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Deficiency in matrix metalloproteinase-2 results in long-term vascular instability and regression in the injured mouse spinal cord

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ABSTRACT

Angiogenesis plays a critical role in wound healing after spinal cord injury. Therefore, understanding the events that regulate angiogenesis has considerable relevance from a therapeutic standpoint. We evaluated the contribution of matrix metalloproteinase (MMP)-2 to angiogenesis and vascular stability in spinal cord injured MMP-2 knockout and wildtype (WT) littermates. While MMP-2 deficiency resulted in reduced endothelial cell division within the lesioned epicenter, there were no genotypic differences in vascularity (vascular density, vascular area, and endothelial cell number) over the first two weeks post-injury. However, by 21 days post-injury MMP-2 deficiency resulted in a sharp decline in vascularity, indicative of vascular regression. Complementary in vitro studies of brain capillary endothelial cells confirmed MMP-2 dependent proliferation and tube formation. As deficiency in MMP-2 led to prolonged MMP-9 expression in the injured spinal cord, we examined both short-term and long-term exposure to MMP-9 in vitro. While MMP-9 supported endothelial tube formation and proliferation, prolonged exposure resulted in loss of tubes, findings consistent with vascular regression. Vascular instability is frequently associated with pericyte dissociation and precedes vascular regression. Quantification of PDGFrβ+ pericyte coverage of mature vessels within the glial scar (the reactive gliosis zone), a known source of MMP-9, revealed reduced coverage in MMP-2 deficient animals. These findings suggest that acting in the absence of MMP-2, MMP-9 transiently supports angiogenesis during the early phase of wound healing while its prolonged expression leads to vascular instability and regression. These findings should be considered while developing therapeutic interventions that block MMPs.

1. Introduction

Angiogenesis is a complex multistep process involving endothelial cell activation, sprouting, regression, and maturation (Wietecha et al., 2013). The matrix metalloproteinases (MMPs) MMP-2 and -9 are integral to these processes (Verslegers et al., 2013). They participate in the degradation of the extracellular matrix (ECM) that in turn facilitates detachment of pericytes, and the subsequent directed migration of endothelial cells along a pro-angiogenic gradient (Carmeliet and Jain, 2011). MMPs also regulate endothelial cell proliferation, differentiation, and unmasking of cryptic sites to release pro/anti-angiogenic factors (Roy et al., 2006) and vascular stabilization (Davis and Saunders, 2006; Zhu et al., 2000). Though their contributions to angiogenesis have been studied in detail in other organ systems (Kessenbrock et al., 2010), there is little known about the participation of these MMPs in angiogenesis during wound healing in the injured spinal cord. We have previously shown that MMP-2 is expressed during the angiogenic phase of wound healing after spinal cord injury (SCI) and deletion of MMP-2 results in poorer functional outcomes (Hsu et al., 2006).

SCI causes direct physical damage to the vascular network resulting in progressive hemorrhagic necrosis beginning in the central gray matter and expanding into the pericentral white matter (Simard et al., 2007). Angiogenesis, presumably resulting from the sprouting of surviving vessels, begins 3–4 days after injury within the central-most part of the damaged cord, and peaks at 7 days, where it reaches control levels or higher (Benton et al., 2008; Casella et al., 2002; Loy et al., 2002; Whetstone et al., 2003). These vessels, surrounded by macrophages, predominantly bear an immature phenotype (Benton et al., 2008; Casella et al., 2002; Loy et al., 2002; Whetstone et al., 2003); they are characterized by abnormal leakiness, the absence of the glucose-1 transporter protein, and paucity of perivascular investments including astrocytes and pericytes (Goritz et al., 2011; Whetstone et al., 2003). In the injured murine spinal cord, these immature vessels are localized to heterodomains, a term used to describe the unique environment

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imposed by focal collections of macrophages that are entrapped by glial scarring (Whetstone et al., 2003). The presence of immature vascular phenotypes in heterodomains contrasts that of the environment within the surrounding glial scar where blood vessels express the glucose-1 transporter, and are associated with astrocytic endfeet that are in close proximity to the endothelial basal lamina (Whetstone et al., 2003).

Inherent to angiogenesis is vascular stabilization (von Tell et al., 2006). Pericytes, which share a common basement membrane with endothelial cells, play a key role in conferring vascular stability (von Tell et al., 2006) through the deposition of matrix and/or by the release, presentation and activation of signals that promote endothelial cell differentiation and quiescence (Armulik et al., 2005). Disruption of pericyte-endothelial cell relationships leads to vascular destabilization, which is characterized by increased permeability, as well as loss of vascular integrity and regression (Simonavicius et al., 2012). While MMP-2 and -9 are implicated in angiogenesis (Verslegers et al., 2013) including vascular stabilization (Davis and Saunders, 2006; Zhu et al., 2000), their relative contributions to this event in the injured spinal cord are unknown.

Here we consider the roles of MMP-2 and -9 in modulating angiogenesis in the injured spinal cord during wound healing when gelatinase activity is notably prominent within angiogenic regions of the contused spinal cord (Goussev et al., 2003; Noble et al., 2002). Our data suggest that vascular stability and vessel maturity are dependent on MMP-2 expression, and that an imbalance in the expression of MMP-2 and MMP-9 results in long-term vascular regression and instability.

2. Materials and methods

2.1. Breeding

To minimize genetic variances, homozygous MMP-2 knockout (KO) mice and their wild-type (WT) littermates were generated by breeding heterozygous males and females on a C57BL/6 background as previously described (Itoh et al., 1997). The genotypes of animals were confirmed by PCR using specific oligonucleotide primers on genomic DNA (Hsu et al., 2006).

2.2. Randomization and blinding

Congenic littermates were genotyped after weaning and housed till adulthood (3 months of age). Since only female mice that had homozygous expression were used in this study, we randomly allocated animals to the different time points and the order of injury. The surgeon was blinded to the genotype. All histological assessments were performed by an observer blinded to injury and genotype of these mice.

2.3. Animal model

All procedures were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. All surgeries were performed blinded to the genotype and randomized across genotypes and post-surgery survival time-points. Adult female WT and KO mice (n = 56) were anesthetized with 2.5% Avertin (0.02 ml/g body weight, intraperitoneally (i.p.), Sigma, St. Louis, MO) and maintained at 37 °C throughout surgery and during recovery by use of a warming pad. A contusive injury was produced as described previously (Hsu et al., 2006). Briefly, using aseptic techniques, the spinous process and the lamina of the 8th thoracic vertebra (T8) were removed and a circular region of the dura mater (~2.4 mm in diameter) was exposed. After stabilization of the vertebral column, a 3-g weight was dropped from a height of 5 cm onto the exposed dura mater. Following injury, the skin was closed with wound clips. Post-operative care included subcutaneous administration of trimethoprim/sulfonamide (Tribrissen), at 30 mg/kg, twice a day for 10 days post-injury and manual expression of the bladder twice/day till the end of the study.

2.4. Systemic BrdU injections

Animals (n = 5/genotype/time point) were administered bromodeoxyuridine [(BrdU, Sigma, St. Louis) (100 mg/kg, i.p.)] 24 h prior to euthanasia at 0, 3, 7, 14, or 21 days post-injury.

2.5. Histological analyses

2.5.1. Tissue preparation

Mice were perfused with 4% paraformaldehyde (PFA) and the spinal cord was excised and post-fixed for 4 h at 4 °C, followed by incubation in 20% sucrose for 3–5 days at 4 °C. A segment, 1.5 cm in length and centered over the site of injury, was cryoprotected in tissue embedding medium (Triangle Biomedical Sciences, Durham, NC). Spinal cords were sectioned longitudinally at 30-μm thickness on a cryostat, then thaw mounted on Superfrost microscope slides (Fisher, Pittsburgh, PA) and stored at −80 °C.

2.5.2. Immunohistochemistry

Every fifth longitudinal section per animal was used for analysis. To minimize variability, replicates from injured animals and one uninjured animal were processed together. Sections were stained with Armenian Hamster anti-platelet endothelial cell adhesion molecule 1 (CD31) Ab (Hamster anti mouse, Millipore, Bellerica, MA; 1:500), anti-bromodeoxyuridine (BrdU Ab, polyclonal sheep, Abcam, Cambridge, MA; 1:1000), Rabbit anti-platelet derived growth factor receptor-beta (PDGFrβ, Rabbit monoclonal anti human, Abcam; 1:100), and Rabbit anti-glial fibrillary acidic protein (GFAP, Rabbit polyclonal anti cow, Dako, Carpinteria, CA; 1:500). Secondary antibodies used were, Cy-3 conjugated Goat x Armenian Hamster IgG (Jackson Immunoresearch, West Grove, PA; 1:200), FITC conjugated Donkey x Sheep IgG (Jackson Immunoresearch; 1:500), Cy-3 conjugated Goat x Rabbit IgG (Jackson Immunoresearch; 1:200), AlexaFluor 488 conjugated Goat x Armenian Hamster IgG (Jackson Immunoresearch; 1:200), and Alexa Fluor-647 conjugated Donkey x Rabbit IgG (Jackson Immunoresearch; 1:200). Nuclei were visualized with DAPI (Invitrogen, Carlsbad, CA; at 300 nM concentration).

2.5.3. Image acquisition

Images were captured at the epicenter, defined as the region of maximal damage. The epicenter was subdivided into 3 zones; a central "core" composed primarily of macrophages, endothelial cells, and PDGFrβ+ pericyte scarring (Figs. 1 and 2), the surrounding glial scar (Zone 1) and the region immediately adjacent to the glial scar (Zone 2), that includes reactive astrocytes (Fig. 1). An internal control consisted of two images, taken at approximately 8 mm rostral and caudal to the epicenter, where the spinal cord showed no overt damage.

All measurements of vascularity as defined by vascular density, area, and number of endothelial cells, were restricted to the core (Fig. 2). Three non-overlapping images per section for a total of 8 sections per animal were captured with a 20× objective using a Zeiss LSM 510 confocal microscope (Zeiss, Thornwood, NY) using the following lasers: 405 nm for DAPI/Hoechst (10%); 488 nm for FITC/Alexa 488 (Argon tube current ~5.5 A 5%); 543 nm for CY3 (80%). The *Z*-stage interval was set at 2 μm intervals. For the uninjured spinal cord, the same numbers of images were captured per section from the same segmental level of the spinal cord. Images were acquired using identical exposures for all groups and imported into the MetaMorph® Microscopy Automation and Image Analysis software (Molecular Devices, Downingtown, PA).

Non-overlapping images (635 μm × 635 μm) within Zones 1 and 2 (Fig. 1) were captured with a Nikon C2 confocal microscope, equipped with 4 lasers (Nikon Inc., Melville, NY) for evaluation of pericyte investment of blood vessels and endothelial apoptosis. The Z-stage interval