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Upregulation of EMMPRIN after Permanent Focal Cerebral Ischemia

Wei Zhu^{a,d}, Steve Khachi^a, Qi Hao^a, Fanxia Shen^a, William L. Young^{a,b,c}, Guo-Yuan Yang^{a,b}, and Yongmei Chen^a

a Center for Cerebrovascular Research, Department of Anesthesia and Perioperative Care, University of California, San Francisco, CA

b Department of Neurological Surgery, University of California, San Francisco, CA

c Department of Neurology, University of California, San Francisco, CA

d Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai, China

Abstract

Elevated activities of matrix metalloproteinases (MMPs) following ischemic stroke have been shown to mediate ischemic injury as well as neurovascular remodeling. The extracellular MMP inducer (EMMPRIN) is a 58-kDa cell surface glycoprotein, which has been known to play a key regulatory role for MMP activities. The roles of EMMPRIN in stroke injury are not clearly understood. In this study, we investigated changes of EMMPRIN in a mouse model of permanent focal cerebral ischemia, and examined potential association between EMMPRIN and MMP-9 expression. Adult male CD-1 mice were subjected to permanent focal ischemia by intraluminal occlusion of the left middle cerebral artery (MCAO) under anesthesia. EMMPRIN expression was markedly upregulated in the peri-infarct area at 2–7 days after ischemia compared to the contralateral non-ischemic hemisphere by Western blot analysis. Immunofluorescent double staining demonstrated that EMMPRIN signals co-localized with vWF-positive endothelial cells and GFAP-positive perivascular astrocytes. In contrast, EMMPRIN signal did not co-localize with NeuN-positive neurons, or MPO-positive neutrophils. Dual fluorescent staining revealed that EMMPRIN co-localized with MMP-9. Our data also demonstrated that increased EMMPRIN expression correlated with increased MMP-9 levels in a temporal manner.

In summary, we report for the first time that EMMPRIN expression was significantly increased in a mouse model of permanent focal cerebral ischemia. The spatial and temporal association between increased EMMPRIN expression and elevated MMP-9 levels suggest that EMMPRIN may modulate MMP-9 activity, and participate in neurovascular remodeling after ischemic stroke.

Keywords

EMMPRIN; MMP-9; stroke

Correspondence: Yongmei Chen, MD, PhD, University of California, San Francisco, Department of Anesthesia and Perioperative Care, 1001 Potrero Ave, Room 3C-38, San Francisco, CA 94110, Tel: 415-206-4795, Fax: 415-206-8907, E-mail: meichen@anesthesia.ucsf.edu.

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1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that degrade the extracellular matrix (ECM) (Rosenberg, 2002). Elevated activities of MMPs, such as MMP-9 following ischemic stroke, have been shown to disrupt the integrity of ECM, leading to blood-brain barrier leakage and brain hemorrhage (Fujimura et al., 1999; Machado et al., 2006; Rosenberg et al., 2001). It has also been reported that activities of MMPs promote functional recovery by facilitating post-ischemic neurovascular remodeling during the delayed phases after ischemic stroke (Zhao et al., 2006).

Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) is a 58-kDa cell surface glycoprotein, which has been described as having a key regulatory role in MMP activity (Gabison et al., 2005). EMMPRIN was originally identified on the tumor cell surface as an inducer of MMPs, and has been reported to induce several MMPs, including collagenase (MMP-1), gelatinase A (MMP-2), stromelysin (MMP-3), gelatinase B (MMP-9), membrane type (MT) 1-MMP (MMP-14), and MT2-MMP (MMP-15). Various biological mediators such as cytokines and free radicals have been shown to enhance the activities of EMMPRIN and MMPs. EMMPRIN is upregulated by amphiregulin and epidermal growth factor (EGF) at both mRNA and protein levels through EGF receptor (EGFR) tyrosine kinase activation (Menashi et al., 2003). Studies have reported that Phorbol 12-myristate 13-acetate induces EMMPRIN expression in tumors (Zhang et al., 2006), and that transforming growth factor- β (TGF- β) upregulates EMMPRIN expression in human corneal epithelial cells (Gabison et al., 2005).

As an MMP inducer, EMMPRIN has been shown to mediate tissue remodeling in both pathological and physiological conditions, such as myocardial infarction and cancer progression (Schmidt et al., 2006; Toole, 2003). However, its roles in stroke injury are not clearly understood. In the present study, we examined the changes of EMMPRIN in a mouse model of permanent focal brain ischemia, and also investigated the spatial and temporal correlations between EMMPRIN expression and MMP-9 levels after stroke.

2. Materials and methods

2.1. Chemicals and reagents

Rat anti-EMMPRIN monoclonal antibody was purchased from Serotec (Raleigh, NC). Rabbit anti-vwf antibody, mouse anti-mouse NeuN antibody, and zymographic standards were obtained from Chemicon (Temecula, CA). Mouse anti-GFAP-CY3 conjugated antibody was from Sigma (St. Louis, MO). Coomassie Brilliant Blue was from Bio-Rad (Richmond, CA), and the nitrocellulose membrane was from Amersham (Piscataway, NJ). Kodak films were obtained from the Eastman Kodak Company (Rochester, NY). Ten percent (10%) zymogram gel was from Invitrogen (Carlsbad, CA), Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rat IgG from Molecular Probes, and biotinylated goat anti-rat secondary antibody from Vector Laboratories.

2.2. Animal stroke model

The study was approved by the University of California, San Francisco Committee of Animal Research and conformed to NIH Guidelines for use of animals in research. Adult male CD-1 mice (30–35g) were subjected to permanent focal ischemia by intraluminal middle cerebral artery blockade (Shen et al., 2006). Surface cerebral blood flow (sCBF) was monitored during MCAO using a laser Doppler flowmetry (Vasamedics). Mice were excluded from the experiment if sCBF in the ischemic core region was more than 15% of the baseline. Stroke mice and sham-operated control mice were sacrificed at 1 day, 2 days, 5 days, and 7 days after

MCAO. In our study, more than 50% animals survived up to 7 days after permanent MCAO model.

2.3. Immunohistochemistry

Immunohistochemical staining was performed as described (Chen et al., 2006b). Briefly, frozen sections (20 μ m thick) were incubated with primary antibodies at the following concentrations: rat anti-EMMPRIN monoclonal antibody, 1:200; rabbit anti-vwf antibody, 1:200; mouse anti-mouse NeuN antibody, 1:500; and mouse anti-GFAP-CY3 conjugated antibody, 1:2000. After incubating at 4°C overnight and washing, the sections were incubated with biotinylated goat anti-rat at 1:5000 dilutions. The sections were treated with the ABC streptavidin detection system. For dual fluorescent staining after incubating with primary antibodies, sections were incubated with Alexa Fluor 594-conjugated goat anti-mouse or Alexa Fluor 488-conjugated goat anti-rat IgG at 1:500 dilutions. Negative controls were performed by omitting the primary antibodies during the immunostaining.

2.4. Evaluation of cerebral infarction

The mice were sacrificed at 1 day, 2 days, 5 days, and 7 days after MCAO for evaluation of infarct volume (Shen et al., 2006). Serial coronal sections (200 μ m apart) were stained with cresyl violet for assessing infarct volume. Sections were digitized, and infarct area was measured using NIH Image J analysis system. The ischemic lesion area was calculated as the difference between the area of the non-ischemic hemisphere and the normal area of the ischemic hemisphere. The infarct volume was calculated by multiplying the infarct areas by the thickness of sections.

2.5. Western blotting

Western blot was performed as previously reported (Chen et al., 2006a). Different samples with an equal amount of proteins were loaded on 10% acrylamide gel for electrophoresis, and were electroblotted onto a PVDF membrane. The membranes were then probed with rat anti-EMMPRIN monoclonal antibody, 1:200, followed by incubation with horseradish-peroxidase (HRP)-conjugated sheep anti-rat IgG. Protein expression was detected with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech Inc). β -actin was used as a loading control.

2.6. Gelatin zymography

Zymography was performed as previously described (Lee et al., 2005). Protein samples (40 μ g) were separated under non-reducing conditions in a 10% zymogram gel containing 0.1% gelatin as a substrate. Following electrophoresis, gels were washed, incubated in developing buffer overnight at 37°C, stained with 0.5% Coomassie Blue R-250, and de-stained. Protein bands were quantified using scanning densitometry with KODAK image analysis software.

2.7. Statistical analysis

Data are presented as mean $\pm$ SD, and were analyzed using one-way ANOVA followed by the Fisher's PLSD test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Increased EMMPRIN expression after stroke

Cerebral infarctions were confirmed by Nissl staining, and representative Nissl-stained brain coronal sections showed ischemic infarct at 1 day (Figure 1b), 2 days (Figure 1c), 5 days (Figure 1d), and 7 days (Figure 1e) after MCAO. Normal brains (Figure 1a) were stained blue with the Nissl staining method, but the cerebral infarcts in the brains of MCAO mice displayed

decreased staining delineating the pale ischemic region. EMMPRIN expression was strongly increased in the peri-infarct region of the ischemic cortex and striatum at 2 to 7 days after stroke (Figure 1g, 1h, 1i, 1j). Higher magnification image of EMMPRIN positive signal is shown in the inset in Figure 1h. EMMPRIN signal was weak in the non-ischemic hemisphere (Figure 1f), and at day 1 after MCAO (Figure 1g). We also noted that EMMPRIN signal was primarily located around microvessel-like structures. To further determine quantitatively increased EMMPRIN expression after stroke and to exclude cross reactivity by immunohistochemical staining, we performed Western blot analysis. As shown in Figure 2, EMMPRIN expression was significantly increased at 2–7 days after stroke compared to expression in the contralateral non-ischemic and normal brain.

3.2. Cellular localization of EMMPRIN

To examine which cells exhibit the increased amount of EMMPRIN, we performed immunofluorescent double staining with cell type specific markers: GFAP as astrocyte marker, vWF as endothelial cell marker, NeuN as neuronal marker, and MPO as neutrophil marker. As shown in Figure 3, EMMPRIN signals co-localized with GFAP-positive peri-vascular astrocytes (Figure 3c), and vWF-positive endothelial cells (Figure 3f). However, EMMPRIN signal did not co-localize with NeuN-positive neurons (Figure 3i), or MPO positive neutrophils (Figure 3m).

3.3. Increased MMP-9 levels after stroke

EMMPRIN has been shown to modulate MMP activities in tumor cells and cardiovascular cells (Schmidt et al., 2006; Toole, 2003). We next examined whether ischemic injury also correspondingly enhanced MMP-9 activities. MMP levels were assayed using substrate gel electrophoresis with gelatin as a substrate. As shown in Figure 4, MMP-9 levels were dramatically increased at 2–5 days after MCAO compared to the contralateral nonischemic side, or normal controls. After MCAO, the induced MMP-9 levels remained elevated up to day 7, but no obvious enhancement of MMP-2 levels was noted.

3.4. Association between EMMPRIN expression and MMP-9 levels after stroke

To further examine the proximity of EMMPRIN expression to MMP-9 upregulation, we determined whether EMMPRIN co-localized with MMP-9. Double immunofluorescent staining showed that EMMPRIN and MMP-9 were co-localized around microvessels (Figure 5A). In addition, linear regression analysis demonstrated that enhanced EMMPRIN expression correlated positively with elevated MMP-9 levels (Figure 5B, $R^2 = 0.84$, $P < 0.05$). These data indicate that enhanced expression of EMMPRIN may induce MMP-9 levels in adjacent cells in the ischemic brain.

4. Discussion

In this study, we demonstrated that: 1) EMMPRIN expression was significantly increased after permanent focal cerebral ischemia; 2) EMMPRIN was predominantly expressed by endothelial cells and peri-vascular astrocytes (in contrast, EMMPRIN immunoreactivity was not observed in neurons or infiltrating neutrophils); and 3) changes in EMMPRIN expression were correlated with MMP-9 levels in a temporal and spatial manner.

The results from our study regarding the upregulation of EMMPRIN expression after focal cerebral ischemia were, in part, consistent with the previous report demonstrating that EMMPRIN level was enhanced after transient MCAO in rats (Burggraf et al., 2005). In their study, Burggraf et al found that EMMPRIN was dramatically increased at day 1 after ischemia, whereas in our study, EMMPRIN expression was significantly increased at 2–7 days after permanent MCAO. The difference in the time course of EMMPRIN induction could have been

caused by the different pathological mechanisms between permanent and transient ischemia. EMMPRIN expression has been found in many types of normal tissues including the brain, suggesting that it plays a physiological role in normal tissue remodeling (Sameshima et al., 2000; Seulberger et al., 1990). It can be enhanced by many stimuli, including cytokines, free radicals, and oxidized low-density lipoproteins (Gabison et al., 2005; Haug et al., 2004). Excessive expression of EMMPRIN has been demonstrated to increase the invasiveness of tumor cells and play a role in the pathophysiology of various disease processes, such as rheumatoid arthritis, asthma, and acute myocardial infarction (Gwinn et al., 2006; Schmidt et al., 2006; Zhu et al., 2006). Enhanced expression of EMMPRIN following focal cerebral ischemia may participate in ischemic brain injury and remodeling, possibly by modulating MMP activities. Moreover, our data showed that ischemia-induced EMMPRIN signals were preferentially located around microvessels, suggesting that EMMPRIN may be associated with blood brain barrier (BBB) permeability by stimulating MMP secretion from endothelial cells or peri-vascular astrocytes.

The role of EMMPRIN in MMP activation was first found in tumor cells (Caudroy et al., 2002). High EMMPRIN expression correlates with poor outcome in various tumors by enhancing MMP production and tumor cell invasion (Bordador et al., 2000; Davidson et al., 2004). Additionally, EMMPRIN has been reported to regulate MMP-9 activity in cardiovascular cells and modulate cardiovascular pathology (Schmidt et al., 2006). Our double-labeled immunostaining showed that EMMPRIN and MMP-9 were co-localized, and furthermore, correlation analysis confirmed that increased MMP-9 activity was closely related to the elevated expression of EMMPRIN. The data indicate that EMMPRIN may function as an upregulator in local MMP-9 activities following ischemic injury. MMPs, especially MMP-9, have been implicated in the pathogenesis of several neurological diseases, including cortical spreading depression, and cerebral ischemia (Gursoy-Ozdemir et al., 2004; Rosell et al., 2006). The early appearance of activated MMPs can have a deleterious role in cerebral ischemia by promoting ECM degradation, leading to brain edema and hemorrhage (Fujimura et al., 1999). MMP-9 has been shown to be re-upregulated at 7–14 days after permanent focal cerebral ischemia (Zhao et al., 2006). Delayed activation of MMPs can exhibit a different role from early activated MMPs, which may participate in neurovascular remodeling and promote functional recovery. In our study, we found that EMMPRIN and MMP-9 expression simultaneously increased in the delayed phase, indicating that EMMPRIN upregulation might contribute to neurovascular remodeling. In supporting this notion, EMMPRIN has been reported to induce the expression of vascular endothelial growth factor (VEGF), which is a prototypical molecule, in promoting angiogenesis (Tang et al., 2006). Angiogenesis is a critical component of neurovascular remodeling following stroke.

In summary, this is the first study to demonstrate that EMMPRIN expression is significantly increased in a mouse model of permanent middle cerebral artery occlusion. Our data show that EMMPRIN upregulation correlated with enhanced MMP-9 levels. This study underscores the potential of EMMPRIN as a novel target for stroke therapy. However, further investigation is needed to determine whether anti-EMMPRIN monoclonal antibody or antagonists of EMMPRIN can suppress ischemia-induced MMP production and tissue remodeling.

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Abbreviations

ECM

extracellular matrix

EMMPRIN

Extracellular Matrix Metalloproteinase Inducer

MCAO

middle cerebral artery occlusion

MMPs

matrix metalloproteinases

sCBF

surface cerebral blood flow

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Figure 1.

Increased EMMPRIN signal after stroke. Representative Nissl-stained brain coronal sections show ischemic infarct at 1 day (b), 2 days (c), 5 days (d), and 7 days (e) after MCAO. A representative brain section from sham-operated mice is shown in (a). Boxed area indicates location of EMMPRIN immunostaining in the peri-infarct cortex. Immunostaining shows low baseline levels of EMMPRIN in the sham operated mice cortex (f). Representative EMMPRIN immunostaining at 1 day, 2 days, 5 days and 7 days after stroke are shown in g, h, i, and j respectively. Higher magnification image of EMMPRIN signal is shown in the inset in (h). Arrows indicate positive EMMPRIN signal. Size bar: 100μm.

Figure 2.

Western blot analysis of EMMPRIN expression. (A) Detection of EMMPRIN at 58kDa. The basal expression of EMMPRIN was observed. EMMPRIN levels were significantly increased after MCAO. Ischemic side: i; contra-lateral non-ischemic side: c; sham-operated control: N. (B) Densitometric analysis. Protein loading was normalized to the actin bands. OD: optical density; *: $P < 0.05$ vs contralateral nonischemic side; $n=8$ per group. The data are representative of three separate experiments.

Figure 3.

Co-localization of EMMPRIN with cellular markers. Cellular localization of EMMPRIN (green, b, e, h, k) with cellular markers (red): astrocyte GFAP (a); endothelial cell: vwF (d); neuron: NeuN (g), and neutrophil: MPO (j). EMMPRIN immunoreactivity is observed in astrocytes and endothelial cells (yellow, c, f). Arrows indicate co-localization of EMMPRIN with GFAP and vwF (yellow color). EMMPRIN does not co-localize with neurons (i) or neutrophils (m). Size bar: 50μm.

Figure 4. Increased MMP-9 levels after stroke determined by gelatin gel zymography. **(A)** Representative gelatin zymograms indicate that MMP-9 level was strongly enhanced after stroke. Recombinant mouse MMP-9 standards were loaded as molecular size markers (M). Ischemic side: i; contra-lateral nonischemic side: c; sham operated control brain: N. **(B)** Histogram shows quantification of MMP-9 levels. OD: optical density; *: $P < 0.05$ vs contralateral nonischemic side; $n=8$ per group. The data are representative of three separate experiments.

Figure 5.

Relationship between MMP-9 levels and EMMPRIN expression. (A) A representative image shows double-labeled MMP-9 (red color) and EMMPRIN (green color) immunostaining. Arrows indicate co-localization of MMP-9 with EMMPRIN (yellow color). Bar scale: 50μm. (B) Scatter graph shows the correlation of MMP-9 levels and EMMPRIN expression after stroke. The relationship between MMP-9 levels and EMMPRIN expression was analyzed using simple regression ($R^2 = 0.84$, $P < 0.05$). OD, optical density.