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Caspase-9 activation and Apaf-1 cleavage by MMP-3

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ABSTRACT

We have previously demonstrated that matrix metalloprotease-3 (MMP-3) can act inside the cell to trigger apoptosis in response to various cell stresses in dopaminergic neuronal cells. However, the mechanism by which MMP-3 activity leads to caspase-3 activation in apoptotic signaling was not known. In the present study, we found that MMP-3 acts upstream of caspase-9. Overexpression of wild type MMP-3, but not mutant MMP-3, generated the enzymatically active 35 kD caspase-9. The caspase-9 activation was absent in MMP-3 knockout cells, but was present when these cells were transfected with wild type MMP-3 cDNA. It was elevated in cells that were under a MMP-3-inducing ER stress condition, and this was attenuated by pharmacologic inhibition and gene knockdown of MMP-3. Incubation of recombinant catalytic domain of MMP-3 (cMMP-3) with procaspase-9 was not sufficient to cause caspase-9 activation, and an additional cytosolic factor was required. cMMP-3 was found to bind to the cytosolic protein Apaf-1, as determined by changes in surface plasmon resonance, and to cleave Apaf-1. Pharmacological inhibition, knockout, and knockdown of MMP-3 attenuated the cleavage. Taken together, the present study demonstrates that MMP-3 leads to caspase-9 activation and suggests that this occurs indirectly via a cytosolic protein, possibly involving Apaf-1.

1. Introduction

Matrix metalloproteinase (MMP)-3, an enzyme responsible for degradation of extracellular matrix, has been associated with the pathogenesis of Parkinson's disease and other neurodegenerative diseases (PD) [1]. Previously, we reported an additional intracellular role of MMP-3 in neurodegeneration. That is, in response to cellular stress, MMP-3 enzyme activity was increased inside the cell and triggered apoptotic signaling, resulting in caspase-3 activation and cell demise [2,3]. Therefore, elucidation of the MMP-3-elicited apoptotic pathway will help understand the cellular mechanism leading to neurodegeneration. MMP-3 activity itself does not cleave procaspase-3 to its active form [2], which suggests involvement of intracellular proteins upstream of procaspase-3.

Caspase-9 directly cleaves procaspase-3, generating the catalytically active caspase-3 [4]. The enzyme normally stays in the cytosol as an inactive 50 kD precursor procaspase-9, and becomes activated upon cleavage of its N-terminal 15 kD. The activation requires Apaf-1, the 130 kD cytosolic protein whose assembly into apoptosome provides the platform on which procaspase-9 is converted to the active form. The assembly is known to be elicited by cytochrome c, the mitochondrial protein that is translocated into the cytosol under a cell stress condition.

Because MMP-3 acts somewhere upstream of caspase-3, it was possible that the MMP-3-induced apoptosis might be mediated by caspase-9 activation. In the present study, we show that MMP-3 leads to caspase-9 activation, that this occurs indirectly via an additional cytosolic protein, and that this is accompanied by cleavage of Apaf-1.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), horse serum, RPMI 1640, Dulbecco's modified Eagle medium (DMEM), Hank's balanced salt solution (HBSS), trypsin/EDTA, and penicillin-streptomycin were from Life Technologies (Seoul, Korea). Brefeldin A (BFA) was purchased from Calbiochem (Darmstadt, Germany) and N-isobutyl-N-(4-methoxyphenylsulfonyl) glycyl hydroxamic acid (NNGH) from Enzo Life Sciences (Plymouth Meeting, PA). Primary antibodies used were rabbit polyclonal anti-mouse caspase-9 (Cell Signaling Technology,

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Beverly, MA), rabbit polyclonal anti-human Apaf-1 (AnaSpec, San Jose, CA) and sheep polyclonal anti-cytochrome c (Sigma-Adrich, St. Louis, MO). Peroxidase-conjugated secondary antibody was from Sigma-Aldrich, and Enhanced Chemiluminescence kit from Pierce (Rockford, IL). Trizol reagent, superscript II reverse transcriptase, and Lipofectamine™ 2000 were from Invitrogen (Carlsbad, CA). Caspase-9 fluorometric assay kit and recombinant procaspase-9 were purchased from BioVision (Mountain View, CA). pcDNA5/FRT/TO-TOPO and pcDNA3.1 *myc*-His vectors were obtained from Life Technologies. Recombinant human MMP-3 catalytic domain (cMMP-3) and rat Apaf-1 were from Calbiochem (Darmstadt, Germany) and Enzo Life Sciences, respectively. Recombinant glutathione S-transferase (GST)-tagged cMMP-3 protein was produced by Bioprogen (Daejeon, Korea).

2.2. Cell cultures

CATH.a cells were grown and plated as previously described [3]). Fibroblasts from MMP-3 knockout (KO) and wild type (WT) animals were obtained and primary cultured as follows. The dermis was removed from postnatal day 1 mice, minced, and incubated in HBSS containing 0.01% trypsin for 30 min at 37 °C. After vigorous vortexing, the cells were pelleted and resuspended in DMEM containing 10% FBS and 10 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. All experiments were performed on cells of passages 3–5.

2.3. Animals

MMP-3 KO mice (C57BL/6_129SvEv), developed by Mudgett et al. [5], and their WT were purchased from Taconic Farms (Germantown, NY). For primary culture experiments, adult female mice (5–7 weeks old, both MMP-3 KO and WT) were superovulated by intraperitoneal injections of pregnant mare serum gonadotropin (5 IU) and human chorionic gonadotropin (5 IU), 48 h apart. The animals were allowed to mate with fertile male mice, and postnatal day 1 offspring were obtained.

2.4. Protein preparations and Western blot analysis

Cell lysate was obtained as previously described by us [3]. To prepare the soluble and mitochondrial fractions, cells were processed as previously reported [6]. Western blot analysis was performed as described previously [6], after loading an equal amount of protein (30 μg) in each lane of SDS polyacrylamide gel. Appropriate antibodies (anti-caspase-9, 1:1000; anti-Apaf-1, 1:2000; anti-cytochrome c, 1:1000) and Enhanced Chemiluminescence were used to detect specific protein bands.

2.5. Caspase-9 activity assay

Casapse-9 activity was measured using a caspase-9 fluorometric assay kit following the manufacturer's instructions. Samples were resuspended in 50 μl of chilled cell lysis buffer and transferred to 96 wells, and 50 μl of 2X reaction buffer (containing 10 mM dithiothreitol) and 5 μl of 1 mM LEHD-7-amino-4-trifluoromethyl coumarin were added. After incubation for 1 h at 37 °C, the plates were read continuously in a fluorescence microplate reader (Molecular Devices), at Ex/Em = 400/505.

2.6. Construction of DNA and transfection

Catalytic domain of human MMP-3 (Phe^{100}-Pro^{273}) was PCR-amplified from cDNA prepared from human SY5Y neuroblastoma. The PCR product was gel-extracted and cloned into the pcDNA5/FRT/TO-TOPO vector. cDNA for catalytically inactive, mutant MMP-3 was generated by substitution of glutamate at amino acid 219 with alanine (E219A) by site-directed mutagenesis using PCR. That the product of this cDNA has no MMP-3 catalytic activity was confirmed. For construction of the full-length caspase-12 cDNA, PCR was performed on a pCAGGSmCASP12 plasmid DNA (kindly provided by Martine Vanhoucke, Universiteit Gent, Belgium) using the following primers: sense, TACGGATCCGGCG GCCAGGAGGACACAT; and antisense, GGCCTCGAGATTCCCGGG AAAAAGGTA. The PCR product was cloned into the pcDNA3.1 *myc*-His vector. 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.

2.7. Preparation of siRNA and transfection

Sense and anti-sense oligonucleotides corresponding to the following cDNA sequences of mouse MMP-3 were used: AAUU CCAACUGCGAAGAUCCACUGA (MMP-3 #1) and AUACCAUCUAC AUCAUCUUGAGAGA (MMP-3 #2). Double stranded siRNA was generated by annealing thee sense and anti-sense oligonucleotides at final concentration of 20 μM. CATH.a cells were transfected in the presence of 4 μl of 20 μM siRNAs (final concentration 40 nM) and 5 μl of Lipofectamine™ 2000. The culture medium was changed after 6 h, and the cells were analyzed 18 h thereafter.

2.8. Surface plasmon resonance

Surface plasmon resonance experiments were performed on a Biacore T100 instrument (GE Healthcare, Uppsala, Sweden) at 25 °C. Anti-glutathione S-transferase (GST) antibody was first immobilized on sensor chip CM5 using the amine coupling kit and GST capture kit (GE Healthcare). At a flow rate of 10 μl/min, each flow cell was activated for 7 min with an equal mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 0.1 M N-hydroxysuccinimide. The anti-GST antibody (30 μg/ml) in 10 mM sodium acetate (pH 5.0) was injected for 5 min, resulting in immobilization level of about 7500 resonance units. The surface on the sensor chip was then deactivated for 7 min with 1 M ethanolamine (pH 8.5). Purified GST-tagged cMMP-3 was then captured by injecting the protein (100 nM) at a flow rate of 10 μl/min for 180 s over the immobilized anti-GST antibody. Full length Apaf-1 was diluted in running buffer (PBS pH 7.4). Binding experiments for various concentrations of full length Apaf-1 were performed in the running buffer at a flow rate of 30 μl/min for 260 s, followed by dissociation for 260 s. At each cycle, the sensor chip was regenerated in 50 mM NaOH at a flow rate of 30 μl/min for 180 s. The recorded sensorgrams were analyzed using BIAevaluation software. Prior to calculations, the binding data were corrected for nonspecific interactions by subtracting the reference surface data (running buffer only) from the reaction surface data. The data thus obtained were globally fitted using a 1:1 binding model to calculate the dissociation constant (K_D).

2.9. Data analyses

ANOVA and Newman–Keuls multiple comparisons tests were used to make comparisons, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. MMP-3 acts upstream of caspase-9 in the apoptotic signaling

We first sought to ascertain whether MMP-3 acts upstream of caspase-9. For this, whether MMP-3 activity might lead to caspase-9 activation was tested in CATH.a cells, a dopaminergic neuronal cell line that we had previously observed MMP-3-mediated apoptosis [2,3]. Western blot analysis on lysate of untreated cells showed two bands that were immunoreactive to caspase-9: the 50 kD procaspase-9 and 35 kD caspase-9. Overexpression of WT MMP-3 resulted in an increase in the 35 kD caspase-9 (Fig. 1A), and this was accompanied by increased caspase-9 enzymatic activity (1.6 ± 0.2 fold) (Fig. 1B). On the other hand, transfection with a mutant MMP-3 cDNA that produces enzymatically inactive MMP-3 yielded no apparent change ($p > 0.05$ vs mock-transfected) in the levels of caspase-9 protein (Fig. 1A) or enzyme activity (Fig. 1B). Therefore, MMP-3 activity was required for the cleavage of procaspase-9 to the enzymatically active caspase-9.

As MMP-3 acts downstream of caspase-12 in apoptotic signaling [3], the MMP-3-elicited caspase-9 activation should also occur downstream of caspase-12. We tested this by comparing the effects of caspase-12 overexpression on caspase-9 in fibroblasts obtained from MMP-3 KO and WT mice (Fig. 1C). In the WT cells, caspase-12 overexpression led to cleavage of the 50 kD procaspase-9 to the 35 kD caspase-9 (10.3 ± 0.5 fold of mock-transfected control). On the other hand, in the MMP-3 KO cells, no change in caspase-9 was noted ($p > 0.05$ vs mock-transfected). Conversely, transfection of the KO cells with WT MMP-3 cDNA resulted in generation of caspase-9 (Fig. 1D). Taken together, catalytically active MMP-3 was required for the generation of caspase-9 from procaspase-9, and this occurred downstream of caspase-12.

3.2. MMP-3 leads to caspase-9 activation in response to ER stress

We had previously observed that treatment of CATH.a cells with brefeldin A (BFA), the drug that induces ER stress by inhibiting intracellular protein transport [7], increased intracellular MMP-3 activity [3]. We therefore utilized this condition to test whether the endogenous MMP-3 could cause caspase-9 activation. As shown in Fig. 2A, exposure of cells to BFA resulted in a dramatic

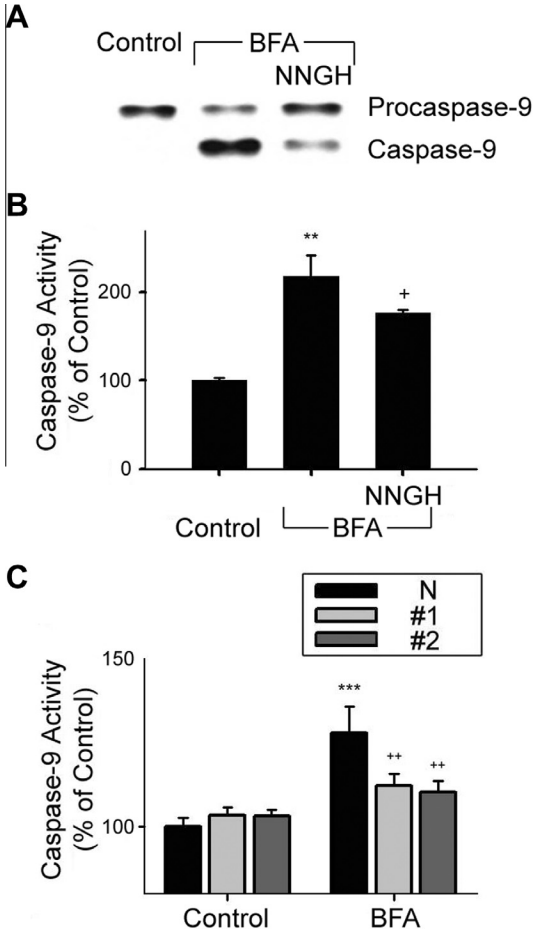

Fig. 2. MMP-3 leads to caspase-9 activation in response to ER stress. CATH.a cells were treated with 10 μM NNGH for 1 h followed by 1 μM BFA for 24 h. The same amounts of cell lysate protein were subjected to (A) western blot against caspase-9 and (B) caspase-9 enzymatic activity assay. $^{**}p < 0.01$ vs control; $^{+}p < 0.05$ vs BFA-alone. (C) CATH.a cells were transfected with MMP-3 siRNA (#1 or #2) or negative siRNA control (N) and subsequently treated with BFA. The same amounts of cell lysate protein were subjected caspase-9 enzymatic activity assay. $^{***}p < 0.001$ vs untreated negative control, $^{++}p < 0.01$ vs BFA-treated negative control.

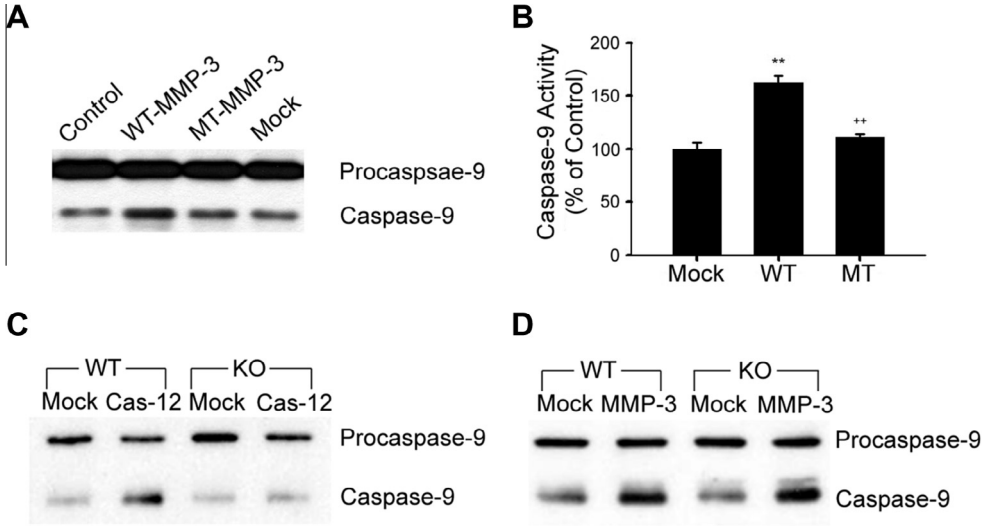

Fig. 1. MMP-3 acts upstream of caspase-9 in the apoptotic signaling. CATH.a cells were transfected with WT or mutant MMP-3 cDNAs or mock-transfected. The same amounts of cell lysate protein were subjected to (A) western blot against caspase-9 and (B) caspase-9 enzymatic activity assay. $^{**}p < 0.01$ vs mock-transfected control; $^{++}p < 0.01$ vs WT-transfected. Primary cultured MMP-3 KO or WT fibroblasts were transfected with (C) caspase-12 cDNA or (D) WT or mutant MMP-3 cDNAs, and the same amounts of cell lysate protein were subjected to western blot against caspase-9.

increase in the 35 kD caspase-9 band. This was attenuated by cotreatment with NNGH, a pharmacological inhibitor of MMP-3 [8]. The caspase-9 catalytic activity was also altered accordingly: it was increased by BFA (218 ± 23% of untreated control) and attenuated by the NNGH cotreatment (177 ± 3%) (Fig. 2B).

We had previously identified two different siRNAs (#1 and #2), designed based on the known mouse MMP-3 sequence [9], that effectively suppressed the BFA-induced MMP-3 induction at both mRNA and enzyme activity levels [3]. Using these siRNAs, we determined the effect of MMP-3 knockdown in the BFA-treated cells. As shown in Fig. 2C, the caspase-9 activity, which was increased by BFA in the mock transfected cells, was dramatically attenuated by the siRNA expression (by 88 ± 8% and 86 ± 7% for #1 and #2, respectively).

3.3. The MMP-3-induced caspase-9 activation requires (a) cytosolic factor(s)

Since MMP-3 is a protease, it was possible that it might proteolytically cleave procaspase-9 to produce the active form. Incubation of cMMP-3 (catalytic domain of MMP-3) with purified procaspase-9, however, did not alter the specific enzyme activity of caspase-9 (Fig. 3A). We therefore tested the possibility that the activation might require other cellular factor(s). Interestingly, incubation of cMMP-3 with the cytosolic fraction of untreated CATH.a cells resulted in a rise in caspase-9 activity (Fig. 3B). The increase was dependent on the amount of cMMP-3, and was prevented in the presence of NNGH. Western blot analysis also showed generation of the caspase-9 band along with a decrease in the procaspase-9 band (Fig. 3C). The cytosolic fraction used in the present study had no contamination by cytochrome c (Fig. 3D), suggesting that the MMP-3-induced caspase-9 activation occurred independently of mitochondria.

3.4. MMP-3 can bind to and cleave Apaf-1

Because caspase-9 activation is known to require the cytosolic protein Apaf-1, it was possible that Apaf-1 might play a role in the MMP-3-elicited caspase-9 activation. We tested whether MMP-3 might physically interact with Apaf-1 by detecting changes in surface plasmon resonance. For this, cMMP-3 was immobilized on a sensor chip, and full length recombinant Apaf-1 in free solution was allowed to interact with cMMP-3. The change in mass of cMMP-3 upon binding with Apaf-1 was followed. The resulting sensorgrams indicated a real-time interaction between the two proteins occurring in a dose-dependent manner (Fig. 4A). The K_D value for binding was 2.45E-05, suggesting the presence of a direct interaction.

We then tested whether Apaf-1 might be a proteolytic substrate of MMP-3. For this, CATH.a cytosolic fraction was incubated with cMMP-3, and western blot analysis was performed against Apaf-1. Indeed, the 130 kD full length Apaf-1 was truncated to about 37 kD and 20 kD proteins (Fig. 4B). Unfortunately, because of lack of information on the epitope of this commercially available Apaf-1 antibody, the possible cleavage region in the 130 kD Apaf-1 protein could not be deduced. In the subsequent experiments, we followed the decrease in the 130 kD protein to assess the cleavage.

We tested whether the Apaf-1 cleavage might be a phenomenon that actually occurs inside the cell by observing the decrease of the full length Apaf-1 protein under a condition that elevates intracellular MMP-3 activity. As shown in Fig. 4C, upon treatment with BFA, the level of Apaf-1 was dramatically decreased, and this was blocked by the MMP-3 inhibitor NNGH ($p > 0.05$ vs control), indicating involvement of MMP-3. Whether an increase in intracellular MMP-3 alone might lead to a similar phenomenon was then tested. As shown in Fig. 4D, overexpression of WT MMP-3 indeed led to disappearance of the full length Apaf-1. On the other hand, in the cells transfected with mutant MMP-3 cDNA, the amount of uncleaved Apaf-1 remained unaltered. We also asked whether MMP-3 knockdown would attenuate the Apaf-1 cleavage. As shown in Fig. 4E, the BFA treatment caused Apaf-1 to decrease to 63 ± 1% in the cells transfected with a negative control siRNA. In comparison, there was no decline in the cells transfected with MMP-3 siRNA (#2; $p > 0.05$ vs respective control).

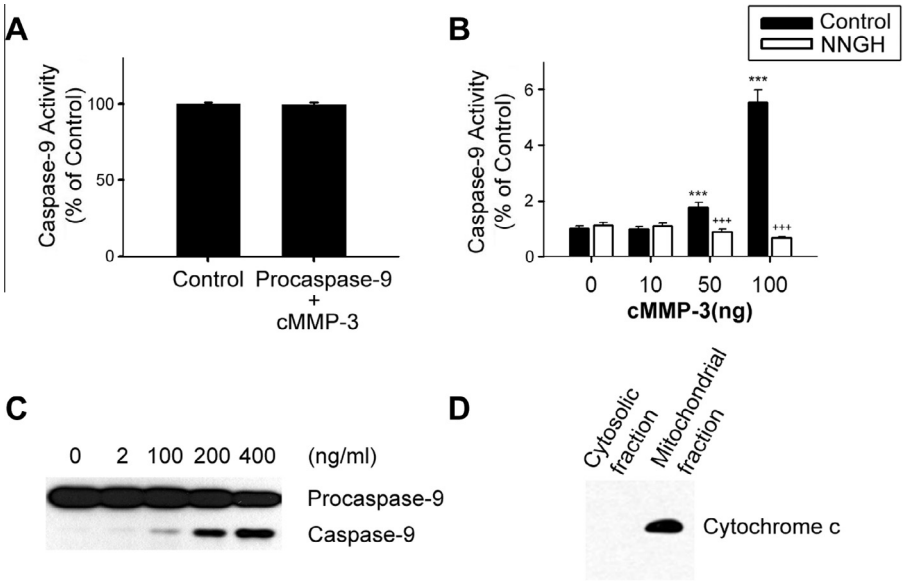

Fig. 3. The MMP-3-induced caspase-9 activation requires (a) cytosolic factor(s). (A) Recombinant procaspase-9 (1.5 μg) was incubated with recombinant cMMP-3 (100 ng/ml) for 2 h and subjected to caspase-9 activity assay; (B and C) 30 μg CATH.a cell cytosolic fraction was incubated with various amounts of recombinant cMMP-3 with or without 10 μM NNGH for 2 h, and the samples were subjected to caspase-9 enzymatic activity assay (B, $^{***}p < 0.001$ vs control, $^{+++}p < 0.001$ vs cMMP-3 alone) and western blot (C); (D) 30 μg of CATH.a cell cytosolic and mitochondrial fractions were subjected to western blot against cytochrome c.

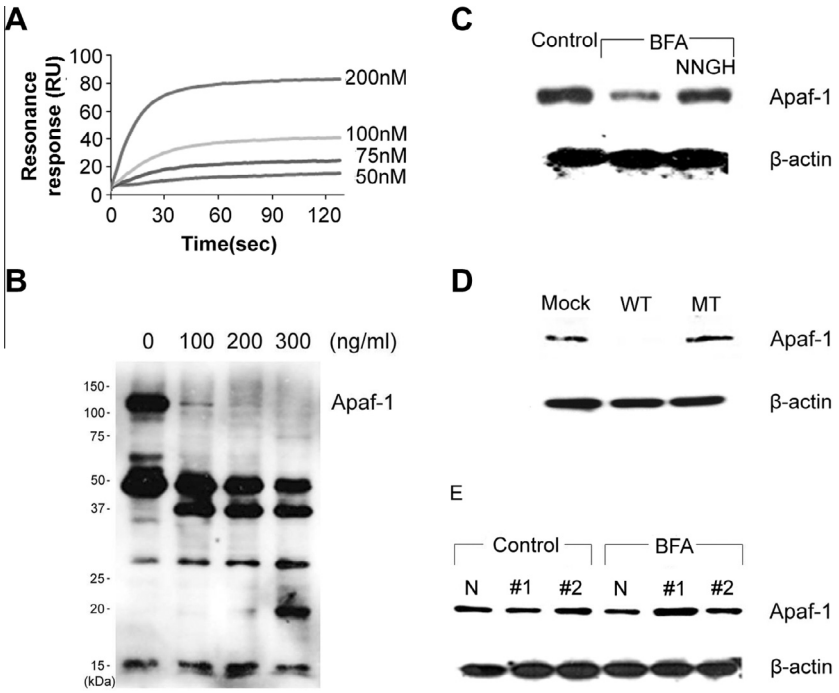

Fig. 4. MMP-3 can bind to and cleave Apaf-1. (A) Changes in SPR were followed after passing various concentrations of Apaf-1 over cMMP-3 immobilized on Biacore sensor chip. The data are shown as a sensorgram of resonance response as a function of time; (B) 30 μg of CATH.a cytosolic fraction was incubated with various amounts of cMMP-3 for 1 h, and then subjected to western blot against Apaf-1. (C–E) CATH. a cells were treated with 10 μM NNGH for 1 h followed by 1 μM BFA for 24 h (C); transfected with WT MMP-3 cDNA or mutant MMP-3 cDNA (D); and transfected with MMP-3 siRNA (#1 or #2) or negative siRNA control (N) and subsequently treated with 1 μM BFA for 24 h (E). The cell lysates were subjected to western blot against Apaf-1.

4. Discussion

In the present study we show that MMP-3 acts upstream of caspase-9 in apoptotic signaling and leads to caspase-9 activation. Procaspase-9 was not a proteolytic substrate of MMP-3; instead, a cytosolic component appeared to mediate the activation. MMP-3 physically associated with and cleaved the cytosolic protein Apaf-1.

MMP-3 participates in neuronal apoptotic signaling. Cellular MMP-3 activity was elevated under oxidative stress or ER stress, and this was attenuated by blocking MMP-3 [2,3]. During apoptotic signaling, MMP-3 acts downstream of caspase-12 [3], and upstream of caspase-3 [2]. Based on these findings, it had been speculated that MMP-3 may act on other cellular components that lead to caspase-3 activation. We show in the present study that MMP-3 causes caspase-9 activation. This was evident by the findings that (1) overexpression of WT, but not mutant, MMP-3 led to caspase-9 activation; (2) caspase-9 was activated by overexpression of caspase-12, which acts upstream of MMP-3, in WT cells but not in MMP-3 KO cells; and (3) caspase-9 was activated in MMP-3-elevated cells, and this was prevented by pharmacological inhibition or knockdown of MMP-3.

Recent studies have reported the role of MMP-3 leading to cell death and neurodegeneration as related to PD. As a protease, the enzyme seems to cleave a number of proteins intracellularly. For example, α-synuclein, the main constituents of Lewy bodies of PD [10] and the protein whose aggregation is known to play a key role in PD pathogenesis [11], is a substrate of MMP-3. Upon truncation by MMP-3, α-synuclein is left with the highly hydrophobic N-terminal region, which readily forms aggregates and exerts cytotoxicity [12–14]. DJ-1, an oxidative stress sensor and a peroxiredoxin-like peroxidase whose gene mutation leads to PD [15], is also cleaved by MMP-3, upon which it loses the protective activity [16]. We show in the present study that MMP-3 cleaves Apaf-1 and leads to capase-9 activation. Therefore, MMP-3 seems to cause a number of biochemical changes inside the cell that contributes to cell death.

It is possible that MMP-3 serves as an important intracellular death signal that orchestrates cell death. In addition, MMP-3 plays a role as a death messenger outside the cell by triggering activation of nearby microglia after its release from dying neurons [17]. Therefore, MMP-3 may induce death of cells that are no longer reparable, so that the damaged cells undergo efficient and timely apoptosis and phagocytosis. This may play a role in the process of neurodegeneration, as MMP-3 KO animals are resistant to the DAergic neurotoxin MPTP [18], transient global ischemia with reperfusion [19], and traumatic nerve injury [20].

The MMP-3-induced caspase-9 activation was observed in the cytosolic fraction isolated from unstressed cells and devoid of mitochondrial proteins, suggesting a possibility that this might occur in a manner independent of cytochrome c. In an attempt to search for the cytosolic factor involved, we focused on Apaf-1, because this protein was the only protein known thus far to be involved in caspase-9 activation. We found that Apaf-1 is a proteolytic substrate of MMP-3. Although we could not directly show that the Apaf-1 cleavage mediates the MMP-3-induced caspase-9, it is possible to speculate that MMP-3 cleaves away the cytochrome c-binding WD40 region in Apaf-1 and consequently induces a conformational change to a form that no longer requires cytochrome c to induce caspase-9 activation. Such cytochrome c-independent activation has previously been reported for WD40-truncated [21] and WD40-absent [22] Apaf-1. It is also feasible that Apaf-1 is cleaved by MMP-3 into a non-functional form and that other unknown mechanisms are involved in mediating the

MMP-3-induced caspase-9 activation. Further studies are necessary to elucidate the exact mechanism by which MMP-3 leads to the caspase-9 activation.

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