ELSEVIER

Neurobiology of Disease

www.elsevier.com/locate/ynbdi
Neurobiology of Disease 30 (2008) 174–185

# Tissue inhibitor of metalloproteinases-3 (TIMP-3) expression is increased during serum deprivation-induced neuronal apoptosis *in vitro* and in the G93A mouse model of amyotrophic lateral sclerosis: A potential modulator of Fas-mediated apoptosis

Jae Keun Lee,[b,c] Jin Hee Shin,[b,c] JaeHong Suh,[b,c] In Sun Choi,[b,c] Keun Sil Ryu,[a] and Byoung Joo Gwag[a,b,c,d,*]

[a]*Departments of Pharmacology, Ajou University School of Medicine, Suwon, South Korea*
[b]*Research Institute for Neural Science and Technology, Ajou University School of Medicine, Suwon, South Korea*
[c]*Interdisciplinary Course for Neuroscience and Technology, Ajou University School of Medicine, Suwon, South Korea*
[d]*Neurotech Pharmaceuticals Company, Ajou University School of Medicine, Suwon, South Korea*

Received 22 July 2007; revised 1 December 2007; accepted 8 January 2008
Available online 4 February 2008

**Cortical neurons deprived of serum undergo apoptosis that is sensitive to inhibitors of macromolecule synthesis. Proteomic analysis revealed differential expression of 49 proteins in cortical neurons 8 h after serum deprivation. Tissue inhibitor of metalloproteinases-3 (TIMP-3), a pro-apoptotic protein in various cancer cells, was increased during serum deprivation-induced apoptosis (SDIA), but not during necrosis induced by excitotoxicity or oxidative stress. Levels of TIMP-3 were markedly increased in degenerating motor neurons in a transgenic model of familial amyotrophic lateral sclerosis. The TIMP-3 expression was accompanied by increase in Fas–FADD interaction, activated caspase-8, and caspase-3 during SDIA and in vulnerable spinal cord of the ALS mouse. SDIA and activation of the Fas pathway were prevented by addition of an active MMP-3. *Timp-3* deletion by RNA interference attenuated SDIA in N2a cells. These findings provide evidence that TIMP-3 is an upstream mediator of neuronal apoptosis and likely contributes to neuronal loss in neurodegenerative diseases such as amyotrophic lateral sclerosis.**


*Keywords:* Neuronal apoptosis; Proteomics; TIMP-3; MMP-3; Fas; Amyotrophic lateral sclerosis

## Introduction

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that mediate degradation of the proteinaceous components of extracellular matrix (Sternlicht and Werb, 2001; Visse and Nagase, 2003). The MMPs play dynamic roles in developmental morphogenesis and in wound healing and repair during progression of tissue injury and pathologic diseases such as arthritis, cancer, and diabetes (Woessner, 1998; Visse and Nagase, 2003; Nagase et al., 2006). The activity of MMPs is regulated by four tissue inhibitors of matrix metalloproteinases (TIMPs), endogenous inhibitors of MMPs (Docherty et al., 1985; Boone et al., 1990; Apte et al., 1994; Greene et al., 1996).

Evidence has accumulated showing a potential role of TIMPs in neuronal and non-neuronal degeneration. Levels of TIMP-1 expression were found to be increased in the hippocampal formation after transient forebrain ischemia or seizure and in the retinal ganglion cell layer after elevation of intraocular pressure (Rivera et al., 1997, 2002; Jourquin et al., 2005). Manipulations increasing TIMP-1 were shown to protect neurons in dissociated and organotypic hippocampal cultures from excitotoxicity but not from apoptosis induced by withdrawal of nerve growth factor or chemical-induced ischemia (Tan et al., 2003). Developmental regulation of TIMP-2 was demonstrated in neural progenitor and neuroblastoma cell lines treated with neurotrophic factors or retinoic acid (Jaworski and Perez-Martinez, 2006). TIMP-2 promoted differentiation and neurite outgrowth in PC12 cells and cortical neurons (Pérez-Martínez and Jaworski, 2005). TIMP-3 was increased in degenerating cortical neurons following focal cerebral ischemia (Wallace et al., 2002) and modulated neuronal death induced by the chemotherapeutic drug doxorubicin (Wetzel et al., 2003). Less is known about the role of TIMP-4 in the brain.

We have performed proteomic analysis of cultured cortical neurons undergoing apoptosis after serum deprivation and identified

* Corresponding author. Department of Pharmacology, Ajou University School of Medicine, Suwon 442-749, South Korea. Fax: +82 31 215 9986.
*E-mail address:* bjgwag@ajou.ac.kr (B.J. Gwag).
**Available online on ScienceDirect (www.sciencedirect.com).**

0969-9961/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2008.01.004

TIMP-3 as a potential mediator of apoptosis. Interestingly, expression of TIMP-3 was increased in the vulnerable spinal motor neurons in the transgenic mouse model of amyotrophic lateral sclerosis (ALS). The present study was performed to delineate the putative role of TIMP-3 in neuronal apoptosis after serum deprivation and in the ALS mice.

## Materials and methods

### *Materials*

*N*-methyl-D-aspartic acid (NMDA) and MK-801 were purchased from RBI (Natick, MA), Trolox was purchased from Aldrich (Milwaukee, WI), active catalytic domain of MMP-3 was purchased from Calbiochem (San Diego, CA), and recombinant TIMP-3 was purchased from R&D Systems (Minneapolis, MA). All other reagents were purchased from Sigma (St. Louis, MO), unless otherwise indicated.

### *Mice*

G93A transgenic mice carrying the G93A human SOD1 mutation were obtained from the Jackson Laboratory (Bar Harbor, ME). Male G93A transgenic mice were crossbred with B6SJLF1/J hybrid females, as previously described (Gurney, 1994). Nontransgenic litter mates were used as controls for biochemical or histological experiments.

### *Neurotoxicity in mouse cortical cell cultures*

Mixed cortical cell cultures containing neurons and glia were prepared as previously described (Ryu et al., 1999). For neuron-rich cortical cell cultures, 2.5 μM cytosine arabinoside was added to cultures at 3 days *in vitro* (DIV 3) to halt the growth of non-neuronal cells. Excitotoxicity or oxidative stress was induced by addition of 30 μM NMDA or 30 μM $FeCl_2$, respectively, to mixed cortical cell cultures (DIV 12–14). Neuronal death was determined 24 h later by measuring LDH release into the bathing media; levels were scaled to the mean LDH value after 24-h exposure to 500 μM NMDA (100%) or sham control (0%). Neuronal apoptosis was induced by serum deprivation of neuron-rich cortical cell cultures and analyzed 24 h later by counting viable neurons excluding trypan blue.

### *Cytotoxicity in Neuro2A cell cultures*

The mouse neuroblastoma Neuro2A (N2a) cell line was a generous gift from Dr. Shin, Sungkyunkwan University. Cells were seeded in 60-mm dishes (TPP, Switzerland) at a density of $1 \times 10^6$ cells/dish and grown in Dulbecco's modified Eagle's medium (JBI, Seoul, Korea) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% fetal bovine serum. Cells were maintained in a humidified atmosphere containing 5% $CO_2$ at 37 °C. For induction of apoptosis by serum deprivation, the medium containing Dulbecco's modified Eagle's medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% fetal bovine serum was change to serum-deprived medium, and cells were incubated for 36 h. Serum-deprived cell death was determined using trypan blue staining. The mode of cell death was further assessed with TUNEL staining and an *in situ* cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany), as described previously (Jover et al., 2002). In brief, N2a cultures grown on ACLAR film (Ted Pella Inc.) were washed 3 times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min at 37 °C. In each following step, samples were washed 3 times with PBS. Fixed cultures were permeablized by 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 2–8 °C. N2a cultures were then incubated with TUNEL reaction mixture for 1 h at 37 °C in the dark.

### *Two-dimensional gel electrophoresis and image analysis*

Cultures were replaced with 500 ml lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2 mM tributylphosphosine, 40 mM Tris, and 100 mM DTT. Cells were harvested, incubated for 1 h, and briefly homogenized in a 1 ml syringe. Samples were added with 0.5 ml endonuclease, incubated for 1 h, and centrifuged at 4 °C for 15 min. Supernatants were collected, and protein levels analyzed using a Bradford assay solution. Samples were stored at −80 °C. Two-dimensional gel electrophoresis (2-DE) of each sample was performed using the immobiline/polyacrylamide system. Approximately 200–300 μg total protein was used. Isoelectric focusing was performed on immobiline strips providing a nonlinear pH 3–10 gradient (IPG strip, GE Healthcare). The second dimension was performed on 7–17% gradient polyacrylamide gels and stained with silver or Coomassie blue. Stained gel images were digitized using a UMAX scanner, and gel matching was performed with Proteomweaver 2 software (Definies, Germany). Protein identification by MALDI-TOF mass fingerprinting was performed by the Yonsei Proteome Research Center.

Qualitative analyses were performed with Proteomweaver 2 image analysis software on average gels from each control or injury group. The relative abundance of proteins in primary neuronal cultures from at least 3 cases in each control or injury group was estimated as the integrated density of the protein spot, determined using Proteomweaver 2. In order to rule out individual variation, spots, whose intensity was always higher or lower in all subjects from one group compared to subjects from the other group, were taken into account. Changes 2-fold or greater in magnitude were considered important.

### *Western blotting and immunoprecipitation*

Cultured cells, lumbar spinal cords, and human brains were lysed in a lysis buffer (RIPA buffer) containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), and 1× protease inhibitors cocktail (Calbiochem, 0.5 mM EDTA, 500 μM AEBSF, 150 nM aprotinin, 1 μM E-64, and 1 μM leupeptin). Protein samples were electrophoresed on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was preincubated with 5% nonfat dry milk, reacted with primary antibodies, and incubated with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Cell Signaling, Beverly, MA). Target proteins were detected with enhanced chemiluminescence reagents (GE Healthcare) on X-ray film or with an LAS 1000 image analyzer (Fuji Photo Film Co., Ltd.). The intensity of the bands was quantified using Image Gauge 3.12 (Fuji Photo Film Co., Ltd.). The primary antibodies were cleaved caspase-3, caspase-8 (1 μg/ml, Cell Signaling, Beverly, MA), TIMP-3 (2 μg/ml, R&D systems, Minneapolis, MN), MMP-3 (1 μg/ml, Oncogene Research Products, San Diego, CA), Fas, and FADD (250 μg/ml, BD Bioscience, Franklin Lakes, NJ).

For immunoprecipitation, protein samples were incubated overnight at 4 °C with 1 μg anti-Fas antibody or anti-TIMP-3 antibody,