The Role of VEGF and VEGFR2/Flk1 in Proliferation of Retinal Progenitor Cells in Murine Retinal Degeneration

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PURPOSE. To analyze the role of VEGF and its receptors, VEGFR2/Flk1 and VEGFR1/Flk1, on retinal progenitor cells (RPCs) in a murine model of inherited retinal degeneration (rd1 mice).

METHODS. After proliferating RPCs in the retina of rd1 mice were labeled with bromodeoxyuridine (BrdU), expressions of VEGFR2/Flk1 and VEGFR1/Flt1 were immunohistochemically analyzed. To examine its effect on the proliferation of BrdU-positive RPCs in rd1 mice, VEGF was administered into retinal culture medium with or without blocking agents against VEGFR2/Flk1 or VEGFR1/Flt1 in vitro or injected into vitreous cavity in vivo.

RESULTS. BrdU-labeled RPCs in rd1 mice expressed VEGF/Flk1 but not VEGFR1/Flt1. These cells later expressed retinal neuronal markers such as Pax6 and rhodopsin. Exposure of the retinas from postnatal day (P) 9 rd1 mice to VEGF increased the number of proliferating RPCs by 61% in vitro. This effect was blocked by concomitant administration of VEGFR2/Flk1 kinase inhibitor. In vivo, a single intravitreal injection of VEGF in rd1 mice at P9 increased by 158% the number of RPCs and cells that developed from RPCs in the peripheral retina at P18.

CONCLUSIONS. VEGF stimulates the proliferation of RPCs through VEGFR2/Flk1 in rd1 mice. The observed proliferation of RPCs that have the potential to differentiate into retinal neurons may enhance the regeneration of the degenerating retina. (Invest Ophthalmol Vis Sci. 2007;48:4315–4320) DOI: 10.1167/iovs.07-0354

Vascular endothelial growth factor (VEGF) is a potent growth factor known to play a major role in the formation and maintenance of vascular structures.1,2 These functions are mediated through two of its tyrosine kinase receptors, VEGFR1/Flt1 and VEGFR2/Flk1, both expressed on vascular endothelial cells.3 However, VEGF has recently been reported to be an important signaling molecule for neuroprotection and neurogenesis.4-10 For example, reduced expression of VEGF in a slower loss of cone photoreceptors.29 Here we show that VEGF expression increases the risk for ALS.7,8 On the other hand, VEGF injected into the central nervous system through the ventricles in rodent models of ALS proved one of the most effective treatments for this disease.9,10 In addition, in vitro and in vivo analyses have suggested that VEGF can stimulate the proliferation of neural stem/progenitor cells in the brain, including those from the cerebral cortex and the hippocampus. This proliferation was mediated through VEGFR2/Flk1 signaling.11-14

In the eye, VEGF plays a significant role in the normal development of the retinal and choroidal vascular systems and in pathologic changes such as diabetic retinopathy.15,16 Choroidal revascularization in age-related macular degeneration,19 and retinopathy of prematurity.5,20,21 Treatments aimed at blocking VEGF signaling to overcome these diseases are under evaluation in humans and have already led to promising results in several forms of retinal and choroidal vascular diseases.22-26

Although the importance of VEGF in neurogenesis of developing retinas in wild-type rats27 and chickens28 has been reported, the role of VEGF in eyes with retinal diseases other than the vasculopathy remains unclear. In particular, it is unclear whether VEGF has the potential to prevent retinal degeneration by protecting retinal neurons against apoptosis or by stimulating the proliferation of retinal progenitor cells (RPCs). To analyze the role of RPCs and the effect of VEGF on RPCs, we studied the rd1 mouse, which has an inherited retinal degeneration, as a model of human hereditary retinal degeneration. During the early period after birth, the normal mouse retina is morphologically immature, and RPCs form a neuroblast layer. By postnatal day (P) 9, the retina differentiates to form a highly complex structure with three distinct neural layers. In rd1 mice, however, rod photoreceptors degenerate rapidly between P9 and P21; this degeneration is followed by a slower loss of cone photoreceptors.29 Here we show that proliferating RPCs are capable of differentiating into cells that express retinal neural markers in postnatal rd1 mouse. VEGF stimulates the proliferation of these RPCs through VEGFR2/Flk1 in vitro. Moreover, a single intravitreal injection of recombinant VEGF in young rd1 mice promotes the proliferation of RPCs and results in an increased number of these cells or cells that developed from RPCs in the peripheral retina. These suggest that VEGF may have the potential to stimulate regeneration of the retinal neurons in the degenerating retina.

METHODS

Animals

We used the C3H/HeJ strain of mice, homozygous for the rd1 mutation, as a murine model of an inherited retinal degeneration.50-51 The C57BL/6j strain of mice was used as the wild-type control. Mice were kept on a 12-hour light/12-hour dark cycle. All experimental procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines for the use of animals at Nagoya University School of Medicine.
Immunohistochemical Analyses

To label cells that are in the S-phase of the cell cycle, mice were injected once intraperitoneally with BrdU (150 mg/kg) and were killed after periods ranging from 2 hours to 21 days. Corneas were removed from the enucleated eyes, and the eyecups were fixed in 4% paraformaldehyde (PFA) for 2 hours at room temperature followed by cryoprotection in 30% sucrose overnight at 4°C. We embedded the eyecups in OCT compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan) and cut 12-μm frozen sections through the dorsal to ventral meridian.

Sections were stained with primary antibodies for rhodopsin (1: 1500; Chemicon, Temecula, CA), VEGFR1/Fit1 (1:30; R&D Systems, Minneapolis, MN), VEGFR2/Flik1 (1:30; R&D Systems), Pax 6 (1:1000; Developmental Studies Hybridoma Bank, Iowa City, IA), or BrdU (1: 1000 [Developmental Studies Hybridoma Bank] and 1:1000 [Oxford Biotechnology, Raleigh, NC]), followed by Alexa 488-, 568-, or 647-conjugated secondary antibodies (all at 1:1500; Molecular Probes, Eugene, OR). A TUNEL assay kit (Roche, Basel, Switzerland) was used, in accordance with the manufacturer’s instructions, to detect apoptotic cells.

Next the flat mount specimens were subjected to BrdU-staining, as follows: after the PFA-fixed eyecups were flattened with radial incision, they were permeabilized in 0.5% phosphate-buffered saline Triton X-100 (PBST) for 2 hours, incubated in 2 N HCl for 45 minutes, and neutralized with 0.1 M Na₂B₄O₇ for 15 minutes. After blocking with 5% goat serum in PBS for 1 hour, the eyecups were incubated with anti-BrdU antibodies for 12 hours and with secondary antibody for 9 hours.

Retinal Explant Culture

Retinal explants were cultured as described in detail with slight modifications. Briefly, the retina was peeled from the RPE of P9 eyes under an ophthalmic surgical microscope. Four radial incisions were made to flatten the retina, and the retina was placed on a chamber filter (Millicell; Millipore, Bedford, MA) with the photoreceptor side up. The chamber was placed in a culture plate with 1 mL culture medium that contained 50% minimal essential medium with HEPES (Gibco, Grand Island, NY), 25% Hanks balanced salt solution (Gibco), 25% heat-inactivated horse serum (Gibco), antibiotics mixture (25 U/mL penicillin, 25 μg/mL streptomycin, 62.5 ng/mL amphotericin B; Gibco), and 200 μM L-glutamine (Sigma). This medium was supplemented with 20 to 200 ng/mL VEGF (PeproTech, Rocky Hill, NJ), 0 to 100 ng/mL anti-VEGFR1/Flit1-neutralizing antibody (R&D Systems), or 0 to 100 nmol/mL SU1498, a potent inhibitor of VEGFR2/Flik1 kinase (Calbiochem, La Jolla, CA). Cultures were maintained at 34°C in 5% CO₂. The medium was changed 24 and 72 hours after the culture was begun, and BrdU (50 μg/mL) was added at 96 hours. Culturing of the retinal explant was stopped at 120 hours, and the retinas were fixed, cryoprotected, frozen, and sectioned as described. We analyzed five retinas from five animals for the different combinations of VEGF and SU1498 protected, frozen, and sectioned as described. We analyzed five retinas from each age group were studied. Student’s paired t-test was used for statistical analysis. All experiments were conducted in duplicate.

RESULTS

RPCs Differentiate into Cells Committed to Rod Photoreceptor Lineages in rd1 Mice

In wild-type rodents, RPCs are capable of differentiating into rhodopsin-positive cells, presumably destined to become rod photoreceptors. To determine whether postnatal RPCs in mice that experience retinal degeneration are similarly capable of differentiating into a photoreceptor lineage, we analyzed the fate of the cells labeled with BrdU at P6 by histologic evaluation at P18.

At P6, the retina is still developing, and BrdU-positive cells that are in the S-phase of the cell cycle form a layer of neuroblasts at the peripheral retina. Later at P18, when the retina is degenerating, the cells that incorporated BrdU also express Pax6 (a marker for multipotent RPCs) or rhodopsin (a marker for rod photoreceptors; Fig. 1). Even if these dividing cells were labeled with BrdU at P12, when the neuroblast layer disappeared and the retina developed into three neuronal layers, a small number of BrdU-positive cells still expressed these markers at P18 in the peripheral retina (data not shown). We failed to find BrdU-positive cells that developed into cells of cone photoreceptor lineage in the retina. However, rare BrdU-positive cells that expressed a marker for a subset of cone photoreceptors, M-cone opsin, were identified in the adjacent ciliary body (data not shown). These findings suggest that BrdU-positive cells in rd1 mouse retina are RPCs capable of differentiating into the rod photoreceptor lineage.

RPCs Express VEGFR2/Flik1

In the mammalian brain, VEGF participates in neurogenesis by stimulating the proliferation of neural stem/progenitor cells through VEGFR2/Flik1. An earlier study on dissociated retinal cells from wild-type mice retinas showed that VEGFR2/Flik1 is expressed on BrdU-positive RPCs. To determine whether this was also true of RPCs in rd1 mice, we examined the in situ expression of two ligands of VEGF, VEGFR1/Fit1 and VEGFR2/Flik1, in P6 rd1 mice. RPCs were labeled with BrdU 2 hours before the eyes were removed. As expected, BrdU-positive RPCs showed staining for VEGFR2/Flik1. BrdU incorporation and VEGFR2/Flik1 expression were more pronounced in RPCs at the peripheral retina (Fig. 2); remaining cells in the neuroblast layer expressed these markers to a lesser degree. We also identified cells that were positive for BrdU but negative for VEGFR2/Flik1 or vice versa. On the other hand, VEGFR1/Fit1 showed a staining pattern not related to the

Intravitreal Injection and Counting of BrdU-Positive Cells

In P9 rd1 mice, 50 ng human recombinant VEGF (PeproTech) in 0.5 μL PBS was injected into the right eye, and 0.5 μL PBS was injected into the left. Injections were performed with a 33-gauge needle through the corneal limbus into the vitreous cavity under an ophthalmic surgical microscope. At P12, the mice were injected intraperitoneally with BrdU (150 mg/kg). At P30, the mice were killed immediately after fundus examination under a microscope to exclude the eyes with retinal detachment, cataract, or opaque vitreous. After the eyes were histologically processed into cilioretinal flat mounts, the specimens were stained for BrdU as described. Digital images of the dorsal and ventral cilioretinal margins containing the peripheral retina and the entire ciliary body were taken from each flat mount specimen. Numbers of BrdU-positive cells were determined by averaging the counts from dorsal and ventral images, obtained with the operator masked to the previous treatment. Student’s paired t-test was used for statistical analysis. All experiments were conducted in duplicate.
BrdU-positive cells; the rare BrdU-positive cells with VEGFR1/Flt1 expression were vascular endothelial cells (Fig. 2). These BrdU-positive cells were negative for TUNEL staining (data not shown), indicating that BrdU incorporation does not represent DNA synthesis associated with apoptosis.37

**VEGFR2/Flk1 Mediates Retinal Stem Cell Proliferation In Vitro**

To determine whether the level of VEGF expression in rd1 mouse retinas has any impact on retinal neurogenesis (proliferation of RPCs) or on neuroprotection (decrease in photoreceptors apoptosis), we analyzed the effect of different concentrations of VEGF in the culture media on BrdU incorporation and apoptosis of the photoreceptors in retinal explants.

The number of BrdU-positive cells in the ONL increased with the addition of VEGF for 6 days, and we observed a maximal increase of these cells by 61.2% at a concentration of 10 ng/mL (P < 0.05; Fig. 3A). On the other hand, the number of TUNEL-positive cells in the ONL was not altered by the VEGF (Fig. 3A). This experiment was repeated once, with similar results (data not shown). These findings indicated that VEGF can stimulate neurogenesis in vitro, but it did not protect neurons from apoptosis under our experimental conditions.

We then studied the role of the two VEGF receptors using this in vitro retinal explant by adding different concentrations of SU1498, an agent that blocks the kinase activity of VEGFR2/Flk1, or by adding anti–VEGFR1/Flt1-neutralizing antibodies to the culture medium containing 10 ng/mL VEGF. The number of BrdU-positive RPCs in the ONL was decreased with exposure to SU1498 at a concentration of 10 to 20 nmol/mL (P < 0.01; Fig. 3B), whereas exposure to anti–VEGFR1-neutralizing antibodies under similar experimental conditions did not alter...
VEGF Expression Is Reduced in Developing rd1 Mouse Retina

Reduced expression of VEGF is associated with neurodegeneration in the brains and spinal cords of mice and humans.7,8 To determine whether the level of VEGF is reduced in the retina of rd1 mouse, we measured its expression in mice between P6 and P18. From P6 to P9, when the morphology of the retina was still normal, the VEGF level expressed in the retina of wild-type controls. However, at P12, after the rapid photoreceptor degeneration had begun, the VEGF expression level was 47.2% lower (P < 0.001) in rd1 retinas than in wild-type retinas and remained lower thereafter (Fig. 4).

VEGF Stimulates Proliferation of RPCs In Vivo

Because VEGF has the potential to stimulate the proliferation of RPCs in vitro and its expression was reduced in the rd1 mouse, we sought to determine whether increasing the VEGF level in the retina by an intravitreal injection of VEGF in rd1 mice would also increase the number of RPCs, as in retinal cultures, or would protect retinal neurons from degenerating, similar to its observed effect on the neurodegenerative disease in the brain in vivo. A single 50-ng intravitreal injection of VEGF in 0.5 μL PBS (right eye) or 0.5 μL PBS (left eye) was given at P9. Proliferating RPCs were then labeled with BrdU at P12, after the initiation of photoreceptor degeneration. The eyes from these mice were later collected at P30 and studied histologically. Only minor portions of the eyes were included in the study, especially those treated with VEGF, because of the high rate of the induced retinal detachments.

Because few BrdU-positive proliferating RPCs were found exclusively at the peripheral retina in the P12 histologic sections, which continued to decline in number (Fig. 5), we analyzed flat mount specimens and focused on the RPCs at the peripheral retina. As expected, the P12-labeled BrdU-positive cells identified at P30, most likely either RPCs or cells that developed from RPCs, were almost exclusively found in the peripheral retina. The number of BrdU-positive cells in the peripheral retina was significantly increased by 138% in the VEGF-treated eyes compared with the contralateral eyes injected with PBS (P = 3.9 × 10⁻⁵; Fig. 6). We also observed a significantly increased number of BrdU-positive cells in the adjacent ciliary body of eyes injected with VEGF (data not shown).

However, at P6 or P9, intravitreal injection of VEGF into rd1 mice did not show any evidence of neuroprotection of the degenerating retina. In other words, no significant difference...
preparations that VEGFR2/Flk1 is expressed on RPCs in rd1 mouse retinas. This expression was most pronounced in the peripheral retina, where RPCs persist exclusively after retinal development.

The ability of VEGF to stimulate the proliferation of RPCs was confirmed in the in vitro analyses using retinal explants from rd1 mice. The number of BrdU-positive RPCs increased with the addition of VEGF to the culture medium. We observed a maximal increase of 61.2% in the number of RPCs after the addition of 10 ng/mL VEGF. A further increase in the VEGF concentration did not increase the number of RPCs. These results are in close agreement with the results of an in vitro study that assessed the potential of VEGF to stimulate the proliferation of neural stem/progenitor cells from the brain.11

In addition, the in vitro analyses indicated that VEGF acts through VEGFR2/Flk1 expressed by RPCs to stimulate neurogenesis. This observation is in agreement with results from neural stem/progenitor cells in the brain.11 However, we were unable to detect any reduction in the rate of photoreceptor apoptosis by adding VEGF to the culture medium. Therefore, it appears that VEGF does not protect retinal neurons from apoptosis, at least with the in vitro culture and in vivo intravitreal injection conditions and the rd1 mice used in the present study.

In the murine brain and spinal cord, reduced expression of VEGF is associated with ALS-like neurodegeneration.7,8 To determine whether VEGF is also reduced in the retina of rd1 mice, we measured its level of expression between P6 and P18. From P6 to P9, when the rd1 retina is morphologically intact, the level of VEGF expression in the retinas from rd1 mice was comparable to that of wild-type controls. However, at P12, when the photoreceptors are rapidly degenerating,29 VEGF expression was significantly lower in the rd1 retinas and remained reduced thereafter. A simple explanation for this observation is that the reduction in VEGF expression resulted from the decreased number of VEGF-expressing photoreceptors. However, given that VEGF is reported to be mainly expressed in the ganglion cell and inner nuclear layers in the retina,39 the decrease in cell number caused by photoreceptor degeneration may be insufficient to explain the observed degree of reduction in VEGF expression. In addition, because a significant reduction in the expression of VEGF, a potent angiogenic factor, in the retina is likely to result in an antiangiogenic state, our results are also in agreement with the previous report that in retinal degeneration, vessels respond poorly to angiogenic stimulation.40

The importance of VEGF as a potential target for the treatment of neurodegeneration was recently highlighted by the observation that an intraventricular injection of VEGF in a rodent model of neurodegenerative disease was one of the most effective treatments.9,10 These reports, together with the results of our in vitro analyses, the coincidence of the reduced VEGF expression and the progression of retinal degeneration, prompted us to administer VEGF as a potential treatment for retinal degeneration. Our results indicated that intravitreal administration of VEGF had no detectable therapeutic effect on the progression of photoreceptor degeneration. However, we found that a single intravitreal injection of VEGF in P9 rd1 mice resulted in an increased number of RPCs or cells that developed from RPCs at P18, further validating that VEGF can stimulate the proliferation of RPCs and may have the potential to enhance retinal regeneration. However, the effect of VEGF was modest considering the limited number of RPCs observed exclusively in the peripheral retina and the larger degree of photoreceptors lost throughout the retina. Nonetheless, these results provide us with a rationale to continue our study of VEGF and to investigate the mechanism for neural regeneration as a treatment of retinal degeneration.

**DISCUSSION**

In mammals, previous studies have shown the significance of VEGF to stimulate the proliferation of neural stem/progenitor cells in the brain. Our results show that VEGF stimulates the proliferation of RPCs through VEGFR2/Flk1 in vitro and further provide in vivo evidence that VEGF promotes the proliferation of RPCs in rd1 mice.

To determine the role of RPCs in rd1 mice, we studied the expression of several neural markers on BrdU-positive cells to track their fate after differentiation. In particular, few RPCs labeled with BrdU at P6 developed into cells that expressed rhodopsin, a marker specific for rod photoreceptors, at P18. However, given that the rd1 mice carry genetic defects in rod photoreceptor-specific Pdeo6b,30,31 the RPC-derived rhodopsin-positive cells may not ultimately contribute to functional vision. Nonetheless, loss of rod photoreceptors induces secondary loss of cone photoreceptors in this mouse model29 and in many forms of hereditary retinal degeneration in humans.38 Therefore, an effort to promote the regeneration of the rod photoreceptors from these RPCs as a possible treatment of these diseases is reasonable. Earlier studies have shown that VEGFR2/Flk1 is expressed on BrdU-positive RPCs of dissociated retinal cells from wild-type mouse retinas and that VEGF binds to these cells.36 In this study, we confirmed in situ
Based on the results of our experiments, we believe there is only a minute concern on the use of anti-VEGF therapy in adult patients, in whom presumably few, if any, retinal stem cells exist at the peripheral retina. However, caution must be exercised regarding its application in children, especially before their retinas are fully developed, because VEGF appears to regulate retinal neurogenesis in various ways.27,28

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