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Activating Notch signaling post-SCI modulates angiogenesis in penumbral vascular beds but does not improve hindlimb locomotor recovery

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Abstract

Manipulation of Notch signaling has led to significant tumor shrinkage as well as recovery from several traumatic and ischemic injury models indicating its potential clinical application. We have tested both an agonist and antagonist of Notch signaling to study the effects of Notch-mediated angiogenesis on spinal cord vascular pathology following traumatic injury. Initial neonatal retinal vascularization assays showed their respective bioactivities *in vivo*. Mice were treated with either the antagonist Jagged1-Fc chimera (Jag1-Fc) or agonist Notch1 antibody (N1 Ab) immediately following a mid-thoracic contusive injury through an initial jugular bolus and tail vein injections for 3 days post-injury. After 14 days, activating Notch signaling decreased the overall vascular density within the penumbral gray matter compared to controls while maintaining the density of perfused vessels. Inhibiting Notch signaling did not change the density or perfusion of microvessels within the lesion penumbra. Furthermore, neither activation nor inhibition of Notch signaling significantly altered inflammation, hypoxia, and lesion volume in the epicenter and penumbra. Importantly, neither treatment changed locomotor function. In postnatal retinal vascular assays, administration of Jag1-Fc and N1 Ab increased and decreased both tip cell numbers and branch points in each treatment, respectively. However, these agents did not modulate primary CNS EC proliferation *in vitro* in spite of sufficient Notch ligand expression. We conclude that Notch signaling, while an important part of developmental angiogenesis, may play a lesser role in mediating vascular recovery following traumatic injury to the CNS.

Keywords

spinal cord injury; Notch; angiogenesis; locomotion; endothelial cell

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Introduction

Notch signaling is vital to vascular development and stability in the adult mammal. Mutations in Notch or its ligands in humans causes vascular conditions such as Allagile syndrome, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and aides in tumorigenesis by regulating sprouting angiogenesis (Joutel, et al., 1996, McDaniell, et al., 2006, Noguera-Troise, et al., 2006, Warthen, et al., 2006). Notch signaling can be effectively modified to either enhance or diminish perfusion of a tumor and the post-natal retina (Benedito, et al., 2009, Li and Harris, 2005). Pathological disruption of CNS vasculature, such as an ischemic stroke or spinal cord injury (SCI), leaves the neural parenchyma without adequate perfusion. In spite of a period of angiogenesis between days 3 and 14 following injury, several markers of normal vascular functionality are absent in a majority of microvessels, including intravascular lectin binding, glucose-transporter 1 expression, and disorganization of tight junction components (Benton, et al., 2008, Whetstone, et al., 2003). Notch signaling may be an effective way to restore the functional status of these angiogenic microvessels post-SCI.

Notch is a type 1 transmembrane receptor that requires cell-to-cell contact for activation by its five transmembrane ligands Jagged (Jag 1 and 2) and Delta-like ligand (Dll1, 3, and 4). The ubiquitin ligase mind bomb regulates ligand endocytosis which triggers cleavage of the Notch extracellular domain by TNF-alpha converting enzyme (TACE) and subsequent receptor processing (Itoh, et al., 2003, Mumm, et al., 2000). Gamma-secretase cleavages by nicastrin and presenilin allows the Notch intracellular domain (NICD) to translocate to the nucleus where it binds CSL (CBF1/Su(H)/Lag-1) to initiate transcription of Notch target genes, notably Hairy/Enhancer of Split (Hes) and HEs-related Repressor Protein (Herp) families (Kopan and Goate, 2002, Kurooka and Honjo, 2000, Wu, et al., 2000). Notch target genes control EC proliferation through inhibition of retinoblastoma phosphorylation (Nosedá, et al., 2004) and downregulation of minichromosome maintenance proteins (Nosedá, et al., 2005), suggesting Notch may be an important target to acutely push ECs to an angiogenic phenotype after SCI.

Following SCI, the lesion epicenter fills with hemorrhage due to dramatic shearing of the microvascular network (Noble and Wrathall, 1989). Over the next three days, the majority of endothelial cells (ECs) are lost leading to an angiogenic response lasting until day 14 post-injury (Benton, et al., 2008, Loy, et al., 2002, Whetstone, et al., 2003). Although seventy percent of microvessels in the injured mouse spinal cord recover by day 14, half of these vessels are insufficiently perfused, many exhibit abnormal tight junction marker expression (Benton, et al., 2008), express aberrant markers of fenestration (Mozer, et al., 2010), and have glycosylation patterns that suggest activation (Benton, et al., 2008). In pathological models, including hindlimb ischemia (Takeshita, et al., 2007) and choroidal neovascularization (Dou, et al., 2007), Notch controls sprouting and organization of the vascular tree leading to adequate perfusion of the ischemic or injured tissue. In the mouse, angiogenesis post-SCI is inadequate and Notch signaling modulation could enhance microvascular sprouting and maturation leading to improved tissue perfusion and controlled inflammation.

The current study manipulates Notch signaling through intravenous infusion of either a known pharmacological agonist (N1 Ab) or antagonist (Jag1-Fc) (Conboy, et al., 2003) immediately and for several days following contusive SCI. Although Notch activation diminished microvascular density in the lesion penumbra, the density of intravascular lectin binding was comparable to control values, suggesting the proportion of surviving microvessels has increased perfusion with Notch activation. Nevertheless, there were no apparent differences in inflammation, hypoxia, and locomotor function. This study

demonstrates that post-SCI Notch signaling diminishes the population of non-patent microvasculature, but does not contribute to functional recovery.

Materials and Methods

Mice

Wild type mice were obtained from Harlan Laboratories (Indianapolis, IN) at 6–8 wks of age for SCI experiments. For postnatal experiments, timed pregnant females at embryonic day 15 were obtained and pups utilized at postnatal day 1 (P1). All animal studies were completed in compliance with University of Louisville IACUC regulations (IACUC #07018 and #10040).

Postnatal Retina

Mice were given subcutaneous injections of 10 $\mu\text{L/g}$ of body weight of 0.7 $\mu\text{g}/\mu\text{L}$ Jagged1-Fc chimera (Jag1-Fc; R&D Systems), 0.7 $\mu\text{g}/\mu\text{L}$ human Fc control (Fc; Sigma, St. Louis, MO), 1:2 dilution hamster IgG control (IgG; Invitrogen, Carlsbad, CA), 1:2 dilution anti-Notch1 antibody (N1 Ab; Upstate/Millipore, Billerica, MA), or vehicle (DPBS) on days P1–P5 with a Hamilton syringe. Eyes were removed on P6, fixed in 4% paraformaldehyde for 1 hr, then retinas were dissected in 0.1 M PBS. Whole retinas were blocked at 4°C overnight in 0.5% Triton in 0.1M PBS (0.5% PBST) with 10% donkey serum and 0.5% BSA. Primary antibody was applied in 0.5% PBST with 5% donkey serum and 0.5% BSA and left for 4 days at 4°C. Secondary antibody was applied in the same solution and incubated overnight at 4°C. Negative controls for each antibody staining were done by parallel substitution of species-matched pre-immune IgG and resulted in no staining.

Retinal quantitative analyses

Photomicrographs at 20 $\times$ of whole mounted retinas were captured using an Eclipse C1 laser confocal microscope (Nikon, Melville, NY). Per retina, 3–4 images were captured and quantified (Hellstrom, et al., 2007). Tip cells were manually counted as PECAM⁺ blind-ended protrusions with filopodial extensions within a 200 $\times$ 300 μm area (DPBS n = 5; Fc n = 7; IgG n = 5; Jag1-Fc n = 6; N1 Ab n = 6). Branch points were counted as 3 intersecting capillary segments, with 4 intersecting segments counted as 2 points, within a 200 $\times$ 200 μm area (DPBS n = 5; Fc n = 7; IgG n = 5; Jag1-Fc n = 8; N1 Ab n = 8).

Parameters for contusion injury of the mouse spinal cord

Following induction of anesthesia (1.2% Avertin, 2-2-2-tribromoethanol, 240 mg/kg given IP), the T8–T10 vertebrae of female mice, 6–8 weeks of age (17–20 g) were exposed and a single laminectomy at the T9 vertebral level performed to expose the spinal cord. After the T8 and T10 vertebrae were secured by immobilized forceps, the impactor (Infinite Horizons; Lexington, KY) (Scheff, et al., 2003) was centered over the exposed spinal cord (level T10), and a moderate 50 kilodyne injury was performed. Injury severity as indicated by displacement of spinal cord tissue was not significantly different between groups. Immediately after contusion, 50 μL of either 0.7 $\mu\text{g}/\mu\text{L}$ Jag1-Fc, 0.7 $\mu\text{g}/\mu\text{L}$ human Fc, 1:2 dilution IgG, or 1:2 dilution N1 Ab was infused into the surgically exposed left external jugular vein. After drug infusion and confirmation of the SCI site under the surgical microscope, the musculature and skin incisions were sutured. Postoperative care included normal saline during surgical procedures, gentamicin every 48 hrs for 5 days, buprenorphine twice a day for 48 hrs, and manual expression of bladders twice a day for seven to ten days or until spontaneous voiding returned. Daily tail vein injections of the appropriate drug and dose followed for 3 days post-injury. All surgical and post-operation procedures were completed according to NIH and IACUC guidelines.

BMS

The baseline BMS score was obtained following 4 days of gentling and subsequently scored days 7 and 14 days post-injury by two testers trained at the Ohio State University and blinded to treatment groups (Basso, et al., 2006).

Treadscan

Mice were run on the Treadscan® (Columbus Instruments; Columbus, OH) day 14 post-injury before the final BMS session prior to sacrifice (Beare et al., 2009). Briefly, a high-speed digital video camera was mounted to record the ventral view of a transparent treadmill belt reflected off the angled mirror mounted below. Digital video images of the underside of the mouse were recorded at 100 frames/second. An adjustable compartment measuring 17 cm × 5 cm was mounted over the treadmill belt, ensuring that the mouse would remain in the view of the camera at all times. TreadScan® software (CleverSys, Inc, Reston, VA) identified each individual paw of the mouse in each frame as it walked on the treadmill as well as initial foot contact, stance duration, stride duration, foot liftoff, swing duration, stride length, track width, and toe spread data for each foot. The outline of each paw was drawn on the computer screen using the software's built-in tracing system. Mice walked on the treadmill for 20-second sessions resulting in 2000 captured frames.

Terminal systemic intravascular injections

Animals were sacrificed 14 days following SCI following the terminal TreadScan® session. After induction of anesthesia as described above, pimonidazole HCl (60 mg/kg in saline; HPI, Inc, Burlington, MA) was infused systemically into the surgically exposed right external jugular vein at a rate of 1.2 ml/hr using a syringe pump (model #: 780100, KD Scientific, Holliston, MA) (Benton, et al., 2005). This hypoxypore conjugates to thiol groups on proteins under hypoxic conditions where $O_2 < 10$ mm Hg (Arteel, et al., 1995). After 15 minutes of hypoxypore circulation, FITC-conjugated *Lycopersicon esculentum* agglutinin lectin (LEA; 100 µg (50 µL at 2 mg/ml); Vector Laboratories, Inc., Burlingame, CA) was infused systemically at the same rate as the hypoxypore into the surgically exposed left external jugular vein. Intravascular lectin is a measure of the functional perfusion status of the microvasculature. Prior to perfusion with 0.1M PBS, FITC-LEA is allowed to circulate for 10 minutes for adequate visualization of the spinal microvasculature (Benton, et al., 2008). The hypoxypore circulates for a total of 30 min before transcardial perfusion. Spinal cords were dissected and frozen on dry ice.

Immunohistochemistry

One centimeter blocks centered on the epicenter of fresh frozen spinal cords were longitudinally sectioned at 20 µm on a cryostat. Sections were dried at 37°C for a minimum of 30 minutes then post-fixed in -20°C methanol for 10 min. Sections were blocked in 0.1M Tris-buffered saline (TBS; pH 7.4), 0.1% Triton X-100, 0.5% bovine serum albumin (BSA), and 10% normal donkey serum for 1 hour at room temperature or overnight at 4°C. Negative controls for each antibody staining were done by parallel substitution of species-matched pre-immune IgG and resulted in no staining. Primary antibodies were applied in 0.1M TBS (pH 7.4), 0.1% Triton X-100, 0.5% BSA, and 5% normal donkey serum for 48 hrs in a humidified chamber at 4°C. The following primary antibodies were used: polyclonal goat anti-FITC (1:1000; Vector Labs) to enhance the signal of the FITC-conjugated lectin; Rat anti-PECAM (1:50; BD Pharmingen) to detect all endothelial cells; rabbit anti-laminin (1:100; Sigma) to identify the vascular heterodomain (Whetstone, et al., 2003); polyclonal rabbit anti-occludin (1:100; Zymed, Carlsbad, CA) to detect endothelial tight junctions; mouse anti-CD68 identifies microglia and macrophages (1:100; Chemicon, Billerica, MA); rat anti-CD45 (1:100; Chemicon, Billerica, MA) to identify all cells of hematopoietic origin.

Sections were incubated with TRITC- or AMCA-conjugated secondary Fab' fragment antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature in a humidified chamber. Excess secondary antibody was eliminated by 3 washes in 0.1M TBS (pH 7.4) then coverslipped.

Primary mouse cortical EC cultures

Using methods developed previously by Nunes et al. (2010), 15 mice (6 wks old) were anesthetized and cortices extracted and placed in L15 with 2% FBS and 1% pen/strep. Briefly, cortices were rinsed with 1% BSA in PBS and minced. Tissue was incubated 20 min at 37°C in 2.5 mL collagenase/mL of tissue and 0.1 mg DNase I/mL. Digested tissue was resuspended in 15% Dextran and centrifuged at 4000 $\times$ /g at 4°C for 20 min. Supernatant was removed and the vascular pellet resuspended then passed through a 500 μ m screen. The filtrate was passed through a 20 μ m screen and vascular fragments retained on the screen were retrieved by soaking it in 1% BSA in PBS. Microvascular fragments were seeded at 100,000 vessels/mL in collagen coated wells in DMEM/F12 with 20% FBS, EC growth serum (Millipore), heparin, and puromycin (2 ng/ μ L) to kill smooth muscle cells. After 2.5 days, the puromycin is removed. When ECs migrating out of the microvascular fragments reach confluency, cells are passaged using a brief trypsinization into 96 well plates (30,000/well) in media without puromycin.

BrdU administration and detection

To detect proliferating cells, 5-bromodeoxyuridine (BrdU) was administered at 50 mg/kg intraperitoneally once daily on days 3–13 post-injury or added to cultures for 24 hrs. BrdU was dissolved in saline (pH 7.35) then filtered just prior to administration. Tissue sections and culture wells were treated as above through application of the primary rat anti-PECAM and the appropriate secondary antibody. After the excess secondary antibody was eliminated, sections were fixed again in cold methanol. BrdU was detected by acid treating (2N HCl at 37°C for 30 min) tissue sections following fixation. The acid was neutralized in 0.1M boric acid (pH 8.5) before the sheep anti-BrdU (1:100; Biotools, Cincinnati, OH) was applied overnight at 4°C. Secondary antibody was applied as above.

PCR

Reverse transcription was performed using 500 ng of RNA isolated from passage 0 (P0) primary cortical ECs using the First Strand Synthesis reaction (SA Biosciences, Frederick, MD). The resulting cDNA was used for end point polymerase chain reaction (PCR) using the GoTaq® DNA polymerase products (Promega, Madison, WI). Each reaction contained 4 μ L of 5 $\times$ PCR buffer, 2 μ L MgCl₂, 0.5 μ L of 10 mM dNTPs, 0.1 μ L 100 μ M Forward primer, 0.1 μ L 100 μ M Reverse primer, 0.2 μ L GoTaq®, 11.1 μ L DNase, RNase-Free H₂O, and 2 μ L cDNA. Primer sequences are as follows (Invitrogen, Carlsbad, CA): Notch1 forward—5' CAT CAC TGT GAG ACC AAC ATC 3'; Notch1 reverse—5' CCC TGT GGT TCC CTT GAG 3' (114 bp, T_a = 54.2°C); Hes1 forward—5' CCT CTG AGC ACA GAA AGT CAT 3'; Hes1 reverse—5' CCC ACT GTT GCT GGT GTA G 3' (674 bp, T_a = 53.7°C); Jag1 forward—5' CCT GCG AGC CAA GGT GTG 3'; Jag1 reverse—5' GAC CTC GGC CAG GCG A 3' (324 bp, T_a = 59.2°C); Dll1 forward—5' GCC CTG CTG TGC CAG GTC 3'; Dll1 reverse—5' GGC TGA TGA GTC TTT CTG GGT 3' (394 bp, T_a = 59.2°C); Dll4 forward—5' CGA GAG CAG GGA AGC CAT GA 3'; Dll4 reverse—5' CCT GCC TTA TAC CTC TGT GG 3' (379 bp, T_a = 55.0°C).

Quantitative analyses

Longitudinal sections from LEA-infused animals were used to assess the density and location of blood vessels, rostral-caudal extent of inflammation and hypoxia, extent of

laminin deposition, and integrity of the blood-spinal cord-barrier. Photomicrographs were captured at 10× with a Nikon TiE 300 inverted microscope equipped with a DXM-1200C coded digital camera and NIS Elements software (Nikon, Melville, NY). The percent area containing LEA⁺ or PECAM⁺ vessels was determined by detecting green and red pixels above of a certain threshold of intensity within the region of interest (ROI) drawn around the area of significant extravascular laminin deposition or area of cleared laminin, where laminin was again associated with the microvasculature. Penumbral vascularity analyses (Fig. 1) were accomplished by expanding the epicenter (e) ROI surrounding significant extravascular laminin (asterisks) by 500 μm then isolating the gray matter (g) from the white (w).

The pan-leukocyte marker CD45 immunostaining, indicating the extent of inflammation, and the hypoxyprobe, indicating hypoxia, in the lesion epicenter and penumbral area were expressed as a density within a defined rostral-caudal distance centered on the epicenter and limited to the area of the cord. Lesion volume was calculated by taking the area of the ROI encompassing the epicenter only and averaging its volume with the adjacent section volume (100 μm apart) to account for the space between sections. This average was added to the next section volume and the process repeated for all sections across the injured cord, i.e. $(\text{Area 1}) \times 20 \mu\text{m} + ((\text{Area 1} + \text{Area 2})/2) \times 80 \mu\text{m} + (\text{Area 2}) \times 20 \mu\text{m} + ((\text{Area 2} + \text{Area 3})/2) \times 80 \mu\text{m} + \dots \text{etc.}$

In vitro studies included counting all Hoescht-stained nuclei that had neither cleaved nor condensed chromatin, within 10 fields per 96 well (3 wells per treatment) at 20× magnification and BrdU⁺ nuclei. The BrdU index was calculated as the fraction of BrdU⁺ cells within the total population (n = 3 experimental replicates).

Statistical analyses

Average BMS and Treadscan scores were analyzed using either independent t-tests of right and left sides averaged or repeated measures ANOVA with the between groups factor to examine differences between the groups. Mixed Measures ANOVA determined significance of BMS scores between groups. Following significant effects with ANOVA analysis, either Bonferroni post hoc t-test following mixed measures ANOVA or Tukey post hoc t-test following repeated measures ANOVA were performed. Vascularity analyses were assessed with independent t-tests of individual epicenter values averaged across 600 μm, sampling every 100 μm or every 200 μm in tissue. Retinal assays were assessed with independent t-tests of averages from 3–4 fields per retina, 5–8 retinas per treatment. *In vitro* analyses were assessed with independent t-tests of 3 wells each drug, 10–20 fields each well, across 3 replicate experiments.

Results

Jag1-Fc and N1 Ab modify tip cell differentiation and vessel branching in the postnatal retinal vasculature

Notch signaling in the mouse retinal vasculature has been studied extensively as it develops over the first postnatal week. Notch and its ligands regulate tip cell differentiation which determines sprout formation and vessel branching (Benedito, et al., 2009, Hellstrom, et al., 2007, Suchting, et al., 2007). To confirm the biological activity of the pharmacological agents used in this study, we treated mice postnatal days 1–5 (P1–5) subcutaneously with vehicle, control, Notch agonist (N1 Ab) or antagonist (Jag1-Fc). The retinas were then dissected at P6 and stained for PECAM to detect the developing retinal vasculature. Jag1-Fc inhibition of Notch resulted in significantly more branch points and tip cell numbers, while N1 Ab activation of Notch robustly decreased both branch points and tip cell numbers (Fig.

2F,G). It can be concluded from these data that these pharmacological agents modify Notch signaling in a way consistent with previously published literature, where Notch inhibition or activation increases or decreases vascular density, respectively.

Temporal expression of Notch and Notch ligands 3–14 days post-SCI

The temporal expression patterns of Notch1 protein and signaling activation were examined following mid-thoracic contusion SCI. Confocal microscopy reveals Notch1 in the membranes of ECs and activated NICD concentrated in the EC nuclei of LEA⁺ microvessels in the sham spinal cord (Fig. 3A,E; arrows). Decreased expression of transmembrane Notch1 occurs after 3 dpi (Fig. 3B) and persists 14 dpi (Fig. 3C–D; arrows). NICD expression is no longer concentrated in the nuclei as in sham animals (Fig. 3E) but is found in the cytoplasm in few ECs 3–14 dpi (Fig. 3F–H; arrows). It is clear that other cells, presumably predominantly inflammatory, also have increased Notch activation post-SCI. Ligand Dll4 is expressed in all microvessels in the sham spinal cord (Fig. 3I) but is highly concentrated in some ECs and decreased in others 3 dpi post-injury (Fig. 3J; arrows). By 14 dpi, Dll4 expression increases to control levels in most ECs (Fig. 3K,L; arrows). On the other hand, EC membranes are minimally stained for Jag1 in sham animals (Fig. 3M; arrow) with more ECs expressing Jag1 3–14 dpi (Fig. 3N–P; arrows). Negative IgG antibody controls shows little staining in microvessels and surrounding cells (Fig. 3Q,R).

Notch1 activation post-SCI increases the proportion of perfused to non-perfused microvessels in the penumbral gray matter

Administration of a Notch inhibitor did not modify total (Fig. 4E,F) and perfused (Fig. 4G,H) vascular density compared to control levels 14 dpi. However, activation of Notch1 by a stimulating antibody decreased the population of non-perfused microvessels in the penumbral gray matter alone (Fig. 4I,J). The decreased density of PECAM-labeled vessels (Fig. 4I; $p < 0.05$) coupled with maintenance of the perfusion index (Fig. 4K), as visualized by intravascular LEA binding, in the penumbral gray matter indicates a modification of the angiogenic response that favors perfused vessels. The decreased density of PECAM-labeled vessels was not significant but showed a clear trend in the white matter penumbra as well (Fig. 4J; $p = 0.08$) along with stable perfusion (Fig. 4L). The already low vascular density in the white matter of the spinal cord may have affected statistical power associated with counting vessels within a relatively large area. In addition, the volume of the lesion epicenter as well as penumbral gray and white matter, were not different between groups, potentially indicative of stable perfusion levels in Notch agonist and antagonist treated mice (Fig. 4M–R).

Microvessel activation and maturation indicated by caveolar protein PV-1 and occludin expression within the lesion epicenter is not affected by manipulation of Notch signaling post-SCI

The plasmalemma vesicle-associated protein 1 (PV-1) is expressed normally in fenestrated endothelium of the lung and kidney (Stan, et al., 1999), and is an indicator of blood-brain barrier disruption and leukocyte trafficking (Keuschnigg, et al., 2009, Shue, et al., 2008). Following SCI, PV-1⁺ vessels appear in the lesion epicenter by 12 hrs post-injury and increase dramatically by 24 hrs remaining elevated through 14 dpi (Mozer, et al., 2010). By day 14 in Notch agonist- or antagonist-treated animals, PV-1⁺ vessels are restricted to the epicenter but are not different from control-treated animals (Fig. 5E). Likewise, expression of tight junction marker occludin is similar in PV-1⁺/LEA⁺ microvessels between control and treated mice 14 dpi (Fig. 5F). Surprisingly, modification of Notch signaling did not change microvascular activation and maturation post-SCI using these markers.

Modification of penumbral gray matter angiogenesis does not influence the magnitude of inflammation or hypoxia

Inflammatory cell infiltration through activated, leaky microvessels contributes to the chronic inflammation in both rodent and human SCI (Fleming, et al., 2006, Popovich, et al., 1996, Sroga, et al., 2003). Manipulation of Notch signaling does not impact the activation status of the angiogenic vessels (Fig. 5E) although non-perfused vessels are lost with Notch activation (Fig. 4J). Additionally, in spite of literature demonstrating Notch signaling in immune cells (Maillard, et al., 2008, Murata-Ohsawa, et al., 2005, Santos, et al., 2007, Singh, et al., 2000), the magnitude of inflammation indicated by the pan-leukocyte marker CD45 also does not change 14 dpi (Fig. 6E,F). The magnitude of macrophage infiltration assessed by CD68 immunohistochemistry also was not different between groups at 14 dpi (data not shown) in spite of Notch, Jagged1, 2 and DLL1, 4 expression (Fung, et al., 2007, Yamaguchi, et al., 2002). Furthermore, the hypoxyprobe pimonidazole HCl showed no difference in conjugation to protein thiol groups under hypoxic conditions (<10 mm Hg) in control and Notch-manipulated animals (Fig. 6L,M). This indicates that oxygen levels were similar in all animals, which corroborates results that show no change in perfusion index in the penumbra.

Locomotor recovery is not enhanced with Notch inhibition or activation

Secondary pathology associated with chronic inflammation and hypoxia is an impediment to locomotor recovery post-SCI due to loss of spared white matter. Targeting vascular pathology would presumably alleviate tissue damage and spare functional axons. With no change in overall perfusion to the spinal cord in spite of decreased angiogenesis of non-functional vessels, there appeared to be little impact on the inflammatory or hypoxic response post-injury. Not surprisingly then, there was no change in locomotor recovery at 7 and 14 dpi as assessed by BMS scoring (Fig. 7). A few differences in gait characteristics were detected with Treadscan®, including foot base and longitudinal deviation from midline for the Notch agonist and run speed for the Notch antagonist, but not the main parameters identified to change with SCI, namely rear track width, hindlimb toe spread, and hindlimb swing time (Beare, et al., 2009).

Primary mouse cortical ECs express Notch1 but do not have significant activation of Notch signaling

When Jag1-Fc and N1 Ab (10 µg/mL) are administered to passage 1 primary murine cells *in vitro*, there is no change in proliferation (Fig. 8E) in spite of previous reports with human umbilical vein EC (HUVEC) and human aortic EC (HAEC) lines (Harrington, et al., 2007, Limbourg, et al., 2007). NICD staining in the nucleus and perinuclear regions and is similar in all treatment groups (Fig. 8F,I). That the staining is not localized to the cell membrane may indicate a post-translational defect in processing since the antibody detects both the intracellular portion of the transmembrane receptor and the gamma-secretase-cleaved intracellular domain. To confirm Notch and Notch ligand expression in CNS ECs, RNA from cortical ECs isolated from mouse brains was isolated for standard, end point PCR. Primary ECs express *notch1* and the Notch ligands *jagged1*, *dll1*, and *dll4* (Fig. 8K,M). Mouse glial-restricted precursor cells (mGRPs) known to express Notch signaling components were used as positive controls to monitor PCR conditions. Robust expression of *notch1*, *jag1*, *dll1*, and *dll4* was observed, as well as decreased *hes1* upon differentiation to oligodendrocytes with thyroid hormone treatment (Wu, et al., 2003), indicating the validity of the RNA primers (Fig. 8J,L). The expression of Notch1 ligands *jag1*, *dll1*, and *dll4* and would suggest that primary cortical ECs retain the potential to activate Notch signaling *in vitro*. However, lack of changes in proliferation with the Notch1 agonist and antagonist suggest that primary mouse ECs react differently *in vitro* than do EC lines.

Discussion

Modulators of Notch signaling mediate the organization of vascular networks in both developmental and pathological contexts. In retinal and tumor vasculature, Notch activation leads to decreased angiogenesis due to decreased tip cell differentiation, whereas Notch inhibition increases angiogenesis (Benedito, et al., 2009, Hellstrom, et al., 2007). Notch activation by N1 Ab also had this effect in the postnatal mouse retina where tip cell number and branch points were decreased and likewise Notch inhibition via Jag1-Fc increased tip cells and branch points. These data support the biological activity of these effectors *in vivo*. Further, immunoglobulin (IgG) 1 half-life studied in Balb/c and severe combined immunodeficient (SCID) mice was similar between species and was found to be approximately 144 hrs (about 6 days) in the whole body (Zuckier, et al., 1994). With intravenous administration immediately and 3 days following injury, we expect that the IgG₁ antibodies for Jag1 and Notch1 gained sufficient concentration in the blood during the targeted period of angiogenesis. Still, we recognize that immediate administration of a therapy for SCI is of little clinical relevance and future studies would address a time course more applicable to the human condition pending results demonstrating significant behavioral recovery.

Following a traumatic SCI in the mouse, Notch activation by N1 Ab increases the proportion of total to perfused microvessels in the gray matter immediately surrounding the epicenter. In spite of losing a portion of the dysfunctional vasculature, the inflammatory response and level of hypoxia are unchanged. Coupled with the similarity in lesion volume and perfusion index, it appears that Notch agonist or antagonist-treated animals and control animals are similarly susceptible to reperfusion injury (Guth, et al., 1999). This suggests that the progression of secondary injury is not modified by Notch signaling, and the lack of locomotor differences support this observation. Quantification of lesion volume in its entirety and differentiating penumbral gray and white matter resulted in no significant differences. Moreover, a lack of enhanced behavioral recovery indicates there was likely no difference in gray and white spared matter. Although we cannot completely exclude this possibility without further investigation, we feel it is unlikely that Notch activation significantly modified the histopathological progression of thoracic SCI.

In the context of SCI, inhibiting Notch signaling should increase penumbral angiogenesis and activating Notch would decrease it. Administration of N1 Ab activated Notch on angiogenic microvessels presumably by preventing ECs from becoming tip cells leading to decreased formation of PECAM-labeled microvascular sprouts in the penumbral gray matter. No difference in microvessel formation occurred with administration of the Notch inhibitor, suggesting differential effects in these distinct microvascular populations.

Developmentally, Notch signaling stabilizes new vascular sprouts by switching the vessel phenotype from angiogenic to quiescent, thus promoting the maturation and stabilization of the vascular tree (Taylor, et al., 2002). This switch is thought to be primarily driven by Notch-mediated inhibition of retinoblastoma protein phosphorylation which prevents cell cycling (Nosedá, et al., 2004). Consistent with this hypothesis, Notch agonist administration decreased penumbral gray matter PECAM density while maintaining the perfusion index (Fig. 4). Fewer microvessels indicates less EC proliferation, and fewer microvessels with the same amount of perfused microvessels indicates that Notch activation increases the efficiency of blood supply to vulnerable spinal cord tissue. These microvessels that are not perfused may be pruned due to lack of flow or a dysregulation of Notch signaling during remodeling (Karsan, 2005, Kurz, 2000).

Activation of Notch1 protects serum-starved HUVECs from apoptosis (Liu, et al., 2003, Taylor, et al., 2002). Additional literature with HUVECs and HAECs has suggested that Notch regulates arteriogenesis and angiogenesis (Harrington, et al., 2007, Limbourg, et al., 2007). In contrast, our mRNA expression data suggest that in spite of Notch ligands and target expression, primary CNS ECs are different with respect to their *in vitro* behavior. Application of the Notch antagonist or the agonist did not affect primary cortical EC proliferation as previously shown in passaged cells *in vitro*. The retina model confirmed the action of these pharmacological agents *in vivo*. Decreased expression ofDll4 inhibits Notch signaling in the retina and results in enhanced vessel branching and tip cell differentiation (Suchting, et al., 2007) a result also seen in P6 retinas with administration of the Notch inhibitor. Conversely, gain-of-function Jagged1 expression decreases vessel branching and severely limits development of the vascular tree by decreasing tip cell formation (Benedito, et al., 2009). This was also, to a lesser extent, observed in the retina with administration of the Notch agonist. These data suggest that *in vitro* and post-SCI microvessel ECs differentially respond to Notch signaling. Alternatively, during *in vivo* SCI experiments spinal cord parenchymal access may have been limited. However, the data shown in Fig. 4 argue against this latter possibility. Moreover, the blood-spinal cord-barrier is permeable to large molecules for up to 21 days in the mouse (Popovich, et al., 1996) and given that these effectors were administered IV immediately following and for 3 days after injury, the animals were treated during a time course of significant barrier permeability.

Conclusions

We pursued Notch as a potential therapeutic target to modify angiogenesis post-SCI to produce a functional, mature microvascular network. Although Notch activation resulted in a more efficient microvascular population, this did not translate to enhancement of locomotor recovery. The lack of locomotor improvement due to the modification in the vasculature suggests that Notch is not an appropriate therapeutic target in SCI. Nonetheless, vascular-mediated secondary pathology post-SCI is an important problem to address acutely after injury (Loy, et al., 2002, Noble and Wrathall, 1989, Norenberg, et al., 2004). Pro-inflammatory mediators and dysregulation of tight-junction proteins lead to a massive immune cell infiltration and vascular leakage (Popovich, et al., 1996, Whetstone, et al., 2003). Additionally, hypoxia due to loss of ECs early following injury may be a major impediment to endogenous repair processes and clinical treatment. In fact, rescuing the microvasculature after contusive SCI does enhance functional recovery (Han, et al., 2010). We believe that a combination of vascular protection and functional angiogenesis is necessary for spinal cord repair following injury. Unlike tumorigenesis and vascular development, Notch signaling is not a part of that therapeutic equation.

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Abbreviations

(SCI)	Spinal cord injury
(ECs)	endothelial cells
(NICD)	Notch intracellular domain

(Jag)	Jagged
(Dll)	Delta-like ligand
(Hes)	Hairy/Enhancer of Split
(dpi)	days post-injury
(N1 Ab)	anti-Notch1 antibody
(Jag1-Fc)	Jagged1-Fc chimeric protein
(LEA)	Lycopersicon esculentum agglutinin
(PV-1)	plasmalemma vesicle associated protein 1
(BrdU)	5-bromodeoxyuridine
(ROI)	region of interest

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Fig. 1.

The percent area containing LEA⁺ or PECAM⁺ vessels was determined by detecting green and red pixels above of a certain threshold of intensity within the region of interest (ROI) drawn around the area of significant extravascular laminin deposition or area of cleared laminin, where laminin was again associated with the microvasculature. Penumbral vascularity analyses were accomplished by expanding the epicenter (e; red) ROI surrounding significant extravascular laminin (blue; asterisks) by 500 µm then isolating the gray matter (g; yellow) from the white (w; green). The magnification of the photomicrographs is 10×.

Fig. 2. Treatment of postnatal mouse pups days 1–5 (P1–5) with vehicle (A), Fc (B), or IgG (C) controls results in similar branching patterns and tip cell differentiation (F, G). Notch inhibition by Jag1-Fc (D) increases branching (F; * $p < 0.05$) and tip cell numbers (G; * $p < 0.05$), while Notch activation by N1 Ab (E) suppresses branching (F; *** $p < 0.001$) and tip cell differentiation (G; *** $p < 0.001$). The magnification of the photomicrographs is 20 $\times$ and the quantitative data are means $\pm$ SD.

Fig. 3.

Notch1 expression with activated NICD is detected at low levels in the sham animal (A,E; arrows) in LEA⁺ vessels and begins to decrease after 3 dpi (B,F; arrows). One to two weeks post-injury (C–D,G–H; arrows) Notch1 and activated NICD are detected in few ECs. NICD expression is apparent in the cytoplasm post-injury (F–H) whereas in the sham spinal cord it is concentrated in the nucleus (E). Dll4 expression is detectable in all ECs in the sham spinal cord (I; arrows). Post-injury Dll4 expression is decreased although it persists in most ECs 3–14 dpi (J–L; arrows). Jag1 is detected in few ECs in the sham animal (M) but its expression increases through 2 weeks post-injury (N–P; arrows). Rabbit and mouse IgG control show no staining 7 and 14 dpi (Q,R). The magnification of the photomicrographs is 80 $\times$.

Fig. 4.

Notch signaling activation decreases microvascular density in the lesion penumbra yet maintains the density of intraluminal LEA binding 14 days post-SCI. Representative pictures of lesion epicenters from animals treated with either Fc control (A), antagonist Jag1-Fc (B), IgG control (C), or agonist N1 Ab (D). Inhibition of Notch signaling by infusion of Jag1-Fc does not change the area of PECAM expression (E–F) in the penumbral areas surrounding lesion epicenter (Fc $n = 8$; Jag1-Fc $n = 9$). Similarly, there is no change in intraluminal LEA binding (G–H) in either the penumbral gray or white matter (Fc $n = 8$; Jag1-Fc $n = 9$). Infusion of N1 Ab tpost-SCI decreases the density of PECAM-labeled microvessels in the penumbral gray (I; $**p < 0.01$) but not penumbral white matter (J; $p = 0.08$; IgG $n = 10$; N1 Ab $n = 10$). In spite of decreased angiogenesis, the similarity in perfusion index would indicate that the control group has more non-functional, i.e. non-perfused, microvessels in the penumbra (K–L; IgG $n = 8$; N1 Ab $n = 7$). Additionally there was no change in epicenter or penumbral gray and white matter volumes (M–R). The magnification of the photomicrographs is 10 $\times$ and the quantitative data are means $\pm$ SD.

Fig. 5.

Plasmalemma vessel associated protein 1 (PV-1) and occludin expression in the lesion epicenter is similar to controls in spite of changes in microvascular density in the lesion penumbra. Representative pictures of lesion epicenters containing PV-1⁺ microvessels (A–D, 10× magnification; inset is 60× magnification showing LEA⁺ and PV-1⁺ microvessels). The number of microvessels expressing PV-1 counted within the lesion epicenter is not changed with either Jag1-Fc (A,B,E) or N1 Ab (C–E) treatment post-SCI. In (F), the XZ and YZ confocal planes are indicated to confirm co-localization. Expression of the tight junction marker occludin appears to be similar in PV-1⁺/LEA⁺ vessels in all treatment groups (F). The quantitative data are means $\pm$ SD (n = 7–10 / treatment).

Fig. 6.

Decreased immature microvessels in penumbral gray matter alone does not influence the magnitude of inflammation or hypoxia 14 days post-SCI. Neither inhibition (A, B, E; Fc treated $n = 10$; Jag1-Fc treated $n = 12$) nor activation (C, D, F; IgG $n = 10$; N1 Ab treated $n = 10$) of Notch signaling changes the percent area of CD45 expression after contusion SCI. Insets in A–D (10 $\times$) are 20 $\times$ magnification of CD45⁺ cells in the penumbra. Area of hypoxyprobe conjugation to proteins under hypoxic conditions does not change with either inhibition (G, H, L; $n = 6$; $n = 6$) or activation (I, J, M; $n = 4$; $n = 3$) of Notch signaling. The negative control is pictured to show lack of staining with a rabbit IgG (K). The quantitative data are means $\pm$ SD.

Fig. 7. Locomotor behavior does not change with Notch signaling manipulation following thoracic SCI. Locomotor function assessed by the BMS is not significantly altered at 7 and 14 days post-injury with Notch signaling inhibition (Jag1-Fc) or activation (N1 Ab) as compared to the appropriate control molecule Fc and IgG, respectively. The quantitative data are means $\pm$ SD (n = 9–10 / treatment).

Fig. 8.

EC proliferation is not affected when primary cultures are treated with a Notch signaling agonist or antagonist. Primary cortical ECs were isolated from adult mice and treated for 24 hrs with BrdU and either vehicle, Fc control, Jag1-Fc, or N1 Ab (A–D; 20× magnification). The BrdU index was calculated from counting BrdU positive cells and dividing by the total number of Hoescht stained cells in 10 random fields in 3–4 wells per group (E; n = 3). Cells were stained for NICD (F–I) to qualitatively assess Notch activation in one experimental replicate. Mouse glial-restricted precursors (mGRPs) express *notch1*, *hes1*, and *jag1* (J) as well as *dll1* and *dll4* (L). Thyroid hormone-treated mGRPs show decreased Notch activation which induces differentiation to oligodendrocytes, i.e. *hes1* expression decreases with exposure to differentiation medium days 0 through 2 (J). This indicates the validity of mouse Notch primers. Primary cortical mouse ECs (mECs) express *notch1* and *jag1* (K) as well as *dll1* and *dll4* (M) but show little to no *hes1* (K) expression at baseline. *Cyclophilin1* (*cyclo1*) was used as housekeeper (J–M).