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TIMP-3 and MMP-3 contribute to delayed inflammation and hippocampal neuronal death following global ischemia

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Abstract

Hippocampal neuronal death following transient global ischemia in the mouse takes days to occur, providing a potential timeframe for therapeutic intervention. Since matrix metalloproteinase-3 (MMP-3) enhances inflammation and tissue inhibitor of metalloproteinases-3 (TIMP-3) promotes apoptosis in ischemia, we hypothesized that they are involved in neuronal death secondary to transient global ischemia. *Timp-3* knockout (T3KO) and wild type (T3WT) mice underwent 30 min bilateral carotid artery occlusion (BCAO), which causes hippocampal neuronal death 7 days after reperfusion. Mice lacking the *Timp-3* gene have significantly less astrogliosis, microglial reactivity, MMP-3 activity and neuronal cell death. In addition, T3KO mice had decreased tumor necrosis factor (TNF) receptor-1 (TNFR1) expression and increased TNF- α converting enzyme (TACE) activity. *Mmp-3* KO mice with a similar BCAO showed significantly fewer microglial cells, reduced TNF- α expression, and less neuronal death than the *Mmp-3* WT. To see if TIMP-3 and MMP-3 cell death pathways were independent, we blocked MMPs with the broad-spectrum MMP inhibitor, BB-94, on days 3 through 6 of reperfusion in T3WT and T3KO mice. BB-94 rescued hippocampal neurons at 7 days in both T3WT and T3KO mice, but significantly fewer neurons died in T3KO mice treated with BB-94. Our results indicate a novel additive role for TIMP-3 and MMP-3 in delayed neuronal death, and show that MMP inhibitors can be used to reduce delayed hippocampal death.

Keywords

MMP inhibition; BB94; neuroinflammation; tumor necrosis factor receptor; TNF- α ; neuronal death

Introduction

Global ischemia reduces blood flow to the brain, causing hypoxic hypoperfusion injury to vulnerable, poorly perfused areas as would occur with cardiac arrest or hypotension during a surgical procedure (Back, et al., 2004, Harukuni and Bhardwaj, 2006, Lo, et al., 2003). During

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global ischemia neurons in the CA1 and CA2 regions of the hippocampus, which are selectively vulnerable, undergo apoptosis after 3 to 7 days (Chen, et al., 1998, Magnoni, et al., 2004, Oguro, et al., 2001). Various acute mechanisms have been proposed for this type of injury, including intracellular mitochondrial dysfunction and DNA degradation (Deierborg, et al., 2008, Kumaran, et al., 2008). The proteases, extracellular tissue plasminogen activator (tPA) and matrix metalloproteinases (MMPs), also play an important role (Lee, et al., 2007, Lee, et al., 2004, Zalewska, et al., 2002). There is evidence that MMP-9 causes early cell death in hippocampal neurons after global ischemia (Lee, et al., 2004). However, the mechanisms involved in the delayed cell death seen after 3 days (Oguro, et al., 2001) remain to be established.

MMPs are involved in neuroinflammation (Rosenberg, 2002), extracellular matrix (ECM) degradation (Gu, et al., 2002), cleavage and activation of other MMPs (Rosenberg, et al., 2001), as well as shedding of death receptors (Wetzel, et al., 2003). They are rapidly upregulated after cerebral ischemia in rats, mice, nonhuman primates, and humans. Research in our lab has previously shown that stromelysin-1 (MMP-3) is involved in blood brain barrier (BBB) breakdown (Gurney, et al., 2006) with expression preceding inflammatory mediator tumor necrosis factor- α (TNF- α) expression in an LPS model of inflammation (Mun-Bryce, et al., 2002). MMP-3 has also been implicated in microglial activation and inflammation in a Parkinson's disease model (Kim, et al., 2005).

Tissue inhibitor of metalloproteinases-3 (TIMP-3) has many roles in ischemic cell death through inhibition of membrane-type 1-MMP (MT1-MMP), MMP-3, and TNF- α converting enzyme (TACE) (Wei, et al., 2005, Wetzel, et al., 2003, Will, et al., 1996). MMP-3 and TACE act as sheddases on the cell surface, releasing death receptors and ligands in the TNF superfamily. By inhibiting sheddase activity, TIMP-3 allows stabilization of death receptors and subsequent apoptosis in a variety of models, including focal ischemia (Wallace, et al., 2002), doxorubicin-induced injury *in vitro* (Wetzel, et al., 2003), and more recently in oxygen-glucose deprivation (OGD) models in cell culture and mild focal ischemia in the *Timp-3* KO (T3KO) mouse (Wetzel, et al., 2008). Tumor necrosis factor α receptor-1 (TNFR1) stabilization on the cell surface is regulated by TACE. TACE activity leads to TNFR1 cleavage and reduced apoptosis (Smith, et al., 1997). TIMP-3 regulates the activity of TACE (Amour, et al., 1998) and can lead to reduced TNFR1 cleavage.

As MMP-3 and TIMP-3 both appear to play a role in cell death and inflammation, we hypothesized that both were involved in delayed hippocampal neuronal death following global ischemia. We demonstrate a delayed inflammatory response and regional selective neuronal death following global ischemia in the mouse. Utilizing both *Mmp-3* KO (M3KO) and T3KO mice, we investigated cell-specific inflammation, death receptor expression, enzyme activation, and neuronal loss in the hippocampus. Using a combination of genetic and pharmacological interventions, we inhibited both TIMP-3 and MMP-3 by genetic knockout and delayed drug treatment in the same animal and were able to prevent hippocampal neuronal death.

Materials and Methods

Transient Global Cerebral Ischemia

All experiments were approved by the University of New Mexico (UNM) Animal Care Committee and conformed to the National Institutes of Health Guide for the Care and Use of Animals in research. Transient bilateral common carotid artery occlusion (BCAO) was conducted on the C57BL/6 mouse, T3KO, M3KO and corresponding wild-types from colonies maintained in the Animal Research Facility at UNM. The T3KO mice were backcrossed seven times or more then bred on a C57BL/6 background and were a generous gift from R. Khokha.

The M3KO mice were backcrossed three times then bred on a B10RIII.H2 background from Taconic with the kind permission of J. Mudgett. The animals were anaesthetized in an incubation chamber via 2% isoflurane by inhalation and injected with 0.1 mg/kg of analgesic (buprenorphine) IP with animal temperature constantly maintained. The skull was exposed and cerebral blood flow measured with a Laser Doppler blood flow monitor (Moore Instruments Limited, Devon, UK) 3 mm caudal and 3 mm lateral to Bregma. Both common carotid arteries were exposed, isolated, and clipped with metal aneurysm clips for 12, 18, or 30 minutes to determine optimal occlusion time. Animals were allowed to recover in their cage for 1–2 hours with a heating lamp. Following reperfusion, the animals were anaesthetized with 2% isoflurane by inhalation and injected IP with 30 mg/kg of sodium pentobarbital at 3, 7, or 30 days. Perfusion with PBS/0.1% Procaine followed by 2% PLP (2% paraformaldehyde, 0.1 M sodium periodate, 0.075 M lysine in 100 mM phosphate buffer at pH 7.4) was conducted intracardially. Brains were removed and immersed in 2% PLP overnight. The brains were cryoprotected with 30% sucrose in PBS, and frozen in OCT. They were stored at -80°C until sectioned. For activity assays and immunoblotting, brains were removed and quickly frozen in 2-methylbutane.

Immunohistochemistry (IHC)

For fluorescence IHC, 10 μm sections were rehydrated through alcohols and PBS containing 0.1% Tween-20 (PBT). Nonspecific binding sites were blocked with PBT containing 1% bovine serum albumin (BSA), and 5% normal serum. Slides were incubated overnight at 4°C with primary antibodies. Slides were washed in PBT and incubated for 90 min at room temperature with secondary antibodies conjugated with FITC and Cy-3. Slides were incubated with 4'-6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Inc., Carlsbad, CA) prior to coverslipping to label cell nuclei. All IHC slides were viewed on an Olympus BX-51 bright field and fluorescence microscope (Olympus America Inc., Center Valley, PA) equipped with an Optronics digital camera and Picture Frame image capture software (Optronics, Goleta, CA). Antibodies used for IHC included TIMP-3 (1:1000; Chemicon, Temecula, CA), glial fibrillary acidic protein (GFAP) for astrocytes (1:400; Sigma-Aldrich, St. Louis, MO), Iba-1 for microglia/macrophages (1:400; Wako Pure Chemical Industries, Richmond, VA), neuron specific nuclear protein (NeuN) (1:400 mouse; Chemicon), p55TNF Receptor 1 (1:1000 Abcam, Cambridge, MA), MMP-3 (1:200 R&D Systems Inc., Minneapolis, MN), TNF- α (10 $\mu\text{g}/\text{ml}$; R&D Systems). Fluoro-Jade C staining (HistoChem Inc., Jefferson, AZ) of 10 μm sections was performed per company protocol.

Fluorescence Intensity Analysis

To compare various reperfusion time points and regions of interest, slides stained with IHC were analyzed for fluorescence staining intensity. All slides to be compared were stained together. Images were taken at the same exposure time for each section and changed to grayscale. Using NIH Image J software, the region of interest was outlined and the total mean fluorescence intensity of each region was recorded and compared to control staining and other reperfusion time points. Values are reported as fluorescence intensity.

Hippocampal Neuronal Stereology

To assess neuronal injury following transient global ischemia, 20 μm sections taken every 100 μm were rehydrated through alcohols, rinsed in PBS and water, and stained with cresyl violet acetate (CVA). Slides were rinsed in tap water, dehydrated through alcohols and xylene, then coverslipped using DPX (Sigma-Aldrich). Stereology was performed using the optical fractionator function of StereoInvestigator software (Version 6, MicroBrightField Inc., Williston, VT) controlling a motorized stage equipped Olympus BX-51 microscope. Viable neurons in the CA2 region of the hippocampus over a total distance of 3.0 mm were counted as this was the area determined most sensitive to injury. The region of interest was delineated

and overlaid with a grid size of $120 \times 60 \mu\text{m}$ and counting frame set at $35 \times 35 \mu\text{m}$ for stereological quantification of cells. Histological sections were blinded to the investigator as to animal identity and reperfusion time.

Immunoblotting for TNFR1

Hippocampus regions of freshly isolated non-fixed brain homogenate were collected by punch biopsy at -20°C and analyzed for protein expression of TNFR1 by Western blot. $50 \mu\text{g}$ of total protein was separated on 10% acrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes, blocked, and incubated overnight at 4°C with primary antibodies against TNFR1 (1:1400 Abcam). Membranes were incubated with HRP conjugated secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and blots developed using chemiluminescent detection with the West Pico Kit (Pierce Biotechnology, Inc., Rockford, IL). Protein bands were visualized on X-ray film and densitometric analysis was used for semiquantitation of band intensities with AlphaEase image software (Alpha Innotech Corp., San Leandro, CA). Actin staining (1:7500 Sigma-Aldrich) on the same PVDF membranes was used to normalize protein loading and transfer. Results are reported as normalized band intensity for quantifying relative protein expression.

TACE Activity Assay

TNF- α converting enzyme (TACE) activity was measured using a fluorimetric assay with a SensoLyte™ 520 TACE (α -Secretase) Activity Assay Kit (AnaSpec Inc., San Jose, CA) according to the operation manual. The SensoLyte™ 520 TACE Activity Assay Kit used a 5-carboxyfluorescein (5-FAM) (fluorophore) and QXL™ 520 (quencher) labeled fluorescence resonance energy transfer (FRET) peptide substrate for continuous measurement of enzyme activity. In the intact FRET peptide, the fluorescence of 5-FAM was quenched by QXL™ 520. Upon cleavage of the FRET peptide by the active enzyme, the fluorescence of 5-FAM was recovered, and continuously monitored at excitation/emission = $490 \text{ nm}/520 \text{ nm}$. Fresh hippocampus tissue punches were homogenized in lysis buffer containing 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM CaCl_2 , 0.05% Brij-35, 0.02% NaN_3 , and 1% Triton X-100, and centrifuged at $12,000 \times g$ for 10 min at 4°C . Equal amount of total protein ($100 \mu\text{g}$) was mixed with assay buffer and TACE substrate to a final volume of $100 \mu\text{l}$. The change in fluorescence was monitored for 60 min at room temperature with a luminescence spectrometer (model LS55; PerkinElmer Instruments, Buckinghamshire, UK). FL WinLab software was used to express data as relative fluorescence units.

MMP-3 Activity Assay

MMP-3 enzymatic activity was measured with a fluorimetric assay using a 5-FAM/QXL™ 520 FRET peptide (Anaspec). In the intact FRET peptide, the fluorescence of 5-FAM was quenched by QXL™ 520. Upon cleavage into two separate fragments by the MMP-3 present in the sample, the fluorescence of 5-FAM was recovered and monitored at excitation/emission = $490 \text{ nm}/520 \text{ nm}$ for 60 min at room temperature using a luminescence spectrometer (PerkinElmer LS55). Homogenized hippocampus tissue samples with $100 \mu\text{g}$ protein (the same ones used for the TACE activity assay above) were mixed with assay buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM CaCl_2 , $20 \mu\text{M}$ ZnSO_4 , and 0.05% Brij-35) and fluorogenic substrate ($1 \mu\text{M}$ final concentration) to a final volume of $200 \mu\text{l}$. FL WinLab software was used to express data as relative fluorescence units.

Statistical Analysis

Statistical comparisons among groups were done using ANOVA with post-hoc analysis for multiple t -tests (Prism 4.0, GraphPad Software Incorporated). All data were presented as mean $\pm$ standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

Results

Delayed hippocampal neuronal injury

Global ischemia was induced in the C57BL/6 mouse by 12, 18, and 30 min occlusion with 3, 7, and 30 days of reperfusion. Blood flow was monitored before, during, and following carotid occlusion with $11.2 \pm 1.1\%$ of original flow during the occlusion period, and flow returning to $44.9 \pm 6.3\%$ following clip removal. Neuronal loss after different occlusion times was compared at 7 days (Fig. 1A). CVA and Fluoro-Jade C staining of the hippocampus at various time points showed that damage was predominantly in the hippocampal CA2 region 7 days after BCAA (Fig. 1B). The 7 day time point with 30 min occlusion was selected for further investigation.

TIMP-3 involved in hippocampal neuronal death

Previous studies have shown TIMP-3 to be involved in neuronal death in the cortex following focal ischemia (Wallace, et al., 2002), and with increased expression in the astrocytes (Liu, et al., 2007). To determine the cellular source of TIMP-3 in our model, we used immunohistochemistry (IHC) and found co-localization of TIMP-3 with the astrocyte marker, GFAP at 7 days reperfusion (Fig. 2A). Quantification of TIMP-3 fluorescence intensity in the hippocampus showed a significant rise in TIMP-3 at 7 days of reperfusion as compared to sham values (Fig. 2B).

To determine the effect of TIMP-3 on cellular injury, we used T3KO and T3WT mice to compare neuronal survival following global ischemia. Stereological analysis of viable hippocampal neurons of the CA2 region showed no cell death at 3 days reperfusion in either genotype. Following 7 days reperfusion, both genotypes showed significant neuronal loss ($p < 0.001$) as compared to sham. More importantly, there was attenuated cell death in the T3KO mice as compared to the T3WT ($p < 0.01$) (Fig. 3).

Inflammation and death receptor stabilization in the T3WT hippocampus

To better understand the role of TIMP-3 in neuronal death, we analyzed hippocampal tissue in the T3KO and T3WT mice for signs of inflammation and death receptor expression. Astrocytes were stained with GFAP at various time points in both the T3KO and T3WT mice and analyzed for fluorescence intensity. There was an increase in astrogliosis in the T3WT as compared to the T3KO at 3 days ($p < 0.01$) and 7 days ($p < 0.001$) of reperfusion (Fig. 4A,B). There was also a significant increase in the Iba-1 microglial staining in the T3WT mice at 3 days ($p < 0.05$) and 7 days ($p < 0.001$) reperfusion as compared to the T3KO (Fig. 4C,D).

We then investigated the expression of TNFR1 in the T3WT and T3KO mice to determine the involvement of this death receptor. With TNFR1 expression on the cell surface, TNF- α can bind and lead to apoptotic cell death. Following 3 days reperfusion, there was co-localization of TNFR1 with NeuN-positive neurons (Fig. 5A), with the T3WT mice having significantly higher TNFR1 protein expression ($p < 0.05$) as compared to the T3KO (Fig. 5B). This correlated at the same time point with decreased TNF- α converting enzyme (TACE) activity in the T3WT as compared to the T3KO (Fig. 5C).

MMP-3 involved in inflammation and neuronal loss

With neuronal loss occurring in both the T3WT and T3KO mice at 7 days reperfusion, we hypothesized an additional mechanism contributed to the observed hippocampal injury. Using IHC, we found increased MMP-3 staining in both the T3WT and T3KO mice at 3 days, co-localized with Iba-1 positive microglia (Fig. 6A). In addition, we performed a fluorometric enzymatic activity assay and found significantly increased MMP-3 activity at 3 days in both

T3WT and T3KO mice ($p<0.01$ and $p<0.001$) with T3WT mice having more MMP-3 activity than the T3KO mice at all time points (Fig. 6B).

To determine the role of MMP-3 in the delayed death of neurons in the hippocampus, we then compared global ischemic injury in the M3KO and M3WT mice. Neuronal stereology of the CA2 region of the hippocampus showed significant protection in the M3KO mice at 7 days reperfusion ($p<0.001$), implicating a role for MMP-3 in neuronal loss (Fig. 7A). In addition, there was a decrease in the microglial staining intensity (Fig. 7C) and TNF- α expression in the M3KO as compared to the M3WT (Fig. 7B).

Pharmaceutical MMP inhibition in T3WT and KO mice

To determine if this delayed neuronal loss could be reduced through pharmacological intervention, we tested the effect of administering a broad-spectrum MMP inhibitor, BB-94, as a selective MMP-3 inhibitor is not available. Since we were interested in the delayed neuronal death with inflammation, we administered the drug 3 through 6 days after reperfusion. Staining the T3WT and T3KO mice tissue with CVA, we performed neuronal stereology at 7 days of reperfusion and saw a dramatic increase in cellular survival in both genotypes ($p<0.001$) (Fig. 8), further implicating a delayed MMP contribution to hippocampal neuronal death. T3KO mice treated with BB94 had neuronal counts equivalent to those seen in the sham mice.

Discussion

Our data show that TIMP-3 and MMP-3 contribute independently to delayed hippocampal neuronal loss following transient global ischemia with reperfusion in the mouse. Neuronal cell death was reduced in both the T3KO and M3KO mice, suggesting that the presence of TIMP-3 and MMP-3 promote cell death at 7 days. With TIMP-3 affecting TACE activity and TNFR1 expression, and MMP-3 affecting inflammation and TNF- α expression in microglia, the regulation of this death receptor pathway by both TIMP-3 and MMP-3 may be an important mechanism in delayed apoptotic cell death in global ischemia. An inhibitor to MMPs might be anticipated to mimic a TIMP-3 pro-apoptotic effect. However, we found the opposite; BB-94, a broad-spectrum MMP inhibitor, had an additive effect, increasing protection of neurons in the T3KO mice that already had partial protection. With neuronal rescue occurring through TIMP-3 knockout in addition to MMP inhibition, these two pathways appear to be independent and additive mechanisms of delayed neuronal death.

With increased neuronal survival and decreased inflammation in the T3KO mice, we demonstrate TIMP-3 involvement in delayed death. This finding supports earlier studies, implicating TIMP-3 in cell death in the rat with focal ischemia induced by middle cerebral artery occlusion and in neuronal cell cultures (Wallace, et al., 2002, Wetzel, et al., 2003). TIMP-3 inhibits the sheddase activity of TACE (Amour, et al., 1998, Drynda, et al., 2005). By a mechanism distinct from MMP inhibition (Wei, et al., 2005), TIMP-3 blocks removal of TNFR1 from the cell surface by TACE, leading to cell death in human colon carcinoma cells (Smith, et al., 1997). Our data agree with these findings showing increased TACE and decreased TNFR1 expression in the neurons of the T3KO mice. The loss of TNFR1 has been shown to decrease inflammation using a TNFR1 knockout mouse (Smookler, et al., 2006) and increase immune hyperreactivity in a TNFR1 knock-in mouse (Xanthoulea, et al., 2004). Our results support the role of TIMP-3 inhibiting TACE and stabilizing TNFR1 in the ischemic mouse hippocampus. In addition, our data further implicate a role for TIMP-3 in the increased reactivity of astrocytes and microglia, exacerbating the delayed inflammatory response.

In an earlier study with doxorubicin treated neuronal cultures, TIMP-3 facilitated cell death through inhibition of MMP-3, which blocked the cleavage of Fas ligand from the cell surface (Wetzel, et al., 2003). TIMP-3 also facilitates neuronal death via death receptor stabilization

in ischemia (Wetzel, et al., 2008), arthritic fibroblasts (Drynda, et al., 2005) and in a transgenic model of amyotrophic lateral sclerosis (Lee, et al., 2008). With an inhibitory domain distinct for TACE as compared to other MMPs (Wei, et al., 2005), TIMP-3 can stabilize death receptors such as TNFR1 on the cell membrane, leading to apoptotic cell death.

The interaction of TIMPs with MMPs can influence various pathways that regulate cell death and survival (Crocker, et al., 2004, Cunningham, et al., 2005). TIMP-3 inhibits several MMPs in addition to TACE. *In vitro* studies demonstrate TIMP-3 can promote cell death through inhibiting the protective effect of MMP-3 cleavage of the death receptor ligand, Fas-L (Wetzel, et al., 2003) and through stabilization of the TNFR1 receptor (Smookler, et al., 2006). Increased TNFR1 stabilization may be the mechanism whereby TIMP-3 promotes neuronal death as MMP-3 appears to be detrimental in our *in vivo* BCAA model.

Recent evidence has implicated MMP-3 as a key modulator of inflammation and cell death in various disease models. Intracellular expression of MMP-3 triggers a neuroinflammatory cascade in a Parkinson's model with microglial induction of TNF- α and other cytokines (Choi, et al., 2008, Kim, et al., 2005). MMP-3 upregulation precedes the onset of disease in a demyelinating transgenic mouse (D'Souza, et al., 2002) and LPS-induced inflammation leads to increased IL1 β stimulation and astrocyte expression of MMP-3 (Crocker, et al., 2006). Previous findings from our lab support a role for MMP-3 in the inflammatory cascade and BBB opening following LPS-induced injury (Gurney, et al., 2006) with MMP-3 upregulation preceding TNF- α expression in reactive microglia (Mun-Bryce, et al., 2002). We therefore hypothesized a role for MMP-3 expression in microglial inflammation and ischemic injury to the mouse neurons following global ischemia. Our data demonstrate an increase in the activity of MMP-3 parallel with increased microglial reactivity, TNF- α expression, and eventual neuronal death in the M3WT compared to M3KO mice. In addition, the T3WT mice had significantly higher MMP-3 activity levels than T3KO mice, suggesting the absence of TIMP-3 may lead to a compensatory increase in MMP-3.

MMP inhibitors have been considered for use in therapeutic applications. Testing these agents in rodent models has shown some promise in reducing injury following ischemia. However, determining the optimal time to administer these drugs is difficult as early inhibition (0–3 days) has shown protection (Cho, et al., 2006, Lee, et al., 2004), but later inhibition in the rat following focal ischemia (7–14 days) reduced recovery through inhibition of neurovascular remodeling (Zhao, et al., 2006). We are unable to determine from our data whether the MMPs in this model are involved in repair processes at the 7 day time point. As inhibition of MMPs has led to reduced inflammation and decreased death, it seems the MMPs are playing a detrimental role in this delayed death. Finding the appropriate time for MMP inhibition is essential to inhibit cell death. The broad spectrum MMP inhibitor, BB-94, showed neuroprotection in the mouse hippocampus when given from 0–3 days following global ischemia (Lee, et al., 2004), so we chose to investigate the delayed effect of MMPs and administered BB-94 from 3–6 days of reperfusion. Our results show significant attenuation of neuronal loss in the hippocampus, demonstrating that delayed MMP inhibition can still protect neurons and extend the therapeutic window for treatment of injury.

Inhibiting MMPs in the T3KO mouse allowed us to alter two potential mechanisms of injury in the same animal. With cellular survival increasing additively in the T3KO mice with BB-94, it appears that MMPs and TIMP-3 are independently involved in delayed neuronal loss (Fig. 9). The effect of BB-94 protection may also be due to other MMPs, with BB-94 showing acute neuronal protection through MMP-9 inhibition in both focal ischemia (Asahi, et al., 2000) and global ischemia from 0–3 days reperfusion (Lee, et al., 2004). MMP-9 did not appear to be involved in our observed delayed death as we found no difference in MMP-9 expression between experimental and controls at 7 days when neuronal loss was observed (data not

shown). Our data has implicated MMP-3 in the observed delayed inflammation and neuronal death, demonstrating that multiple MMPs appear to be involved in neuronal injury.

In conclusion, using a combination of genetic and pharmacological approaches, we provide evidence for the novel role of both TIMP-3 and MMP-3 in delayed hippocampal injury following global ischemia. With decreased inflammation in both the T3KO and M3KO mice, we demonstrate neuronal protection. Further investigation uncovered a role of the TNF death receptor pathway, with both TIMP-3 and MMP-3 contributing independently. Delayed inhibition of MMPs also shows neuroprotection and supports the use of MMP inhibitors for clinical treatment of delayed injury following hypoxic hypoperfusion from cardiac arrest or hypotension following surgery.

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Figure 1. Delayed hippocampal injury. A) Cresyl violet staining of hippocampal neurons at various occlusion times with 7 days reperfusion. Compared to sham, 12 and 18 min occlusion had minor injury, with 30 min showing significant hippocampal damage. B) Reperfusion time points showed minimal CA2 injury at 3 days, with marked neuronal loss by 7 days. Fluorojade staining (right column inset) labeled degenerating neurons at 7 days reperfusion. Scale Bars: all 50 μ m, except B) left column: 300 μ m.

Figure 2. TIMP-3 delayed expression in astrocytes. A) GFAP-positive astrocytes (FITC, green) do not express TIMP-3 (Cy3, red) in the hippocampus of Sham animals (top row), but show co-localization at 7 days reperfusion (bottom row). DAPI (blue) stained nuclei become disrupted at 7 days. Scale Bar: 50µm. B) TIMP-3 quantification of fluorescence intensity by immunohistochemistry using Image J software shows significant increase of TIMP-3 expression at 7 days reperfusion (**p<0.01)(n=8 for Sham, 7d, 30d; n=10 for 3d).

Figure 3.

Stereology of hippocampal neurons in T3WT and T3KO mice. Cresyl violet stained viable neurons of the CA2 region of the hippocampus were counted with stereology. 3 days reperfusion showed no significant cell death or difference between T3WT and T3KO. 7 days reperfusion showed significant death in both groups as compared to sham (***p<0.001), with T3KO mice having significantly less neuronal loss than the T3WT (**p<0.01) (n=12 for WT Sham, n=10 for KO Sham, n=10 for both 3d, n=8 for WT 7d, n=9 for KO 7d).

Figure 4.

Inflammation of astrocytes and microglia in T3WT mice. A) GFAP-positive astrocytes (green) at 7 days reperfusion. B) Quantification of fluorescence intensity demonstrates T3WT mice had more GFAP reactivity than Sham values at 7 days ($***p<0.001$) and compared to T3KO mice at 3 days ($**p<0.01$) and 7 days reperfusion ($***p<0.001$) ($n=8$ for WT Sham and WT 30d; $n=9$ for KO 3d, KO 7d, and KO 30d; $n=10$ for KO Sham, WT 3d, and WT 7d). C) Iba-1 positive microglia/macrophages at 7 days reperfusion. D) Quantification of fluorescence intensity demonstrates T3WT mice had more Iba-1 reactivity than Sham values at 3 days ($**p<0.01$) and 7 days reperfusion ($***p<0.001$) and compared to the T3KO mice at 3 days ($*p<0.05$) and 7 days reperfusion ($***p<0.001$) ($n=8$ /group). Scale Bars: 50 μ m.

Figure 5.

TNFR1 expression and TACE activity. A) NeuN-positive neurons (green) co-localize with TNFR1 expression (red) more in the T3WT than T3KO in the hippocampus at 3 days reperfusion. DAPI counterstain for nuclei. Scale Bar: 50 μ m. B) Western blot quantification of TNFR1 protein at 55kDa confirms increased expression at 3 days reperfusion in the T3WT vs. T3KO (* p <0.05) (n =6/group). Protein loading normalized with actin. C) TACE activity measured with a fluorometric assay shows increased activity in the T3KO hippocampus at 3 days reperfusion (* p <0.05) (n =7 for Shams, n =9 for 3d, n =5 for 7d).

Figure 6.

MMP-3 activity in microglia. A) Iba-1 positive microglia/macrophages (green) co-localize with MMP-3 (red) in both T3WT and T3KO hippocampi at 3 days reperfusion. DAPI counterstain for nuclei. Scale Bar: 50 μm. B) MMP-3 activity assessed with a fluorometric assay shows increased activity in the T3WT hippocampus, with both T3WT and T3KO having increased activation at 3 days reperfusion (** $p < 0.01$, *** $p < 0.001$). T3WT mice had constitutively greater MMP-3 activity than T3KO mice (### $p < 0.001$) ($n = 10$ /group for WT, $n = 8$ for KO Sham, $n = 10$ for KO 3d and KO 7d).

Figure 7.

M3KO vs. M3WT inflammation and cell loss at 7 days. A) Cresyl violet stained neurons were counted by stereology with the M3KO having attenuated cell loss in the CA2 region of the hippocampus (** $p < 0.001$) ($n = 4$ for Shams, $n = 8$ for 7d). B) Iba-1 positive microglia/macrophages (green) co-localize with TNF- α (red) in the M3WT hippocampus. DAPI counterstain for nuclei. Scale Bars: 50 μ m. C) Increased Iba-1 microglial fluorescence intensity in the M3WT vs. M3KO (* $p < 0.05$) ($n = 4$ for Shams, $n = 8$ for WT 7d, $n = 7$ for KO 7d).

Figure 8.

Delayed BB-94 inhibition of MMPs 3–6 days after reperfusion significantly rescued neuronal loss in the hippocampus of both T3WT and T3KO compared to the original cell death observed in Figure 3, with T3KO plus BB-94 neuronal counts returning to sham levels (**p<0.001) (n=8 for T3WT, n=11 for T3WT+BB94, n=9 for T3KO, n=10 for T3KO+BB94).

Figure 9. Proposed mechanism of MMP-3 and TIMP-3 induced neuronal death. Inflammation is induced by two pathways in this model: MMP-3 leads to reactive microglia releasing TNF- α , while TIMP-3 inhibits TNF-R removal by TACE. TNF- α can then bind to TNF-R on the cell surface leading to neuronal cell death.