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Crosstalk between oligodendrocytes and cerebral endothelium contributes to vascular remodeling after white matter injury

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Abstract

After stroke and brain injury, cortical gray matter recovery involves mechanisms of neurovascular matrix remodeling. In white matter however, the mechanisms of recovery remain unclear. In this present study, we demonstrate that oligodendrocytes secrete matrix metalloproteinase-9 (MMP-9), which accelerates the angiogenic response after white matter injury. In primary oligodendrocyte cultures, treatment with the pro-inflammatory cytokine interleukin-1 β (IL-1 β) induced an upregulation and secretion of MMP-9. Conditioned media from IL-1 β -stimulated oligodendrocytes significantly amplified matrigel tube formation in brain endothelial cells, indicating that MMP-9 from oligodendrocytes can promote angiogenesis in vitro. Next we asked whether similar signals and substrates operate after white matter injury in vivo. Focal white matter injury and demyelination was induced in mice via stereotactic injection of lysophosphatidylcholine (LPC) into corpus callosum. Western blot analysis showed that IL-1 β expression was increased in damaged white matter. Immunostaining demonstrated MMP-9 signals in MOBP (myelin-associated oligodendrocytic basic protein)-positive oligodendrocytes. Treatment with an IL-1 β -neutralizing antibody suppressed the MMP-9 response in oligodendrocytes. Finally, we confirmed that the broad spectrum MMP inhibitor GM6001 inhibited angiogenesis around the injury area in this white matter injury model. In gray matter, a neurovascular niche promotes cortical recovery after brain injury. Our study suggests that an analogous oligovascular niche may mediate recovery in white matter.

Keywords

oligodendrocyte; white matter injury; matrix metalloproteinase-9; vascular remodeling; cerebral endothelial cell

Introduction

The adult mammalian brain can be surprisingly plastic, especially after stroke and brain injury (Chopp et al. 2008; Cramer and Riley 2008; Moskowitz et al. 2010). Under normal

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conditions, newborn neurons in the subventricular and subgranular zones migrate to olfactory regions and the hippocampus. After brain injury, the birth rate of new cells seems to increase and neuroblasts are rerouted toward damaged tissue (Arvidsson et al. 2002; Nakatomi et al. 2002). Along with neurogenesis, the recovering brain also exhibits complex patterns of vascular remodeling and neural plasticity. In the so-called neurovascular niche, cell-cell signaling between cerebral endothelium and neuronal precursor cells mediate angiogenesis and repair of damaged gray matter (Iadecola 2004; Ohab et al. 2006; Zacchigna et al. 2008; Zhang and Chopp 2009; Zlokovic 2010). The underlying mechanisms of this neurovascular recovery involve multiple signals and substrates. Several angiogenic and neurogenic mediators are involved, e.g. VEGF, FGF and SDF-1 (Ohab et al. 2006; Yoshimura et al. 2001; Zhang et al. 2000). And in concert with this trophic response, matrix remodeling is also essential, especially that involving the matrix metalloproteinase (MMP) family of extracellular proteases (Larsen et al. 2003; Lee et al. 2006; Reeves et al. 2003).

More recently, it has been recognized that white matter recovery is also essential for the brain to repair itself after injury and neurodegeneration (Benowitz and Carmichael 2010). In vivo imaging studies in animal models of stroke recovery show an intricate interplay between remyelination and microvessel regrowth and as white matter tracts rewire (Jiang et al. 2010). In contrast to gray matter however, the molecular mechanisms of white matter recovery after brain injury remain relatively undefined. The neurovascular niche provides a cell-cell signaling framework for remodeling gray matter. Is it possible that similar cell-cell signaling niches may also contribute to recovery in white matter? In this study, we use a combination of primary cell culture methods and in vivo mouse models to test the hypothesis that after focal injury, white matter recovery is mediated in part by crosstalk between oligodendrocytes and cerebral endothelium that promotes angiogenesis.

Experimental Procedures

Cell Culture

Oligodendrocyte precursor cells were prepared as previously described (Arai and Lo 2009a). Briefly, cerebral cortices from 1–2 day old Sprague-Dawley rats were dissected, minced, and digested. Dissociated cells were plated in poly-D-lysine-coated 75-cm² flasks, and maintained in Dulbecco's Modified Eagle's medium containing 20% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. After the cells were confluent (~10 days), the flasks were shaken for 1 hour on an orbital shaker (220 rpm) at 37°C to remove microglia. The medium was changed with a new medium and shaken overnight (~20 hours). The medium was then collected and plated on non-coated tissue culture dishes for 1 hour at 37°C to eliminate possible contamination by astrocytes and microglia. The non-adherent cells were collected and replated in Neurobasal Media containing glutamine, 1% penicillin/streptomycin, 10 ng/mL PDGF, 10 ng/mL FGF, and 2% B27 supplement onto poly-DL-ornithine-coated plates. Four to 5 days after plating, the OPCs were then differentiated into mature oligodendrocytes by switching culture media to Dulbecco's Modified Eagle's medium containing 1% penicillin/streptomycin, 10 ng/mL CNTF, 50 ng/mL T3, and 2% B27 supplement. Rat brain microendothelial cell line (RBE.4) were maintained in EGM-2MV containing EGM-2MV SingleQuots kit onto collagen-coated 25-cm² flasks.

Immunocytochemistry

After the cells reached 70–80% confluence, they were washed with ice-cold PBS (pH 7.4), followed by 4% paraformaldehyde for 30 min. After being further washed three times in PBS containing 0.1% Triton X-100, they were incubated with 1% bovine serum albumin (BSA) in PBS for 1 h. Then cells were incubated with primary antibodies against the oligodendrocyte markers MBP (1:200) and olig2 (1:200) at 4°C overnight. After washing

with PBS, they were incubated with secondary antibodies conjugated with fluorescein isothiocyanate for 1 h at room temperature. Finally, nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI: H-1200 from Vector Laboratories), and cells were rinsed and coverslips were placed. Immunostaining was analyzed with a fluorescence microscope (Olympus BX51) interfaced with a digital charge-coupled device camera and an image analysis system.

Gelatin Zymography

Forty eight hours after IL-1 β treatment, the culture medium was collected and centrifuged at 10,000 g for 5 min at 4°C to remove cells and debris. The cleared medium was concentrated 10-fold using Microcon (Millipore) with a 10 kDa pore diameter cutoff, then each sample was mixed with equal amounts of SDS sample buffer (Novex) and electrophoresed on 10% SDS-polyacrylamide gels (Novex) containing 1 mg/ml gelatin as the protease substrate. Following electrophoresis, gels were placed in 2.7% Triton X-100 for 1 h to remove SDS, and then incubated for 10 h at 37°C in developing buffer (50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM CaCl₂, and 0.2% Brij 35; Novex) on a rotary shaker. After incubation, gels were stained in 30% methanol, 10% acetic acid, and 0.5% w/v coomassie brilliant blue for 1 h followed by destaining. Mixed human MMP-2 and MMP-9 standards (Chemicon) were used as positive controls.

Cell death assay

To ensure that our secreted MMP-9 measurements were not confounded by nonspecific protein release due to cell damage, cytotoxicity was quantified by a standard measurement of lactate dehydrogenase (A LDH assay kit from Roche).

Preparation of oligodendrocytes conditioned media

When cells were 90–95% confluent, the cells were once washed with PBS followed by culturing in Dulbecco's Modified Eagle's medium containing, 50 ng/ml IL-1 β , 1% penicillin/streptomycin and 2% AO-free B27 supplement for 24 hours. Then the culture medium was collected and filtered using 0.20 μ m filter. The oligodendrocytes conditioned medium was kept in –80°C until use.

In vitro tube formation assay

The standard Matrigel assay was used to assess the spontaneous formation of capillary-like structures by the rat brain endothelial cell line (RBE4). Standard 24-well plates were coated with 150 μ L of cold Matrigel and allowed to solidify at 37°C for 30 mins. Cells (2×10^4 cells/cm²) were seeded in that plates, and incubated at 37°C for 18 hr with or without oligodendrocyte-conditioned medium. The degree of tube formation was determined by counting the number of tubes in four random fields from each well. Data were analyzed as a percentage of the tube numbers in untreated control wells.

In vivo white matter injury model

All experiments were performed following an institutionally approved protocol in accordance with National Institutes of Health guidelines. Male C57BL6 mice (11–12 weeks, Charles River Laboratories) were deeply anaesthetized with isoflurane (1% to 2%) in 30%/70% oxygen/nitrous oxide. Lysophosphatidylcholine (LPC: obtained from Sigma) at a concentration of 10 μ g/ μ l or 0.5 μ l saline was injected through a 30-gauge needle over 5 minutes into the left corpus callosum. The placement coordinates were anterior: 0.5 mm from bregma, lateral: 1.0 mm from bregma, depth: 2.3 mm from the skull surface. Mouse monoclonal IL-1 β antibody or mouse control IgG was injected in left intra-cerebro-ventricular (anterior: –1.5 mm, lateral: 0.8 mm, depth: 2.5 mm) on 2 days after LPC

injection. GM6001 (50 mg/kg, Millipore) or saline (0.1 ml/10 g) was injected intraperitoneally on days 2 and days 4 after LPC injection.

Immunohistochemistry

Mice brain were taken after perfusion with PBS (pH 7.4) at 5 days after LPC injection and quickly frozen in liquid nitrogen. Coronal sections of 12- μ m thicknesses were cut on cryostat at -20°C and collected on glass slides. Sections were fixed by 4% PFA or acetone, and rinsed three times in PBS (pH 7.4). After blocking with 3% BSA, sections were then incubated at 4°C overnight in a PBS solution containing the primary antibodies in PBS, 0.1% Tween 20, 0.3% BSA. Staining was performed for mature oligodendrocytes (MOBP, 1:100), endothelial cells (CD31, 1:100), MMP-9 (1:1000) and Ki67 (1:200). The sections were washed and incubated for 1 h with secondary antibodies with fluorescence conjugations. Subsequently, the slides were covered with VECTASHIELD mounting medium with DAPI (H-1200 from Vector Laboratories). Three random areas around injured white matter tract induced by LPC were chosen in each group. MOBP and MMP-9 double positive area was calculated as a percentage of total MOBP positive area. The number of double positive cells for Ki67 and CD31 was counted by means of Image J analysis.

Western Blotting

Tissue samples of corpus callosum were dissected in Pro-PREPTM Protein Extraction Solution (BOCA SCIENTIFIC). Samples were heated with equal volumes of SDS sample buffer (Novex) and 10 mM DTT at 95°C for 5 min, then each sample (20 μg per lane) was loaded onto 4–20% Tris-glycine gels. After electrophoresis and transferring to polyvinylidene difluoride membranes (Novex), the membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 0.2% I-block (Tropix) for 90 min at room temperature. Membranes were then incubated overnight at 4°C with monoclonal anti-IL-1 β antibody (1:1000), anti-CD31 antibody (1:1000) or anti- β -actin antibody (1:5,000) followed by incubation with peroxidase-conjugated secondary antibodies and visualization by enhanced chemiluminescence (Amersham).

Statistical analysis

Quantitative data were analyzed with t-tests for parametric data and Mann-Whitney for non-parametric data. Data are expressed as mean $\pm$ SD. A value of $p < 0.05$ was considered significant.

Results

Oligodendrocyte precursor cells (OPCs) were derived from neonatal rat brains, then differentiated into primary oligodendrocytes following standard protocols (Arai and Lo 2009a; Hayakawa et al. 2011). This system was stable and reproducible. OPCs did not express myelin-basic-protein (MBP), a marker of mature oligodendrocytes (Fig. 1a). But after stimulating cells with CNTF and T3, they started to differentiate into mature oligodendrocytes and express MBP over time (Fig. 1a). Immunostaining confirmed that our cultured oligodendrocytes were positive for the mature oligodendrocyte markers MBP and olig2 (Fig. 1b).

Using our oligodendrocyte culture system, we first asked whether these cells could produce matrix metalloproteinase-9 (MMP-9), a well-known angiogenic factor. Gelatin zymography showed that oligodendrocytes did not secrete MMP-9 under normal conditions. But after stimulation with IL-1 β , they produced MMP-9 in a concentration dependent manner (Fig. 2a). MMP-2 was also detected but did not appear to be significantly affected by IL-1 β (Fig. 2a). It has been previously shown that the MEK/ERK pathway is important for MMP-9

regulation in astrocytes (Arai et al. 2003; Hsieh et al. 2010). Therefore, we next asked whether this pathway could be involved in mature oligodendrocytes as well. The MEK inhibitor U0126 but not the inactive analog U0124 blocked MMP-9 secretion (Fig. 2b), indicating that the MEK/ERK signaling cascade plays an essential role in oligodendrocytic MMP-9 secretion under stimulated conditions. A standard LDH assay confirmed that the IL-1 β treatment did not induce cell death and nonspecific protein release in our oligodendrocyte cultures (Fig. 2c).

To demonstrate that oligodendrocyte-derived MMP-9 was biologically important, we used a media transfer approach that was previously used to dissect soluble factor coupling in the neurovascular niche (Guo et al. 2008). Rat oligodendrocytes and rat brain endothelial cells (RBE.4) were grown separately, then conditioned media from IL-1 β -stimulated oligodendrocytes were collected and added to the endothelial cells (Fig. 3a). The standard Matrigel tube formation assay was then used as an in vitro measure of angiogenesis. When oligodendrocytes were stimulated with IL-1 β , their conditioned media amplified endothelial tube formation (unstimulated: 215.3 $\pm$ 70.5% versus stimulated: 314.8 $\pm$ 117.3%, $p < 0.05$). Next, we asked whether the ability of stimulated oligodendrocytes to induce angiogenesis was dependent on MMP-9. Control endothelial cells were treated with the same medium containing IL-1 β derived from empty wells without oligodendrocytes. Treatment with IL-1 β -stimulated-oligodendrocyte-conditioned media significantly amplified tube formation in the brain endothelial cells (Fig. 3b). Importantly, the broad spectrum MMP inhibitor GM6001 reduced endothelial tube formation, consistent the idea that IL-1 β -stimulated-oligodendrocytes may augment angiogenesis by releasing MMP-9 (Fig. 3c).

To extend these in vitro observations, lysophosphatidylcholine (LPC) was used to produce focal white matter injury in adult C57BL6 mice in vivo. Stereotaxic injection of LPC into the mouse corpus callosum induced focal demyelination in the damaged white matter tracts (Fig. 4a). Western blot analysis showed that IL-1 β expression around the white matter lesion was increased at 5 days after LPC injection (Fig. 4b). In situ zymography demonstrated that gelatinases (MMP-2 and MMP-9) were correspondingly activated in the damaged white matter areas (Fig. 4c). Immunostaining confirmed that some of the MMP-9 positive cells were oligodendrocytes identified with the oligodendrocyte marker MOBP (Figs. 4d–e). Importantly, treatment with a neutralizing antibody for IL-1 β reduced oligodendrocytic MMP-9 expression (Figs. 4d–e), suggesting that IL-1 β indeed regulates MMP-9 production in oligodendrocytes in this in vivo white matter injury model.

Thus far, our data show that IL-1 β mediates the ability of oligodendrocytes to produce pro-angiogenic MMP-9 in vitro and in vivo. But is this phenomenon functionally relevant for white matter homeostasis and repair after injury in vivo? In our LPC white matter injury model, an angiogenic response with increased vascular density was detectable by 5 days after injury in the demyelinated zones. In these areas, CD31-positive microvessels were closely associated with MOBP-positive oligodendrocytes (Fig. 5a), consistent with the idea of an oligovascular niche in recovering white matter. To further characterize angiogenic recovery in white matter, CD31/Ki67 double-staining was used to identify newly formed microvessels. The overall number of CD31/Ki67 double-positive cells was clearly increased in the damaged regions, suggesting a focal angiogenic response during the repair phase (Figs. 5b–c). Notably, these angiogenic responses were blocked by the potent MMP inhibitor GM6001 (Figs. 5b–c). Furthermore, western blot analysis revealed that GM6001 treatment reduced the total expression of CD31 in the LPC damaged area, indicative of a decrease in endothelial cell density (Figs. 5d–e).

Discussion

Our data demonstrate that oligodendrocytes are an important source of MMP-9 that can help facilitate vascular remodeling in white matter. Oligodendrocytes do not express MMP-9 at a high level under normal conditions. But after injury, they respond to inflammatory IL-1 β signaling and produce MMP-9 to enhance angiogenesis. Therefore, oligodendrocytes may play as a sensor for white matter injury and maintain white matter homeostasis by secreting angiogenic factors.

The concept of the neurovascular unit was proposed to understand pathophysiologic responses after stroke and other CNS diseases. This concept emphasized that brain function and dysfunction arise from integrated interactions between neurons, astrocytes, and vascular compartments. Neurovascular responses after injury underlie a transition from acute injury to delayed repair as the brains to initiate endogenous angiogenesis that facilitates neuroplasticity and remodeling (Arai et al. 2009; Arai et al. 2011; Carmichael 2006). Angiogenesis and vasculogenesis have been detected in rodent models of cerebral ischemia (Ding et al. 2008) as well as in human stroke (Krupinski et al. 1996). It is now recognized that in gray matter, angiogenic and neurogenic responses are tightly co-regulated after brain injury, comprising cell-cell signaling within the neurovascular niche (Ohab et al. 2006). The idea of the neurovascular niche primarily guide research in cell-cell trophic coupling in gray matter. But, those interactions are likely to be important in white matter as well. In this regard, an analogous oligovascular niche may also exist and support white matter remodeling through both oligodendrogenesis and angiogenesis (Arai and Lo 2009b).

The MMP family consists of endopeptidases with common motifs comprising propeptide and zinc-binding catalytic regions (Nagase and Woessner 1999). MMPs, especially MMP-9, are now well accepted as key neurovascular proteases to damage the blood-brain barrier (BBB) and cause neuronal death in the acute injury phase. However, recent studies demonstrate that these same proteases have a beneficial role in accelerating neurovascular remodeling in the recovery phase after injury (Lo 2008). This concept of biphasic responses may also apply to white matter. MMPs degrade almost all extracellular matrix molecules including myelin in white matter (Kieseier et al. 1999). Hence, during acute stages of white matter injury, MMPs play deleterious roles through myelin destruction or BBB breakdown. But our findings here suggest that during delayed stages of recovery, MMPs may promote white matter remodeling by accelerating angiogenesis. Finding ways to augment these endogenous remodeling signals should be a promising therapeutic approach for repairing both gray and white matter compartments in the damaged brain (Zhang and Chopp 2009).

Our present findings suggest a novel role of an oligovascular niche, wherein vascular remodeling occurs during white matter recovery after brain injury. Nevertheless, there are a few issues that warrant further studies. First, our experiments only focused on endothelial-oligodendrocyte interactions. In recent years, it has become clear that dynamic cell-cell interactions contribute to brain homeostasis. Hence, other glial types such as astrocytes and pericytes may also contribute to white matter function. In fact, we and others have demonstrated that astrocytes nourish oligodendrocyte lineage cells (Arai and Lo 2010; Corley et al. 2001). How trophic signals mediate multi-cellular interactions should be carefully dissected. Second, although our hypothesis centers on MMP-9, our pharmacology is limited to the broad spectrum MMP inhibitor GM6001 that can inhibit MMP-1, 2, and -8, in addition to MMP-9. Future studies should carefully examine roles of the larger MMP network in white matter homeostasis and remodeling. Finally, our focus on oligodendrocyte-derived MMP-9 is consistent with previous studies reporting that oligodendrocytes produce MMP-9 in response to extracellular stress (Uhm et al. 1998). But, oligodendrocytes may secrete other angiogenic factors such as vascular endothelial growth factor (VEGF). The role

of the VEGF family is well established in terms of common neuronal, glial, and vascular functions in gray matter. Moreover, recent studies demonstrate that VEGFs may also participate in white matter homeostasis (Hayakawa et al. 2011; Le Bras et al. 2006). Our oligodendrocyte cultures can also produce VEGF (data not shown). How MMPs may synergize with trophic factors should be further examined.

In conclusion, our findings demonstrate that oligodendrocytes produce MMP-9 after white matter injury, and this may underlie an oligovascular niche that promotes angiogenic recovery. Hence, cell-cell trophic coupling is essential for brain vascular plasticity not only in gray matter but also in white matter. Similar to the neurovascular niche, an oligovascular niche may provide an important mechanism for functional remodeling after brain injury. Further studies are warranted to more fully characterize how oligovascular signaling may be modulated to augment white matter recovery after stroke, brain injury or neurodegeneration.

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Figure 1.

a. Western blot confirmed that oligodendrocyte precursor cells were functional and matured into myelin-basic-protein (MBP)-positive oligodendrocytes over time. **b.** Immunostaining demonstrated that mature oligodendrocytes expressed olig2 (green) and MBP (green), oligodendrocyte markers. Nuclei were stained with DAPI (blue).

Figure 2.

a. Gelatin zymography showed that IL-1 β increased the level of MMP-9 in the conditioned media. p.c. indicates positive control for MMP-2 and -9. **b.** The MEK inhibitor U0126 but not an inactive analog U0124 blocked the MMP-9 secretion by IL-1 β . **c.** A standard LDH assay confirmed that the treatment of IL-1 β did not induce cell death in oligodendrocyte cultures. Experiments were performed in duplicate, repeated 3–6 times independently.

Figure 3.

a. Schematics for our media transfer experiments. **b.** Conditioned media (CM) from IL-1 β -stimulated oligodendrocytes (OL) increased the number of rings in the matrigel tube formation assay. Images are representative pictures for the tube formation assay. **c.** The broad spectrum MMP inhibitor GM6001 (1 μ M) reduced tube formation by IL-1 β -stimulated-oligodendrocyte-conditioned media. Experiments were performed in duplicate, repeated 4–6 times independently. *P<0.05.

Figure 4.

a. Stereotaxic injection of LPC into the corpus callosum induced focal demyelination in white matter tracts assessed by myelin staining (green). N=4. **b.** Western blot showed that IL-1 β expression increased in the LPC-injected region. C: contralateral side. I: ipsilateral side. N=4. **c.** In situ zymography detected significant MMP-2/9 activities in the damaged area. N=4. **d-e.** Immunostaining confirmed that oligodendrocytes (MOBP: red) produced MMP-9 (green). A neutralizing antibody for IL-1 β blocked the oligodendrocytic MMP-9 expression. N=5 per group, *P<0.05.

Figure 5.

a. Oligodendrocytes (MOBP; red) and brain endothelial cells (CD31; green) are closely associated in recovering white matter tracts of the LPC model. N=4. **b–c.** The MMP inhibitor GM6001 reduced the number of double positive cells for CD31 (an endothelial marker; green) and Ki67 (a proliferation marker; red). N=6 per group, *P<0.05. **d–e.** Western blot analysis confirmed that total expression of CD31 was also decreased by the GM6001 treatment, indicating that MMP inhibition suppresses angiogenesis in white matter. N=3 per group, *P<0.05.