Matrix Metalloproteinase-3 Is Increased and Participates in Neuronal Apoptotic Signaling Downstream of Caspase-12 during Endoplasmic Reticulum Stress*

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Although endoplasmic reticulum (ER) stress-induced apoptosis has been associated with pathogenesis of neurodegenerative diseases, the cellular components involved have not been well delineated. The present study shows that matrix metalloproteinase (MMP)-3 plays a role in the ER stress-induced apoptosis. ER stress induced by brefeldin A (BFA) or tunicamycin (TM) increases gene expression of MMP-3, selectively among various MMP subtypes, and the active form of MMP-3 (actMMP-3) in the brain-derived CATH.a cells. Pharmacological inhibition of enzyme activity, small interference RNA-mediated gene knockdown, and gene knock-out of MMP-3 all provide protection against ER stress. MMP-3 acts downstream of caspase-12, because both pharmacological inhibition and gene knockdown of caspase-12 attenuate the actMMP-3 increase, but inhibition and knock-out of MMP-3 do not alter caspase-12. Furthermore, independently of the increase in the protein level, the catalytic activity of MMP-3 enzyme can be increased via lowering of its endogenous inhibitor protein TIMP-1. Caspase-12 causes liberation of MMP-3 enzyme activity by degrading TIMP-1 that is already bound to actMMP-3. TIMP-1 is decreased in response to ER stress, and TIMP-1 overexpression leads to cell protection and a decrease in MMP-3 activity. Taken together, actMMP-3 protein level and catalytic activity are increased following caspase-12 activation during ER stress, and this in turn plays a role in the downstream apoptotic signaling in neuronal cells. MMP-3 and TIMP-1 may therefore serve as cellular targets for therapy against neurodegenerative diseases.

The endoplasmic reticulum (ER) is the organelle responsible for proper synthesis and folding of proteins as well as maintenance of intracellular calcium homeostasis. Various cellular stresses cause disruption of normal ER functions, leading to ER stress, and excessive and prolonged ER stress leads to accumulation of misfolded and/or unfolded proteins, and ultimately apoptosis (1).

Neurons are particularly vulnerable to ER stress, and ample evidence exists in the literature that links ER stress with neurodegeneration (2–4). Therefore, identification of cellular components and elucidation of the sequence of events following ER stress in neuronal cells would be of great importance in understanding the mechanism of neurodegeneration. The molecular connection between the ER stress response and apoptotic signaling, however, is not clearly understood. Although caspase-12, residing outside of ER membrane, has been shown to be specifically involved in the apoptotic signaling that results from ER stress (5, 6), the exact mechanism by which this occurs has not been elucidated and immediate downstream targets of caspase-12 remain to be identified.

Matrix metalloproteinase (MMP)-3 belongs to a family of MMP enzymes known to participate in degradation of components of the extracellular matrix. MMP-3 has been associated with pathogenesis of a number of diseases such as Alzheimer disease, Parkinson disease, stroke, brain trauma, neuroinflammation, multiple sclerosis, glia, and arthritis (7–10). Although the enzyme was previously thought to be active only outside the cell, we have recently reported its novel, additional role inside the cell. That is, the catalytically active, cleaved form of MMP-3 (actMMP-3) is produced intracellularly in neurons that are under oxidative stress and plays a role in apoptotic signaling (11).

The findings that MMP-3 activity participates in apoptotic signaling led us to investigate its possible involvement in the ER stress–induced cell demise. Specifically, we asked whether MMP-3 expression and activity might be altered during ER stress and whether MMP-3 might participate in the subsequent cell death signaling, and if so, to elucidate the mechanism by which this occurs in relation to caspase-12.
EXPERIMENTAL PROCEDURES

**Materials**—Fetal bovine serum, horse serum, RPMI 1640, Dulbecco's modified Eagles medium, l-glutamine, trypsin/EDTA, penicillin-streptomycin, neurobasal medium, and B-27 were from Invitrogen (Gaithersburg, MD). Poly-l-lysine, p-aminophenylmercuric acetate, tunicamycin (TM), and brefeldin A (BFA) were purchased from Sigma. N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid (NNGH) and an MMP-3 fluorescence assay kit were purchased from Biomatol (Plymouth Meeting, PA). An in situ cell death detection kit for TUNEL staining was from Roche Diagnostics GmbH (Penzberg, Germany). Z-ATAD-FMK was purchased from BioVision (Mountain View, CA). Primary antibodies used were goat polyclonal anti-mouse MMP-3 antibody (R&D Systems Inc., Minneapolis, MN), goat polyclonal anti-human MMP-3 antibody (BioVision), goat polyclonal anti-mouse TIMP-1 antibody (R&D Systems Inc.), mouse monoclonal anti-human TIMP-1 antibody (Calbiochem), rabbit polyclonal anti-mouse caspase-12 antibody (Cell Signaling Technology Inc., Beverly, MA), and rabbit anti-mouse cleaved caspase-3 (Asp-175) antibody (Cell Signaling Technology Inc.). Anti-goat IgG and anti-rabbit IgG were from Sigma, and Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 488 goat anti-rabbit IgG, and Alexa Fluor 546 goat anti-rabbit IgG were from Molecular Probes (Eugene, OR). An Enhanced Chemiluminescence kit was from Pierce. [3H]Butyric acid was from Amersham Biosciences, and Tag polymerase was from Roche Applied Science. Laminin, TRlZol reagent, supercript II reverse transcriptase, and Lipofectamine™ 2000 were purchased from Invitrogen. Recombinant human MMP-3 and TIMP-1 were from R&D Systems Inc. and Calbiochem, respectively. A nickel-nitritriacetic acid column and a Hi-TrapQ column were from Qiagen (Chatsworth, CA) and Amersham Biosciences, respectively. Protein G PLUS-Agarose was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other chemicals were reagent grade and were purchased from Sigma or Merck (Rahway, NJ).

**CATH.a Cell Culture**—Cells were grown in RPMI 1640 containing 8% horse serum, 10% fetal bovine serum, 100 IU/liter penicillin, and 10 μg/ml streptomycin at 37 °C in 95% air and 5% CO₂ in humidified atmosphere. For experiments, the cells were plated on polystyrene tissue culture dishes at a density of 3 × 10⁵ cells/well in 24-well culture plates or 5.5 × 10⁵ cells/well in 6-well culture plates. After 24 h, the cells were fed with fresh medium and treated.

**Animals**—MMP-3 knock-out (KO) mice (C57BL/6 × 129SvEv), originally developed by Mudgett et al. (12), and their wild-type (WT) were obtained from Taconic Farms (Germantown, NY) and bred at the specific pathogen-free animal facility at the University of Ulsan Asan Institute for Life Science. To obtain mouse embryos for primary culture experiments, adult female mice (5–7 weeks old, both MMP-3 KO and WT) were superovulated by injecting 5 IU of pregnant mare serum gonadotropin intraperitoneally, followed by administration of 5 IU of human chorionic gonadotropin 48 h later. The animals were mated with fertile male mice. The presence of vaginal plug was determined on the following day, which was considered embryonic day zero.

Primary Culture of Cortical Neurons of MMP-3 KO and WT Mice—All procedures were preapproved by the Animal Experiment Review Committee of the Asan Institute for Life Science and performed in compliance with the guidelines set forth by the Society for Neuroscience. The cortex was removed from a 13-day gestation embryo and incubated with 0.01% trypsin in Hanks' balanced salt solution for 15 min at 37 °C. After trituration, 3 × 10⁵ cells were plated on each polystyrene coverslip that had been precoated with 100 μg/ml poly-l-lysine and 4 μg/ml laminin and placed in a 24-well culture plate. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ in neurobasal medium supplemented with B-27, 2 mM glutamine, 100 IU/liter penicillin, and 10 μg/ml streptomycin. On days 5 or 6 in vitro, the neurons were fed with fresh medium and treated.

Construction of DNA—For construction of the full-length caspase-12 cDNA, PCR was performed on a pCAGGS-mCASP12 plasmid DNA (kindly provided by Martine Vanhoucke, Universiteit Gent, Belgium) using the following primers: sense, TACGGATCCGGCCAGGAGGACCACAT; and antisense, GCCCTCAGATATCCTCCGGGAAAAAGGT. For construction of full-length TIMP-1 cDNA, PCR was performed on pGEM-hTIMP-1 plasmid DNA, which was kindly donated by Dylan Edwards of the University of East Anglia, using the following primers: sense, CCGGAATTCCGGGAGAGGACACATG-GCCCCCTTTTGGCCCTGGC; and antisense, CCGCTCGACGGGCTACGTATCTGGGACCCCGG. The PCR products of caspase-12 and TIMP-1 were cloned into the pcDNA3.1 myc-His vector and pcDNA 3.0 vector, respectively.

Purification of Recombinant Caspase-12—Caspase-12 was overexpressed in Escherichia coli BL21(DE3)pLysS in the presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside at 30 °C for 3 h. The protein was purified by passing through a nickel-nitritriacetic acid column and a Hi-TrapQ column, as described before (13). Approximately 1 mg of protein was obtained from a 1-liter culture.

Transfection—CATH.a cells cultured on 6-well culture plates were subjected to transfection by the addition of 10 μl of Lipofectamine™ 2000 and 4 μg of DNA. After 6 h of incubation, the culture medium was changed, and the cells were maintained for additional 18 h before analysis.

LDH Assay—Degrees of cell death were assessed by activity of lactate dehydrogenase (LDH) released from the culture medium using the method previously described (14). Aliquots (50 μl) of cell culture medium were incubated at room temperature in the presence of 0.26 mM NADH, 2.87 mM sodium pyruvate, and 100 mM potassium phosphate buffer (pH 7.4) in a total volume of 200 μl. The rate of NAD⁺ formation was monitored for 5 min at 2-s intervals at 340 nm using a microplate spectrophotometer ( Molecular Devices, Menlo Park, CA).

Immunostaining—CATH.a cells grown on a coverslip were treated with BFA after which they were fixed in cold 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS), pH 7.4, for 30 min at room temperature. After washing twice in PBS, the cells were incubated for 1 h in blocking solution (0.1% PBS containing 5% fetal bovine serum and 0.3% Triton X-100). The cells were then incubated overnight with appropriate primary antibody diluted in incubation solution (0.1% PBS con-
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taining 5% fetal bovine serum and 0.2% Triton X-100) at 4 °C and washed twice in PBS. The samples were incubated at room temperature for 1 h with appropriate fluorescence-labeled secondary antibody diluted in the incubation solution. For cleaved caspase-3, rabbit polyclonal anti-cleaved caspase-3 antibody (1:100) and Alexa Fluor 488 goat anti-rabbit IgG (1:200) were used. For caspase-12, rabbit polyclonal anti-caspase-12 antibody (1:150) and Alexa Fluor 546 goat anti-rabbit IgG (1:200) were used. The samples were also incubated with respective secondary antibodies alone to confirm specificity. The cells were washed in PBS, mounted on a glass slide, and counted under a confocal microscope (TCS-ST2, Leica, Wetzlar, Germany).

**TUNEL Staining**—TUNEL staining was performed to assess apoptotic cells according to the manufacturer’s protocol. The cells labeled with tetramethylrhodamine red were counted under a confocal microscope.

**Western Blot Analysis**—Western blot analysis was performed as described previously (15). Cells were washed with ice-cold PBS and lysed on ice in lysis buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 100 μM molybdc acid, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml apro- tinin, and 1 mM phenylmethylsulfonyl fluoride). The soluble fraction was obtained, and an equal amount of protein (30 μg) was subjected to transfection by adding 5 and 1 μl (4 μM final concentration) of the substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2). The plates were read continuously in a confocal microscope (TCS-ST2, Leica, Wetzlar, Germany).

**MMP-3 Activity Assay**—MMP enzyme activity was measured by using an MMP-3 fluorescence assay kit following the manufacturer’s instructions. Oligonucleotide primers were designed based on GenBank™ entries for mouse MMP-1 (sense, 5’-CTGAGACCTATGAAGAGCCAGC-3’; antisense, 5’-TCTGTGATTG-GGATGGGATTG-3’), MMP-3 (sense, 5’-GATCTTCTC-ATTGGCCATCTCTTC-3’; antisense, 5’-CCTCAGTATT-TGTCCTCATAAAAGA-3’), MMP-7 (sense, 5’-GTTGGAG-GAGCGGAGGTGAA-3’; antisense, 5’-ACAGGTTCAG-CCTAGGAAAG-3’), MMP-9 (sense, 5’-CCTTACCAGGCAG-CAGCAGGC-3’; antisense, 5’-GAAAGGGCTTGTCGCCAGAAGG-3’), and β-2-microglobulin (sense, 5’-GGGAAGCCCGA-ACATCTGAA-3’; antisense, 5’-CGGCCCATCTGTCTAG-CTTA-3’). PCR mixes contained 10 μl of 2×PCR buffer, 1.25 mM of each dNTP, 100 pmol of each forward and reverse primer, and 2.5 units of Taq polymerase in the final volume of 20 μl. Amplification was performed in 38 cycles for 1 min at 95 °C, 1 min at 58.5 °C, and 1 min at 72 °C. After the last cycle, all samples were incubated for an additional 7 min at 72 °C. PCR fragments were analyzed on 1.5% agarose gel in 1×TAE (40 mM Tris acetate, pH 8.3, and 2 mM Na2 EDTA) containing ethidium bromide. Amplification of β-2-microglobulin, a relatively invariant internal reference RNA, was performed in parallel, and cDNA amounts were normalized against β-2M mRNA levels. Each primer set specifically recognized only the gene of interest as indicated by amplification of a single band of expected size.

**γ-Aminobutyric Acid Uptake Assay**—Primary cultured cortical neurons were rinsed with Hanks’ balanced salt solution containing 10 μM aminooxyacetic acid, 2 mM β-alanine, and 1.3 mM EDTA at room temperature. The neurons were further incubated in the presence of 50 nM [3H]butyric acid for 20 min. The cells were then washed with Hanks’ balanced salt solution and lysed with 0.1 N NaOH for 1 h. Radioactivity was measured using a liquid scintillation counter (Beckman, Fullerton, CA).

**Immunoprecipitation**—Protein G PLUS-Agarose beads were washed three times with 0.1 M Tris-HCl (pH 8.5), and then incubated with 1 μg of TIMP-1 antibody for 2 h at 4 °C with rotation. The beads/antibody complex was sequentially washed with 0.1 N Tris-HCl (pH 8.5), 50 mM Tris-HCl (pH 7.5)/150 mM NaCl, and finally RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 0.5% Triton X-100). Cell lysate (100 μg) of untreated CATH.a cells was incubated with or without caspase-12 and then with the beads/antibody complex for at least 2 h at 4 °C. The sample was then washed twice with RIPA buffer and twice with PBS. The pellet was boiled for 7 min, and the proteins in the supernatant were resolved by SDS-PAGE.

**Preparation of siRNA and Transfection**—Sense and antisense oligonucleotides corresponding to the following cDNA sequences of mouse MMP-3 and caspase-12 were used: AAU-UCCAACUGCAAGAAGCCAGUG (MMP-3 #1), AAUCC-AUCUCAAUCAUCUGAAGAG (MMP-3 #2), AGCGAGGC-GCUAUCCCUUUGUUUGUG (caspase-12 #1), and AAGUG-AAGGGAACAGCGUCUGCC (caspase-12 #2). The sense and antisense oligonucleotides were annealed following the manufacturer’s protocol (Invitrogen) to generate double-stranded siRNAs at the final concentration of 20 μM. CATH.a cells grown to 80% confluency in 6- or 24-well culture plates were subjected to transfection by adding 5 and 1 μl of Lipofectamine™ 2000 and 4 and 1 μl of 20 μM siRNAs (final concentration, 40 nM), respectively. After 6 h of incubation, the culture medium was changed, and the cells were maintained for additional 18 h before analysis.

**Data Analyses**—Comparisons were made using analysis of variance and Newman-Keuls multiple comparisons test. p < 0.05 was considered statistically significant for all analyses.

**RESULTS**

**ER Stress-induced Cell Damage Is Accompanied by Elevated MMP-3 Protein Level in Neuronal Cells**—To establish a relationship between ER stress-induced neuronal death and MMP-3,
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FIGURE 1. Cell death and MMP-3 protein level are increased in CATH.a cells exposed to BFA. Degrees of cell death determined by LDH activity in the medium of cells exposed to various amounts of BFA (A) and 1 μM BFA for various durations (B). Typical Western blots against MMP-3 on lysates of cells exposed to various doses of BFA for 24 h (C), and 1 μM BFA for various durations (D). The intensity of each band was determined by densitometry and expressed as fold induction relative to the corresponding untreated control. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 versus control.

FIGURE 2. MMP-3 gene expression is induced in response to ER stress specifically among MMP subtypes. A, a representative RT-PCR performed on various MMP members from RNA preparation of CATH.a cells treated with 1 μM BFA or 1.5 μg/ml TM for 6 h. B, densitometric analysis of the RT-PCR results, normalized against β-2M and expressed as fold induction compared with respective untreated control. ***, p < 0.001.

We also tested the effect of another ER stress-inducing agent TM, an inhibitor of glycosylation (supplemental Fig. 1). TM also caused cell death in a dose-dependent manner between 0.2 μg/ml and 1.5 μg/ml, reaching 290.8 ± 11.8% of untreated control cells at 1.5 μg/ml TM. The cell death was apparent as early as 6 h and continued to rise with time. This was accompanied by an increase in actMMP-3 level (4.9 ± 0.2-fold after 24 h at 1.5 μg/ml). The degree of induction was similar to that caused by 1 μM BFA (4.9 ± 0.3-fold).

ER Stress Induces Gene Expression of MMP-3 Selectively among MMP Subtypes—Whether this increase in MMP-3 protein might involve a rise in its mRNA level was tested by RT-PCR (Fig. 2). BFA (1 μM) and TM (1.5 μg/ml) caused increases in MMP-3 mRNA level to 4.3 ± 0.2- and 3.9 ± 0.2-fold, respectively, in 6 h. We determined whether this induction was a phenomenon specific to MMP-3 among various MMP enzymes. For this, RT-PCR was performed on the same RNA preparations for MMP-1, MMP-7, and MMP-9, which belong to the collagenase, matrilysin, and gelatinase subtypes, respectively. The results showed that, unlike MMP-3, the mRNAs for these other MMP types were not altered by BFA or TM (Fig. 2, A and B). Because BFA and TM treatments consistently led to similar results, the subsequent experiments were carried out using BFA (1 μM).

MMP-3 Participates in the ER Stress-induced Cell Death—We tested whether the active MMP-3 thus produced might play a role in the death signaling itself. Pretreatment of cells with NNGH, an inhibitor of MMP-3 enzyme activity (22), attenuated the BFA-induced cell death by 33.1 ± 10.4% (Fig. 3A). The effect of MMP-3 KO was also assessed using pri-
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BFA exposure to BHA was lower (62.1 ± 1.5% and 63.4 ± 3.4% of cells transfected with negative control siRNA for #1 and #2, respectively) (Fig. 3E). These data taken together indicated that MMP-3 played a role in the BFA-induced cell death.

MMP-3 Acts Downstream of Caspase-12 in the ER Stress-induced Apoptotic Signaling—We sought to determine the relationship of MMP-3 with caspase-12, the enzyme known to be activated by ER stress in other cells. We first confirmed that BFA caused an increase in caspase-12 in CATH.a cells: procaspase-12 (55 kDa) was elevated to 5.6 ± 0.1-fold of untreated control (Fig. 4A, compare lanes 1 and 3), along with the cleaved, active form. This was not affected by the MMP-3 inhibitor NNGH (Fig. 4A, compare lanes 3 and 4). In addition, the number of caspase-12-immunopositive cells was not altered when MMP-3 expression was knocked down (Fig. 4C). Taken together, the data indicated that the caspase-12 induction was not under the control of MMP-3. On the other hand, the MMP-3 knockdown caused a decrease in the number of cells immunopositive for the active form of caspase-3 (67.0 ± 4.6% and 63.1 ± 4.9% of the cells transfected with the negative control siRNA for #1 and #2, respectively) (Fig. 3D). Under this condition, the BFA-induced apoptotic death, as determined by the number of TUNEL-positive cells, was attenuated by 35.7 ± 0.1% and 38.7 ± 3.4% by #1 and #2, respectively (Fig. 6A). These data together indicated that MMP-3 was downstream of caspase-12 in the ER stress-induced cell death.

For further verification, we examined the effect of MMP-3 knockdown mediated by siRNA. Two different siRNAs (#1 and #2), designed based on the known mouse MMP-3 sequence (23), effectively suppressed the BFA-induced increase in MMP-3 mRNA (Fig. 3D). Under this condition, the number of TUNEL-positive apoptotic cells following pretreatment of cells with the caspase-12 inhibitor Z-ATAD-FMK (24) prior to BFA resulted in attenuation of the actMMP-3 induction (50.3 ± 0.1% of BFA-alone control) (Fig. 5A) and a decrease in cell death by 43.5 ± 8.1% (Fig. 5B). Because of the low specificity of Z-ATAD-FMK for caspase-12, we also determined the effect of knocking down caspase-12 expression. Two different siRNA sequences (#1 and #2), designed based on the known mouse caspase-12 sequence (25), efficiently silenced the caspase-12 expression at the mRNA level (Fig. 6A). Under this condition, the BFA-induced apoptotic death, as determined by the number of TUNEL-positive cells, was attenuated by 35.7 ± 8.8% and 38.7 ± 3.4% by #1 and #2, respectively (Fig. 6B), and the actMMP-3 level was lower (0.4 ± 0.1- and 0.5 ± 0.1-fold for #1 and #2, respectively) (Fig. 6C), when compared with the cells transfected with the negative control siRNA. This together indicated that MMP-3 was downstream of caspase-12 in the ER stress-induced cell death.

primary cultured cortical neurons isolated from MMP-3 KO mice (Fig. 3B), and degrees of cell survival were assessed by the neurons’ ability to take up γ-aminobutyric acid, an index of plasma membrane integrity. The results showed that, whereas BFA caused dramatic reduction in γ-aminobutyric acid uptake to 12.6 ± 0.5% of untreated control in the WT, the effect was significantly less (46.0 ± 0.3%) in the KO, demonstrating that cells deficient in MMP-3 were less vulnerable to ER stress. Cell death assessed by LDH activity released into the medium also revealed similar results (Fig. 3C): 157.0 ± 24.8% and 118.8 ± 7.3% of untreated cells for the WT and KO, respectively.

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Caspase-12 Is Sufficient to Increase MMP-3 Activity in the Presence of Cell Lysate—It was possible that, as a protease, caspase-12 might also exert an effect on MMP-3 by a cleavage mechanism. Incubation of purified, recombinant pro-MMP-3 in the presence of various amounts of recombinant active caspase-12, however, did not result in cleavage of pro-MMP-3 or an increase in MMP-3 enzyme activity (not shown). Therefore, pro-MMP-3 was found not to be a substrate of caspase-12. We tested the possibility that caspase-12 might indirectly alter MMP-3 enzyme activity via a mechanism involving other cellular component(s). For this, we performed the same experiments but in the presence of lysate of untreated CATH.a cells. p-aminophenylmercuric acetate, the chemical known to cleave and activate pro-MMP-3 (26), was used as a positive control. This resulted in no cleavage of pro-MMP-3, as expected (Fig. 7A), but interestingly, significant elevation of MMP-3 activity was observed (1.8 ± 0.1-fold, Fig. 7B). Thus, caspase-12 caused an increase in MMP-3 enzyme activity, and this mechanism required one or more cellular components already present in unstressed cells and did not accompany pro-MMP-3 cleavage.

TIMP-1 Is a Substrate of Caspase-12—Because MMP-3 activity was known to be regulated by its endogenous inhibitor TIMP-1 (27), it was possible that caspase-12 might be degrading TIMP-1. Indeed, incubation of TIMP-1 in the presence of recombinant active caspase-12 resulted in disappearance of TIMP-1 (Fig. 8A).

Whether MMP-3 activity might actually be increased by the TIMP-1 degradation was tested. For this, we used commercially available catalytic domain of MMP-3 (cMMP-3), which con-
contains the TIMP-1 binding region (27). Addition of recombinant TIMP-1 to the solution of cMMP-3 at 1:1 molar ratio caused almost complete inhibition of MMP-3 activity (Fig. 8B). On the other hand, when TIMP-1 that had been previously incubated with caspase-12 was added to cMMP-3, the MMP-3 activity remained at 84.1 ± 0.4% of the original MMP-3 activity.

Caspase-12 Can Degrade TIMP-1 That Is in Complex with actMMP-3—Because most MMP-3 exists bound to TIMP-1, the action of caspase-12 on TIMP-1 would be physiologically important only if it can degrade TIMP-1 that is already bound to MMP-3. To test this, the lysate of untreated CATH.a cells was incubated with active caspase-12 and then immunoprecipitated with TIMP-1 antibody. When this immunoprecipitate was subjected to Western blot analysis against MMP-3, the 48-kDa actMMP-3, but not the 55-kDa pro-MMP-3, was detected, indicating that it is mainly the actMMP-3 form that complexes with TIMP-1 (Fig. 9A). The exposure to caspase-12 resulted in a decrease in the amount of actMMP-3 in the immunoprecipitate (Fig. 9A). Because actMMP-3 and TIMP-1 were known to form a complex at a 1:1 molar ratio (28–30), and caspase-12 did not degrade MMP-3 itself, the results indirectly suggested that the decrease in actMMP-3 in the immunoprecipitate was due to degradation of TIMP-1 in the complex by caspase-12. Furthermore, incubation of cMMP-3/TIMP-1 complex with active caspase-12 resulted in an increase in MMP-3 activity to 2.3 ± 0.1-fold (Fig. 9B). This further supported the notion that caspase-12 can degrade TIMP-1 that is already bound to MMP-3 and thereby liberate MMP-3 activity.

Cellular TIMP-1 Is Protective and Is Decreased in Response to ER Stress—If TIMP-1 is a substrate of caspase-12, then the cellular level of TIMP-1 should be changed under ER stress. As shown in Fig. 10A, BFA treatment indeed caused down-regulation of TIMP-1 in a time-dependent manner. Densitometric analysis revealed a significant decrease in the TIMP-1 level as early as 3 h (55.6 ± 0.8% of time zero, p < 0.01) and almost complete degradation within 24 h.

The BFA-induced down-regulation of TIMP-1 was attenuated in the case of caspase-12 knockdown: transfection with caspase-12 siRNAs caused the TIMP-1 level to remain at the control level (Fig. 10B). This was also the case in the presence of the caspase-12 inhibitor Z-ATAD-FMK (Fig. 10C). Interestingly, the caspase-12 inhibition seemed to cause an increase in TIMP-1 compared with the untreated control.

To test the protective role of TIMP-1, CATH.a cells were allowed to overexpress TIMP-1 by transfection with TIMP-1...
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FIGURE 10. Cellular TIMP-1 is protective and decreases in response to ER stress. A, typical Western blot of TIMP-1 on lysate of CATH.a cells that has been exposed to 1 μM BFA for various time periods. B, typical Western blot against TIMP-1 on lysates of CATH.a cells transfected with caspase-12 siRNA (#1 or #2) or negative siRNA control (N) and subsequently treated with BFA. C, TIMP-1 Western blot on lysate of CATH.a cells that was pretreated with 1 μM Z-ATAD-FMK for 1 h followed by 1 μM BFA for 24 h. D and E, CATH.a cells transfected with TIMP-1 cDNA were exposed to BFA, extent of cell death was monitored by TUNEL staining, and TUNEL-positive cells were counted and expressed as the percentage of total cells (D), and MMP-3 enzyme activity was measured (E). *p < 0.05; **p < 0.01 versus respective untreated control; † p < 0.05; ††, p < 0.01 versus mock transfected BFA-treated.

cDNA. Under this condition, the BFA-induced cell death was attenuated by 65.3 ± 5.3% of mock transfected control (Fig. 10D). This was accompanied by suppression of the BFA-induced MMP-3 activity increase (50.6 ± 19.1%) (Fig. 10E).

DISCUSSION

Evidence that ER stress is involved in the pathogenesis of various neurodegenerative diseases has previously been presented. ER stress markers are up-regulated in postmortem brains and cell culture models of Parkinson disease, Alzheimer disease, amyotrophic lateral sclerosis, and Huntington disease (3). In the present study we show for the first time that intracellular MMP-3 activity plays an important role in the neuronal cell death that occurs in response to ER stress. MMP-3 activity is increased under ER stress via induction of gene expression of MMP-3, an increase in actMMP-3 protein, and degradation of the inhibitory protein TIMP-1. The MMP-3 activity in turn participates in the ER stress-induced apoptotic signaling.

Because the discovery that caspase-12 specifically participates in the apoptotic signaling induced by ER stress, attempts have been made to identify the immediate downstream targets of caspase-12 to determine the cellular components and sequence of events following ER stress. The results of the current study provide the first evidence that MMP-3 activity links caspase-12 with the apoptotic signaling.

It seems that the MMP-3 expression in normal, healthy neuronal cells is kept low, because MMP-3 mRNA was barely detectable in untreated cells. Under ER stress condition, on the other hand, the mRNA level was dramatically increased. Although the mechanism by which ER stress leads to MMP-3 gene expression is unclear at present, it is possible that JNK is involved in this induction process. It has been shown that ER stress results in activation of JNK (31) and that JNK activity can induce MMP-3 transcription via phosphorylation of c-Jun (32).

We have previously noted that inhibition of JNK activity causes suppression of MMP-3 induction (11). It is also possible that caspase-12 activity itself may lead to MMP-3 gene expression by acting on specific transcription factor(s). Relevant to this speculation, activated caspase-12 has been shown to enter the nucleus in response to ER stress (33, 34).

We have found that ER stress also leads to generation of actMMP-3. This was found to occur downstream of caspase-12 activity, because both pharmacological inhibition and gene knockdown of caspase-12 led to suppression of the actMMP-3 generation. The cleavage of pro-MMP-3 to actMMP-3, however, does not involve caspase-12, because caspase-12 incubated alone with pro-MMP-3 did not generate actMMP-3. We have observed that this cleavage involves a serine protease in the case of oxidative stress (11). It is possible that ER stress also leads to induction/activation of this protease, which in turn causes the cleavage of pro-MMP-3. The molecular connection between caspase-12 and actMMP-3 generation is currently under investigation in our laboratory.

The present study demonstrates that caspase-12 can lead to an increase in MMP-3 activity by yet another mechanism: via degradation of TIMP-1, the endogenous inhibitor protein of MMP-3 (27) known to bind to MMP-3 in a 1:1 stoichiometry (28–30). This was evident by the following observations: 1) exposure to active caspase-12 resulted in degradation of TIMP-1; 2) TIMP-1 was degraded in cells under ER stress, which was blocked by caspase-12 knockdown and pharmacological inhibition; 3) enzyme activity of MMP-3 in the lysate of untreated cells was increased after exposure to caspase-12; and 4) MMP-3 activity was liberated from the MMP-3-TIMP-1 complex upon exposure to caspase-12.

Therefore, the degradation of TIMP-1 following ER stress would result in liberation of any pre-existing actMMP-3 and also allow the newly generated actMMP-3 freely render its catalytic activity. By inhibiting MMP-3 activity, TIMP-1 seems to play an important role in regulation of neuronal survival/death. Indeed, we have shown that overexpression of TIMP-1 results in suppression of the MMP-3 activity increase and provides cell protection in CATH.a cells exposed to BFA. Protective roles of...
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TIMP-1 have previously been reported in other systems (35–40).

We have previously observed that MMP-3 activity acts upstream of caspase-3 in apoptotic cell death following oxidative stress (11). As shown in the present study, this was also the case with ER stress, because the generation of cleaved caspase-3 is suppressed when MMP-3 expression was knocked down. However, procaspase-3 is not a direct substrate of MMP-3, because purified procaspase-3 incubated alone with cMMP-3 did not undergo any cleavage (data not shown). Therefore, MMP-3 seems to act on other cellular components that lead to caspase-3 cleavage, and the identity of MMP-3 targets is currently being investigated.

In addition to the well known extracellular activation and function of MMP-3 as an enzyme that exerts its action on extra-cellular matrix (41), we present the evidence that MMP-3 serves intracellularly as an important participant in the caspase-12-triggered apoptosis in neuronal cells under ER stress. Although caspase-12 seems to be lost in humans, the human homologue, caspase-4, is thought to be controlled in a similar manner (5, 42, 43). In light of the fact that ER stress-induced apoptosis is related to the pathogenesis of neurodegenerative diseases, MMP-3 and TIMP-1 may serve as cellular targets with which therapy against neurodegenerative diseases may be designed.

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