OPEN

Journal of Cerebral Blood Flow & Metabolism (2013) 33, 1041–1049

www.jcbfm.com

ORIGINAL ARTICLE

TIMP-1 attenuates blood–brain barrier permeability in mice with acute liver failure

Feng Chen[1], Evette S Radisky[2], Pritam Das[1], Jyotica Batra[2], Toshiyuki Hata[3], Tomohide Hori[3], Ann-Marie T Baine[1], Lindsay Gardner[3], Mei Y Yue[1], Guojun Bu[1], Gregory del Zoppo[4], Tushar C Patel[3] and Justin H Nguyen[3]

Blood–brain barrier (BBB) dysfunction in acute liver failure (ALF) results in increased BBB permeability that often precludes the patients from obtaining a life-saving liver transplantation. It remains controversial whether matrix metalloproteinase-9 (MMP-9) from the injured liver contributes to the deregulation of BBB function in ALF. We selectively upregulated a physiologic inhibitor of MMP-9 (TIMP-1) with a single intracerebroventricular injection of TIMP-1 cDNA plasmids at 48 and 72 hours, or with pegylated-TIMP-1 protein. Acute liver failure was induced with tumor necrosis factor-α and D-(+)-galactosamine in mice. Permeability of BBB was assessed with sodium fluorescein (NaF) extravasation. We found a significant increase in TIMP-1 within the central nervous system (CNS) after the administration of TIMP-1 cDNA plasmids and that increased TIMP-1 within the CNS resulted in an attenuation of BBB permeability, a reduction in activation of epidermal growth factor receptor and p38 mitogen-activated protein kinase signals, and a restoration of the tight junction protein occludin in mice with experimental ALF. Pegylated TIMP-1 provided similar protection against BBB permeability in mice with ALF. Our results provided a proof of principle that MMP-9 contributes to the BBB dysfunction in ALF and suggests a potential therapeutic role of TIMP-1 in ALF.

Journal of Cerebral Blood Flow & Metabolism (2013) **33,** 1041–1049; doi:10.1038/jcbfm.2013.45; published online 27 March 2013

Keywords: acute liver failure; BBB permeability; tight junction; TIMP-1

INTRODUCTION

Blood–brain barrier (BBB) dysfunction results in increased BBB permeability and subsequent brain edema[1] in patients with acute liver failure (ALF). Brain edema remains a major cause of death and a major impediment to liver transplant eligibility for patients with ALF.[2] In the early stages of hepatic encephalopathy in ALF, the BBB becomes permeable to small polar molecules.[1] If the BBB dysfunction persists, then brain edema will end with herniation and death. Dysfunction of BBB in ALF is reversible when the ALF is timely corrected by either spontaneous regeneration or successful liver replacement. However, the molecular mechanisms of BBB dysfunction in ALF are poorly understood.

Blood–brain barrier and its tight junctions restrict free movement of polar molecules, nonpolar molecules, and water in and out the central nervous system (CNS). Blood–brain barrier is made of endothelial cells that line the lumen of the brain capillary and use tight junction complexes to seal the paracellular cleft between the two cellular edges of its own cells or between two adjacent cells. It rests on the basal lamina, which is engulfed with pericytes, astrocytic endfoots, and is surrounded with microglial cells and neurons. This collectively makes up a neurovascular unit.[1] It is estimated that the brain contains 100 billion capillaries that spread over 400 miles in length and cover 215 square feet of surface area.[3] Thus, a perturbation in BBB integrity can have a major reverberating impact on CNS function.

The pattern of brain injury in ALF is different from that in brain ischemia or trauma. In traumatic and ischemic brain injuries, the BBB is usually structurally damaged, and the predominant sources of injury are derived from the constituents within the CNS and the neurovascular unit.[4] In contrast, BBB injury in ALF is associated with minimal changes in the ultrastructural architecture of the BBB.[5] It is also believed that injurious factors and neurotoxins, including matrix metalloproteinase-9 (MMP-9) and inflammatory cytokines, are released from the failing liver into the systemic circulation and target the brain capillary endothelial cells, leading to BBB dysfunction.[6,7] Data have shown that MMP-9, proteolytic factors,[5,8] and inflammatory cytokines, including tumor necrosis factor-α (TNF),[6] are released into the systemic circulation shortly after liver injury. However, it remains poorly understood how the CNS is injured and whether factors like MMP-9 or others derived from distal organs injure the BBB and lead to an increased permeability to small molecules like water, ammonium, and amino acids in ALF.

Matrix metalloproteinase-9 is an independent risk factor in the development of stroke.[9] It is intimately involved in BBB injury caused by brain ischemia[10] and brain trauma.[11] In 2006, we found that the monoclonal antibodies specific for active MMP-9 or GM6001, a synthetic broad-spectrum MMP-9 inhibitor, ameliorates the increased BBB permeability in mice and rats with experimentally induced ALF.[5,12] Similar findings have been recently reported by others.[13] We have shown that the brain is not the source of MMP-9 in ALF subjects. Rather, liver-derived MMP-9, which is present in the lumen of systemic circulation, directly influences brain capillary endothelial cells.[5] Matrix metalloproteinase-9 is released into the systemic circulation from the injured liver within 2 hours of the chemical induction

[1]Department of Neuroscience, Mayo Clinic, Jacksonville, Florida, USA; [2]Department of Cancer Basic Science, Mayo Clinic, Jacksonville, Florida, USA; [3]Department of Transplantation, Mayo Clinic, Jacksonville, Florida, USA and [4]Division of Hematology, Department of Medicine, School of Medicine, University of Washington, Seattle, Washington, USA. Correspondence: Professor JH Nguyen, Division of Transplant Surgery, Department of Transplantation, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, USA.
E-mail: nguyen.justin@mayo.edu
The work of JHN was supported by the Deason Foundation, Sandra and Eugene Davenport, Mayo Clinic CRT-II, and R01NS051646-01A2.
Received 21 September 2012; revised 28 February 2013; accepted 3 March 2013; published online 27 March 2013

of ALF.[5,8] Using RT-PCR and zymographic assays, there was no upregulation of MMP-9 within brains of mice with ALF.[5] We subsequently found significant alterations in tight junctional proteins, particularly occludin, in brain endothelial cells exposed to MMP-9 *in vitro*, and the alterations were reversed with GM6001 or tissue inhibitors of MMP (TIMP)-1 upregulation. We also found marked alterations in tight junctional proteins in the brains of mice with ALF and the changes were reversed with GM6001.[14] We identified that epidermal growth factor receptor (EGFR) and p38 mitogen-activated protein kinases (MAPK) mediated the derangement of occludin in brain endothelial cells *in vitro* and that both EGFR and p38 MAPK are activated and reversed with GM6001 in the brains of mice with ALF.[15] These results support the concept that excess MMP-9 in the brain capillary lumen, particularly when its physiologic inhibitor TIMP-1 is markedly decreased,[12] attack the endothelial cell, including its tight junctional proteins, particularly occludin. Endothelial receptor-mediated signals also lead to an increase in BBB permeability to small polar molecules. However, it remains unclear how MMP-9 or its associates contribute to the development of the BBB dysfunction or how we can reverse its effect when MMP-9 is both ubiquitous and does not have a clinically effective inhibitor.[16]

In this study, we propose that an upregulation of TIMP-1, the physiologic and specific tissue inhibitor of MMP-9, within CNS would mitigate the BBB dysfunction as it attenuates the BBB permeability in mice with experimentally induced ALF. The intracerebral induction of TIMP-1 will avoid confounding the effect of the inhibitor since a systemic administration of the inhibitor may affect the disease process of ALF as MMP-9 or TIMP-1 participates in the liver injury and regeneration.[8,17] By this approach, the results will provide a proof of principle on the role of MMP-9 in BBB dysfunction in ALF. The results may strengthen the role of TIMP-1 as an effective therapeutic agent.

MATERIALS AND METHODS

Animals

C57BL/6 mice (male, 12 to 16 weeks of age, 20 to 30 g body weight) were used (Harlan Laboratories, Indianapolis, IN, USA). Mice were housed in a specific pathogen-free condition with 12-hour light/dark cycles, food, and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic (Protocols A22411 and A24907).

Antibodies and Reagents

Mouse anti-TIMP-1 antibody was from Calbiochem (Billerica, MA, USA; IM63), and anti-CD31 (PECAM-1) antibody from Cell Signal Technology (Danvers, MA, USA; 3568). Anti-phospho p38 MAPK (sc9211), anti-p38 MAPK (sc9212), and anti-MMP-9 antibodies (sc-13520) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-occludin (Zy71-1500) from Invitrogen-Zymed Laboratories (Carlsbad, CA, USA); and anti-phospho-EGFR (Tyr 1069) was from EMD Millipore (Billerica, MA, USA; 09-310). Recombinant mouse TNF was purchased from R&D Systems (Minneapolis, MN, USA; 410-MT-050), D-(+) Galactosamine hydrochloride (G1639-16), sodium fluorescein, and FITC dextrans (FD10S, FD 40S, FD 150S) from Sigma (Houston, TX, USA).

Blood–Brain Barrier Permeability

Sodium fluorescein (NaF, 376 daltons (D)) was used to assess the BBB permeability as we previously described.[18] Mice received an intravenous injection of 8 mL/kg of 0.6% NaF 2 hours after the induction of ALF. Thirty minutes later, mice were perfused with 10 mL of normal saline. Brains were resected and brain stems were removed. Half hemisphere of cerebrum was homogenized in 30% trichloroacetic acid, and centrifuged at 10,000 *g* for 5 minutes. From the supernatant, NaF concentration was measured with a fluorometer at 460 nm excitation and 515 nm emission with a standard curve. NaF was quantitated and expressed as pg/mg of brain tissue. Permeability ratio was obtained by dividing the NaF in pg/mg of the study case to that of the normal control.

Fluorescent Microscopy for Sodium Fluorescein Extravasation

Fluorescent microscopy was performed as previously described.[5] The brains were perfused via ascending aorta with 5 mL of saline, followed by 5 mL of 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline pH 7.4. The brains were removed and kept overnight in the same solution. The brains were immersed in 30% sucrose in 0.1 mol/L phosphate-buffered saline for 48 hours, then frozen in OCT (Sigma) and stored at -70°C. The NaF extravasation was observed in the cryostat sections (20 μm) using a fluorescent microscope (Leica DMLB, Wetzlar, Germany) with a N2.1 filter, 100 W mercury lamp, MicroMax monochrome camera, and MCID/M5 (Imaging Research, Ontario, Canada).

Intracerebroventricular Administration of Tissue Inhibitors of Matrix Metalloproteinase 1 cDNA Plasmid

Tissue Inhibitors of Matrix Metalloproteinase 1 cDNA plasmid, PCMV6 vector or saline, was administered intracerebroventricular (ICV) to C57Bl/6 mice in a total volume of 5 μL as we previously described.[19] The mice were anesthetized by isoflurane (Abbott Lab, North Chicago, IL, USA) inhalation and positioned in a stereotactic instrument (Stoelting Co., Wood Dale, IL, USA). A small incision was made, the skull exposed, and a small burr hole was drilled using a Dremel rotary tool (Dremel Company, Racine, WI, USA). TIMP-1 cDNA plasmid, control cDNA, or saline was injected using a Flexifil microsyringe (10 μl) at a rate of 0.5 μL/min controlled by a syringe pump (Harvard Apparatus, Holliston, MA, USA) into the left lateral ventricle at the coordinates: 0.4 mm caudal to bregma, 1.0 mm lateral to sagittal suture, and 2 mm in depth. The incision was closed with Vet-Bond, and the mice were injected 0.9% saline 1 mL and were placed on an isothermal pad at 37°C and observed after surgery until recovery.

Immunohistochemical Localization and Staining

Mouse brain slides were prepared from formalin-fixed, paraffin-embedded brain tissues.[15] Rabbit antibody against phospho-EGFR (Tyr 1069), a mouse antibody against TIMP-1, and a rabbit antibody against CD31 were used according to the respective manufacturer's instructions. Briefly, deparaffinized slides were steamed for 30 minutes. Nonspecific binding sites were blocked with Dako's All Purpose Blocker (Dako, CA, USA). Primary antibodies were prepared with Dako's Antibody Diluent Solution at 1:50 to 1:100 dilutions and were incubated overnight in humidifier at 4°C. After washing, slides were applied with Alexa Fluor dye-labeled secondary antibodies (Invitrogen): Alexa Fluor 488 goat anti-mouse (green) and Alexa Fluor 568 goat anti-rabbit (red). Images were obtained using Zeiss 510 META confocal microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA).

Protein Isolation and Western Blot Analysis

Brain specimens were homogenized in lysate buffer (50 mmol/L Tris-HCl pH 7.3, 150 mmol/L NaCl, 3 mmol/L MgCl, 1 mmol/L DTT, 1 mmol/L EDTA, 1 mmol/L EGTA, 1.0% Triton X-100) containing protease and phosphatase inhibitors. Equal amounts of proteins (30 μg) from each sample were resolved on 4% to 20% SDS–PAGE gels, transferred, and immunoblotted onto nitrocellulose membrane. Antibodies for TIMP-1, occludin, phosphorylated p38 MAPK, p38 MAPK, MMP-9, and GAPDH antibodies were used. Band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

To determine the level of EGFR activation, the brain specimens were prepared with ice-cold immunoprecipitation buffer (50 mmol/L HEPES, pH 7.5, 50 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 1 mmol/L Na_3VO_4, 30 mmol/L 2-(*p*-nitrophenyl) phosphate, 100 mmol/L NaF, 10% glycerol, 1.5 mmol/L $MgCl_2$, and protease inhibitor cocktail). Lysates were centrifuged at 14,000 *g* for 5 minutes, and the supernatant was immunoprecipitated with anti-EGFR and Dynabeads M-280 magnetic protein bead separation system (Invitrogen) overnight at 4°C.

Data Analysis

The results were expressed as mean $\pm$ standard deviation. All statistics were performed using Graphpad Prism (GraphPad Software, Inc., La Jolla, CA, USA, Version 5.0). Comparisons of multiple groups were performed by one-way ANOVA followed by Tukey's *post hoc* testing. A P value of <0.05 was considered as statistically significant.

RESULTS

Increased Blood–Brain Barrier Permeability in Mice with Tumor Necrosis Factor-α/Gal-Induced Acute Liver Failure

Mice that were treated with D-(+)-galactosamine (Gal) and TNF reproduced ALF as we and others previously reported.[8,15,20] The TNF/Gal-induced ALF is similar to the azoxymethane-induced ALF.[5] However, TNF/Gal-induced mice experienced a much shorter course of the acute manifestations of ALF without a loss of body temperature control.[20] The TNF/Gal-treated mice manifested ALF maintaining body temperature at 36.2 ± 0.1°C without an event of hypothermia, Figure 1A. Thus, using TNF/Gal model reduces the confounding factor hypothermia since it influences the CNS injury and the liver disease process.[21] Tumor necrosis factor-α is a main mediator of ALF in Gal/LPS-induced ALF in mice.[22] The use of TNF is consistent with the findings that TNF is markedly elevated in the systemic circulation of the human patients with ALF.[23] Therefore, TNF/Gal-induced ALF is a suitable model.

Tumor necrosis factor-α is commonly administered at 0.3 μg together with 20 mg Gal in a mouse. However, at this dose usually only 67% of the treated mice experience mortality of ALF.[8,22] We found that at the dose of 1.0 μg of TNF and 20 mg Gal/mouse, >90% of the TNF/Gal-treated mice showed lethality, consistent with azoxymethane-induced ALF.[5] Therefore, we used this dose in our study. Since TNF is known to influence the BBB,[22] we examined the BBB permeability using NaF permeability in mice that received saline vehicle, 1.0 μg TNF alone, versus 1.0 μg TNF with 20 mg Gal. After 2 hours, NaF was injected intravenously. The brain was perfused with phosphate-buffered saline, homogenized, and NaF was quantitated and expressed as pg/mg of brain tissue. The brain extravasated NaF was 664 ± 211, 738 ± 175, and 1,262 ± 296 pg/mg for vehicle control, TNF alone, and TNF/Gal-treated mice, respectively ($n = 3$ each). The permeability ratio against the control was 1, 1.11, and 1.90, respectively. Only TNF/Gal-treated mice had significant increase in NaF permeability ($P = 0.046$). We observed that TNF induces a slight increase in BBB permeability, and its impact on the BBB did not differ statistically from that control mice. In the absence of TNF/Gal-induced ALF, the impact of TNF alone seems relatively small. However, in the combination of TNF/Gal, the BBB permeability was markedly increased well above the level of BBB permeability that was induced by TNF alone. Therefore, TNF/Gal combination is a valid model for investigations of BBB permeability in experimental ALF.

We assessed the increased BBB permeability in ALF mice using fluorescent markers at varying molecular weights including NaF of 376 D and FITC dextrans at 10, 40, and 150 kD. As shown in Figure 1B, the mice with ALF had the highest permeability to NaF. The permeability was markedly decreased as the molecular weights of the permeability markers were increased. The permeability ratio of NaF was 2.01 ± 0.40 (n = 10) as compared with the permeability ratio of FITC dextrans having molecular weights of 10, 40, and 150 kD ($n = 3$/group) being 1.14 ± 0.07, 0.93 ± 0.15, and 0.95 ± 0.10, respectively. These results suggested that the BBB was most permeable to small molecules that were <10,000 D, then slightly permeable to molecules ~10 kD, and not permeable to molecules that are 40 kD or larger.

Intracerebroventricular Administration of Tissue Inhibitors of Matrix Metalloproteinase 1 cDNA Plasmids Increased Intracranial Tissue Inhibitors of Matrix Metalloproteinase 1 Expression

We reported that TIMP-1 is significantly decreased in the sera of patients with ALF[24] and in rats with experimental ALF.[12] It can be logically considered to increase TIMP-1 level in the systemic circulation in ALF. Therefore, we administered TIMP-1 cDNA plasmids according to the hydrodynamics technique as previously described.[25] Although we found that TIMP-1 is upregulated in the brains of these mice, we also found that the upregulation of TIMP-1 interfered with the development of ALF (Not shown). Our findings are consistent with previous reports.[17] Thus, intravenous replenishment of TIMP-1 would confound the purpose of the experiment. Therefore, we used the ICV approach for the upregulation of TIMP-1 for the proof of concept.[11]

We previously transfected mouse-derived brain endothelial cells, bEnd3 cells, *in vitro* with TIMP-1 cDNA plasmids resulting in significant expression of TIMP-1.[14] In this study, we administered TIMP-1 cDNA plasmids ICV. As shown in Figure 2, we found a marked increase in the expression of TIMP-1 in the brains of ALF mice that received ICV administration of TIMP-1 cDNA plasmids. At both 48 and 72 hours, although the mice that received the control DNA, i.e., PCMV-6 vector, had increase in the levels of TIMP-1/GAPDH, these changes were not statistically different from saline controls. In contrast, the ALF mice that received ICV TIMP-1 cDNA had significant increase in TIMP-1 upregulation as compared with the ALF mice that received the PCMV6 vector or saline at both 48 and 72 hours with $P = 0.006$ and $P = 0.002$, respectively (Figures 2A–2D). The upregulation of TIMP-1 was confirmed with confocal fluorescent microscopy showing TIMP-1 present within CNS parenchymal cells and CD31-positive endothelial cells along the microvessels, Figures 2E–2H.

We also determined whether MMP-9 would be upregulated in the brains of the mice that received ICV injections. We found no

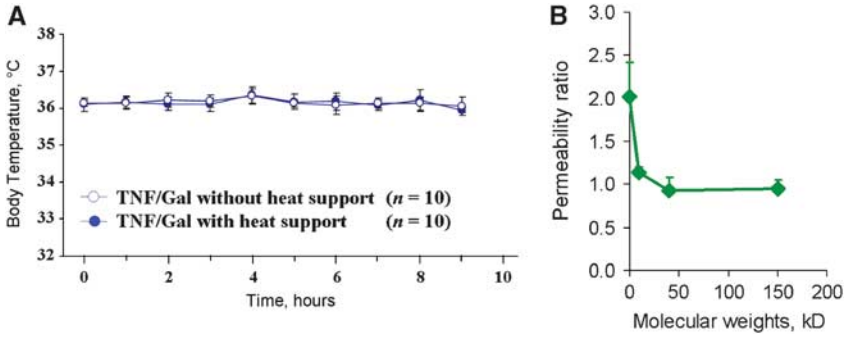

Figure 1. Experimental model of acute liver failure (ALF) in mice using tumor necrosis factor-α (TNF)/Gal induction. (**A**) Normothermia is sustained in mice having TNF/Gal-induced ALF. Each mouse ($N = 10$/group) received 1.0 μg TNF and 20 mg Gal. Body temperature was monitored hourly. With or without heating pad support, the ALF mice maintained their body temperature at 36.2 ± 0.1°C. (**B**) Selective blood–brain barrier (BBB) permeability in TNF/Gal-induced ALF mice. Fluorescent markers including sodium fluorescein (NaF) (376 D), and FITC dextrans (10, 40, and 150 kD) were intravenously administered. Extravasated fluorescent marker in the perfused brains of the study mice was quantitated and expressed as pg/mg of brain tissue. Permeability ratio expressed the permeated fluorescent marker in the study mice above the control mice. The permeability ratio was 2.01 ± 0.40, 1.14 ± 0.07, 0.93 ± 0.15, and 0.95 ± 0.01 for NaF, FITC dextrans of 10, 40, and 150 kD, respectively.

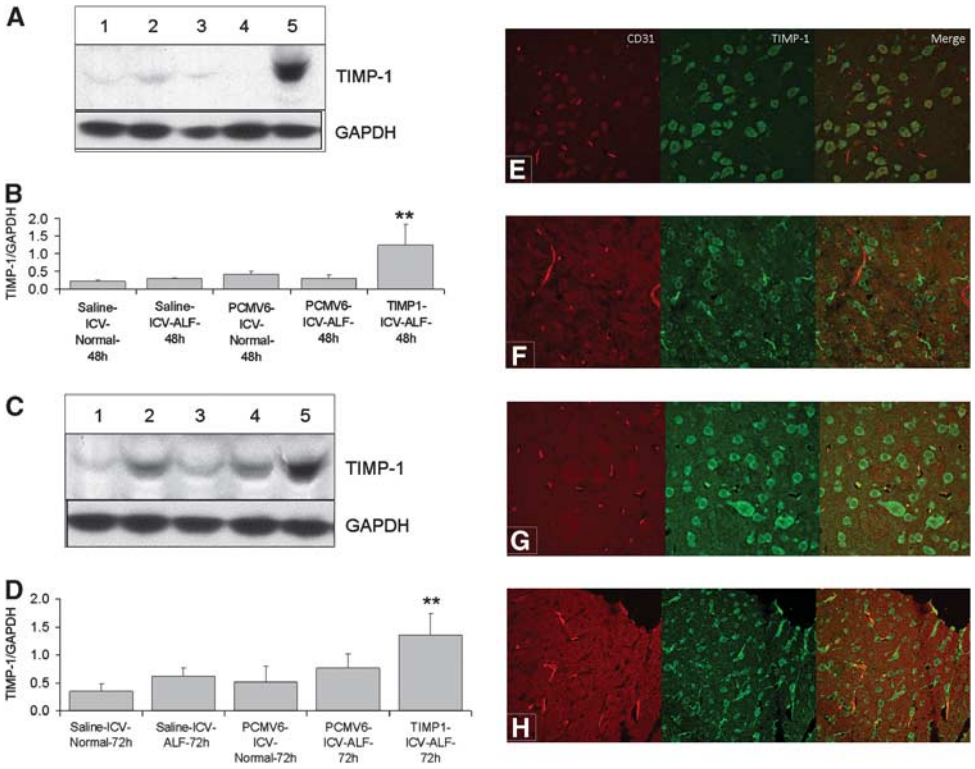

Figure 2. Intracerebroventricular (ICV) administration of tissue inhibitors of MMP (TIMP)-1 cDNA plasmids increased intracerebral TIMP-1 expression in acute liver failure (ALF) mice. (**A**, **B**) Western blotting of TIMP-1 expression level in the brains of mice in the study groups at 48 hours ($N = 3$ each): (1) normal control mice with ICV injection of saline; (2) ALF mice with ICV injection of saline; (3) normal control mice with ICV injection of PCMV6 vector; (4) ALF mice with ICV injection of PCMV6 vector; (5) ALF mice received ICV TIMP-1 cDNA. The TIMP-1/GAPDH levels were 0.22 ± 0.04, 0.30 ± 0.02, 0.40 ± 0.09, 0.30 ± 0.09, and 1.24 ± 0.61, respectively. The ALF mice that received ICV TIMP-1 cDNA had significant increase in TIMP-1 as compared with those that received cDNA control, i.e., PCMV6 vector or saline (**$P = 0.006$). (**C**, **D**) Western blotting of TIMP-1 expression level in the brains of mice in the study groups at 72 hours (N = 3 each): (1) normal control mice with ICV injection of saline; (2) ALF mice with ICV injection of saline; (3) normal control mice with ICV injection of PCMV6 vector; (4) ALF mice with ICV injection of PCMV6 vector; and (5) ALF mice received ICV TIMP-1 cDNA. The TIMP-1/GAPDH levels were 0.36 ± 0.13, 0.62 ± 0.15, 0.52 ± 0.28, 0.76 ± 0.26, and 1.34 ± 0.39, respectively. The ALF mice that received ICV TIMP-1 cDNA had significant increase in TIMP-1 as compared with those that received cDNA control, i.e., PCMV6 vector or saline (**$P = 0.002$). (**E**–**H**) Confocal immunofluorescent microscopy showed the upregulation of TIMP-1 in the brains of ALF mice that had ICV TIMP-1 cDNA plasmids. Each figure included endothelial marker CD31 (red), TIMP-1 (green), and the colocalization of TIMP-1 with CD31 (merge): (**E**) normal controls receiving ICV saline; (**F**) normal controls receiving ICV TIMP-1 cDNA plasmids; (**G**) ALF mice receiving ICV saline; (**H**) ALF mice receiving ICV TIMP-1 cDNA at 48 hours. TIMP-1 was found to be upregulated throughout the central nervous system (CNS), particularly in the endothelial cells along vessels in the ALF mice with ICV TIMP-1 cDNA.

increase in MMP-9 expression in the brains of mice that received TIMP-1 plasmids as compared with control mice. The western blot analysis showed a normalized MMP-9 level of 1.38 ± 0.85, 1.89 ± 1.36, 2.34 ± 1.19, and 1.30 ± 0.45 ($P > 0.100$) in normal control, ALF mice received ICV saline, ALF mice received ICV TIMP-1 cDNA 48 hours prior, and ALF mice received ICV TIMP-1 cDNA 72 hours before TNF/Gal induction, respectively.

Thus, ICV administration of TIMP-1 cDNA plasmids before ALF induced significant upregulation of TIMP-1 within the brain parenchyma and vasculature without a significant increase in MMP-9.

Increased Intracerebral Tissue Inhibitors of Matrix Metalloproteinase 1 Attenuated Blood–Brain Barrier Permeability in Mice with Acute Liver Failure

We studied the BBB permeability in mice that received TIMP-1 cDNA plasmids 48 and 72 hours before ALF induction. At 48 and 72 hours, the mice that received ICV injection of either PCMV6 vector or saline before ALF induction served as disease control; and normal controls received saline injection in place of ALF induction. The mice having ALF and ICV injection of either PCMV6 vector or saline had a significant increase in NaF permeability. The brain NaF extravasations in mice received saline-ICV control, saline-ICV ALF, PCMV6-ICV control, PCMV6-ICV ALF, and PCMV6-TIMP1-ICV ALF were $3,004 \pm 442$ ($n = 5$), $12,125 \pm 1,978$ ($n = 5$), $3,648 \pm 470$ ($n = 3$), $11,320 \pm 1,855$ ($n = 3$), and $6,814 \pm 675$ ($n = 6$) pg/mg, respectively, at 48 hours, Figure 3A; and the corresponding brain NaF extravasations were $3,092 \pm 726$ ($n = 5$), $11,029 \pm 2,017$ ($n = 5$), $4,329 \pm 831$ ($n = 3$), $11,152 \pm 1,034$ ($n = 3$), and $6,378 \pm 1,033$ ($n = 7$), respectively, at 72 hours, Figure 3B. The mice that received intracerebral upregulation of TIMP-1 for 48 and 72 hours before ALF induction showed significant decrease in NaF permeability by 39.8% ($P = 0.024$) and 42.8% ($P = 0.026$), respectively. It is noted that although there was an increase in TIMP-1 in the mice with ALF that received ICV PCMV6 vector or saline at 72 hours, there was no reduction in NaF permeability in these mice suggesting a minimal critical level of TIMP-1 for an effect.

We next sought to determine whether an ICV administration of TIMP-1 protein itself would provide the same protection against BBB permeability in mice with ALF. In this study, TIMP-1 was modified with pegylation, extending its circulation half-life and

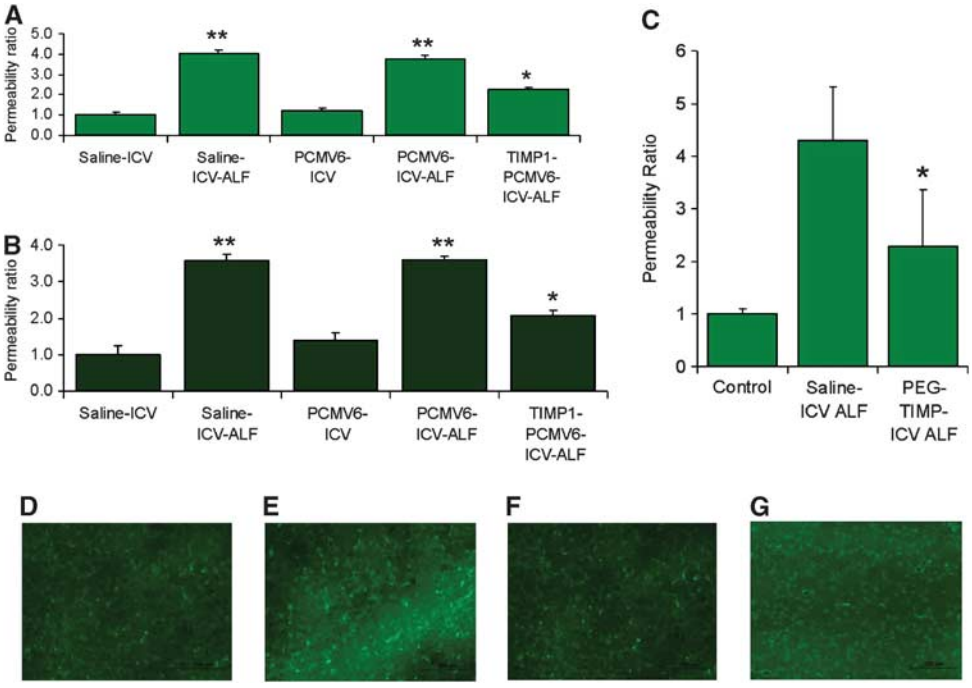

Figure 3. Upregulated tissue inhibitors of MMP (TIMP)-1 within central nervous system (CNS) attenuated blood–brain barrier (BBB) permeability in mice with acute liver failure (ALF). (**A**) ALF mice with intracerebroventricular (ICV) injection of TIMP-1 cDNA plasmids at 48 hours before ALF induction had significant reduction in the sodium fluorescein (NaF) permeability. Permeability ratio was 1.0 ± 0.1 ($n = 5$), 4.0 ± 0.2 ($n = 5$), 1.2 ± 0.1 ($n = 3$), 3.8 ± 0.2 ($n = 3$), and 2.3 ± 0.1 ($n = 6$) for saline-ICV, saline-ICV ALF, PCMV6-ICV, PCMV6-ICV ALF, and PCMV6-TIMP1 ICV ALF, respectively. The control mice receiving saline or PCMV6 had no difference in NaF permeability; the ALF mice that received saline or PCMV6 had similar increases in permeability ratio's ($P = 0.793$) that were significantly increased above the corresponding control animals (**$P = 0.002$ and 0.016, respectively). Treatment with TIMP-1 cDNA plasmids resulted in significant reduction of 39.8% in the permeability ratio in the ALF mice (*$P = 0.023$). (**B**) ALF mice with ICV injection of TIMP-1 cDNA plasmids at 72 hours before ALF induction had significant reduction in the NaF permeability. Permeability ratio was 1.0 ± 0.2 ($n = 5$), 3.6 ± 0.2 ($n = 5$), 1.4 ± 0.2 ($n = 3$), 3.6 ± 0.1 ($n = 3$), and 2.1 ± 0.2 ($n = 7$) for saline-ICV, saline-ICV ALF, PCMV6-ICV, PCMV6-ICV ALF, and PCMV6-TIMP1 ICV ALF, respectively. Treatment with TIMP-1 cDNA plasmids resulted in significant reduction of 42.8% in the permeability ratio in the ALF mice (*$P = 0.026$). (**C**) ICV administration of PEG-TIMP-1 significantly attenuated the BBB permeability in ALF mice. The ALF mice that were treated with pegylated TIMP-1 (PEG-TIMP-ICV ALF, $n = 5$) had NaF brain extravasation of $4,048 \pm 1,907$ pg/mg as compared with $7,610 \pm 1,828$ pg/mg for ALF mice that received vehicle (Saline ICV ALF, $n = 4$) (*$P = 0.013$). (**D**–**G**) Brain extravasation of NaF was assessed with fluorescent microscopy in (**D**) control mice with ICV injection of saline, (**E**) ALF mice with ICV injection of saline, (**F**) ALF mice with ICV injection of TIMP-1 cDNA plasmids, and (**G**) PEG-TIMP-1.

preserving MMP inhibitory activity as we recently described.[26] PEG-TIMP-1 showed a plasma half-life in mice of 28 hours, compared with a half-life of 1.1 hours for unmodified human recombinant TIMP-1. The mice that had ICV administration of PEG-TIMP-1 (75 μg/mouse, $n = 5$) immediately before ALF induction had significant reduction in NaF permeability ($P = 0.013$), Figure 3C.

We confirmed the NaF extravasation using fluorescent microscopy. We observed an increased extravasation of NaF in the brains of mice with ALF as compared with those of the control mice, Figures 3D and 3E. The increased permeability of NaF was ameliorated in the brains of the ALF mice that received TIMP-1 cDNA plasmids and that received PEG-TIMP-1, Figures 3F and 3G. The results collectively suggested that an increased TIMP-1 protein within CNS provided protection against BBB permeability in mice with TNF/Gal-induced ALF.

Upregulation of Tissue Inhibitors of Matrix Metalloproteinase 1 Reverses the Loss of the Tight Junction Protein Occludin, the Activations of p38 Mitogen-Activated Protein Kinase and Epidermal Growth Factor Receptor in Brains of Mice with Acute Liver Failure

Since the increased TIMP-1 within CNS attenuates the BBB permeability, we asked whether TIMP-1 will reverse the loss of the tight junction protein occludin[14] and the associated activation of EGFR and p38 MAPK pathways[15] that were observed to be MMP-9 dependent. At each time point 48 and 72 hours before ALF induction, saline or TIMP-1 plasmid was injected. We pooled the study animals from the two time points so that we had six normal control, i.e., the mice received ICV saline injection without ALF (Vehicle) that had the occludin/GAPDH level of 2.76 ± 0.85, and six ICV-saline injected ALF mice that had occludin level decreasing to 1.45 ± 0.33 ($P = 0.007$). The occludin level was restored to 2.53 ± 0.57 ($P = 0.002$) and 2.83 ± 0.80 ($P = 0.003$) in ALF mice that received ICV TIMP-1 cDNA at 48 and 72 hours ($n = 6$/group), respectively, Figures 4A and 4B.

From the same study mice above, we found that the ALF mice had an increased activation of p38 MAPK, i.e., p-p38 MAPK as compared with that in the brains of normal control, 1.12 ± 0.14 versus 0.62 ± 0.11, respectively ($P = 0.001$). However, the ALF mice that received TIMP-1 cDNA at 48 and 72 hours before ALF induction had significantly decreased level of activated p38 MAPK, 0.77 ± 0.16 and 0.89 ± 0.06, respectively ($P = 0.013$), Figures 4C and 4D.

To assess the activation of EGFR, we performed immunohistochemical staining for phosphorylated-Tyr EGFR as we previously reported[15] on the brain specimens of the study mice that received ICV saline without ALF (Figure 4E), ICV TIMP-1 cDNA without ALF (Figure 4F), ICV saline with ALF (Figure 4G), and ICV TIMP-1 cDNA with ALF (Figure 4H) at 48 hours. As compared with the controls in

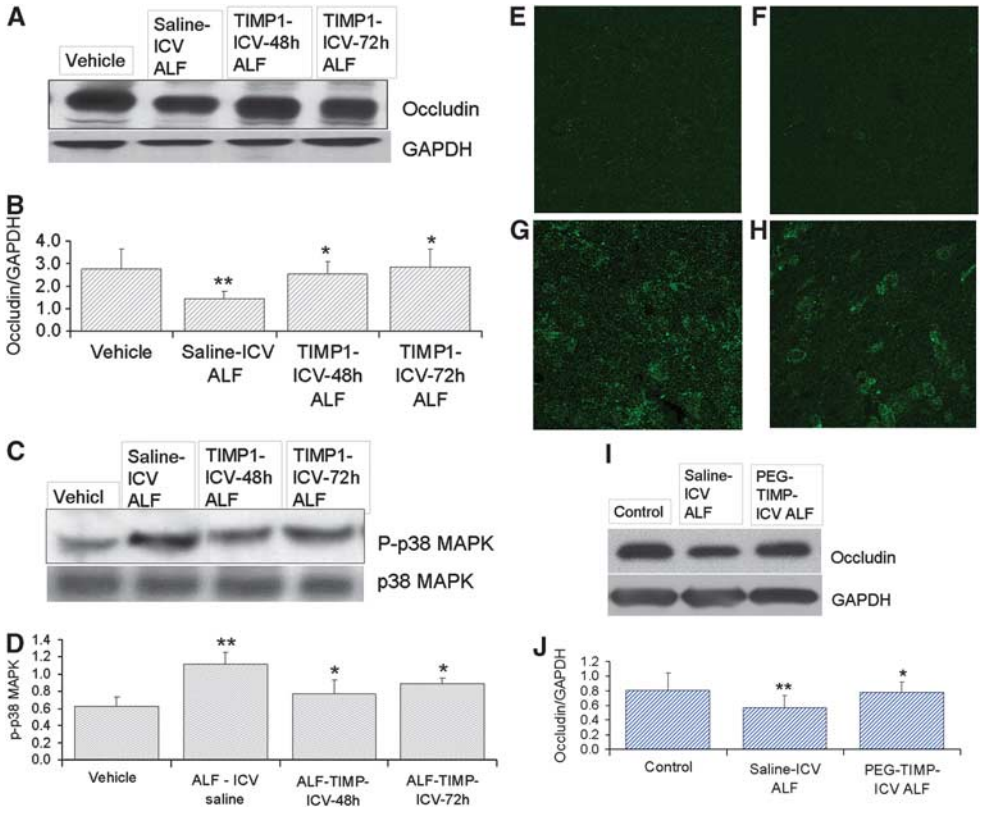

Figure 4. Upregulation of tissue inhibitors of MMP (TIMP)-1 reversed the loss of the tight junction protein occludin, the activation of p38 mitogen-activated protein kinase (MAPK) and epidermal growth factor receptor (EGFR) in brains of mice with acute liver failure (ALF). (**A**, **B**) Western blotting and histogram of occludin in brains of mice with intracerebroventricular (ICV) injection of saline (vehicle), of ALF mice receiving ICV injection of saline (Saline-ICV ALF), of ALF mice receiving ICV injection of TIMP-1 cDNA plasmids at 48 hours (TIMP1-ICV-48 hours ALF), and of ALF mice receiving ICV injection of TIMP-1 cDNA plasmids at 72 hours (TIMP1-ICV-72 hours ALF). The occludin level was significantly decreased in ALF mice as compared with the normal control (1.45 ± 0.33 versus 2.76 ± 0.85, $^{**}P = 0.007$). Treatment with TIMP-1 cDNA plasmids at 48 and 72 hours reversed the occludin to near normal level (2.53 ± 0.57 and 2.83 ± 0.80, $^{*}P = 0.003$, respectively) ($N = 6$ per study group). (**C**, **D**) Immunoblotting of activated p-p38 MAPK and p38 MAPK in brains of mice in the study groups as above: vehicle, saline-ICV ALF, TIMP1-ICV-48 hours ALF, and TIMP1-ICV-72 hours ALF. The p-p38 MAPK was significantly increased in ALF mice as compared with the normal control (1.12 ± 0.14 versus 0.62 ± 0.11, $^{**}P = 0.001$). Treatment with TIMP-1 cDNA plasmids at 48 and 72 hours significantly reduced the p-p38 MAPK as compared with the saline-ICV ALF (0.77 ± 0.16 and 0.89 ± 0.01, $^{*}P = 0.013$, respectively). (**E**–**H**) Immunohistochemical staining of phosphorylated Tyr-EGFR in brains of control mice with ICV injected saline (**E**), of control mice with TIMP-1 ICV injected with cDNA plasmids (**F**), of ALF mice that received ICV injected saline (**G**), and of ALF mice that received ICV injected TIMP-1 at 48 hours (**H**). There was no immunofluorescence in control mice and in mice with ICV TIMP-1 in (**E**) and (**F**). In contrast, ALF showed immunofluorescent staining for activated EGFR in (**G**), which was attenuated in ALF mice that were treated with TIMP-1 cDNA plasmids in (**H**). (**I**–**J**) Western blotting and histogram of occludin in brains of normal mice with ICV injection of saline (vehicle), of ALF mice receiving ICV injection of saline (Saline-ICV ALF), and of ALF mice receiving ICV injection of PEG-TIMP-1 (PEG-TIMP-ICV ALF). The occludin level was decreased in ALF mice receiving saline as compared with the normal control (0.57 ± 0.16 versus 0.80 ± 0.23, $^{**}P = 0.038$). Treatment with PEG-TIMP-1 reversed the occludin to near normal level in ALF mice (0.78 ± 0.13, $^{*}P = 0.015$) ($N = 6$ per study group).

Figures 4E and 4F, we observed an increase in phosphorylated-Tyr EGFR activation in the brains of ALF mice, Figure 4G, and the activation of EGFR was attenuated with the pretreatment of TIMP-1 cDNA plasmids, Figure 4H.

Each study group consisted of six mice; to have enough samples for western blot assay for phosphorylated-Tyr EGFR, we pooled every two of the six brain specimens into three samples for each group and performed immunoprecipitation to extract phosphorylated-Tyr EGFR. We immunoblotted for the level of phosphorylated-Tyr EGFR. We found phosphorylated-Tyr EGFR level was 0.79 ± 0.07, 2.21 ± 0.71, 1.06 ± 0.09, and 1.43 ± 0.31 in normal control mice, ALF mice that received ICV injected saline, ALF mice that received ICV injected TIMP-1 at 48 hours, and ALF mice that received ICV injected TIMP-1 at 72 hours, respectively. The activation of EGFR was significant above the normal control ($P = 0.013$), and the activation was significantly attenuated by TIMP-1 cDNA pretreatment at 48 hours ($P = 0.025$); although, the activation of EGFR was attenuated but not statistically significant by TIMP-1 treatment at 72 hours ($P = 0.080$).

Among the control and ALF mice that received the ICV administration of vehicle or PEG-TIMP-1 ($n = 6$/group), we only assessed the level of occludin in addition to the determination of BBB permeability above. We found that, in agreement with the study using TIMP-1 cDNA plasmids, ICV administration of PEG-TIMP-1 protein significantly reduced the loss of occludin in mice with ALF ($P = 0.015$), Figures 4I and 4J.

These results collectively suggest that the increase in TIMP-1 either via cDNA plasmids or PEG-TIMP-1 protein administration into the brain ventricle restores BBB integrity as it reverses the deregulation of tight junction occludin and mitigates the activation of the EGFR and p38 MAPK cascades, resulting in attenuation of BBB permeability in the brains of mice with ALF.

DISCUSSION

The concept that in ALF the brain is an innocent bystander relentlessly irritated by MMP-9 and other inflammatory factors from the brain capillary lumen remains controversial. Since MMP-9 has been implicated in the initiation phase of the BBB dysfunction in ALF, we sought to provide a proof of concept by selectively inducing an upregulation of TIMP-1 within CNS, thus avoiding the confounding effect of TIMP-1 on the liver injury process. In this study, we showed that an increase in the local defense by upregulating TIMP-1 within the CNS increases the expression of TIMP-1, which attenuates the BBB permeability, restores occludin in the tight junction, and reduces EGFR and p38 MAPK activation in mice with TNF/Gal-induced ALF.

Dysfunction of BBB in ALF is associated with a permeability that is selective only to small molecules. A derangement in tight junction integrity in the brain capillary resulting in increased paracellular permeability is likely the mechanism for the BBB dysfunction.[1,27] Occludin integrity is essential for normal barrier function.[28] Disturbance and interference in the occludin may result in a reversible disruption of the tight junction barrier resulting in an increase in barrier permeability. The loss of occludin integrity has been observed in the brains of mice with azoxymethane-induced ALF,[14] in rats with ALF that was surgically induced with portacaval shunt and hepatic artery ligation,[29] and in mice with ALF that was induced with TNF/Gal or LPS/Gal.[14,22] In this study, we found that increase in TIMP-1 within the CNS attenuates BBB permeability, and its associated loss of occludin is mediated at least partly via the EGFR and p38 MAPK pathways. These results together suggest that occludin deregulation is a major element of the BBB dysfunction in ALF.

When BBB dysfunction occurs in ALF, the resultant brain edema can be lethal. Brain edema tends to progress rapidly, preventing an opportunity for transplant candidacy evaluation and disallowing for enough time to wait for a donor liver. The window of time between a successful transplantation versus death without transplantation can be as short as 48 hours.[2] Therefore, the window of opportunity for a life-saving chance is very limited. Moreover, the BBB dysfunction is reversible once the diseased liver is replaced or regenerated. Thus, the therapy for BBB dysfunction needs to be effective, but it does not have to be long lasting or continued during the future lifetime of the patients. The severity of organ failure and the rapidity of the disease progression in ALF coupled with brain injury collectively make the experimental approaches to dissect the molecular mechanisms of brain injury in ALF difficult.

One of the main objectives in the investigations into the mechanisms of BBB permeability in ALF is not to interfere with the liver disease process to understand the byproduct impact of the liver failure on the brain and its BBB. Our earlier work suggests that MMP-9 and TIMP-1 have an important part in the pathogenesis of BBB dysfunction leading to brain edema in ALF. However, it remains controversial whether MMP-9 is important in BBB dysfunction in ALF at all. Since a systemic blockade of MMP-9 will interfere with the liver failure process, we apply the use of an intracerebral approach to illustrate a proof of principle on the role of MMP-9 and its potential impact on BBB permeability in ALF.[11]

At the present time, there is no effective inhibitor against MMP-9 that is clinically available.[16] TIMP-1 is a potent physiologic inhibitor for MMP-9, but it has a short half-life. To ensure large and sustained production of a desired protein, a viral carrier is usually used. However, the incorporation of viral genes increases the risk of viral immunogenicity and is not recommended in transplant and immunosuppressed recipients.[30] In addition, the occurrence of BBB dysfunction followed by brain edema will last only a few days since it will either progress to brain herniation and death, or resolve once the ALF is timely corrected by a new liver replacement. For these reasons, we use naked cDNA plasmids of TIMP-1, as this approach has no vector immunogenicity or risk of insertional mutagenesis.[25] The upregulation of TIMP-1 expression attenuates BBB permeability in mice with TNF/Gal-induced ALF. However, after TIMP-1 cDNA injection, it requires 2 or more days for TIMP-1 to be synthesized and produced. In real life, patients with ALF may not likely have time. Therefore, we explored the use of TIMP-1 protein itself in a manner similar to when recombinant enzyme is administered intracerebroventricularly as a treatment for stroke[31] and metabolic inborn disease.[32] Pegylated TIMP-1 has a prolonged half-life, and it ameliorates BBB permeability in mice with TNF/Gal-induced ALF. Pegylated TIMP-1, thus representing a potentially promising therapy to combat BBB dysfunction in ALF, and further investigations are warranted.

In this study, we found that when TIMP-1 is produced at an adequate level within CNS, it protects against BBB permeability, reverses the loss of occludin, and attenuates the activation of EGFR and p38 MAPK signaling in mice with ALF. These findings suggest that intracerebral TIMP-1 provides protection against exogenous and injurious factors bombarding the brain capillary endothelial cells, and its tight junction corroborates an important role of MMP-9 in the BBB dysfunction in ALF. We observed a generalized expression of TIMP-1. We suspected that TIMP-1 was produced by CNS cells particularly the capillary endothelial cells. TIMP-1 is thus likely to be present at the capillary cellular surface to directly counteract the incoming MMP-9 and its partners. Previous work suggested that intracerebral TIMP-1 may enter the blood circulation at a rate of 1% per minute,[33] suggesting that intracerebral TIMP-1 can be effective against extracranial MMP-9-mediated influence. However, further investigations are required to understand how TIMP-1 acts within the CNS and at the interface of BBB against the inflammatory factors within the capillary lumen.

In traumatic and ischemic brain injuries, MMP-9 is produced within CNS by its cellular components. Therefore, overexpressing TIMP-1 using a metallothionein-1 promoter provides protection in both traumatic and ischemic brain injuries.[11] The adenoviral-mediated gene transfer of TIMP-1 and -2 into striatum 3 days before global cerebral ischemia reduced neuronal damage.[34] TIMP-1 and -2 also protects against BBB disruption in focal ischemic injury.[35] By contrast, in ALF, little MMP-9 is found in the brain.[5] Since MMP-9 is a large molecule, 85 to 95 kD, it is unable to penetrate BBB in ALF. MMP-9 is tightly controlled by TIMP-1 at 1:1 molar ratio. In ALF, we previously reported that TIMP-1 in the serum is markedly reduced when we measured with gelatin zymography for MMP-9 activity and reverse gelatin zymography for TIMP-1 activity against MMPs.[12,24] Therefore, the MMP-9 to TIMP-1 balance is altered to a relative increase in MMP-9. How MMP-9 acts on the BBB in ALF remains largely unknown. However, the constant bombarding on the BBB by the inflammatory factors including TNF, interleukin 1, and MMP-9 is detrimental, as they exert their actions on various receptors on the endothelial cell cellular surface, activating or deactivating various signaling cascades in the brain capillary. Moreover, their impact on BBB is likely transmitted deep into CNS. For example, we have learned that MMP-9 may directly act on the tight junction components and/or it can activate EGFR with subsequent activation of p38 MAPK, IKKβ, NFκB leading to a deregulation of occludin resulting in an increased paracellular permeability.[15] Therefore, intracerebral upregulation of TIMP-1 may reverse the injurious process from downstream upward. In this study, we found that when TIMP-1 was upregulated within CNS, the activation of EGFR, p38 MAPK, and its cascade was attenuated. These results support the influence of MMP-9 on the endothelial cells and its reverberating impact on the signaling at BBB regulating its paracellular permeability.

The findings in this study represent a novel affirmation on the role of vascular etiology in the pathogenesis of BBB dysfunction in ALF.[5,13] The BBB vascular dysfunction has been recently recognized to precede various neurologic diseases, including

seizures[36] and Alzheimer's disease.[37] The BBB dysfunction in ALF may represent an opposite manifestation of BBB dysfunction in a manner that resembles the BBB dysfunction found in epilepsy.[36] That is, excitatory toxins that permeate through BBB will induce neuronal epileptogenesis. On the opposite spectrum, hepatic encephalopathy and astrocytic swelling and edema might be a result of inhibitory toxins such as ammonia and gamma-aminobutyric acid that penetrate the BBB. Therefore, it is essential to understand the vascular contribution to BBB dysfunction to achieve a successful prevention and control of the development of brain dysfunctions in ALF.

There are limitations in the current study. First, although TIMP-1 has a strong affinity to MMP-9, we did not examine the non-MMP effects of TIMP-1 since TIMP-1 is known to promote cellular proliferation, growth, and tumor formation.[30] In addition, the roles of other TIMPs and MMPs were not investigated; although, we did not observe an increase in MMP-2, 3, and 8 in the brains of ALF mice. Second, we cannot entirely rule out the effects of inflammatory cytokines including TNF, interleukin 1, and tissue plasminogen activator that may penetrate into CNS in ALF since these factors can induce astrocytic release of MMP-9.[38,39] Third, the lack of a significant reduction of the activated EGFR in the brains of ALF mice that received TIMP-1 cDNA plasmids at 72 hours will need further examination since it suggests an involvement of an MMP-independent path. Fourth, since neuroinflammation is an important element in ALF, we assessed the neuroinflammatory condition in the brains of ALF mice using the western blot marker for microglial activation with OX-6.[40] We noted a trend of a small increase in OX-6 levels in brains of mice that received ICV injection of the cDNA control and TIMP-1 cDNA plasmids. However, there was no statistical significance. These results appear to suggest that effect of TIMP-1 on BBB permeability may be independent of neuroinflammation. However, the exact role of neuroinflammation and its interaction with TIMP-1 in ALF require further investigation.

Overall, the results from this study provide the proof of principle that MMP-9 contributes to the mechanisms that are important in BBB dysfunction in ALF. To our knowledge, these data are the first in the field of ALF to identify the potential use of the physiologic inhibitor TIMP-1 for local and regional protection against a continuing systemic attack by MMP-9 and its partners on the integrity of the brain capillary. The results represent potential therapeutic tool and target for effective control of BBB dysfunction in ALF. Moreover, this approach may be useful for other conditions wherein brain injury is a byproduct of peripheral organ dysfunctions including pancreatic encephalopathy[41] and neonatal encephalopathy.[42]

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Ms Kelly Viola and Ms Victoria L Jackson, MLIS, of the Mayo Clinic for their editorial assistance and Dr Wenlang Lin of the Department of Neuroscience at the Mayo Clinic for his assistance on the immunohistochemical localization and staining.

REFERENCES

1 Nguyen JH. Blood-brain barrier in acute liver failure. *Neurochem Int* 2012; **60**: 676–683.
2 Lee WM, Squires Jr. RH, Nyberg SL, Doo E, Hoofnagle JH. Acute liver failure: Summary of a workshop. *Hepatology* 2008; **47**: 1401–1415.
3 Zlokovic BV. Neurovascular mechanisms of Alzheimer's neurodegeneration. *Trends Neurosci* 2005; **28**: 202–208.
4 Rosenberg GA. Neurological diseases in relation to the blood-brain barrier. *J Cereb Blood Flow Metab* 2012; **32**: 1139–1151.
5 Nguyen JH, Yamamoto S, Steers J, Sevlever D, Lin W, Shimojima N *et al.* Matrix metalloproteinase-9 contributes to brain extravasation and edema in fulminant hepatic failure mice. *J Hepatol* 2006; **44**: 1105–1114.
6 Jalan R, Pollok A, Shah SH, Madhavan K, Simpson KJ. Liver derived pro-inflammatory cytokines may be important in producing intracranial hypertension in acute liver failure. *J Hepatol* 2002; **37**: 536–538.
7 Lidofsky SD, Bass NM, Prager MC, Washington DE, Read AE, Wright TL *et al.* Intracranial pressure monitoring and liver transplantation for fulminant hepatic failure. *Hepatology* 1992; **16**: 1–7.
8 Wielockx B, Lannoy K, Shapiro SD, Itoh T, Itohara S, Vandekerckhove J *et al.* Inhibition of matrix metalloproteinases blocks lethal hepatitis and apoptosis induced by tumor necrosis factor and allows safe antitumor therapy. *Nat Med* 2001; **7**: 1202–1208.
9 Barr TL, Latour LL, Lee KY, Schaewe TJ, Luby M, Chang GS *et al.* Blood-brain barrier disruption in humans is independently associated with increased matrix metalloproteinase-9. *Stroke* 2010; **41**: e123–e128.
10 Rosenberg GA, Estrada EY, Dencoff JE. Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. *Stroke* 1998; **29**: 2189–2195.
11 Tejima E, Guo S, Murata Y, Arai K, Lok J, van Leyen K *et al.* Neuroprotective effects of overexpressing tissue inhibitor of metalloproteinase TIMP-1. *J Neurotrauma* 2009; **26**: 1935–1941.
12 Yamamoto S, Nguyen JH. TIMP-1/MMP-9 imbalance in brain edema in rats with fulminant hepatic failure. *J Surg Res* 2006; **134**: 307–314.
13 Cauli O, Lopez-Larrubia P, Rodrigo R, Agusti A, Boix J, Nieto-Charques L *et al.* Brain region-selective mechanisms contribute to the progression of cerebral alterations in acute liver failure in rats. *Gastroenterology* 2011; **140**: 638–645.
14 Chen F, Ohashi N, Li W, Eckman C, Nguyen JH. Disruptions of occludin and claudin-5 in brain endothelial cells in vitro and in brains of mice with acute liver failure. *Hepatology* 2009; **50**: 1914–1923.
15 Chen F, Hori T, Ohashi N, Baine AM, Eckman CB, Nguyen JH. Occludin is regulated by epidermal growth factor receptor activation in brain endothelial cells and brains of mice with acute liver failure. *Hepatology* 2011; **53**: 1294–1305.
16 Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002; **295**: 2387–2392.
17 Duarte S, Hamada T, Kuriyama N, Busuttil RW, Coito AJ. TIMP-1 deficiency leads to lethal partial hepatic ischemia and reperfusion injury. *Hepatology* 2012; **56**: 1074–1085.
18 Shimojima N, Eckman CB, McKinney M, Sevlever D, Yamamoto S, Lin W *et al.* Altered expression of zonula occludens-2 precedes increased blood-brain barrier permeability in a murine model of fulminant hepatic failure. *J Invest Surg* 2008; **21**: 101–108.
19 Chakrabarty P, Jansen-West K, Beccard A, Ceballos-Diaz C, Levites Y, Verbeeck C *et al.* Massive gliosis induced by interleukin-6 suppresses Abeta deposition in vivo: evidence against inflammation as a driving force for amyloid deposition. *FASEB J* 2010; **24**: 548–559.
20 Hori T, Chen F, Baine A-MT, Gardner LB, Nguyen J. Fulminant liver failure model with hepatic encephalopathy in the mouse. *Ann Gastroenterol* 2011; **24**: 294–306.
21 Vaquero J, Belanger M, Blei AT, Butterworth RF. Lack of assessment of body temperature in mice with acetaminophen toxicity. *Hepatology* 2006; **44**: 279–280.
22 Lv S, Song HL, Zhou Y, Li LX, Cui W, Wang W *et al.* Tumour necrosis factor-alpha affects blood-brain barrier permeability and tight junction-associated occludin in acute liver failure. *Liver Int* 2010; **30**: 1198–1210.
23 Nagaki M, Iwai H, Naiki T, Ohnishi H, Muto Y, Moriwaki H. High levels of serum interleukin-10 and tumor necrosis factor-alpha are associated with fatality in fulminant hepatitis. *J Infect Dis* 2000; **182**: 1103–1108.
24 Nguyen JH. Reply to: Palenzuela *et al.* Matrix metalloproteinase-9 in fulminant hepatic failure. *Hepatology* 2010; **51**: 1475–1476, author reply 6.
25 Richard M, Arfi A, Seguin J, Gandolphe C, Scherman D. Widespread biochemical correction of murine mucopolysaccharidosis type VII pathology by liver hydrodynamic plasmid delivery. *Gene Ther* 2009; **16**: 746–756.
26 Batra J, Robinson J, Mehner C, Hockla A, Miller E, Radisky DC *et al.* PEGylation extends circulation half-life while preserving in vitro and in vivo activity of tissue inhibitor of metalloproteinases-1 (TIMP-1). *PLoS ONE* 2012; **7**: e50028.
27 Sandoval KE, Witt KA. Blood-brain barrier tight junction permeability and ischemic stroke. *Neurobiol Dis* 2008; **32**: 200–219.
28 Bamforth SD, Kniesel U, Wolburg H, Engelhardt B, Risau W. A dominant mutant of occludin disrupts tight junction structure and function. *J Cell Sci* 1999; **112**: 1879–1888.
29 Sawara K, Desjardins P, Chatauret N, Kato A, Suzuki K, Butterworth RF. Alterations in expression of genes coding for proteins of the neurovascular unit in ischemic liver failure. *Neurochem Int* 2009; **55**: 119–123.
30 Stetler-Stevenson WG. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. *Sci Signal* 2008; **1**: re6.

31 Guerra-Crespo M, Gleason D, Sistos A, Toosky T, Solaroglu I, Zhang JH *et al.* Transforming growth factor-alpha induces neurogenesis and behavioral improvement in a chronic stroke model. *Neuroscience* 2009; **160**: 470–483.
32 Chang M, Cooper JD, Sleat DE, Cheng SH, Dodge JC, Passini MA *et al.* Intraventricular enzyme replacement improves disease phenotypes in a mouse model of late infantile neuronal ceroid lipofuscinosis. *Mol Ther* 2008; **16**: 649–656.
33 Maness LM, Kastin AJ, Banks WA. Relative contributions of a CVO and the microvascular bed to delivery of blood-borne IL-1alpha to the brain. *Am J Physiol* 1998; **275**: E207–E212.
34 Magnoni S, Baker A, Thomson S, Jordan G, George SJ, McColl BW *et al.* Neuroprotective effect of adenoviral-mediated gene transfer of TIMP-1 and -2 in ischemic brain injury. *Gene Ther* 2007; **14**: 621–625.
35 Fujimoto M, Takagi Y, Aoki T, Hayase M, Marumo T, Gomi M *et al.* Tissue inhibitor of metalloproteinases protect blood-brain barrier disruption in focal cerebral ischemia. *J Cereb Blood Flow Metab* 2008; **28**: 1674–1685.
36 Marchi N, Angelov L, Masaryk T, Fazio V, Granata T, Hernandez N *et al.* Seizure-promoting effect of blood-brain barrier disruption. *Epilepsia* 2007; **48**: 732–742.
37 Bell RD, Winkler EA, Singh I, Sagare AP, Deane R, Wu Z *et al.* Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. *Nature* 2012; **485**: 512–516.
38 Lee SR, Guo SZ, Scannevin RH, Magliaro BC, Rhodes KJ, Wang X *et al.* Induction of matrix metalloproteinase, cytokines and chemokines in rat cortical astrocytes exposed to plasminogen activators. *Neurosci Lett* 2007; **417**: 1–5.
39 Tejima E, Zhao BQ, Tsuji K, Rosell A, van Leyen K, Gonzalez RG *et al.* Astrocytic induction of matrix metalloproteinase-9 and edema in brain hemorrhage. *J Cereb Blood Flow Metab* 2007; **27**: 460–468.
40 Jiang W, Desjardins P, Butterworth RF. Cerebral inflammation contributes to encephalopathy and brain edema in acute liver failure: protective effect of minocycline. *J Neurochem* 2009; **109**: 485–493.
41 Farkas G, Marton J, Nagy Z, Mandi Y, Takacs T, Deli MA *et al.* Experimental acute pancreatitis results in increased blood-brain barrier permeability in the rat: a potential role for tumor necrosis factor and interleukin 6. *Neurosci Lett* 1998; **242**: 147–150.
42 Bednarek N, Svedin P, Garnotel R, Favrais G, Loron G, Schwendiman L *et al.* Increased MMP-9 and TIMP-1 in mouse neonatal brain and plasma and in human neonatal plasma after hypoxia-ischemia: a potential marker of neonatal encephalopathy. *Pediatr Res* 2012; **71**: 63–70.