Role of chaperone-like activity of High Mobility Group Box 1 : its role in reducing the formation of polyglutamine aggregates

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Directed by Professor Jeon-Soo Shin

The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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December 2012

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December 2012

ACKNOWLEDGEMENTS

1999년 연세대학교 의과대학 의예과를 입학한 지가 엊그제 같은데 어느덧 10년 이상의 시간이 흘러 의과대학 의과학 박사과정을 마치게 되었습니다. 이비인후과 레지던트 과정을 마친 뒤 시작한 박사과정에 어려움도 많고 힘든 시기도 있었지만 과학이라는 학문에 한 발자국 내밀 수 있도록 지도해주신 신전수 교수님께 진심으로 감사 드립니다. 그리고 바쁘신 와중에 논문 지도에 시간을 아끼지 않으신 김호근, 김종선, 윤호근, 김창훈 교수님께도 깊은 감사를 드립니다. 또한 이비인후과 레지던트 기간 내내 또 그 후에도 저를 이 길로 인도해주신 저의 멘토 윤주헌 교수님께도 깊은 감사 드립니다. 박사님으로서 모범을 보여주신 곽만섭 선생님, 함께 실험을 진행한 고은애, 기쁨과 슬픔 그리고 생활을 함께 했던 김솔 박사님, 김숙영 선생님, 권민경, 손민아, 이은정, 장선미, 김아름, 이용준, 이아람 모두 감사드립니다.

박사과정 내내 실험실 생활에 집중할 수 있도록 끝없는 지지를 아끼지 않았던 남편 이세빈님과 아들 이채운, 사랑하는 나의 아버지, 어머니, 시아버지, 시어머니께 끝없는 감사와 사랑의 마음을 전합니다.

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High mobility group box 1 (HMGB1), which mainly exists in the nucleus, can be secreted into extracellular area and functions as a DAMP molecule through TLR 2, 4, and RAGE. Recently, the function of HMGB1 in cytoplasm has been reported as a sentinel molecule for viral nucleic acid sensing and a autophagy regulator. In this study, we aimed at discovering unknown function of cytoplasmic HMGB1 and identified the chaperone-like activity of HMGB1. We 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 luciferase activity as a reporter protein in O23 cells after co-transfection of firefly luciferase with HMGB1. Next, we demonstrated that HMGB1 prevented the formation of aggregates and toxicity caused by expanded polyglutamine (polyQ), the main cause of Huntington's disease. HMGB1 interacted with polyQ in immunofluorescence and co-immunoprecipitation assay, while the overexpression of HMGB1 or exogenous administration of recombinant HMGB1 proteins remarkably reduced polyQ induced aggregates in SHSY5Y cells and *hmgb1*-deficient mouse embryonic fibroblasts upon filter trap and immunofluorescence assay. Finally, overexpressed HMGB1 proteins in mouse primary embryonic striatal neurons also bound to polyQ and decreased the formation of polyQ aggregates. To this end, we have demonstrated that HMGB1 has chaperone-like activity and suggest the possibility of HMGB1 as a therapeutic agent in polyQ disease.

Keywords: HMGB1, chaperone, polyglutamine

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I. INTRODUCTION

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 known 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 Toll-like receptor (TLR) -2, -4, and receptor for advanced glycation end products (RAGE)[5,6] and promotes inflammation. HMGB1 can act as a late mediator of endotoxemia and sepsis in animal models and human patients.[7-10] HMGB1 moves to the cytoplasm in a redox-dependent mechanism and plays a role as a regulator between macroautophagy and apoptosis in the cytoplasm by binding to beclin-1 but dissociating from bcl-2.[11]

Some proteins form aggregates under stressful conditions such as increase of reactive oxygen species, metal ions or aging.[12,13] Aggregated proteins can be cleared by proteasome or macroautophagy[14,15] and chaperones modify these proteostasis strongly.[16,17] Polyglutamine (polyQ) diseases such as spinobulbar atrophy, dentatorubral-pallidoluysian atrophy, Huntington's disease, have been known to be caused by the aggregation of polyglutamine proteins within neuronal cells.[18] The pathologic mechanisms underlying such diseases seem to derive from the common signature present in all genes, amplification of GAC repeat encoding the amino acid glutamine (Q) in the context of different proteins. The hallmark of these diseases is the

accumulation of aggregates containing fragment of polyQ proteins. The polyglutamine expansion interferes with basic cellular processes such as transcription, protein degradation and survival/death signaling.[19] Huntington's disease (HD) is a autosomal dominant neurodegenerative disorder caused by the aggregation of an expanded polyglutamine (polyQ) of the mutant huntingtin protein (mHtt), which is a cytosine-adenine-guanine repeat of over 35 in the gene coding for the protein huntingtin.[20] The aggregates formed by polyQ induce neuronal cell toxicity by several mechanisms such as proteasomal dysfunction,[21] sequestration of transcriptional factors.[22] Therefore the clearance of aggregates formed by polyQ is a main therapeutic target in Huntington's disease.

Molecular chaperones are defined as any protein that interact with and stabilize another proteins to acquire its functionally active conformation, without being present in its final structure.[23] Chaperones like HSP70 can suppress the formation of polyQ aggregates and alleviate the toxicity in cultured cells and animal model.[24,25] Up-regulation of chaperones decreases the formation of aggregates therefore, chaperone molecules were considered as therapeutic targets in many aggregate-prone diseases.[26] It was reported that in response to

oxidative stress, cytoplasmic Hsp72, well known chaperone molecule, translocates and interacts with nuclear proteins, including HMGB1.[27,28] Therefore, we aimed at finding a novel role of HMGB1 as a chaperone-like molecule in cytoplasm just like in the nucleus, where HMGB1 works as a DNA chaperone, in similar way with well known chaperone molecules.

We investigated the chaperone-like function of HMGB1 using physical stresses on target proteins and intracytoplasmic stress on luciferase and polyQ proteins. We show that HMGB1 inhibited the chemically-induced aggregation of insulin and lysozyme as well as the heat-induced aggregation of citrate synthase *in vitro*. In O23 cells, HMGB1 also decreased heat induced inactivation of the luciferase protein. In a disease model of polyQ, we demonstrated that HMGB1 proteins could bind to polyQ and reduce the formation of aggregates and diminished the cytotoxicity caused by polyQ aggregates.

II. MATERIALS AND METHODS

1. Cell culture and DNA transfection

Human neuroblastoma SHSY5Y, human embryonic kidney epithelial HEK293, Chinese hamster O23, mouse fibroblast NIH3T3 cells were cultured at 37^{o}C under 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco by Invitrogen, Carlsbad, California, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine. The cells were transfected with polyQ and/or HMGB1 plasmid with the aid of Lipofectamine 2000 (Invitrogen). Electroporation was used for transfection in MEF cells using microporator (Invitrogen). Primary mouse embryonic striatal neuron cells were prepared from embryonic day 14. Dissociated striatal neurons were plated at a density of 3.5 hemispheres per 10 ml of media onto poly-D-lysine (100 μg/ml) and laminin (100 μg/ml) coated plates. The cultures were maintained with antibiotics in Neurobasal medium (Gibco by Invitrogen) supplemented with B27 (Invitrogen). The cells were used for transfection after 7 days of culture.

2. Plasmids and recombinant proteins

Human huntingtin plasmid containing exon 1 with 25 glutamine or 97 glutamine repeats followed by a GFP sequence (25Q-GFP or 97Q-GFP) was used for the aggregation study. pRSVLL/V encoding cytoplasmic luciferase plasmid (a kind gift of Dr. Harm H. Kampinga, University of Groningen, Netherlands) was used for intracytoplasmic protein refolding measurement after heat shock treatment.[29] Myc-tagged HMGB1 wild type, HMGB1 C106A mutant and HMGB1 boxes A (aa 1-79) and B (aa 88-162) plasmids were used for the transfection study.[30] We used recombinant HMGB1 proteins produced in insect High Five cells (Insect HMGB1; ATGen Co., Seongnam, South Korea), mouse myeloma cell line of NS0 (R&D Systems, Minneapolis, Minnesota, USA) or human cells (Euk HMGB1, Sino Co., Beijing, China).

3. Measurement of chaperone-like activity of HMGB1 *in vitro*

A turbidity measurement was used to measure the level of protein aggregation following a protocol with minor modifications.[31] To check the chaperone-like effect of HMGB1 in chemical induced protein aggregation, 0.8 mg/ml of insulin (Sigma-Aldrich, St. Louis, Missouri, USA) and 1 mg/ml of lysozyme (Sigma-Aldrich) in 10 mM PBS buffer

(pH 7.4) were incubated at 37^{o}C with 20 mM DTT. For the heat induced aggregation, a citrate synthase protein solution (0.25 μM) was prepared in 50 mM HEPES buffer (pH 8.0) and incubated for 1.5 h at 43^{o}C in the thermostatic cell holder. Insect origin HMGB1 proteins were added in same conditions and checked the light absorbance induced by target protein aggregation. In both chemical and heat induced aggregation experiments, total volume of protein solutions was 50 μL. Absorbance was monitored at 320 nm with spectrophotometer under UV-on condition (Beckman, Urbana, Illinois, USA).

4. Measurement of luciferase activity

Chinese hamster O23 cells were transiently transfected with luciferase plasmid of pRSVLL/V and HMGB1 plasmids[29,30] to check the chaperone-like activity of HMGB1 in cell system. 5 X 10^{5} number of O23 cells were seeded in 60 mm culture dish and transiently co-transfected with cytoplasmic luciferase and HMGB1 plasmids with the aid of Lipofectamine 2000. The cells were split into 24 well plates 24 h after transfection and incubated at 37^{o}C for 24 h. The plates were treated with cycloheximide (20 μg/ml) for 30 min to prevent further translocation and

heat shock-treated at 43^{o}C for 30 min. After heat shock treatment, cells were washed three times with PBS and then lysed to measure the change of luciferase activity. Luciferase activity per μg protein was measured following the manufacture's protocol of Dual luciferase reporter assay system (Promega, Madison, Wisconsin, USA). pcDNA and pCMV-HSP70 was transfected as a negative and positive control of chaperone-like function.

5. Treatment of ROS scavengers

To figure out that ROS generated by polyQ aggregates influence on the cytoplasmic translocation of HMGB1 protein, we pretreated ROS scavengers and compared the number of cells which contain cytoplasmic HMGB1 protein. 5 X 10^4 number of SHSY5Y cells were seeded in 4 chamber slide and maintained for 24 hours. Cells were pretreated with 10 mM of N-acetyl-L-cysteine (NAC, Sigma-Aldrich) and 25 nM of Mito-TEMPO (Enzo Life Sciences, Farmingdale, New York, USA) 1 h before 97Q-GFP transfection. 48 h after transfection, endogenous HMGB1 was immunostained with anti-HMGB1 antibody (Abcam, Cambridge, UK) and cytoplasmic HMGB1 proteins were observed by confocal microscopy (Olympus, Center Valley, Pennsylvania, USA). We counted

100 GFP-positive cells in multiple random visual fields in each condition and compared the number of cytoplasmic HMGB1 containing cells.

6. Western blot analysis

To detect the level of protein expression, cells were lysed using Pro-prep protein extraction solution (Intron, Seoul, South Korea) including a mixture of protease inhibitors (Sigma-Aldrich). Certain amount of protein samples were loaded on 12% SDS-PAGE and transferred to nitrocellulose membrane. A western blot analysis was performed using primary antibodies of rabbit anti-HMGB1 (Abcam), mouse anti-firefly luciferase (Abcam), and mouse anti-GFP (SantaCruz Biotechnology, Santa Cruz, California, USA) and secondary antibodies of HRP-labeled goat anti-rabbit, goat anti-mouse Ig (Jackson Labs, Bar Harbor, Maine, USA). ECL was used to reveal the signals (Pierce, Rockford, Illinois, USA). Relative band intensities were measured using Image J program (NIH).

7. Immunoprecipitation

To identify the binding of HMGB1 with 97Q-GFP, cells were transiently co-transfected with the indicated plasmid of 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^{o}C for 1 h. The precleared extracts were incubated with mouse anti-GFP antibody (Abcam) overnight, and then the protein G-Sepharose was added for 2 h at 4^{o}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.

8. Immunofluorescence and confocal imaging

Cells were cultured in 4 chambers (Nunc, Roskilde, Denmark) and fixed with 4% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4 mM $MgSO_4$, pH 7.0) for 20 min at RT. After fixation, cells were washed three times with cold PBS and permeabilized with 0.2% Triton X-100 for 10 min, and then blocked with 1% BSA-PBS for 1 h at RT. A primary antibody in 1% BSA-PBS was added and incubated overnight at 4^{o}C. The cells were washed three times with cold PBS and Alexa 488- or Alexa 594-conjugated secondary antibody (Invitrogen) in 1% BSA-PBS was added to the cells for 1 h at RT. The cells were washed three times with cold PBS and mounted with

Vecta shield mounting solution (Vector laboratories, Burlingame, California, USA) and observed under a FV1000 confocal microscope (Olympus).

9. Cell viability measurement

CCK8 assay was performed to measure the cytotoxicity by polyQ overexpression. Briefly, cells at a density of 5 X 10^3 cells per well in 96 well plate were cultured for 24 hours and transfected with 25, 97Q-GFP plasmid. 48 after transfection, 10 μL of CCK-8 reagent was added to each well and incubated for 2 h at 37℃. Cell viability was evaluated by measuring absorbance at 450 nm. For propidium iodide (PI) staining, working solution was made by adding 2 μl of 1 mg/ml PI solution (Sigma-Aldrich) and 20 μL of 1 mg/ml RNase A (Bio Basic INC) into 2 ml PBS. PI working solution was added to culture slide for 20 min at 37℃ and the remaining solution was washed with PBS Trypan blue staining was performed by mixing same volume of trypan blue and cell suspension. Trypan blue staining positive cells were counted 48 h after transfection.

10. Filter trap assay

SHSY5Y cells were overexpressed with a construct of 97Q-GFP for 48 h in the presence of HMGB1 or control plasmids. Cells were sonicated in 100 μL of PBS containing 1 mM PMSF, and the cell lysates were harvested after centrifugation. Total of 200 μg of proteins were taken and filtered through a 0.22 μm cellulose acetate membrane. The membrane was pre-equilibrated with PBS containing 1% SDS. A dot blotter apparatus (Millipore, Billerica, Massachusetts, USA) was used for the application of samples. After samples were passed through the membrane, the membrane was washed twice with PBS containing 1% SDS buffer. Then, the membrane was immunoblotted with an anti-GFP antibody.

11. Proximity ligation assay (PLA)

Molecular interaction was evaluated with the aid of Duolink PLA kit (Olink Bioscience, Uppsala, Sweden). Briefly, cells were cultured in 4 chambers (Nunc) and fixed with 4% paraformaldehyde in PHEM buffer for 20 min at RT. After fixation, cells were washed three times with cold PBS and incubated with a blocking agent for 1 h. Primary antibodies against HMGB1 and GFP were added and incubated overnight

and then washed three times (washing buffer: 150 mM NaCl, 1 mM Tris base, and 0.05% Tween 20, pH 7.4). Duolink PLA probes were applied and incubated for 1 h in a humidity chamber at 37^{o}C. Unbound Duolink PLA probes were removed with wash buffer, and the samples were incubated in the ligation solution for 1 h. For amplification, diluted polymerase was applied for 100 min and the amplified probe was detected with diluted Duolink Detection stock. Images were taken by confocal microscopy.

12. Statistical analysis

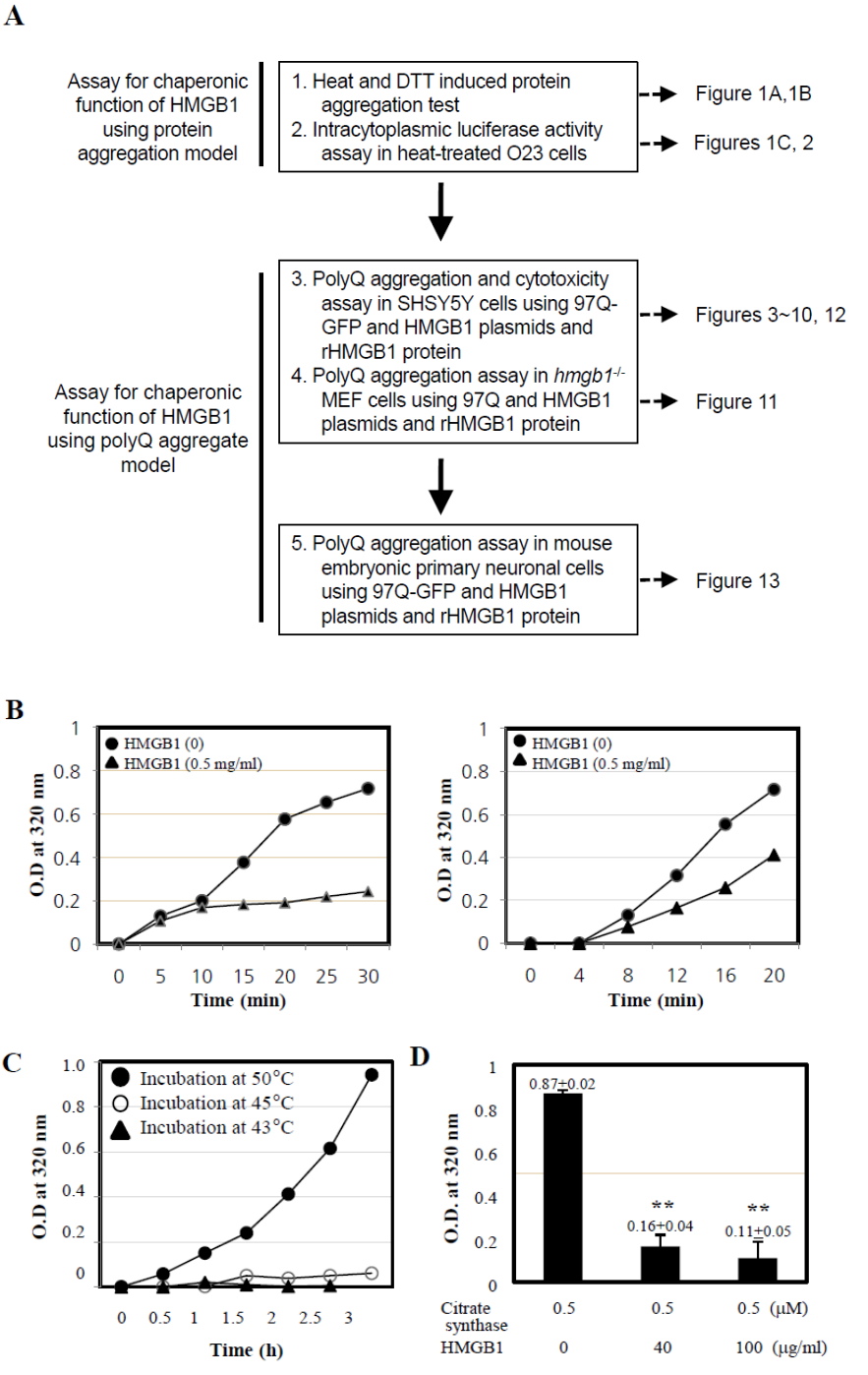
Statistical analysis was performed by analysis of variance (ANOVA) followed by the Bonferroni test or student's t-test. All data were presented as mean ± S.E.M.

III. RESULTS

1. HMGB1 reduces physical stress induced protein denaturation *in vitro*.

To investigate whether HMGB1 has chaperone-like activity, the ability to prevent chemical and heat induced target protein aggregations was evaluated by measuring turbidity *in vitro*. After that, chaperone-like activity of HMGB1 in cells were checked using luciferase overexpressed O23 and *hmgb1*$^{-/-}$ MEFs. Finally, to check the ability of HMGB1 to reduce polyglutamine aggregates, primary mouse neuronal cells were used (Fig 1A). Insulin and lysozyme were used as target protein for the chemical (DTT) induced aggregation. Insulin and lysozyme in 10 mM phosphate buffer (pH 7.4) were incubated with 20 mM DTT in the presence or absence of HMGB1 protein at 37^{o}C for the indicated time, and the degree of light scattering caused by aggregation was measured. As shown in Fig. 1B, DTT induced aggregations, indicated by the absorbance of light scattering at 320 nm, of both insulin and lysozyme were increased with time, however, the level of aggregation was decreased with the addition of rHMGB1 protein. In the heat shock

induced protein aggregation experiments, citrate synthase was incubated with rHMGB1 protein at 43^{o}C. In our previous study, HMGB1 protein became turbid at high temperature (Fig. 1C), citrate synthase was used since it is sensitive to high temperature. [32] Heat induced aggregation of 0.5 μM of citrate synthase was significantly decreased to 0.16 and 0.11 from 0.86 O.D. with the addition of 40 and 100 μg/ml of HMGB1, respectively (Fig. 1D).


A
Assay for chaperonic function of HMGB1 using protein aggregation model
1. Heat and DTT induced protein aggregation test
2. Intracytoplasmic luciferase activity assay in heat-treated O23 cells
Figure 1A,1B
Figures 1C, 2
Assay for chaperonic function of HMGB1 using polyQ aggregate model
3. PolyQ aggregation and cytotoxicity assay in SHSY5Y cells using 97Q-GFP and HMGB1 plasmids and rHMGB1 protein
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4. PolyQ aggregation assay in hmgb1-/- MEF cells using 97Q and HMGB1 plasmids and rHMGB1 protein
Figure 11
5. PolyQ aggregation assay in mouse embryonic primary neuronal cells using 97Q-GFP and HMGB1 plasmids and rHMGB1 protein
Figure 13
B
HMGB1 (0)
HMGB1 (0.5 mg/ml)
O.D at 320 nm
Time (min)
C
Incubation at 50°C
Incubation at 45°C
Incubation at 43°C
O.D at 320 nm
Time (h)
D
O.D. at 320 nm
0.87±0.02
0.16±0.04
0.11±0.05
**
Citrate synthase 0.5 0.5 0.5 (μM)
HMGB1 0 40 100 (μg/ml)

Figure 1. HMGB1 suppresses chemical and heat induced aggregation of target proteins. (A) Summarized study design. (B) Aggregation curve of insulin (0.8 mg/ml, left pannel) and lysozyme (1 mg/ml, right pannel) in the absence of rHMGB1 (circle) and in the presence of rHMGB1 protein (0.5 mg/ml, triangle). (C) rHMGB1 protein (0.5 mg/ml) was incubated at different temperature for 3 h and the aggregation was measured by optical density (O.D) at 320 nm. Dark circle, 50^{o}C; open circle, 45^{o}C; dark triangle, 43^{o}C. (D) Heat induced aggregation of citrate synthase. Samples were incubated at 43^{o}C for 90 min with or without rHMGB1 protein.

2. HMGB1 prevents heat induced inactivation of luciferase activity in O23 cells.

In order to confirm that HMGB1 has chaperone-like activity not only *in vitro*, but also in viable cells, we checked heat induced inactivation of luciferase activity. Chinese hamster O23 cells were used as a model for determining intracellular chaperone-like function by overexpressing luciferase plasmid.[29] O23 cells were transiently co-transfected with luciferase plasmid together with CMV-myc or HMGB1-myc or HSP 70-myc plasmids. Luciferase activity after thermal stress, which exposed to the high temperature condition at 43^{o}C for 30 min, was observed by measuring light absorbance. When O23 cells were co-transfected with luciferase and HMGB1 plasmids, luciferase activity protected from direct thermal stress was increased compared to the controls (Fig. 2A). CMV-myc was co-transfected with luciferase plasmid as a negative control and HSP70 plasmid was co-transfected as a positive control, respectively.

HMGB1 contains cysteine residue at 106 (C106), which is important for the intermolecular interaction.[33] O23 cells were co-transfected with luciferase and HMGB1 C106A plasmids to deterimine if cysteine 106 of HMGB1 plays an important role in the chaperone-like

activity. Under same condition, there was no significant difference between wild type HMGB1 and mutant HMGB1 C106A, suggesting that the chaperone-like effect of HMGB1 is not related to intermolecular di-sulfide interaction (Fig. 2B).

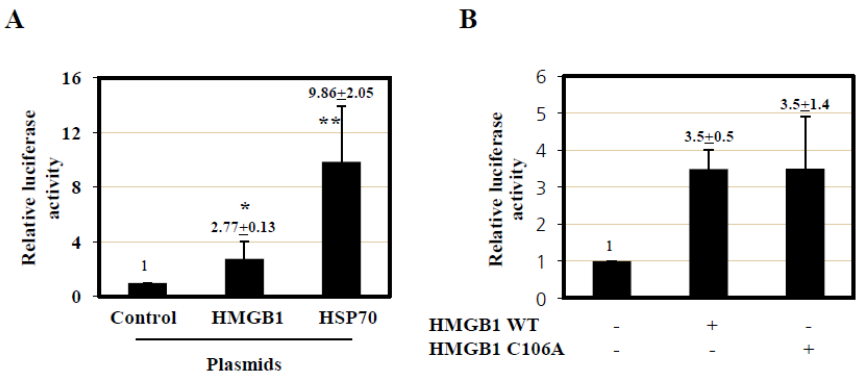
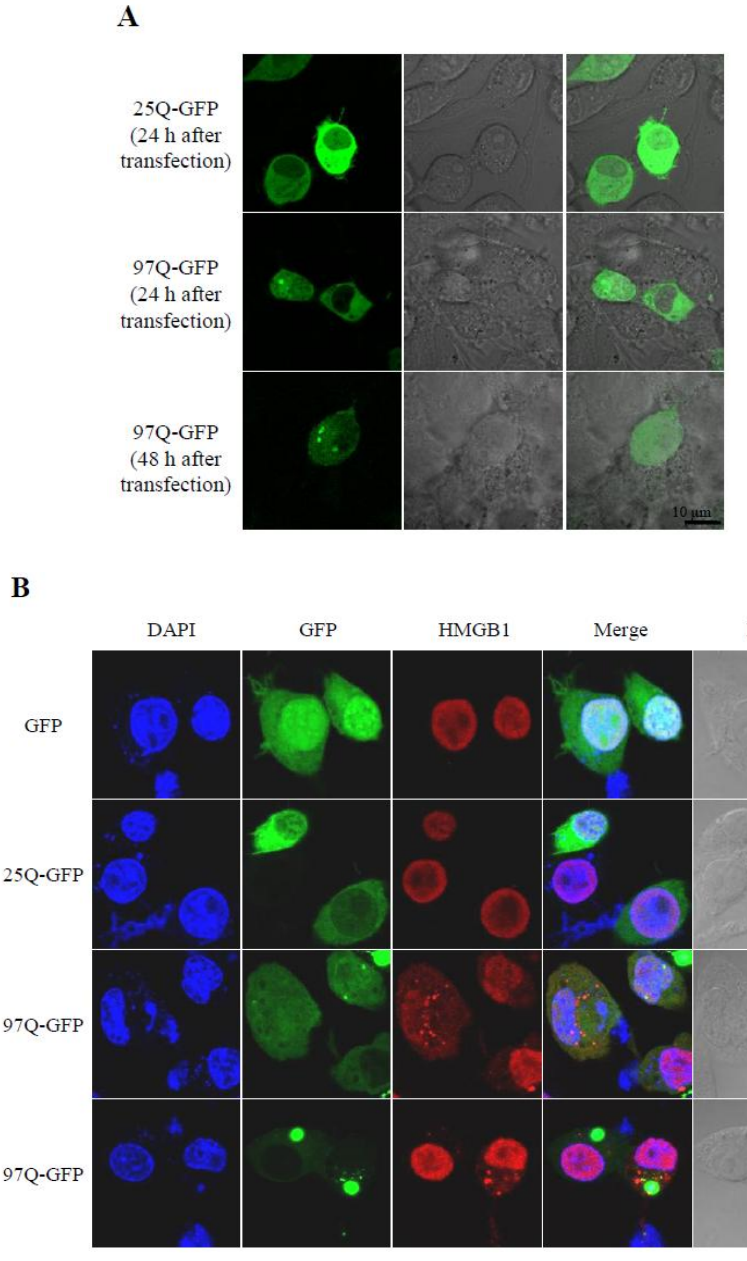


Figure 2. HMGB1 prevents heat induced luciferase inactivation. (A) O23 cells were transiently co-transfected with luciferase and HMGB1 plasmids. HSP70 plasmid and empty vector pcDNA were used as a positive and a negative controls, respectively. Cells were pretreated with cycloheximide for 30 min and heat shock-treated for 30 min at 43℃ to inactivate luciferase. (B) Cysteine 106 mutant HMGB1 shows similar function to wild type HMGB1 in O23 cells. O23 cells were transiently co-transfected with luciferase and HMGB1-myc or cysteine 106 mutant HMGB1 plasmids. pcDNA was used as a control plasmid. Data shown as mean ± S.E.M (N=3). * $p < 0.05$, ** $p < 0.01$ (N=3).

3. Overexpression of 97Q-GFP induces nucleocytoplasmic translocation of HMGB1.

To check whether the expanded polyQ induces HMGB1 translocation, we first tested the aggregates formation of 97Q-GFP in SHSY5Y cells. When SHSY5Y cells were transfected with 97Q-GFP construct, distinct aggregates could be observed after 48 h, but this was rare in 25Q-GFP (Fig. 3A). For 97Q-GFP transfected cells, nucleocytoplasmic translocation of HMGB1 was clearly observed (Fig. 3B). Aggregation of a fragment of huntingtin protein directly causes free radical production *in vivo*,[34] and oxidative potential is important for the nucleocytoplasmic translocation of HMGB1.[18] To test whether ROS scavenging inhibits the translocation of HMGB1, NAC, a precursor of glutathione which acts as a scavenger for the OH radical and Mito-tempo, mitochondria-targeted antioxidant with superoxide and alkyl radical scavenging properties, were treated. As shown in Fig. 3C, scavenging of ROS generated from overexpressed 97Q-GFP with NAC and Mito-Tempo decreased the translocation of HMGB1.


A
25Q-GFP
(24 h after
transfection)
97Q-GFP
(24 h after
transfection)
97Q-GFP
(48 h after
transfection)
10 μm
B
DAPI
GFP
HMGB1
Merge
DIC
GFP
25Q-GFP
97Q-GFP
97Q-GFP
10 μm

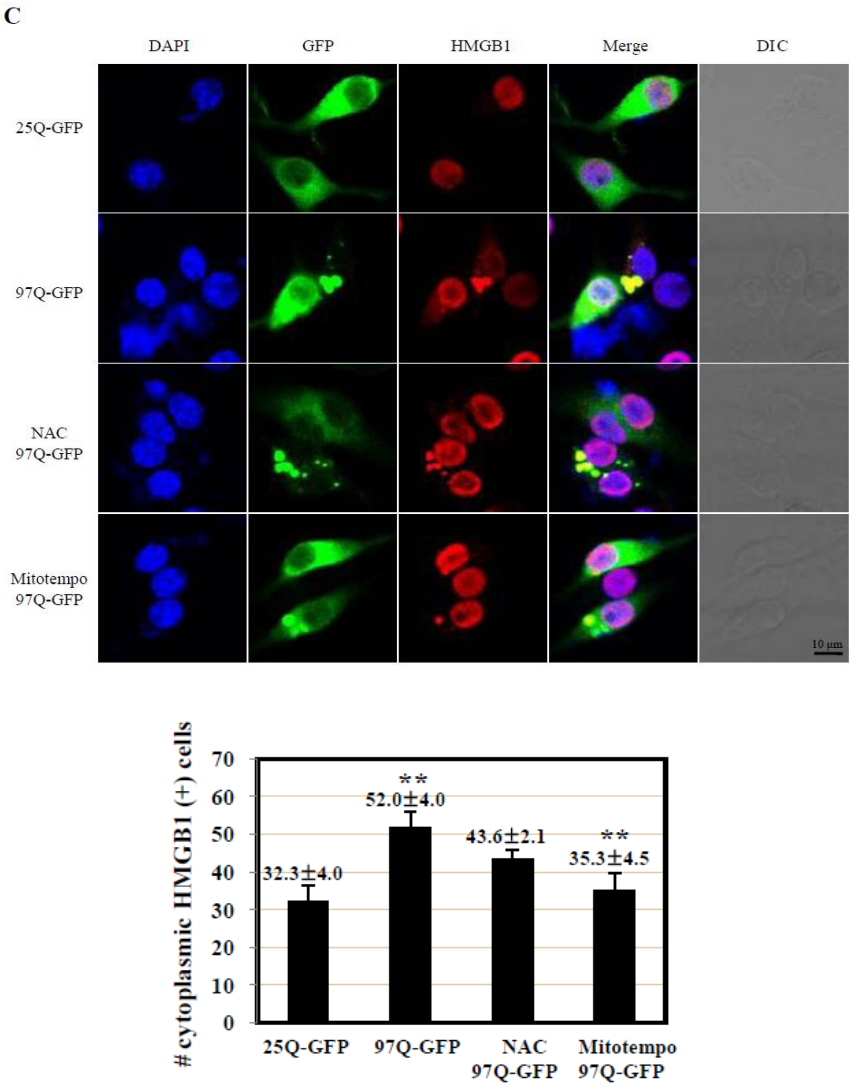


Figure 3. Aggregation of overexpressed 25Q-GFP and 97Q-GFP in SHSY5Y cells. (A) SHSY5Y cells were transfected with 25Q-GFP or 97Q-GFP plasmid and incubated for 24 h or 48 h. Formation of aggregates was observed with confocal microscopy. (B) SHSY5Y cells were transfected with 25Q-GFP or

97Q-GFP plasmid and incubated for 48 h. Endogenous HMGB1 was immunostained with anti-HMGB1 antibody and alexa 594-conjugated secondary antibody. Aggregation of 97Q-GFP and the translocation of HMGB1 was observed with confocal microscopy. (C) SHSY5Y cells were transfected with 25Q-GFP or 97Q-GFP plasmid and incubated for 48 h. Antioxidants NAC (10 mM) and Mito-TEMPO (25 nM) were applied 1 h before transfection and endogenous HMGB1 was immunostained to compare degree of translocation. The number of cytoplasmic HMGB1 containing cells was counted. * $p < 0.05$, ** $p < 0.01$ (N=3).

4. HMGB1 interacts with 97Q in SHSY5Y cells.

To observe whether HMGB1 binds to 97Q-GFP, SHSY5Y cells were transfected with 97Q-GFP and endogenous HMGB1 proteins were immunostained with anti-HMGB1 antibody. Aggregates of 97Q-GFP were co-localized with HMGB1, which were translocated to the cytoplasm (Fig. 4A). The bindings of HMGB1 with 97Q-GFP and 25Q-GFP were also proved by co-immunoprecipitation assay in SHSY5Y and HEK 293 cells (Fig. 4B). To confirm the interaction, SHSY5Y cells were transfected with 97Q-GFP plasmid and Duolink PLA was performed. As shown in Fig. 4C, colocalization of endogenous HMGB1 protein with 97Q-GFP, which signals as red light, was observed in confocal microscopic analysis and a quantitative comparison was shown (Fig. 4C right panel). When we investigated the binding domains of HMGB1 to 97Q-GFP protein, both box A and B proteins showed the binding to 97Q-GFP by co-immunoprecipitation analysis in both SHSY5Y and HEK293 cells (Fig. 5).

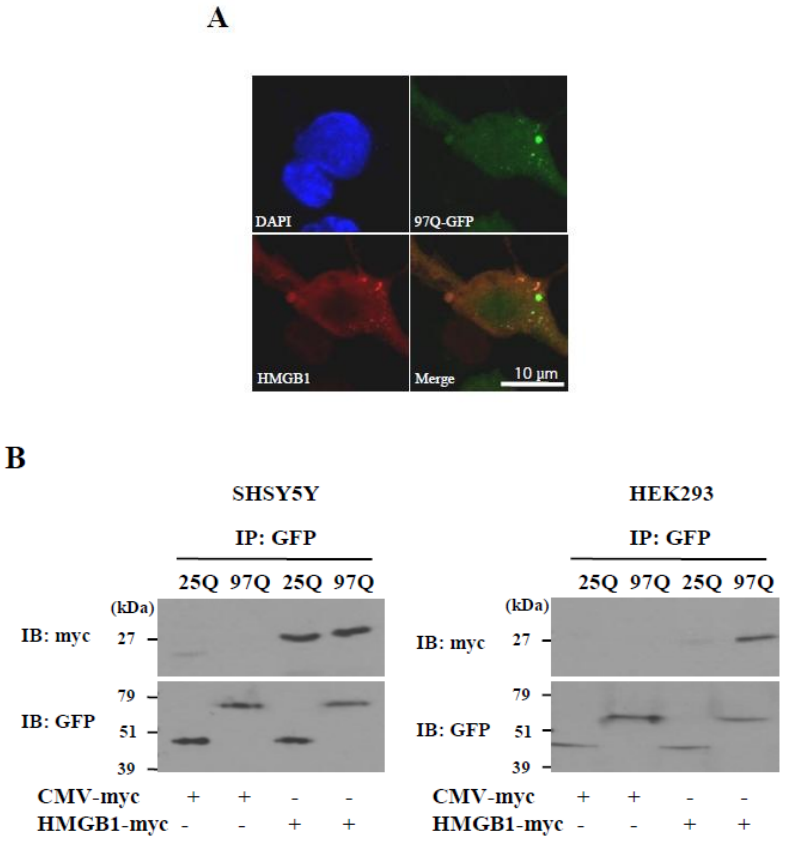


Figure 4. HMGB1 interacts with 97Q-GFP. (A) Immunofluorescent confocal analysis showing colocalization of HMGB1 with 97Q-GFP. SHSY5Y cells were transfected with 97Q-GFP and endogenous HMGB1 was immunostained with anti-HMGB1 antibody to observe the co-localization of 97Q-GFP and endogenous HMGB1. (B) SHSY5Y (left panel) and HEK293 cells (right panel) were co-transfected with 25, 97Q-GFP and HMGB1-myc plasmids, and the cell lysates were immunoprecipitated with anti-GFP antibody followed by immunoblotting with anti-myc antibody to detect HMGB1. pCMV-myc was used

control plasmid. (C) Duolink proximity ligation assay (PLA). SHSY5Y cells were transfected with 97Q-GFP and PLA was performed to observe the colocalization between 97Q-GFP and endogenous HMGB1 protein. PLA signal were counted per 50 GFP-positive cells (right panel).

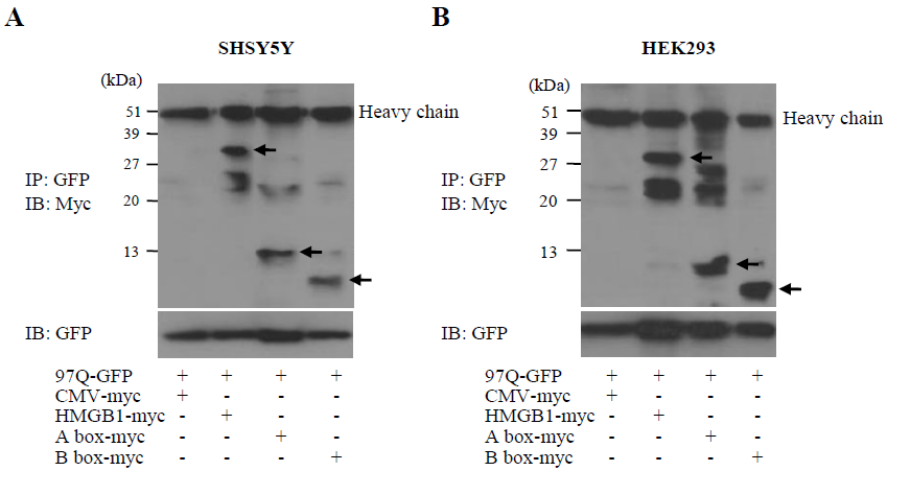
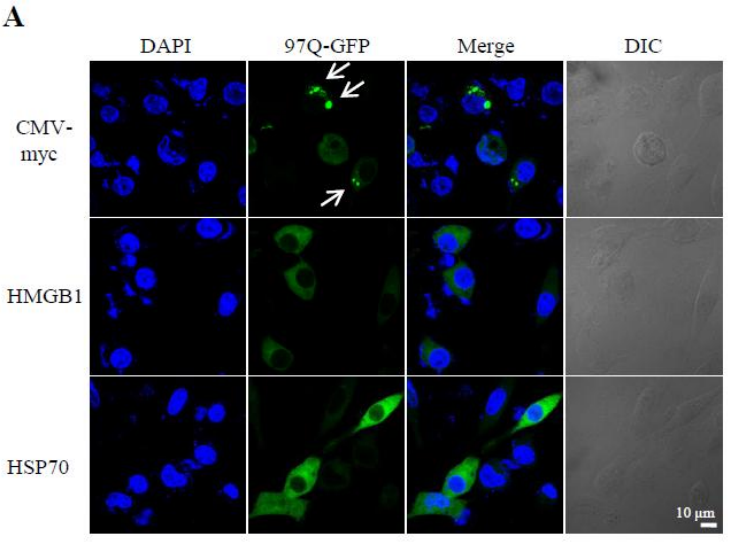


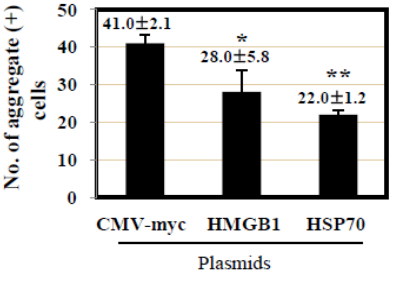
Figure 5. Interaction of WT HMGB1, box A and B proteins with 97Q-GFP. SHSY5Y (A) and HEK 293 cells (B) were transiently co-transfected with 97Q-GFP and each plasmid coding HMGB1, box A and B. pcCMV-myc was used as a negative control. The cell lysates were co-immunoprecipitated to check the interaction between 97Q-GFP and HMGB1 proteins. Lysates were immunoprecipitated with anti-GFP antibody for 97Q, and then immunoblotted with anti-Myc antibody for various types of HMGB1. Expression of 97Q-GFP protein was examined for loading control. Arrows indicates WT HMG1, box A and B proteins.

5. HMGB1 suppresses the formation of polyQ aggregates.

To investigate the effect of HMGB1 on the aggregation of polyQ, SHSY5Y cells were transfected with a construct of 97Q-GFP and incubated for 48 h in the presence or absence of HMGB1. We counted 100 GFP-positive cells in multiple random visual fields in each condition and compared the number of aggregates containing cells among GFP-positive cells.

The number of aggregates containing cells per 100 GFP positive cells was significantly reduced when the cells were co-transfected with HMGB1 plasmid (Fig. 6A, B) and the size of the aggregates was decreased in HMGB1 co-transfected cells (data not shown). When the amount of aggregates were 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 of overexpressed HMGB1 proteins (Fig. 6C). The protein expression of transfected HMGB1 plasmid was confirmed by western blot assay (Fig. 6C lower panel).


A
DAPI
97Q-GFP
Merge
DIC
CMV-myc
HMGB1
HSP70
10 μm


B
No. of aggregate (+) cells
50
40
30
20
10
0
41.0±2.1
*
28.0±5.8
**
22.0±1.2
CMV-myc
HMGB1
HSP70
Plasmids

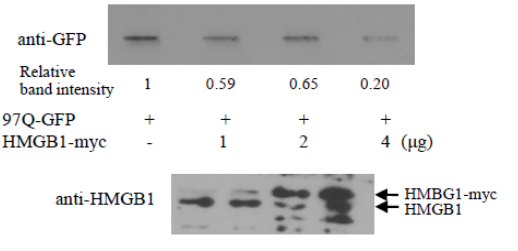

C
anti-GFP
Relative band intensity
1
0.59
0.65
0.20
97Q-GFP
+
+
+
+
HMGB1-myc
-
1
2
4 (μg)
anti-HMGB1
HMBG1-myc
HMGB1

Figure 6. Aggregation of polyQ fragments is suppressed by HMGB1. (A) SHSY5Y cells were co-transfected with 97Q-GFP and HMGB1-myc plasmids and cultured for 48 h. pcDNA and HSP70 plasmids were used as a negative and a positive controls, respectively. Arrows indicate aggregates formed by 97Q-GFP. (B) Number of aggregate containing cells was counted per 100 GFP-positive cells. The experiments were repeated three times. * $p < 0.05$, ** $p < 0.001$ to the control (CMV-myc). (C) Analysis of 97Q-GFP aggregates using filter trap analysis. SHSY5Y cells were co-transfected 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 and expression of transfected plasmids was checked by western blot analysis.

6. Cysteine 106 of HMGB1 has no significant effect on the chaperone-like function.

As cysteine 106 mutant HMGB1 showed similar chaperone-like activity in O23 cells, we checked the effect of cysteine 106 mutant HMGB1 in this system. When we co-transfected C106A mutated HMGB1 (C106A HMGB1) with 97Q-GFP plasmid, the number of aggregate containing cells was found to be significantly reduced compared to control sample in immunofluorescence assay (Fig. 7A) and the amount of aggregates were reduced in filter trap assay (Fig. 7B). We could not find statistically important difference between wild type and C106 mutant HMGB1 in chaperone-like function. The interaction between C106A HMGB1 and 97Q-GFP was detected by immunoprecipitation assay (Fig. 7C).

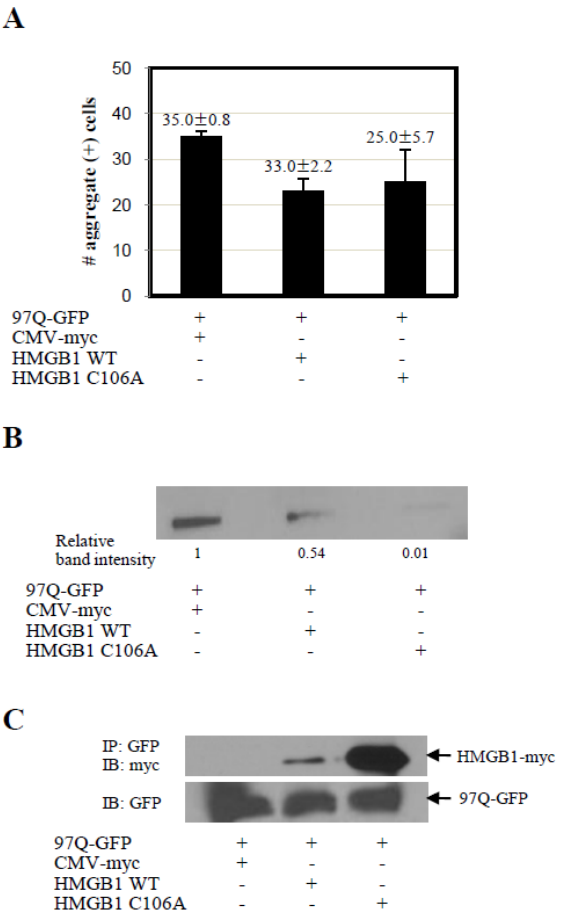


Figure 7. Chaperone-like activity of HMGB1 is not dependent on cysteine 106. SHSY5Y cells were co-transfected with 97Q-GFP and HMGB1-myc or cysteine 106 mutated HMGB1 plasmids and cultured for 48 h. pcCMV-myc was used as a negative control. The number of aggregate containing cells was counted per 100 GFP-positive cells. These experiments were repeated three times, and mean ± S.E.M. was depicted. (B) Filter trap analysis was performed using the cell lysates of each group. Relative band intensities were measured. (C) SHSY5Y

cells were transiently co-transfected with 97Q-GFP and WT HMGB1 or C106A mutant HMGB1 plasmids and co-immunoprecipitated to check the interaction of two molecules. Immunoprecipitation was performed with anti-GFP antibody, and immunoblotted with anti-myc antibody.

7. Exogenously administered recombinant HMGB1 proteins penetrate plasma membrane and enter into the cytoplasm.

Next, we evaluated the effect of rHMGB1 protein treatment on the cells overexpressing 97Q-GFP since exogenous HMGB1 proteins can enhance the gene delivery between the cells.[19] For this, SHSY5Y cells were transfected with 97Q-GFP and then treated with rHMGB1 proteins at 4 μg/ml concentration. First, we found previously unknown information as far as we know that the exogenously treated rHMGB1 proteins can penetrate the cell membrane and be detected in cell lysates as shown in western blot analyses (Fig. 8A). Rhodamine-conjugated rHMGB1 proteins penetrated the plasma membrane and were observed in the cytoplasm of cells. This finding was not inhibited by cytochalasin B pre-treatment, which prevents actin polymerization, suggesting that not only endocytotis but other mechanisms may be involved (Fig. 8B).

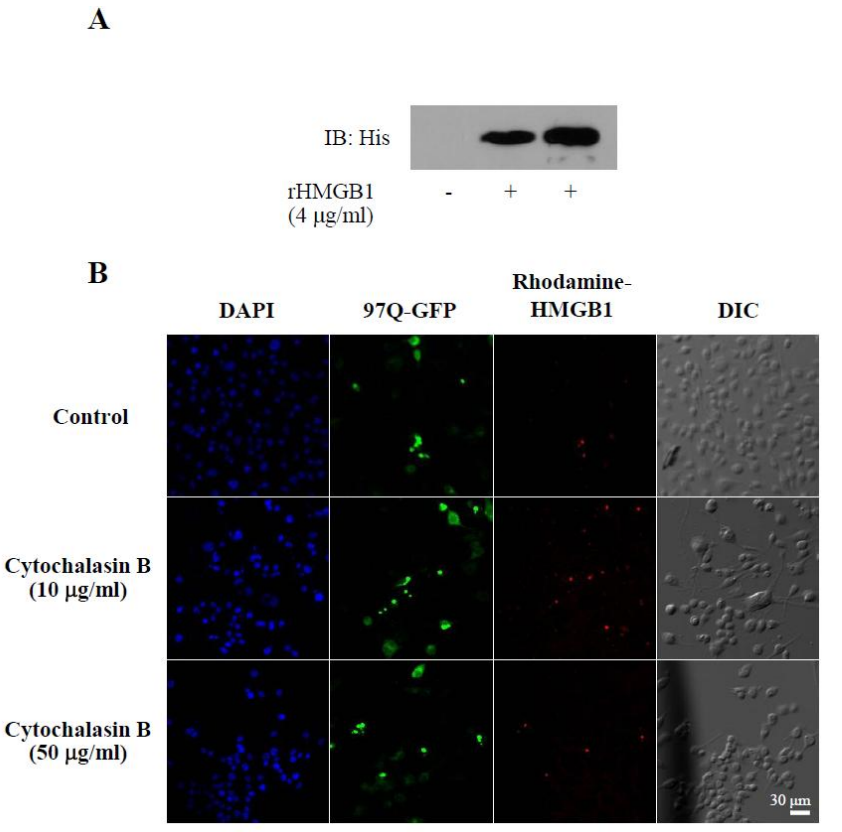


Figure 8. Exogenously administered rHMGB1 proteins were observed in the cytoplasm. (A) His-tagged rHMGB1 proteins were added into the culture medium of HEK 293 cells for 24 h. Western blot was performed with the cell lysates using anti-His antibody. rHMGB1 proteins were treated into two separate dishes #1 and #2. (B) SHSY5Y cells were transfected with 97Q-GFP and incubated for 24 h. Then cytochalasin B was pretreated to the culture medium for 1 h and rhodamine-conjugated rHMGB1 proteins were added in the culture medium. Rhodamine signal was observed under confocal microscopy.

8. Treatment of recombinant HMGB1 proteins into the culture media reduces the formation of polyQ aggregates.

We added rHMGB1 proteins purified from Hi Five insect cells to the culture media of SHSY5Y cells and incubated for 24 h and found that the formation of 97Q-GFP aggregates was significantly reduced following a dose-dependent relationship with rHMGB1 (Fig. 9A and B). The average number of aggregate containing cells per 100 GFP positive cells was reduced from 35.6±6.7 to 15.0±2.2 at 4 μg/ml of rHMGB1 protein treatment (Fig. 9B upper) and the average pixel density of 30 aggregates was reduced to 137.3 from 230.16 (Fig. 9B lower). Similar result was observed in filter trap assay (Fig. 9C). Unexpectedly, higher concentration of rHMBG1 protein treatment inversely increased the amount of aggregates (Fig. 9C).

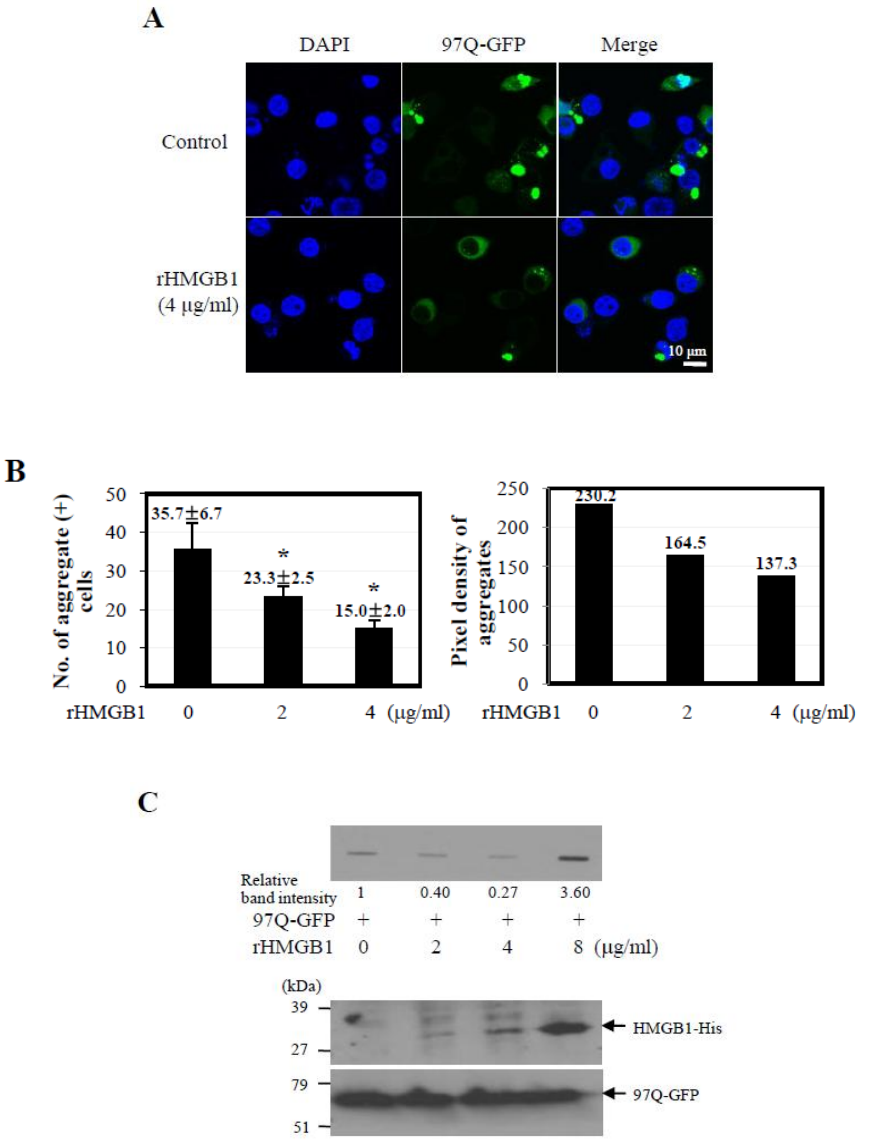


Figure 9. Effect of rHMGB1 proteins on the formation of 97Q-GFP aggregates (A and B). SHSY5Y cells were transiently transfected with 97Q-GFP plasmid and then treated with rHMGB1 proteins at the concentration of 4 μg/ml.

Distribution of 97Q-GFP aggregates was observed (A) and the number of aggregate containing cells was counted per 100 GFP-positive cells (B upper panel). * $p < 0.05$ to control group (N=3). The pixel density of 30 aggregates was measured after the treatment of rHMGB1 proteins (B lower panel). (C) SHSY5Y cells were transfected with 97Q-GFP plasmid and were incubated with rHMGB1 proteins. The formed aggregates were analyzed by filter trap assay. The membrane was immunoblotted with anti-GFP antibody to check the amount of aggregates (C upper panel). Exogenous rHMGB1 proteins which penetrated the cells were analyzed using anti-His antibody by western blot assay (C lower panel).

9. Exogenously administered recombinant HMGB1 proteins colocalize with 97Q-GFP in the cytoplasm.

We hypothesized that co-incubated rHMGB1 proteins might interact with polyQ and reduce the formation of aggregates. To check the binding of exogenously treated rHMGB1 proteins with the aggregates of 97Q-GFP, co-immunoprecipitation assay was performed in 97Q-GFP overexpressed SHSY5Y cells, which were treated with His-tagged rHMGB1 proteins (Fig. 10A). The binding of rHMGB1 proteins to 97Q-GFP was observed after immmunofluorescence by staining of the His (Fig. 10B). When rhodamine-conjugated rHMGB1 proteins were used to detect the interaction with 97Q-GFP, the binding could be clearly observed in confocal microscopy (Fig. 10C). These data suggest that co-incubated extracellular rHMGB1 proteins enter into the cytoplasm of the cells and interact with the aggregates.

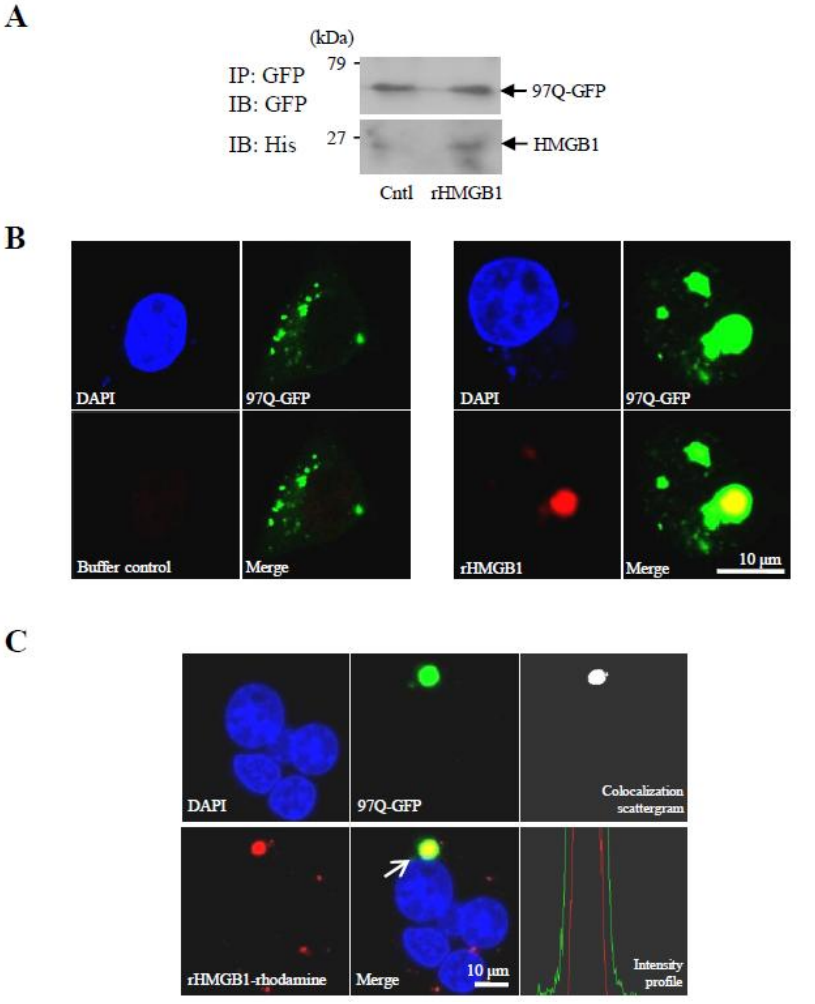


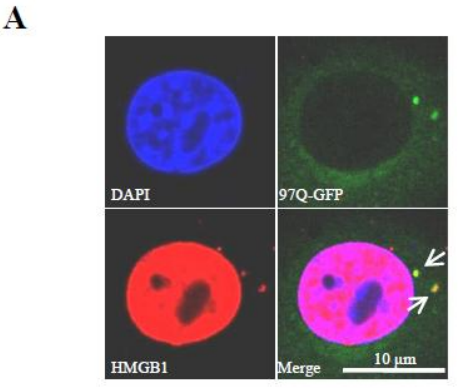
Figure 10. Exogenously administered rHMGB1 proteins colocalized with 97Q-GFP. (A) SHSY5Y cells were transiently transfected with 97Q-GFP plasmid and then rHMGB proteins were added to the culture medium at 4 μg/ml. SHSY5Y cell lysates were immunoprecipitated with anti-GFP and the binding of rHMGB1 proteins to 97Q-GFP was observed with anti-His antibody. (B) SHSY5Y cells

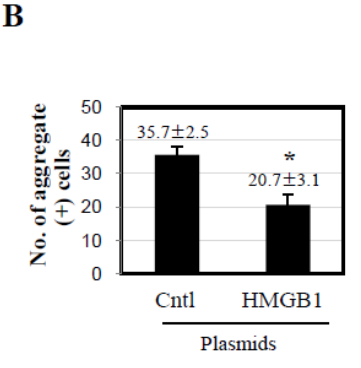
were transiently transfected with 97Q-GFP plasmid and then rHMGB proteins were added to the culture medium at 4 μg/ml. The cells were immunostained with anti-His antibody for intracellular staining of rHMGB1 proteins (low panel). Buffer-treated control is upper panel. (C) SHSY5Y cells were transiently transfected with 97Q-GFP and then treated with rhodamine-conjugated rHMGB1 proteins at 4 μg/ml concentration to detect the interaction with 97Q-GFP protein. The colocalization of 97Q-GFP and rhodamine-rHMGB1 proteins was observed using colocalization scattergram and intensity profile analysis which was measured across the aggregates (arrow).

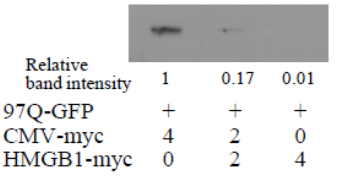
10. HMGB1 reduces the formation of mHtt aggregates in *hmgb1*$^{-/-}$ MEF cells

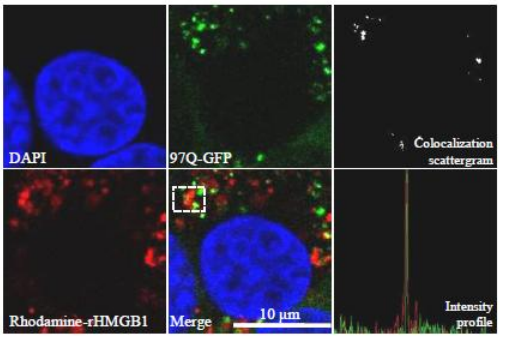
Next, we tested the effect of HMGB1 in *hmgb1*$^{-/-}$ MEF cells to confirm that endogenous HMGB1 assumes these functions. When *hmgb1*$^{-/-}$ MEF cells were co-transfected with 97Q-GFP and HMGB1-myc plasmids, the 97Q-GFP protein and its aggregates were observed as expected. Most of the expressed HMGB1 proteins were observed in the nucleus while some were in the cytoplasm. Reconstituted HMGB1 proteins were colocalized with 97Q-GFP aggregates in the cytoplasm (Fig. 11A). The number of aggregate positive SHSY5Y cells was significantly lower in *hmgb1* reconstituted cells (Fig. 11B), and the amount of 97Q-formed aggregates was lower in *hmgb1* reconstituted cells by filter trap analysis (Fig. 11C). When *hmgb1*$^{-/-}$ MEF cells transfected with 97Q-GFP were treated with rhodamine conjugated rHMGB1 proteins, colocalization of HMGB1 with 97Q-GFP was observed in confocal microscopy (Fig. 11D) and the number of aggregate containing cells was decreased (Fig. 11E). The band intensity of filter trap analysis was significantly weaker (Fig. 11F) which means there were fewer 97Q-formed aggregates in the rHMGB1 treated cells. We found that much amount of rHMGB1 proteins at concentration of 8 μg/ml, however,

slightly increased formation of aggregates by filter trap analysis (Fig. 11F).


A
DAPI
97Q-GFP
HMGB1
Merge
10 μm


B
No. of aggregate (+) cells
50
40
30
20
10
0
35.7±2.5
*
20.7±3.1
Cntl
HMGB1
Plasmids


C
Relative band intensity 1 0.17 0.01
97Q-GFP + + +
CMV-myc 4 2 0
HMGB1-myc 0 2 4


D
DAPI
97Q-GFP
Colocalization scattergram
Rhodamine-rHMGB1
Merge
10 μm
Intensity profile

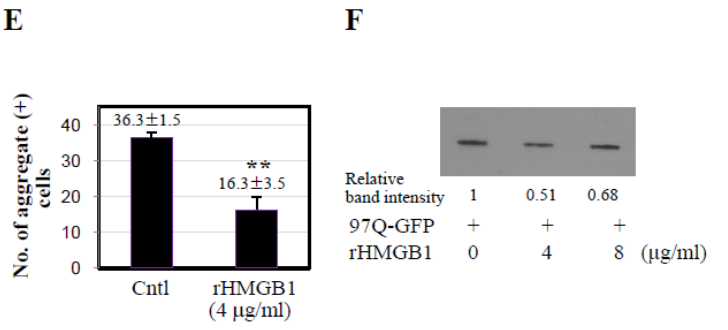


Figure 11. Effect of HMGB1 on the formation of 97Q-GFP aggregates in *hmgb1*$^{-/-}$ MEF cells (A and B). *Hmgb1*$^{-/-}$ MEF cells were transfected with a mixture of 97Q-GFP and HMGB1 plasmids, and the colocalization of 97Q-GFP and HMGB1 (arrow) was observed by immunostaining of HMGB1 protein. The number of aggregate containing cells was counted among 100 GFP-positive cells. Mean ± SEM, * p<0.05 (N=3). (C) Filter trap analysis of whole cell lysates of *hmgb1*$^{-/-}$ MEF cells, which were co-transfected with 97Q-GFP and HMGB1 plasmids. Relative band intensity was measured (D and E). *Hmgb1*$^{-/-}$ MEF cells were treated with rhodamine-conjugated rHMGB1 proteins at 4 μg/ml and the colocalization was observed using colocalization scattergram and intensity profile analysis. The number of aggregate containing cells was counted amoung 100 GFP-positive cells. ** p<0.01 (N=3). (F) Filter trap analysis of whole cell lysates of *hmgb1*$^{-/-}$ MEF cells, which were transfected with 97Q-GFP plasmid and then treated with rHMGB1 proteins.

11. HMGB1 reduces 97Q-GFP-induced cytotoxicity

We speculated whether the chaperone-like activity of HMGB1 could reduce the cytotoxicity caused by polyQ aggregates. SHSY5Y cells were transiently co-transfected with 97Q-GFP and HMGB1 plasmids for 48 h and the change of cell viability was measured. As shown in Fig. 12A, the number of propidium iodide (PI) positive cells, indicating cell death, decreased when the cells were co-transfected with HMGB1 plasmid. HSP70 is a well known chaperone molecule in Huntington's disease,[35] so we used HSP70 as a positive control. In the CCK8 cell viability assay, the cell viability of 97Q-GFP transfected cells restored to 45.8% from 29.5% when the cells were co-transfected with HMGB1 plasmid (Fig. 12B). The results of the trypan blue staining analysis, which stains the dead cells, showed that the transfection of HMGB1 plasmid decreased the number of cell deaths to 25.0$\pm$1.0 from 40.6$\pm$4.0 (Fig. 12C). These data demonstrate that HMGB1 reduces the 97Q-GFP aggregates induced cell death.

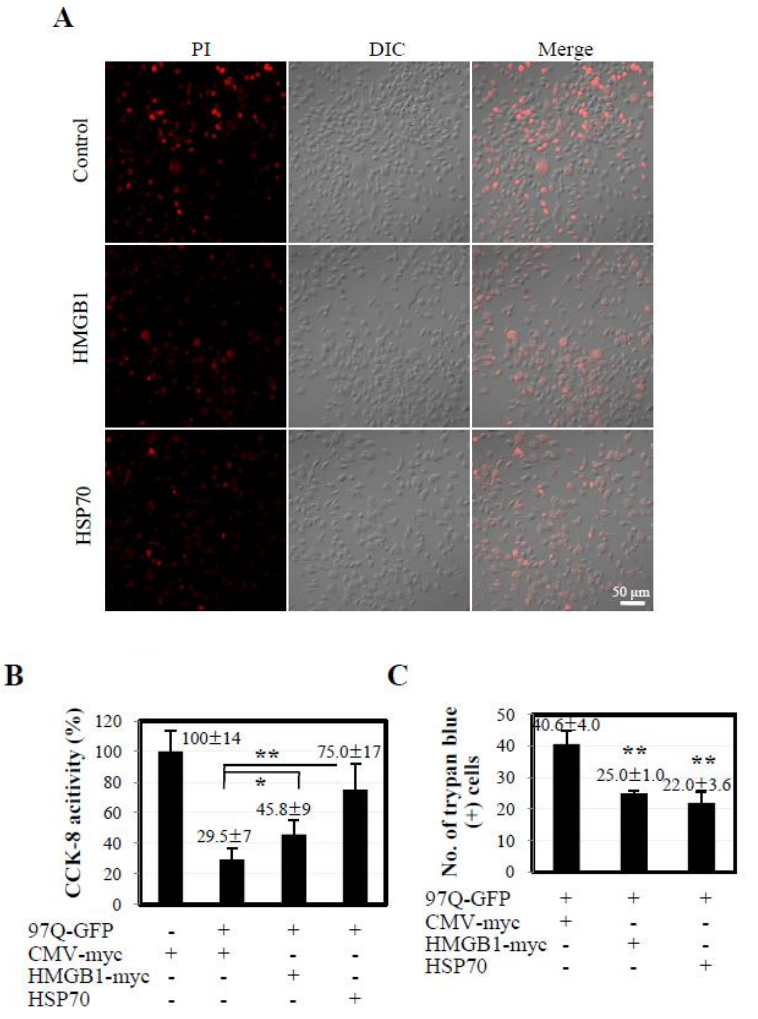
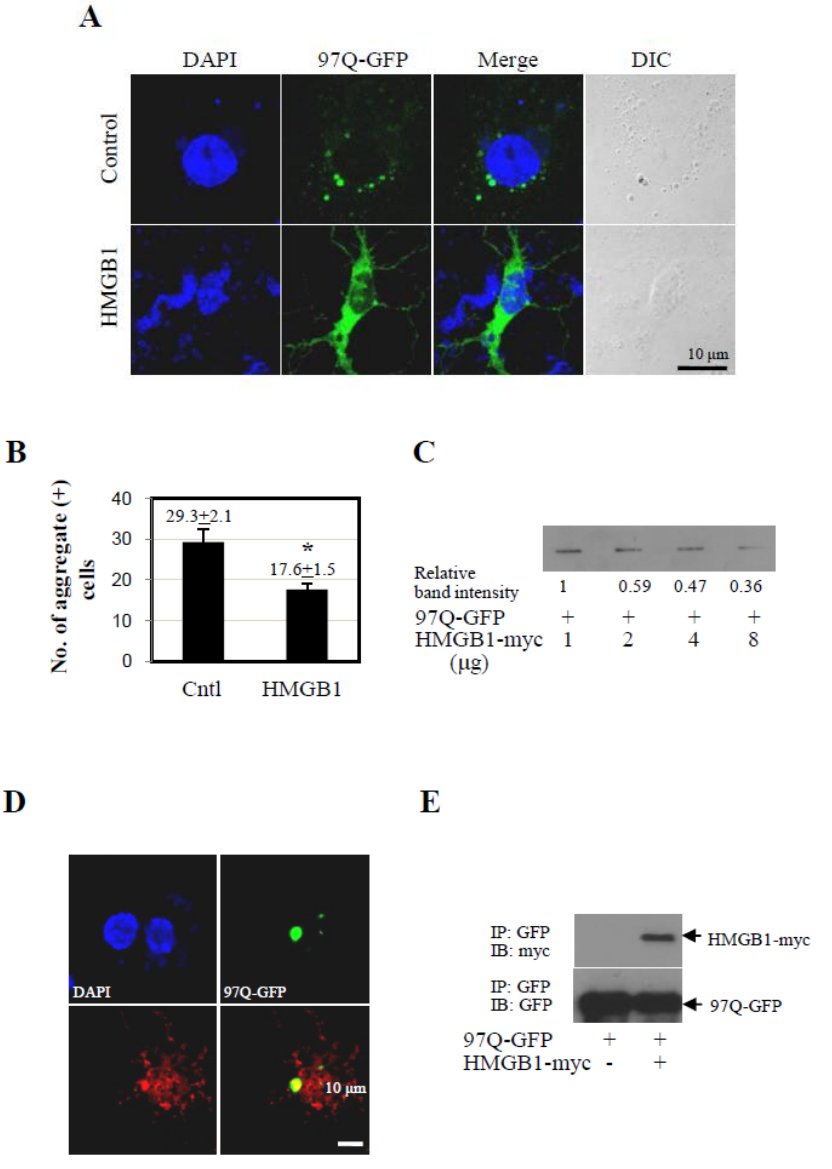


Figure 12. Effect of HMGB1 on the 97Q induced cytotoxicity. SHSY5Y cells were co-transfected with 97Q-GFP and HMGB1-myc plasmids and incubate for 48 h. Cell viability was observed by propidium iodide (PI) staining (A), CCK8 assay (B) and trypan blue exclusion assay (C). * $p<0.05$, ** $p<0.01$ (N=3).

12. HMGB1 reduces the formation of polyQ aggregates in mouse primary embryonic striatal neuron cells.

Finally, we observed the chaperone-like activity of HMGB1 using mouse primary neuronal cells. For this, mouse primary embryonic striatal neurons at embryonic day 14 were isolated and transfected with 97Q-GFP in the presence or absence of HMGB1 plasmid to observe the effect on polyQ aggregates formation. HMGB1 reduced the number of polyQ aggregate positive cells (Fig. 13A and B) and the size of aggregates (data not shown). The results of the filter trap analysis showed that the band intensity of polyQ formed aggregates was decreased in a dose-dependent manner of HMGB1 (Fig. 13C). HMGB1 protein colocalized with 97Q-GFP aggregates in immunofluorescence assay (Fig. 13D) and HMGB1 was bound to 97Q-GFP in the immunoprecipitation analysis (Fig. 13E).

When mouse primary embryonic striatal neurons were treated with rHMGB1 proteins, the number of polyQ aggregate positive cells and the band intensity of filter trap aggregates were significantly lower (Fig. 13F-H). The binding of rHMGB1 protein to polyQ-GFP was observed by immunostaining of His tag which is conjugated to rHMGB1 protein and co-localization was observed under confocal microscopy (Fig. 13I).


A
DAPI
97Q-GFP
Merge
DIC
Control
HMGB1
10 μm
B
No. of aggregate (+) cells
40
30
20
10
0
29.3±2.1
*
17.6±1.5
Cntl
HMGB1
C
Relative band intensity
1
0.59
0.47
0.36
97Q-GFP + + + +
HMGB1-myc (μg) 1 2 4 8
D
DAPI
97Q-GFP
10 μm
E
IP: GFP
IB: myc
HMGB1-myc
IP: GFP
IB: GFP
97Q-GFP
97Q-GFP + +
HMGB1-myc - +

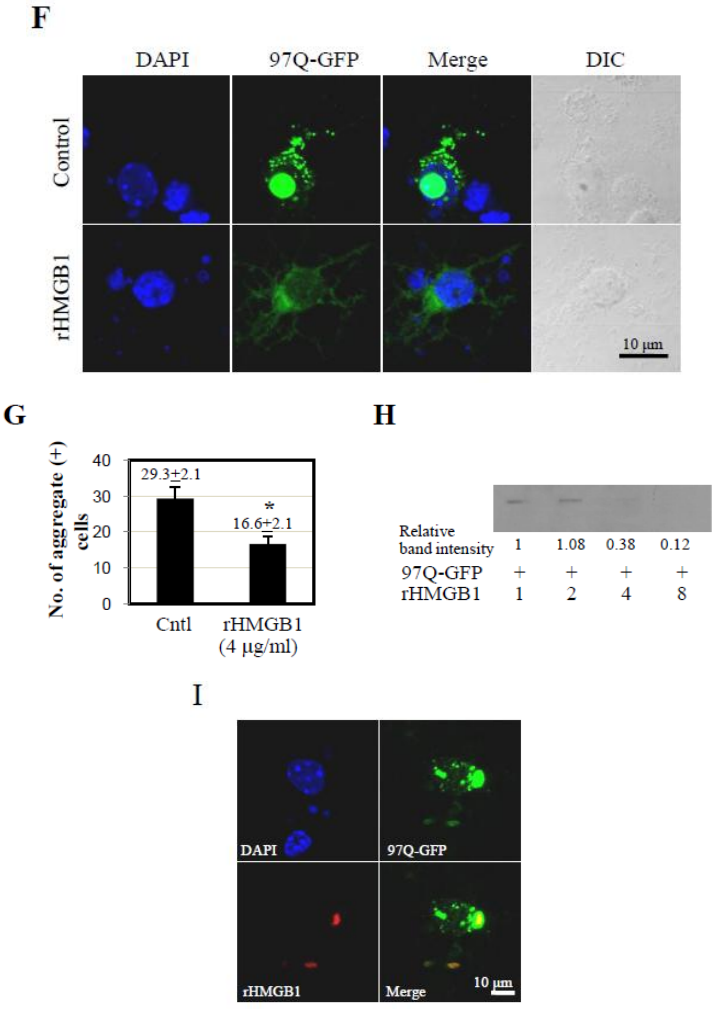


Figure 13. Effect of HMGB1 on the formation of 97Q-GFP aggregates in mouse brain primary striatal cells. (A) Mouse embryonic striatal neuronal cells were isolated at day 14 and then co-transfected with 97Q-GFP and HMGB1 plasmids. The distribution of 97Q-GFP aggregates was observed under confocal microscopy. (B) The number of aggregate containing cells was counted among

100 GFP-positive cells. Empty plasmid was transfected as a control group. (C) Filter trap analysis was performed after co-transfection of 97Q-GFP and HMGB1 plasmids in primary striatal neurons. (D) Colocalization of 97Q-GFP with HMGB1 after co-transfection of both plasmids. (E) Co-immunoprecipitation analysis of the binding between HMGB1 and 97Q-GFP in primary neuronal cells. (F) Mouse embryonic striatal neuronal cells were transfected with 97Q-GFP plasmid. After 24 h, rHMGB proteins (4 μg/ml) were added to the culture media and distribution of 97Q-GFP aggregates was analyzed by confocal microscopy. (G) The number of aggregate containing cells was counted among 100 GFP-positive cells, and empty plasmid was used for control group (Cntl). (H) Filter trap analysis was performed using the cells which were transfected with 97Q-GFP and incubated with rHMGB proteins. (I) Primary neuronal cells were transfected with 97Q-GFP plasmid and rhodamine conjugated rHMGB1 proteins were added to the culture media. The colocalization of 97Q-GFP with rHMGB1 protein was observed by confocal microscopy. * $p<0.05$ (N=3).

In our experiments, HMGB1 was translocated to the cytoplasm under proteotoxic stress condition such as generation of ROS, and translocated HMGB1 interacted with polyQ and decreased the formation of aggregates, and finally reduced the cytotoxicity.

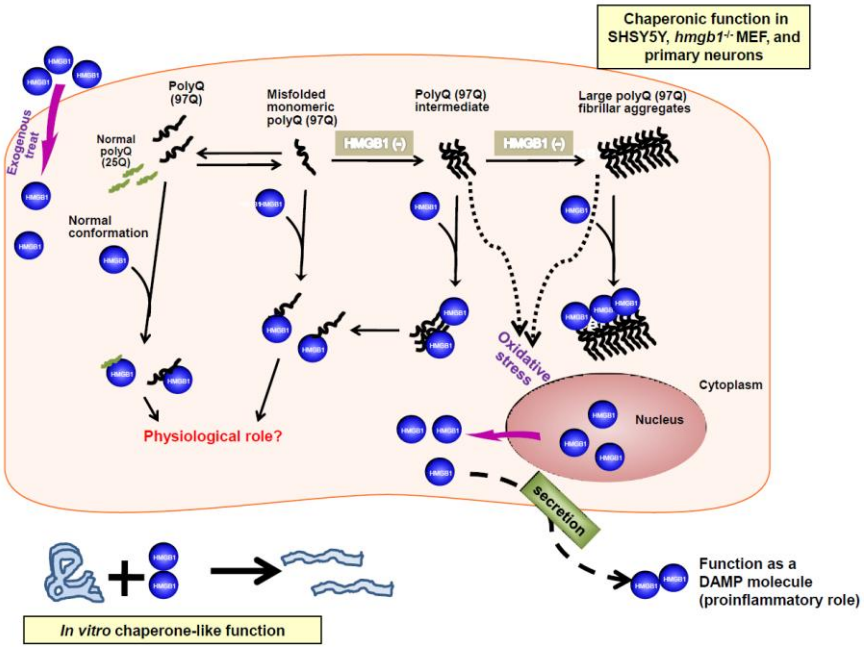


Figure 14. Conceptual function of HMGB1 in polyQ disease model. HMGB1 translocates to the cytoplasm under some proteotoxic condition such as increase of reactive oxygen species. In cytoplasm, HMGB1 interacts with polyQ aggregates and reduces the formation of aggregates and aggregates induced cytotoxicity.

IV. DISCUSSION

Although HMGB1 is diffusely distributed in the cytoplasm of hepatocyte and neuronal cells, HMGB1 is mainly located in the nucleus.[36] It is well known that 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 in the nucleus.[37,38] When HMGB1 is secreted into the extracellular space, it also functions as a pro-inflammatory cytokine molecule through interaction with TLR-2, 4, and RAGE.[6] In addition, HMGB1 can play an intracytoplasmic role as a regulator between macroautophagy and apoptosis [11,39] and a sentinel molecule for viral nucleic acid sensing.[40]

In this study, we have demonstrated the novel finding that HMGB1 has a chaperone-like activity *in vitro,* reducing protein aggregation induced by heat or chemical stress. During this experiment, we found that high temperature induced self aggregation of HMGB1 proteins, suggesting different function of monomeric and oligomeric HMGB1 proteins. The other functions of oligomerized form of HMGB1 should be studied. The chaperone-like function of HMGB1 was also

proved in O23 cells decreasing heat shock induced inactivation of cytoplasmic luciferase.

Interactions of HMGB1/2 protein with polyQ have been reported by proteomic analysis.[38] On the contrary, the positive interaction of HMGB1 to normal 25Q protein in our result was not agreed with a previous report of negative binding to 20Q protein.[38] 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.

Well known chaperone molecules such as HSP70 and HSP40 also have been reported to interact with mutant huntingtin and suppress aggregation formation and cellular toxicity by the correction of misfolded proteins or degradation.[41] HSP70 increases soluble polyQ proteins by directly associating with polyQ aggregates in a dynamic and transient manner.[42,43] HSP40 (DNAJ) associates with aggregated but not soluble mutant huntingtin and suppresses aggregation by chaperone modification of high molecular weight complexes.[26] In this study, we found that HMGB1 interacts with polyQ and reduces the formation of aggregate. HMGB1 increased soluble form of polyQ and finally reduced polyQ induced cytotoxicity. These results show similar pattern with HSP70, a

well known strong chaperone molecule suggesting the possibility that HMGB1 might act as a chaperone molecule. It is also possible that HMGB1 might act as a co-chaperone molecule which helps other chaperone molecules. Previously, HSP 72 chaperone was reported to interact with HMGB1 in the nucleus and now we are further studying the interaction of HMGB1 with other chaperone molecules. Therefore, the possibility of HMGB1 as a co-chaperone molecule should be considered.

Cysteine 106 of HMGB1 is an important residue reflecting redox status and its cytokine activity.[33,44] In this study, however, HMGB1 C106 mutant plasmid showed similar chaperone-like activity to wild type HMGB1, suggesting that the SH residue of HMGB1 C106 plays no significant role in chaperone-like activity. We assume that the interaction of HMGB1 with polyQ might not be disulfide bond dependent. Also, we think that the binding of HMGB1 with polyQ might not sequence specific because A and B box were all interacting with polyQ in co-immunoprecipitation assay. The biochemical mechanism of binding needs to be further evaluated.

Expansion of polyQ repeats leads to early increased ROS coinciding with polyQ aggregation. Aggregation of a fragment of huntingtin protein directly causes free radical production *in vivo*[45] and

inhibition of polyQ aggregation suppresses ROS.[46,47] Oxidative potential is important for the nucleocytoplasmic translocation of HMGB1 and redox status of HMGB1 also affects its specific function.[18] In our study, ROS generated by an overexpression of 97Q-GFP induced cytoplasmic translocation of nuclear HMGB1. This translocation was decreased by the treatment of ROS scavengers, N-acetyl-l-cysteine and Mito-Tempo, demonstrating the role of ROS in HMGB1 translocation. It is possible that ROS generated by polyglutamine expansion changed the redox status of HMGB1, especially 106 cysteine, and induced cytosolic movement. Our previous finding shows that posttranslational modification of phosphorylation is also responsible for the nucleocytoplasmic transport of HMGB1, and Ca^{++}-dependent classical protein kinase C (cPKC) is an effector kinase of HMGB1 phosphorylation.[48] Considering that expanded polyQ expression induces Ca^{++}-dependent protein kinase C via metabotropic glutamate receptor-mediated cell signaling,[49] it is possible that cPKC enzyme (α, βI, βII, and γ) family are main kinases for nucleocytoplasmic transport of HMGB1 to interact with polyQ. Further investigation is necessary whether phosphorylation of HMGB1 could be induced by overexpression of expanded polyQ.

Interestingly, overexpression of HMGB1 is not always beneficial

in reducing polyQ aggregation. A high concentration of HMGB1 rather aggravated the formation of polyQ aggregates both in the overexpressed and recombinant HMGB1 treated conditions. In our other study, we found that HMGB1 proteins tend to be aggregated by itself (Data not published), therefore the amount of HMGB1 over the limit may be co-aggregated with polyQ or high concentration of HMGB1 may be self-aggregated and have adverse influence on polyQ aggregation. This negative phenomenon was also observed in HSP70, whose overexpression is not always beneficial for the survival of motor neurons.[50] These observations suggest that the concentration of HMGB1 level is very critical for its function.

Exogenously added recombinant HMGB1 proteins were transduced into the cytoplasm, co-localized with polyQ aggregates, and reduced the formation of polyQ aggregates. The penetration of the HMGB1 protein into the cytoplasm was not inhibited by the cytochalasin B, suggesting that the internalization of HMGB1 is not only due to endocytosis. These actions are similar to the HSP70 protein, which also enters human motor neurons[51] and protects motor neurons subjected to the oxidative stress by decreasing aggregates positive cells,[52,53] although the mechanism is not clearly explained. Since intracellular penetration of

exogenous HSP70 was time- and dose-dependent,[35] under the other circumstances, such as different concentration of HMGB1 or different incubation time, the function of exogenously applied HMGB1 could be changed. Repeated experiments in *hmgb1*$^{-/-}$ MEF cells confirmed that endogenous HMGB1 has a chaperone-like activity.

Together, these findings demonstrate that HMGB1 can work as a chaperone-like molecule *in vitro* and HD primary neuronal cell model.

V. CONCLUSION

The aim of this study was to find out the novel function of cytoplasmic HMGB1. We identified a previously unknown function of HMGB1 proteins as a chaperone-like molecule *in vitro* which reduce the stress induced aggregation of tested target proteins. Using luciferase activity, we also detected that HMGB1 proteins reduced the heat induced inactivation of lucieferase activity. Finally, in the Huntington's disease cell model, HMGB1 moved to the cytoplasm when co-transfected with 97 polyglutamine and reduced the formation of polyglutamine aggregates. These findings were also proved when recombinant HMGB1 proteins were added exogenously. We detected the chaperone-like activity of HMGB1 protein for the first time and discovered that exogenously added recombinant HMGB proteins can function as chaperone-like molecules in the cytoplasm through penetration of cell membranes. These new findings propose another method to deal with the aggregates prone diseases.

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ABSTRACT (IN KOREAN)

High Mobility Group Box 1 단백질의 샤페론 기능:

polyglutamine 집적체 형성 억제에 미치는 영향

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민 현 진

HMGB1 단백은 주로 핵에 존재하는 DNA-샤페론 단백질로 기능이 잘 알려져 있고 핵에서 세포질을 통해 세포 밖으로 이동하여 사이토카인과 유사하게 다양한 역할을 수행한다는 것이 보고되어 왔으나 세포질에서의 기능에 대해서는 연구가 아직 부족한 상태이다. 본 연구에서는 세포질에서 HMGB1 의

새로운 역할을 밝히고자 하였고 다른 논문에서 HMGB1 단백질이 HSP70 과 같은 샤페론 분자와 핵 안에서 결합함을 보고한 바 있어 HMGB1 단백질이 핵에서와 마찬가지로 세포질에서도 샤페론 분자와 같은 역할을 수행할 수 있는지 확인하는 것을 본 연구의 목적으로 하였다. 이를 확인하는 과정으로 첫째, 재조합 HMGB1 단백질을 이용하여 화학적으로 라이소자임과 인슐린을 응집시키는 조건하에서 HMGB1 단백이 목표 단백질의 응집을 억제함을 밝혔으며, 사이트레이트 형성 효소를 열적으로 응집시키는 조건하에서 또한 목표 단백질의 응집을 억제함을 밝혔다. 둘째, 루시퍼레이즈 플라스미드를 O23 세포에 과발현하고 열처리를 통해 루시퍼레이즈 효소의 활성을 억제하였을 때 HMGB1 플라스미드를 함께 과발현시킨 경우 대조군에 비해 루시퍼레이즈 효소의 활성 억제 정도가 감소한 것을 확인하였다. 다음으로 신경세포종 SHSY5Y, 생쥐에서 분리한 배아 일차 뇌 선조 신경세포에 트랜스펙션에 의해 HMGB1 단백질을 과발현시키거나 외부에서 재조합 HMGB1 단백질을 처리해준 경우 세포질 내에서 polyglutamine 에 의해 생기는 직접체 형성을

감소시키고, polyglutamine 집적체에 의한 세포 독성을 감소시키며, 이러한 현상은 *hmgb1*$^{-/-}$ MEF 을 이용한 실험에서도 반복되어 확인되었다. 이상의 결과로 세포질 내에서 HMGB1의 샤페론으로서 새로운 기능을 밝히고 세포 내에서 polyglutamine에 의한 집적체 형성을 억제하는 조절 기능을 할 수 있음을 제시하였다.

핵심되는 말: HMGB1, 샤페론, polyglutamine

PUBLICATION LIST

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