The regulatory mechanism of TNF-α-stimulated *S100B* expression and the inhibitory role of intracytoplasmic HMGB1 in NF-κB-mediated TLR3 signaling

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The regulatory mechanism TNF-α-stimulated *S100B* expression and the inhibitory role of intracytoplasmic HMGB1 in NF-κB-mediated TLR3 signaling

Directed by Professor Jeon-Soo Shin

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늘 실험실에서 편안하게 연구할 수 있도록 배려해 주신 나의 남편 정경용님과 나의 사랑스런 아들 정현우에게 감사와 사랑의 맘을 전합니다. 마지막으로 너무 부족한 저를 늘 품에 안아주시는 하나님께 감사 드립니다.

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ABSTRACT

The regulatory mechanism TNF-α-stimulated *S100B* expression and the inhibitory role of intracytoplasmic HMGB1 in NF-κB-mediated TLR3 signaling

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(Directed by Professor Jeon-Soo Shin)

It has been known that Receptor Advanced Glycation End product (RAGE) is a member of the immunoglobulin superfamily of cell surface proteins interacting with a range of ligands, including advanced glycation end products (AGE), amyloid- peptide, HMGB1 and S100B. S100B and HMGB1 proteins are known to be physiological ligands for RAGE in migratory and inflammatory cellular responses.

Here, we report two results (1) the mechanism by which TNF-α represses the S100B expression via Sp1. (2) The negative role of intracytoplasmic HMGB1 in NF-κB-mediated TLR3 signaling.

S100B, a calcium-binding protein predominantly produced and secreted by astrocytes in the central nervous system (CNS), takes part in the development of the brain and in neurodegenerative disorders. The serum level of TNF-α is elevated in the brain disease. In this study, we found that TNF-α repressed S100B expression in primary human astrocytes and U373 astrocytoma cells dose- and time-dependently. Transcriptional repression of S100B gene by TNF-α was not affected by cycloheximide or NF-κB inhibitors, but was abrogated by treatment with two GC-rich DNA-binding protein inhibitors, mithramycin A and its analog, chromomycin A_3. Transcription of S100B promoter lacking the Sp1 binding site (pGBSΔ-168/+697) was increased by TNF-α treatment, suggesting that Sp1 is involved in the repression of S100B transcription by TNF-α. Furthermore, an electrophoretic mobility supershift assay showed that Sp1 binding was reduced by treatment with TNF-α. These results suggest that the repression of S100B expression by TNF-α is mediated by Sp1.

High mobility group box protein 1 (HMGB1), a nuclear DNA-binding protein, is present in almost all eukaryotic cells. We show that HMGB1 inhibits poly(I:C)-induced NF-κB but not ISRE (the interferon-stimulated response element of the IFNA/B-inducible gene) activation. Furthermore, HMGB1 inhibits NF-κB activity in dose dependent manner, in both TLR3 and TLR4 table cell line. Cytoplasmic HMGB1 inhibits phosphorylation of IκBα but did

not affect that of IRF-3. When we treated cells with poly(I:C), phosphorylation of ERK level was decreased in HMGB1-tranfected cells. These date suggest that HMGB1 specifically inhibits poly(I:C)-mediated NF-κB. Cytoplasmic HMGB1 decreased p65 translocation into the nucleus. Furthermore, ectopic HMGB1 did also increase the binding to p65 in the cytosol. After poly(I:C) treatment, p65 strongly bind to all types of karyopherins and karyopherin α3/α5 were bound to endogenous HMGB1 at the that time. In competition assay, increasing amount of recombinant HMGB1 decreased the binding of p65 and karyopherin α3/α5. Overall our data indicated that HMGB1 inhibits poly(I:C)-mediated TLR3 signaling.

Key words : S100B, TNF-α, Sp1, HMGB1, p65, poly(I:C) and TLR3

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CHAPTER I.

TNF-α represses S100B in human brain cells via Sp1

I. INTRODUCTION

S100B, a glia-derived protein of the EF-hand type, is involved in a variety of cellular functions, including protein phosphorylation, regulation of cytoskeletal composition, cell cycle dynamics, and Ca^{2+} homeostasis [1-3]. Extracellular S100B exerts neurotrophic or neurotoxic effects depending on its concentration. Nanomolar concentrations of S100B have been shown to stimulate neurite

outgrowth and enhance neuronal survival during development and after injury [4-5], whereas micromolar concentrations cause neuronal death by downregulation of bcl-2 [6] and trigger astrocyte death by increasing the expression of inducible nitric oxide synthase and the secretion of nitric oxide [7].

In vivo, transgenic mice overexpressing S100B exhibited impaired hippocampal long-term potentiation and learning memory [8-9] and increased susceptibility to perinatal hypoxia-ischemia [10]. In contrast, S100B-null mice showed strengthened synaptic plasticity due to enhanced long-term potentiation in the hippocampal CA1 region, with no detectable abnormalities in brain cytoarchitecture [11]. Human studies have detected high levels of S100B in the brains of Down's syndrome patients (Trisomy 21), most likely associated with the localization of the S100B gene at 21q22.3 [12-13]. Patients with Down's syndrome often show brain pathologies resembling Alzheimer's disease, which has also been associated with S100B overexpression [14]. In Alzheimer's disease, activated astrocytes associated with neuritic plaques have been found to contain elevated levels of S100B [13, 15]. Collectively, these observations suggest that S100B might have a role in the synaptic plasticity mediated by glial-neuronal interaction [11] and in the pathogenesis of neurodegenerative disorders. S100B, which has both autocrine and paracrine cytokine-like actions, participates in brain inflammation by activating astrocytes, microglia and neurons [16-18], as well as by triggering production of IL-6 in neurons [17]. In terms of molecular

expression, the human S100B gene is under complex transcriptional control and contains both positive and negative regulatory regions [19]. However, the transcriptional mechanisms regulating S100B expression are poorly understood.

Tumor necrosis factor (TNF)-α is a well-studied cytokine in the peripheral immune system that acts as a key effector molecule in the pathogenesis of many inflammatory and autoimmune diseases [20]. In the CNS, TNF-α has been reported to have an important role in glial-neural communication [21] and in neuroinflammatory diseases such as bacterial meningitis [22], multiple sclerosis [23], and other neurodegenerative diseases [24-26]. TNF-α shows diverse cell type- and target molecule-specific actions; for example, it is cytotoxic to oligodendrocytes in mixed glial cultures ex vivo [27-28] but has a proliferative effect on astrocytes [29]. however, no previous report has directly tested the effect of TNF-α on S100B expression levels.

In the present study, we found that TNF-α represses S100B mRNA and protein expression in primary human astrocytes and in the human astrocytoma cell line, U373. We further provide evidence that the novel role of TNF-α in transcriptional repression of S100B is mediated via Sp1, not by NF-κB.

II. MATERIALS AND METHODS

1. Materials

Human recombinant TNF-α, IL-18 and TGF-β were obtained from R&D Systems (Minneapolis, MN), interferon (IFN)-γ was from PharMingen (San Diego, CA), and LPS (*E. coli* 055:B5) was from Difco (Detroit, MI). The NF-κB inhibitors pyrrolidine dithiocarbamate (PDTC), caffeic acid phenethyl ester (CAPE)[30], and BAY11-7082 [31] were purchased from Sigma-Aldrich (St. Louis, MO). The blocking monoclonal antibody (mAb) against TNF-α and the anti-glial fibrillary acidic protein (GFAP) antibody were obtained from PharMingen (San Diego, CA), and the anti-S100B mAb was purchased from Labfrontier Co. (Seoul, Korea). The Sp1 inhibitors mithramycin A and chromomycin A3 were purchased from Sigma-Aldrich and Calbiochem (La Jolla, CA), respectively.

2. Cell Cultures

Primary human astrocytes and U373 human astrocytoma cells (American Type Culture Collection, Manassas, VA) were used in this study. Primary astrocytes were isolated from three human fetuses (therapeutic abortions) aged from 20 to 23 wks; the use of tissue samples was approved by our Institutional Review Board. Astrocytes were isolated as described previously [32] and cultured at 37^{o}C under 5% CO_2 in DMEM containing 10% FBS (Life Technologies,

Grand Island, NY), 1% nonessential amino acids (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The culture medium was changed every week. Indirect immunofluorescence staining for GFAP revealed that most of the cultured cells (over 98%) were astrocytes. U373 cells were cultured in MEM containing 10% FBS, 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate.

3. Northern blot analysis

Total RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen, Santa Clara, CA) or the TRI_{ZOL} reagent (Life Technologies). Ten to twenty micrograms of total RNA from each sample were separated on a 1% agarose gel containing 6% formaldehyde, and then transferred to a Hybond^{TM}-N nylon membrane (Amersham Life Science, Buckinghamshire, England). For probe preparation, an *Eco*RV-*Bam*HI fragment of the human S100B cDNA (nucleotides 73 to 351, GenBank Accession No. NM_006272) was labeled with [α-^{32}P] dCTP (3000 Ci/mM; NEN Life Science Products, Inc., Boston, MA) using a Megaprime^{TM} DNA Labeling System (Amersham). The labeled fragment was purified with Sephadex G-50 and used as a probe at $< 2\times10^7$ cpm/μg DNA. Membranes were prehybridized and hybridized overnight at 42^{o}C with 2 x 10^6 cpm/ml of [^{32}P]-labeled probe in the ExpressHyb^{TM}

hybridization buffer (Clontech, Palo Alto, CA). After hybridization, membranes were washed twice at room temperature for 10 min in 1× SSC, 0.1% SDS and twice at 55°C for 10 min in 0.2× SSC, 0.1% SDS. Signals were detected by autoradiography with an intensifying screen at –70°C, and the mRNA levels were quantified with a Fujix BAS2500 bioimage analyzer (Fuji Photo Film Co., Tokyo, Japan). The intensity of β-actin mRNA expression or 28S rRNA was used to normalize the relative abundances of each sample.

4. RT-PCR

Reverse transcription was performed using random primers and murine Molony leukemia virus-reverse transcriptase (Life Technologies). RT-PCR was performed using 100 ng of cDNA, 0.8 mM dNTPs, 0.4 pm of each primer, and 1 U *Taq* polymerase (TaKaRa, Shiga, Japan) in a 25 μl final volume. The primers used for human S100B were 5′-ACT ACT GCC TGC CAC GAG TT-3′ (forward), and 5′-GGC CAG TCA GCT TAC ACA CA-3′ (reverse). The PCR conditions consisted of 27 cycles of 30 sec at 94°C, 30 sec at 62°C, and 1 min at 72°C, followed by an additional extension of 10 min at 72°C. To provide normalization controls for mRNA input, β-actin or GAPDH mRNA levels were determined for each sample in separate reactions, with PCR performed over 25 cycles to ensure that the amplification was completed within the linear range. The signal ratio of S100B to β-actin or GAPDH was determined based on the

ratio of the intensity of the PCR product compared with that of β-actin or GAPDH. The relative OD of each band was measured for analysis.

5. Intracytoplasmic staining

Cells were detached after trypsinization, washed with PBS containing 0.1% BSA, fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and then permeabilized in 0.1% Triton X-100 in Tris-buffered saline (pH 7.4) with 1% FBS and 0.1% NaN_3 for 5 min. Subsequently, the fixed, permeabilized cells were blocked with 5% normal goat serum (JRH Biosciences, Lenexa, KS), reacted with anti-S100B mAb or anti-GFAP mAb for 20 min on ice, washed and then incubated with FITC-conjugated goat anti-mouse Ig for 20 min. GFAP was used as a marker for astrocytes, and an isotype control Ab was used as a negative control. The results were analyzed with a FACstar flow cytometer (Becton Dickinson, San Jose, CA).

6. Transient transfection and luciferase assay

Target cells were plated to 70-80% confluence on 24-well plates, and a DNA transfecting complex mixture (0.2 μg) containing human S100B promoter-luciferase constructs [19] and β-gal expression vectors was transfected into the cells using FuGENE™ 6 (Roche) according to manufacture's protocol. Twenty-four h after transfection, the medium was changed and the cells were

treated with TNF-α at a final concentration of 10 ng/ml for another 24 h. Cells were lysed with 50 µl of 1× reporter lysis buffer, and luciferase activity was measured with the Luciferase Reporter Assay System (Promega) and a BIOLUMAT luminometer (Berthold, Germany). β-gal activity was measured in the same lysates, and the luciferase activity of each sample was normalized with respect to β-galactosidase activity for calculation of the activation values.

7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from U373 cells were prepared as previously described [33]. Briefly, $5x10^6$ cells were lysed with 1 ml of EMSA buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2, 0.2% Nonidet P-40, 1 mM DTT, and 0.5 mM PMSF), incubated on ice for 5 min, and centrifuged at 10,000 x g for 5 min. The resulting pellet was resuspended in 50 µl of EMSA extraction buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM DTT and 0.5 mM PMSF), the supernatant was collected and the protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA). For EMSA, double stranded oligodeoxynucleotides (ODN, 5'-CAG TCC TGC CGC CCA GGA CCC G-3') containing the GC rich region of the S100B promoter region were end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Promega, Madison, WI). The reaction mixtures were incubated for 30 min at 4^{o}C and were then resolved on a 4% non-denaturing

polyacrylamide gel in 0.25% TBE buffer at 4°C for 2 h at 180 V. The resulting products were detected by autoradiography. For competition experiments, a 100-fold excess of unlabeled probe was added to the reaction mixture before the addition of labeled probe. For supershifting experiments, 4 μg of antibodies against Sp1 or Sp3 (Santa Cruz) were added.

8. Knockdown of Sp1 and Real-time PCR

Cells were transfected with 200 nM of Sp1 siRNA (Santacruz) using Lipofectamine 2000. After 24h, transfected cells were treated with 10 ng/ml of TNF-α for 24 h. RNA isolation was performed using the RNeasy kit (Qigen) according to manufacturer's instructions. Isolated RNA was used to synthesize cDNA using SuperScript II cDNA synthesis (Invitrogen) and qPCR was performed on ABI PRISM 7700 Sequence Detection System and software (PE Biosystems) using SyberGreen (ABI). Primers sequences used were S100B forward: CCTCATCGACGTTTTCCAC, S100B reverse: GCTCATTGTTGATGAGCTCC [Molecular and cellular probes 2001, 15 71-73]; GAPDH forward: CCCCTTCATTGACCTCAACTAC; GAPDH reverse: TCTCGCTCCTGGAAGATGG.

III. RESULTS

1. Repression of S100B expression by TNF-α in human primary astrocytes.

In our previous study, we found that TNF-α represses the S100B expression in human primary astrocytes[66]. Primary human astrocytes were treated with IFN-γ (1,000 U/ml), TNF-α (10 ng/ml), LPS (100 ng/ml), IL-18 (10 ng/ml), or TGF-β (10 ng/ml) for 24 h. Northern blotting revealed that untreated primary astrocytes expressed abundant S100B mRNA. Addition of IFN-γ and TGF-β did not alter the basal S100B mRNA expression. In contrast, treatment with LPS or IL-18 increased the S100B mRNA levels by 40-60%, whereas addition of TNF-α reduced S100B mRNA expression by over 70% after 24 h (Fig. 1A). These data suggest that TNF-α is an important regulatory factor for S100B transcription. As TNF-α has pronounced effects in neuroinflammatory and autoimmune diseases [20, 22, 24],

In order to confirm that TNF-α decreased the S100B expression, we firstly examined the dose-response changes in S100B mRNA following TNF-α treatment. S100B mRNA level was reduced to 70%, 50%, 25%, and 13% when primary astrocytes were treated with 0.01, 0.1, 1, and 10 ng/ml of TNF-α for 24 h, respectively (Fig. 1B). Second, to investigate the time effect of TNF-α on S100B expression, cells were treated with 10 ng/ml of TNF-α for the indicated times and the S100B mRNA level was measured by RT-PCR. As a result of

Figure 1C, S100B mRNA levels showed little change over the initial 8 h after addition of TNF-α. Thereafter, the expression levels slowly declined between 8 and 24 h to a low level that was sustained for over 48 h (Fig. 1C). To determine whether the TNF-α-mediated repression of S100B mRNA leads to a decrease in S100B protein, intracellular S100B protein expression was evaluated by flow cytometric analysis of mean fluorescence intensity (MFI). As shown in Figures 1D and 1E, untreated cells had an MFI of 2.25 $\pm$ 0.10 for S100B protein expression. The MFI levels were reduced to 1.47 $\pm$ 0.15 and 1.31 $\pm$ 0.08 after treatment with 1 and 10 ng/ml of TNF-α for 24 h, respectively. These results collectively show that TNF-α decreased S100B expression at both the mRNA and protein levels.

In this report, I focused our subsequent experiments on examining the mechanism involving TNF-α-induced repression of S100B expression.

2. TNF-α-mediated repression of S100B expression is blocked by anti-TNF-α antibody.

To confirm that TNF-α-mediated repression of S100B expression is TNF-α specific, human primary astrocytes were treated with TNF-α in the presence or absence of an anti-TNF-α mAb. The expression of S100B mRNA was not decreased when cells were treated with TNF-α in the presence of the

anti-TNF-α Ab (Fig. 1F), indicating the specificity of the TNF-α on repression of S100B mRNA expression. We also tested the reversibility of the effect of TNF on S100B expression. Cells were treated with TNF-α for 36 h, then the TNF-α was removed from half of the cultures, and S100B levels were examined at various times thereafter. S100B mRNA levels were increased following TNF-α withdrawal and returned to basal levels within 72 h after the removal, whereas S100B mRNA levels remained repressed for 132 h in the presence of TNF-α (Fig. 1G).

3. The effect of TNF-α on S100B expression in human astrocytoma cell line, U373.

To identify a relevant cell line for additional work, we used RT-PCR to examine S100B mRNA expression level in four human astrocytoma cell lines: A172, U87MG, U373 and T98G. S100B mRNA levels were abundant in U373 cells (Fig. 2A), and flow cytometric analysis showed that intracytoplasmic S100B protein expression was correlated with the levels of S100B transcripts in these cells (Fig. 2B). In contrast, there was little or minimal mRNA or protein expression of S100B in A172, U87MG and T98G cells (Fig. 2A and 2B). In U373 cells, TNF-α treatment repressed the expression of S100B mRNA consistent with the effect seen in primary astrocytes (Fig. 2C), so we chose human astrocytoma cell line, U373 for the further study.

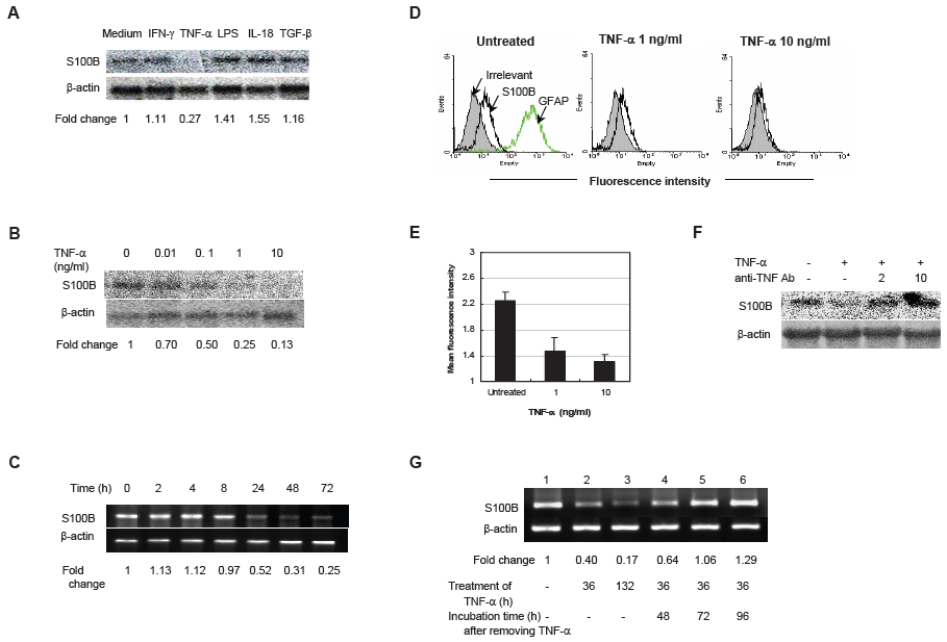

Fig. 1. TNF-α represses S100B expression in primary astrocytes and TNF-α-mediated repression of S100B expression is TNF-α specific. Human astrocytes from primary cultures were incubated with medium alone (lane 1), 1,000 U/ml IFN-γ (lane 2), 10 ng/ml TNF-α (lane 3), 100 ng/ml LPS (lane 4), 10 ng/ml IL-18 (lane 5), and 10 ng/ml TGF-β (lane 6) for 24 h and processed for Northern blotting (A). Cells were incubated with various concentrations of TNF-α (0, 0.01, 0.1, 1 and 10 ng/ml) for 24 h and S100B mRNA levels were analyzed by Northern blotting (B). Cells were incubated with 10 ng/ml of TNF-α for the indicated periods of time, and S100B mRNA was analyzed by RT-PCR (C). Cells were treated with 1 and 10 ng/ml TNF-α, and

intracytoplasmic S100B protein levels were determined by flow cytometry. Isotype-matched irrelevant antibodies (filled area) and anti-GFAP antibodies (gray line) were used as negative and positive astrocyte markers, respectively (D). The expression of S100B protein was determined by the relative mean fluorescence intensity values (E). Addition of an anti-TNF-α mAb blocked the TNF-α-induced decrease of S100B mRNA expression (F). Primary astrocytes were incubated with the indicated concentrations of anti-TNF-α-mAb in the presence of 10 ng/ml of TNF-α for 24 h, and S100B mRNA expression was determined by Northern blotting. Expression of β-actin was monitored as a control. The data shown are representative of two independent experiments. Reversibility of S100B mRNA after withdrawal of TNF-α treatment (G). Primary astrocytes were incubated without TNF-α (lane 1) or with 10 ng/ml TNF-α for 36 h (lane 2) and 132 h (lane 3). After treatment with TNF-α for 36 h, the culture dishes were washed and further incubated without TNF-α for 48 h (lane 4), 72 h (lane 5), and 96 h (lane 6). The relative S100B mRNA levels are presented as fold-changes. The data shown are representative of three independent experiments

4. TNF-α-mediated repression of S100B expression does not require new protein synthesis or NF-κB.

To determine whether protein synthesis was required for the observed TNF-α-induced S100B mRNA repression, U373 cells were pretreated with the protein synthesis inhibitor, cycloheximide (CHX) (2 μg/ml), and then treated with TNF-α (10 ng/ml) for 24 h. As shown in Figure 3A, the TNF-α-mediated repression of S100B mRNA was not abrogated by CHX pretreatment. These data suggest that TNF-α decreased the S100B expression without new protein synthesis.

To study whether the NF-κB pathway was involved in TNF-α-mediated repression of S100B, we preincubated cells with the NF-κB inhibitors such as CAPE, PDTC, or BAY11-7082, and then treated cells with 10 ng/ml of TNF-α for 24 h. The cell viabilities were over 90% in treated cultures, but addition of the inhibitors did not prevent the repression of S100B by TNF-α at RNA levels (Fig. 3B and 3C). These results strongly suggest that repression of S100B expression by TNF-α is not mediated by the transcription factor, NF-κB.

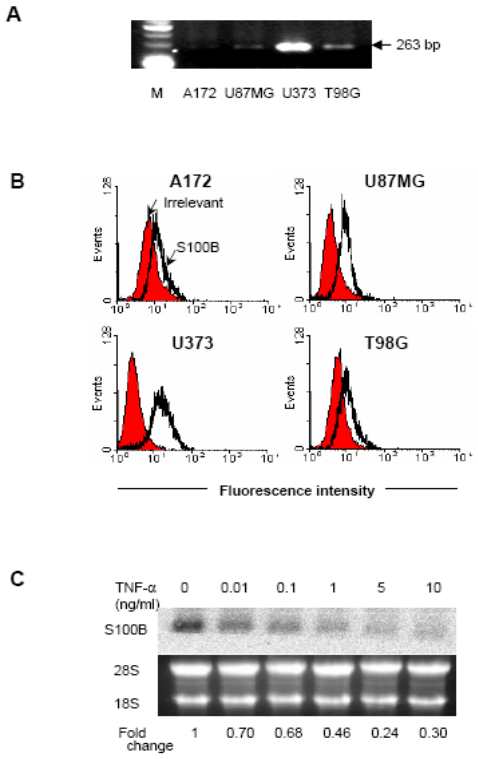

Fig. 2. Expression of S100B in human astrocytoma cell lines and the effect of TNF-α on this expression. RT-PCR analysis of S100B mRNA in A172, U87MG, U373 and T98G cells showed a single band corresponding to the expected PCR product size; S100B mRNA was highly expressed in U373 cells (A). Intracytoplasmic S100B protein levels were determined by flow cytometry; S100B protein was highly expressed in U373 cells (B). Dose-dependent decrease of S100B mRNA expression following TNF-α treatment of U373 cells (C). Cells were incubated with various concentrations of TNF-α for 24 h and S100B mRNA was analyzed by Northern blotting. The results shown are representative of three independent experiments.

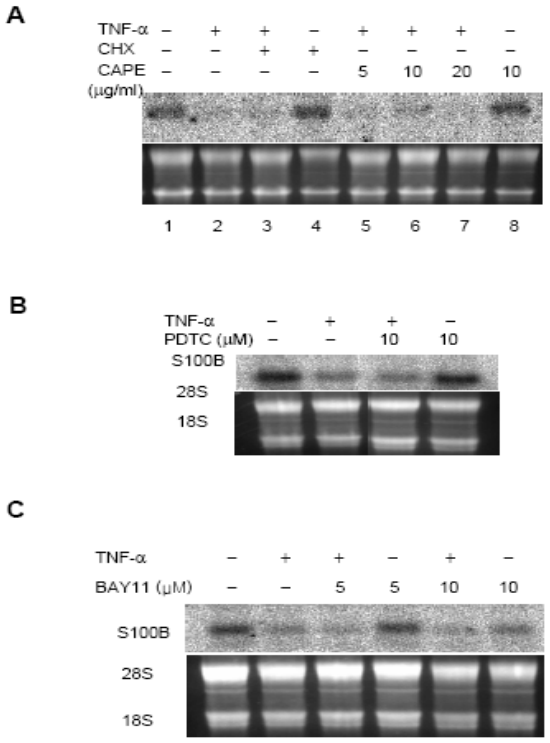

Fig. 3. TNF-α-mediated repression of S100B expression does not require new protein synthesis or NF-κB. Cycloheximide (CHX) did not inhibit the TNF-α-mediated repression of S100B mRNA expression in U373 cells. U373 cells were treated with 2 μg/ml of cycloheximide (CHX) and then treated with 10 ng/ml of TNF-α for 24 h (A, lanes 3 and 4). To examine the effects of NF-κB inhibitors on S100B mRNA repression, U373 cells were treated with CAPE (A, lanes 5-8), PDTC (B), or BAY11-7082 (C) and then treated with 10 ng/ml of TNF-α for 24 h. Total RNA was subjected to Northern blot analysis. TNF-α-induced repression of S100B mRNA was not blocked by the NF-κB inhibitors. The data shown are representative of three independent experiments.

5. Mithramycin A and chromomycin A_3 inhibit TNF-α-mediated repression of S100B mRNA.

To examine whether the Sp1 transcription factor is involved in mediating the TNF-α effects, we tested the effect of mithramycin A on TNF-α-mediated S100B repression. We used mithramycin A because it is known to bind to double-stranded DNA of GC rich promoters, such as Sp1 and Egr-1, via minor groove with GC base specificity [34]. Pretreatment with mithramycin A inhibited TNF-α-mediated repression of S100B mRNA expression in a concentration-dependent manner (Fig. 4A and 4B). Specifically, treatment with 10 ng/ml TNF-α repressed S100B mRNA levels in U373 cells to about 50% of the control, whereas cells treated with mithramycin A at 100, 500 and 1000 nM exhibited S100B mRNA levels that returned to basal values in a concentration-dependent manner. We similarly tested chromomycin A_3, a structural analog of mithramycin A, and found that it also inhibited the repression of S100B mRNA expression in a concentration-dependent manner (Fig. 4C). These data suggest that mithramycin A and chromomycin A_3 are potential inhibitors of TNF-α-mediated S100B mRNA repression, possibly via the binding of these GC-rich DNA-binding proteins to the promoter of the S100B gene.

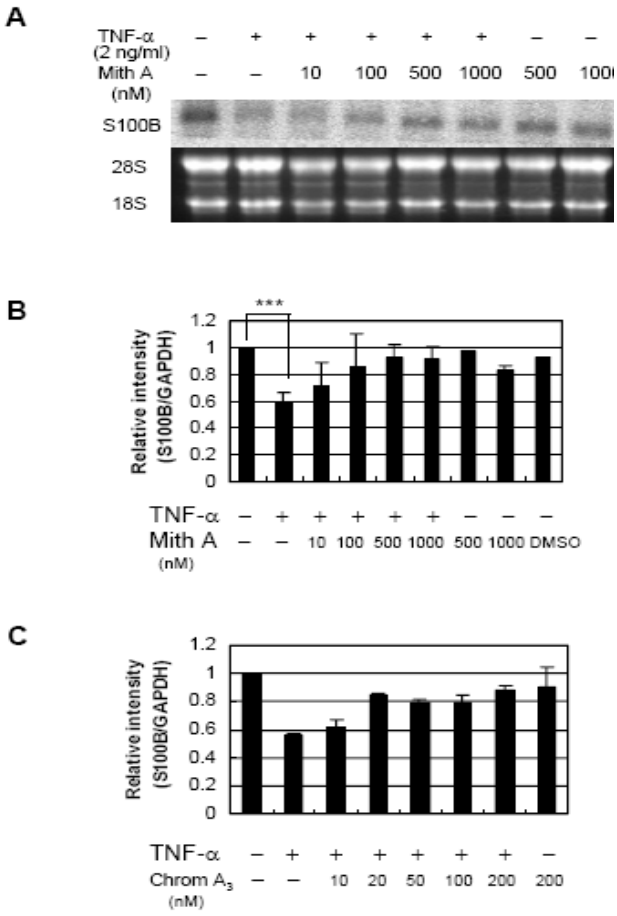

Fig. 4. Mithramycin A and chromomycin A3 inhibit TNF-α-mediated repression of S100B mRNA. U373 cells were pretreated with the indicated concentrations of mithramycin A (A) or chromomycin A_3 (C) for 2 h, and then treated with 10 ng/ml of TNF-α for 24 h. Total RNAs isolated from the cells were subjected to Northern blots or RT-PCRs. Densitometric analyses are presented as the relative ratio of S100B mRNA to the control buffer-treated cells in Mith A-pretreated cells (B). Three independent experiments of Northern blot were performed, respectively, and the results were similar.

6. Sp1 is involved in the TNF-α-mediated repression of S100B mRNA.

To further determine whether Sp1 transcription factor binding site is involved in the regulation of S100B mRNA expression in response to TNF-α, S100B promoter activities were measured. The S100B promoter contains an Sp1-binding site at position +2 in exon 1 (Fig. 5A) [35]. We used four previously described plasmid constructs [19] in these experiments; three of them (pGBS-4437, pGBS-788 and pGBS-168) contained Sp1-binding sites along with different lengths of the promoter region, while the fourth (pGBSΔ-168/+697) contained the entire promoter region without the Sp1 binding site. U373 cells were transiently transfected with the plasmids, and luciferase activity was measured in cells treated with or without TNF-α. In cells transfected with plasmids pGBS-4437, pGBS-788 and pGBS-168, the luciferase activities were unchanged following TNF-α treatment. However, the luciferase activity increased about 3-fold in U373 cells transfected with pGBSΔ-168/+697 and treated with TNF-α (Fig. 5B), suggesting that the Sp1-binding site located near the transcription initiation site is required for the regulation of S100B expression by TNF-α.

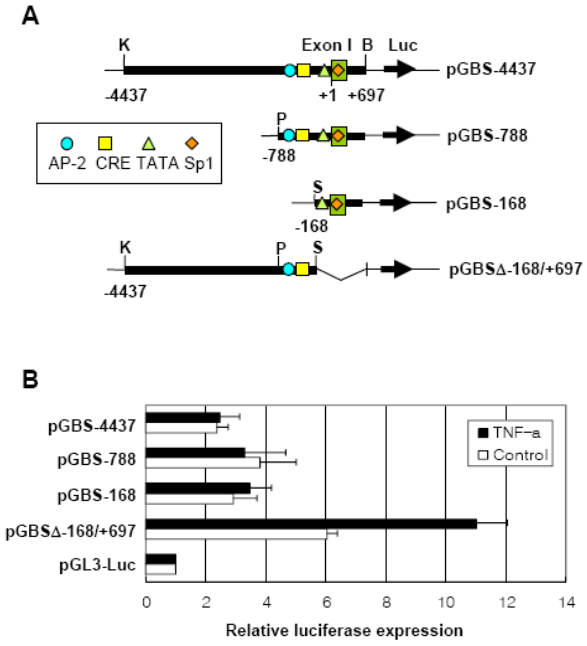

Fig. 5. Human S100B promoter activity in U373 cells after TNF-α treatment. Schematic human S100B promoter-luciferase constructs [19] used in this study (A). The key TNF-α-responsive element is located in the S100B promoter from -168 to +697. B. *Bam*HI; K, *Kpn*I; P, *Pvu*II; S, *Sma*I. Numbering is relative to the transcription initiation site designated +1. B. Cells were transiently transfected with 0.2 μg of the indicated constructs and treated or not treated with 10 ng/ml of TNF-α for 24 h. Luciferase activities were determined, and normalized versus β-galactosidase expression (B). Four independent experiments were performed.

7. TNF-α inhibits Sp1 binding activity to Sp binding site of S100B promoter.

To investigate whether Sp1 transcription factor can bind to Sp1 binding site of S100B promoter, we performed EMSA assay. Nuclear extracts were prepared from U373 cells treated with 10 ng/ml of TNF-α. Three protein-DNA complexes interacted with a double-stranded cis-element 22-mer ODN probe corresponding to the region around the wild-type GC box of S100B promoter in the absence or presence of TNF-α (lanes 2, 7 and 8 of Fig. 6A). The addition of unlabeled ODNs as a decoy completely abolished the formation of all three complexes (lane 6 of Fig. 6A and 6B). As expected, all three complexes were diminished in nuclear extracts from the cells pretreated with TNF-α (Fig. 6A lane 8). We performed supershift assays using an anti-Sp1 antibody to confirm that Sp1 was involved in the binding to the S100B promoter. The Sp1 complexes were supershifted by the anti-Sp1 antibody in TNF-α-treated U373 cells (Fig. 6B lane 3 and 5), but not by an anti-Sp3 antibody (Fig. 6B lane 4). To confirm that endogenous Sp1 binding to Sp1 binding site on S100B promoter can decrease upon TNF-α stimulation, Consistent with EMSA result, Chip assay showed that TNF-α decreased Sp1 binding activity to Sp1 binding site on S100B promoter (Fig. 6D).

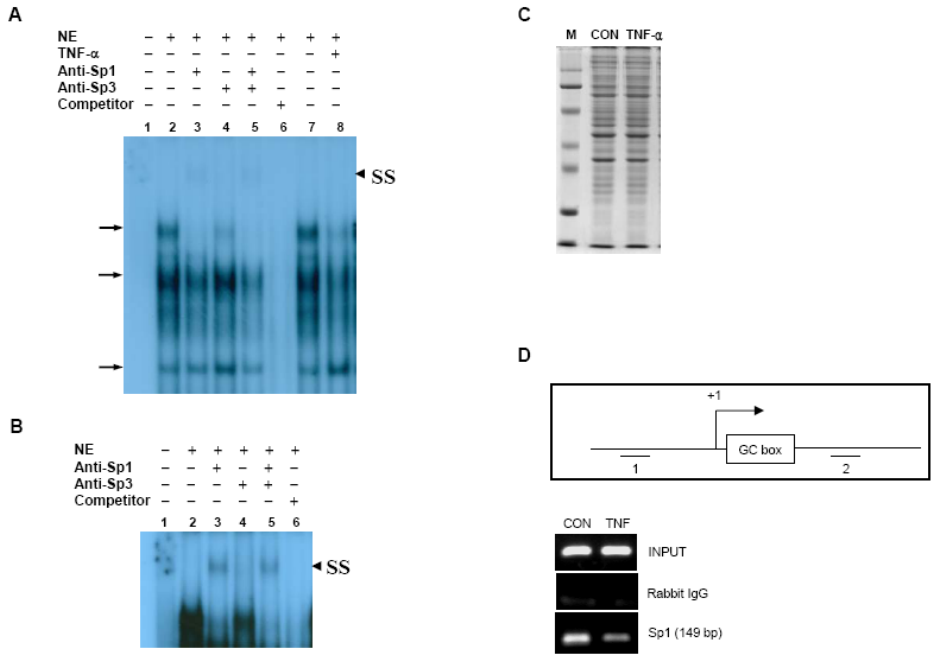

Fig. 6. TNF-α inhibits Sp1 binding activity to S100B promoter region. U373 cells were pretreated with mithramycin A (Mith A) and then treated with 10 ng/ml of TNF-α for 24 h. The binding activities of Sp1 in nuclear extracts (5 μg) were assayed by EMSA (A). Supershift analysis of protein-DNA complexes using polyclonal antibodies to Sp1 and Sp3 transcriptional factors (B). The protein-DNA complexes are designated by arrows. The supershifted band is identified as black triangle. Coomassie brilliant blue staining shows the same amount of nuclear extracts in EMSA assay (C). Chip assay was performed with anti-Sp1 or normal rabbit IgG antibody. Immunoprecipitated DNA was amplified by using primer for Sp1 binding site (-45 to +76) of S100B promoter region (D). NE, nuclear extracts; TSA, trichostatin A. Competition experiments were performed with a 100-fold excess of the unlabeled oligonucleotides.

8. TNF-α represses S100B expression via Sp1 and increases the association of HDAC-1 and Sp1.

To confirm that Sp1 is critical response element for TNF-α stimulation, cells were transfected with siRNA of Sp1. When the Sp1 protein expression level was decreased by siRNA of Sp1 transfection, S100B transcription level was not decreased by TNF-α treatment (Fig. 7A). To examine the mechanism How can Sp1 binding activity reduce upon TNF-α, we study weather HDAC-1 is involved in TNF-α-mediated suppression of S100B expression, we performed immunoprecipitation using anti-HDAC-1 antibody. As shown in Figure 7B, After TNF-α stimulation, the binding of Sp1 and HDAC-1 was increased but TNF-α did not affect Sp1 acetylation.

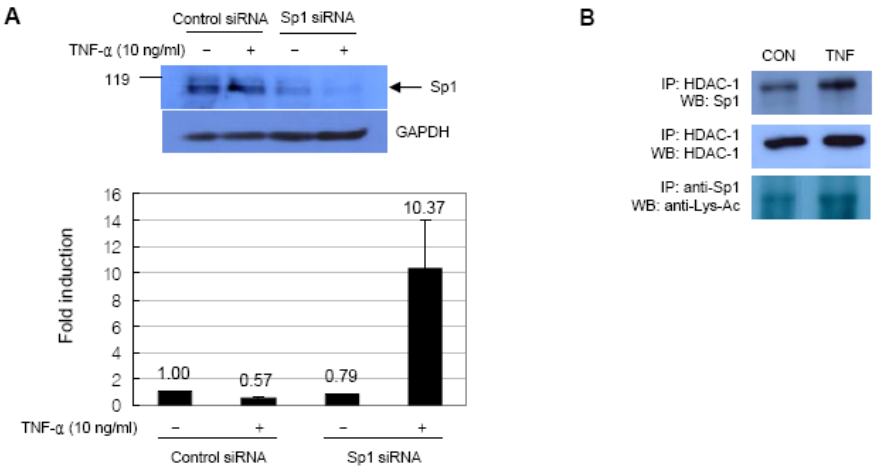

Fig. 7. Sp1 is essential to TNF-α-mediated repression of S100B and interacts with HDAC-1. U373 cells were transfected with siRNA of Sp1. After 24 h, cells were treated with 10 ng/ml of TNF-α for 24 h and the level of S100B mRNA was measured by Real time PCR (A). Immunoprecipitaion was performed with anti-HDAC-1 or anti-Sp1 antibody and anti-Sp1, HDAC or acetyl lysine antibody were used in immunoblotting (B).

IV. DISCUSSION

S100B takes some part not only in the pathophysiology of neurodegenerative disorders typical of Alzheimer's disease and Down's syndrome, but also in brain inflammatory diseases[36]. TNF-α plays critical roles in the development and progression of CNS diseases, including Alzheimer's disease and CNS infections[22]. It has been reported that TNF-α reduced intracellular expression of GFAP and S100B[37-38]. However, the molecular mechanisms that how can TNF-α suppress intracellular expression of GFAP and S100B in brain are still unknown. In this study, we have identified a mechanism for TNF-α-induced repression of S100B gene expression, via inhibition of Sp1 binding in human primary astrocytes and an astrocytoma cell line.

Time course analyses showed that TNF-α-induced S100B repression in primary astrocytes required over 24 h to achieve maximal effect, and was then maintained for at least 48 h (Fig. 1). Previous work has been shown that TNF-α treatment for long period decreased S100B protein level as well as GFAP. Many articles report that TNF-α activates the S100B secretion and it's accumulation may be detrimental to astrocytes and neurons. In that case, These TNF-α was treated for short time so it can be explained the role of TNF-α on S100B expression in early stage of brain disease. In contrast to it, our experimental condition was similar to other's group. More exposing to TNF-α, more decreasing the S100B protein. These results collectively suggest that the effects

of TNF-α on S100 proteins may differently act on development of brain disease.

TNF-α-induced pro-inflammatory gene transcription is primarily related to the activation and translocation of NF-κB. However, NF-κB inhibitors did not block the TNF-α-induced repression of S100B mRNA expression, and the S100B promoter sequence does not contain a direct binding site for NF-κB [35]. Thus, it appears that NF-κB is not directly involved in the TNF-α-induced decrease in S100B expression. In contrast, the promoter regions in the human [39] and mouse [40] S100A8/A9 genes contain putative NF-κB binding sites. The S100B promoter-dependent transcription has been observed in the mouse [41] and the rat [42]. Thus, cell type specificity and differences in the promoter regions of S100B and S100A8/A9 may explain the different responses of S100B and S100A8/A9 to TNF-α.

We have identified that Sp1 may play an important role in TNF-α-mediated repression of S100B expression in cultured astrocytoma cells. Transcriptional repression can be mediated by alterations in the activity or DNA binding properties of *trans*-activators or repressor or by epigenetic mechanisms that alter DNA structure. Our data indicate that transcriptional repression of S100B by TNF-α requires binding of Sp1 to the S100B promoter (Fig. 6). Consistent with this result, real binding of Sp1 to NF-κB binding site in DNA was decreased. Sp1 is a direct target for histone deacetylase 1 (HDAC1)-mediated

transcriptional repression. In figure 7B, HDAC1 and Sp1 binding was increased in TNF-α-treated cells suggesting that HDAC may mediate the transcriptional repression of S100B by binding to Sp1 [43]. Furthermore, alteration of the chromatin structure in the transcribed region of a gene has been shown to be associated with histone modification [44]. We are currently investigating the effect of histone modification on S100B expression.

The cells associated with extracellular plaques within the brains of Alzheimer's disease patients produce a variety of cytokines and other related proteins that can ultimately influence plaque and tangle formation. Cytokines typically associated with amyloid plaques (*e.g.*, IL-1, IL-6 and TNF-α) may influence the expression of additional factors that participate in the pathogenesis of Alzheimer's disease. For example, elevated levels of IL-1, such as those found in the brain tissues of Alzheimer's disease patients, can upregulate S100B expression in astrocytes [13, 15, 45]. In addition, S100B stimulates IL-1β production in microglia [46-47] and β-amyloid precursor protein levels in neurons [48], and both S100B and IL-1β can enhance the ability of amyloid-beta (Aβ)1-42 to activate astrocytes [49]. The interrelationships among these various glial activating proteins, as well as the finding that overexpression of S100B in Alzheimer's disease correlates with the degree of neuritic amyloid plaques [14], suggest that high levels of S100B may be an important factor in the pathogenesis of Alzheimer's disease. Altogether, these results and our present findings suggest

that S100B expression in neuropathological conditions is likely to be dependent on the net effect of cytokine interactions.

In summary, we have shown that TNF-α represses S100B expression in primary astrocytes and astrocytoma cells via the Sp1 transcriptional factor. However, the combined effects of TNF-α and other cytokines on S100B expression, and their influence on pathological brain conditions, remain to be elucidated.

CHAPTER II.

HMGB1 inhibits NF-κB activation mediated poly(I:C)-TLR3 signaling

I. INTRODUCTION

HMGB1 is a ubiquitous nuclear protein and its amino acid sequence is highly conserved across species. Structurally, HMGB1 has two DNA binding motifs of approximately 80 amino acids termed the A and B boxes respectively, and a highly acidic C-terminal domain. In most cells, HMGB1 is located in the nucleus where it acts as an architectural protein that stabilizes nucleosomes and facilitates transcription[50]. HMGB1 is released actively by macrophages and monocytes[51-52] and passively by necrotic cells[53]. HMGB1 leaves to the extracellular space when monocytes/macrophages actively secrete it following acetylation/phosphorylation and transfers to secretory endolysomal compartments[54].

In this study, we investigate two questions. The first question is whether HMGB1 can be localized in intracytoplasmic space or not. Interestingly, recent reports suggest that HMGB1 localization is not limited in the nuclei[55]. This information lead us to study that the role of HMGB1 following according to it's localization. The second question is what the role of intracytoplasmic HMGB1 is.

Toll-like receptor 3 (TLR3) recognizes polyriboinosinic:polyribocytidylic acid poly(I:C), a synthetic analog of dsRNA, and transduces signals to activate NF-κB and IFN-β promoters via MyD88-independent and TRIF dependent manner[56-57]. dsRNA activates four transcription factors: IRF-3, c-Jun, ATF-2, and NF-κB through TLR3 and TRIF dependent pathway[58]. TLR4 recognizes bacterial lipopolysaccharide and triggers both MyD88- and TRIF-dependent pathways[59]. It has been reported that endogenous and viral inhibitors of TLR3 regulate NF-κB or IRF3 activation. Many signaling molecules such as TRIF, TRAF6, RIPs, TBK1/IKKε, and IRF3 were targeted by several inhibitors[60].

Here we report that intracytoplasmic HMGB1 specifically inhibits NF-κB activation induced by TLR3 signaling, which is mediated by p65 transcription factor.

II. MATERIALS AND METHODS

1. Cell culture and antibodies

HEK293-hTLR3 and HEK293-hTLR4/MD2-CD14 (HEK293-hTLR4) stable cell lines (InvivoGen, San Diego, CA, USA), which were stably expressing TLR3 and TLR4 in HEK293 cells, respectively, and HEK293 cells were used in this study. The cells were cultured at 37^{o}C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Ten μg/ml blasticidin for HEK293-hTLR3 cells and both 10 μg/ml blasticidin and 50 μg/ml HygroGold for HEK293-hTLR4 cells were added to maintain the respective cell lines.

The antibodies used in the experiments were anti-HMGB1 from Abcam (abcam, Cambidge, UK), anti-human TLR3 antibody from R&D (R&D, Minneaolis, MN), anti-GFP antibody (sc-9996) from SantaCruz Biotechnology (SantaCruz, CA), and anti-GAPDH antibody from AbFrontier Inc. (Seoul, Korea).

2. Luciferase reporter assay

To investigate the role of intracytoplasmic HMGB1 in TLR3-mediated NF-κB signaling, HEK293-hTLR3 cells were grown in 24 well plates with 10% FBS-DMEM, containing 10 μg/ml blasticidin. The cells were co-transfected

with pHMGB1-EGFP-N1[54], pIL6-κB-Luc plasmid and pCMV-β-galactosidase (Promega) using the FuGene6 transfection reagent (Roche). After 24 h incubation, the cells were stimulated with 10 μg/ml of poly(I:C) (Amersham Bioscience) for 4 h. Luciferase activity was measured by a Luciferase Reporter Assay System (Promega), and β-galactosidase activity was measured O-nitrophenyl-β-D-galactopyranoside as a substrate. Luciferase activity of each sample was normalized with respect to β-galactosidase activity for calculation of the activation value. To observe the effect of HMGB1 on IRF-3 activation, pISRE-Luc plasmid (Stratagene, La Jolla, CA) was used for the transfection.

To investigate the role of intracytoplasmic HMGB1 in LPS-stimulated NF-κB activity in TLR4 signaling, HEK293-hTLR4 cells were also used. For this, 0.2 μg/ml of LPS (*E. coli* 0111:B4; Sigma-Aldrich) was used.

3. Immunoprecipitation and Western blotting

HEK293-hTLR3 cells were transiently transfected with pHMGB1-EGFP-N1, the cells were lysed with RIPA buffer after 24 h incubation. The cell lysates were precleared by incubation with 20 μl of 50% protein G-Sepharose (Amersham) slurry for 1 h at 4˚C. The precleared lysates were then incubated with 1 μg of anti-HMGB1 antibody (Abcam) overnight, and then 20 μl of 50% protein G-Sepharose slurry was added to the lysates. After incubation for 1 h at 4˚C, the protein G-Sepaharose was washed twice prior to preparation for

SDS-PAGE. For Western blotting, the samples were boiled with sample loading buffer and electrophoresed in 10% SDS polyacrylamide gel. For the stripping, the membranes were incubated in buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS, 0.1 M 2-mercaptoethanol) at 60˚C for 30 min and washed extensively with PBST containing 0.05% Tween 20.

4. Isolation of nuclear extracts

Nuclear extracts from cells were prepared according to the previous report [61]. In brief, the cells were incubated in 600 μl of hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1% Nonidet P40) for 5 min and then lysed by the addition of 0.3% Nonidet P-40. Cell lysate was centrifuged at 13,000 rpm for 1 min to pellet the nuclei, and the cytosolic fraction was removed. Nuclei were extracted by incubating in 50 μl of extraction buffer (25 mM HEPES pH7.9, 0.3 M NaCl, 1 mM EDTA, 0.1 mM EGTA, and 0.1 mM PMSF) for 30 min followed by centrifugation at 13,000 rpm for 10 min. The supernatant was harvested and aliquoted after centrifugation.

5. Confocal microscope imaging

To observe the localization of HMGB1 and p65 proteins, HEK293-hTLR3 cells were cultured in LabTek II chambers (Nalge Nunc, Naperville, IL, USA).

Cells were transfected with pHMGB1-EGFP-N1 and incubated for 24 h. Cells were treated with 10 μg/ml of poly(I:C) for 30 min, followed by fixation in 4% paraformaldehyde in PBS buffer for 30 min at RT. After fixation, the cells were washed with PBS and incubated for 5 min. Cells were incubated with PBS-based permeabilization buffer containing 0.1% Triton X-100 for 10 min. The cells were incubated with rabbit anti-p65 antibody (Santa Cruz, sc-8008) overnight at 4˚C after blocking with 1% BSA in 0.02% PBST for 20 min. After three washes with blocking solution, Alexa Flour 594-labeled donkey anti-rabbit IgG antibody (Molecular Probes) was added. Cells were observed with a FV1000 confocal microscope (Olympus) after mounting with vectashield mouning medium which contains DAPI (Vector Laboratories, Burlingame, CA, USA).

6. Cytokine production and MAPK activation assay

The effect of intracytoplasmic HMGB1 on the cytokine production, Human Cytokine Array Panel A Array Kit (R&D, Minneapolis, MN, USA) was used. For this, HEK293-hTLR3 cells ($3X10^6$ cells in 100 mm dish) were transfected with pHMGB1-EGFP-N1 and cultured for 24 h in 10 ml of serum-free OPTI-MEM medium. The cells were treated with 10 μg/ml of poly(I:C) for 18 h, and 1 ml of cell supernatant was harvested and applied on the membrane which contains 36 different anti-cytokine antibodies printed in duplicate. Added biotinlyated detection antibody cocktail to sample and incubated overnight at

4˚C. Washed the membrane and incubated with Streptavidin-HRP for 30 min at RT. Washed the membrane and incubated the membrane with chemiluminescent detection reagent. pEGFP-N1 was used as a negative control plasmid.

To observe the effect of intracytoplasmic HMGB1 on MAPK activation, Proteome profiler^{TM} array was used. HEK293-hTLR3 cells were transfected with pHMGB1-EGFP-N1 and were treated with 10 μg/ml of poly(I:C) for 30 min. Cell lysates (500 μg) was used for Human phosphor-MAPK array kit (R&D, Minneapolis, MN, USA).

7. Karyopherin-GST binding assay

To investigate whether p65 is inhibited to translocate into the nucleus, the inhibition of p65 binding to nuclear import protein by HMGB1 was tested using immunoprecipitation assay. For this, GST-fused nuclear import proteins of GST-KAP proteins were prepared as reported previously[54]. Two μg of each GST-KAP protein was coupled to glutathione-Sepharose 4B beads. HEK293-hTLR3 cells were treated with 10 μg/ml of poly(I:C) for 4 h and whole cell lysates were prepared as a source of p65 protein. To test whether HMGB1 inhibits the association between p65 and KAP proteins, we mixed the same amount of GST-KAPα3/5 either recombinant HMGB1 protein or HMGB1-EGFP transfected whole cell lysates. After incubation, immunoblotting was performed with anti-HMGB1, p65 and GST antibodies.

8. Chimera RNA interference of HMGB1

HEK293-hTLR3 ($1X10^6$) cells were grown on 6 well overnight. Cells were co-transfected with 5 nM of chimera RNAi of HMGB1 (Abnova, catalog #H00003146-R01), pIL6-κB-Luc, and pCMV-β-galactosidase plasmids by using Lipofectamine 2000. After 24 h, 10 μg/ml of poly(I:C) was added and incubated for 4 h. Cells were lysed and measured luciferase activity. Naito 1 Pre-design chimera RNAi (Abnova, catalog #R0017) was used as a negative control in HMGB1 knockdown experiments.

III. RESULTS

1. Intracytoplasmic HMGB1 inhibits NF-κB activation in poly(I:C)-induced TLR3 signaling.

To investigate the effect of HMGB1 in poly(I:C)-mediated TLR3 signaling, HEK293-pUNO (null) or HEK293-hTLR3 cells were co-transfected with pHMGB1-EGFP-N1[54] and pIL-6-κB-luciferase plasmids. After 24 h, cells were treated with 10 μg/ml of poly(I:C) for 4 h and NF-κB promoter activity was measured. As a result, poly(I:C)-induced NF-κB luciferase activity was decreased in the presence of HMGB1. These results were not shown in HEK293-pUNO (null) cells which do not express human TLR3 (Fig. 1A). Fig. 1B shows that HMGB1 inhibited poly(I:C)-induced activation of NF-κB in a dose-dependent manner. In contrast, HMGB1 had no effect on ISRE activity induced by poly(I:C). We also observed that HMGB1 did not affect TBK1 mediated ISRE activation and IRF3 phosphorylation (Fig. 1D and 1E). TLR3 and TLR4 signaling share TRIF-dependent pathway which leads to NF-κB activation. To address this, NF-κB luciferase activity was measured by using HEK293-hTLR3 and HEK293-hTLR4 cells. As shown in Fig. 1B and 1C, intracytoplasmic HMGB1 dose-dependently inhibited NF-κB luciferase activity as well as in poly(I:C)-stimulated HEK293-hTLR3 cells and LPS-stimulated HEK293-hTLR4 cells. In the same conditions of poly(I:C) or LPS stimulation,

ISRE promoter activity was not significantly affected by HMGB1. These results suggest that intracytoplasmic HMGB1 specifically inhibits poly(I:C) induced NF-κB activation.

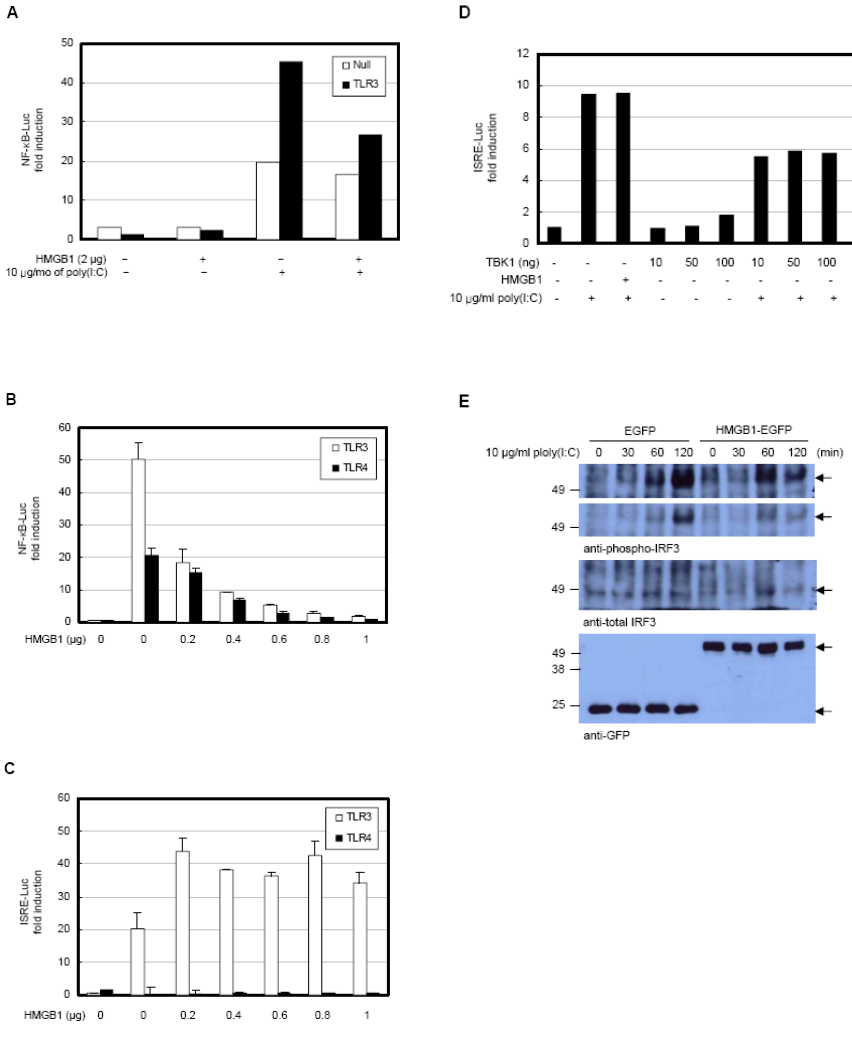

Fig. 1. Intracytoplasmic HMGB1 inhibits poly(I:C)-induced NF-κB but not ISRE activation. HEK293-pUNO (null) or HEK293-hTLR3 (TLR3) cells were transfected with pHMGB1-EGFP-N1 (0.5 μg), β-galactosidase (0.3 μg), and pIL-6-kB-luciferase (0.1 μg) plasmids. After 24 h, cells were treated with 10 μg/ml of poly(I:C) for 4 h (A). HEK293-hTLR3 or HEK293-hTLR4 stable cells were transfected with pHMGB1-EGFP-N1 along with pIL-6-κB-Luc or ISRE-Luc plasmid. After 24 h, cells were treated by 10 μg/ml of poly(I:C) or 0.2 μg/ml of LPS for 6 h (B and C). HEK293-hTLR3 cells were transfected with ISRE-Luc plasmid along with the indicated amounts of TBK1 (10, 50, 100 ng) plasmid and pHMGB1-EGFP-N1 plasmid. After 24 h, cells were treated with 10 μg/ml of poly(I:C) for 4 h and ISRE Luc activity was measured (D). HEK293-hTLR3 cells were transfected with pEGFP-N1 or pHMGB1-EGFP-N1 plasmid. After 24 h, cells were treated with 10 μg/ml of poly(I:C) for the indicated time. Protein extracts were analyzed by immunoblotting with specific antibodies directed to phosphorylated form of IRF3. Protein loading was controlled by reprobing with anti-IRF3 antibody. Protein extracts were simultaneously analyzed for the expression of EGFP-tagged proteins (E).

2. Intracytoplasmic HMGB1 inhibits IκBα phoshporylation in poly(I:C) stimulated HEK293-hTLR3 cells.

To determine the role of HMGB1 in poly(I:C)-TLR3-mediated NF-κB signaling, we examined the effect of intracytoplasmic HMGB1 on IKKα, IKKβ, and IκBα. HEK293-hTLR3 cells were transfected with pEGFP-N1 or pHMGB1-EGFP-N1 plasmid. After 24 h, cells were treated with 10 μg/ml of poly(I:C) for the indicated time. Intracytoplasmic HMGB1 inhibited IκBα phosphorylation in poly(I:C) stimulated HEK293-hTLR3 cells (Fig. 2A). After poly(I:C) stimulation, the level of IκBα phosphorylation was dose-dependently decreased in pHMGB1-EGFP-N1-transfected cells (Fig. 2B). In NF-κB signaling, IκBα is mainly phosphorylated by IKKα and IKKβ. When HMGB1-transfected cells were treated with poly(I:C), we detected the similar pattern of IKKα or IKKβ phosphorylation like in the EGFP only transfected cell (Fig. 2C). These data suggest the intracytoplasmic overexpression of HMGB1 inhibits poly(I:C)-induced NF-κB activation by inhibiting IκBα phosphorylation.

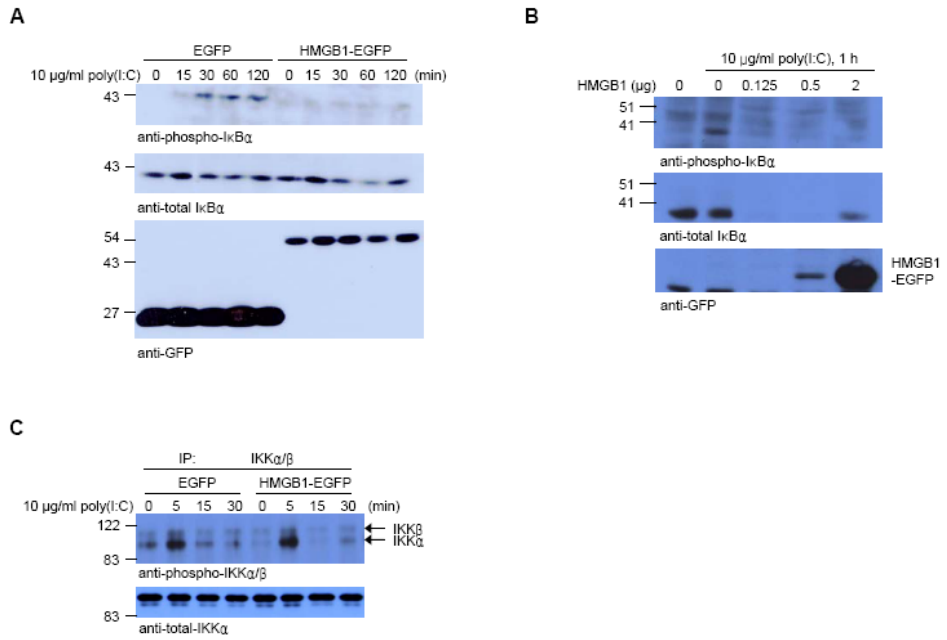

Fig. 2. Intracytoplasmic HMGB1 inhibits IκBα phosphorylation by poly(I:C) stimulation. HEK293-hTLR3 cells were transfected with EGFP or HMGB1-EGFP plasmid. After 24 h, cells were treated with 10 μg/ml of poly(I:C) for the indicated time intervals. Protein extracts were analyzed by immunoblotting with specific antibodies directed to phosphorylated form of IκBα. Protein loading was controlled by reprobing with anti-IκBα antibody. Protein extracts were simultaneously analyzed for the expression of EGFP-tagged proteins (A). Dose effect of intracytoplasmic HMGB1 on IκBα phosphorylation after poly(I:C) treated for 1 h (B). IKKα/β phosphorylation was not changed in pHMGB1-EGFP-N1 (2 μg) plasmid transfected cells after poly(I:C) treatment (C).

3. Intracytoplasmic HMGB1 inhibits mitogen-activated protein kinase (MAPK) activation after poly(I:C) stimulation.

It has been reported that poly(I:C)-induced TLR3 signaling activates MAPK phosphorylaton which results cytokine production[62-63]. To investigate that intracytoplasmic HMGB1 is involved in MAPK activation after poly(I:C) treatment, HEK293-hTLR3 cells were transfected with pEGFP-N1 or pHMGB1-EGFP-N1 plasmid. After 10 μg/ml of poly(I:C) stimulation, cell supernatants and whole cell lysates were harvested to assess cytokine production and phosphorylation of MAPK. In the presence of HMGB1, phospho-ERK1/2 was strongly decreased (Fig. 3A) and SDF-1 cytokine was slightly reduced (Fig. 3B). These results suggest that intracytoplasmic HMGB1 affect poly(I:C)-induced MAPK activation.

4. Intracytoplasmic HMGB1 binds to p65 in cytoplasm by poly(I:C) stimulation.

To understand how HMGB1 inhibits poly(I:C)-induced NF-κB activation, we performed immunoprecipitation with anri-TRIF, TRAF6, TAK1, and p65 antibodies. When whole cell lysates were used, we found that HMGB1 was bound to only endogenous p65 (Fig. 4B). This result prompted the investigation of the binding between HMGB1 and p65 in the cytoplasm which prevents p65 nuclear translocation. When HMGB1-transfected cells were treated with

poly(I:C), binding of HMGB1 and p65 was increased in the cytoplasm (Fig. 4A). After poly(I:C) stimulation, the binding of p65 and HMGB1 in the cytoplasm was increased in pHMGB1-EGFP-N1 plasmid- transfected cells (Fig. 4C). These data suggest HMGB1 inhibits poly(I:C)-induced NF-κB activation by interacting with p65 in the cytoplasm.

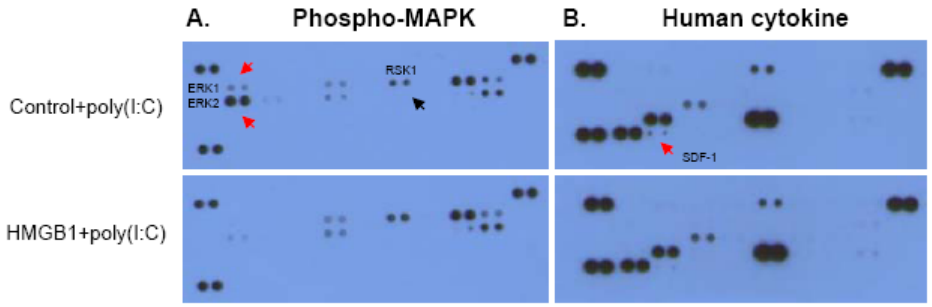

Fig. 3. Intracytoplasmic HMGB1 inhibits poly(I:C)-induced ERK1/2 phosphorylation. HEK293-hTLR3 cells were transfected with pEGFP-N1 and p HMGB1-EGFP-N1 for 24 h and cells were treated with 10 μg/ml of poly(I:C). Whole cell lysates and supernatants were used for phosphor-MAPK and cytokine array, respectively. Black arrow; increased spot, Red arrow; decreased spot

Next we identified which domain of HMGB1 was responsible for binding to p65. HEK293 cells were transfected with pHMGB1-EGFP-N1, pC-tail deleted HMGB1-EGFP-N1, pCMV-A box-myc, and pCMV-B box-myc plasmids. pEGFP-N1 and pCMV-myc plasmids were used as control vectors. We confirmed that all proteins were effectively expressed in whole cell lysates (Fig. 5A). Then, we reciprocally performed immunoprecipitation with anti-p65 and anti-HMGB1/myc. The interaction between HMGB1 and p65 is mediated by the two HMG boxes, which are both able to bind p65, albeit weakly. The strongest interaction occurred with a truncated HMGB1 protein, which contained both HMG boxes but lacked the acidic tail, suggesting that the acidic tail interfere with the binding surface for p65 (Fig. 5C). When control vector-transfected cell lysates were mixed with anti-HMGB1 antibody, we also detected the strong p65 band. These results indicate that endogenous HMGB1 can be associated with endogenous p65.

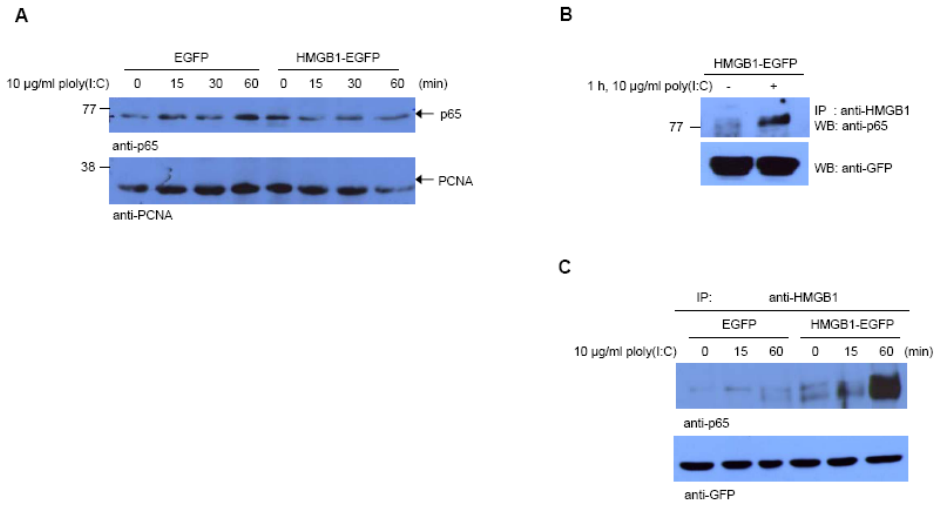

Fig. 4. The association between intracytoplasmic HMGB1 and p65 is increased in poly(I:C)-stimulated HEK293-hTLR3 cells. The translocation of p65 was detected with the anti-p65 antibody in pEGFP-N1 or pHMGB1-EGFP-N1 tranfected cells (A). After poly(I:C) stimulation, HMGB1 bound to p65 in whole cell lysates (B). After poly(I:C) treatment, binding between HMGB1 and p65 was increased in the cytoplasm (C).

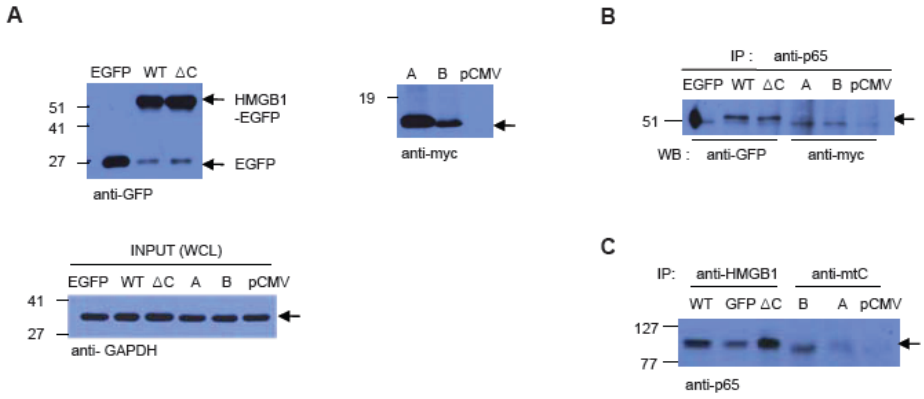

Fig. 5. HMGB1 binds to endogenous p65 in the HEK293 cells. HEK293 cells were transfected with pEGFP-N1, pHMGB1-EGFP-N1, pC-tail deleted HMGB1-EGFP-N1, pCMV-myc, pCMV-HMGB1 A box-myc, pCMV-HMGB1 B box-myc plasmids. Whole cell lysates were immunoblotted with anti-GFP, anti-myc or anti-GAPDH (loading control) antibodies for the expression 24 h later transfection (A). Immunoprecipiation was performed with anti-p65 (B), anti-HMGB1 and anti-myc antibodies (C), and immunoblotted with corresponding Abs to observe the binding interaction. EGFP: pEGFP-N1, WT: pHMGB1-EGFP-N1, ΔC: plasmid of acidic-tail deleted HMGB1-EGFP-N1, pCMV: pCMV-myc, A: pCMV-HMGB1 A box-myc , B: pCMV-HMGB1 B box-myc.

5. Intracytoplasmic HMGB1 inhibits the interaction of p65 and karyopherins.

It has been reported that the NF-κB components of the classical and alternative pathways have different specificities to karyopherin molecules. There is a correlation between binding of karyopherins to different nuclear localization sequences (NLS) and their ability to be imported into the nucleus. In order to investigate the type of karyopherins which interacts with p65 after poly(I:C) stimulation, we prepared GST-karyopherin fusion proteins α1, α2, α3, α4, α5, α6, and β1 from *E. coli* (Fig. 6B). HEK293-hTLR3 cells were treated with poly(I:C) for 1 h and were allowed to bind to Sepharose 4B-immobilized GST-karyopherins α1, α2, α3, α4, α5, α6, and β1 (Fig. 6A). All types of karyopherins bound to p65 upon poly(I:C) stimulation and their binding was increased by poly(I:C) stimulation.

We tested whether HMGB1 could interfere the association between NF-κB and the karyopherins α3/α5. As shown in Fig. 6C and 6D, recombinant HMGB1 inhibited the interaction between NF-κB and karyopherins α3 and α5 in a dose-dependent manner. Furthermore, whole cell lysates from pHMGB1-EGFP-N1 transfected cells show a decreased binding to karyopherin α3/α5 in contrast to the control whole cell lysates (Fig. 6C and 6D). We suggest that intracellular HMGB1 binds and masks the NLS on NF-κB thereby preventing its exposure to karyopherin and its transport into the nucleus.

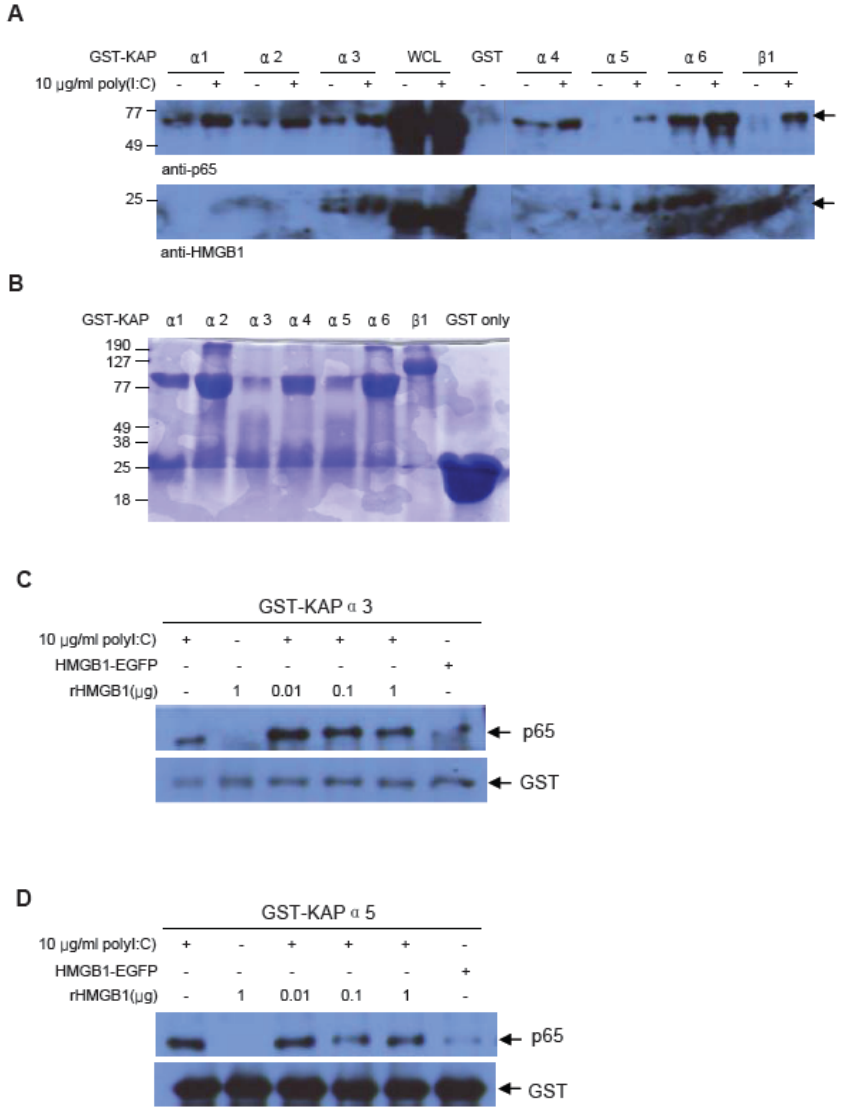
A
GST-KAP
α1
α 2
α 3
WCL
GST
α 4
α 5
α 6
β1
10 μg/ml poly(I:C)
77
49
anti-p65
25
anti-HMGB1
B
GST-KAP
α1
α 2
α 3
α 4
α 5
α 6
β1
GST only
190
127
77
49
38
25
18
C
GST-KAP α 3
10 μg/ml polyI:C)
HMGB1-EGFP
rHMGB1(μg)
p65
GST
D
GST-KAP α 5
10 μg/ml polyI:C)
HMGB1-EGFP
rHMGB1(μg)
p65
GST

Fig. 6. HMGB1 interferes with p65 and karyopherin interaction. (A) HEK293-hTLR3 cells were both untreated and treated with 10 ng/ml of poly(I:C) for 1 h. Whole cell lysates were mixed with Sepharose-immobilized bacterially expressed GST-karyopherins. Sepharose-bound proteins were detected by immunoblotting with anti-p65, anti-HMGB1 antibodies. (B) Coomassie brilliant blue staining of purified GST-karyopherin proteins. (C and D) GST-KAP α3/α5-coupled Sepharose beads were incubated with whole cell lysate as a source of p65 in the presence of various amounts of recombinant HMGB1. WCL (polyI:C): whole cell lysate from poly(I:C)-treated HEK293-hTLR3 cells, WCL (H/E, polyI:C) WCL from poly(I:C)-treated HEK293-hTLR3 cells overexpressed with HMGB1-EGFP.

6. Intracytoplasmic HMGB1 co-localizes with p65 upon poly(I:C) treatment.

To confirm whether HMGB1 is co-localized with p65 after poly(I:C) treatment, HEK293-hTLR3 cells were transfected with pHMGB1-EGFP-N1 plasmid and poly(I:C) was treated for 30 min. As shown in Fig. 7, HMGB1-EGFP (green) was observed in the cytoplasm and co-localized with p65 when HEK293-hTLR3 cells were treated with poly(I:C) (Fig. 7). These data suggest that intracytoplasmic HMGB1 inhibits poly(I:C)-induced NF-κB migration to nucleus by interacting with p65 in the cytoplasm.

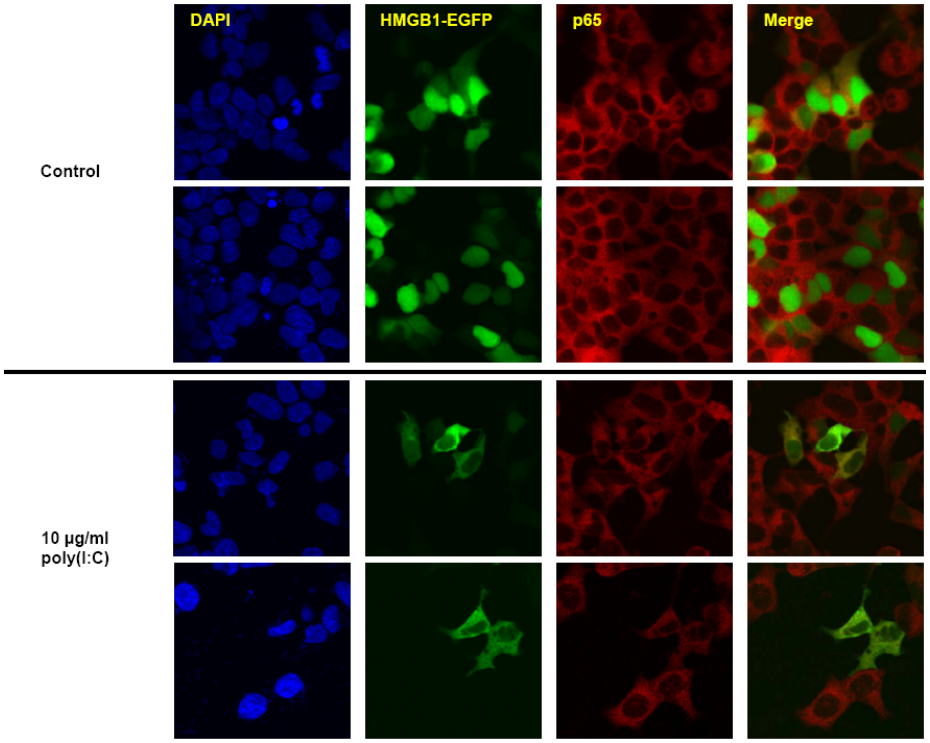

Fig. 7. The binding between HMGB1 and p65 is increased in the cytoplasm after poly(I:C) stimulation. HEK293-hTLR3 cells were transfected with pHMGB1-EGFP-N1 plasmid and treated with 10 μg/ml of poly(I:C) for 30 min. Cells were stained with anti-p65 and Alexa Flour 594-labeled secondary Abs. DAPI was used for nuclear staingin.

7. HMGB1 knockdown increases poly(I:C)-induced NF-κB activation.

Next to confirm whether knockdown of HMGB1 can increase poly(I:C)-induced NF-κB activation since HMGB1 can act as a negative

regulator in poly(I:C)-induced NF-κB activation. For this, HEK293-hTLR3 cells were transfected with 5 nM of chimera RNAi of HMGB1 and were treated with 10 μg/ml of poly(I:C). As shown in Fig. 8A, NF-κB luciferase activity was increased to 6.7 times when HMGB1 expression were decreased (Fig. 8B).

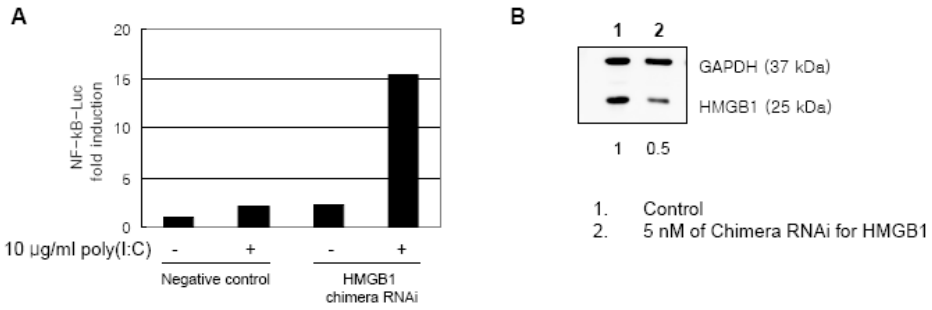

Fig. 8. Knockdown of HMGB1 increases NF-κB activation in HEK293-hTLR3 cells. (A) HEK293-hTLR3 cells were transfected with 5 nM of chimera RNAi of HMGB1 and NF-κB luciferase activity was measured. NF-κB luciferase activity was normalized to β-galactosidase. (B) Whole cell lysate was used from chimera RNAi of HMGB1- or control RNAi-treated cells to observe the expression of HMGB1.

IV. DISCUSSION

HMGB1 is a highly conserved component of eukaryotic nuclei. The role of HMGB1 has been reported by its localization. In the nucleus, HMGB1 acts as a transcriptional regulator. The presence of HMGB1 in the extracellular space implicates two situations. First, HMGB1 leaks from cells when the integrity of membranes is lost during necrosis. Second, HMGB1 can be secreted by monocytes, macrophages following LPS, IL-1 or TNF stimulation. Extracellular HMGB1 is an important necrosis marker and plays a role as a proinflammatory cytokine, however, the role of intracytoplasmic HMGB1 is still unknown. In this study, it is demonstrated that intracytoplasmic HMGB1 specifically inhibits NF-κB activation in poly(I:C)-induced TLR3 signaling. Intracytoplasmic HMGB1 was shown to inhibit NF-κB promoter activity in both poly(I:C)-stimulated HEK293-hTLR3 and LPS-stimulated HEK293-hTLR4 cells. These results indicate that HMGB1 can affect downstream TRIF-mediated NF-κB activation. After poly(I:C) stimulation, TRIF mediates IRF-3, NF-κB, and MAPK activation. Based on these data, intracytoplasmic HMGB1 had little effect on MAPK and IRF-3 activation. Unlike most TLR3 negative regulators, HMGB1 influences more directly NF-κB signaling molecules. As shown in Fig. 2A and B, HMGB1 partially inhibits IκBα phosphorylation and binds to p65 resulting in suppression of NF-κB activation after poly(I:C) stimulation.

The nuclear translocation of p65 is a critical step in NF-κB activation. My study of coimmunoprecipitation experiments indicated that endogenous HMGB1 could interact with endogenous p65 (Fig. 5), and intracytoplasmic HMGB1 inhibits poly(I:C)-induced translocation of p65 into nucleus (Fig. 4A and 7). Taken together, intracytoplasmic HMGB1 inhibits poly(I:C)-induced NF-κB activation by interaction with p65 in the cytoplasm. Karyopherin proteins are transport systems that recognize the nuclear localization signal (NLS) on p65 during its transport into the nucleus[64]. Karyopherin α3 and α5 are known to bind to p65 depending on different stimulations and different cell types[65]. In our data, all types of karyopherin were bound to p65 in HEK293-hTLR3 cells. Intracytoplasmic HMGB1 also binds strongly to p65 in HEK293-hTLR3 cells after poly(I:C) treatment. In addition, wild type HMGB1, acidic-tail deleted HMGB1, A box, and B box of HMGB1 proteins could bind to p65 protein in HEK293 cells. HMGB1 disrupts the association between karyopherin and p65 binding when recombinant HMGB1 was used as an inhibitor. Based on that the recognition of NLS in p65 protein by karyopherin is essential for p65 nuclear translocation, intracytoplasmic HMGB1 led to detain p65 in the cytoplasm by poly(I:C) stimulation (Fig. 7). Further experiments are necessary to show the mechanism how HMGB1 interferes the association between karyopherin and p65 protein.

CONCLUSION

PART I

1. TNF-α downregulates S100B expression in human primary astrocytes and astrocytoma cell line, U373.

2. The downregulation of S100B expression does not require new protein synthesis and NF-κB signaling.

3. Sp1 is involved in TNF-α-mediated suppression S100B expression.

4. TNF-α decreases Sp1 binding to Sp1 binding site on the S100B promoter.

5. TNF-α increases association between Sp1 and HDAC-1.

PART II

1. Intracytoplasmic HMGB1 inhibited NF-κB activation but not ISRE in poly(I:C)-induced TLR3 and LPS-treated TLR4 signaling.

2. Intracytoplasmic HMGB1 decreased poly(I:C)-induced IκBα phosphorylation.

3. Wild type HMGB1, acidic tail-deleted HMGB1 proteins showed the binding to p65 in the co-transfection study using HEK293 cells.

4. The nuclear translocation of p65 protein was inhibited by overexpressed HMGB1 in poly(I:C)-treated cells.

5. After poly(I:C) stimulation, the binding of HMGB1 to p65 was increased in the cytoplasm.

6. HMGB1 inhibited the binding of p65 to karyopherin α3 and α5.

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ABSTRACT IN KOREAN

S100B 발현조절과
TLR3 신호전달에서 세포 내 HMGB1의 억제 기능

<지도교수: 신 전 수>

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김 은 숙

S100B와 HMGB1 분자는 동일한 수용체인 RAGE에 대한 리간드로 알려져 있으며, 여러 세포 주에서 이러한 수용체를 매개로 염증반응에 중요한 역할을 한다. 본 연구에서는 첫째, 대표적인 염증 사이토카인의 하나인 TNF-α에 의한 S100B 단백질의 발현조절 기전을 규명하고, 둘째 TLR3 수용체를 매개로 이루어지는 NF-κB 활성을 억제하는 HMGB1의 새로운 기능을 밝혔다. S100B 단백질은 주로 중추신경계내 성상세포에서 발현되며, 특히, 신경퇴행적장애를 겪는

환자의 경우 S100B의 분비 수준이 매우 높다고 보고 되었다. 따라서, S100B의 발현조절 연구는 이러한 뇌 질환에 대한 이해를 높이는데 매우 중요할 것으로 기대된다. 사람의 성상세포 U373을 TNF-α로 처리한 결과 S100B의 mRNA, 단백질 발현 수준이 모두 감소함을 발견했다. 그리고 이러한 감소는 S100B promoter region내에 위치하는 Sp1 binding site에 대한 전사조절인자 Sp1의 결합 억제에 의한 것임을 확인했다. 또한 TNF-α 자극은 HDAC-1과 Sp1의 결합으로 증가시킴으로 Sp1 단백질의 결합을 억제하는 것으로 나타났다. 따라서 TNF-α에 의한 S100B 발현조절은 전사조절인자, Sp1을 통해 이루어짐을 밝혔다.

HMGB1 단백질은 DNA에 결합하는 핵 단백으로 처음 알려졌으며, 거의 모든 세포에서 발현된다. 먼저 TLR3 수용체가 과발현되는 세포주(HEK293-hTLR3)를 대상으로 HMGB1단백을 과잉발현 시킨 후, TLR3 수용체의 리간드인 poly(I:C)를 처리하여 TLR3 수용체를 통한 신호전달을 조사하였다. 그 결과, HMGB1은 특이적으로 poly(I:C)에 의한 NF-κB 활성을 억제하였으며, 이러한 억제는 HMGB1 단백 농도 의존적임을 밝혔다. 또한 TLR4 와 MD2 단백질을 과발현하는 세포주 (HEK293-hTLR4/MD2)를 LPS로 처리하였을 때도 동일한 결과를 나타냈다. 과발현된 HMGB1 단백은 poly(I:C)에 의한 IκBα의 인산화를

부분적으로 감소시켰다. 야생형 HMGB1, C-말단이 제거된 HMGB1은 세포 내 p65단백질과 결합함을 보였고, poly(I:C) 처리 후, p65의 핵 내로의 이동은 세포질 내 HMGB1 (cytoplasmic HMGB1) 단백 발현의 증가에 의하여 감소하였다. 이러한 결과는 세포질 내에서 poly(I:C) 처리 후, HMGB1과 p65의 증가된 결합을 면역침강법과 공초점 현미경을 통해 재확인되었다. HEK293-hTLR3 세포주에서 전사조절인자 p65가 여러가지 karyopherin 단백과 결합함을 확인하였으며, HMGB1은 p65와 karyopherin α3 및 α5의 결합을 억제함을 확인하였다. 이상의 결과로 세포질내 HMGB1은 세포신호전달에서 NF-κB의 활성을 억제하여 세포의 신호전달 조절에 관여함을 제시하였다.

핵심되는 말 : S100B, TNF-α, Sp1, HMGB1, poly(I:C) and TLR3

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