REVIEW

Targeting Microvasculature for Neuroprotection after SCI

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Summary: Spinal cord injury (SCI) is characterized by secondary degeneration, which leads to tissue loss at the epicenter and subsequent functional deficits. This review provides insight into the pathophysiology of microvascular dysfunction and endothelial cell loss, which are among the earliest responses during the first postinjury day. The enigmatic role of the angiogenic response in the penumbra around the lost tissue, which occurs during the first 2 weeks, is also discussed. The importance of stabilizing and rescuing the injured vasculature is now well-recognized, and several pharmacological and genetic treatments have emerged in the past few years. We conclude with suggestions for future experimental research, including development of vascular-selective treatments and exploitation of genetic models. In summary, vascular dysfunction following SCI is an important contributor to neurological deficits, as proposed long ago. However, there now appears to be new and potentially powerful opportunities for treating acute SCI by targeting the vascular responses. **Key Words:** Spinal cord injury, therapeutics, neuroprotection, vasculature, inflammation.

Acute vascular responses contribute to secondary degeneration after SCI

Following the primary mechanical injury, a secondary injury develops gradually over the first hours to weeks due to disruption in blood flow and progression of detrimental inflammation, leading to white and gray matter loss, cavitation and glial scarring. The concept of the secondary injury in spinal cord injury (SCI) was first hypothesized by Allen [1] in which improvement after spinal cord compression in dogs was enhanced following removal of the hemorrhagic necrotic debris in the lesion epicenter. The good news is that there appears to be a window of opportunity to intervene with therapeutics aimed at minimizing the progression of the secondary injury to limit further loss of function and promote spontaneous recovery. This review will focus on vascular responses in mammals, and the reader is referred to other reviews dealing with other aspects of SCI pathophysiology [2–8].

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The idea that vascular dysfunction might play a critical role in the pathophysiology of SCI is not new. Following Allen's [9] description of vascular pathology in dogs with contusive SCI, others further characterized various vascular aspects of SCI, including reduced perfusion [10–13]. The earliest formulation of ideas about causation appears to be that "the initial vascular damage and subsequent reparative changes within the spinal cord appear to adequately explain the cavitation observed" [14]. Moreover, it was proposed that "The hypoperfusion of the white matter found in this study suggests that ischemia plays a role in paraplegia resulting from experimental compression injury of the spinal cord" [15]. Thus, the idea that vascular protection would be an important component and viable therapeutic strategy following central nervous system (CNS) injuries was born [4, 6, 16–19]. In comparison to humans, different species show a largely similar vascular and ensuing inflammatory response to SCI [20, 21]. However, until a more detailed comparison is performed and/or some of the vascular-targeted therapeutic reagents prove to be beneficial in humans, the clinical relevance of the experimental data reviewed here should be viewed as speculative.

Direct physical damage to the spinal cord shears blood vessels within the lesion epicenter causing a central hemorrhage, starting off as small petechial bleeding and

progressing rostrally and caudally mostly throughout the gray matter [22–24]. The area of this hemorrhagic epicenter was demonstrated by Noble and Wrathall [23] to have a linear relationship with the severity of injury and was further shown to increase in size from 24 h to 1 week postinjury. Most likely due to its iron-containing hemoglobin, blood is toxic to CNS tissue and is thought to contribute to the necrotic changes following SCI [25].

Direct physical disruption of blood vessels also causes a profound disruption of normal blood flow to the spinal cord [22–24]. Furthermore, vasospasm [26] and loss of autoregulation [27, 28] increase with more severity of post-trauma ischemia [4]. Work in monkeys showed that following SCI, gray matter perfusion was obliterated, whereas white matter perfusion was actually increased, suggesting that white matter degenerates through mechanisms other than ischemia [29]. In fact, microvessel density is relatively low in white matter throughout the CNS compared with gray matter, suggesting that axons and glial cells are less sensitive to ischemia. Blood pressure often drops in injuries higher than T4, and this is considered to contribute to worse outcomes in humans. Low mean arterial pressure most likely contributes to low spinal cord perfusion and ischemia [30], and thus, vasopressors are acutely administered to support falling mean arterial pressure in patients [31]. One cautionary note for treatment is that reperfusion injury triggered by immediate relief of ischemia and hypoxia can be deleterious to tissue preservation [32]. This vascular dysfunction is a major contributor to the massive death of spinal cord cells, including oligodendrocytes, neurons, astrocytes, precursor cells, and endothelial cells (ECs), which leaves the injury epicenter necrotic.

ECs and blood vessels show degenerative changes within 30 minutes after SCI [33–35] and are lost during the first 3 days [34, 36–38], exacerbating the ischemia (Fig. 1). In the injury penumbra, Casella et al. [34] show that EC death does not correspond to neuronal and glial death; however, the majority of ECs lost are in the injury epicenter causing significant hemorrhage and disruption of vascular autoregulation, and thus is an indirect contributor to neuronal and glial cell death [4, 23, 39]. The EC loss is caused in part by reactive oxygen species and lipid peroxidation of microvasculature [6, 40]. The hemorrhagic epicenter due to microvascular disruption and EC death is a major cause of the overwhelming presence of free radicals leading to oxidative stress [41, 42]. The activity of lipolytic enzymes increases while antioxidant activity decreases resulting in free radical-induced lipid peroxidation and subsequent apoptosis of affected cells [43, 44].

Integrin binding to extracellular matrix molecules, such as laminin, is important for attachment and survival of various cells, including ECs [45, 46]. ECs detach rapidly following SCI [38, 47], suggesting that their death might result from lack of integrin stimulation through a process called anoikis. The $\alpha v \beta 3$ integrin promotes EC survival during tumor angiogenesis [48]. Moreover, decreases in angiopoietin 1 (Ang1) following SCI [49], the ligand for the EC-selective receptor Tie2, may also contribute to EC death. Tie2 (Tek) promotes EC survival, stabilizes blood vessels, and reduces leakiness, effects observed during both developmental and adaptive angiogenesis in tumors and experimental CNS pathology [50–58]. Angiopoietin 2 (Ang2), on the other hand, destabilizes blood vessels during angiogenesis and in the absence of vascular endothelial growth factor (VEGF) can contribute to vascular regression and EC death [59]. Although VEGF decreases [49], Ang2 increases following SCI (Gene Expression Omnibus profiles entry GDS2159/1448831_at/Angpt2/Mus musculus) and Ang2 might thus contribute to degenerative changes as well as to the angiogenic responses discussed as follows.

Prolonged repercussions of SCI include permeability [60, 61] and the blood spinal cord barrier (BSCB) instability [36, 62] (Fig. 1). The neurovascular unit (NVU) is responsible for maintaining BSCB integrity and constitutes a complex structure within the CNS comprised of ECs, neurons, astrocytes, and pericytes. Investigators continue to unravel the interactions among the cells of the NVU and the therapeutic strategies to target NVU pathology following cerebral and SCI [63, 64]. Pericytes are interesting because they provide structural support and aid in the transmission of small molecules and ions via gap junctions, as well as autocrine and paracrine signaling among ECs, neurons, and astrocytes [65]. They appear to be very important for neuronal structure, function, and overall health [66–68]. Loss of pericytes by 7 days post-SCI may contribute to BSCB instability by loss of direct physical contact and communication via gap junctions [37]. Pericyte death after traumatic brain injury has been shown to be as high as 40%, demonstrating a significant disruption of the NVU following CNS trauma [69]. Therapuetic targeting of pericytes after ischemic stroke has been recognized as another important avenue to support damaged microvessels and prevent neuronal death [70]. The BSCB is formed by the interactions of these cells, as well as the endothelial tight junctions (TJs), in which the occludins, claudins, and junctional adhesion proteins strictly regulate flow of cells and molecules into the CNS. In the normal animal, the functional properties of each family of TJ components and the selective leakiness of the CNS TJs are well-studied [71, 72]. How these processes are disrupted following traumatic and ischemic CNS microvascular injury is a topic under current investigation [36, 62, 73].

Perfusion of damaged and/or immature vessels causes excessive permeability due to dysfunctional TJs [3, 36, 37] (Fig. 1) and can result in functional consequences when

paired with an acute stressor, such as exercise [74]. Disruption of these junctional proteins results in BSCB instability and increased permeability up to 21 days in the mouse and 28 days in the rat [36, 62]. Upregulation of matrix metalloproteinases results in excessive extracellular matrix (ECM) digestion, increased inflammation, and uncontrolled vascular permeability [75]. BSCB pathology plays a major role in secondary injury by promoting edema and inflammatory infiltration and by providing CNS access to potentially neurotoxic molecules [76]. As demonstrated by Nagy

et al. [77, 78], cationic changes in the luminal endothelial glycocalyx increases cerebral permeability to macromolecules, such as horseradish peroxidase. Changes in the glycocalyx post-SCI increases cation content and is an important indicator of EC phenotype and BSCB stability [61]. Lectins are used to define these changes as a means to identify injured or reactive microvessels. For instance, the lectin IB4 binds only a subset of proliferative microvessels maximally at 7 dpi, which illustrates that phenotypic changes in the EC glycocalyx are functionally relevant
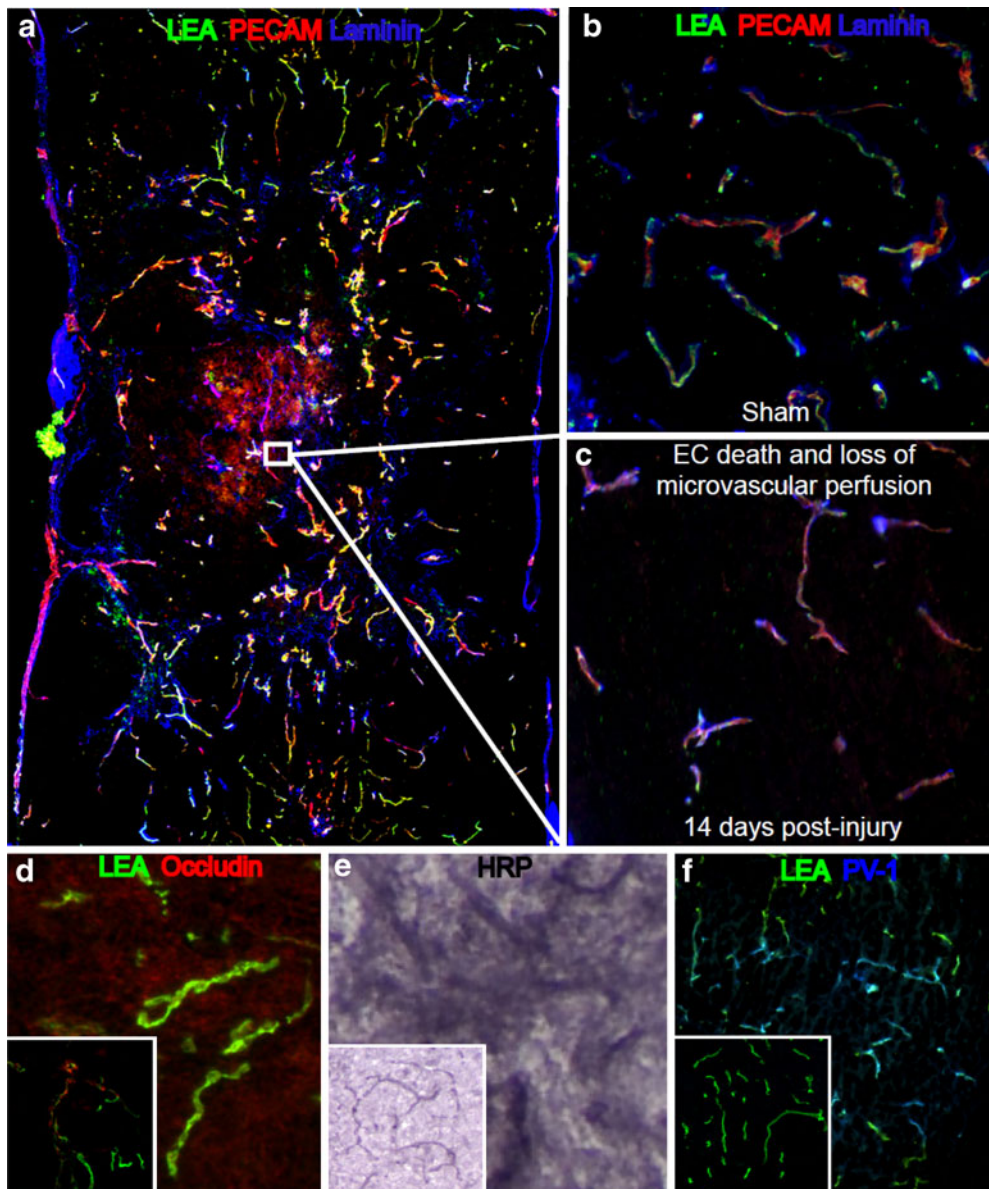

FIG. 1. (a) Fourteen days following traumatic SCI, mechanical disruption of microvessels (box indicates site of microvascular loss displayed in [c]), and endothelial cell (EC) death has resulted in spinal cord hypoxia, and (c) loss of adequate microvascular perfusion compared to the sham spinal cord (b). (d) The neurovascular unit having lost appropriate tight junctional proteins, such as occludin, (e) becomes permeable to large proteins such as horseradish peroxidase (HRP). (f) Microvascular dysregulation is further evidenced by expression of plasmalemmal vesicle-associated protein (PV-1), a marker of fenestrated capillaries. Insets in (D–F) show sham spinal cord microvessels. LEA = *Lycopersicon esculentum* (tomato) agglutinin; PECAM = platelet endothelial cell adhesion molecule.

Table 1. *Therapeutics Leading to Tissue Sparing and Functional Recovery with an Additional Vascular Component in Animal Models of Traumatic SCI*

Target	Treatment	Outcome	Study reference numbers
Vascular protection			
Spinal cord perfusion	Nimodipine plus vasopressor	Blood flow and axon conduction ↑, function modest ↑	[30, 91, 92]
Oxidative damage	Tirilazad, selenium, tempol	Blood flow ↑, stabilized BSCB	[93–95]
Heme oxygenase-1	Genetic HO-1 knockdown, hemin	Inflammation ↓, Stabilized BSCB	[42, 96]
Nonselective ion channels	KO, antisense knockdown	Hemorrhage ↓	[97–99]
Tie2 and αvβ3 integrin	C16 peptide and angiopoietin 1	Vascular density ↑, inflammation ↓, permeability ↓	[83]
Tie2 and VEGFR	AAV-VEGF and AAV-Ang-1	MRI lesion volume ↓, MRI detected vascular stabilization	[100]
Protein tyrosine phosphatases	Small molecule inhibitor (bpV[phen])	Vascular density ↑, apoptosis ↓	[101]
Angiogenesis			
VEGFR	ZFP-VEGFA transcription factor; VEGF-producing neural stem cells	Vascular density ↑, apoptosis ↓	[102, 103]
Semaphorin 3A	Small molecule inhibitor (SM-216289)	Vascular density ↑	[104]
Notch	Agonist	Vascularity ↓, maintained perfusion	[105]
c-Met	Hepatocyte growth factor	Vascular density ↑	[106]
αvβ3 integrin	C16 integrin agonist	Vascular density ↑	[107]
CXCL10	Anti-CXCL10 antibody	Vascular density ↑	[108]
Cell therapy	IV CD133+ cells	Vascular density ↑	[107]

AAV-Ang-1 = adeno-associated virus-Ang-1; AAV-VEGF = adeno-associated virus-VEGF; BSCB = blood-spinal cord barrier; CXCL10 = C-X-C motif chemokine 10; MRI = magnetic resonance image; SCI = spinal cord injury; VEGFR = VEGF receptor; ZFP-VEGFA = zinc finger protein VEGF isoform A.

[37]. Plasmalemmal vesicle-associated protein, a species-conserved protein marker of fenestrated endothelium, also specifically marks ECs undergoing pathological transformation after SCI [79] (Fig. 1). Microvascular permeability is a key factor in spinal cord inflammation and edema. Due to the restricted space within the bony spinal canal, edema further exacerbates the ischemic injury. However, it is still debated whether early or late decompression in humans is beneficial [80].

The degree of white matter-sparing correlates to long-term preservation of locomotor function [81]. Thus, the mechanisms of contributing white matter loss are of great interest. White matter loss ensues during the subacute phase, partially due to activation of FAS and p75 receptors, which triggers apoptosis of glial cells [82]. The finding that the number of blood vessels correlates with white matter-sparing at 7 days postinjury [83] suggests that maintenance of vascular function is a good target for improving function of long-projecting fiber tracts, which are essential for motor and sensory function. The mechanisms involved in this apparent relationship are unknown but may involve reduced inflammation. The inflammatory response following SCI is clearly involved in white matter damage [8, 84], but the molecular vascular mechanisms involved in the recruitment of detrimental leukocytes require further investigation. Mechanical disruption of endothelial basement membranes promotes infiltration of inflammatory cells [85].

Although astrocytes are understudied within the realm of the vascular responses, their primary role may be to reduce leukocyte trafficking and promoting BSCB repair, and thereby functional recovery [86–88].

Treatments targeting acute vascular responses following SCI

Six neuroprotective agents have been taken as far as phase III clinical trials [89], including methylprednisolone, naloxone, tirilazad mesylate, monosialoganglioside GM1, 4-aminopyridine, and tizanidine. Yet, none of these showed efficacy for their primary endpoint and none are approved by the Untied States Food and Drug Administration (FDA) for SCI. Currently, the only neuroprotective treatment for SCI is methylprednisolone, but its use is controversial and its efficacy is debatable [90]. We, and others, have recognized that targeting of the vascular responses might change this dire picture. Several discoveries during the last decade of the molecular mechanisms involved in vascular dysfunction have led to a growing arsenal of experimental treatments. The studies presented in Table 1. have demonstrated enhanced functional angiogenesis or vascular protection along with improvements in tissue-sparing and/or locomotor behavior signifying the clinical potential of a vascular therapeutic for SCI. Interestingly, several studies that show tissue-sparing and functional recovery also report improved vascularity with attenuated inflammation or improved vascularity with decreased apoptosis. Stabilization of the BSCB, as measured by large molecule extravasation postinjury, is another important indicator of vascular protection.

As discussed, low blood pressure contributes to inadequate spinal cord perfusion. Treatment with the

CNS vasodilator nimodipine plus systemic vasopressors, such as adrenaline or volume expansion, but not individually, can maintain spinal cord blood flow and axonal conduction during the acute injury phase in rats [30, 91]. However, this is only accompanied by a moderate functional improvement [92]. One of the underlying problems is the damage and death of ECs caused in part by reactive oxygen species and lipid peroxidation of microvasculature [6, 40], perhaps formed due to reperfusion injury [32]. In fact, treatments with the steroid tirilazad, antioxidants, or selenium maintain spinal cord blood flow and reduce leakage following contusive SCI [93, 95, 109]. Peroxynitrite-induced oxidative damage following SCI can be reduced by the nitroxide antioxidant Tempol [110]. The treatment has to be initiated within the first hour, perhaps consistent with a role for peroxynitrites in reperfusion injury to ECs. Heme-oxygenase (HO) is also a target for antioxidant intervention. HO reduces oxidative injury by catalyzing the conversion of the oxidant heme to the antioxidant biliverdin. HO-1 is the inducible form and is protective following SCI [42]. The pharmacological drug, hemin, induces HO-1 and is neuroprotective following SCI [96]. Hemin has already been approved by the FDA as an intravenous treatment for porphyria. Of interest is the fact that HO-1 activity is stimulated by endoplasmic reticulum stress in vascular smooth muscle cells (VSMCs) [111], suggesting possible connections among these therapeutic interventions and further exemplifying the need to investigate the roles of VSMCs and pericytes in SCI recovery.

Membrane channels also provide targets for therapeutic compounds. ECs greatly increase expression of sulfonylurea receptor 1 (SUR1)–regulated NC_{Ca-ATP} channels, such as transient receptor potential cation channel subfamily M member 4 (Trpm4), and this contributes to the temporal and spatial expansion of secondary damage after SCI [98]. SUR1 is also newly expressed in humans with SCI, making this system a relevant target [97]. Due to ATP depletion in the ischemic spinal cord, channels like Trpm4 open, which causes depolarization leading to edema and EC death. Inhibition of SUR1 by genetic knockout or by treatment with antisense reduces microvascular fragmentation, hemorrhage, white matter loss, and functional deficits in rats and mice with cervical hemi-contusion injuries [97, 98]. The antisense is probably more efficacious than pharmacological inhibitors because it is expected to reduce the *de novo* expression. Trpm4 inhibition by either knockout in mice or antisense in rats has a similar neuroprotective effect [99]. Of note is that glibenclamide and repaglinide are both FDA-approved drugs for type II diabetes, and they also have such neuroprotective properties following SCI [98]. Glibenclamide is a sulfonylurea inhibitor that binds to SUR1 and repaglinide is known to block K_{ATP} channels.

To our knowledge, no published studies have attempted to selectively reduce hemorrhage and the toxicity of hemorrhagic blood, but it is possible that the antioxidants also reduce the toxicity of blood. There is likely a very fine line in trying to reduce bleeding and maintain spinal cord perfusion.

Among the most direct support for the idea that EC dysfunction and death play a prominent role in secondary degeneration after SCI follows from work targeting the Tie2 receptor, which is almost exclusively present in ECs [112, 113]. Intravenous injections of Ang1 during the first week following a contusive SCI reduces white matter loss, inflammation, and locomotor deficits [83]. This is consistent with the known pro-survival role of Ang1 for ECs [50, 52, 114, 115] and the finding that Ang1 levels decrease following SCI [100]. The anti-inflammatory effects may be due to its known down-regulation of ICAM1, which is necessary for leukocyte binding [116]. Importantly, the treatment can be given with a 4-h delay, which would be sufficient time to make a diagnosis in human SCI cases and to allow the start of intravenous infusions.

As discussed, the αvβ3 integrin also contributes to EC survival. Integrins have a reciprocal interaction with growth factor receptors [117], and both αvβ3 integrin and Tie2 can activate the PI3K-Akt pathway in ECs [118, 119]. This may explain why a combined intravenous treatment with Ang1 and an αvβ3 integrin peptide agonist named C16 was even more effective in reducing the detrimental outcomes of contusive SCI in mice [83]. We have also shown that Ang-1 reduces permeability at 72 h following SCI [83], most likely via the capacity of Ang-1 to preserve the integrity of EC tight junctions under pathological conditions [58, 120]. Others have also seen the BSCB stabilizing effects of Ang1 and that a combination of VEGF and Ang1 leads to improved functional recovery [100].

Finally, protection of ECs by a broad spectrum, selective protein tyrosine phosphatase (PTP) inhibitor also seems to result in protection of long-projecting axons [101]. PTPs are known to inhibit tyrosine kinase growth factor receptors and their downstream intracellular signaling tyrosine kinases. It remains to be determined which mechanisms underlie the PTP inhibition effects and whether this might include facilitating Tie2 signaling.

Adaptive angiogenesis following SCI

Angiogenesis is the development of new vessels from pre-existing capillaries whereas vasculogenesis is primarily an embryonic event in which angioblasts form the

primordial vasculature [121]. Following contusive SCI, new blood vessels form at the epicenter and in the penumbra most likely through angiogenesis during days 3 to 14 postinjury [17, 34, 36, 37, 39]. Additionally, circulating angioblasts might contribute to vessel formation during the reparative phase as they do in other systems [122–124]. Vascular repair may also be affected by hemodynamics [122, 125], suggesting that continued preservation of spinal cord blood flow throughout extended times postinjury would be beneficial. Angiogenesis in the CNS is unique, in that during CNS vascular development, rapid vessel production depends on high numbers of angiogenic sprouts with long filopodia instead of the intussusception seen in peripheral vascular beds [122]. Whether the same is true following SCI or whether this can be exploited to develop targeted therapies is unknown.

The angiogenic response has been viewed as a vital process that occurs following pathological and traumatic processes, such as SCI to support regenerating or spared tissue [18, 19, 126]. Despite a 75% loss of ECs in the first hour following contusive rodent SCI, a robust angiogenic response begins during the first week after injury [17, 34, 127]. In the rat, a net vascular regression occurs as cystic cavitation develops during the second week postinjury [17, 126], whereas vascular density reaches 70% of control values by 14 days in the mouse [36, 37]. IB4 binds to a subset of proliferative microvessels at 7 days postinjury [37]. Other markers for angiogenic microvessels are $\alpha1\beta1$ integrin [128] and ADAM8 [129], which increase dramatically at the epicenter following SCI and co-localize with IB4. Molecules that play a role in EC migration, such as MMP14, are also found at the injury site [129]. The new vasculature is not necessarily beneficial as they may be of the fenestrated, "leaky" type as indicated by their expression of plasmalemmal vesicle-associated protein [79].

Angiogenic blood vessels are thought to be leaky, and therefore the question is whether such vessels are beneficial or detrimental. In our recent study, we did observe a correlation between the increased number of vessels at 7 days postinjury and locomotor function in mice treated with C16 peptide [83]. C16 alone did not reduce permeability, suggesting that despite greater permeability, new blood vessels are beneficial. This is supported by the improved function seen in rats treated with VEGF, whereas the permeability at the epicenter was increased [130]. However, other studies have shown VEGF treatment to be ineffective after SCI [131], or detrimental [132].

An additional consideration for therapeutic targeting of the vasculature is remodeling, which stabilizes and matures postangiogenic microvessels [133]. Therapies targeting newly formed microvessels that have not properly undergone vascular remodeling can be tested *in vitro* and *ex vivo* [133, 134], and have been shown to be beneficial in tumor angiogenesis [135]. Mural cell

recruitment as one aspect of vascular remodeling and maturation is an important consideration in the CNS. Astrocytes, pericytes, and VSMCs are integral components of the BSCB, as well as contributing to vessel maturation. An important illustration of this is the syndrome cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy caused by mutations in Notch 3, expressed on VSMCs [136]. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy is the predominant genetic cause of ischemic stroke and an important model of vascular dementia [137]. Progressive deterioration of VSMCs and accumulation of granular osmiophilic material in the VSMC basement membrane are characteristic of the disease [138], which disrupts vascular mechanotransduction causing permanent vasodilation, pooling, and ischemia [137]. In the normal spinal cord, desmin-positive pericytes are detected at vascular branch points, but by 7 days postinjury they are largely absent [37]. Thus, pericytes and VSMCs are additional mediators of CNS blood flow that are important to consider as a vascular therapeutic targeting the injured spinal cord. Such mechanisms have yet to be exploited therapeutically.

Therapeutic Angiogenesis?

It is possible that not all microvessels can be rescued following SCI. It follows from what was previously described that manipulation of sprouting angiogenesis and vascular remodeling should aim at creating a mature, well-perfused microvasculature, but this phenomenon is complex and not well understood in the realm of SCI.

VEGF has been a frequent vascular target in animal studies for several reasons. VEGF is a potent mediator of angiogenesis and vascular permeability, and is upregulated within the lesion epicenter acutely following SCI [139, 140]. The application of VEGF in SCI is a matter of debate and investigators have found apparently conflicting evidence. Intraparenchymal application of $VEGF_{165}$ in the lesion epicenter of rats increases both vascular permeability by 24-h post-SCI and exacerbates the loss of both white and gray matter [132]. Formally known as vascular permeability factor, VEGF increases vascular permeability when administered acutely as seen by magnetic resonance imaging, although it improves function [130]. On the other hand, $VEGF_{165}$ intraparenchymal injection has resulted in significantly increased hind limb locomotor scores, indicating alleviation of secondary damage [141]. Other methods of VEGF treatment include intraparenchymal injection of a zinc finger protein transcription factor designed to activate expression of all isoforms of endogenous VEGF-A [102]. This resulted in enhanced vascularity and better tissue sparing and function. Transplanted neural stem cells

secreting VEGF have also enhanced the spontaneous angiogenic response and have shown beneficial effects [103]. Yet others have found only limited effects of repeated VEGF treatments [131].

Sema3A inhibition with a small molecule is also accompanied by better vascularity and outcomes following SCI [96, 101, 102, 104]. Sema3A shares the neuropilin 1 receptor with VEGF and competitively antagonizes VEGF165 binding. Thus, it is possible that the Sema3A inhibitor enhances endogenous VEGF signaling and angiogenesis. The interpretation of the study is complicated by the lack of showing angiogenesis (an increase compared with earlier times or to normal) and the fact that Sema3A was found on fibroblasts and neuropilin-1 is typically found on axons.

To address the issue of enhanced permeability due to VEGF treatment, Herrera et al. [100] have shown that co-treatment with Ang1 via intraparenchymal infection with adeno-associated virus (AAV) vectors immediately following SCI further improves outcomes. This is consistent with the role of Ang-1 in establishing and maintaining vascular maturation, stabilization, and integrity [50, 52, 56].

Post-SCI dysregulation of VEGF or an inappropriate VEGF gradient would be an important factor in downstream Notch signaling. VEGF receptor 2 activation results in increased Notch signaling, returning ECs to a nonangiogenic state [142, 143], probably because ECs with high Notch expression become quiescent stalk cells [144, 145]. Thus, application of a Notch pharmacological agonist in addition to exogenous VEGF may enhance its protective effects while controlling deleterious increases in vessel permeability. Intravenous administration of a Notch agonist decreases penumbral microvascular density in the gray matter while maintaining perfusion [105]. This suggests that the VEGF-Notch interaction is complex and difficult to therapeutically target.

C-met via its ligand hepatocyte growth factor is a known angiogenic pathway, activated by growing tumors and targeted in potential anti-cancer therapies [146]. C-met signaling also regulates angiogenesis in porcine aortic EC line and proliferation and migration in human umbilical vein endothelial cells (HUVECs) by VEGF induction [147, 148]. Potential exploitation of c-met signaling to promote angiogenesis has been studied in models of ischemic stroke and SCI [106, 149]. Enhanced locomotor recovery and decreased lesion volume was observed with spinal cord intraparenchymal pre-treatment of hepatocyte growth factor, with significantly diminished apoptosis of oligodendrocytes and neurons and increased new microvessel formation [106]. Although interesting, these results would obviously need to be observed in a clinically relevant model in which c-met signaling was activated post-SCI.

The $\alpha v \beta 3$ integrin is known to play a role in angiogenesis [48] and the C16 $\alpha v \beta 3$ integrin agonist promotes angiogenesis in the chick chorioallantoic membrane assay [150, 151]. Our own studies also suggest that this is the case following SCI as C16 treated injured mice had more perfused blood vessels at the epicenter at 7 days than at 1 day postinjury [83]. Co-treatment with Ang1 did not seem to inhibit the effects of C16.

Inhibition of the cytokine C-X-C motif chemokine 10 (CXCL10) promotes upregulation of angiogenic factors resulting in increased density of blood vessels. Although there is significant tissue sparing in anti-CXCL10 antibody-treated animals, the functional implications of this result is unknown [108]. Nevertheless, subsequent studies by the same investigators have revealed decreased apoptosis and increased axonal sparing, further suggesting potential therapeutic benefit [152].

An additional less well-explored approach to target the vascular compartment post-SCI is cellular therapy. Sasaki et al [107] used granulocyte stimulating factor to mobilize and iscolate CD133+ cells for transplantation post-SCI. The CD133+ cells are a subtype of CD34+ endothelial precursors that effect not only angiogenesis but also secrete CNS growth factors and cytokines [153], and when administered to the spinally injured rat, improve locomotor recovery [107].

Future directions

As previously discussed, endothelial targeting of SCI therapies has shown tremendous promise, particularly the neuroprotective efforts during the acute phase. The clear advantage of targeting the microvasculature is the easy, rapid, and direct manner that intravenous drug delivery poses. We are increasingly able to understand the complexities of the vascular responses and the still limited extent to which vasculature can be protected with current experimental and FDA-approved drugs. Thus, a better understanding both how to maximally protect the damaged vasculature and how angiogenesis contributes to recovery of traumatically injured spinal tissue is an essential topic of further research.

Also, as previously discussed, current options for manipulating angiogenic responses after SCI are limited to available pharmacological agents. However, from a translational standpoint, these therapies will be the easiest to move into clinical trials in SCI. We contend that testing drugs that are FDA-approved for other applications for their angiogenic and/or vasoprotective properties after SCI may be the most fruitful approach. The vasoprotective potential of existing drugs can be readily tested in short *in vivo* assays, as most microvessels at the epicenter are dead, nonperfused or dysfunctional 1 day following contusive or compressive SCI.

With respect to understanding molecular mechanism(s) underlying angiogenic responses and their effects on the recovery of function after SCI, mouse models have the

most promise. Transgenic mouse reporter models in which transgenes are specifically and potentially temporally expressed in ECs could help in analyses or purification of ECs. Such genes can be over expressed or conditionally deleted in ECs. The EC-specific receptor tyrosine kinase *tie2* has been used to drive expression of *lacZ* [154] and enhanced green fluorescent protein (EGFP) [155] in the mouse. The one caveat to using the Tie2-GFP mice is that EGFP expression is high in arterial vessels and much weaker in venous vessels (J.B. Hoying, personal communication). Tie2-GFP transgenic rats have also been developed [156]. The advantage of these models is that transgene expression is observed only in the vasculature. One potential problem to control for is the fact that Tie2 can also be expressed by a subclass of pro-angiogenic monocytes [157] and by hematopoietic stem cells [158]. Although these reporters have not been used in SCI models to date, their potential to directly observe vascular responses (either in real time with 2-photon optics or histologically) after SCI is clear. An alternative mouse model is to use the universal reporter ROSA-enhanced yellow fluorescent protein gene (EYFP) mouse [159], which contains an *EYFP* inserted into the *Gt(ROSA)26Sor* locus. Expression of *EYFP* is blocked by an upstream *loxP*-flanked STOP sequence. When bred to mice with a *cre* recombinase gene under the control of a promoter of interest, the STOP sequence of the targeted gene is deleted in the tissue of interest, and *EYFP* expression is observed. If these mice are crossed with a mouse expressing *cre* under the control of the EC-specific vascular endothelial-cadherin promoter [160], all vasculature will express EYFP. Crossing these *cre* mice with *loxP* mice of any gene of interest could produce interesting models for investigating the role of those genes in EC behaviors. Using available tamoxifen-inducible Vascular endothelial-cadherin-CreER^{T2} [161] or Tie2ER^{T2} [162] mice would enable temporal induction of both floxed reporter and/or transgenes, specifically in the vasculature. Studies using these mice would be valuable from a therapeutic standpoint, as a means to identify novel target genes and/or proteins for development of future pharmacological effectors.

Vascular protection and angiogenesis may also become an important vehicle for axonal regeneration as neo-vessels have been observed to exert a potent growth stimulating action on axonal sprouting [163]. Not only do spinal cord microvessels support neural parenchyma by providing adequate blood supply and oxygenation, but the CNS endothelium actively secretes neurotrophins such as BDNF that have been shown to support regenerating spinal cord tissue [64, 164]. In addition, the basement membrane of CNS blood vessels contain ECM molecules like laminin, which have guidance properties. Indeed, regenerating axons have been shown to follow newly formed blood vessels after SCI [17].

Given the central role of vascular dysfunction and the potential of vascular targeting treatments for SCI, there is a great need to develop noninvasive vascular outcome measures, including imaging and biomarkers. It is expected that vascular assessment will be needed for both for diagnostic purposes and for the assessment of therapeutic efficacy. One of the imaging problems that needs to be to resolved is the limited resolution of current technology and the very small dimensions of the mouse and rat spinal cord. This is also still challenging for techniques, such as positron emission tomography, when considering the human spinal cord.

Finally, our studies and the studies of others are currently and continue to be directed by what we learned from the cancer and cardiovascular fields. We have realized that the detrimental and beneficial inflammatory components of SCI are heavily dependent on the status of the microvasculature, and we foresee that the immunology field will help us develop more advanced vascular targeting therapies for SCI.

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