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Chaperone-like Activity of High-Mobility Group Box 1 Protein and its Role in Reducing the Formation of Polyglutamine Aggregates

Hyun Jin Min,**† Eun Ae Ko,**† Jie Wu,**† Eun Sung Kim,* Min Kyung Kwon,* Man Sup Kwak,* Ji Eun Choi,‡ Jong Eun Lee,†§ and Jeon-Soo Shin*†‡§‖

High-mobility group box 1 protein (HMGB1), which mainly exists in the nucleus, has recently been shown to function as a sentinel molecule for viral nucleic acid sensing and an autophagy regulator in the cytoplasm. In this study, we studied the chaperone-like activity of HMGB1 and found that HMGB1 inhibited the chemically induced aggregation of insulin and lysozyme, as well as the heat-induced aggregation of citrate synthase. HMGB1 also restored the heat-induced suppression of cytoplasmic luciferase activity as a reporter protein in hamster lung fibroblast O23 cells with expression of HMGB1. Next, we demonstrated that HMGB1 inhibited the formation of aggregates and toxicity caused by expanded polyglutamine (polyQ), one of the main causes of Huntington disease. HMGB1 directly interacted with polyQ on immunofluorescence and coimmunoprecipitation assays, whereas the overexpression of HMGB1 or exogenous administration of recombinant HMGB1 protein remarkably reduced polyQ aggregates in SHSY5Y cells and hmgbl−/− mouse embryonic fibroblasts upon filter trap and immunofluorescence assay. Finally, overexpressed HMGB1 proteins in mouse embryonic primary striatal neurons also bound to polyQ and decreased the formation of polyQ aggregates. To this end, we have demonstrated that HMGB1 exhibits chaperone-like activity and a possible therapeutic candidate in polyQ disease. The Journal of Immunology, 2013, 190: 000–000.

High-mobility group box 1 (HMGB1) is a highly conserved nuclear protein, and its amino acid sequence is conserved across species (1). In most eukaryotic cells, HMGB1 is located in the nucleus, where it functions as a nucleosome stabilizer and a regulator of transcription (2, 3). HMGB1 recently has been shown to play a role as a cytokine-like molecule when it is released into extracellular space. HMGB1 can be released passively after necrosis and also actively from macrophages and monocytes if they are stimulated (4). HMGB1 binds to TLR-2, -4, and receptor for advanced glycation end products (5, 6) and promotes inflammation. HMGB1 was also shown to act as a late mediator of endotoxemia and sepsis in animal models and human patients (7–10). HMGB1 moves to the cytoplasm via a redox-dependent mechanism and plays a role as a regulator between macroautophagy and apoptosis in the cytoplasm by binding to bcl-2 and dissociating from bcl-2 (11). Some proteins form aggregates under stressful conditions such as increases of reactive oxygen species, metal ions, or aging (11–14). Aggregated proteins can be cleared by macroautophagy (15, 16), and chaperones are known to modify proteostasis strongly (17, 18). Polyglutamine (polyQ) tract is present in wild-type proteins that are from a cytosine-adenine-guanine (CAG) repeat coding for a glutamine. PolyQ expansion disease family includes at least nine heritable disorders of Huntington disease and the spinocerebellar ataxias SCA1, SCA2, SCA3, SCA6, SCA7, and SCA17 caused by expansion of a CAG repeat of affected proteins (19). Huntington disease is a neurodegenerative disorder caused by the aggregation of expanded polyQ of the mutant huntingtin protein (mHtt) over 35 CAG repeats in the gene (20) and expanded polyQ tracts beyond the threshold influence on secondary structure and cause the formation of aggregates (21, 22). The aggregates formed by polyQ induce neuronal cell toxicity by several mechanisms such as proteinaggregation dysfuction (23) and sequestration of transcriptional factors (24). How the mutated proteins in these polyQ cause disease in cellular and molecular mechanisms is still in discussions; however, the clearance of aggregates formed by polyQ is a therapeutic target.

Molecular chaperones are defined as any protein that interacts with and stabilizes another protein to acquire its functionally active conformation, without being present in its final structure (25). Chaperones like HSP70 can suppress the formation of mHtt aggregates, alleviating its toxicity in cultured cells and animal models (26–28). Upregulation of chaperones such as HSP70 and HSP40 decrease the formation of aggregates, and therefore, chaperone molecules have been considered as one of the therapeutic targets in many aggregate-prone diseases (29).

The online version of this article contains supplemental material.
In the current study, we investigated the chaperone-like function of HMGB1 by applying physical stress to target proteins and intracytoplasmic stress to luciferase and poly-Q proteins. We show that HMGB1 inhibits the chemically induced aggregation of insulin and lysozyme as well as the heat-induced aggregation of citrate synthase in vitro. In an in vivo refolding assay using hamster lung fibroblast O23 cells, HMGB1 also decreased the heat-induced activation of the luciferase protein. In an aggregation-prone model of expanded poly-Q, we demonstrated that HMGB1 proteins could bind to poly-Q and reduce the formation of aggregates, diminishing the cytotoxicity caused by poly-Q aggregates.

Materials and Methods

Cell culture and DNA transfection

Human neuroblastoma cell line SH-SY5Y, human embryonic kidney epithelial cells HEK293, hamster lung fibroblasts O23, mouse embryonic fibroblast (MEF) NIH3T3 cells, and hmgb1−/− MEF cells (HMGBiotech) were cultured at 37°C under 5% CO2 in DMEM supplemented with 10% FBS (Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine. The cells were transfected with poly-Q and/or trophotometer (Beckman Coulter).

Cell viability measurement

To detect protein expression, cells were lysed using ProPrep protein extraction solution (Intron) including a mixture of protease inhibitors (Sigma-Aldrich). Certain amounts of protein samples were loaded on 12% SDS-PAGE and transferred to a nitrocellulose membrane. A Western blot analysis was performed using primary Abs of rabbit anti-HMGB1 (Abcam), mouse anti-firefly luciferase (Abcam), and mouse anti-GFP (Santa Cruz Biotechnology), as well as secondary Abs of HRP-labeled goat anti-rabbit and goat anti-mouse Ig (The Jackson Laboratory). ECL was used to reveal the signals (Pierce, Rockford, IL). Relative band intensities were measured using the Image J program (National Institutes of Health).

Immunochemistry

To identify the binding of HMGB1 with 25Q- or 97Q-GFP, cells were transiently cotransfected with the indicated plasmids of 25Q- or 97Q-GFP and HMGB1-Myc. Lysed cell homogenates were centrifuged at 13,000 rpm for 15 min and precleared by incubation with protein G-Sepharose (Sigma-Aldrich) at 4°C for 1 h. The precleared extracts were incubated with mouse anti-GFP Ab (Abcam) overnight and then incubated with protein G-Sepharose for 2 h at 4°C. Immune complexes were collected by centrifugation and washed with cold PBS. Collected complexes were fractionated by SDS-PAGE, and an immunoblot analysis was performed.

Immunoprecipitation

To check the chaperonic effect of HMGB1 in chemical-induced protein aggregation following a previous protocol with minor modifications (33). Myc-tagged wild-type HMGB1 as well as HMGB1 boxes A (aa 1–79) and B (aa 88–162) plasmids were used for the transfection study (31, 32). We used recombinant HMGB1 (rHMGB1) proteins produced in insect High Five cells (Insect HMGB1; ATGen), a mouse myeloma cell line of NS0 (R&D Systems), or human cells (Euk HMGB1; Sino).

Measurement of chaperone-like activity of HMGB1 in vitro

A turbidity measurement was performed to measure the level of protein aggregation following a previous protocol with minor modifications (33). To check the chaperonic effect of HMGB1 in chemical-induced protein aggregation, protein solutions of insulin (0.8 μm) and lysozyme (1 mg/ml) in 10 mM PBS (pH 7.4) were incubated at 37°C with 20 mM DTT. For the heat-induced aggregation, a 0.25 μM citrate synthase protein was prepared in 50 mM HEPES buffer (pH 8) and incubated with HMGB1 proteins for 1.5 h at 43°C in the presence or absence of HMGB1 in a thermostatic cell holder. Absorbance was monitored at 320 nm with a Beckman spectrophotometer (Beckman Coulter).

In vivo refolding assay

Chinese hamster lung fibroblast O23 cells were transiently transfected with luciferase plasmid of PRSVLL/V and HMGB1 plasmid (31, 34), pRSVLL/V encoding cytoplasmic luciferase plasmid (a kind gift from Dr. H. Kampina, University of Groningen, Groningen, The Netherlands) was used for intracytoplasmic protein refolding measurement after heat-shock treatment (34). The cells were split into 24-well plates 24 h after transfection and further incubated at 37°C for 24 h. The plates were treated with cycloheximide (20 μg/ml) for 30 min. heat-shock treated at 43°C for 30 min, washed three times with PBS, and then lysed to measure change in luciferase activity. Luciferase activity per microgram protein was measured following the manufacturer’s protocol of the Dual luciferase reporter assay system (Promega). pcDNA and pCMV-HSP70 was transfected as negative and positive controls, respectively, for chaperonic function.

Treatment of reactive oxygen species scavengers

To investigate the influence of reactive oxygen species (ROS) generated by poly-Q aggregates on the cytoplasmic translocation of HMGB1 protein, we pretreated SH-SY5Y cells with ROS scavengers and subsequently compared the number of cells that contained cytoplasmic HMGB1 protein. SH-SY5Y cells were pretreated with 10 mM N-acetyl-l-cysteine (NAC; Sigma-Aldrich) and 25 mM Mito-TEMPO (Enzo Life Sciences) 1 h before 97Q-GFP transfection. After 48 h of 97Q-GFP transfection, endogenous HMGB1 was immunostained with anti-HMGB1 Ab (Abcam), and cytoplasmic HMGB1 proteins were confirmed by confocal microscopy (Olympus). We counted 100 GFP-positive cells in multiple random visual fields in each condition and compared the number of cells that contained cytoplasmic HMGB1.

Western blot analysis

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Mouse embryonic primary striatal neuron cells

Mouse embryonic primary striatal neuron cells were prepared from embryonic day 14. Dissociated striatal neurons were plated at a density of 3.5 hemispheres/10 ml media onto poly-t-lysine–(100 μg/ml) and laminin-coated (100 μg/ml) plates. The cultures were maintained with antibiotics in Neurobasal medium (Life Technologies) supplemented with B27 (Invitrogen). The cells were used for transfection after 7 d of culture. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Yonsei Laboratory Animal Research Center (2010-0392).

Statistics

Statistical analysis was performed by ANOVA followed by the Bonferroni test or Student t test. All data were presented as means ± SE.

Results

HMGB1 reduces physical stress–induced protein denaturation in vitro and heat shock–induced luciferase inactivation in O23 cells

To investigate whether HMGB1 exhibits any chaperone-like activity, the ability to prevent chemical- and heat shock–induced target protein aggregations was evaluated by measuring turbidity in vitro. Insulin and lysozyme were used for the chemical (DTT)-induced aggregation. Insulin and lysozyme in 10 mM phosphate (pH 7.4) were incubated with 20 mM DTT in the presence or absence of HMGB1 at 37°C for the indicated time, and the degree of light scattering by aggregation was measured. As shown in Fig. 1A1 and 1A2, DTT-induced aggregations of both insulin and lysozyme were increased with time; however, the levels of aggregation decreased with the addition of HMGB1. In the heat shock–induced protein aggregation experiments, citrate synthase was used to incubate with HMGB1 protein at 43°C because citrate synthase is sensitive to thermal treatment >45°C (35), and HMGB1 protein became turbid when it was heat-treated at 50°C (Fig. 1B1). Thermally induced aggregation of citrate synthase was significantly reduced with the addition of HMGB1 (Fig. 1B2, 1B3).

Chinese hamster O23 cells were used as a model for determining in vivo refolding assay for intracellular chaperone-like function using luciferase (34). O23 cells were transiently cotransfected with luciferase plasmid with CMV-myc, HMGB1-myc, or HSP70-myc plasmid. CMV-myc and HSP70 plasmids were cotransfected as negative and positive controls, respectively (Fig. 1C). Luciferase activity after thermal stress at 43°C for 30 min was measured by measuring light absorbance. When O23 cells were cotransfected with luciferase and HMGB1 plasmids, luciferase activity from direct thermal stress was increased compared with the controls.

Overexpression of 97Q-GFP induces nucleocytoplasmic translocation of HMGB1, and cytoplasmic HMGB1 colocalizes to 97Q-GFP

To investigate the effect of HMGB1 on protein aggregation, we introduced an aggregation-prone model of expanded polyQ in SHSY5Y neuroblastoma cell line. The aggregation of the mHtt protein of expanded polyQ results in neuronal cell toxicity, and the clearance of aggregates is considered as a main therapeutic target in Huntington disease. We first tested the aggregate formation of 97Q-GFP in SHSY5Y cells (Supplemental Fig. 1A). When SHSY5Y cells were transfected with the 97Q-GFP construct, distinct aggregates could be observed after 48 h, but this was rare in 25Q-GFP. For 97Q-GFP–transfected cells, nucleocytoplasmic translocation of HMGB1 was clearly observed in confocal microscopy and Western blot analysis of cytosolic fraction of transfected cells (Supplemental Fig. 1A, 1B). We observed that the individual vector of 25Q- and 97Q-GFP expressed well, and the level was not reduced by HMGB1 overexpression (Supplemental Fig. 1C).

To test whether ROS scavenging inhibits the translocation of HMGB1, NAC, a precursor of glutathione that acts as a scavenger for the OH radical, and Mito-tempo, a mitochondria-targeted

FIGURE 1. HMGB1 protein suppresses chemical- and heat-induced aggregation of target proteins. (A) Aggregation curve of insulin [0.8 mg/ml (A-1)] and lysozyme [1 mg/ml (A-2)] in the absence of HMGB1 (circles) and in the presence of HMGB1 (0.5 mg/ml; triangles) or HSP70 (0.5 mg/ml; squares) induced by 20 mM DTT. (B-1) Aggregation of HMGB1 protein by heat treatment. rHMGB1 protein (0.5 mg/ml) was incubated at different temperatures for 3 h, and the aggregation was measured by OD at 340 nm. (B-2 and B-3) Thermal aggregation of citrate synthase. Citrate synthase (0.25 μM) was incubated at 43°C for 90 min with 0.4 μM of HMGB1 protein. Same molecular concentration of HSP70 protein was used as a positive control. (C) O23 cells were transiently cotransfected with luciferase and HMGB1 plasmids. HSP70 plasmid and empty vector pcDNA were used as positive and negative controls, respectively. Cells were treated with cycloheximide for 30 min after 24 h of incubation and heat-shock treated for 30 min at 43°C to inactivate luciferase.

*p < 0.05, **p < 0.01 versus CMV-myc control (n = 3).
antioxidant with superoxide and alkyl radical–scavenging properties, were treated. As shown in Fig. 2A and 2B, NAC and Mito-tempo scavenging of ROS generated from overexpressed 97Q-GFP aggregates decreased the translocation of HMGB1.

To observe whether HMGB1 directly interact with 97Q-GFP, SHSY5Y cells were overexpressed with 97Q-GFP and immunostained with anti-HMGB1 Ab. Aggregates of 97Q-GFP were colocalized with HMGB1, which were translocated to the cytoplasm (Fig. 3A). The direct interactions between HMGB1 and 97Q-GFP or 25Q-GFP were also shown by immunoprecipitation assay in SHSY5Y and HEK 293 cells (Fig. 3B). To confirm again the interaction, SHSY5Y cells were overexpressed with 97Q-GFP, and Duolink PLA was performed. As shown in Fig. 3C, colocalization of endogenous HMGB1 protein with 97Q-GFP was observed in confocal microscopic analysis. When we investigated the bindings of A and B domains of HMGB1 to 97Q-GFP
protein, both domains exhibited the binding to 97Q-GFP upon immunoprecipitation analysis using SHSY5Y and HEK293 cells (Fig. 3D).

**HMGB1 suppresses the formation of mHtt aggregates**

To investigate the effect of HMGB1 on the aggregation of expanded polyQ, SHSY5Y cells were cotransfected with a construct of 97Q-GFP plasmid and incubated for 48 h in the presence or absence of HMGB1 overexpression (Fig. 4A, 4B). The number of polyQ aggregate-containing cells among 100 GFP-positive cells was significantly reduced when the cells were cotransfected with HMGB1 plasmid. When the amount of aggregates was further analyzed using filter-trap assay, the band intensity showing 97Q-GFP aggregates immobilized on cellulose acetate membrane was decreased in a dose-dependent manner with HMGB1 plasmid transfection (Fig. 4C, top panel). The protein expression of HMGB1-myc plasmids was observed by Western blot analysis. *p < 0.05, **p = 0.001 versus CMV-myc control. DIC, Differential interference contrast.

**FIGURE 4.** HMGB1 reduces the formation of 97Q-GFP aggregates. (A and B) SHSY5Y cells were cotransfected with 97Q-GFP and HMGB1-myc plasmids and cultured for 48 h. The 97Q-GFP aggregates were analyzed with confocal microscopy, and the numbers of aggregate-positive cells were counted among 100 GFP-positive cells. Arrows indicate 97Q-GFP aggregates. pcDNA and HSP70 plasmids were used as negative and positive controls, respectively. The experiments were repeated three times. (C) Analysis of 97Q-GFP aggregates using filter-trap analysis. SHSY5Y cells were cotransfected with a construct of 97Q-GFP and HMGB1, and each cell lysate protein (200 µg) was filtered through a 0.22-µm cellulose acetate membrane for Western blot analysis. Relative band intensities were measured using Image J program (National Institutes of Health). Expression of HMGB1-myc plasmids was observed by Western blot analysis. *p < 0.05, **p = 0.001 versus CMV-myc control. DIC, Differential interference contrast.

**FIGURE 5.** Effect of rHMGB1 protein on the formation of 97Q-GFP aggregates. (A–C) SHSY5Y cells were transiently transfected with 97Q-GFP plasmid and then treated with rHMGB1 at the indicated concentrations. Distribution of 97Q-GFP aggregates was observed (A), and the number of aggregate-containing cells was counted among 100 GFP-positive cells (B). (C) The average pixel density of 30 aggregates was measured after treatment with rHMGB1. (D) SHSY5Y cells were transiently transfected with 97Q-GFP plasmid followed by rHMGB1 protein treatment. Cell lysates were analyzed for aggregate formation using filter-trap assay. The filter membrane was immunoblotted with anti-GFP Ab to check the amount of aggregates (top panel). Whole-cell lysates for exogenous HMGB1 protein that penetrated the cell membrane was analyzed using anti-His Ab (bottom panel). *p < 0.05 versus the rHMGB1-untreated group (n = 3).
transfected HMGB1 plasmid was observed by Western blot assay (Fig. 4C, bottom panel).

**Administration of exogenous rHMGB1 protein reduces the formation of mHtt aggregates**

Next, we evaluated the effect of exogenous rHMGB1 protein treatment on the cells overexpressing 97Q-GFP, because the addition of exogenous rHMGB1 protein can increase the binding with TLR9 in the cytoplasm (37). We first tested whether exogenous treatment of rHMGB1 protein can penetrate the cell membrane into cytoplasm. Addition of rhodamine-conjugated rHMGB1 proteins to the medium was able to directly penetrate the cell membrane under the observation of confocal microscopy (Supplemental Fig. 2A). This finding was not inhibited by cytochalasin D and actin filaments.
B pretreatment, which prevents actin polymerization, even at the high concentration of 50 μg/ml, suggesting that this penetrating mechanism is not relevant with endocytosis. The penetration of rHMGB1 to the cytoplasm was also probed in the cell lysates after cell washing by Western blot analysis (Supplemental Fig. 2B).

When rHMGB1 protein was added to the medium and incubated for 24 h in SHSY5Y cells transfected with 97Q-GFP, the formation of 97Q-GFP aggregates was significantly reduced by rHMGB1 protein treatment (Fig. 5A). The average number of aggregate-containing cells among 100 GFP-positive cells was dose-dependently reduced from 35.7 ± 6.7 to 15.0 ± 2.0 at 4 μg/ml of rHMGB1 protein treatment (Fig. 5B). The average pixel density of 30 aggregates was also reduced by the treatment of rHMGB1 protein (Fig. 5C). In a filter-trap assay, rHMGB1 protein treatment inhibited 97Q-GFP aggregation (Fig. 5D). When SHSY5Y cells were treated with trichostatin A, which is an inhibitor of histone deacetylase, to induce acetylation that results in intracytoplasmic translocation of HMGB1 (38), 97Q-GFP aggregate-positive cells among 100 GFP(+) cells were significantly decreased (Supplemental Fig. 2C, 2D).

We hypothesized that rHMGB1 protein might interact with polyQ directly to reduce the formation of aggregates. The binding of exogenously treated rHMGB1 protein with the aggregates of 97Q-GFP was observed using immunoprecipitation assay (Fig. 6A). 97Q-GFP–overexpressed SHSY5Y cells were treated with 4 μg/ml of His-tagged rHMGB1, and the binding of rHMGB1 to 97Q-GFP could be observed after immunofluorescence staining of rHMGB1 using anti-His Ab (Fig. 6B). When rhodamine-conjugated rHMGB1 protein was treated to observe direct binding to intracellular 97Q-GFP, colocalization was clearly observed by confocal microscopy (Fig. 6C). Colocalization was confirmed by colocalization scatter plot and cross-sectional intensity profiles provided by confocal microscopy software (Fig. 6C). These data suggest that exogenous rHMGB1 protein enters the cytoplasmic membrane and interacts with polyQ aggregates.

PolyQ aggregates tend to be eliminated by autophagy or lysosomal pathway (21), and HMGB1 is an important autophagy regulator (11). Therefore, we performed PLA to check the possibility that HMGB1-associated aggregates might be localized in autophagosomes. We observed that HMGB1 is colocalized with LC3B, an autophagy marker molecule, and also with some 97Q-GFP aggregates (Supplemental Fig. 3A, 3B).

**Effect of HMGB1 on the formation of mHtt aggregates in hmgbl−/− MEF cells**

Next, we tested the effect of HMGB1 on hmgbl−/− MEF cells to confirm that exogenous HMGB1 supplement could assume these functions. When hmgbl−/− MEF cells were cotransfected with 97Q-GFP and HMGB1-myc plasmids, the 97Q-GFP protein and its aggregates were observed in the cytoplasm as expected (Fig. 7A). Most of the HMGB1 proteins were observed in the nucleus, because HMGB1 has bipartite nuclear localization signal sequences (38). HMGB1 proteins in the cytoplasm were colocalized with 97Q-GFP aggregates (Fig. 7A). The number of aggregate-positive cells was significantly reduced when HMGB1 was replenished (Fig. 7B), and the production of 97Q-GFP aggregates was decreased in HMGB1-transfected cells on filter-trap analysis (Fig. 7C). 97Q-GFP aggregation decreased cell viability, and cell viability was restored by overexpression of HMGB1 (Fig. 7D).

When hmgbl−/− MEF cells transfected with 97Q-GFP were treated with rhodamine-conjugated rHMGB1 protein, the colocalization of HMGB1 and 97Q-GFP was observed, and the number of 97Q-GFP aggregate-containing cells among 100 GFP-positive cells was significantly decreased (Fig. 7E, 7F). The band intensity of filter-trap analysis also decreased after treatment with 4 μg/ml of rHMGB1 (Fig. 7G). rHMGB1 protein treatment at a high

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**FIGURE 8.** Effect of HMGB1 on 97Q-GFP–induced cytotoxicity. SHSY5Y cells were cotransfected with 97Q-GFP plasmid and HMGB1-myc plasmid and incubated for 48 h. GFP transfection was used for a negative control study. Cell viability was measured using PI staining (A), CCK-8 activity (B), and trypan blue staining (C). *p < 0.05, **p < 0.01 (n = 3). DIC, Differential interference contrast.
concentration of 8 μg/ml, however, slightly increased formation of aggregates (Fig. 7G).

Effect of HMGB1 on 97Q-GFP–induced cellular cytotoxicity

We speculated whether the chaperonic activity of HMGB1 could reduce the cellular cytotoxicity caused by the formation of polyQ aggregates. For this, SH-SY5Y cells were transiently cotransfected with 97Q-GFP and HMGB1 plasmids for 48 h, after which changes in cell viability were measured. As shown in Fig. 8A, the number of PI-positive cells, indicating cell death, decreased when the cells were cotransfected with HMGB1 plasmid. HSP70 is a well-known chaperonic molecule in Huntington disease and was used as a positive control (39). In the CCK8 cell-viability assay, the cell viability of 97Q-GFP–transfected cells was restored from 29.5 to 45.8% when the cells were cotransfected with HMGB1 plasmid (Fig. 8B). Upon trypan blue staining analysis, which stains dead cells, the transfection of HMGB1 plasmid decreased the number of cell deaths from 40.6 ± 4.0 to 25.0 ± 1.0 (Fig. 8C). These data demonstrate that HMGB1 reduces 97Q-GFP aggregate-induced cell death.

HMGB1 reduces polyQ aggregation in mouse embryonic primary striatal neuron cells

Finally, we used mouse embryonic primary neuronal cells for the chaperone-like activity of HMGB1. For this, mouse embryonic primary striatal neuron cells at embryonic day 14 were isolated and cotransfected with 97Q-GFP and HMGB1 plasmids. HMGB1 significantly reduced the number of polyQ aggregate-positive cells (Fig. 9A, 9B). The results of the filter-trap analysis showed that the band intensity of 97Q-GFP aggregates was decreased in a dose-dependent manner with HMGB1 (Fig. 9C). HMGB1 protein was colocalized with 97Q-GFP aggregates by confocal microscopy and immunoprecipitation analysis (Fig. 9D, 9E).

When mouse embryonic primary striatal neurons were treated with rHMGB1 protein, the fluorescence intensity of 97Q-GFP was diffusely observed, and the number of polyQ aggregate-positive cells was significantly decreased (Fig. 9F, 9G). The band intensity of filter-trap aggregates showed similar results (Fig. 9H). Treatment of rhodamine-conjugated rHMGB1 protein to primary striatal cells, which were overexpressed with 97Q-GFP, also exhibited HMGB1 binding to 97Q-GFP (Fig. 9I).

Discussion

HMGB1 is a ubiquitous nuclear protein and is diffusely distributed in the cytoplasm, especially of hepatocytes and neuronal cells (40). In various conditions under proinflammatory signals or oxidative stresses, nuclear HMGB1 can be transported into cytoplasm or secreted into the extracellular space. In the nucleus, HMGB1 plays a role as a DNA chaperone that acts as an architectural chromatin-
binding factor, which bends DNA and promotes protein assembly at specific DNA targets. Extracellular HMGB1 functions as a proinflammatory cytokine-like molecule by direct binding to TLR4 or indirectly binding with other molecules (41). In addition, HMGB1 can play an intracytoplasmic role as a regulator between macroautophagy and apoptosis (11, 42–44) and a sentinel molecule for viral nucleic acid sensing (45).

In this study, we have demonstrated as a novel finding that HMGB1 exhibits a chaperone-like activity. Extracellular HMGB1 protein reduced protein aggregation induced by heat or chemical stress, and this chaperone-like function could be observed in the cytoplasm by the restoration of intracytoplasmic luciferase activity after heat stress. In addition, HMGB1 was able to reduce the formation of aggregation of expanded polyQ and restore the cell viability from aggregation-induced toxicity. Expanded polyQ is a shared feature of all polyQ diseases, forming misfolded proteins that disrupt normal cellular pathways. Compensatory expression of HMGBs ameliorates polyQ-induced pathology of activation of Chk1 and H2AX, cell-cycle checkpoint kinase and histone that marks sites of DNA structural damage, respectively, and in vivo toxicity of mutant polyQ protein by suppressing genotoxic stress in a Drosophila polyQ model (46). On the contrary, the positive interaction of HMGB1 to normal 25Q protein in our result did not agree with a previous report of negative binding to 20Q protein (46). It is possibly due to the number difference of glutamine repeats, 20 or 25, but the clear comparison experiment is necessary to further investigate the physiological meaning of HMGB1 binding to normal polyQ. Classical chaperone proteins of HSP70 and HSP40 family proteins bind to soluble mHtt and suppress aggregation formation and cellular toxicity by correcting the structure of misfolded proteins or degradation of irreversibly damaged polypeptides (47). HSP70 increases the level of soluble polyQ proteins by directly associating with polyQ aggregates in a dynamic and transient manner (48, 49), and HSP40 (DNAJ) associates with aggregates and suppresses aggregation by chaperone modification of high m.w. complexes (29).

Expansion of polyQ repeats leads to early increase of ROS coinciding with polyQ aggregation. Aggregation of a fragment of Htt protein directly causes free radical production in vivo (36), and inhibition of polyQ aggregation suppresses ROS (50, 51). Oxidative potential is important for the nucleocytoplasmic translocation of HMGB1, and redox status of HMGB1 also affects its specific function (52). In our study, ROS generated by overexpression of 97Q-GFP induced translocation of nuclear HMGB1. This translocation was decreased by treatment of ROS scavengers NAC and Mito-tempo, demonstrating the important role of ROS in HMGB1 translocation and binding to expanded polyQ. Our previous finding shows that posttranslational modification of phosphorylation is also responsible for the nucleocytoplasmic transport of HMGB1, and Ca²⁺-dependent classical protein kinase C is an effector kinase of HMGB1 phosphorylation (53). Considering that expanded polyQ expression induces Ca²⁺-dependent protein kinase C via metabotropic glutamate receptor–mediated cell signaling (54), it is possible that members of the classical protein kinase C enzyme (α, β₁, βII, and γ) family are the main kinases for nucleocytoplasmic transport of HMGB1 to interact with polyQ. Further investigation is necessary to determine whether phosphorylation of HMGB1 could be induced by overexpression of expanded polyQ.

Exogenous administration of rHMGB1 proteins reduced the formation of intracellular mHtt aggregates. The penetration mechanism of HMGB1 protein into the cytoplasm is not clear, and this penetration was not inhibited by cytochalasin B, suggesting a phagocytosis-independent mechanism. Previous reports that addition of exogenous rHMGB1 proteins to the medium can increase the binding with TLR9 in the cytoplasm (37) and HMGB1–oligodeoxynucleotide complexes are efficiently taken by cells in the absence of HMGB1 receptor of receptor for advanced glycation end products support intracellular uptake of exogenous HMGB1 protein. In HSP70 protein, it can also enter human motor neurons in time- and dose-dependent manners (39, 55) and protect motor neurons subjected to the oxidative stress by decreasing aggregates-positive cells (56, 57), although the mechanism has not been clearly explained.

Overexpression of HMGB1, however, is not always beneficial to reducing polyQ aggregation. A high concentration of HMGB1 rather aggravated the formation of mHtt aggregates in both HMGB1-overexpressed cells and exogenously rHMGB1-treated cell-culture conditions (data not shown). HMGB1 proteins tended to be oligomerized or aggregated by themselves under oxidative stress conditions (data not shown). Excessive amount of HMGB1 is possibly coaggregated with polyQ aggregates. HSP70 protein also showed a similar effect when the protein was overexpressed, suggesting that the optimal concentration of chaperonic proteins is beneficial for the survival of motor neurons (58). The experiments of addition of HMGB1 in hmg1b⁻/⁻ MEFS cells and primary neuronal cells confirmed that HMGB1 has a chaperone-like activity.

In summary, we identified a new role of HMGB1 as a chaperone-like molecule in vitro and in vivo. Additionally, the chaperone-like activity of HMGB1 could be a new therapeutic choice of polyQ diseases.

Disclosures

The authors have no financial conflicts of interest.

References

Figure S1. Overexpression of 97Q-GFP induces translocation of HMGB1. (A) SHSY5Y cells were transfected with 25Q-GFP or 97Q-GFP plasmid and incubated for 48 h. Aggregation of 97Q-GFP was observed in arrows. Endogenous HMGB1 was immunostained with anti-HMGB1 antibody and Alexa 594-conjugated secondary antibody. The intracytoplasmic translocation of HMGB1 was observed with confocal microscopy. (B)
Cytosolic fractions of SHSY5Y cells were isolated after transfection of 25Q- or 97Q-GFP plasmid and western blot analysis was performed to observe HMGB1 level in cytoplasm and the relative band intensities were measured. * $p$ value < 0.05 (n=3). (C) SHSY5Y cells were transfected with 25Q-GFP and 97Q-GFP plasmids with or without an addition of HMGB1 or HSP70 plasmid to observe the expression of individual plasmid and the effect of HMGB1 to 25Q- or 97Q-GFP expression. Whole cell lysates were prepared and western blot analyses were performed with anti-GFP, anti-GAPDH, and anti-myc antibodies after 48 h.
Figure S2. Exogenous rHMGB1 protein penetration and the effect of HMGB1 migration agonist (TSA) on 97Q-GFP formation. (A-B) HEK293 cells were treated with rhodamine-conjugated HMGB1 protein (4 μg/ml) for 24 h in the presence or absence 1 h pretreatment of cytochalasin B to observe the migration of exogenous HMGB1 into cells. Nuclei were stained with DAPI and observed under confocal microscopy (A). Western blot analysis was performed with the cell lysates to detect the intracellular translocation of rHMGB1 protein using anti-His antibody. Two separate tests were performed. (C-D) Effect
of HMGB1 translocation agonist on the aggregate formation of 97Q-GFP. SHSY5Y cells were transfected with 97Q-GFP plasmid and treated with 10 ng/ml of trichostatin A (TSA), which induces HMGB1 translocation into cytoplasm, for 12 h. Co-transfection of HMGB1 plasmid was used for control. After 48 h, HMGB1 translocation was observed, and 97Q-GFP aggregate (+) cells were counted among 100 GFP-positive cells (D). * p value<0.05.
Figure S3. Autophagosome formation in 97Q-GFP and HMGB1 overexpressed cells. (A) SHSY5Y cells were co-transfected with 97Q-GFP and HMGB1 plasmids and incubated for 48 h, and proximity ligation assay (Duolink) between HMGB1 and LC3B was performed to observe the interaction of HMGB1 and LC3B. HMGB1 and LC3B binding shows red fluorescence and their bindings to 97Q-GFP shows yellow (arrow) in merged images, and PLA signal numbers were evaluated (B). ** $p$ value<0.01 to Duolink only.