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Neutralizing Endogenous VEGF Following Traumatic Spinal Cord Injury Modulates Microvascular Plasticity but not Tissue Sparing or Functional Recovery

Richard L. Benton[1,2,3,*], **Melissa A. Maddie**[1,3], **Mark J. Gruenthal**[1,3], **Theo Hagg**[2,3,4], and **Scott R. Whittemore**[1,2,4]

[1] Kentucky Spinal Cord Injury Research Center, University of Louisville School of Medicine, Louisville, KY 40292, USA

[2] Department of Anatomical Sciences and Neurobiology, University of Louisville School of Medicine, Louisville, KY 40292, USA

[3] Department of Neurological Surgery, University of Louisville School of Medicine, Louisville, KY 40292, USA

[4] Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292, USA

Abstract

Acute loss of spinal cord vascularity followed by an endogenous adaptive angiogenic response with concomitant microvascular dysfunction is a hallmark of traumatic spinal cord injury (SCI). Recently, the potent vasoactive factor vascular endothelial growth factor (VEGF) has received much attention as a putative therapeutic for the treatment of various neurodegenerative disorders, including SCI. Exogenous VEGF exerts both protective and destabilizing effects on microvascular elements and tissue following SCI but the role of endogenous VEGF is unclear. In the present study, we systemically applied a potent and well characterized soluble VEGF antagonist to adult C57Bl/6 mice post-SCI to elucidate the relative contribution of VEGF on the acute evolving microvascular response and its impact on functional recovery. While the VEGF Trap did not alter vascular density in the injury epicenter or penumbra, an overall increase in the number of *Griffonia simplicifolia* isolectin-B4 bound microvessels was observed, suggesting a VEGF-dependency to more subtle aspects of endothelial plasticity post-SCI. Neutralizing endogenous VEGF neither attenuated nor exacerbated chronic histopathology or functional recovery. These results support the idea that overall, endogenous VEGF is not neuroprotective or detrimental following traumatic SCI. Furthermore, they suggest that angiogenesis in traumatically injured spinal tissue is regulated by multiple effectors and is not limited by endogenous VEGF activation of affected spinal microvessels.

Keywords

VEGF Trap; Aflibercept; intravital lectin; neovascularization; endothelial; Basso Mouse Scale for Locomotion (BMS)

*Address correspondence to this author at the Kentucky Spinal Cord Research Center, 511 South Floyd Street, MDR 616, Louisville, KY 40292, USA; Tel: 502-852-1333; Fax: 502-852-5148; rlbent01@louisville.edu.

INTRODUCTION

Traumatic spinal cord injury (SCI) results in both immediate loss of spinal vascular support and the initiation of multiple molecular cascades resulting in continued microvascular plasticity and dysfunction, which persist for days to weeks. The immediate "traumatic phase" of vascular disruption and the subsequent compromise of perfusion contribute to the profound loss of neural elements. This is caused by the high metabolic demand of spinal gray matter and the especially intricate anatomy of gray matter microvascular beds (Ducker and Assenmacher, 1969). Tissue ischemia is caused by physical destruction of microvasculature, and exacerbated by petichial hemorrhage, vasogenic edema (Yang *et al.*, 1994), excitotoxicity (Bullock and Fujisawa, 1992), and loss of penumbral blood flow due to onset of vasospasm/vasoconstriction (Ducker and Assenmacher, 1969). This prolonged ischemia initiates an endogenous pathoangiogenic response lasting for days to weeks post-SCI, giving rise to a neovascularization of affected spinal tissue (Whetstone *et al.*, 2003; Benton *et al.*, 2008a; Beggs and Waggener, 1979; Zhang and Guth, 1997; Imperato-Kalmar *et al.*, 1997). These newly formed vascular networks exhibit multiple pathologic attributes including decreased glucose transport potential, increased blood-spinal cord barrier (BSCB) permeability, abnormal glycocalycal phenotype, and altered integrity of tight-junctions (Noble and Wrathall, 1989; Noble *et al.*, 1996; Whetstone *et al.*, 2003; Popovich *et al.*, 1996; Benton *et al.*, 2008a). A number of vasoactive molecules are thought to participate in this vascular pathophysiology specific to SCI, including the potent vasoactive factor vascular endothelial growth factor (VEGF) (Sharma, 2005). However, the molecular control of these microvascular responses following SCI remains unclear.

Immediately following traumatic SCI, tissue levels of VEGF mRNA (Skold *et al.*, 2000; Bartholdi *et al.*, 1997) and protein (Vaquero *et al.*, 1999; Akiyama *et al.*, 2004) are dramatically upregulated, with levels normalizing by 14 days post-injury (Vaquero *et al.*, 1999). While the temporal profile of this increase in VEGF occurs coincident with vascular plasticity and BSCB pathophysiology (Loy *et al.*, 2002; Casella *et al.*, 2002; Whetstone *et al.*, 2003; Benton *et al.*, 2008a; Bartholdi *et al.*, 1997; Imperato-Kalmar *et al.*, 1997; Beggs and Waggener, 1979), it is unknown to what degree VEGF mediates these vascular events following traumatic SCI. Acute injection of exogenous VEGF following contusive injury in the adult rat can be neuroprotective (Widenfalk *et al.*, 2003) or can exacerbate tissue loss with no enhanced vascular protection (Benton and Whittemore, 2003). More recently, indirect evidence of the deleterious vasoactive actions of VEGF in the context of traumatic SCI has been provided (Akiyama *et al.*, 2004). To date, no report exists regarding the role of endogenous VEGF or the effect of direct antagonism of VEGF in SCI. Thus, the purpose of the current study was to directly antagonize VEGF action in the injured spinal cord using systemic administration of a neutralizing VEGF Trap and examine the subsequent effects on vascular plasticity, tissue sparing and recovery of function following contusive SCI.

MATERIAL AND METHODS

Contusive Mouse SCI

All surgical intervention, perioperative care, and treatment of all animals used in this study were in strict accordance with the PHS Policy on Humane Care and Use of Laboratory Animals and approved by the University of Louisville IACUC committee. Contusive spinal cord injuries (T9/10 spinal segment) of moderate severity (50 kdyn force/500–600 μm displacement) were performed on a total of 26 adult female C57Bl/6 mice (18–20 g, Harlan) as previously described (Benton *et al.*, 2008a).

Administration of VEGF Trap

Injured mice were then randomly divided into two treatment groups receiving a single intravenous bolus of VEGF Trap (Aflibercept, 12.5 mg/kg, n = 13; a generous gift from Regeneron Pharmaceuticals Inc., Tarrytown, NY) or hFc control peptide (12.5 mg/kg, n = 13) at the time of SCI. The VEGF Trap (R1R2) is a fully soluble VEGF decoy created by fusing the immunoglobulin 2 domain of VEGFR1 with the immunoglobulin domain of VEGFR2 (Holash *et al.*, 2002). VEGF Trap also comprises the Fc domain of human IgG1, which induces forced homodimerization of the extracellular VEGF receptor domains. VEGF Trap has a kD of ~pM and superior pharmacokinetic characteristics (t1/2 of ~20 hours in a mouse), ensuring that endogenous VEGF can be neutralized of the first post-injury week with a single loading dose. VEGF Trap exhibits potent anti-tumor effects *in vivo* by interruption of malignant neovascularization and is currently in clinical trials for this application (Rudge *et al.*, 2005). More recently, systemic administration of VEGF Trap has been shown to decrease detrimental aspects of pathoangiogenesis in retinal vascular beds (Shah *et al.*, 2006; Saishin *et al.*, 2003), suggesting efficacy at the level of the blood-CNS barrier.

Intravital Labeling of Spinal Microvasculature

To assess acute spinal microvascular plasticity, neovascularization and tissue sparing, on day 7 post-SCI, a subset of mice in each group (VEGF Trap/n=5, hFc/n=5) were deeply anesthetized. Next, 100 μg/50 μl of FITC-conjugated *Griffonia simplicifolia* isolectin B4 (FITC-IB4, L-9381; Sigma, St. Louis, MO) was delivered systemically by intravenous injection *via* the right external jugular vein and allowed to circulate for 15 minutes. Mice were then transcardially perfused with 10 ml of saline followed by 15 ml of 4% paraformaldehyde (PFA). Spinal cords were dissected and longitudinally sectioned at 20 Nm on a cryostat, slide-mounted, and stored at −80°C until use.

Immunohistochemical analyses and quantitative assessment of microvascular density-To determine vascular density in injury epicenters, vascular endothelial cells (ECs) were identified using a monoclonal rat anti-PECAM-1 antibody (#550274, 1: 50, BD Pharmingen, San Diego, CA). SCI epicenters were identified by quantification of extravascular laminin deposition using polyclonal rabbit anti-laminin (L9393, 1: 100, Sigma, St. Louis, MO)(Benton *et al.*, 2008a; Whetstone *et al.*, 2003). The density of neovascular structure in affected spinal tissue was examined by analyzing the density of luminal binding of FITC-IB4, which identifies activated/angiogenic blood vessels post-SCI (Benton *et al.*, 2008a). Primary antibodies were applied in 0.1M TBS, 0.1% Triton X-100, 0.5% BSA, and 5% normal donkey serum overnight in a humidified chamber at 4°C and epitopes visualized using Rhodamine- (TRITC; 1: 200 dilution) or AMCA- (1: 100 dilution) conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). All photomicrographs demonstrating immunostained spinal cord sections were captured using a Nikon TE 300 inverted microscope equipped with a Spot CCD camera and capture software (Diagnostic Instruments Inc., Sterling Heights, MI). Quantitative assessment of intravascular IB4 binding, vascular EC immunohistochemistry, and lesion area 7 days post-SCI was accomplished as previously described (Benton *et al.*, 2008a) using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Briefly, every 5th longitudinal section from each experimental case was processed for stated markers and the epicenter for each case was defined and converted to an area of interest (AOI; Fig. 1) and pixel area of each AOI was automatically calculated by the software. The density of epicenter binding/staining for each image was then calculated by ratio of the total pixels of binding/staining for each measure over the total pixels in the AOI area. Penumbral data were obtained by calculating the density in a 500 μm area bordering each epicenter AOI. All quantitative data for the 7 day post-SCI analyses are expressed as mean ± s.d. and statistical analysis of these data was accomplished using a Student's *t*-test.

Chronic Assessment of Functional Recovery

The remainder of the mice (n=7/hFc control, n=8/VEGF Trap) were allowed to recover for 6 weeks post-SCI and were assessed weekly for locomotor function using the Basso Mouse Scale (BMS) (Basso *et al.*, 2006). Briefly, locomotor function in spinal cord injured mice was determined during weekly sessions post-SCI by two experienced raters presented with animals in random order. Mice are placed in a round field with a smooth bottom and allowed to freely ambulate for a period of 4 minutes. The low end of the scale (0–4) is characterized by individual joint movements, whereas the intermediate (4–6) and high (6–9) ends of the scale are characterized by weight support and coordination and paw position, respectively. At the end of the experimental period (day 42 post-SCI), mice were euthanized and perfused as described above. A total of 1 cm of spinal cord tissue containing the injury site at its center was dissected, blocked, and sectioned transversely at 20 μm on a cryostat. Spared gray matter was assessed by immunohistochemistry performed as stated above using polyclonal rabbit anti-Map2 (AB5622, 1: 250, Chemicon Inc., Temecula, CA). Every 25th section (≈500 μm resolution) was processed for Map2-immunoreactivity and images captured as stated above. Quantitative per area gray matter preservation was obtained by converting the total spinal cord section to an AOI and calculating the percentage of that area occupied by Map2-immunoreactivity. Spared white matter was assessed eriochrome cyanine staining as described elsewhere (Rabchevsky *et al.*, 2001) in a manner similar to that used to assess gray matter sparing. All quantitative data for the 42 day post-SCI analyses are expressed as mean ± s.d. and statistical analysis of these data was performed by two-way ANOVA with a Tukey HSD post hoc *t*-test.

RESULTS

Previous studies have shown that by 7 days post-SCI, epicenter vascularity is significantly greater than that observed 1 day following injury, consistent with an adaptive angiogenesis (Whetstone *et al.*, 2003; Benton *et al.*, 2008a). VEGF Trap treatment did not alter total vascularity as measured by PECAM-1 immunodensity in the injury epicenter or penumbral zones (Fig. 2A). This result suggests that VEGF expressed at the site of SCI is not essential for the SCI-induced neovascular response in and around the injury site. Despite having no effect on total parenchymal vascularity, significantly more of the regenerated blood vessels both within the lesion/heterodomain proper ($t = -5.61$, df = 7, $P < 0.001$) and penumbral zones ($t = -5.94$, df = 7, $P < 0.001$) expressed luminal affinity for IB4 (Fig. 2B). This result suggests that the VEGF Trap is biologically active in the injured cord and that, surprisingly, endogenous VEGF alters EC plasticity in a pattern that appears to modulate the functional status of neovascular beds in and around the injury site, as luminal IB4 affinity appears to be related to the maturation state of newly formed blood vessels in the injured spinal cord (Benton *et al.*, 2008a).

While little is known about how the maturation of angiogenic CNS microvessels is manifested by luminal phenotype, previous findings have suggested that luminal glycocalycal phenotype mediates inflammation contributing to histopathology following SCI (Noble *et al.*, 1996). To address these possibilities, we analyzed lesion sizes as assessed by the pathologic deposition of extravascular laminin, an area for epicenter histopathology referred to as the "heterodomain" (Whetstone *et al.*, 2003) (Fig. 2C; hatched outline). We found that sub-acute (7 day post-SCI) histopathology is not affected by the VEGF Trap treatment or the increased presence of IB4+ neovascular structure (Fig. 3). Furthermore, VEGF Trap treatment did not appear to alter inflammatory responses at this time point as determined by a quantitative assessment of Iba1-immunoreactive activated microglia/macrophages at the injury sites as well as in penumbral zones of the lesion (data not shown).

Histopathology was also assessed in mice that survived 42 days post-injury. Overall, lesion lengths were found to be appropriate for the C57BL/6 strain at this time point post-SCI (Kigerl

et al., 2006) suggesting consistent injury severities for mice in both experimental groups. Spinal gray matter was nearly completely lost at the injury epicenter, with significant histopathology extending approximately 2 mm rostral and caudal to the epicenter (Fig. 4A), with similar observations were made for the expansion of demyelinated white matter (Fig. 4B). Neither white nor gray matter loss post-SCI was significantly affected by treatment with the VEGF Trap. Terminal locomotor scores were statistically identical in each experimental group (Fig. 4C), consistent with histologic data.

DISCUSSION

While somewhat unexpected, the relative insensitivity of the spinal microvascular plexus to VEGF Trap mediated VEGF antagonism is consistent with previous findings. In peripheral tissues, the responsiveness of vessels to VEGF blockade is attenuated with age (Baffert *et al.*, 2004), an effect that was postulated be related to diminished VEGF receptor expression. Recent data show EC mRNA expression of VEGF-R1 (flt-1) and VEGF-R2 (flk-1) decreased by 24 hours post-SCI (Benton *et al.*, 2008b). Capillaries in the intact adult cortex are the least dependent upon VEGF for their survival/maintenance as compared to vascular beds in other organs (Kamba *et al.*, 2006). Despite this, VEGF-R1 does appear to drive pathoangiogenesis in mild traumatic brain injury (TBI) (Krum *et al.*, 2008). Furthermore, in a model of retinal neovascularization, pathologic choroidal vascular plasticity is also VEGF-dependent (Saishin *et al.*, 2003). However, this dependency appears to be quite complex as exemplified by recent findings in stroke. Administration of VEGF blocking antibodies following focal cortical ischemia ameliorates blood-brain barrier (BBB) leakage (Chi *et al.*, 2007). This therapeutic action appears to be VEGF isoform specific, as addition of exogenous VEGF-B actually reduces stroke infarct volume with no change in BBB permeability (Li *et al.*, 2008). Results from the present study would suggest a similarly complex role for VEGF in vascular remodeling following traumatic SCI. Thus, affected spinal microvessels may not exhibit the same requirement for VEGF activity as is observed following TBI or in other pathologically activated CNS vascular beds and/or its requirement is isoform specific.

Despite having no impact on sub-acute vascularity in injury epicenters, VEGF antagonism alters the anatomy and/or physiology of regenerated blood vessels after SCI. It is possible that the increased affinity for IB4 in affected vasculature is merely the result of enhanced perfusion of neovascular elements in the injured spinal cord. Blockade of VEGF in activated tumor vessels using an approach similar to that employed in the current study results in increased perfusion pressures and improved blood flow in the tumor stroma (Tong *et al.*, 2004). This would be consistent with the “normalization” of plastic microvascular plexi by VEGF antagonism, which is an emerging concept in the context of tumor neovascularization (Fukumura and Jain, 2007). Alternatively, VEGF Trap may alter neovascular phenotype in the absence of functional changes. VEGF is known to alter the functional luminal phenotype of extra-CNS microvessels *in vivo* (Melder *et al.*, 1996) as well as induce disruption of the endothelial glycocalyx (Fu and Shen, 2003). Current results would most directly support this latter explanation.

Fundamentally, present data highlight the complexity of vascular regulation after SCI, a precedent for which exists in peripheral tissue pathology. Specifically, comparable micro-vascular resistance to VEGF-R1 and VEGF-R2 antagonism in pathologic angiogenesis is currently of intense investigation, especially in the context of tumor neovascularization (Shojaei and Ferrara, 2008; Bergers and Hanahan, 2008). To date, several explanations for this phenomenon have been proposed, which fall under the conceptual categories of adaptive/evasive resistance and intrinsic non-responsiveness (Bergers and Hanahan, 2008). Several of these possibilities are quite plausible in the context of SCI. For example, several alternative pro-angiogenic pathways are induced in solid tumors and appear to contribute to the

circumvention of an absolute dependence on VEGF for neovascularization. These include fibroblast growth factor (Fgf) (Casanovas *et al.*, 2005), interleukin 8 (IL8) (Mizukami *et al.*, 2005), and platelet derived growth factor-alpha (PDGFA) (Fernando *et al.*, 2008). Importantly, all of these pro-angiogenic cytokines are upregulated in injured/inflamed spinal tissue (Tripathi and McTigue, 2008; Sun *et al.*, 2008; Ishizu *et al.*, 2005). It is possible that a comparable "redirection" of the endogenous angiogenic response may occur in SCI, with increased activation of these angiogenic pathways in affected microvascular ECs.

Unbiased transcriptional screening of ECs isolated from tumor microvasculature has identified a number of novel regulators of tumor neovascularization (St *et al.*, 2000). Of those identified, Delta-like ligand 4 (DLL4) was robustly enriched in tumor ECs, suggesting a role for Notch signaling in vascular activation in solid tumors. Indeed, this pathway appears to be quite relevant to tumor neovascularization refractory to VEGF blockade (for review see Thurston and Kitajewski, 2008). Blockade of DLL4 reduces tumor size by disrupting microvascular function and had additive effects when combined with anti-VEGF therapy (Ridgway *et al.*, 2006; Noguera-Troise *et al.*, 2006). Interestingly, spinal microvascular ECs express detectable levels of DLL4 acutely following SCI (unpublished observations) suggesting a comparable co-stimulatory role for Notch in angiogenesis in the injured spinal cord. Currently, studies are underway to determine to what extent Notch activation regulates EC survival and/or plasticity following SCI.

Finally, in many pathoangiogenic contexts it is likely that lack of complete efficacy of VEGF-R1 and –R2 blockade may be due to incomplete suppression of VEGF signaling in activated ECs. This is most likely due to expression of neuropilin1 (NRP1) receptor, which effects vascular remodeling both independently and in combination with VEGF-R2 (Pan *et al.*, 2007). Furthermore, very recent evidence has identified a novel VEGF receptor isoform (i.e. VEGF-R3), which appears to be critical for the neovascular response in solid tumors (Su *et al.*, 2008; Tammela *et al.*, 2008; Petrova *et al.*, 2008). To date, no data exist regarding EC expression of NRP1 in the injured spinal cord, although it has recently been implicated in regenerative neuronal responses following SCI (Mire *et al.*, 2008).

In summary, the results from the current study demonstrate the complexity of the molecular control of micro-vascular responses to SCI. Further, they suggest targeting a single molecule as a therapeutic intervention might not result in dramatic modulation of pathoangiogenic cascades. As other molecular effectors of pathoangiogenesis in the injured/diseased CNS are identified, future experiments utilizing combinatorial approaches, of which VEGF signaling will likely be a component, may have enhanced potential as viable therapeutic strategies to stabilize and improve microvascular function in CNS pathology.

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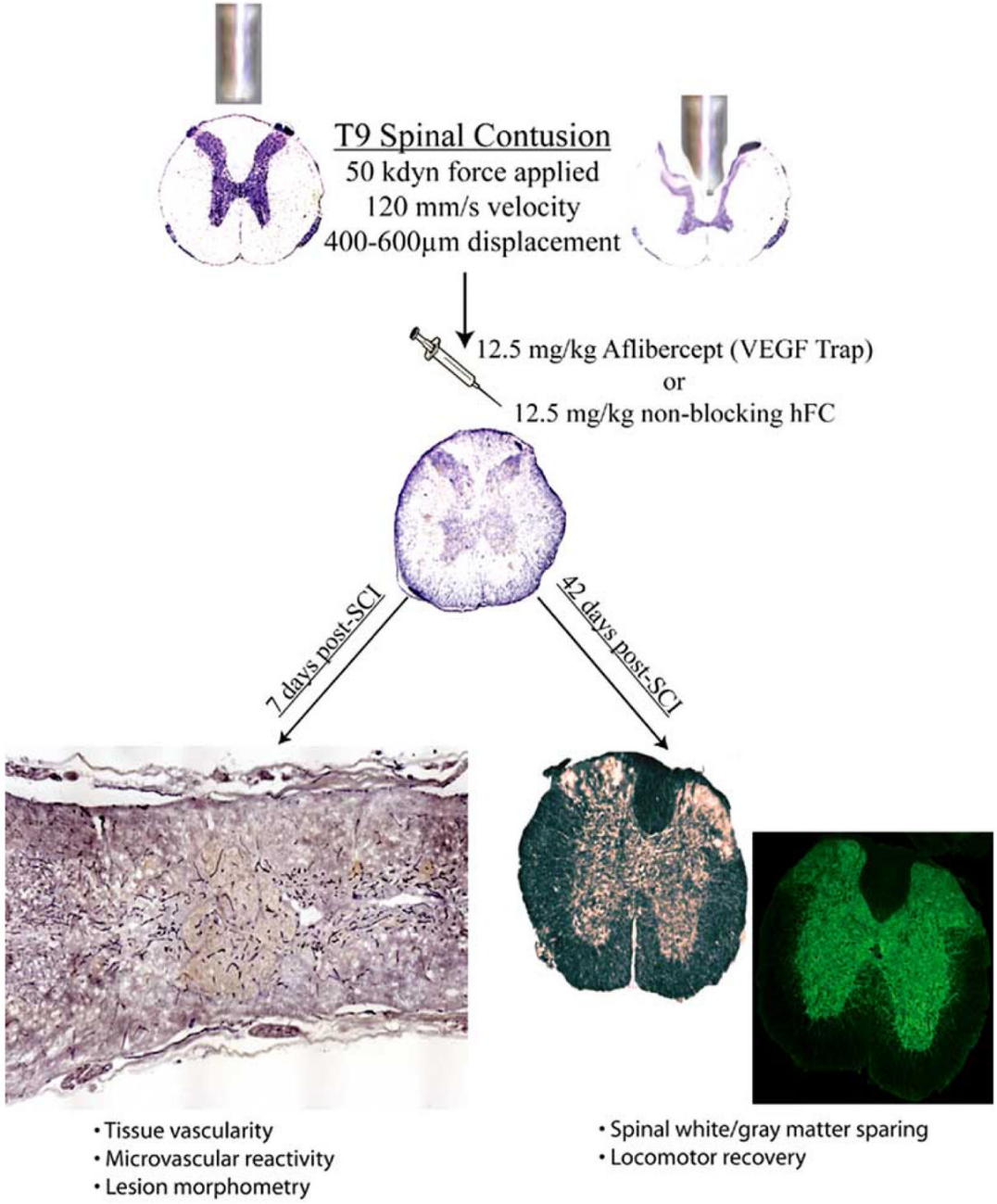

Fig. 1.
Schema of experimental protocol.

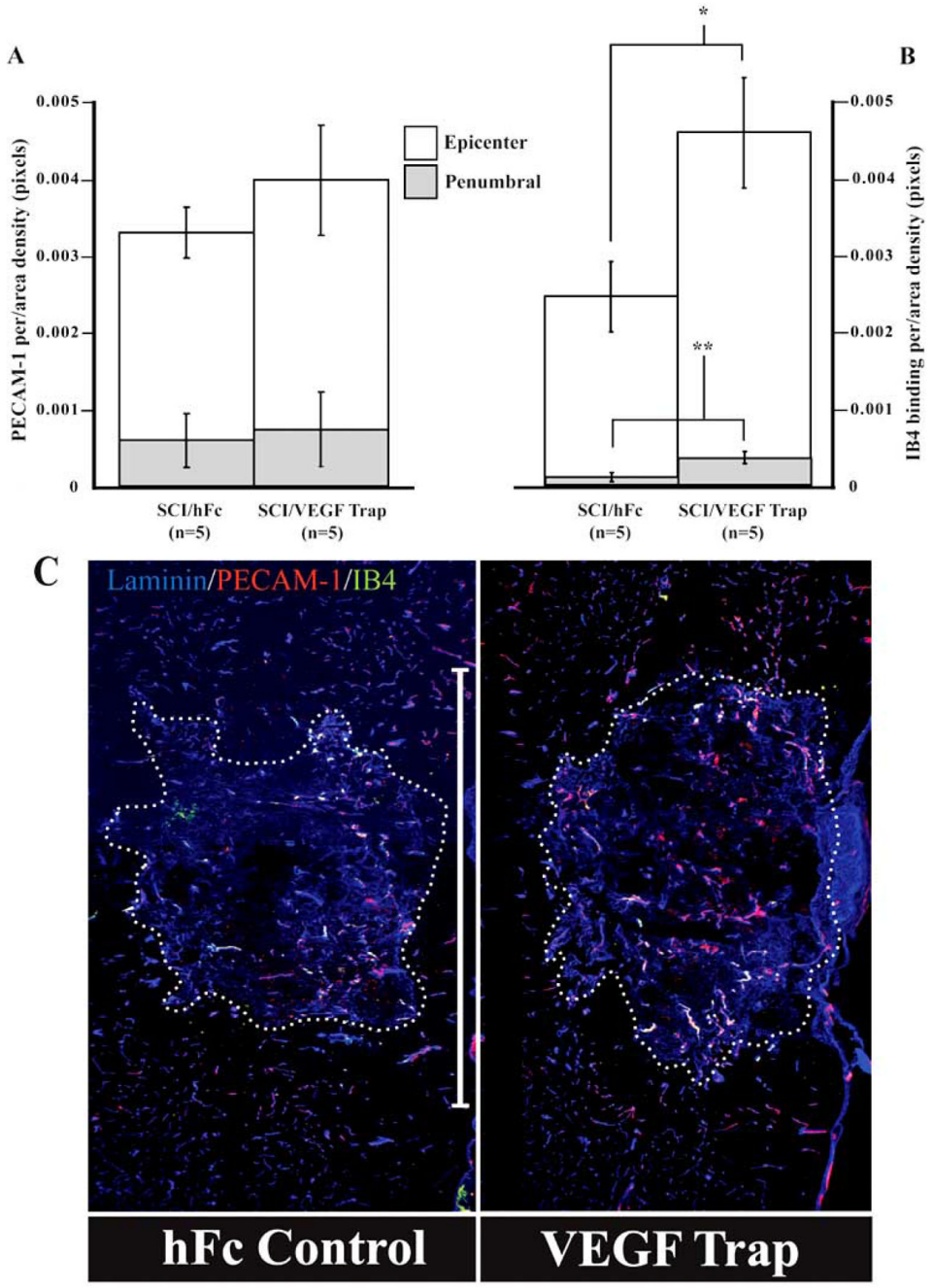

Fig. 2.

Neutralizing endogenous VEGF increases neovascular profiles 7 days post-SCI. Total vascularity (PECAM-1-immunoreactivity) and IB4 binding was assessed in injury epicenters (C; hatched line) and in penumbral tissue (i.e., a 500μm zone surrounding epicenters). Quantitative data show total vascularity to be unaffected by VEGF-Trap 7 days post-SCI (**A**). By contrast, vascular activation/angiogenesis as demonstrated by IB4-bound microvessels is significantly increased in the injury epicenter and penumbra (**B**). All quantitative data are the mean ± s.d. (* P = 0.0008, ** P = 0.0006) Scale bar (**C**) = 1mm.

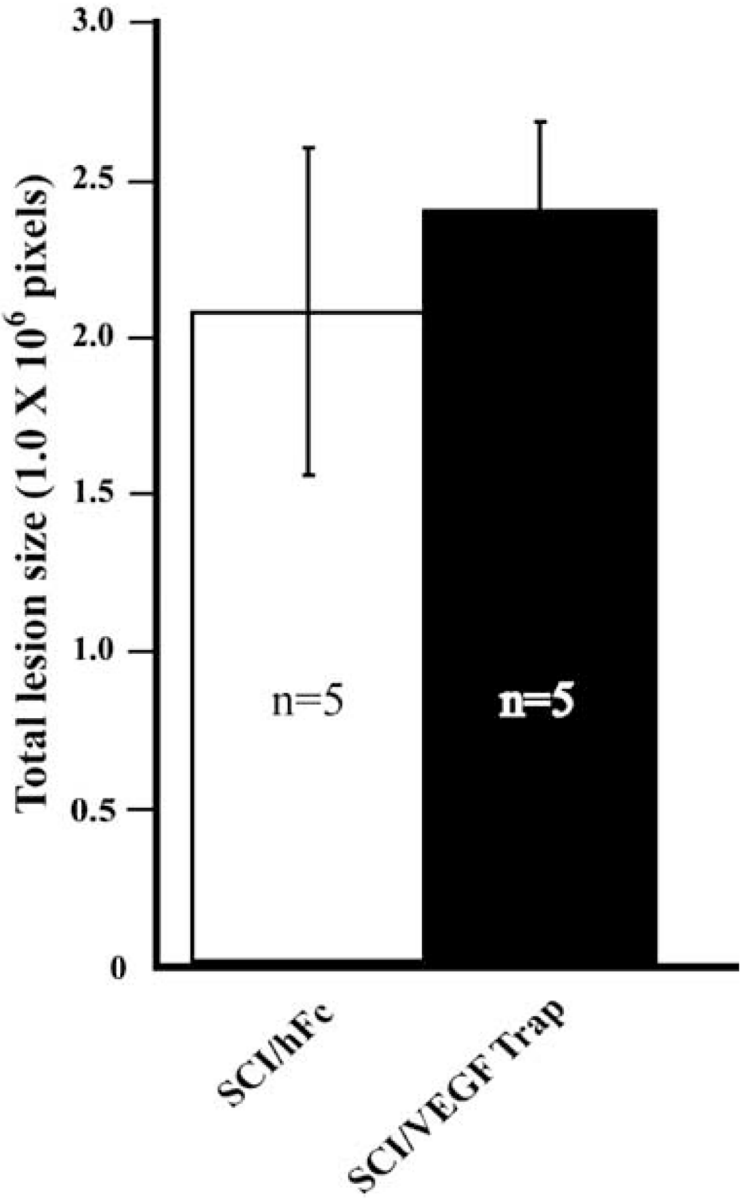

Fig. 3.

Lesion sizes are unaffected by VEGF Trap treatment at 7 days post-SCI. Areas of pathologic tissue transformation (see Fig. (**2**); hatched outline) were quantified. Blockade of endogenous VEGF acutely had no chronic effect on lesion evolution. All quantitative data are represented as the mean ± s.d.

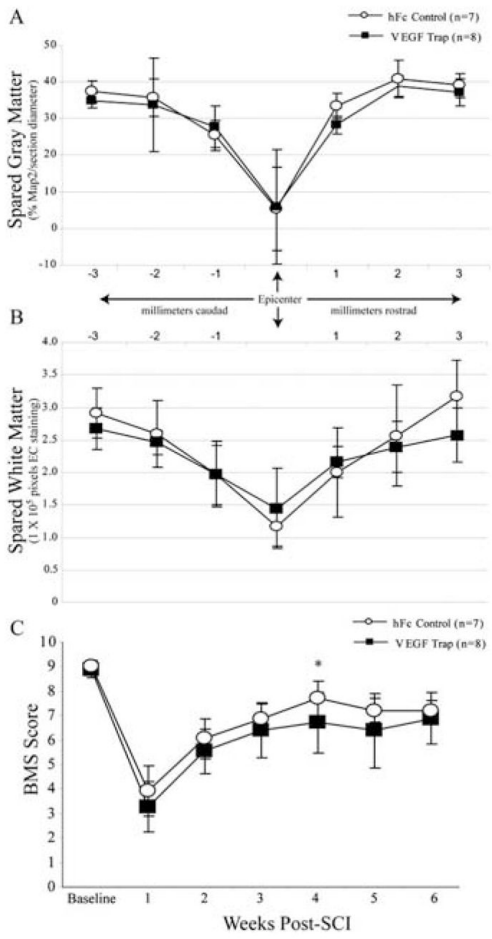

Fig. 4.

Neutralizing endogenous VEGF does not alter chronic histopathology or alter locomotor recovery following SCI. Spared spinal gray (**A**) and white (**B**) matter was analyzed 42 days post-SCI, with no effect observed with VEGF Trap treatment. Similarly, no difference was observed in terminal functional outcome as assessed by BMS evaluation (**C**). The only exception was a statistically significant decrease in BMS scores in the VEGF Trap treated group at 4 weeks post-SCI ($P < 0.05$), which was not observed in subsequent evaluation sessions. All data are represented as the mean ± s.d.