the therapeutic potential of its neuroprotective effect is limited by its potent and general activity in inducing undesired angiogenesis and blood vessel leakage, which is highly detrimental in most conditions.

Compared with the other members of the VEGF family, VEGF-B has received much less attention, and its biological function remains debatable (8, 9). VEGF-B shares a high degree of sequence homology with VEGF and PlGF. It binds to the tyrosine kinase VEGFR-1 and its coreceptor NP-1 (10, 11). VEGF-B is highly expressed in most tissues and organs (12, 13). VEGF-B in most conditions seems to be redundant or inert. In contrast to VEGF-deficient mice (14, 15), VEGF-B−deficient mice are healthy and fertile, with apparently normal developmental and physiological angiogenesis (16, 17). In contrast to VEGF and PlGF, VEGF-B is not required for neovessel formation in proliferative retinopathy (18) and blood vessel remodeling in pulmonary hypertension (19). Adenoviral VEGF-B gene transfer in the hind limb (20) and carotid artery (21) did not induce any angiogenic response, while the other VEGF family members did. Moreover, numerous studies have shown that VEGF-B has no effect on blood vessel permeability (19, 22–24), in contrast to the other VEGF family members.

Current functional analysis of VEGF-B has led to inconsistent and controversial results. While one study reported smaller heart and abnormal coronary artery vasculature caused by VEGF-B deficiency in mice (17), this was not observed in another independent study (16). Several reports have shown an angiogenic effect of VEGF-B (24–26), whereas other studies have observed the lack of angiogenic activity of VEGF-B in different model systems (16, 18, 20, 21, 27). VEGF-B was reported to play a role in pulmonary hypertension (28); however, this was not observed in another independent study (19). In summary, due to various reasons including the distinct gene-targeting approaches used, the different genetic background of the animals in different studies, and experimental variations among different research groups, the reports published thus far with regard to the function of VEGF-B have been inconsistent. Further studies on VEGF-B are therefore especially warranted.

In this study, to determine the biological function of VEGF-B, we performed a genome-wide gene expression profiling assay. We validated our findings in multiple cell lines and animal models. We report here that VEGF-B is a potent apoptosis inhibitor by sup-

Nonstandard abbreviations used: CNV, choroidal neovascularization; ECD, extracellular domain; IB4, isolectin B4; LDH, lactate dehydrogenase; MCA, middle cerebral artery; NP-1, neuropilin 1; ONC, optic nerve crush; PlGF, placenta growth factor; RGC, retinal ganglion cell.

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pressing the expression of the BH3-only protein and other apoptotic/cell death–related genes via VEGFR-1. VEGF-B treatment rescues neurons from apoptosis in both the retina and brain without causing undesired angiogenesis. VEGF-B therefore appears to be the first member of the VEGF family that has been observed to have a potent antiapoptotic effect but lack general angiogenic activity. VEGF-B thus may potentially offer a new therapeutic option for the treatment of neurodegenerative diseases.

Results

Genome-wide gene profiling reveals that VEGF-B inhibits the expression of numerous apoptotic/cell death–related genes. To determine the biological function of VEGF-B, we performed a genome-wide gene expression profiling assay to identify the genes regulated by VEGF-B. We treated mouse primary aortic SMCs with human recombinant VEGF-B167 protein in a hypoxic condition (1% oxygen), because these cells express VEGFR-1 and VEGFR-1 is upregulated in hypoxia (data not shown). We performed the expression profiling analysis using the whole mouse genome microarray containing 41,534 mouse genes and transcripts. Results revealed that VEGF-B treatment significantly downregulated the expression of about 600 genes by more than 2-fold. Among them, the genes downregulated to the greatest extent fall into the apoptosis/cell death–related pathways (Table 1). The 3 most downregulated genes were the ones critically involved in apoptosis: Bmf (29) (about 10-fold downregulated), Trp53inp1 (30) (about 8-fold downregulated), and DCN (31) (about 7-fold downregulated). Thus, the genes that are downregulated to the greatest extent by VEGF-B are the ones critically involved in the apoptosis/cell death–related pathways.

VEGF-B inhibits apoptotic gene expression in multiple cells and cell lines. We next used 5 different types of cells/cell lines to verify the microarray data. We treated the rat retinal ganglion cell–derived (RGC-derived) cell line RGCs, the immortalized rat retinal pericyte cell line TR-rPCT, the immortalized rat retinal Müller cell line TR-MUL, the immortalized rat retinal endothelial cell line TR-iBRB, and mouse primary aortic SMCs (mSMCs) with VEGF-B167 (100 ng/ml) for 6 hours in either normoxic or hypoxic (1% oxygen) conditions, and the expression of the apoptotic/cell death–related genes was investigated by real-time PCR assay. VEGF-R1 expression was detected by real-time PCR assay in all the 5 different types of cells (data not shown). VEGF-B significantly inhibited the expression of many apoptotic/cell death–related genes, consistent with the microarray data. Among these genes, the expression of most of the BH3-only protein genes (Bmf, Hrk, Puma, Noxa (Pmaip1), Bad, Bid, Bik) was inhibited by VEGF-B in the different types of cells (Figure 1, A–E). The BH3-only protein genes are therefore the major target genes suppressed by VEGF-B. VEGF-B also inhibited the expression of p53, the caspases (Casp2, Casp8, Casp9, Casp12), and other apoptotic/cell death–related genes (Bak, Bax, Bel211, TNF-α, Dcn, Olr1, etc.; Figure 1, A–E). Thus, VEGF-B inhibited the expression of the BH3-only protein and other apoptotic/cell death–related genes in all of the 5 types of cells/cell lines investigated, indicating a general role of VEGF-B as an apoptosis inhibitor in different types of cells.

Table 1

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VEGF-B inhibits oxidative stress–, serum deprivation–, and Bmf-induced apoptosis in cultured cells. We next used different approaches to test whether VEGF-B could inhibit apoptosis in the RGC5 cells. We first treated the RGC5 cells with hydrogen peroxide (H2O2), which is known to induce oxidative stress–induced apoptosis, and investigated the apoptosis status using the TUNEL assay. VEGF-B167 treatment (100 ng/ml) significantly decreased the H2O2-induced apoptosis in the RGC5 cells by about 50% (Figure 2, A–D; n = 3, P < 0.01). We next tested whether VEGF-B could inhibit the serum deprivation–induced cell death in the RGC5 cells. We cultured the cells in serum-free medium and treated the cells with recombinant proteins of human VEGF-B167 (100 ng/ml), VEGF (25 and 100 ng/ml), and PIGF (25 and 100 ng/ml), and the viability of the cells was measured at different time point using the MTT assay. VEGF-B167 treatment significantly increased cell survival as potently as 10% FCS at days 1 and 2 (Figure 2E; n = 3, P < 0.01). The survival effect of VEGF-B167 on the RGC5 cells was slightly greater in a hypoxic condition (data not shown). In contrast, PIGF, another VEGFR-1 ligand, did not show such an effect (Figure 2F). VEGF had only a weak survival effect on the RGC5 cells (Figure 2G; n = 3, P > 0.05).

Bmf is an essential apoptosis inducer in response to cellular stresses (29). Overexpression of Bmf led to cellular apoptosis within 24 hours (29). The potent inhibitory effect of VEGF-B on the expression of Bmf indicates that VEGF-B may inhibit Bmf-mediated apoptosis. To test this, we overexpressed the mouse Bmf gene in the rat RGC5 cells and determined the apoptosis rate and the expression of Bmf level in the cells with or without VEGF-B treatment. Bmf overexpression significantly induced cellular apoptosis in the RGC5 cells (Figure 2, H, I, and K; n = 3, P < 0.01). VEGF-B167 treatment (100 ng/ml for 48 hours) inhibited the Bmf-induced apoptosis by about 40% (Figure 2, J and K; n = 3, P < 0.01). Further, VEGF-B treatment inhibited the expression of both the endog-
enous rat and the exogenous mouse Bmf genes (Figure 2L; n = 3, P < 0.01). Thus, VEGF-B inhibited the oxidative stress-, serum deprivation-, and Bmf-induced apoptosis in the RGC5 cells.

VEGF-B inhibits axotomy-induced neuronal apoptosis and apoptotic gene expression in the retina. We next investigated the antiapoptotic effect of VEGF-B in vivo using several different animal models. First, we used the optic nerve crush (ONC) injury mouse model. In this model, it is known that ONC injury results in apoptotic death of the RGCs in the retina after the crush injury. In situ hybridization analysis showed that both VEGF-B and VEGFR-1 are highly expressed in the retina (Figure 3A). A higher level of VEGF-B expression was found in the RGCs and cells in the inner nuclear layer (Figure 3A, left, arrows). VEGFR-1 expression was mainly found in the inner plexiform, part of the inner nuclear layer, and the rod and cone layer (Figure 3A, right). Real-time PCR assay showed that both VEGF-B (Figure 3B) and VEGFR-1 (Figure 3C) were significantly upregulated in the retinas after ONC injury. The upregulation was seen as early as 6 hours after ONC and reached a very high level at 1 week after ONC injury (Figure 3, B and C; n = 4, P < 0.05). To test whether VEGF-B can rescue RGC death, we administered human recombinant VEGF-B167 intravitreally into the mouse eyes after ONC injury and counted viable RGCs after 2 weeks. Treatment with a single dose of VEGF-B167 (500 ng/eye) significantly increased the number of viable RGCs by about 1.7-fold (Figure 3, D–F; n = 8 [vehicle] and n = 12 [VEGF-B167], P < 0.01). On the contrary, intravitreal treatment with VEGF-B neutralizing antibody (32) (5 pmol/eye) decreased the number of the viable RGCs by about 33% (Figure 3F; n = 8, P < 0.05). Since it is known that VEGF-B binds to VEGFR-1 (11), we next tested whether inhibition of VEGF-B by the VEGFR-1 extracellular domain (VEGFR-1 ECD; 5 pmol/eye), acting as a ligand trap, could impair RGC survival. VEGFR-1 ECD treatment decreased the viable RGCs by about 42% (Figure 3F; n = 8, P < 0.05). Thus, VEGF-B is critically required for RGC survival in the injured retina.

To clarify the molecular mechanism underlying the survival effect of VEGF-B in the retina, we investigated the expression of an array of apoptotic/cell death–related genes in the retinae with or without ONC injury and with or without VEGF-B treatment 1 week after the injury. Real-time PCR analysis revealed that VEGF-B treatment (500 ng/eye) inhibited the expression of the BH3-only protein Bmf, Bmf and p53 in both normal and ONC-injured retinae (Figure 3, C–J; n = 4, P < 0.05). It is interesting to note that Noxa expression in the retina was upregulated about 2.7-fold 1 week after the ONC injury,
while no significant difference was observed with the other apoptotic genes (Figure 3, G–J). VEGF-B thus promotes RGC survival, at least partially, by inhibiting the expression of the BH3-only protein and other apoptotic genes in the retina.

**VEGF-B inhibits NMDA-induced neuronal apoptosis and apoptotic/cell death–related gene expression in the retina.** In many neurodegenerative and neurologic disorders, neuronal injury and death are caused by the overstimulation of the excitatory amino acid receptors. We next tested whether VEGF-B could inhibit the excitotoxic-induced apoptosis in the retina using the NMDA injury model. We injected NMDA (20 nmol/eye) with or without VEGF-B 167 (500 ng/eye) intravitreally into mouse eyes. After 24 hours, the retinae were harvested for TUNEL staining and gene expression analysis. VEGF-B 167 treatment significantly reduced the number of apoptotic cells in the RGC, the inner nuclear layer, and the outer nuclear layer (Figure 4A–C; n = 8, P < 0.001 and P < 0.01). Real-time PCR assay revealed that VEGF-B treatment inhibited the expression of a number of the BH3-only protein genes (Bmf, Hrk, Bad, Bid, Bim), as well as other apoptotic/cell death–related genes (TNF-α, Timp3, Timp1, Casp8, Bak, Bax) in the NMDA-injured retina (Figure 4D). VEGF-B thus is a potent inhibitor of excitotoxin-induced apoptosis, at least partially, via suppression of the BH3-only protein and other apoptotic/cell death–related gene expression.

**VEGF-B inhibits ischemia-induced neuronal apoptosis and apoptotic gene expression in the brain.** It has been shown that VEGF-B deficiency led to enlarged brain infarct volume after middle cerebral artery (MCA) occlusion in mouse (33). However, the underlying molecular mechanisms remain unclear. Further, it remains unknown whether VEGF-B protein treatment could rescue ischemia-induced neuronal death in the brain. We next performed the MCA occlusion stroke model using both the VEGF-B–deficient and wild-type mice to address these questions. Immunohistochemical staining showed that both VEGF-B and VEGFR-1 are expressed in the neurons in normal brain (data not shown). VEGF-B expression was highly upregulated in the border zone of the brain after stroke (Figure 5A). VEGF-B deficiency led to about 50% larger brain damage volume as compared with that in the wild-type mice (Table 2, n = 10, P < 0.05). This defect was largely rescued by VEGF-B–deficient and wild-type mice to address these questions. Immunohistochemical staining showed that both VEGF-B and VEGFR-1 are expressed in the neurons in normal brain (data not shown). VEGF-B expression was highly upregulated in the border zone of the brain after stroke (Figure 5A). VEGF-B deficiency led to about 50% larger brain damage volume as compared with that in the wild-type mice (Table 2, n = 10, P < 0.05). This defect was largely rescued by VEGF-B–deficient and wild-type mice to address these questions. Immunohistochemical staining showed that both VEGF-B and VEGFR-1 are expressed in the neurons in normal brain (data not shown). VEGF-B expression was highly upregulated in the border zone of the brain after stroke (Figure 5A). VEGF-B deficiency led to about 50% larger brain damage volume as compared with that in the wild-type mice (Table 2, n = 10, P < 0.05). This defect was largely rescued by VEGF-B–deficient and wild-type mice to address these questions. Immunohistochemical staining showed that both VEGF-B and VEGFR-1 are expressed in the neurons in normal brain (data not shown). VEGF-B expression was highly upregulated in the border zone of the brain after stroke (Figure 5A). VEGF-B deficiency led to about 50% larger brain damage volume as compared with that in the wild-type mice (Table 2, n = 10, P < 0.05). This defect was largely rescued by VEGF-B–deficient and wild-type mice to address these questions. Immunohistochemical staining showed that both VEGF-B and VEGFR-1 are expressed in the neurons in normal brain (data not shown). VEGF-B expression was highly upregulated in the border zone of the brain after stroke (Figure 5A). VEGF-B deficiency led to about 50% larger brain damage volume as compared with that in the wild-type mice (Table 2, n = 10, P < 0.05). This defect was largely rescued by VEGF-B–deficient and wild-type mice to address these questions. Immunohistochemical staining showed that both VEGF-B and VEGFR-1 are expressed in the neurons in normal brain (data not shown). VEGF-B expression was highly upregulated in the border zone of the brain after stroke (Figure 5A).
Figure 5, B and C). TUNEL staining showed that VEGF-B167 treatment significantly decreased the number of the apoptotic cells in the border zone of the stroke, where the surviving neurons were at risk of apoptosis (number of apoptotic cells/field: 31 ± 9 in the control group versus 12 ± 1 in the VEGF-B treatment group, n = 3, P < 0.05; Figure 5, D and E). VEGF-B treatment thus protected neurons from apoptosis in the brain. To understand the underlying molecular mechanisms, we analyzed the expression of the BH3-only protein and other apoptotic genes in the brains with MCA occlusion with or without VEGF-B treatment. VEGF-B treatment markedly downregulated the expression of the BH3-only protein genes Bmf and Hrk and the apoptotic gene Trp53inp1 according to the real-time PCR assay (Figure 5F). Thus, VEGF-B protects the neurons in the brain, at least partially, by inhibiting the expression of the BH3-only protein and other apoptotic/cell death–related genes.

VEGFR-1 mediates the ant apoptotic effect of VEGF-B. It is known that VEGF-B binds to VEGFR-1 (11). To verify whether VEGFR-1 could be activated by VEGF-B in different types of cells. Using Western blot assay, VEGFR-1 was detected in the cortex neurons isolated from neonatal mice, in the bEnd.3 cell line (transformed mouse cerebral cortex–derived endothelial cells), and in the RGC5 cells (Figure 6, A and B). Immunoprecipitation using an anti–VEGFR-1 antibody followed by Western IB assay using an anti-phosphotyrosine antibody showed that VEGF-B167 stimulation (100 ng/ml) resulted in VEGFR-1 activation in the bEnd.3 cells, cortex neurons, and the RGC5 cells (Figure 6, A and B).

**Figure 3** VEGF-B inhibits axotomy-induced apoptosis in the retina. (A) VEGF-B is highly expressed in the retina as shown by the in situ hybridization assay. A high level of VEGF-B expression was found primarily in the RGCs and the inner and outer nuclear layers (INL and ONL, arrows). VEGFR-1 expression was mainly found in the inner plexiform layer, part of the inner nuclear layer, and the inner and outer segment layers (IS, OS). Scale bar: 50 μm. (B and C) Real-time PCR assay showed that VEGF-B and VEGFR-1 expression were upregulated in the retinae after ONC injury. The upregulation was seen as early as 6 hours after ONC and reached a high level after 1 week. (D–F) A single dose of VEGF-B167 intravitreal treatment increased the number of viable RGCs by about 1.7-fold. VEGF-B neutralizing antibody intravitreal treatment decreased the number of viable RGCs by about 33% (F). VEGFR-1 ECD treatment decreased the number of viable RGCs by about 42% (F). Scale bar: 10 μm. (G–J) Real-time PCR analysis revealed that VEGF-B167 treatment inhibited the expression of the BH3-only protein genes Noxa (G) and Bmf (H), as well as Bak (I) and p53 (J) expression in both normal and ONC-injured retinae. *P < 0.05, **P < 0.01.

**Figure 4** VEGF-B inhibits excitotoxin-induced apoptosis in the retina. (A) NMDA intravitreous treatment led to massive apoptosis in the retina as shown by TUNEL staining. (B and C) VEGF-B167 treatment significantly reduced the number of apoptotic cells in all the 3 layers of the retina. Scale bar: 20 μm. (D) Real-time PCR assay revealed that VEGF-B167 treatment inhibited the expression of the BH3-only protein (Bmf, Hrk, Bad, Bid, Bim) and other apoptotic/cell death–related genes in the NMDA-injured retina. **P < 0.01, ***P < 0.001.
The cell lysate was further subjected to Western blot assay using antibodies against phosphorylated and total ERK1/2 or Akt. In both the bEnd.3 cells and the cortex neurons, VEGF-B treatment led to ERK1/2 activation (Figure 6C). In TR-iBRB cells, VEGF-B treatment induced Akt phosphorylation (Figure 6D). However, in the other types of cells tested, VEGF-B treatment did not induce significant Akt phosphorylation (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI33673DS1), suggesting that there could be other intracellular pathways mediating the activity of VEGF-B in these cells. Thus, VEGF-B treatment activated VEGFR-1 followed by the activation of the ERK1/2 or Akt pathways in different types of cells.

We next tested whether VEGFR-1 blockade could abolish the inhibitory effect of VEGF-B on the expression of the apoptotic/cell death–related genes in the RGCs cells. VEGFR-1 neutralizing antibody treatment completely abolished the inhibitory effect of VEGF-B on the expression of Bmf, Bax, and Bik, indicating that the effect of VEGF-B indeed was mediated by VEGFR-1 (Figure 6, E–H; \( n=3, P<0.001 \) and \( P<0.05 \) respectively). Moreover, VEGFR-1 neutralizing antibody treatment upregulated the expression of Bmf, Bax, and Bik (Figure 6, F–G), indicating that VEGFR-1 ligands other than VEGF-B may also contribute to the inhibition of their expression via VEGFR-1. In addition, VEGFR-1 neutralizing antibody treatment abolished the inhibitory effect of VEGF-B on the expression of Bax1 to a lesser extent (Figure 6H; \( n=3, P<0.05 \)). Thus, the inhibitory effect of VEGF-B on the expression of the BH3-only protein and other apoptotic/cell death–related genes is largely mediated by VEGFR-1. Indeed, this observation was confirmed in vivo using the ONC retinal injury model. When the VEGF-B protein was administered together with the VEGFR-1 neutralizing antibody into the mouse vitreous with ONC injury, VEGF-B–induced RGC survival was largely abolished (Figure 6, I–L; \( n=8, P<0.05 \)). Thus, in vivo data confirmed that the anti-apoptotic effect of VEGF-B is mediated by VEGFR-1.

VEGF-B at a dose effective for neuron survival did not cause undesired angiogenesis in the eye. Overgrowth of blood vessels in the eye may lead to severe vision damage or loss. We therefore investigated whether intravitreal administration of VEGF-B treated could affect normal retinal vasculature and pathological angiogenesis. We sectioned the retinae with or without VEGF-B–treated treatment and stained them with isoclectin B4 (IB4), which labels the vascular endothelial cells, and blood vessel density was determined in different locations of the retina (central, middle, and peripheral). No difference was observed in terms of blood vessel density and morphology 14 days after VEGF-B treatment (Figure 7, A–C; \( n=6, P<0.05 \)). Thus, VEGF-B protein treatment at a dose effective for neuron survival did not affect normal blood vessels in the retina. To investigate whether VEGF-B affects pathological angiogenesis, we used the laser-induced choroidal neovascularization (CNV) model. We administered VEGF-B intravitreally immediately after laser treatment. One week after VEGF-B treatment, when CNV reaches the peak level, the eyes were harvested and the CNV area measured after IB4 staining. No difference was observed between the VEGF-B–treated and the control groups (Figure 7, D–F; \( n=8, P<0.05 \)). Thus, VEGF-B protein treatment at a dose effective for neuron survival did not affect pathological CNV. To test whether VEGF-B is involved in blood vessel permeability, we performed the modified Miles assay using the VEGF-B–deficient and wild-type mice. No difference was found between the 2 groups (OD\(_{620} =0.17 \pm 0.01\) in VEGF-B–deficient and \(0.19 \pm 0.02\) in wild-type mice, \( n=10, 12, P>0.05 \)). Thus, at a dose effective for neuron survival, VEGF-B treatment did not seem to affect normal or pathological angiogenesis, and VEGF-B deficiency did not affect blood vessel permeability according to the modified Miles assay. However, our data do not exclude the possibility that VEGF-B in other conditions may affect the blood vessel system. Further studies are needed to look into this.

**Discussion**

VEGF-B was discovered many years ago and has a high sequence homology to the other VEGF family members. However, the function of VEGF-B is still a debatable issue. We reveal here for the first time to our knowledge a novel function of VEGF-B as a potent apoptosis inhibitor. VEGF-B treatment rescues neurons...
family that has a potent antiapoptotic effect while lacking a genetic survival. VEGF-B thus appears to be the first member of the VEGF family members, VEGF-B does not have a general effect on angiogenesis and blood vessel permeability. This nature of VEGF-B is of particular importance for the potential clinical usage of VEGF-B as a neuroprotective factor. VEGF-B thus has an unusual safety profile, with minimal side effects, as a survival molecule. It may therefore be used to treat a broad array of neurodegenerative diseases involving neuronal death, such as ocular neurodegenerative diseases, stroke, autism, multiple sclerosis, Alzheimer disease, amyotrophic lateral sclerosis, fibromyalgia, tinnitus, Parkinson disease, Huntington disease, etc. VEGF-B therefore could potentially offer a new and better therapeutic option for the treatment of neurodegenerative diseases.

Most of the VEGF family members have been shown to play critical roles in development or physiological/pathological angiogenesis (4, 15, 35, 36). It is therefore surprising that VEGF-B did not seem to have a major impact during development, physiological or pathological angiogenesis (8, 9), even though it has a high sequence similarity to the other VEGF family members and uses the same receptors. Indeed, in contrast to VEGF- and VEGF-C-deficient mice (15, 35, 36), which are embryonic lethal, VEGF-B-deficient mice are largely normal in terms of lifespan, fertility, angiogenesis, etc. (16, 17). In contrast to PlGF, which is critically required for pathological angiogenesis (4), VEGF-B is not required for neovessel formation in different pathological conditions (16, 18–21, 27). In contrast to the other VEGF family members, which all stimulate blood vessel permeability (37–39), VEGF-B has no effect on blood vessel permeability (19, 22–24). In contrast to VEGF, which is a potent stimulator for vascular endothelial cell proliferation and migration, VEGF-B has little effect on endothelial cell proliferation and migration (data not shown) (9). Several studies have reported an angiogenic activity of VEGF-B (24–26). However, this has not been observed by other investigators (16, 18, 20, 21, 27). Thus, studies on VEGF-B have led to debatable and inconsistent results, and the function of VEGF-B remains elusive.

In this study, we demonstrated that VEGF-B signaling is critical for cell survival. Using a genome-wide gene profiling approach, we revealed that the genes most significantly downregulated by VEGF-B are the genes that are critically involved in the apoptosis/cell death–related pathways. We validated our results both in vitro and in vivo using multiple cell lines and animal models. Among the genes downregulated by VEGF-B, the BH3-only proteins, which are critical regulators in initiating apoptosis, were consistently inhibited by VEGF-B in different experimental settings. The BH3-only proteins are therefore the main targets suppressed by VEGF-B, suggesting a major role of VEGF-B in inhibiting apoptosis. Indeed, from apoptosis in both the retina and brain. Mechanistically, we demonstrate that VEGF-B inhibits the expression of the BH3-only protein and other apoptotic/cell death–related genes via VEGFR-1. It is worth noting that the survival effect of VEGF-B is not associated with undesired angiogenesis at a dose effective for neuron survival. VEGF-B thus appears to be the first member of the VEGF family that has a potent antiapoptotic effect while lacking a general angiogenic activity.

Even though VEGF has also been shown to be a potent neuroprotective factor (34), the therapeutic potential of its neuroprotective effect is limited because of its potent and general angiogenic and permeability-promoting effect. In contrast to the other VEGF family members, VEGF-B does not have a general effect on angiogenesis and blood vessel permeability. This nature of VEGF-B is of particular importance for the potential clinical usage of VEGF-B as a neuroprotective factor. VEGF-B thus has an unusual safety profile, with minimal side effects, as a survival molecule. It may therefore be used to treat a broad array of neurodegenerative diseases.

**Figure 6**
VEGFR-1 mediates the effect of VEGF-B. (A) VEGF-B treatment induced VEGFR-1 activation in the bEnd.3 cells and the cortex neurons using the immunoprecipitation assay and anti–VEGFR-1 antibody, followed by the Western blot assay using anti-phosphotyrosine antibody (anti-pTyr). VEGF-B was detected in bEnd.3 cells and cortex neurons using Western blot assay. (B) VEGF-B treatment induced VEGFR-1 activation in RGC5 cells using Western blot assay and antibodies against phosphotyrosine and VEGFR-1. (C) VEGF-B treatment led to ERK1/2 activation in both bEnd.3 cells and cortex neurons using Western blot assay and antibodies against phosphorylated and total ERK1/2. (D) VEGF-B treatment induced Akt phosphorylation in TR-iBRB cells using Western blot assay and antibodies against phosphotyrosine and VEGFR-1. (E) VEGFR-1 neutralizing antibody treatment abolished to various degrees the inhibitory effect of VEGF-B on the expression of Bmf (E), Bax (F), Bik (G), and Bak1 (H) in RGC5 cells. (I, J, and K) VEGF-B protein treatment increased RGC survival in the ONC-injured retina. Scale bar: 20 μm. (I, K, and L) VEGF-B neutralizing antibody treatment largely abolished the VEGF-B–induced RGC survival in the ONC-injured retina. *P < 0.05, **P < 0.01, ***P < 0.001.
Viable RGCs by about 42% in the ONC injury model, suggesting that systemic anti-VEGF treatment may lead to thromboembolic events, such as stroke, and increase the risk of neurodegeneration is ischemia and lead subsequently to neuronal apoptosis (41). Due to its potent antiapoptotic nature, VEGF-B may potentially be used clinically as an adjunct to the current anti-VEGF therapies to provide a balance between preventing neuronal apoptosis and inhibiting angiogenesis.

VEGF-B binds to VEGFR-1 and NP-1 (10, 11). VEGF-B is highly expressed in the retina and is critically required for the maintenance and survival of the retinal vasculature (42). VEGF-1 is expressed in the brain and upregulated after brain ischemia (43), suggesting a role of VEGF-1 in neuronal survival. Indeed, the survival effect of VEGF-1 has been shown in numerous cell types (44–46). In this study, VEGF-1 ECD treatment decreased the viable RGCs by about 42% in the ONC injury model, suggesting the critical role of the VEGFR-1 ligands in RGC survival. Indeed, VEGF-B stimulation led to VEGFR-1 and ERK MAPK activation in RGC5, bEnd.3, and cortex neuronal cells. Furthermore, VEGF-1 neutralizing antibody largely abolished the inhibitory effect of VEGF-B on the expression of the apoptotic/cell death–related genes in the RGC5 cells in vitro and decreased VEGF-B–dependent RGC survival in the retina in vivo. Thus, the antiapoptotic/survival effect of VEGF-B is mainly mediated by VEGFR-1. The other receptor used by VEGF-B, NP-1, is also expressed by neurons in the retina and brain (47, 48) and is upregulated after focal cerebral ischemia (48). NP-1 has been shown to promote the survival of various types of cells (49). NP-1 could therefore also be involved in mediating the survival effect of VEGF-B. Further studies are needed to verify this possibility.

In summary, using multiple approaches involving the genome-wide gene profiling assay, cultured cells, and animal models, we show for the first time to our knowledge that VEGF-B is a potent apoptosis inhibitor. VEGF-B rescues neurons from apoptosis in both the retina and brain without causing undesired angiogenesis. Moreover, the survival effect of VEGF-B is achieved by inhibiting the expression of the BH3-only protein and other apoptotic/cell death–related genes via VEGFR-1. VEGF-B appears to be the first member of the VEGF family that has a potent antiapoptotic effect but lacks general angiogenic activity. VEGF-B thus could potentially provide a new therapeutic option for treating different types of neural degenerative diseases.

Methods

Microarray analyses. Mouse aortic artery SMCs were isolated and cultured as described previously (50). SMCs were grown to 80% confluence and synchronized by overnight serum starvation followed by stimulation with human VEGF-B167 (100 ng/ml) for 6 hours in a hypoxic (1% oxygen) condition. Total RNA was isolated using the TRIzol reagent (Invitrogen) followed by RNEasy Mini Kit (QiAGEN) purification according to the manufacturer’s instructions. Total RNA (10 μg) was subjected to cDNA preparations using Cy3-dUTP or Cy5-dUTP (Amersham Biosciences) and an oligo-dT primer. Microarray assay was performed using the Whole Mouse Genome Oligo Microarray (Agilent Technologies). This array is composed of 41,534 (60-mer) oligonucleotide probes representing more than 41,000 mouse genes and transcripts compiled from a broad sequence collection. For the first slide, the VEGF-B167–treated cDNA was labeled with the Cy3 fluorescent dye, and the control cDNA was labeled with the Cy5 fluorescent dye. For the second slide, the VEGF-B167–treated cDNA was labeled with the Cy5 fluorescent dye, and the control cDNA was labeled with the Cy3 fluorescent dye. The whole experiment, including the treatment of the SMCs with VEGF-B167, was repeated independently twice. Thus, 4 Agilent slides were used, and 4 sets of original signals were obtained with each RNA sample. Two-tailed Student’s t test was used for the statistical analysis of the expression data. The threshold of differential gene expression was set at greater than 2-fold upregulation or 50% downregulation of the genes. Functional grouping of the differentially expressed genes was performed using several different tools, including WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt) and Ingenuity Pathways Analysis (https://analysis.ingenuity.com/pa/login/login.jsp).

Cell culture, cell viability, Bnf overexpression, and real-time PCR assay. Cells of the rat RGC-derived cell line RGC5, the immortalized rat retinal pericyte cell line (TR-rPCT), and the immortalized rat retinal Müller cell line (TR-MUL) were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 33°C with 5% CO2. The immortalized rat retinal capillary endothelial cell line (TR-IBRB) and the bEnd.3 cell line derived from the transformed...
mouse cerebral cortex endothelial cells were cultured in DMEM supplemented with endothelial cell growth factors (1-033484; Roche Diagnostics). For the viability assay, RGC5 cells were starved in serum-free medium for 3 days. The cells were then seeded in 96-well tissue culture plate (BD Biosciences) and allowed to attach for 24 hours in serum-free medium. The medium was then replaced with 100 µl of serum-free medium supplemented with recombinant human VEGF-B (100 ng/ml). Serum-free medium with 100 ng/ml BSA was used as a negative control. Cell culture medium with 10% FBS was used as a positive control. All the experiments were performed in triplicate. Cell viability was evaluated using the MTT method according to the manufacturer’s protocol (MTT Cell Viability Assay Kit; Invitrogen) at different time points. After lysis of the cells, the formazan dye was quantified by measuring the absorbance at 540 nm using a photometer (Multiscan EX Microplate Photometer; Thermo Electron Corp.).

The mouse Bmf expression construct was a kind gift from Andreas Strauss at The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia. The DNA plasmid was transfected into the RGC5 cells using the FuGENE Transfection Reagent (Roche Diagnostics) according to the manufacturer’s instructions. Apoptosis of the transfected cells was analyzed 2 days after transfection. For VEGFR-1 neutralizing antibody (R&D Systems) treatment, the RGC5 cells were treated with the antibody at 500 ng/ml overnight, followed by VEGF-B recombinant protein treatment at 100 ng/ml for 6 hours. For the real-time PCR assay, total RNA was isolated using the RNeasy Mini-kit (Qiagen) according to the manufacturer’s instructions. Three micrograms of total RNA was used for cDNA synthesis using the SuperMix Kit (Invitrogen) and used for the real-time PCR reaction.

Cerebral ischemic stroke model. Focal cerebral ischemia was produced by permanent occlusion of the MCA as described previously (51). VEGF-B-deficient mice were described previously (16). Littermates from the mice that were backcrossed onto the C57BL/6 background for more than 6 generations were used for the experiments. Briefly, 8-week-old male mice were anesthetized, and a U-shape incision was made between the left ear and the left eye. A small opening (1–2 mm in diameter) was made in the region over the MCA with a handheld drill. The MCA was ligated and transected distally to the ligation point. After 24 hours, the mice were killed with an overdose of Nembutal (500 mg/kg; Abbott Laboratories) and perfused with 1% PFA. The brains were removed, postfixed in 1% PFA overnight, and subjected to MAP-2 immunohistological staining. The infarct volume was defined as the sum of the MAP-2 negative areas of the sections multiplied by their thickness using Axiosvision software (Carl Zeiss Inc.). For VEGF-B protein treatment, a skin incision was made at the top of the head of the anesthetized mouse. Four small openings (1 mm in diameter) were made in the skull at −1, 0, 1, 2 mm from the bregma to the lambda and 1 mm left from the bregma. A fine needle (33 gauge) was inserted to the depth of 1.2 mm from the skull surface, and 1 µl of 0.55 µg/µl recombinant human VEGF-B (75) was injected over 5 minutes into each opening and the skin sutured. MCA ligation was then performed as described above.

Laser-induced CNV model and Angiography. Ten-week-old female mice were anesthetized and pupils dilated. Mice were positioned on a rack connected to a slit lamp delivery system. Four photocoagulation spots were made (75-µm spot size, 75 ms, 90 mW power; Oculight Infrared Laser System 810 nm; IRIDEX Corp.) in the area surrounding the optic nerve in each eye. The sites were visualized through a handheld contact lens and a viscous surface lubricant. Only laser-induced burns with a bubble formation were included in the study. The mice were given lubricant ophthalmic ointment after laser treatment. Seven days after laser treatment, the eyes were removed and fixed, the retina dissected, and choroids isolated and stained with IB4 conjugated with Alexa Fluor 568 (Invitrogen). The eyecups were flat-mounted in Aquamount with the sclera facing down and the total neovascular area measured using Axiosvision software (Carl Zeiss Inc.) and the mean value per burn presented for each eye. Miles assay was performed as described previously (24).


34. Nishijima, K., et al. 2007. Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the...