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p38 MAP kinase mediates transforming-growth factor- β 1-induced upregulation of matrix metalloproteinase-9 but not -2 in human brain pericytes

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Abstract

Pericytes are vascular mural cells embedded within the basal lamina of blood micro-vessels. Within the neurovascular unit, pericytes play important roles in regulating neurovascular homeostasis by secreting soluble factors, such as matrix metalloproteinases (MMPs). However, little is known about the regulatory signaling pathways in brain pericytes. Here we show that transforming growth factor- β 1 (TGF- β 1) induces MMP-9 upregulation in pericytes via p38 mitogen-activated protein (MAP) kinase signaling. Cultured human brain vascular pericytes were used in this study. When the brain pericytes were treated with purified human TGF- β 1 (0.1–10 ng/mL for 24 h), the levels of MMP-2 and MMP-9 in culture media were significantly increased in a concentration dependent manner as measured by gelatin zymography. WST assay confirmed that TGF- β 1 did not affect cell survival of the brain pericytes. A TGF- β -receptor inhibitor SB431542 (0.5 – 5 μ M) decreased the TGF- β 1-induced upregulation of MMP-2 and MMP-9. To assess the underlying intracellular mechanisms, we focused on p38 MAP kinase signaling, which is one of the major downstream kinases for TGF- β 1. A well-validated p38 MAP kinase inhibitor SB203580 (0.5 – 5 μ M) cancelled the effect of TGF- β 1 in upregulation of MMP-9 but not MMP-2. Western blotting confirmed that TGF- β 1 treatment increased the level of p38 MAP kinase phosphorylation in pericytes, and again, the TGF- β -receptor inhibitor SB431542 (0.5 – 5 μ M) blocked the TGF- β 1-induced phosphorylation of p38 MAP kinase. Both TGF- β 1 and MMP-9 are major neurovascular mediators, and therefore, our current finding may suggest a novel mechanism for how pericytes regulate neurovascular homeostasis.

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Disclosures: none

Keywords

pericyte; MMP-9; p38 MAP kinase; neurovascular unit

1. Introduction

The neurovascular unit is now relatively well accepted as a conceptual model to understand mechanisms of physiology and pathophysiology of CNS diseases. Fundamentally, this concept shows that all compartments of the neurovascular unit cooperate with each other to maintain normal brain function. Within the neurovascular unit, pericytes attach to cerebral endothelial cells via basal lamina (Diaz-Flores et al., 2009; Stratman et al., 2009), and play critical roles in regulating neurovascular homeostasis (Bell et al., 2010; Sa-Pereira et al., 2012; Winkler et al., 2011). For example, pericytes secrete multiple soluble factors to modulate blood vessel structure and enhance blood-brain barrier (BBB) tightness (Armulik et al., 2010; Winkler et al., 2011). However, mechanisms on how pericytes produce neurovascular mediators are still mostly unknown.

Matrix metalloproteinases (MMPs) are one of the major mediators for cell-cell or cell-matrix interaction in the neurovascular unit. MMPs comprise a family of zinc endopeptidases, and play an important role in regulating extracellular matrix signaling (Nagase et al., 2006). Since MMPs can degrade almost all extracellular matrix molecules, MMPs may contribute to neurovascular homeostasis through modulating axonal growth/regeneration, myelin formation, and vascularization (Verslegers et al., 2013; Yong, 2005). On the other hand, uncontrolled expression/activation of MMPs may result in neurovascular damage such as BBB dysfunction (Lo, 2008; Maki et al., 2013; Moskowitz et al., 2010). Among the MMP superfamily, gelatinases (MMP-2 and MMP-9) have been extensively studied as a therapeutic target for several neurological diseases including stroke, multiple sclerosis, Alzheimer's diseases, and cerebral hemorrhage (Avolio et al., 2003; Davis and Senger, 2005; Kook et al., 2013; Nagase et al., 2006; Rosell et al., 2008).

Although MMP-2 and MMP-9 are secreted from all the cells that contribute to the neurovascular unit, little is known about their regulatory signaling pathways in brain pericytes. Since it has been shown previously that p38 mitogen-activated protein (MAP) kinase signaling contribute to cytokine-induced MMP-9 upregulation in astrocytes (Arai et al., 2003; Wu et al., 2004), we tested if the p38 MAP kinase signaling also mediates MMP-2 and MMP-9 upregulation in brain pericytes. In this study, upregulation of MMP-2 and MMP-9 was induced with transforming-growth factor- β 1 (TGF- β 1), as TGF- β 1 regulates MMP-2 and MMP-9 expression in several types of cells such as astrocytes, meningeal cells, and smooth muscle cells (Hsieh et al., 2010; Okamoto et al., 2009; Zhang et al., 2013). Then p38 MAP kinase phosphorylation and pericyte secretion of MMP-2 and MMP-9 were measured to determine whether p38 MAP kinase plays a critical role in the secretion of these metalloproteinases from pericytes.

2. Results

Primary cell culture systems may get contaminated with other kinds of cells. Therefore, we first confirmed that the cultured human brain vascular pericytes were all positive for pericyte markers PDGF-R- β and α -SMA, assessed by immunocytochemical staining (Figure 1). This was important because many types of brain cells are known to be potent sources of MMP-9. Next, we examined whether the brain pericytes secreted MMP-2 and MMP-9 in vitro. Gelatin zymography showed that baseline MMP-2 and MMP-9 were both detectable in conditioned media from human pericytes under normal conditions as previously reported (Fig. 2A) (Xing et al., 2010). Treatment of TGF- β 1 (0.1 –10 ng/mL for 24 h) resulted in an increase of MMP-2 and MMP-9 secretion in a concentration dependent manner (Figure 2B–C). The WST assay showed that the TGF- β 1 treatment induced neither cell proliferation nor cell death in pericyte cultures (Figure 2D), suggesting that the TGF- β 1-induced increase of MMP-2/9 level in the culture media was not due to an increase of cell numbers or to non-specific release from damaged cellular membranes.

We then examined the intracellular signaling pathway in TGF- β 1-induced MMP-2/9 upregulation in pericytes. TGF- β 1 activates the TGF- β type1 receptor, which then signals to downstream pathways (Massague, 2000). Therefore, we first confirmed that brain pericytes indeed expressed TGF- β 1 receptor. Immunocytochemistry studies showed that the TGF- β type 1 receptor is strongly expressed in our brain pericyte cultures (Figure 3A). Next, we used SB431542, a selective inhibitor of TGF- β type1 receptor, to further confirm that TGF- β 1-induced MMP-2/9 upregulation was in fact mediated by the TGF- β type 1 receptor. As expected, co-treatment of pericytes with SB431542 (0.5 – 5 μ M) reduced the TGF- β 1-mediated MMP-2/9 upregulation (Figure 3B–D) without affecting cell survival (Figure 3E).

Next, we examined whether p38 MAP kinase was upregulated in pericytes after TGF- β 1 treatment, as p38 MAP kinase contributes to MMP-2/9 upregulation in several types of cells. Western blots using anti-phospho-p38 MAP kinase antibody showed that TGF- β 1 treatment in fact increased the phosphorylation level of p38 MAP kinase in pericytes, and importantly, the TGF- β type1 receptor inhibitor SB431542 ameliorated the phosphorylation (Figure 4A–B). Finally, we tested if SB203580, a well-validated inhibitor for p38 MAP kinase, decreased the TGF- β 1-induced MMP-2/9 upregulation in pericytes. Co-treatment of pericytes with SB203580 (0.5 – 5 μ M) inhibited the upregulation of MMP-9 by TGF- β 1, but unexpectedly, the MMP-2 level was not changed (Figure 5A–C). WST assay confirmed that SB203580 treatment did not change the cell viability (Figure 5D). These data indicate that different signaling cascades may be involved in the TGF- β 1-induced MMP-2/9 upregulation in pericytes.

3. Discussion

Our current study demonstrates that in cultured human brain vascular pericytes, (i) TGF- β 1 increases the secreted levels of MMP-2 and MMP-9, (ii) TGF- β type1 receptor mediates the TGF- β 1-induced MMP-2/9 upregulation, and (iii) TGF- β 1-mediated phosphorylation of p38 MAP kinase leads to an increase in secreted MMP-9 but not MMP-2. TGF- β is a multipotent cytokine and participates in a number of physiological and pathological processes, such as

inflammation, wound healing, cell proliferation/differentiation (Bottner et al., 2000; Walshe et al., 2009). Within the neurovascular unit, TGF- β is produced and released from neurons, cerebral endothelial cells, glial cells, and pericytes (Gomes et al., 2005; Vivien et al., 1998). TGF- β may play important roles in pericyte function (Jakobsson and van Meeteren, 2013). Previous reports show that cerebral endothelium-derived TGF- β regulates cell constriction and induces extracellular matrix expression in pericytes (Li et al., 2011; Sieczkiewicz and Herman, 2003). Our findings complement these findings and may provide novel insights into the mechanisms on how TGF- β regulates pericyte function in the neurovascular unit.

Regulating MMP-2/9 function is critical for maintaining neurovascular homeostasis (del Zoppo, 2010; Fernandez-Cadenas et al., 2012; Kono et al., 2014). Under physiologic conditions, MMP-2/9 activity is tightly controlled in the brain whereas dysregulation of MMP-2/9 leads to neurovascular damage. These regulatory mechanisms include transcriptional control, zymogen activation, and dynamic inhibition by tissue inhibitors of metalloproteinase (del Zoppo, 2010). During the acute phase of neuroinflammation after brain injury, dysregulated or over-activated MMP-2/9 activity leads to neurovascular dysfunction (Lo et al., 2003). But during the recovery phase after injury, MMP-2/9 may play important roles in neurovascular remodeling and repairing (Lo, 2008; Maki et al., 2013; Moskowitz et al., 2010). Thus far, all the cells in the neurovascular unit including pericytes are known to produce MMP-2/9 (Takata et al., 2011; Zozulya et al., 2008). However, their regulatory mechanisms in brain pericytes are still mostly unknown. In the neurovascular unit, pericytes attach to cerebral endothelial cells via basal lamina, and also contact with astrocytic end-feet (Sa-Pereira et al., 2012). The gap between cerebral endothelium and pericyte is very short (~20 nm), and therefore, vascular function may be regulated by pericyte-derived soluble factors (Dohgu et al., 2005; Sa-Pereira et al., 2012; Winkler et al., 2012). Our finding on pericytes secreting MMP-2/9 in response to TGF- β 1 would support the idea that pericytes play an important role in the neurovascular unit in regulating vascular homeostasis.

There are a few caveats in the present study. First and foremost, our in vitro model may not fully predict what happens in vivo. Our cell culture model is simplified and optimized to maintain brain pericytes healthy in vitro, while conditions around pericytes in the brain are more heterogeneous and dynamic. Can TGF- β 1 induce MMP-2 and MMP-9 upregulation in brain pericytes in vivo as well? Will pathological stress change the response of pericytes to TGF- β 1 (e.g. TGF- β 1 receptor expression level/phosphorylation status)? These questions should be carefully tested in in vivo studies. Second, we focused on only p38 MAP kinase to examine the intracellular signaling pathway in TGF- β 1-induced MMP-2/9 upregulation in pericytes. TGF- β 1 signaling is initiated by an activation of the type 2 receptor kinase followed by an activation of the type1 receptor (Derynck and Zhang, 2003). p38 MAP kinase is one of the major downstream kinases for TGF- β 1, but TGF- β 1 is known to activate other MAP kinases such as extracellular signal-regulated kinase (ERK) or c-jun N-terminal kinase (JNK) (Derynck and Zhang, 2003). Because the promoter region of the MMP-9 gene contains AP-1 sites (Clark et al., 2008), ERK and JNK are implied to also be critical for signal transduction cascades in MMP-9 upregulation. In fact, previous studies show that ERK and JNK mediate TGF- β 1-induced MMP activation in astrocytes, meningeal cells, smooth muscle cells (Hsieh et al., 2010; Okamoto et al., 2009; Zhang et al., 2013). In

addition, ERK and JNK may contribute to MMP-2 regulation as well (Cui et al., 2014; Kuo et al., 2006; Milkiewicz et al., 2007). Therefore, future studies are warranted to examine the roles of ERK and JNK in TGF- β 1-induced MMP-2 and -9 upregulation. Third, many other MMPs besides MMP-2/9 may be involved in neurovascular physiology and pathophysiology. Pericyte can produce a wide spectrum of these proteases including MMP-1 and MMP-3 (Arihiro et al., 2001; Yang et al., 2013). It would be critical for future studies to examine how multiple MMPs interact in pericytes in response to stimuli and injury. Finally, our current finding that TGF- β 1 regulates MMP-2/9 activation in pericytes provides clarification on only one aspect of pericyte function. Non-cell autonomous mechanisms are also critical for regulating pericyte function. What types of cells in the neurovascular unit produce TGF- β 1 and increase MMP-2/9 secretion from pericytes? How does pericyte-derived MMP-2/9 modulate vascular function such as BBB tightness? To answer these questions, future studies would require co-culture experiments in vitro or a cell-specific gene targeting approach in vivo.

In conclusion, we have demonstrated that the p38 MAP kinase signaling pathway is essential for MMP-9 upregulation in pericytes in response to TGF- β 1 stimulation. Both TGF- β 1 and MMP-9 are major mediators for cell-cell interaction in the neurovascular unit, and therefore, further investigations into their roles of in the regulation of pericytes are warranted to achieve a better understanding of neurovascular function and dysfunction.

4. Experimental Procedure

4.1. Cell culture

Human brain vascular pericytes were purchased from ScienCell (Carlsbad, CA, USA). These cells were originated from human brain tissue and were successfully passaged. The cells were seeded in poly-L-lysine-coated 12 well plates and maintained in Pericyte-Medium (ScienCell, USA) containing pericyte growth supplement (ScienCell, USA), 5% fetal bovine serum and 1% penicillin/streptomycin. The pericyte culture plates were kept in 5% CO₂ incubator at 37°C. The culture medium was changed every other day until the cells were ready for experiments (e.g. 80–90% confluency). Human brain pericytes within passage 9 were used for this study.

4.2. Reagent

Recombinant human TGF- β 1 was purchased from PeproTech. TGF- β 1 was dissolved in citric acid solution according to the manufacturer's instruction. The final concentration of citric acid in all the groups for Figures 2, 3, 4, and 5 was 0.1%. SB431542 was purchased from Sigma, and SB203580 was from Calbiochem. Both SB431542 and SB203580 were dissolved in DMSO. The final concentration of DMSO in all the groups for Figures 3, 4, and 5 was 0.1%.

4.3. Immunocytochemistry

The cells were washed with PBS (pH 7.4), followed by incubation with 4% paraformaldehyde for 15min. After being further washed three times in PBS containing 0.1% Triton X-100, they were incubated with 3% bovine serum albumin in PBS for 1 h.

Then the cells were incubated with primary antibodies against platelet-derived growth factor-receptor- β (PDGF-R- β : purchased from Abcam, USA, 1:100), α -smooth muscle actin (α -SMA: purchased from Abcam, USA, 1:100), or TGF- β type 1 receptor (purchased from Abcam, USA, 1:100) at 4°C overnight. After washing with PBS, they were incubated with secondary antibodies conjugated with fluorescein isothiocyanate for 1h at room temperature. Nuclei were counterstained with DAPI.

4.4. MMP Gelatin Zymography

Twenty-four hours after cells were treated with TGF- β 1 in serum-free medium, the culture medium was collected and centrifuged at 2000g for 5 min to remove cells and debris. The supernatant was then concentrated 30-fold using Vivaspinn (Sartorius Stedim Biotech, Germany). Then each sample was mixed with equal amount of 2 $\times$ SDS sample buffer (Novex, USA). Samples were incubated at 37°C for 30 min before electrophoresis. After the electrophoresis, gelatinolytic activity was determined as previously described (Arai et al., 2003). Human purified MMP-2 and MMP-9 standards (Chemicon, USA) were used as positive controls.

4.5. Cell death/survival assay

Pericyte proliferation/survival was assessed by water-soluble-tetrazolium (WST) assay (Dojindo, Japan) according to the manufacturer's instruction.

4.6. Protein extraction and western blot analysis

Cells were stimulated with TGF- β 1 for 5 minutes after 6-hour serum starvation. Inhibitors were added to the culture media 30 minutes before TGF- β 1 stimulation. After removing the culture medium, the cells were collected into cell lysis buffer (Pro-PREPTM Protein Extraction Kit, iNtRON Biotechnology) and samples were mixed with equal volumes of sample buffer containing 91% 2 $\times$ SDS sample buffer (Novex) and 9% 2-mercaptoethanol (Sigma). Subsequently, samples were heated at 95°C for 5 min, and each sample (20 μ l per lane) was loaded onto 4–20% Tris–glycine gels. After electrophoresis and transferring to polyvinylidene difluoride membranes (Novex), the membranes were blocked in 0.2% I-block (Tropix) for 1 h at room temperature. Then they were incubated overnight at 4°C with anti-phospho-p38 MAP kinase antibody (purchased from Cell Signaling Tech, USA, 1:1000) and β -actin (purchased from Sigma, USA, 1:5000), followed by incubation with peroxidase-conjugated secondary antibodies. Signals were visualized by enhanced chemiluminescence (Amersham).

4.7. Statistical analysis

Experiments were performed in duplicate, repeated 3–5 times independently. Quantitative data were analyzed using ANOVA followed by post hoc Tukey test or Tukey-Kramer test. All values are expressed as mean $\pm$ SD. A value of $P < 0.05$ was considered statistically significant.

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Highlights

1. TGF- β 1 increased MMP-2 and MMP-9 secretion from cultured human brain vascular pericytes.
2. A TGF- β receptor SB431542 suppressed TGF- β 1-induced MMP-2/9 upregulation in pericytes.
3. TGF- β 1 increased p38 MAP kinase phosphorylation in pericytes.
4. A p38 MAP kinase inhibitor SB203580 suppressed TGF- β 1-induced MMP-9 but not MMP-2 upregulation in pericytes.

Figure 1. Fluorescent-stained cells of cultured human pericytes

Cells were stained for the pericyte markers PDGF-R- β (FITC; green) and α -SMA (FITC; green). Nuclei were stained by DAPI (blue). Negative control indicates a representative image obtained from immunostaining of secondary antibody only. Scale bar indicates 50 μ m. These data demonstrate that our cultured pericytes express pericyte marker proteins.

Figure 2. TGF- β 1-induced MMP-2/9 upregulation in cultured human pericytes

A: Gelatin zymogram of pericytes treated with TGF- β 1 (0.1–10 ng/mL for 24 h). “p.c.” indicates positive controls loaded with human MMP-2 and MMP-9 standards. **B–C:** Graphs represent data obtained from gelatin zymograms. TGF- β 1 treatment increased MMP-2 and MMP-9 levels in culture medium in a dose-dependent manner. Data are mean $\pm$ SD of $n=3$ independent experiments. **D:** TGF- β 1 (0.1–10 ng/mL for 24 hours) was not cytotoxic to pericytes. There were no changes in cell viability assessed by WST assay. Data are expressed as mean $\pm$ SD with $n=4$ independent experiments.

Figure 3. TGF-β type 1 receptor mediates TGF-β1-induced MMP-2/9 upregulation in cultured human pericytes

A: Immunostaining confirmed that our pericyte cultures express TGF-β type 1 receptor. Cells were stained with anti-TGF-β type 1 receptor antibody (green). Nuclei were stained by DAPI (blue). Scale bar indicates 50 μm. **B:** Gelatin zymogram of pericytes treated with TGF-β1 (5 ng/mL for 24 h) with co-treatment of a TGF-β type 1 receptor inhibitor SB431542. “p.c.” indicates positive controls loaded with human MMP-2 and MMP-9 standards. **C–D:** Graphs represent data obtained from gelatin zymograms. Co-treatment of SB431542 significantly reduced TGF-β1-induced MMP-2/9 upregulation (TGF-β1: 5 ng/mL for 24 h). Data are expressed as mean ± SD with n=4–5 independent experiments. *P<0.05 vs TGF-β1 treatment only. **E:** TGF-β1 with or without SB431542 (TGF-β1: 5 ng/mL, SB431542: 0.5 or 5 μM for 24 h) was not cytotoxic to pericytes. There were no changes in cell viability assessed by WST assay. Data are expressed as mean ± SD with n=4 independent experiments.

Figure 4. TGF- β type 1 receptor mediates TGF- β 1-induced p38 MAP kinase phosphorylation in cultured human pericytes

A: Western blot shows that after exposure to TGF- β 1 (5 ng/mL for 5 min), phosphorylation level of p38 MAPK was increased. Co-treatment with SB431542, a TGF- β type 1 receptor inhibitor, reduced TGF- β 1-induced p38 phosphorylation. **B:** Graph represents data obtained from western blots. Co-treatment of SB431542 significantly reduced TGF- β 1-induced p38 phosphorylation in a dose-response manner. Data are mean $\pm$ SD of n=4 independent experiments. *P<0.05 vs TGF- β 1 treatment only

Figure 5. p38 MAP kinase mediates TGF-β1-induced MMP-9 but not MMP-2 upregulation in cultured human pericytes

A: Gelatin zymogram of pericytes treated with TGF-β1 (5 ng/mL for 24h) with co-treatment of a p38 MAPK inhibitor SB203580. “p.c.” indicates positive controls loaded with human MMP-2 and MMP-9 standards. **B–C:** Graphs represent data obtained from gelatin zymograms. Co-treatment of a p38 MAPK inhibitor SB203580 significantly reduced TGF-β1-induced MMP-9 upregulation. Data are expressed as mean ± SD with n=4–5 independent experiments. *P<0.05 vs TGF-β1 treatment only. **D:** TGF-β1 with or without SB203580

(TGF- β 1: 5 ng/mL, SB203580: 0.5 or 5 μ M for 24 hours) was not cytotoxic to pericytes. There were no changes in cell viability assessed by WST assay. Data are expressed as mean $\pm$ SD with n=4 independent experiments.