Vascular Endothelial Growth Factor Enhances Axonal Outgrowth in Organotypic Spinal Cord Slices via Vascular Endothelial Growth Factor Receptor 1 and 2

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Enhancing adult nerve regeneration is a potential therapeutic strategy for treating spinal cord injury. Vascular endothelial growth factor (VEGF) is a major contributor to angiogenesis, which can reduce the spinal cord injury by inhibiting the inflammation and improve recovery after spinal cord injury. We have previously demonstrated that exogenous VEGF has neurotrophic effects on injured spinal nerves in organotypic spinal cord slice cultures. However, the mechanisms underlying the neurite growth by exogenous VEGF remain to be explored in spinal cord. In this study, we found out that exogenous VEGF mediated axonal outgrowth through VEGF receptor 1 (VEGFR1) and VEGFR2, both of which were expressed on organotypic spinal cord slices. Although VEGFR1 and VEGFR2 were constitutively expressed in some cells of control spinal cord slices, VEGF treatment upregulated expression of VEGFR1 and VEGFR2. Both VEGFR1 and VEGFR2 were expressed in neuronal cells as well as glial cells of organotypic spinal cord slices. We also observed that VEGF-induced axonal outgrowth was attenuated by a specific mitogen-activated protein kinase (MAPK) inhibitor PD98059 and a specific phosphoinositide 3-kinase (PI3K) inhibitor wortmannin. Thus, these findings suggest that these MAPK and PI3K pathways have important roles in regulating VEGF-induced axonal outgrowth in the postnatal spinal cord.

Tissue Eng Regen Med 2016;13(5):601-609

Key Words: Vascular endothelial growth factor; Neurotrophic factor; Spinal cord; Organotypic culture; Receptors

INTRODUCTION

Spinal cord injury (SCI) causes axon degeneration, loss of neurons and glia, and demyelination around the lesion site, leading to functional deficits [1,2]. Primary mechanical spinal cord insults lead to the generation of secondary events, including ischemia, inflammation, and vascular changes [3]. Axon regeneration in the central nervous system (CNS) is inhibited by myelin-associated inhibitors [4] and the formation of glial scars [5]. However, there are no effective repair strategies because of the limited capacity for regeneration in the CNS.

Vascular endothelial growth factor (VEGF) is best known for its role in regulating vessel growth during development and ischemia [6]. VEGF primarily exerts its biological actions through two high-affinity tyrosine kinases, VEGFR1 (also known

as Flt-1: fms-like tyrosine kinase) and VEGFR2 (also known as KDR/Flk-1: fetal liver kinase 1-murine homologue/kinase insert domain containing receptor-human homologue) [6]. We previously reported that VEGF is associated with the induction of axonal growth in an injured organotypic spinal cord slice culture [7]. Recent studies have also shown that VEGF attenuates the SCI by inhibiting the inflammation [8] and enhances axonal outgrowth and survival of neuronal cells [9]. VEGF expression increases in the spinal cord following damage [10]. In addition, VEGF treatment increases endogenous repair and improves functional recovery in animal models of spinal cord contusion injury [11]. However, it is unclear how axonal growth is regulated by VEGF in the spinal cord.

Thus, we sought to investigate the underlying mechanisms mediating exogenous VEGF-induced axonal outgrowth in the spinal cord using an organotypic spinal cord slice culture derived from post-natal day 16 rats previously developed by our group [7]. We examined the expressions of VEGFR1 and VEGFR2 as well as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways in VEGF-in-

Received: Marh 23, 2016

Revised: May 13, 2016

Accepted: June 1, 2016

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duced axonal growth in organotypic spinal cord cultures. The results demonstrated that exogenous VEGF significantly promoted axonal outgrowth and upregulated VEGFR1 and VEGFR2 in organotypic spinal cord cultures. Inhibiting these receptors reduced this VEGF-mediated axonal outgrowth. Moreover, both the MAPK kinase (MEK) inhibitor PD98059 and the PI3K inhibitor wortmannin significantly inhibited VEGF-induced axonal outgrowth in the spinal cord cultures, suggesting that these two pathways are involved in regulating VEGF-induced axonal growth in the postnatal spinal cord.

MATERIALS AND METHODS

Organotypic spinal cord slice culture

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University (Permit Number: SNU-101105-6). Organotypic spinal cord slice cultures were prepared as previously described [7,12]. Sixteen-day-old Sprague-Dawley rats were anaesthetized with avertin, and their lumbar spinal cords were collected under sterile conditions. Nerve roots and excess connective tissue were removed in cold Hank's balanced salt solution (HBSS; Gibco, Carlsbad, CA, USA) containing 6.4 mg/mL glucose. The spinal cords were cut into 400- μ m slices with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, UK). Four slices were carefully placed on a membrane insert (Millicell-CM; Millipore, Billerica, MA, USA) and placed into a 6-well plate with 1 mL of culture media consisting of 50% Eagle's minimum essential medium (Gibco), 25% HBSS, 25% horse serum (Gibco, Carlsbad, CA, USA), 6.4 mg/mL glucose and 20 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA). Slices were cultured at 37°C with 5% CO₂, and the media were changed twice per week. After 7 days of culturing in the spinal cord slice culture media, recombinant human VEGF₁₆₅ (R&D Systems, Minneapolis, MN, USA) of 12.5, 25, 50, or 100 ng/mL were added to the cultures directly to the medium immediately. This range of doses has previously been used *in vitro* to study neurotrophic effects [7]. Control slices received vehicle alone. All slice cultures were incubated under standard conditions for 3 days.

Immunohistochemistry

The slice cultures were fixed with 4% paraformaldehyde overnight at 4°C, permeabilized with 0.5% Triton X-100 in 1% bovine serum albumin (BSA) for 10 min, and blocked with 0.1% Triton X-100 in 3% BSA for 1 hour at room temperature. They were incubated overnight at 4°C with the following pri-

mary antibodies: rabbit polyclonal anti-neurofilament-M (NF-M) (1:500; Chemicon, Temecula, CA, USA), rabbit polyclonal anti-VEGFR1 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rabbit polyclonal anti-VEGFR2 (1:50; Santa Cruz Biotechnology). After washing with PBS, the slices were incubated with Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA). The cultures were then counterstained with 4,6-diamidino-2-phenylindole (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for nuclear staining and mounted with fluorescent mounting medium (Dako, Glostrup, Denmark). Immunofluorescence was examined using a laser-scanning confocal microscope, Fluoview FV300 (Olympus, Tokyo, Japan). The Z-stack images were captured using a laser-scanning confocal microscope at 4–5 μ m intervals.

Receptor neutralizing antibody treatment

Neutralizing polyclonal goat antibodies (immunoglobulin G, IgG) against VEGFR1 (16 μ g/mL) (R&D Systems, Minneapolis, MN, USA) or VEGFR2 (4 μ g/mL) (R&D Systems, Minneapolis, MN, USA) were used to determine the involvement of VEGFR1 and VEGFR2 in axonal outgrowth following VEGF administration. Neutralizing antibodies were added to the organotypic spinal cord culture medium containing 50 ng/mL VEGF. Control slices received vehicle alone. The slice cultures were maintained for 3 days, followed by fixation in 4% paraformaldehyde and processing for immunohistochemistry and confocal microscopy, as described previously.

Signal transduction experiments

To study the role of the MAPK and PI3K pathways in organotypic spinal cord cultures, either a specific MEK inhibitor PD98059 (Promega, Madison, WI, USA) or a specific PI3K inhibitor wortmannin (Sigma-Aldrich) were introduced into VEGF-treated slices and examined for neurite growth by NF-M immunoeexpression. PD98059 (50 μ M) or wortmannin (100 nM) was added to spinal cord slice cultures that also received 50 ng/mL VEGF. Control slice cultures received vehicle alone. All slice cultures were incubated under standard conditions for 3 days, followed by fixation in 4% paraformaldehyde and processing for immunohistochemistry and confocal microscopy, as described previously.

VEGFR1 or VEGFR2 co-localization with cell-specific markers

The VEGFR1 and VEGFR2 expressions in neurons, astrocytes or oligodendrocytes were examined in vehicle or VEGF (50 ng/mL) treated organotypic spinal cord slices using laser-scanning confocal microscopy. The antibodies used were rabbit polyclonal anti-VEGFR1 (1:50), rabbit polyclonal anti-VEGFR2

(1:50), mouse monoclonal anti-neuronal class III β -tubulin (TuJ1) (1:200; Chemicon, Temecula, CA, USA), mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:500; Millipore, Billerica, MA, USA), and mouse monoclonal anti-galactocerebroside (GalC) (1:200; Sigma-Aldrich, St. Louis, MO, USA). The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA).

Image analysis

Each immunostaining was visualized under the Z-stack laser-scanning confocal microscope, digitally captured and quantified by morphometric image analysis (integrated optical density, IOD) with the use of Image J software (National Institutes of Health, Bethesda, MD, USA), as described previously [7,12]. Values were expressed in percentage of controls.

Statistical analysis

All statistical analyses were performed with the R programming language. Data were analyzed by either Student's t-test or the analysis of variance followed by post hoc comparisons with Tukey test. p values <0.05 were considered statistically significant. All data were expressed as the mean $\pm$ standard error of mean.

RESULTS

VEGF induces axonal outgrowth in organotypic spinal cord slice cultures

Spinal cord slices were cultured with at the concentrations of

0, 12.5, 25, 50, and 100 ng/mL for 3 days and immunostained for the NF-M neuronal marker to examine whether VEGF induces axonal outgrowth in the organotypic spinal cord slice cultures (Fig. 1). Substantial axonal growth was observed by robust NF-M immunorexpression in spinal cord slices treated with 50 or 100 ng/mL VEGF (Fig. 1A). IOD of the NF-M-immunostained axons was quantified to identify changes in axonal outgrowth (Fig. 1B). VEGF concentrations of 50 and 100 ng/mL produced significant increases of 73% and 92% respectively in NF-M-expressing axons when compared with those in control slices ($p<0.05$) (Fig. 1B). NF-M-expressing axons did not increase significantly at lower doses of VEGF (e.g., 12.5 or 25 ng/mL). Interestingly, there was no significant difference in axonal outgrowth between 50 and 100 ng/mL of VEGF. These results indicate that a certain concentration of VEGF increased axonal outgrowth in organotypic spinal cord slice cultures.

VEGFR1 and VEGFR2 are involved in VEGF-induced axonal outgrowth

VEGF exerts its multiple functions largely through two high-affinity tyrosine kinases, VEGFR1 and VEGFR2 [13]. To investigate which VEGF receptor(s) were involved in VEGF-induced axonal outgrowth in the organotypic spinal cord slice cultures, we first determined VEGFR1 expression in spinal cord slices treated with or without 50 ng/mL VEGF (Fig. 2A). VEGFR1 was constitutively expressed in some cells of control spinal cord slices (Fig. 2A). However, the relative IOD of VEGFR1 expression increased significantly in VEGF-treated slices ($100.0 \pm 6.0\%$ in Control vs. $124.4 \pm 6.2\%$ in V50; $p<0.05$) (Fig. 2B). Intact re-

Figure 1. VEGF induces axonal outgrowth in organotypic spinal cord slice cultures. (A) Representative confocal images of NF-M immunostaining in spinal cord slices treated with VEGF at the concentrations of 0, 12.5, 25, 50, and 100 ng/mL for 3 days. The zero concentration group was used as the control. Scale bars, 200 μ m. (B) The relative integrated optical density (IOD) of NF-M-immunostained axons after VEGF application to the spinal cord slice cultures. The IOD was normalized to the control. The bars represent mean $\pm$ standard error of mean of at least six slices. * $p<0.05$ (analysis of variance followed by post hoc Tukey test). VEGF: vascular endothelial growth factor, NF-M: neurofilament-M.

Figure 2. Effect of VEGF on VEGFR1 and VEGFR2 expression in organotypic spinal cord cultures. (A and C) Representative confocal images of VEGFR1 (A) or VEGFR2 (C) immunostaining in spinal cord slices treated with vehicle (control) or 50 ng/mL VEGF (V50). VEGFR1 or VEGFR2 were detected with Alexa Fluor 546-conjugated antibodies (red). The nuclei were visualized by DAPI staining (blue). Scale bars, 20 μ m. (B and D) The relative IOD of VEGFR1 (B) or VEGFR2 (D) immunostaining in spinal cord slices. The IOD is normalized to control. The bars represent mean $\pm$ standard error of mean of at least six slices. * p <0.05 (Student's *t*-test). VEGF: vascular endothelial growth factor, VEGFR: VEGF receptor, IOD: integrated optical density, DAPI: 4,6-diamidino-2-phenylindole.

ceptor tyrosine kinases such as VEGFRs can translocate to intracellular destinations including nucleus and cytoplasm and such intracellular receptor trafficking events activate signal transduction pathways [14]. Consistent with previous reports [15,16], we found that VEGFR1 was predominantly present in the nuclei of the cells in both untreated and VEGF-treated spinal cord slices (Fig. 2A), suggesting that most of the VEGFR1 is translocated into the nucleus. We also determined VEGFR2 expression in spinal cord slices treated with or without 50 ng/mL VEGF (Fig. 2C). Similar to pattern of VEGFR1 expression, VEGFR2 was also constitutively expressed in some cells of control spinal cord slices (Fig. 2C). The relative IOD of VEGFR2 immunostaining appeared to increase slightly in VEGF-

treated slices compared with that in control slices ($100.0 \pm 2.4\%$ in Control vs. $114.0 \pm 3.7\%$ in V50; $p < 0.05$) (Fig. 2D). In contrast to VEGFR1 translocation, VEGFR2 was partially translocated to the nucleus via VEGF treatment.

To further examine the implications of receptors in VEGF-induced axonal outgrowth in the spinal cord slice cultures, we blocked VEGFR1 or VEGFR2 with a neutralizing anti-VEGFR1 polyclonal antibody or a neutralizing anti-VEGFR2 polyclonal antibody (Fig. 3A). The relative IOD of NF-M-immunostained axons showed that axonal outgrowth was notably inhibited in VEGF-treated slices in the presence of anti-VEGFR1 antibody ($120.9 \pm 13.3\%$; $p < 0.05$) or anti-VEGFR2 antibody ($93.5 \pm 5.9\%$; $p < 0.001$) as compared with that in VEGF-treated slices ($174.9 \pm$

Figure 3. Receptor mediation and signal transduction pathways involved in VEGF-induced axonal outgrowth in spinal cord slices. (A) Representative confocal images of NF-M immunostaining in spinal cord slices treated with vehicle (control), 50 ng/mL VEGF (V50), 50 ng/mL VEGF together with neutralizing anti-VEGFR1 antibody, or 50 ng/mL VEGF together with neutralizing anti-VEGFR2 antibody. Scale bars, 200 μ m. (B) The relative IOD of NF-M-immunostained axons in spinal cord slice cultures. The IOD is normalized to control. The bars represent mean $\pm$ SEM of at least twenty two slices. The IOD is normalized to control. The bars represent mean $\pm$ SEM of at least thirteen slices. * p <0.05, ** p <0.01 (ANOVA followed by post hoc Tukey test). (C) Representative confocal images of NF-M immunostaining in spinal cord slices treated with vehicle (control), 50 ng/mL VEGF (V50), 50 ng/mL VEGF together with mitogen-activated protein kinase signaling pathway inhibitor PD98059, or 50 ng/mL VEGF together with phosphoinositide 3-kinase signaling pathway inhibitor wortmannin (WORT). Scale bars, 200 μ m. (D) The relative IOD of NF-M-immunostained axons in spinal cord slice cultures. The IOD is normalized to control. The bars represent mean $\pm$ SEM of at least thirteen slices. * p <0.05 (ANOVA followed by post hoc Tukey test). VEGF: vascular endothelial growth factor, VEGFR: VEGF receptor, NF-M: neurofilament-M, IOD: integrated optical density, SEM: standard error of mean, ANOVA: analysis of variance.

13.2%) (Fig. 3B). The relative IOD of NF-M-positive axons in the slices with neutralizing antibodies were comparable to those of control slices (Fig. 3B). These results indicate that both VEGFR1 and VEGFR2 play important roles in VEGF-induced axonal outgrowth in organotypic spinal cord slice cultures.

MAPK and PI3K signaling pathways are involved in VEGF-induced axonal outgrowth

We next applied the specific inhibitors of MAPK and PI3K signaling inhibitors, PD98059 and wortmannin, in VEGF-treated spinal cord slice cultures to examine the implication of potential signaling pathways involved in axonal outgrowth th-

rough VEGFRs (Fig. 3C). PD98059 non-competitively inhibits activation of the MAPK pathway by Raf-1 [17]. The analysis of relative IOD of NF-M-immunostained axons demonstrated that adding PD98059 markedly blocked VEGF-induced axonal outgrowth when compared with that in VEGF-treated spinal cord slices ($97.3 \pm 17.8\%$ in V50+PD98059 vs. $188.2 \pm 14.9\%$ in V50; $p < 0.05$) (Fig. 3D). VEGF-treated slices in the presence of PD98059 appeared comparable with untreated control slices (Fig. 3D). The specific inhibitor wortmannin was added to block the PI3K pathway [18]. Confocal images of NF-M immunostaining showed that NF-M positive axonal outgrowth was significantly blocked when wortmannin was added to

VEGF-treated spinal cord slices ($100.2 \pm 21.0\%$) ($p < 0.05$) (Fig. 3D). VEGF-treated slices in the presence of wortmannin appeared comparable with untreated control slices (Fig. 3D). These findings suggest that both MAPK and PI3K pathways are involved in VEGF-induced axonal outgrowth in spinal cord slices.

VEGFR1 and VEGFR2 expression in neurons and glial cells of spinal cord slices

The organotypic slice culture model consists of all cellular components in a near-normal anatomical architecture. Thus, it is possible that glial cells stimulated by VEGF could support axonal outgrowth. To compare the difference between the cell types

Figure 4. VEGFR1 and VEGFR2 expression in neurons and glial cells of VEGF-treated spinal cord slices. (A, C, and E) Double immunostaining of VEGFR1 or VEGFR2 (red) and TuJ1 (A), GFAP (C), or GalC (E) (green) in spinal cord slices treated with vehicle (control) or 50 ng/mL VEGF. Each arrow shows either VEGFR1 or VEGFR2 expression in TuJ1-, GFAP-, or GalC-positive cells. Scale bars, 20 μ m. (B, D, and F) Comparison of VEGFR1-positive cells and VEGFR2-positive cells in TuJ1- (B), GFAP- (D), and GalC-positive cells (F) in vehicle (control)- or VEGF-treated organotypic spinal cord slices. The bars represent mean $\pm$ standard error of mean of at least three slices. * $p < 0.05$, *** $p < 0.001$ (Student's t-test). NS: not significant, VEGF: vascular endothelial growth factor, VEGFR: VEGF receptor, TuJ1: class III β -tubulin, GFAP: glial fibrillary acidic protein, GalC: galactocerebroside.

expressing VEGF1 and the cell types expressing VEGF2 following VEGF application, the spinal cord slice cultures were double-immunostained for the neuronal marker TuJ1, the astrocytic marker GFAP or the oligodendrocytic marker GalC, and either VEGFR1 or VEGFR2. Double immunostaining of TuJ1 and VEGFR1 or VEGFR2 showed no significant difference between VEGFR1 and VEGFR2 expression in neurons of spinal cord slices regardless of the presence or absence of VEGF (50 ng/mL) (Fig. 4A and B). Although not statistically significant, VEGF administration slightly increased the expression of VEGFR1 and VEGFR2, respectively, in TuJ1-positive cells (Fig. 4B). Interestingly, while $60.27 \pm 2.29\%$ and $71.86 \pm 2.66\%$ of the GFAP-expressing cells were VEGFR1-positive cells in control slices and VEGF-treated slices respectively, most GFAP-positive cells did not express VEGFR2 in both control and VEGF-treated slices (Fig. 4C and D). This remarkable difference may represent preferential expression of VEGFR1 in GFAP-positive glial cells. VEGF treatment significantly increased VEGFR1 expression in GFAP-positive cells (Fig. 4D). Similar to the result observed in Figure 4A and B, there was no significant difference between the percentage of GalC/VEGFR1-double positive cells and GalC/VEGFR2-double positive cells in control slices and VEGF-treated slices respectively (Fig. 4E and F). However, VEGFR2 expression was significantly elevated in GalC-positive cells following administration of VEGF (Fig. 4F). Taken together, our findings indicate that VEGFRs are expressed in glial cells as well as neurons of spinal cord slices.

DISCUSSION

In this study, we demonstrated that VEGF induces axonal outgrowth in a postnatal organotypic spinal cord slice culture through VEGFR1 and VEGFR2. The neurotrophic function of VEGF is mediated by the consequent triggering of the MAPK and PI3K pathways. VEGF is known as an angiogenic factor that serves a role in organogenesis, tissue repair, and tumor growth and is composed of at least six isoforms containing 121, 145, 165, 183, 189, and 206 amino acids. VEGF₁₆₅ is the most abundant and biologically active form [19,20]. We previously showed that VEGF₁₆₅ has neurotrophic effects including cell protection and axonal growth in the demyelinated spinal cord [7]. VEGF expression increases in the spinal cord following damage to suggest that the upregulated VEGF could lead to neovascularization to ameliorate metabolic distress and may have protective effects [10].

The organotypic spinal cord slice model remarkably preserves the original cytoarchitecture [21,22]. We used rat organotypic spinal cord slice cultures trimmed blood vessels and meninges off [23,24] to characterize neurotrophic properties

of VEGF on the postnatal spinal cord, which also allowed us to evaluate VEGF functions independently from vascularization. VEGF treatment can alter angiogenesis rapidly in the brain, triggering a cellular signaling cascade or brain-derived neurotrophic factor secretion from endothelial cells [25,26]. VEGF can then regulate axonal growth directly or indirectly by activating astrocytes and other cell type, such as oligodendrocytes.

The potential interaction between glia and nerve cells can provide a microenvironment exerting VEGF-induced neurotrophic effects in the spinal cord. The present study demonstrated that VEGFR1 and VEGFR2 levels were upregulated by exogenous VEGF in the organotypic spinal cord culture. Since VEGFR1 and VEGFR2 expressions were observed in both spinal neurons and glial cells, it will be interesting to investigate the molecular interactions among these cells in the spinal cord in response to VEGF. The signaling events involved in the postnatal spinal cord treated with exogenous VEGF have not been detailed previously, although some studies have shown seminal pleiotrophic roles for VEGF actions in embryonic rat brain [9,27]. For example, it has been suggested that VEGF-induced neuritic growth is mediated by VEGFR2 and both the MAPK and PI3K pathways in embryonic rat brain explants. Inhibiting VEGFR1 did not attenuate VEGF-induced TuJ1-positive neuritic growth [9]. Similarly, the present results show that both the MAPK and the PI3K signaling pathways are implicated in VEGF induced NF-M-positive axonal outgrowth in an organotypic spinal cord culture (Fig. 5). Interestingly, our neutralizing antibody results indicated that both VEGFR1 and VEGFR2 inhibit

Figure 5. A molecular model for axonal outgrowth in response to VEGF in spinal cord. Exogenous VEGF induces axonal outgrowth in spinal cord through VEGFR1 and VEGFR2 in conjunction with MAPK and PI3K signaling pathways. VEGF interacts with both two receptors which expression is upregulated. VEGF-induced axonal outgrowth is abrogated by inhibition of MAPK and phosphoinositide 3-kinase pathway, respectively. VEGF: vascular endothelial growth factor, VEGFR: VEGF receptor, MAPK: mitogen-activated protein kinase.

ited axonal growth mediated by exogenous VEGF in an organotypic spinal cord culture. These results may have been caused by developmental differences between the prenatal and postnatal stages or tissue-specific properties of the brain and spinal cord. In addition, we observed that more than a half of spinal neurons and most oligodendrocytes expressed both VEGFR1 and VEGFR2 whereas astrocytes mostly expressed VEGFR1 in VEGF-treated spinal cord slices. Thus, VEGFR1-expressing astrocytes may play an important role in VEGF-induced axonal outgrowth in our spinal cord slice cultures.

VEGFR1 and VEGFR2 are VEGF-activated main receptors with tyrosine kinase catalytic activity. VEGFR2 increases cell survival of endothelium and CNS [6,28], and its expression is observed in motor neurons in the human spinal cord and is attenuated in patients with amyotrophic lateral sclerosis [29]. Mouse motor neurons and adult rat motor neurons also express VEGFR2 [30,31]. In contrast, the precise function of VEGF and VEGFR1 in the nervous system is unclear. Some studies have demonstrated that VEGFR1 is upregulated in astrocytes of neocortical explants by VEGF treatment [27] and in activated glial cells of rat hippocampus following injury [32]. We also observed that around 72% of astrocytes expressed VEGFR1 in the spinal cord slices. Although the functional relevance of VEGFR1 is controversial [33,34], our findings suggest that exogenous VEGF exerts its neurotrophic role through VEGFR1 in the spinal cord slices.

In conclusion, our findings demonstrate that exogenous VEGF promotes axonal outgrowth via not only VEGFR1-dependent signaling but also VEGFR2-dependent signaling in organotypic spinal cord slice culture, and that this mechanism includes activation of VEGFR1 and VEGFR2 localized in spinal neurons and glial cells. In addition, VEGF-induced axonal growth was mediated by both the MAPK and the PI3K signaling pathways. Although these findings point to a mechanistic role for VEGF in spinal axon growth, how VEGF acts on other cell types, including astrocytes, oligodendrocytes, microglia, and neural progenitors remains to be elucidated. Therefore, additional study on diverse cell signaling pathways related to VEGF could provide important therapeutic insights.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (M-SC, 2012R1A1A2007945, NRF-2015R1D1A1A01056950, and H-WP, 2015R1A5A1009701) funded by the Ministry of Education, and by a grant of the Korean Health Technology R&D Project (M-SC, A120476), Ministry of Health & Welfare, Republic of Korea.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University (Permit Number: SNU-101105-6).

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