Effect of VEGF Treatment on the Blood-Spinal Cord Barrier Permeability in Experimental Spinal Cord Injury: Dynamic Contrast-Enhanced Magnetic Resonance Imaging

Chirag B. Patel,¹ David M. Cohen,² Pallavi Ahobila-Vajjula,¹ Laura M. Sundberg,¹
Tessy Chacko,¹ and Ponnada A. Narayana¹

Abstract

Compromised blood-spinal cord barrier (BSCB) is a factor in the outcome following traumatic spinal cord injury (SCI). Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis and vascular permeability. The role of VEGF in SCI is controversial. Relatively little is known about the spatial and temporal changes in the BSCB permeability following administration of VEGF in experimental SCI. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) studies were performed to noninvasively follow spatial and temporal changes in the BSCB permeability following acute administration of VEGF in experimental SCI over a post-injury period of 56 days. The DCE-MRI data was analyzed using a two-compartment pharmacokinetic model. Animals were assessed for open field locomotion using the Basso-Beattie-Bresnahan score. These studies demonstrate that the BSCB permeability was greater at all time points in the VEGF-treated animals compared to saline controls, most significantly in the epicenter region of injury. Although a significant temporal reduction in the BSCB permeability was observed in the VEGF-treated animals, BSCB permeability remained elevated even during the chronic phase. VEGF treatment resulted in earlier improvement in locomotor ability during the chronic phase of SCI. This study suggests a beneficial role of acutely administered VEGF in hastening neurobehavioral recovery after SCI.

Key words: BBB score; blood-spinal cord barrier; DCE-MRI; spinal cord injury; VEGF

Introduction

FOLLOWING MECHANICAL TRAUMA TO SPINAL CORD, a series of pathobiological events ensue, leading to the so-called “secondary injury.” It is generally thought that much of the neurologic deficit in SCI is the result of secondary injury. It is therefore not surprising that major therapeutic efforts have been directed towards slowing down or reversing the progression of secondary events in SCI (Bradbury and McMahon, 2006; Rossignol et al., 2007). Dynamic vascular changes are thought to play an important role in the evolution of secondary injury in SCI (Shingu et al., 1989; Blight, 1991; Popovich et al., 1996; Tator and Koyanagi, 1997; Mautes et al., 2000; Bilgen et al., 2001; Westergren et al., 2001; Casella et al., 2002; Loy et al., 2002; Narayana et al., 2004; Maikos and Shreiber, 2007). Among others, some of the specific vascular changes include angiogenesis and disruption of the blood-spinal cord barrier (BSCB).

Vascular endothelial growth factor (VEGF) has multiple and diverse functions that include endothelial cell mitogen-

esis, endothelial cell survival via antiapoptotic effects, vasodilatation, and increased vascular permeability (Sledge, 2002). Recent studies also indicate that VEGF exerts direct neurotrophic effects (Jin et al., 2000; Matsuzaki et al., 2001; Facciano et al., 2002; Svensson et al., 2002), plays a role in axonal growth (Kawakami et al., 1996), and stimulates proliferation of stem cells (Jin et al., 2002; Zhu et al., 2003). Studies by Bartholdi et al. (1997) and Skold et al. (2000) have demonstrated that cells within the lesioned spinal cord area are capable of expressing VEGF. Krum et al. (2002) demonstrated that VEGF infusion produced a remarkable localized neovascularization, leading to a 100% increase in the index of cerebral vascular proliferation at 3 days following VEGF delivery. Similarly, Harrigan et al. (2002) reported that intraventricular infusion of VEGF resulted in an increase in capillary permeability and vessel density in a dose-dependent manner.

Investigations into the role of VEGF in experimental SCI have produced conflicting results that are summarized in Table 1. The detrimental effect of VEGF in SCI was reported

¹Department of Diagnostic and Interventional Imaging, University of Texas Medical School at Houston, Houston, Texas.

²Anaplerosis Associates, Inc., Omaha, Nebraska.

TABLE 1. SUMMARY OF *IN VIVO* RODENT STUDIES INVESTIGATING THE ROLE OF VEGF IN SCI

Authors	Source of VEGF	VEGF's effect in SCI	Suggested mechanism
Vaquero et al., 1999	Endogenous (detected via monoclonal antibody)	Negative	Edema
Facchiano et al., 2002	Exogenous (human recombinant VEGF or adenovirus coding for VEGF)	Positive	Angiogenic and neurotrophic effects
Widenfalk et al., 2003	Exogenous (human recombinant VEGF)	Positive	Protection/repair of blood vessels and decreased apoptosis
Benton and Whittemore, 2003	Exogenous (human recombinant VEGF)	Negative	Increased blood-spinal cord barrier permeability
Xiaowei et al., 2006	Endogenous (upregulation via post-injury hypoxia)	Positive	Hypoxia tolerance and vascularity of injured spinal cord
Choi et al., 2007	Endogenous (hypoxia-inducible VEGF plasmid)	Positive	Decreased apoptosis
Sakanaka et al., 2007	Endogenous (upregulation via intravenous injection of dihydrogensenoside Rb1)	Positive	Protects against ischemia
Kao et al., 2008	Endogenous (upregulation via administration of umbilical cord blood-derived CD34 + cells)	Positive	Reversing SCI-induced spinal cord infarction and apoptosis

by Benton and Whittemore (2003). In these studies, a single dose of VEGF was injected into the injured cord in a rat model of contusion SCI. Profound and acute changes in the microvascular permeability were observed in gray matter (GM). By 6 weeks post-SCI, a profound exacerbation of lesion volume was apparent. These authors concluded that "intraparenchymal application of VEGF may exacerbate SCI, likely through the effect on vessel permeability" (Benton and Whittemore, 2003). In contrast, at least two other studies have observed beneficial effects of VEGF in SCI. In one study, Facchiano et al. (2002) investigated the effect of short- and long-term administration of VEGF to the injured cord in a rat model of transection SCI. A significant reduction in the extent of retrograde corticospinal tract (CST) axonal degeneration was observed, and evidence of axonal regeneration was present. The reduced degeneration and increased regeneration of axons was more evident when short-term VEGF treatment was combined with long-term treatment. These improvements appear to be the result of increased angiogenic activity and possible neurotrophic effect exerted by VEGF (Facchiano et al., 2002). In a rat model of contusion SCI, Widenfalk et al. (2003) administered a single injection of VEGF directly into the cord on the day of injury. At 6 weeks post-injury, VEGF-treated animals showed improved behavioral scores relative to controls. Widenfalk et al. (2003) concluded that the beneficial effect seen in VEGF-treated animals could be the result of increased blood vessels, protection and repair of vasculature, decreased apoptosis, and possible protective effects on other cells (Widenfalk et al., 2003).

As pointed out earlier, VEGF has an important effect on BSCB permeability. It has been postulated that VEGF exacerbates injury by increasing the BSCB permeability and promoting edema (Vaquero et al., 1999; Benton and Whittemore, 2003). However, relatively little is known about spatial and temporal evolution of the BSCB permeability following the administration of VEGF in SCI. Such an understanding is critical for evaluating the role of VEGF in SCI.

Traditionally, the BSCB permeability has been assessed *ex vivo*, using histological techniques. While such studies provide a wealth of information about the BSCB permeability at very high spatial resolution, histological approaches provide only static "snap shots" of the state of the BSCB permeability and require large numbers of animals for statistically reliable results. Therefore, noninvasive *in vivo* techniques for evaluating the BSCB permeability are highly desirable. Such techniques allow both spatial and temporal changes in the BSCB permeability to be tracked in the same group of animals. This methodology requires fewer animals and, more importantly, is clinically relevant.

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is a powerful noninvasive technique for gaining valuable information about the BSCB permeability in SCI (Runge et al., 1997; Bilgen et al., 2001; Bilgen and Narayana, 2001). DCE-MRI involves repeated acquisition of T1-weighted MR images following intravenous administration of paramagnetic contrast agents such as gadopentate dimeglumine (Gd-DTPA, or Gd, for short) (Tofts et al., 1999). Normally, the BSCB is impermeable to large molecules such as Gd (molecular weight of 938 Da). However, when the BSCB is compromised, Gd leaks out of the systemic vasculature into the spinal cord, rendering these areas hyperintense on T1-weighted MRI scans. In SCI, one generally observes two types of enhancements on MRI: (1) diffuse enhancements (DE) that are mainly the result of mechanical disruption of the vasculature and are confined to the epicenter of injury and (2) focal enhancements (FE) that are thought to represent neovasculature and are generally seen away from the SCI epicenter (Bilgen et al., 2001). In addition, areas that appear normal on post-contrast T1-weighted images, termed non-enhancing (NE), but with somewhat compromised BSCB, are also observed (Cohen et al., 2009). Using an appropriate pharmacokinetic model (Bilgen and Narayana, 2001), the temporal changes in the contrast agent-induced signal increase can be quantitatively related to the BSCB permeability (Bilgen et al.,

2001, 2002, 2007; Bilgen and Narayana, 2001; Cohen et al., 2009).

In this study, we used DCE-MRI to investigate the effects of acutely administered VEGF on the spatial and temporal evolution of the BSCB permeability up to post-injury day 56. In addition, the behavioral status of the animals was assessed using the Basso-Beattie-Bresnahan (BBB) score (Basso et al., 1995). Our studies show that the BSCB permeability is higher in VEGF-treated animals relative to saline-treated controls at all time points. These longitudinal studies also provide evidence of reduced BSCB permeability with time. Our studies also suggest that acute VEGF treatment may be beneficial in hastening neurobehavioral recovery after SCI.

Methods

The protocol employed in these studies was approved by the institutional Animal Welfare Committee. All animal procedures were performed according to the *NIH Guide for the Care and Use of Laboratory Animals*.

Spinal cord injury

Male Sprague-Dawley rats (300–350 g) were anesthetized with spontaneous inhalation of isoflurane (4%). They were then intubated and maintained under anesthesia by mechanical ventilation. The spinal cord at the level of T7 was exposed by removing the T7 spinous process and the corresponding laminae, and a moderately severe contusion injury was produced using an in-house designed computer-controlled injury device that has been shown to produce consistent injuries (Cohen et al., 2009; Narayana et al., 2004). Briefly, the injury device was designed to produce contusive-type injury and is based on a linear motor (P01-23x80; LinMot Inc., Zurich, Switzerland) mounted on an *x-y-z* platform (Sherline Products Inc., Vista, CA). The tip of the injury bit measured 0.5 mm × 0.5 mm. The mechanical parameters were as follows: impact speed of bit = 1.5 m/sec; cord compression = 1.7 mm; duration of compression = 80 ms. These mechanical parameters were shown to produce a consistent and moderately severe injury to the spinal cord (Narayana et al., 2004). As judged by the BBB locomotor score, which, as a behavioral outcome measure, is one of the best ways to validate the severity of

injury across different SCI models (Stokes and Jakeman, 2002), our device produces a moderately severe injury that is comparable to that produced by commonly used SCI devices (Table 2).

A piece of Gelfoam (Pharmacia & Upjohn Company, Kalamazoo, MI) soaked with saline (40 μ L of 0.9% saline; Hospira Inc., Lake Forest, IL) or VEGF (4 μ g; R&D Systems, Minneapolis, MN; diluted in 40 μ L of 0.1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]), was placed on the contusion site immediately after SCI. The VEGF dose of 4 μ g was selected based on previous results from Widenfalk et al. (2003), who studied doses of 1 μ g (low-dose), 4 μ g, and 20 μ g (high-dose) of VEGF in a rodent model of SCI. They found that 4 μ g led to better outcomes than 1 μ g, and that 20 μ g did not show significantly improved outcome compared to 4 μ g.

For improved signal-to-noise ratio (SNR) in MRI, a RF coil was implanted subcutaneously over the injury site without touching the spinal cord (Fenyves and Narayana, 1998). The wound was closed in two layers. These serial studies required repeated administration of contrast agent on different days. For intravenous delivery of Gd (Magnevist; Berlex Laboratories, Montville, NJ) during the DCE-MRI scans, the right jugular vein was cannulated, and a vascular port with silicone tubing (Instech Solomon, Plymouth Meeting, PA) was implanted, and the incisions were closed. To ensure patency of the jugular port and connected tubing over the 8-week duration of the serial studies, 0.2 mL of 0.9% saline was injected into the port before and after each scan. If resistance on the plunger of the syringe was felt during administration of the saline bolus, the port or tubing was assumed to be blocked and the animal was excluded from further MRI scanning. Final confirmation of patency of the jugular port was assessed after the DCE-MRI scan, in which contrast agent could be readily observed in the vasculature.

Animal care

Animals were allowed to recover in warmed cages, and saline was administered subcutaneously. Animals also received Baytril-100 (2.5 mg/kg; Bayer Healthcare LLC Animal Division, Shawnee Mission, KS) and Buprenex (0.01 mg/kg;

TABLE 2. A COMPARISON OF BBB SCORES ACROSS STUDIES USING DIFFERENT SCI CONTUSION DEVICES IN RATS. DATA IS PRESENTED AS MEAN $\pm$ STANDARD DEVIATION

Study	SCI contusion level	Injury device	Injury severity	Control "treatment"	Day 28 BBB score	Day 42 BBB score	Day 56 BBB score
Current study	T7	In-house impactor	Moderately severe	Saline	12.4 $\pm$ 1.5	14.2 $\pm$ 2.4	16.7 $\pm$ 2.4
Jakeman et al., 1998	T8	OSU ESCID ¹	0.9 mm cord displacement	Vehicle (PBS)	17.0 $\pm$ 2.4	18.2 $\pm$ 1.5 ²	–
Ankeny et al., 2001	T8	OSU ESCID ¹	1.0 mm cord displacement	Vehicle (PBS)	10.9 $\pm$ 1.0	11.2 $\pm$ 0.5	12.5 $\pm$ 2.3
Widenfalk et al., 2003	T9	NYU ³ impactor	25 mm WD height	Ringer solution	10.4 $\pm$ 2.2	10.3 $\pm$ 2.8	–
Cao et al., 2005	T9	IH ⁴ impactor	150 kdyn	None	13.5 $\pm$ 2.0	–	–
Mills et al., 2001	T10	NYU ³ impactor	12.5 mm WD height	None	16.3 $\pm$ 2.7	–	–
Scheff et al., 2003	T10	IH ⁴ impactor	150 kdyn	None	13.6 $\pm$ 2.8	13.5 $\pm$ 1.8	–

¹Ohio State University electromagnetic SCI device, ²day 43 BBB score, ³New York University, ⁴Infinite Horizon.

Hospira Inc., Lake Forest, IL) subcutaneously. Animals' bladders were manually expressed every 12 h until the return of spontaneous urination.

Behavioral assessment

Prior to MRI scans, open-field BBB locomotor assessments (Basso et al., 1995) were performed by two independent observers who were blinded to the treatment, and BBB scores are reported as the average of their two scores.

Magnetic resonance imaging protocol

The same group of animals underwent the DCE-MRI scans on days 3, 7, 14, 28, 42, and 56 post-SCI. All MR studies were performed on a 7-Tesla Bruker scanner (70/30 USR; Bruker Biospec, Karlsruhe, Germany) using a 116-mm shielded gradient insert that is capable of producing maximum gradient amplitude of 400 mT/m with 80- μ s rise time. Animals were placed in the supine position on a Plexiglas bed, and a 35 mm $\times$ 40 mm coil that was inductively coupled to the implanted RF coil was placed under the rat. On the day of MRI scan, animals were anesthetized with an induction dose of 4% isoflurane, and were then intubated and mechanically ventilated with 2–2.5% isoflurane, 30% oxygen, and 67.5–68% air through a rodent ventilator (model 683; Harvard Apparatus, Holliston, MA) for the duration of the scan (approximately 3 h). Silicone tubing (Instech Solomon) was attached to the jugular port with the other end of the tubing attached to a two-way valve. Each of the two ports of the valve was connected to a syringe, one filled with Gd (Magnevist; Berlex Laboratories, Montville, NJ) at a concentration of 287 mg/kg and the other with 0.9% saline. Prior to performing each MRI scan, a quality assurance scan that included SNR and magnetic field homogeneity assessment was performed, as described previously (Cohen et al., 2009).

The respiratory rate and rectal temperature were monitored throughout the experiment with a physiologic monitoring unit (model 1025; SA Instruments, Inc., Stonybrook, NY). A pulse oximeter (model 8600V; Nonin Medical Inc., Plymouth, MN) was used to monitor heart rate and oxygen saturation levels. For the duration of the experiment, a heating system (model 11007B, SA Instruments, Inc.) was used to maintain the body temperature at 37°C. Following the acquisition of a tri-pilot scan (for locating the spinal cord) and high-resolution anatomical images, pre-contrast T1-weighted spin echo, axial images were acquired (acquisition parameters: repetition time [TR] = 500 ms, echo time [TE] = 10.4 ms, field-of-view [FOV] = 2.6 cm $\times$ 2.6 cm, slice thickness = 1 mm, and acquisition matrix = 256 $\times$ 128 [zero-filled to 256 $\times$ 256]). Then, without moving the animal, a 0.2-mL bolus of Gd was injected in less than 5 sec into the jugular vein via the vascular port. Immediately following the administration of Gd, T1-weighted images were continuously acquired at 30 time points with a temporal resolution of 2 min, as part of the DCE-MRI scan.

Mathematical model of Gd distribution in the rat spinal cord

The model for analyzing the DCE-MRI data in rat spinal cord was described previously (Bilgen and Narayana, 2001). For quantification of Gd leakage through the compromised

BSCB, a two-compartment model was employed. One compartment represents the systemic circulation (intravascular) and the second compartment represents the extravascular extracellular space (EES) within the spinal cord. The adequacy of a two-compartment model in spinal cord has been demonstrated in Bilgen et al. (2001, 2002) and Cohen et al. (2009). The concentration of Gd in the spinal cord EES at each time point t , $[Gd(t)]_{EES}$, was estimated according to the following equation (Cohen et al., 2009):

$$[Gd(t)]_{EES} = K_{ps} \times \left[\left[\frac{0.4}{0.6 - K_{sp}} + \frac{0.16}{0.04 - K_{sp}} \right] e^{-K_{sp}t} - \left[\frac{0.4}{0.6 - K_{sp}} \right] e^{-0.6t} - \left[\frac{0.16}{0.04 - K_{sp}} \right] e^{-0.04t} \right]$$

The parameters K_{ps} and K_{sp} represent the transfer rates of Gd from systemic circulation to the EES and from the EES back to systemic circulation, respectively. The rate of influx of Gd from the systemic circulation into the EES is $K_{ps} \times [GD(t)]_{Systemic\ Circulation}$ and the rate of disappearance of Gd from the EES is $K_{sp} \times [GD(t)]_{EES}$. The values of $Gd(t)_{EES}$ at each time point (based on DCE-MRI data) were fitted to the above equation using Matlab (MathWorks, Inc., Natick, MA) to estimate K_{ps} and K_{sp} . Because of the uncertainty in the exact time of Gd injection and because it improved the ability of the model to fit the data, the time of contrast injection (t_0) was also estimated as a part of the curve fitting routine.

Tissue processing and histology

Immunohistochemistry (IHC) was performed to detect albumin extravasation into the spinal cord parenchyma for BSCB permeability assessment and to confirm the DCE-MRI results. Previous studies have directly (Cohen et al., 2009; Gordh et al., 2006; Maikos and Shreiber, 2007) or indirectly (Sharma et al., 2006) assessed albumin extravasation as an indicator of BSCB integrity in rodent models of spinal cord injury.

Following the terminal MRI scans on day 56 post-SCI, four saline control animals, four VEGF-treated animals, and one uninjured animal were transcardially perfused with saline followed by 4% paraformaldehyde (PFA) in PBS. The spinal cords were then removed, postfixed overnight in 4% PFA, and immersed in 30% sucrose-PBS (0.1 M PBS) for 2–3 days at 4°C. Spinal cords were sectioned into three segments of 3 mm length each: an epicenter segment (centered around the lesion site), a rostral segment, and a caudal segment. Each segment was embedded in tissue freezing medium and frozen at -20°C . Spinal cord segments were sectioned at 35 μ m using a cryostat (CM1800; Leica Microsystems, Inc., Bannockburn, IL) and stored at -20°C in tissue storing media.

Spinal cord sections were immunofluorescently stained for albumin as described previously (Cohen et al., 2009). Tissue sections were viewed and captured using a Spot Flex digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a Leica RX1500 upright microscope (Leica Microsystems, Inc.). 10 $\times$ fluorescence images were captured with SPOT Advanced imaging software (Diagnostic Instruments, Inc.). For comparisons between sections, the exposure time was held constant for a given magnification level. The primary antibody in one set of spinal cord sections (negative control) was omitted to confirm that the observed signal in the

other spinal cord sections that were exposed to the primary antibody is from albumin.

Data analysis

Exclusion criteria. Data were excluded if the rat died during the 56-day period as the result of an observable illness (e.g., bloody urine, lack of appetite, or porphyrin rings around eyes). However, if an otherwise healthy rat died after the completion of the MRI scan, the corresponding data was not excluded.

MRI region of interest (ROI) analysis. Data were separately analyzed for FE, DE, and NE areas. As described earlier by Cohen et al. (2009), the enhancing (FE and DE) areas were identified in an unbiased manner using the statistical decision mechanism that is based on histogram analysis as described elsewhere (Bilgen et al., 2001). FE areas were identified as localized hyperintense regions on axial spinal cord slices whose signal intensity was at least double that of the mean signal intensity for the entire slice. DE areas were identified as hyperintense patches on post-contrast MR images of the spinal cord. This was realized following the consensus between two independent observers. If FE or DE areas were present in a MRI slice, the NE area for that slice was determined by subtracting the enhancing areas from the total spinal cord cross section. If no FE or DE areas were present, then the NE area for that slice was the ROI of the entire spinal cord. Thus, it was possible for a single slice of the spinal cord to simultaneously contain NE, FE, and DE areas. Due to insufficient number of DE areas (which are mainly confined to the epicenter region), it was not possible to adequately analyze DE data or to compare DE data with NE and FE data. Thus, results for DE areas are not presented here. In addition, due to the appearance of FE areas predominantly in the chronic period (after day 14 post-SCI [Bilgen et al., 2001]), the results for K_{ps} in the FE areas during the acute and subacute periods were not included in the analysis.

Estimation of K_{ps} and K_{sp} . Even though both K_{ps} and K_{sp} were determined, we have focused mainly on K_{ps} because this is the parameter that represents leakage of Gd from the systemic circulation and is a measure of the BSCB permeability. Further, the estimation of K_{sp} tends to be noisy because it is computed from the decaying part of the curve (Bilgen and Narayana, 2001). Due to the longitudinal nature of our study, we had six time points. In addition, three spatial regions were under consideration. In order to provide meaningful interpretations of spatio-temporal changes, the key focus of our studies, in accordance with our strict adherence to Bonferroni correction, we collapsed the temporal data into three time periods: acute (day 3 post-SCI), subacute (days 7–14 post-SCI), and chronic (days 28–56 post-SCI). This is consistent with the time periods used in our previously published study of longitudinal data that used the same time points for data acquisition (Cohen et al., 2009). Therefore, in addition to comparing the values of K_{ps} by individual days, data was also analyzed by these three post-SCI time periods. For the same reasons, the spinal cord locations were grouped into three spatial regions: caudal (slices that were 4–8 mm caudal to the injury epicenter), epicenter (slices that were ≤ 2 mm away from the injury epicenter, including the epicenter slice), and rostral (slices that were 4–8 mm rostral to the injury epicenter). For each MRI scan, the SCI

epicenter was determined independently by two observers as the slice with the largest lesion. If the observers' choice of epicenter slice did not match, the observers conferred until the epicenter slice was agreed upon.

Statistical analysis

Statistical analyses were performed using STATA (Intercooled Stata 9.2 for Windows; StataCorp LP, College Station, TX). The longitudinal data were analyzed using generalized estimating equations (GEEs) for assessment of longitudinal temporal association between BBB scores and K_{ps} . Testing for the significance of the relationship between the BBB scores and K_{ps} was based on a population-averaged, panel-data model (Zeger and Liang, 1986). Unlike multiple analysis of variance (MANOVA), which requires the imputation of missing data by using options such as last value carried forward, longitudinal interpolation, and longitudinal regression, GEE can handle cases with missing data without the need for imputation (Twisk and de Vente, 2002). Using various imputation methods to run MANOVA and GEE on an incomplete longitudinal dataset, Twisk and de Vente (2002) concluded that not imputing missing data would result in superior GEE results, while imputing missing data would result in superior MANOVA results. Because no imputation technique is without bias to the resulting complete dataset, we chose to not impute any missing data and employed GEE analysis.

All other statistical procedures used the Wilcoxon signed-rank test. MANOVA analysis assumes intra-group normal distribution. Wilcoxon signed-rank tests, like the *F*-test (modified MANOVA), are more robust in the analysis of experimental data that cannot be assumed to be normally distributed. All values are reported as mean $\pm$ standard deviation unless otherwise stated. Statistical significance was defined as $p < 0.05$, with corrections for multiple comparisons (Bonferroni adjustment).

Results

Table 3 summarizes the number of animals that were scanned at each time point for the saline and VEGF cohorts.

Non-enhancing areas

Figure 1a,b displays K_{ps} values for both groups, averaged over the three spatial regions, plotted by post-SCI time period. For VEGF animals, K_{ps} was significantly smaller (i.e., lower BSCB permeability) in the chronic period compared to both acute ($p < 0.0001$) and subacute ($p < 0.001$) periods (Wilcoxon rank-sum test, corrected $\alpha = 0.0167$). For saline animals, K_{ps} was significantly greater in the acute period compared to both subacute ($p < 0.01$) and chronic ($p < 0.0001$) periods. In general, the value of K_{ps} is greater in the VEGF animals relative to saline controls, but this difference reached statistical

TABLE 3. SUMMARY OF NUMBER OF ANIMALS IN EACH TREATMENT GROUP ON EACH POST-SCI MRI SCAN DAY

	Day 3	Day 7	Day 14	Day 28	Day 42	Day 56
Saline	5	6	6	5	5	5
VEGF	4	6	8	6	5	6

FIG. 1. K_{ps} values and Basso-Beattie-Bresnahan (BBB) scores plotted over time, for both treatment groups. (a) K_{ps} in non-enhancing (NE) areas of saline controls. (b) K_{ps} in NE areas of vascular endothelial growth factor (VEGF)-treated animals. (c) K_{ps} in NE areas of saline controls versus VEGF-treated animals. (d) BBB scores of saline controls versus VEGF-treated animals. * $p < 0.01$, § $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. Error bars represent standard error of the mean.

significance only in the subacute period ($p < 0.01$). Table 4 summarizes these results as well as results for the pair-wise individual post-SCI day comparisons of K_{ps} for each cohort.

Figure 2 shows the values of K_{ps} at each spatial region of the spinal cord, for the three post-SCI time periods. In the epicenter region, K_{ps} was significantly greater in the VEGF cohort compared to the saline cohort ($p < 0.01$, two-tailed unpaired Student *t*-test) during both the subacute and chronic periods. Whereas K_{ps} in the epicenter region during the subacute time period contributed to the overall difference in K_{ps} during this time period (Figs. 1c and 2b), this was not the case during the chronic time period (Figs. 1c and 2c). This could be due to significant reduction in the K_{ps} values between the subacute and chronic phases in the VEGF cohort (Fig. 1b).

Focally enhancing areas

As mentioned earlier, FE areas on DCE-MRI represent underlying angiogenesis, and these areas do not usually appear on DCE-MRI until after day 14 post-SCI (Bilgen and

Narayana, 2001). During the chronic period, the value of K_{ps} in the FE areas was $0.0254 \pm 0.0234 \text{ min}^{-1}$ in VEGF-treated animals and $0.0328 \pm 0.0209 \text{ min}^{-1}$ in saline controls. These values were not significantly different from each other ($p < 0.053$, Wilcoxon rank-sum test, corrected $\alpha = 0.0167$). Also during the chronic period, we investigated whether the values of K_{ps} were different between the FE and NE areas, for each cohort. During the chronic time period, only the saline cohort exhibited a significant difference in K_{ps} between the FE ($0.0328 \pm 0.0209 \text{ min}^{-1}$) and NE ($0.0174 \pm 0.0102 \text{ min}^{-1}$) areas ($p < 0.0001$, Wilcoxon rank-sum test, corrected $\alpha = 0.0167$).

BBB scores

The significantly increased BBB score in the VEGF cohort compared to the control cohort during the chronic phase (Fig. 1d) is attributed to day 28 post-SCI, on which the BBB score was significantly greater in the VEGF cohort (16 ± 1.7) compared to the saline cohort (12.4 ± 1.5 ; $p < 0.0077$, Wilcoxon rank-sum test, corrected $\alpha = 0.0083$). While neurobehavioral

TABLE 4. SUMMARY OF STATISTICALLY SIGNIFICANT RESULTS FOR PAIR-WISE TIME PERIOD OR INDIVIDUAL DAY COMPARISONS OF K_{ps} IN NON-ENHANCING (NE) AREAS, WITHIN SALINE AND VEGF GROUPS

Comparison for K_{ps}		Saline		VEGF	
		p-value	Significant?	p-value	Significant?
Time Period Comparisons	acute vs. subacute	$p < 0.0026$	Yes ^a	$p < 0.0808$	No ^a
	acute vs. chronic	$p < 0.0001$	Yes ^a	$p < 0.0001$	Yes ^a
	subacute vs. chronic	$p < 0.0239$	No ^a	$p < 0.0008$	Yes ^a
Individual Day Comparisons	day 3 vs. day 14	$p < 0.0001$	Yes ^b	$p < 0.003$	Yes ^b
	day 3 vs. day 28	$p < 0.0001$	Yes ^b	$p < 0.0001$	Yes ^b
	day 3 vs. day 42	$p < 0.001$	Yes ^b	$p < 0.0011$	Yes ^b
	day 3 vs. day 56	$p < 0.0001$	Yes ^b	$p < 0.0026$	Yes ^b
	day 7 vs. day 14	$p < 0.019$	No ^b	$p < 0.0002$	Yes ^b
	day 7 vs. day 28	$p < 0.0063$	No ^b	$p < 0.0001$	Yes ^b
	day 7 vs. day 42	$p < 0.0787$	No ^b	$p < 0.0001$	Yes ^b
	day 7 vs. day 56	$p < 0.001$	Yes ^b	$p < 0.0001$	Yes ^b

Corrected α for multiple comparisons = 0.0166^a or 0.0033^b .

FIG. 2. Changes in blood-spinal cord barrier (BSCB) permeability in non-enhancing (NE) areas over time, for both groups. K_{ps} in NE areas versus spatial region for both groups during the acute (a), subacute (b), and chronic (c) time periods. * $p < 0.01$. Error bars represent standard error of the mean.

recovery was significantly accelerated in the VEGF cohort by day 28 post-SCI, the BBB scores on day 56 post-SCI were not significantly different between the saline (16.7 ± 2.4) and VEGF (16.9 ± 1.7) cohorts ($p < 0.92$).

Relationship between BBB scores and the BSCB permeability

The BBB scores were correlated with K_{ps} in the previously defined areas (NE and FE), using the GEE procedure for a population-averaged model (α corrected for multiple comparisons = 0.0167). For multiple correlations, the GEE procedure matched K_{ps} values by treatment group, time period, and spatial region. Table 5 summarizes the results of statistical significance testing for the associations between BBB scores, and K_{ps} values in the NE and FE areas. Figure 3 displays the associations between NE area K_{ps} values and the BBB scores, for both cohorts.

The association between K_{ps} and BBB scores was tested for saline and VEGF cohorts separately. We first tested the association irrespective of spatial region. In NE areas, only the saline cohort showed a significant overall association ($p < 0.001$) between K_{ps} and BBB score, while in FE areas only the VEGF cohort showed a significant overall association ($p < 0.016$) between K_{ps} and BBB score. Second, we tested the association within each of the three previously defined regions of the spinal cord, for each treatment. In NE areas, the association between K_{ps} and BBB score was statistically significant in all three regions ($p < 0.001$ in each region) for the saline cohort, while statistical significance was not achieved in any individual region for the VEGF cohort. In FE areas, the association between K_{ps} and BBB score was statistically significant in the caudal and rostral regions ($p < 0.001$ in each region) for the saline cohort while statistical significance was not achieved in any individual region for the VEGF cohort.

Histological verification of DCE-MRI results

Histology was evaluated qualitatively. As shown in Figure 4, albumin staining was prominent in the epicenter region/segment, with more albumin extravasation seen in the VEGF-treated animal compared to saline control. In the caudal region/segment, albumin extravasation persisted, although to a lesser extent and comparably between both groups. The albumin staining in the uninjured control spinal cord was similar to that observed for the negative control sections that were not treated with the primary antibody (data not shown).

Discussion

In these studies, we have investigated the spatio-temporal changes in the BSCB permeability with DCE-MRI in a rodent model of contusion SCI treated with VEGF. To the best of our knowledge, this is the first study to longitudinally track changes in the BSCB permeability in SCI with VEGF treatment.

Effects of VEGF on locomotor recovery

The most important observation in the present studies is the improved BBB scores on day 28 post-SCI in the VEGF-treated animals relative to the saline controls. However, this difference disappeared by day 56 post-SCI. Thus, VEGF appears to accelerate neurobehavioral recovery, but a persistent improvement in BBB scores in VEGF-treated animals compared to saline controls was not observed. This is somewhat different from the observation by Widenfalk et al. (2003), who reported significant improvements in the BBB scores on days 7, 35, and 42 post-SCI of rats treated with the same dose of VEGF. This difference in neurobehavioral improvement observed in our studies may be due to a number of factors; Widenfalk et al. (2003) used a 25-g weight-drop (WD) at the T9 level, while we used a moderately severe contusive injury at the T7 level. Yet, the plateau effect on BBB scores observed in our study was also present in the work of Widenfalk et al. (2003), the difference being that the onset of plateau occurred on day 28 post-SCI in our study and on day 14 post-SCI in Widenfalk et al.'s study. The other difference in post-SCI neurobehavioral improvement may be due to the method of administration of treatment. Whereas Widenfalk et al.

TABLE 5. SUMMARY OF TEMPORAL ASSOCIATIONS BETWEEN BBB SCORES AND K_{ps} VALUES IN THE NE AND FE AREAS, FOR BOTH COHORTS

Tissue area	Spatial region	Saline cohort	VEGF cohort	Corrected α value
Non-enhancing (NE)	all combined	$p < 0.001$ (*)	$p < 0.078$	0.05
	caudal	$p < 0.001$ (*)	$p < 0.144$	0.0167
	epicenter	$p < 0.001$ (*)	$p < 0.083$	0.0167
	rostral	$p < 0.001$ (*)	$p < 0.206$	0.0167
Focal enhancements (FE)	all combined	$p < 0.117$	$p < 0.016$ (*)	0.05
	caudal	$p < 0.001$ (*)	$p < 0.628$	0.0167
	epicenter	$p < 0.309$	$p < 0.999$	0.0167
	rostral	$p < 0.001$ (*)	$p < 0.047$	0.0167

injected VEGF into the lesioned area of the spinal cord directly after SCI, we placed a piece of Gelfoam soaked with VEGF on top of the spinal cord directly after SCI. Longer duration treatment with VEGF could possibly sustain the improvement in the locomotor recovery.

Effects of VEGF on BSCB permeability

Three tissue areas of relevance were identified and studied in DCE-MRI scans. While NE areas do not display visible contrast enhancement and may appear normal upon visual inspection, our quantitative analysis has demonstrated that these areas of the spinal cord contain compromised BSCB. The

lack of visible enhancement in these areas suggests subtle changes in the BSCB permeability of either the old vasculature or neovasculature that is difficult to visualize on the post-contrast T1-weighted images. Nevertheless, quantitative analysis of the DCE-MRI studies suggests the presence of compromised BSCB in the NE areas. However, the BSCB permeability in these NE areas appears to significantly decrease with time in both cohorts, perhaps reflecting endogenous recovery over time. While there is a trend of increased NE area BSCB permeability in VEGF-treated animals compared to saline controls, a significant treatment difference in K_{ps} , across all spatial regions, was only detected in the subacute time period. The lack of a significant difference in K_{ps} between both cohorts during the other two time periods may be due to (1) increased edema in NE areas observed on MRI (Narayana et al., 1999) and increased hemorrhage (Bilgen et al., 2000) in the acute phase, and (2) endogenous semi-restoration of the BSCB in the chronic phase (Cohen et al., 2009). Our spatio-temporal analysis also indicates significant treatment differences in the NE areas only in the epicenter region during the subacute (days 7–14 post-SCI) and chronic (days 28–56 post-SCI) periods. The lack of a significant difference in NE area BSCB permeability at the injury epicenter between the VEGF and saline cohorts possibly reflects increased angiogenesis and increased permeability during the early phases of angiogenesis (Dvorak et al., 1995, 1999). Therefore, differences in NE area BSCB permeability during the acute phase may be indistinguishable regardless of treatment. For the chronic period, the results of BSCB permeability as determined by DCE-MRI were verified through immunohistochemistry for albumin on spinal cords from animals sacrificed at 56 days post-SCI. While VEGF-treated animals demonstrated increased BSCB permeability compared to saline controls in the epicenter region, albumin extravasation was comparable between the two groups in the caudal region.

The values of K_{ps} in FE areas were significantly larger compared to the NE areas, but only in the subacute and chronic time periods. The lack of a significant difference in the BSCB permeability between FE and NE areas during the acute period may be due to the relative absence of FE areas compared to NE areas during this time period (Bilgen and Narayana, 2001).

Consistent with Vaquero et al.'s finding that endogenous VEGF (measured via immunoreactivity in the arterioles of pia mater and GM) decreased by day 14 post-SCI (Vaquero et al., 1999), we have shown a statistically significant decrease in the BSCB permeability from day 3 to day 14 post-SCI in saline controls ($p < 0.0001$; Table 4).

FIG. 3. Association between blood-spinal cord barrier (BSCB) permeability and Basso-Beattie-Bresnahan (BBB) scores. K_{ps} of the non-enhancing (NE) areas are plotted over time as closed squares. BBB locomotor scores are concurrently plotted as open squares. K_{ps} versus time and BBB score versus time for saline (a) and vascular endothelial growth factor (VEGF; b) cohort. ** $p < 0.001$. Acute, subacute, and chronic time periods represent 3 days, 7–14 days, and 28–56 days post-spinal cord injury (SCI), respectively. Error bars represent standard error of the mean.

FIG. 4. Extravasation of albumin into the spinal cord parenchyma in the epicenter and caudal regions at 56 days post-spinal cord injury (SCI). The increased albumin extravasation in the epicenter region in the vascular endothelial growth factor (VEGF)-treated animal (**b**) compared to the saline control (**a**) can be seen. However, no significant difference in the albumin extravasation was observed in the caudal region of the VEGF-treated animal (**d**) compared to the saline control (**c**). For comparison, the uninjured spinal cord does not exhibit the extravasation of albumin (**e**). Three cords from each group were qualitatively assessed and representative images are shown.

Association between BSCB permeability and locomotor recovery

Cohen et al. (2009) found a significant inverse correlation in untreated injured animals between NE area K_{ps} values and BBB scores. In the current study, we found a similar significant inverse temporal association in the control cohort. The lack of a significant inverse correlation in the VEGF cohort of the current study suggests that VEGF treatment does not exacerbate the injury through the mechanism of compromised BSCB permeability. This is consistent with the finding of Benton and Whittemore (2003), of almost no significant immunostaining differences in microvascular architecture (blood vessel count, density, and diameter) at 6 weeks post-SCI between VEGF and control cohorts. Interestingly, Benton et al. observed a significant VEGF treatment effect on microvasculature at 6 weeks post-SCI only in the ventral white matter that is 2 mm rostral to the SCI epicenter (Benton and Whittemore, 2003). In our studies, during the chronic time period, the only spatial region of the spinal cord that demonstrated a significant difference in K_{ps} between VEGF and saline cohorts was the epicenter region, which contains slices 2 mm rostral to the epicenter of injury. The absence of any significant differences in lesion area between the saline and VEGF cohorts at day 56 post-SCI in our study is somewhat

different from the findings of Benton et al., who reported a significantly increased cavitation size of the spinal cord 6 weeks post-SCI due to VEGF treatment, both at 2 mm rostral and 2 mm caudal to the injury epicenter (Benton and Whittemore, 2003). Some of the differences between the results of Benton et al. and those presented in our study may be due in part to the timing of VEGF administration. While VEGF was administered immediately after SCI in our study, Benton et al. administered VEGF 3 days post-SCI (Benton and Whittemore, 2003).

We observed a significant correlation between BSCB permeability in the FE areas and BBB scores (1) in the VEGF cohort when all three regions were considered together and (2) in the saline cohort in the rostral and caudal regions. We believe the lack of a significant correlation in the epicenter region in both cohorts was due to the fact that most FE areas were observed distal to the site of injury. In addition, the DE areas that are due to mechanically damaged BSCB may have overwhelmed the FE areas in the epicenter region, leading to a diminution in the number of FE areas detected in this region.

Persistent increase in BSCB permeability over time

As previously reported by Cohen et al. (2009), the value of K_{ps} remained elevated during the chronic phase of the current

study, even in regions away from the epicenter. This finding in the context of chronically injured spinal cord could have implications for longer-term interventions that employ small therapeutic agents to penetrate the still compromised BSCB (Baptiste and Fehlings, 2006).

Based on our studies, compromised BSCB permeability does not appear to be the primary driving factor for VEGF's observed treatment effects in SCI. This finding might have implications for combination therapies with particular emphasis on the effects of VEGF other than promoting vascular permeability. The neuroprotective role of VEGF is thought to be related to enhanced neurovascular perfusion that reduces neurodegeneration (Oosthuysen et al., 2001; Storkebaum et al., 2004). VEGF has also been shown to promote neurite outgrowth from cerebral cortical neurons (Jin et al., 2006) and retinal explants (Bocker-Meffert et al., 2002). Another option for the use of VEGF in SCI is in combination with other angiogenic agents. For example, angiopoietin-1 has been shown to stabilize adult vasculature (Thurston et al., 2000) and, in combination with VEGF, has been shown to stabilize neurovasculature in a rodent model of stroke (Zacharek et al., 2007). Thus, VEGF in combination with other therapies, with particular emphasis on timing of administration, may prove beneficial in SCI.

Caveats

We have been using saline as a control in our previous studies and simply continued that practice. BSA would have been a better control. Rat albumin has previously been shown to have a therapeutic effect in the context of experimental SCI (Cain et al., 2007). However, the concentration of rat albumin in Caine's study was fivefold higher than the BSA vehicle used in our VEGF cohort. While we cannot totally rule out the effect of BSA vehicle (which was delivered via Gelfoam) on BBB scores in the VEGF-treated group in our study, we believe that because of its low concentration, the BSA vehicle in our study did not significantly confound the effects of VEGF on BBB score.

While the number of animals used in this study (Table 3) may be considered small and thus predispose the results of BBB score comparisons to the potential for type II error, this number of animals has been shown to be sufficient for comparisons of MRI metrics such as K_{ps} (Cohen et al., 2009).

Conclusion

These studies demonstrate that the BSCB permeability was greater at all time points in VEGF-treated animals compared to saline controls, most significantly in the region containing the SCI epicenter. Although a significant temporal reduction in the BSCB permeability was observed in both saline and VEGF cohorts ($p < 0.0001$), BSCB permeability remained elevated even during the chronic phase. Our studies indicate that acutely administered VEGF hastens neurobehavioral recovery by day 28 post-SCI but that this improvement over saline controls does not persist by day 56 post-SCI. We have demonstrated that NE areas on DCE-MRI scans represent areas of compromised BSCB despite their normal-looking appearance. These NE areas may play a significant role in the secondary injury of SCI, and thereby functional outcome. Changes in the BSCB permeability do not appear to be the primary driving factors for VEGF's observed treatment effects in SCI. This

finding might have implications for combination therapies with particular emphasis on the effects of VEGF other than its effect on promoting vascular permeability via angiogenesis (e.g., neuroprotection via anti-apoptotic pathways, promotion of neurite growth).

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Ponnada A. Narayana, Ph.D.

Department of Diagnostic and Interventional Imaging
University of Texas Medical School at Houston
6431 Fannin Street
Houston, TX 77030

E-mail: Ponnada.A.Narayana@uth.tmc.edu