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Original Article

Reduced Vascular Endothelial Growth Factor Expression in Contusive Spinal Cord Injury

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Abstract

Vascular endothelial growth factor (VEGF) is being investigated as a potential interventional therapy for spinal cord injury (SCI). In the current study, we examined SCI-induced changes in VEGF protein levels using Western blot analysis around the epicenter of injury. Our results indicate a significant decrease in the levels of $VEGF_{165}$ and other VEGF isoforms at the lesion epicenter 1 day after injury, which was maintained up to 1 month after injury. We also examined if robust $VEGF_{165}$ decrease in injured spinal cords affects neuronal survival, given that a number of reported studies show neuroprotective effect of this VEGF isoform. However, exogenously administered $VEGF_{165}$ at the time of injury did not affect the number of sparred neurons. In contrast, exogenous administration of VEGF antibody that inhibits actions of not only $VEGF_{165}$ but also of several other VEGF isoforms, significantly decreased number of sparred neurons after SCI. Together these results indicate a general reduction of VEGF isoforms following SCI and that isoforms other than $VEGF_{165}$ (e.g., $VEGF_{121}$ and/or $VEGF_{189}$) provide neuroprotection, suggesting that $VEGF_{165}$ isoform is likely involved in other pathophysiological process after SCI.

Key words: contusion; expression; growth factor; immunofluorescence; isoforms; neuroprotection; spinal cord injury; vascular; Western

Introduction

Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis and a mediator of vascular permeability (Connolly et al., 1989; Ferrara et al., 2003; Leung et al., 1989; Senger et al., 1983). In addition to the angiogenic and vascular permeability properties, VEGF is also considered to have neuroprotective effects (Facchiano et al., 2002; Oosthuyse et al., 2001; Svensson et al., 2002) and thus may be a critical mediator in the recovery after spinal cord injury (SCI).

The VEGF family consists of six different members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF) (Ferrara et al., 2003). The most predominant form is VEGF-A, which is alternatively spliced into six different isoforms identified as 121, 145, 165, 183, 189, and 206 (Robinson and Stringer, 2001). $VEGF_{165}$ is the most predominant of the six different isoforms and is found both diffusible, and bound to the cell surface and extracellular matrix (Park et al., 1993). VEGF isoforms 121 and 145 are freely diffusible, while 183 and 189 are strongly bound to the extracellular matrix (Ferrara et al., 2003; Jingjing et al., 1999; Poltorak et al., 1997; Zachary, 2001). Most biological effects of VEGF are mediated via two receptor tyrosine kinases, VEGFR1 (KDR/Flk-1) and VEGFR2 (Flt-1) (Ferrara et al., 2003; Neufeld et al., 1999; Zachary, 2003), but specific VEGF isoforms also bind neuropilins 1 and 2, non-tyrosine kinase receptors originally identified as receptors for semaphorins, polypeptides with essential roles in neuronal patterning (Gluzman-Poltorak et al., 2000; Makinen et al., 1999; Migdal et al., 1998; Soker et al., 1998).

Recent studies have examined the effect of acute administration of VEGF in SCI, albeit with different outcomes (Benton and Whittemore, 2003; Widenfalk et al., 2003). Studies by Widenfalk et al. (2003) indicated that acute administration of VEGF improved behavioral outcome and reduced the lesion volume and level of apoptosis following SCI (Widenfalk et al., 2003), while the study by Benton and Wittenmore (2003) indicated that acute VEGF administration exacerbated lesion volume resulting in poor functional recovery (Benton and Whittemore, 2003). To date, the use of VEGF treatment following SCI is controversial. Additionally, the expression and role of VEGF in injured spinal cord are not well understood. Therefore, the major focus of our study was to examine the

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expression profile of $VEGF_{165}$ following SCI. We examined the protein levels of $VEGF_{165}$ via Western analysis and observed a significant decrease at the lesion site 1 day after injury that persisted up to 1 month post-injury. Additionally, we determined that suppressing VEGF expression by acute administration of a neutralizing antibody reduced the number of neuronal cells around the lesion at chronic time points.

Methods

Animal subjects and surgery

All surgical procedures and subsequent care and treatment of all animals used in this study were in strict accordance with NIH guidelines for animal care. Our institutional animal welfare committee approved these studies.

To examine the VEGF protein expression profile at 1, 7, 14, and 28 days post-injury, a total of 18 male Sprague-Dawley rats (300–350 g) were used and compared to six sham controls. SCIs were performed as described previously (Ramu et al., 2007; Scheff et al., 2003). Briefly, animals were anesthetized with 4% isoflurane, and maintained under anesthesia with a mixture 2% isoflurane, air, and oxygen, administered through a Harvard rodent ventilator (model 683; Harvard Apparatus, South Natic, MA) during the entire procedure. A laminectomy was performed at the 7th thoracic vertebra (T7), and the T6 and T8 vertebral process were clamped to stabilize the vertebral column. A 150-kDyne force was delivered to the exposed cord to produce a moderate level of injury using an Infinite Horizon Impactor (Precision System and Instrumentation, Lexington, KY). The animals were allowed to recover in warmed cages and received subcutaneous injections of Baytril-100 (2.5 mg/kg, Bayer Healthcare LLC Animal Division, Shawnee Mission, KS) twice a day for 3–5 days, and Buprenex (0.01 mg/kg, Hospira, Inc., Lake Forest, IL) twice a day for 5 days. Animals were also administered subcutaneous injections of saline twice daily for 5 days. The animals' bladders were manually expressed twice daily by the method of Crede until the return of spontaneous urination. Animals had free access to food and water.

$VEGF_{165}$ exogenous administration

To examine the neuroprotective role of VEGF, a total of 32 adult male Sprague-Dawley rats (300–350 g) were used. Rats were divided into four different groups ($n = 8$/group). Groups 1–3 received a single injection (1.5 μl) of either saline, human recombinant $VEGF_{165}$ (4 μg/ml; catalog no. 293-VE, R&D Systems; Minneapolis, MN), or anti-VEGF (4 μg/ml; catalog no. AF564, R&D Systems), respectively, immediately after injury. The bioactivity of both the protein and antibody were tested, and specific details are provided by R&D Systems. Sham controls receiving only a laminectomy served as Group 4. Injections were delivered at a depth of 1.2 mm below the surface directly into the contusion site at a rate of 0.5 μl/min through a glass pulled needle driven by a picospritzer (Parker Hannifin Corporation, Fairfield, NJ). We used the same concentration of VEGF that was employed in a previous study (Widenfalk et al., 2003). Animals were sacrificed after 56 days for the neuroprotection analysis. Analysis of the behavioral data, histology, and magnetic resonance imaging (MRI) analysis from the above groups is in progress and will be reported in a subsequent manuscript.

Tissue processing for Western analysis

For Western analysis, animals were transcardially perfused with saline, and the endogenous expression of VEGF was determined at days 1, 7, 14, and 28 after injury. Spinal segments were processed for Western analysis as described previously (Nesic et al., 2006) with a few minor modifications. Briefly, the lesion site consisting of a 2–3-mm spinal segment was removed and homogenized in a solution of T-PER® (Tissue Protein Extraction Reagent; Pierce, Rockford, IL) supplemented with Complete, EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). The buffer and sample mix were homogenized at 4°C using a hand-held glass homogenizing pestle. After the homogenization, all samples were centrifuged at 4°C for 10 min at 10,000 rpm. Supernatant samples were frozen and stored at −20°C. All samples were processed on the same day to avoid any changes in sample preparations.

Western analysis

Western analysis was performed on all samples as described previously with a few minor modifications (Nesic et al., 2006). Briefly, the amount of protein in each sample was measured using the Bradford assay employing bovine serum albumin (BSA) as a standard. Equal amounts of protein (40 μg) were then boiled for 10 min with appropriate volume of 6× sample buffer (350 nM Tris-HCL, pH 6.8, 1 M Urea, 1% 2-mercaptoethanol, 9.3% DTT, 13% sodium dodecyl sulfate [SDS], 0.06% bromophenol blue, and 30% glycerol). Samples were then resolved on a 12% SDS–polyacrylamide gel and separated at 150 v for 4 h. The gel was then transferred overnight to a polyvinyl difluoride (PVDF) membrane at 4°C. The membrane was then reversibly stained with Ponceau S to confirm transfer of proteins and then destained in distilled water. The membranes were blocked for 1 h at room temperature in 5% BSA in Tris-buffered saline with Tween-20 (TBST; 10mM Tris-HCl, pH 7.9, 150 mM NaCl, and 0.05% Tween-20). The following primary antibodies were used: rabbit anti-VEGF (sc-507, 1:300; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-Beta actin (1:80,000; Sigma-Aldrich, St. Louis, MO) was used as a loading control. Both primary and secondary antibodies were diluted in blocking solution, and washed with Tris-buffered saline containing 0.2% Tween-20. Peroxidase activity was detected using the Amersham enhanced chemiluminescence system (ECL; RPN2209, Amersham Biosciences, Piscataway, NJ).

Preincubation of anti-VEGF with SCI sample

The expression of the $VEGF_{165}$ isoform was confirmed by performing a preincubation of the 24-h sample with a recombinant human $VEGF_{165}$ (catalog no. 293-VE; R&D System), with a volume ratio of 1:10, respectively. The control peptide was reconstituted as recommended by the manufacturer. The antibody/sample solution was left for overnight mixing at 4°C. The samples were then boiled and processed for Western analysis as described above.

Immunohistochemistry and image acquisition

Animals receiving acute spinal injections after injury were perfused with saline followed by 4% paraformaldehyde in phosphate-buffered saline (0.1 M PBS) at 56 days post-injury.

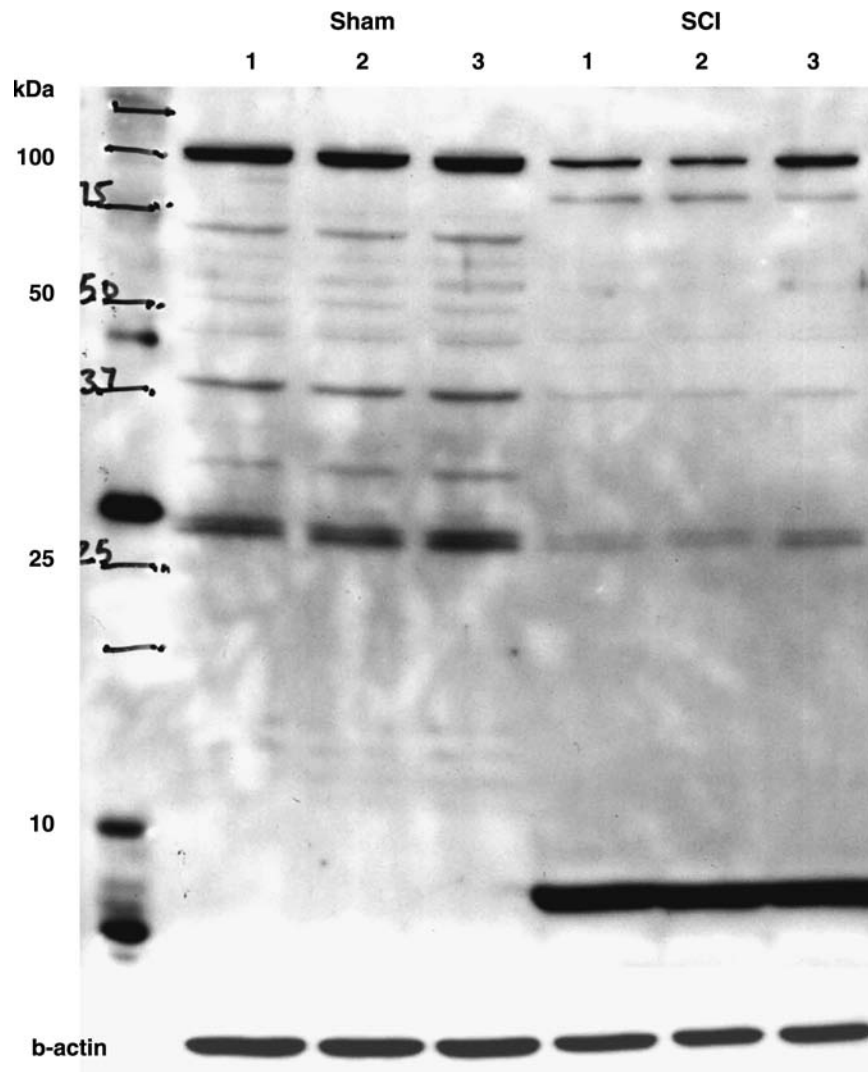

FIG. 1. Western analysis examining the expression of vascular endothelial growth factor (VEGF) at the lesion epicenter 1 day after spinal cord injury (SCI). VEGF was reduced in SCI samples compared to sham controls. Multiple bands may indicate the multiple VEGF isoforms in spinal cord tissue.

The spinal cords were removed, post-fixed overnight in 4% paraformaldehyde, and then immersed in 30% sucrose/0.1 M PBS for 2–3 days at 4°C. The spinal cord was divided into epicenter, rostral, and caudal segments. Each segment was 3 mm in length. Segments were then sectioned coronally at a thickness of 40 μm using a cryostat (model CM1800; Leica, Bannockburn, IL), and sections were stored at −20°C in tissue-storing media.

Spinal cord sections were processed as free floating and were incubated in the following antibodies: neuronal nuclei (NeuN; Millipore, Billerica, MA), glial fibrillary acidic protein (GFAP; Millipore), and VEGF (Santa Cruz Biotech). The primary antibody was diluted with blocking solution (0.1 M PBS containing 5% goat serum and 0.3% Triton X-100). For controls, only secondary antibodies were applied to determine the antibody specificity.

Appropriate secondary antibody was used at a dilution of 1:500 in 0.1 M PBS containing 5% goat serum and 0.3% Triton X-100. The following Alexafluor® dye conjugated secondary antibodies were used: goat anti-mouse IgG Alexa Fluor® 488 (Invitrogen, Carlsbad, CA) and goat anti-rabbit IgG (H + L) Alex Fluor® 568 (Invitrogen). Tissue sections for NeuN$^+$ nuclei quantification and co-labeling with VEGF were viewed and captured using a Spot Flex digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a Leica RX1500 upright microscope, and the images were collected using the Spot software. Operator acquiring the images was blinded to the groups.

Neuroprotection analysis

Spinal cord sections ($n = 10$ sections/animal) were analyzed from both rostral and caudal segments of all animals ($n = 8$ animals/group). The epicenter segment was not analyzed due to the large amount of tissue damage. The numbers of NeuN$^+$ neuronal nuclei were quantified in the entire coronal section using ImagePro Plus software (Media Cybernetics, Inc., Silver Spring, MD). Threshold levels were

determined from intact spinal cord sections and secondary control sections. These levels were applied to other groups. The nuclei were quantified in ImagePro and verified by manually counting neuronal nuclei. Operator quantifying cell nuclei was blinded to the groups.

Statistical analysis

Statistical analysis for Western and neuroprotection study was performed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test, and differences were considered significant if $p < 0.05$. Statistical analysis was performed using GraphPad Prism4 software (GraphPad Software, San Diego, CA).

Results

Quantitative assessments of VEGF protein level changes over time after SCI were based on Western blot analaysis and a polyclonal antibody that recognizes three VEGF isoforms: 121, 165, and 189. As mentioned earlier, VEGF isoform 121 is freely diffusible, 165 is both bound to matrix and freely diffusible, and 189 is strongly bound to the extracellular matrix (Ferrara et al., 2003). As shown previously, all three VEGF isoforms are expressed in the rat spinal cords, similar to human spinal cords (Ferrara et al., 2003).

As shown in Figure 1, representative VEGF Western blot indicates several bands (27–100 kD), which likely correspond to different VEGF isoforms expressed in spinal cord tissue. All VEGF-bands were reduced in injured spinal cords at 1 day post-SCI ($n = 3$), suggesting that SCI induced decrease in the expression levels of all three VEGF isoforms.

Since VEGF isoform 165 is most often studied in SCIs (Benton and Whittemore, 2003; Widenfalk et al., 2003), we identified the band in the Western blot that was 165-specific, using a standard competition experiment shown in Figure 2A ($n = 2$). We preincubated VEGF antibody with rat $VEGF_{165}$ peptide (1:10), and after subsequent Western blotting, the 27-kD band was significantly reduced, in contrast to other bands with higher molecular weight, which may represent SDS-resistant multimers of other VEGF isoforms or are non-specific bands. Furthermore, we also loaded or "spiked" purified rat $VEGF_{165}$ at two different concentrations (0.2 and 0.02 mg) and found that a band of 25 kD (Fig. 2B) that closely corresponds to the strongest band of 27 kD in the spinal cord samples. Discrepancy between those two molecular bands likely indicates glycosylation of $VEGF_{165}$ in spinal cord tissue (Brandner et al., 2006).

Based on these results, the 27-kD band appears to be $VEGF_{165}$ specific. We have, therefore, performed quantitative analyses of 27-kD bands in sham and SCI samples at different post-SCI time points at 2–3-mm segments surrounding site of injury. As shown in Figure 3, SCI induced a significant decrease in $VEGF_{165}$ levels as early as 1 day ($p < 0.05$; $n = 5$) that persisted for 1 month after SCI ($p < 0.05$; $n = 5$), in both rostral and caudal regions.

VEGF expression was observed in both gray and white matter in normal spinal cord (Fig. 4). Neuronal cell bodies in the gray matter were identified by NeuN labeling (Fig. 4B)

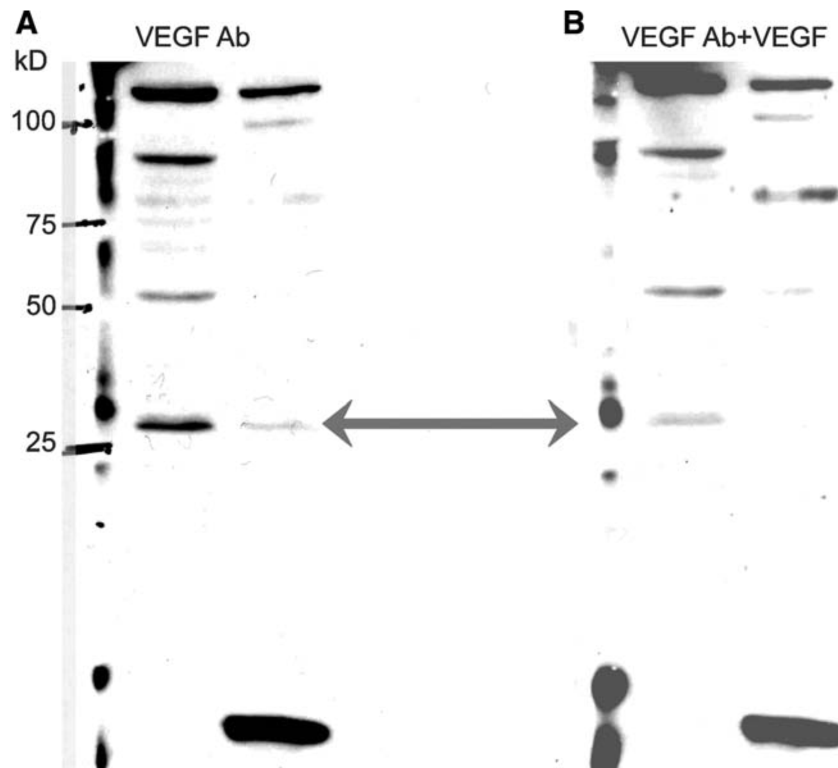

FIG. 2. One-day post–spinal cord injury (SCI) samples preincubated with vascular endothelial growth factor (VEGF) antibody. (**A**) Western analysis of 24-h samples indicating a reduction in the VEGF165 isoform. (**B**) A Western blot of VEGF165 samples at 2 and 0.02 mg/ml concentration in each lane, respectively, preincubated with an anti-VEGF antibody. VEGF165 isoform was reduced in the preincubated Western blot.

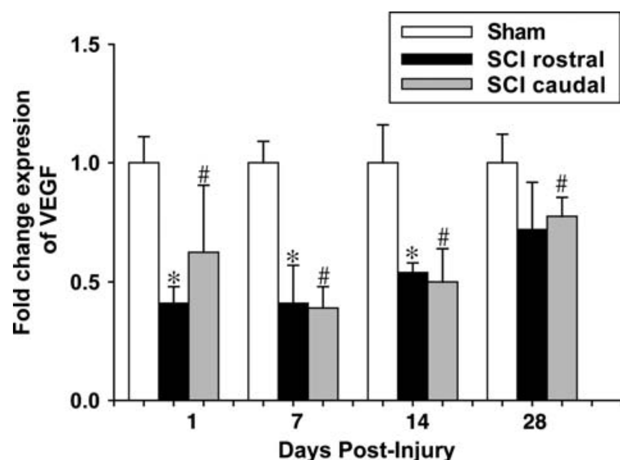

FIG. 3. Temporal vascular endothelial growth factor (VEGF) expression profile in regions around the lesion epicenter after spinal cord injury (SCI) compared to sham controls. VEGF165 isoform was significantly reduced 1 day after injury and remained reduced out to 28 days post-injury. Error bars represent standard error of the mean. Significance levels were set at $p > 0.05$.

and were observed co-labeling with VEGF (Fig. 4C). Astrocytic populations observed in the white matter determined by GFAP labeling (Fig. 4E) also demonstrated co-labeling with VEGF (Fig. 4F) in uninjured cord. Normal spinal cord sections indicate a clearly defined gray and white matter regions with the most predominant VEGF labeling in the gray matter (Fig. 5A). The uninjured spinal cord displayed typical GFAP labeling (Fig. 5B). However, when examining GFAP labeling in an injured section taken from around the lesion epicenter, there appears a typical upregulation of GFAP (Fig. 5D). VEGF expression was also observed (Fig. 5C), but there was a significant loss of tissue and disruption of tissue intergrity in the lesion epicenter.

We hypothesized, based on the VEGF expression levels observed from the Western analysis and histologic staining of injured spinal cord, that supplementing the injured environment with $VEGF_{165}$ may increase potential neuroprotective effects of VEGF as observed in other studies (Choi et al., 2007; Facchiano et al., 2002; Oosthuyse et al., 2001; Svensson et al., 2002; Widenfalk et al., 2003). We therefore exogenously delivered 1.5 μl of 4 μg/ml $VEGF_{165}$ via direct injection over 5 min into the lesion site at the time of injury as described previously by Widenfalk et al. (2003). As shown in Figure 6, exogenous administration of $VEGF_{165}$ did not affect the number of neurons, quantified using the neuronal nuclei specific marker NeuN.

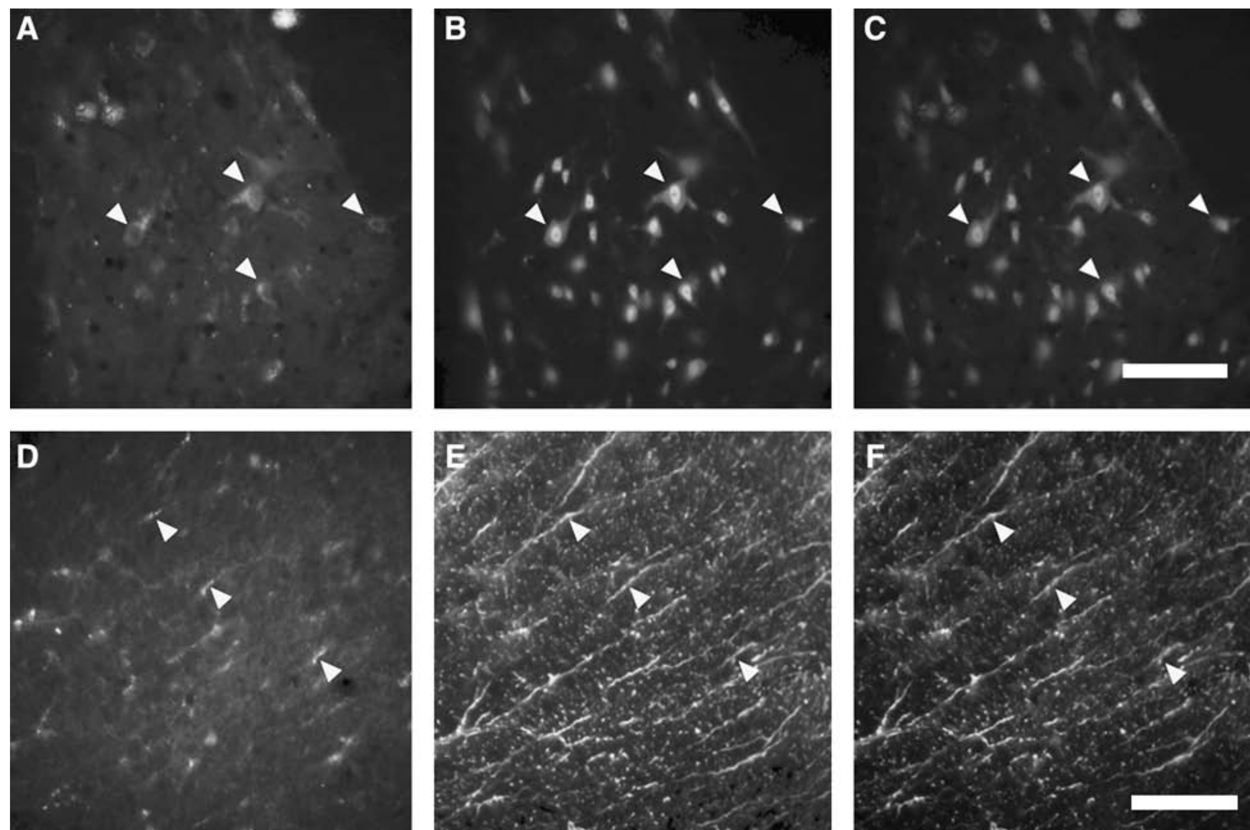

FIG. 4. Immunofluoresence labeling of neurons and astrocytes expressing vascular endothelial growth factor (VEGF) in uninjured spinal cord. VEGF expression (**A**) was observed in neuronal cell bodies in the ventral gray matter identified by NeuN labeling (**B**). Neurons double labeled for neuronal nuclei (NeuN) and VEGF are identified by arrowheads (**C**). VEGF expression (**D**) was also observed in astrocytic cells labeled with glial fibrillary acidic protein (GFAP) (**E**) in the lateral white matter. (**F**) Represents astrocytic process double labeled with GFAP identified with arrowheads. Scale bar = 100 μm.

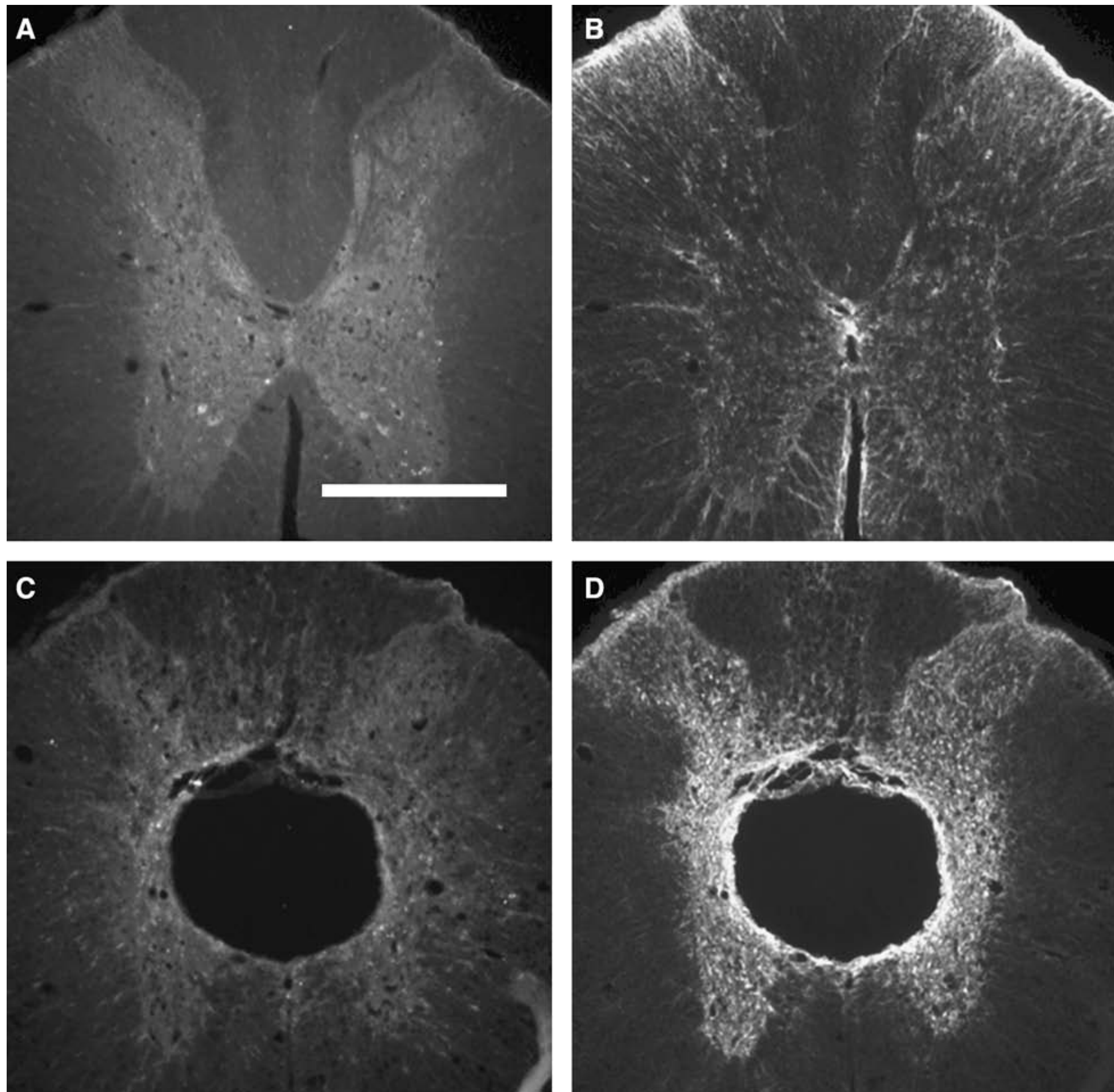

FIG. 5. Vascular endothelial growth factor (VEGF) immunolabeling of coronal sections from uninjured and injured spinal cord. VEGF expression is observed in both gray and white matter regions in uninjured spinal cord (**A**). Astrocytes labeled with glial fibrillary acidic protein (GFAP) also identify gray and white matter regions in uninjured tissue (**B**). A 56-day post-injured spinal cord section showing VEGF labeling around the lesion cavity with a loss of defined gray and white matter regions (**C**). Astrocytic labeling with GFAP indicates an increase around the lesion epicenter (**D**). Scale bar = 500 μm.

Interestingly, the delivery of anti-VEGF antibody (direct injection of 1.5 μl at a 4 μg/ml concentration) caused a significant decrease in the number of $NeuN^+$ cells in injured animals compared with VEGF, saline, or controls (Fig. 6). This VEGF antibody identified the same isoforms as the antibody used in Western blot experiments (Fig. 1). This ensured that the same VEGF isoforms whose protein levels were reduced after SCI (likely $VEGF_{121}$ and $VEGF_{189}$) are also the ones whose actions were blocked with VEGF antibody administration. This result suggests that VEGF isoforms other than $VEGF_{165}$ (such as $VEGF_{121}$ or $VEGF_{189}$) may have a role in sparing neurons after SCI.

Discussion

Decreased VEGF protein levels after SCI

To the best of our knowledge, VEGF protein levels following contusive SCI have not been previously analyzed, especially not in regard to different VEGF isoforms. We demonstrated a significant decrease in $VEGF_{165}$ isoform and likely other VEGF isoforms 1 day after spinal cord contusion with a sustained significant decrease in protein levels up to 1 month after injury. The preincubation assay confirmed that the $VEGF_{165}$ isoform was significantly decreased after injury. Interestingly, a recent study also observed a significant de-

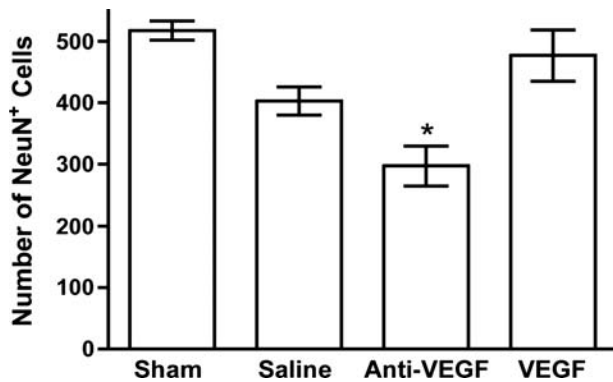

FIG. 6. A neuroprotection study was performed on four groups that received an acute epicenter injection of the following: vascular endothelial growth factor–165 (VEGF165), anti-VEGF, or saline. Neuroprotection was assessed by quantifying the number of neuronal nuclei (NeuN+) cells 56 days post-injury. A significant decrease was observed in the anti-VEGF-treated group (*) compared to VEGF165, saline, and sham controls. No significant difference was observed in the VEGF165-treated group. Error bars represent standard error of the mean. Significance levels were set at $p > 0.05$.

crease in VEGF protein expression following ischemic SCI (Savas et al., 2008). In that study, no attempt was made to examine which particular VEGF protein isoforms were decreased. While previous SCI studies have demonstrated a significant increase in the expression of VEGF mRNA and VEGF receptors after injury (Choi et al., 2007; Skold et al., 2000) to the best of our knowledge, VEGF protein levels following contusion SCI have not been quantified.

A number of contributing factors may play a role in the observed decreases in VEGF protein level. We showed that VEGF was normally expressed in neurons and astrocytes (Fig. 4A,D). The dramatic loss of neurons at the site of injury 1 day after SCI (Nesic-Taylor et al., 2005) may contribute to the overall decrease in VEGF observed in this study. Additionally, roughly 50% of astrocytes die 1 day after contusion SCI at the epicenter of injury (Grossman et al., 2001). This may imply that the loss of VEGF-expressing neurons and astrocytes contributes to decreased VEGF synthesis in acutely injured spinal cords. While the initial loss of both astrocytic and neuronal populations appears to result in the reduction of VEGF protein levels, astrocytic replacement, and activation in the chronic phase of injury (Grossmann et al., 2001) does not seem to contribute to the restoration of VEGF levels. Possible downregulation of VEGF levels by activated astrocytes in chronically injured cords remains to be determined.

Although the loss of VEGF-expressing cells is likely to be the main cause of decreased VEGF protein levels in injured spinal cords, it may not be the only one. All our VEGF Western blots at all time points analyzed (1 day to 1 month post-SCI; $n = 5$ per time point; Figs. 1 and 3) showed a very strong band (<10 kD) observed only in injured spinal cords that may represent degraded VEGF. The VEGF immunolableing observed in the injured spinal cord may be that of the degraded VEGF (Fig. 5C). Previous studies have demonstrated that proteolysis of VEGF is increased in chronic wounds and that proteases such as plasmin are involved (Lauer et al., 2000). Interestingly, when plasmin is injected into the brain, it results in considerable edema from a possible effect on the endogenous VEGF which in turn may have an effect on the blood-brain barrier both acutely and chronically (Armao et al., 1997; Figueroa et al., 1998; Xue and Del Bigio, 2001). Thus, SCI-induced degradation of VEGF may be a novel mechanism that regulates protein levels and the effectiveness of VEGF in injured spinal cord.

Neuroprotective effects of $VEGF_{165}$

Significant decreases in the VEGF protein levels in injured spinal cord may affect the various roles of VEGF in the CNS: neurogenesis and angiogenesis that take place in the secondary and chronic phase after SCI and neuronal protection that is impaired in the acute phase post-injury. In this report, we focused on putative neuroprotective effects of VEGF and possible consequences of SCI-induced reduction in VEGF on neuronal survival. Studies on the effects of VEGF on neurogenesis and angiogenesis in SCI are underway.

Recent data from several groups indicate that one of the important roles of VEGF in the CNS is in the regulation of neuronal survival. For example, Jin et al. (2000) demonstrated that the application of exogenous VEGF promotes survival of rat cerebral neurons in culture and rescues HN33 hippocampal cells from death by serum withdrawal. It has been reported that VEGF also has neuroprotective effects in different CNS injury models (Facchiano et al., 2002; Lambrechts et al., 2003, 2004; Oosthuyse et al., 2001; Zachary, 2005). In these studies, it was determined that the survivial effects of VEGF are mediated through VEGFR2 triggering the anti-apoptotic pathway involving the phospatidylinositol 3′-kinase (PI3K)–dependent activation of the serine/threonine kinase, Akt, or PKB (Zachary, 2005). Thus, we hypothesized that acute exogenous administration of $VEGF_{165}$ would counteract SCI–induced reduction in $VEGF_{165}$ and affect neuronal survival after SCI. Additionally, it has been previously shown that spinal neurons express VEGF receptors (Choi et al., 2007); so, exogenous administration of VEGF in acutely injured spinal cords would predictably affect the survival of spinal neurons by the activation of the PI3K/Akt pathway. Our results, however, indicated that supplementing the injured environment with $VEGF_{165}$ showed no significant effect in neuronal survival. The lack of neuroprotection may seem intriguing since recent reports demonstrated that transfection of neuronal and astrocytes with plasmids expressing $VEGF_{165}$ has a beneficial effect on the motor recovery after SCI (Choi et al., 2007). However, it can also mean that VEGF autocrine effect may differ from paracrine VEGF effects in regulating neuronal survival. VEGF exerting an autocrine effect on neuronal survival has been shown in a previous study (Ogunshola et al., 2000).

Our result also suggests that other VEGF isoforms might be more important for direct protection of neuronal survival early on after SCI. For example, it has been shown that $VEGF_{121}$ may also have a neuroprotective role by binding to VEGFR-2 and triggering PI3K/Akt pathway (Zachary, 2005). As to which VEGF isoform in injured spinal cords is involved in neuronal protection remains to be determined. Our result shown in Figure 6 is consistent with VEGF isoforms other than 165, which is important for neuroprotection; however, suppression of endogenous VEGF with a neutralizing antibody (used in all our Western blots), which blocks the activity of all

VEGF isoforms, resulted in greater reduction in neuronal numbers after SCI compared to other groups. Similar to our results, anti-VEGF when injected into the lateral ventricle of rat brain after ischemia markedly increases infarct volume (Bao et al., 1999).

Taken together, our results suggest that SCI induces decreases in different VEGF isoforms, and that one of those isoforms is important for early neuronal protection after SCI. It is unlikely that $VEGF_{165}$, primarily involved in angiogenesis and neurogenesis as reported previously, is the isoform involved with neuroprotection. Therefore, the roles of other VEGF isoforms in injured spinal cords and the one that has direct neuroprotective effect remains to be determined. This knowledge should help in designing more effective VEGF treatments that would include several isoforms so that activated VEGF receptors on different cell types can exert their full therapeutic potential.

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Author Disclosure Statement

No competing financial interests exist.

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