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The Box A Domain of High Mobility Group Box-1 Protein as an Efficient siRNA Carrier With Anti-Inflammatory Effects

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ABSTRACT

High mobility group box-1 (HMGB-1) is a DNA binding nuclear protein and pro-inflammatory cytokine. The box A domain of HMGB-1 (rHMGB-1A) exerts an anti-inflammatory effect, inhibiting wild-type HMGB-1 (wtHMGB-1). In this study, HMGB-1A was evaluated as an siRNA carrier with anti-inflammatory effects. HMGB-1A was expressed and purified by consecutive nickel chelate chromatography, cationic exchange chromatography, and polymixin B chromatography. Purified rHMGB-1A demonstrated an anti-inflammatory effect, reducing tumor necrosis factor-α (TNF-α) in wtHMGB-1 or lipopolysaccharide (LPS) activated macrophages. In gel retardation assay, rHMGB-1A formed a stable complex with siRNA at or above a 1:2 weight ratio (siRNA:rHMGB-1A). A heparin competition assay showed that an siRNA/rHMGB-1A complex released siRNA more easily than an siRNA/polyethylenimine (PEI, 25 kDa) complex. Luciferase siRNA/rHMGB-1A reduced firefly luciferase expression at a similar level as luciferase siRNA/PEI complex. Furthermore, TNF-α siRNA/rHMGB-1A synergistically reduced TNF-α expression in LPS activated macrophages. Therefore, rHMGB-1A may be useful as an siRNA carrier with anti-inflammatory effects in siRNA therapy for various inflammatory diseases. J. Cell. Biochem. 113: 122–131, 2012.

KEY WORDS: HIGH MOBILITY GROUP BOX-1; GENE THERAPY; ANTI-INFLAMMATION; RECOMBINANT PEPTIDE; siRNA DELIVERY

Gene therapy may be used to treat a number of diseases by delivering therapeutic genes intracellularly using viral or nonviral vectors [Han et al., 2000; Kang et al., 2005]. Viral vectors generally have higher gene delivery efficiencies than nonviral vectors, but intrinsic problems with viral vectors, such as high cytotoxicity and immunogenicity, have limited their applications [Kang et al., 2005]. In contrast, nonviral vectors have low cytotoxicity and immunogenicity [Han et al., 2000; Lee and Kim, 2002; Kang et al., 2005], and do not integrate DNA into the host chromosome, eliminating the possibility of oncogenic recombination. Therefore, improving transfection efficiency is one of the goals in developing nonviral vectors. Recently, nonviral vectors have been found to be more useful since they can deliver siRNA [Ikeda and Taira, 2006; Kim et al., 2006b]. The most widely used nonviral gene delivery systems are polyethylenimine (PEI, 25 kDa) and poly-L-lysine (PLL) [Benns et al., 2000; Nishikawa and Huang, 2001; Kang et al., 2005]. However, both PEI and PLL are cytotoxic under these conditions [Kang et al., 2005; Lee and Kim, 2005]. Therefore, developing a gene carrier with low cytotoxicity is another goal of nonviral carrier research.

In developing the carriers, nuclear proteins, especially fragmented peptides of histone 1 (H1) and histone 2A (H2A), have shown promise for DNA delivery, but have low transfection efficiency and specificity [Kaneda et al., 1989; Balicki et al., 2000; Puebla et al., 2003; Kaouass et al., 2006; Wagstaff et al., 2007]. Another nuclear scaffold protein investigated as a gene delivery carrier is high mobility group box-1 (HMGB-1), which binds the DNA minor groove nonspecifically, inducing a strong DNA bend [Thomas, 2001; Andersson et al., 2002; Kim et al., 2008a, 2008c; Han et al., 2009]. We previously evaluated derivatives of the HMGB-1 domains: box A, box B, and acidic tail [Thomas, 2001]. Box A and box B are DNA binding domains, while the acidic tail has a high aspartate and glutamate content with negative charge that interferes with DNA and peptide interaction. Therefore, eliminating the acidic tail from HMGB-1 improved the stability and transfection efficiency of HMGB-1/DNA complexes [Kim et al., 2008a; Han et al., 2009].

HMGB-1 also functions in the extracellular space as a pro-inflammatory cytokine [Mantell et al., 2006; Qiu et al., 2008; van Zoelen et al., 2009]. HMGB-1 binds to receptor for advanced

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glycation end products (RAGE) or toll-like receptors 2 and 4 (TLRs) [van Zoelen et al., 2009]. Box B is associated with binding RAGE or TLRs [Andersson et al., 2002]. The pro-inflammatory effects of HMGB-1 cause rheumatoid arthritis, atherosclerosis, and diabetic retinopathy [Kokkola et al., 2003; af Klint et al., 2005; Mantell et al., 2006]. In addition, HMGB-1 is related to angiogenesis that contributes to cancer progression [Pullerits et al., 2003; Taniguchi et al., 2003; Logsdon et al., 2007; Sasahira et al., 2007; Wang et al., 2008]. Therefore, eliminating box B from a HMGB-1 gene carrier may minimize side effects. Recombinant HMGB-1 box A (rHMGB-1A), without box B or the acidic tail, was evaluated as a DNA carrier [Kim et al., 2008a; Han et al., 2009]. The results suggest that rHMGB-1A is a promising gene carrier, since it exhibited no cytotoxicity and moderate gene delivery efficiency. However, rHMGB-1A has not been evaluated as a small interfering RNA (siRNA) carrier. High mobility box peptides formed stable complexes with short double stranded DNAs [Stott et al., 2006], suggesting that HMGB-1A may form stable complexes with siRNA. Therefore, rHMGB-1A may be more effective with short nucleic acids, such siRNA, than long plasmid DNA.

rHMGB-1A has been reported to be an antagonist to wild-type rHMGB-1 (wtHMGB-1) [Andersson et al., 2002; Zhang et al., 2008]. In an animal model, rHMGB-1A reduced the severity of rheumatoid arthritis [Kokkola et al., 2003]. Additionally, inhaling rHMGB-1A prevented inflammation in an acute lung injury animal model [Gong et al., 2009]. Therefore, rHMGB-1A may be a possible therapeutic peptide to treat various inflammation diseases.

In this study, we evaluated rHMGB-1A as an siRNA carrier. rHMGB-1A was produced and purified by consecutive nickel affinity chromatography, cationic chromatography, and polymixin-B chromatography. The anti-inflammatory effect of rHMGB-1A was evaluated, showing that HMGB-1A reduced the effect of lipopolysaccharides (LPS) as well as wtHMGB-1. In addition, siRNA delivery efficiency of HMGB-1A was evaluated and compared with PEI and PLL. The results suggest that the anti-inflammatory effect of HMGB-1A may make it a useful siRNA carrier for gene therapy of various inflammatory diseases.

MATERIALS AND METHODS

CLONING rHMGB-1A

rHMGB-1A cDNA (amino acids 1–87) was amplified by PCR from a wtHMGB-1 expression plasmid, pET21a-HMGB-1 [Kim et al., 2008b]. The PCR primers were as follows: forward 5′-CCGGAATTCATGGGCAAAGGAGATCCTAAG-3′, reverse 5′-CCCAAGCTTTTTTGTCTCCCCTTTGGG-3′. For convenience, EcoRI and HinDIII sites (underlined), respectively, were inserted to the primers. The amplified rHMGB-1A cDNA was digested with EcoRI and HinDIII and purified by agarose gel electrophoresis. pET-21a (Novagen, Madison, WI,) vector was digested with EcoRI and HinDIII, and the rHMGB-1A cDNA was inserted, creating pET21a-rHMGB-1A. This recombinant plasmid produces rHMGB-1A peptide with a 6 histidine repeat at the C-terminus for purification by nickel chelate affinity chromatography. pET-21a-rHMGB-1A was confirmed by direct sequencing.

EXPRESSION OF rHMGB-1A

pET21a-rHMGB-1A was transformed into *E. coli* BL21 (λDE3) for expression. A single colony of *E. coli* was selected and cultured overnight in 10 ml in Luria–Bertani (LB) medium containing 50 μg/ml ampicillin (LB-amp) in a shaking incubator at 37°C and 250 rpm. This bacterial culture was transferred to 4 L LB-amp and incubated under the same condition until reaching an optical density of 0.6–0.8 at 600 nm. Then IPTG was added to a concentration of 500 μM to induce rHMGB-1A expression. The cells were incubated for an additional 6 h and harvested by centrifugation at 6,000*g* for 15 min at 4 °C. The supernatant was removed and the bacterial pellet was resuspended in chilled lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 6 M urea (pH 8.0)) containing 1 mM PMSF. The resuspended bacterial cells were lysozyme treated (0.3 mg/ml) for 20 min and lysed by sonication (3 sec × 30 bursts) with 5 min on ice between bursts. The lysate was cleared by centrifugation at 10,000*g* for 30 min at 4°C and the supernatant was used for rHMGB-1A purification.

PURIFICATION OF rHMGB-1A

The C-terminal 6 histidine stretch confers a high affinity to nickel ions. Nickel chelate affinity chromatography was the first purification step for rHMGB-1A. Cell extracts containing rHMGB-1A were loaded into a nickel (Probond resin, Invitrogen, Carlsbad, CA) column (Glass Econo Column, BioRad, Hercules, CA) pre-equilibrated with equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, 6M urea, and 20 mM imidazole, pH 8.0). Unbound proteins were removed by washing with equilibration buffer and bound proteins were eluted by step gradient with imidazole (100, 150, 200, 250, 300 mM) in elution buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, and 6 M urea) at 1 ml/min. Each eluted fraction was assayed with a BCA assay kit (Pierce, Rockford, IL). The protein was collected and dialyzed against cationic exchange buffer (16 mM NaH_2PO_4, 1 mM Na_2HPO_4, 0.1 mM sodium azide, pH 5.7) containing 0.2 mM PMSF using a membrane with a 6,000–8,000 Da molecular weight cut-off (Spectra/Por dialysis membrane, Millipore, Billerica, MA) at 4°C overnight.

After dialysis, the protein was loaded into a CM sepharose (GE Healthcare, Waukesha, WI) column pre-equilibrated with equilibration buffer (16 mM NaH_2PO_4, 1 mM Na_2HPO_4, 0.1 mM sodium azide, 200 mM NaCl, pH 5.7). The unbound proteins were removed by washing with equilibration buffer, and rHMGB-1A was eluted using step gradient of NaCl (350, 400, 450, 500, 550 mM) in elution buffer (16 mM NaH_2PO_4, 1 mM Na_2HPO_4, and 0.1 mM sodium azide, pH 5.7) at 1 ml/min. Each eluted fraction was assayed with a BCA assay kit (Pierce, Rockford, IL). The purified protein fractions were collected and dialyzed as described above, and analyzed by electrophoresis on a 15% (w/v) SDS–PAGE.

Finally, to remove any contaminating endotoxin such as LPS, rHMGB-1A was subjected to a polymyxin B column (Pierce, Rockford, IL).

GEL RETARDATION ASSAY

siRNA condensation with rHMGB-1A was verified by a gel retardation assay. Firefly luciferase siRNA for this assay was

purchased from Ambion (Austin, TX). siRNA/rHMGB-1A complexes were prepared by mixing 50 pmol siRNA with increasing amounts of rHMGB-1A in 5% (w/v) glucose. siRNA/rHMGB-1A complex was incubated for 30 min at room temperature. The complexes were analyzed by electrophoresis on a 10% (w/v) acrylamide gel for 70 min at 100 V in 1 × TBE. After ethidium bromide staining for 30 min, siRNA was visualized using a UV transilluminator.

HEPARIN COMPETITION ASSAY

A heparin competition assay was performed to dissociate siRNA/carrier (rHMGB-1A or PEI) complexes. siRNA/rHMGB-1A and siRNA/PEI complexes were prepared at 1:5 and 1:1 weight ratios in 5% (w/v) glucose, respectively. After incubating for 30 min at room temperature, increasing concentrations of heparin were added to the siRNA/carrier complex solution. The complexes were analyzed by electrophoresis on a 10% (w/v) acrylamide gel for 60 min at 100 V in 1 × TBE. After ethidium bromide staining for 30 min, siRNA was visualized using a UV transilluminator.

CELL CULTURE AND TRANSFECTION

Human embryonic kidney 293 (HEK 293) cells and mouse macrophage (Raw 264.7) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) containing 10% (v/v) FBS at 37°C in a 5% CO_2 incubator.

For the dual luciferase assays, HEK 293 cells and Raw 264.7 were seeded in 10 mm dishes. The cells were washed with fresh serum-free DMEM. psiCHECK-2 (Invitrogen, Grand Island, NY)/PEI complexes were prepared at a 1:1 weight ratio and added to the cells. The cells were incubated for 4 h at 37°C in a 5% CO_2 incubator. After incubating, media containing transfection mixtures were removed and 10 ml of fresh DMEM containing 10% (v/v) FBS was added to the cells. The cells were incubated for an additional 24 h at 37°C in a 5% CO_2 incubator. HEK 293 cells and Raw 264.7 transfected with psiCHECK-2 were harvested and seeded in 24-well plates at 3.5×10^4 and 5.0×10^4 cells/well, respectively. The cells were incubated for 24 h at 37 °C. The cells were then washed with fresh serum-free DMEM. Fifty picomoles firefly luciferase siRNA (Ambion, Austin, TX) were complexed with rHMGB-1A at a 1:5 weight ratio. PEI and poly-L-lysine (PLL, 20 kDa) were used as controls in silencing and cytotoxicity assays. siRNA/PEI and siRNA/PLL complexes were prepared at 1:1 and 1:2 weight ratios, respectively, based on previous reports [Lee et al., 2001, 2003]. siRNA/carrier complex was added to each well. The cells were then incubated for 4 h at 37°C in a 5% CO_2 incubator. After 4 h, media containing transfection mixtures were removed and 500 μl of fresh DMEM containing 10% (v/v) FBS was added to each well. The cells were incubated for an additional 48 h at 37°C in a 5% CO_2 incubator.

For fluorescence microscopy study, a green fluorescence protein (GFP) expression plasmid, pcDNA-EFGP, was transfected into the 293 cells as described above in the dual luciferase assay. After 24 h of incubation, GFP siRNA/carrier complexes were prepared and added to the pcDNA-EGFP transfected cells. The sequences of GFP siRNA were 5′-CAAGCUGACCCUGAAGUUCTT-3′ (upstream) and 5′-GAACUUCAGGGUCAGCUUGTT-3′ (downstream). The GFP siRNA was chemically synthesized (Samchully Pharm, Seoul, Korea). The cells were then incubated for 4 h at 37°C and the medium containing transfection mixtures were replaced with fresh DMEM containing 10% (v/v) FBS. The cells were incubated for an additional 24 h at 37°C. GFP expression in the cells was verified by fluorescence microscopy.

DUAL LUCIFERASE ASSAY

After transfection and incubation, DMEM was removed from the wells. Cells were washed with 500 μl of PBS, and 80 μl of passive lysis buffer (Promega, Madison, WI) was added to each well. Cells were lysed at room temperature for 15 min and transferred to microcentrifuge tubes. The lysates were vortexed for 15 sec and centrifuged at 13,000 rpm for 3 min by microcentrifuge. The supernatant was transferred to a new tube. Luciferase activity was then measured in relative light units (RLU) using a 96-well plate luminometer (Berthold, Pforzheim, Germany). The final luciferase activities were calculated as: (firefly RLU/renilla RLU) × 100 (%).

CYTOTOXICITY ASSAY

Cytotoxicity was evaluated using the MTT assay and MultiTox-Glo cytotoxicity assay (Promega, Madison, WI). HEK 293 cells were seeded at 3.5×10^4 cells/well and 5.0×10^4 cells/well in 24-well microassay plates and incubated for 24 h before transfection. Luciferase siRNA/rHMGB-1A complex was prepared at a 1:5 weight ratio. Luciferase siRNA/PEI and luciferase siRNA/PLL complexes were prepared at 1:1 and 1:2 weight ratios, respectively, as controls. The siRNA/carrier complexes were added to cells, and incubated for 4 h at 37 °C in serum free medium. After the transfection mixtures were removed, 500 μl of fresh DMEM containing 10% (v/v) FBS was added to each well. The cells were incubated for an additional 48 h at 37°C. At the end of the transfection experiment, 40 μl of 5 mg/ml MTT reagent in PBS was added. The plates were incubated for an additional 4 h at 37°C. Medium containing MTT solution was removed and 750 μl of DMSO was added to dissolve formazan crystals formed by live cells. Absorbance was measured at 570 nm. Cell viability (%) was calculated according to the following equation:

$$\text{Cell viability}\,(\%) = \left(\mathrm{OD}_{570\,(\text{sample})}/\mathrm{OD}_{570\,(\text{control})}\right) \times 100,$$

where $OD_{570(sample)}$ is the measurement from wells treated with carrier/DNA complex and $OD_{570(control)}$ is the measurement from wells treated with 5% glucose. MultiTox-Glo cytotoxicity assay was performed as described in the manufacturer's manual (Promega, Madison, WI).

TUMOR NECROSIS FACTOR-α ASSAY

Anti-inflammatory effect of rHMGB-1A was evaluated with the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α). Raw 264.7 were cultured in DMEM containing 10% (v/v) FBS. The cells were seeded at 2.0×10^5 cells/well in 24-well plate and incubated at 37 °C in a 5% CO_2 incubator for 24 h. TNF-α siRNA was synthesized chemically, based on the previous report (upstream, 5′-GACAAC-CAACUAGUGGUGCTT-3′; downstream, 5′-GCACCACUAGUUG-GUUGUCTT-3′, Samchully Pharm, Seoul, Korea) [Sioud, 2005]. TNF-α siRNA/rHMGB-1A complexes were added to Raw 264.7 cells

for pre-incubation. siRNA/rHMGB-1A complexes were prepared at a 1:5 weight ratio. After pre-incubation, wtHMGB-1 or LPS was added to Raw 264.7 and the cells were incubated for 24 h.

ELISA was performed using a mouse TNF-α ELISA kit (Biosource, Camarillo, CA). Briefly, a flat-bottom 96-well ELISA plate was coated with capture antibody and left overnight. The plate was washed with wash buffer and blocked by incubating 1× assay diluent for 1 h. After washing, the samples were loaded into the wells. After 2 h of incubation, the plate was washed and detection antibody was placed into each well. The plate was incubated for 1 h and then washed with wash buffer. Avidin-HRP was added to each well and the plate was incubated for 30 min. After washing, substrate solution was placed into each well and the plate was incubated for 15 min at room temperature. After adding stop solution, the absorbance was measured at 450 nm.

RT-PCR was performed to measure the TNF-α mRNA level. The sequences of the primers for RT-PCR were as follows: forward primer, 5′-CTACTCCCAGGTTCTCTTCAA-3′ and reverse primer, 5′-GCAGAGAGGAGGTTGACTTTC-3′. One microgram of total RNA was amplified using Access RT-PCR system (Promega, Madison, WI) as suggested in the manufacturer's manual.

RESULTS

DESIGN, EXPRESSION, AND PURIFICATION OF RECOMBINANT HMGB-1A PEPTIDE

wtHMGB-1 consists of box A and box B, which are DNA binding domains, and an acidic tail with a high negative charge (Fig. 1A). Previous reports showed that box B had pro-inflammatory effects as a RAGE or TLR ligand [Andersson et al., 2002; Logsdon et al., 2007; van Zoelen et al., 2008]. The acidic tail weakens the charge interaction between DNA and HMGB-1 [Kim et al., 2008b]. To avoid the pro-inflammatory effects of box B and negative charges of the acidic tail, only box A of wtHMGB-1 was expressed, purified, and evaluated for siRNA delivery (Fig. 1B).

The purification strategy is described in Figure 1C. rHMGB-1A expression was induced by IPTG. After lysis, cell extracts were subjected to nickel chelate affinity chromatography, and rHMGB-1A was eluted at 150 mM of imidazole. This rHMGB-1A was purified further by cationic exchange chromatography, and eluted at 450 mM of NaCl. The purified rHMGB-1A was analyzed by SDS–PAGE (Fig. 1D). The cationic exchange chromatography removed contaminants in the fractions from nickel chelate affinity

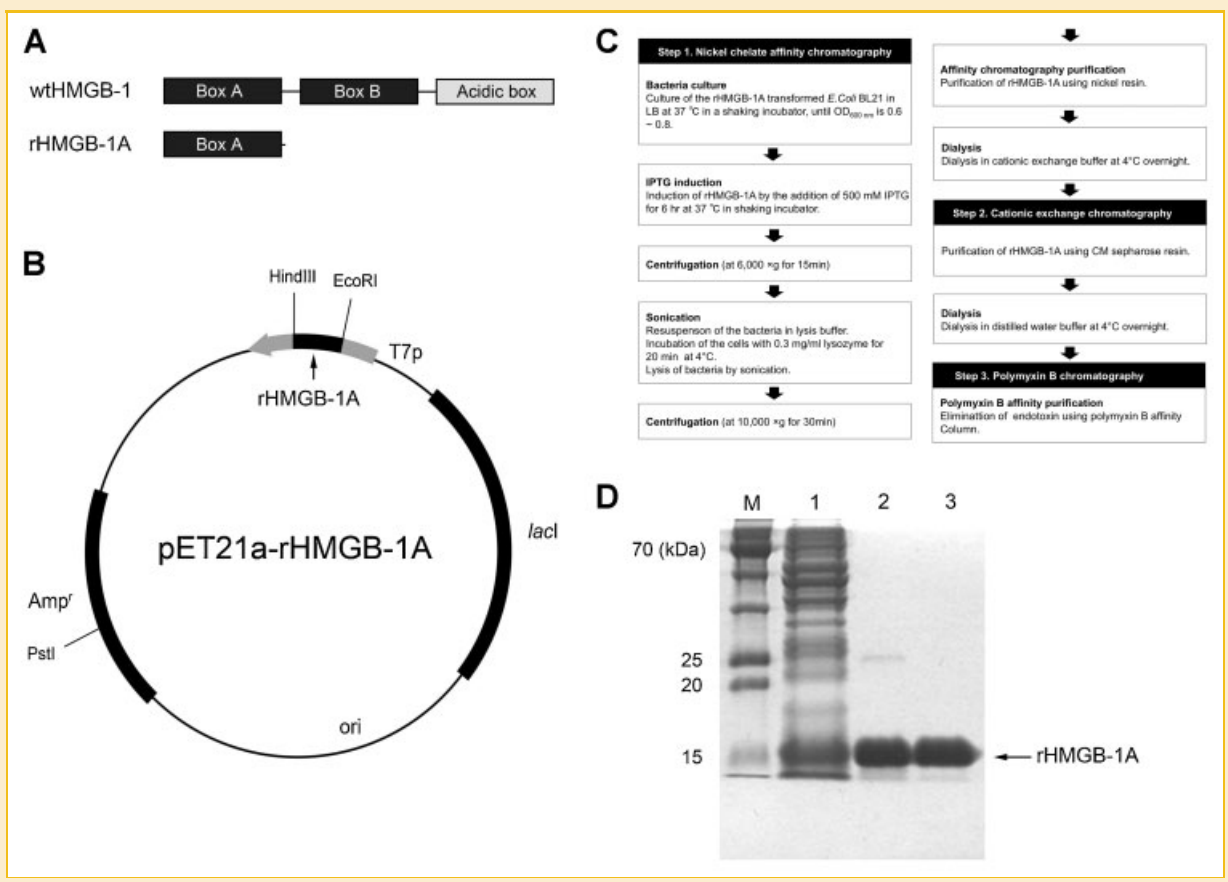

Fig. 1. Purification of rHMGB-1A. A: Structures of wtHMGB-1 and rHMGB-1A, (B) the map of the rHMGB-1A expression plasmid, (C) steps for rHMGB-1A purification, and (D) SDS–PAGE analysis. Purified rHMGB-1A protein was analyzed by SDS–PAGE. M, molecular weight markers; Lane 1, crude bacterial lysates after induction; lane 2, rHMGB-1A purified by nickel chelate affinity chromatography; lane 3, rHMGB-1A purified by cationic exchange chromatography.

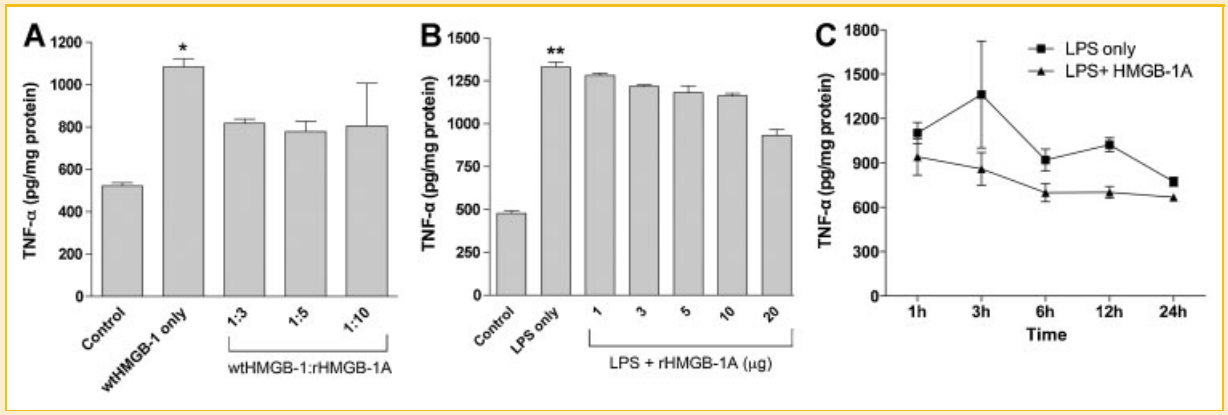

Fig. 2. Reduced TNF-α secretion by rHMGB-1A. Raw 264.7 cells were preincubated with rHMGB-1A for 60 min before adding wtHMGB-1 (A) or LPS (B). The TNF-α level was measured by mouse TNF-α ELISA. The data are expressed as the mean values (±standard deviation) of quadruplicate experiments. $^{*}P < 0.01$ as compared with 1:3 and 1:5. $^{**}P < 0.05$ as compared with 3, 5, 10, and 20. C: The TNF-α level was measured at various time points by mouse TNF-α ELISA.

chromatography. To remove any contaminating endotoxins, purified rHMGB-1A was subjected to polymyxin B affinity chromatography. wtHMGB-1 was also produced and purified by the same method as rHMGB-1A.

ANTI-INFLAMMATORY EFFECT OF rHMGB-1A

The anti-inflammatory effect of rHMGB-1A was evaluated using activated Raw 264.7 macrophage cells. Raw 264.7 cells were pre-incubated with increasing amounts of rHMGB-1A for 1 h. After incubation, Raw 264.7 cells were activated by adding wtHMGB-1. The rHMGB-1A preincubation reduced TNF-α secretion in the cells activated by wtHMGB-1 (Fig. 2A), confirming that rHMGB-1A was a wtHMGB-1 antagonist.

To determine whether rHMGB-1A reduced TNF-α in Raw 264.7 cells activated by LPS, the cells were incubated with rHMGB-1A for 1 h prior to adding LPS (Fig. 2B). rHMGB-1A reduced TNF-α level significantly in the LPS treated cells. In addition, the TNF-α level was measured in a time-dependent manner (Fig. 2C). The results showed that the TNF-α level increased and reached its peak at 3 h and decreased gradually along with time. The TNF-α level was reduced by the treatment with rHMGB-1A at all the tested points, compared with the controls (Fig. 2C). These results suggest that HMGB-1A reduces inflammatory cytokines in the cells activated by LPS or wtHMGB-1.

CHARACTERIZATION OF siRNA/rHMGB-1A COMPLEX

A gel retardation assay was performed (Fig. 3) to confirm complex formation between siRNA and rHMGB-1A. Thirty picomoles luciferase siRNA was mixed with increasing amounts of rHMGB-1A. The siRNA was significantly retarded at a 1:2 weight ratio (siRNA:rHMGB-1A). However, additional rHMGB-1A did not completely retard siRNA. This indicates that siRNA/rHMGB-1A complex had a slight negative charge, even at a 1:10 weight ratio.

Heparin is a highly sulfated glycosaminoglycan that can effectively interfere with electrostatic interactions between cationic reagents and siRNA. Therefore, a heparin competition assay was performed to evaluate the stability of siRNA/rHMGB-1A complexes. An siRNA/PEI complex was used as a control. Free siRNA was released from siRNA/PEI or siRNA/rHMGB-1A (Fig. 4) complexes with increasing heparin. Free siRNA was released from rHMGB-1A with less heparin than PEI, suggesting that the siRNA/rHMGB-1A complex is less stable than the siRNA/PEI complex. A previous report showed that PEI formed a tight complex with siRNA and did not release siRNA efficiently in cells [Malek et al., 2009]. This inefficient release is one problem in PEI mediated siRNA delivery, thus weaker binding between siRNA from rHMGB-1A may be beneficial for gene silencing.

DELIVERY OF LUCIFERASE AND GFP siRNAs WITH rHMGB-1A

To evaluate the efficiency of siRNA delivery by rHMGB-1A, an in vitro knock-down assay was performed with siRNA/rHMGB-1A complex. The assay was performed in HEK 293 (Fig. 5A) and Raw 264.7 cells (Fig. 5B), and PEI and PLL were used as controls. Firefly luciferase siRNA was complexed with rHMGB-1A, PEI, or PLL and transfected into luciferase expressing cells. The silencing efficiencies of the delivered siRNAs were measured by dual-luciferase assay. Renila luciferase activity was used as an internal control and relative activity of firefly luciferase was measured. siRNA/rHMGB-1A reduced luciferase expression with a silencing efficiency similar to

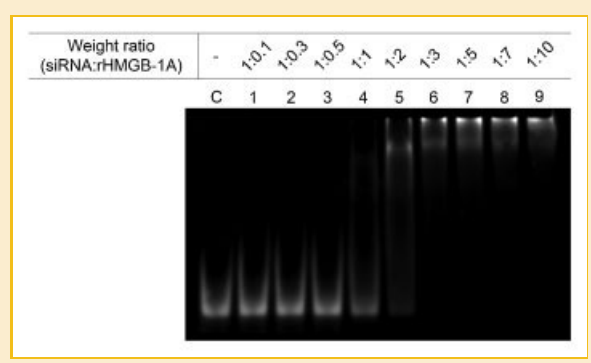

Fig. 3. Gel retardation assay. Thirty picomoles siRNA was mixed with increasing amounts of rHMGB-1A to form complexes. siRNA/rHMGB-1A complexes were analyzed by 10% polyacrylamide gel.

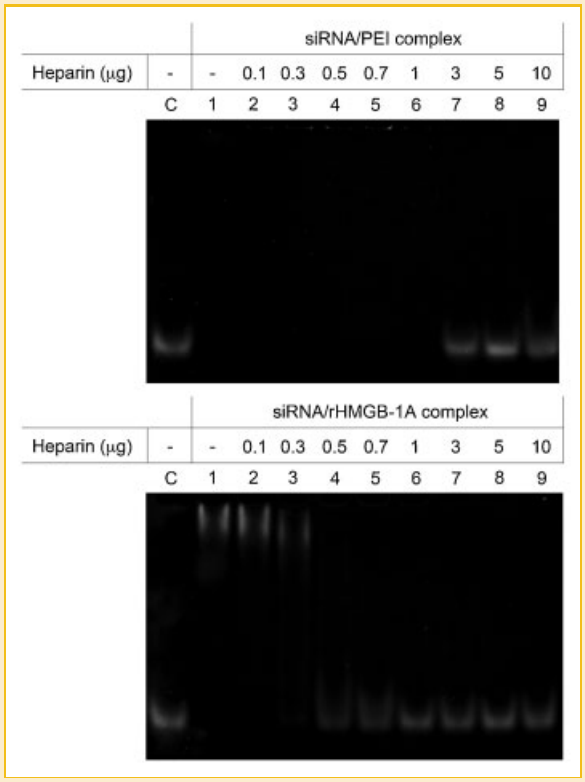

Fig. 4. Heparin competition assay. siRNA/PEI and siRNA/rHMGB-1A complexes were prepared at 1:1 or 1:5 weight ratios, respectively. After 30 min, the complexes were mixed with increasing heparin. Samples were analyzed in a 10% polyacrylamide gel.

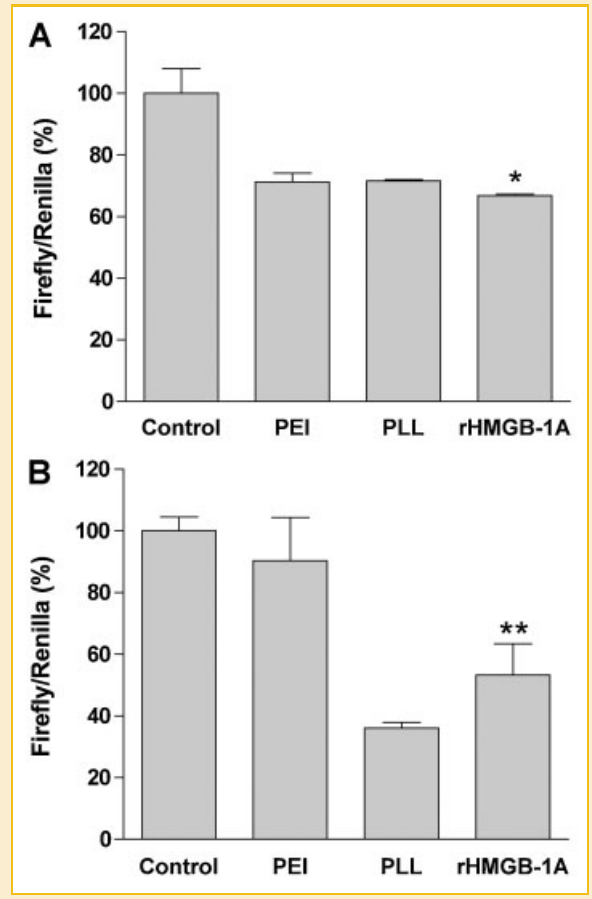

Fig. 5. Luciferase silencing efficiency of siRNA/rHMGB-1A complex. siRNA/carrier complexes were prepared as described in the Materials and Methods section. siRNA/carrier complexes were added to HEK 293 (A) and Raw 264.7 (B) cells. Gene silencing efficiency was measured by dual luciferase assay. The data are expressed as mean values (± standard deviation) of quadruplicate experiments. $^{*}P < 0.01$ as compared with control, but no significance as compared with PEI and PLL. $^{**}P < 0.05$ as compared with control, PEI, and PLL.

siRNA/PEI and siRNA/PLL in HEK 293 cells (Fig. 5A). rHMGB-1A was more efficient than PEI in Raw 264.7 cells (Fig. 5B).

GFP expression was also reduced by the delivery of GFP siRNA by rHMGB-1A (Fig. 6). This result showed that GFP siRNA/rHMGB-1A complex reduced GFP expression in HEK 293 cells, compared with control. Again, the reduction caused by HMGB-1A is similar to PEI (Fig. 6).

CYTOTOXICITY OF rHMGB-1A

To evaluate rHMGB-1A cytotoxicity, an MTT assay was performed. HEK 293 cells were incubated with siRNA/rHMGB-1A, siRNA/PEI, or siRNA/PLL for 48 h. rHMGB-1A was not toxic to HEK 293 cells (Fig. 7A). PEI and PLL were considerably toxic to HEK 293 cells. The toxicity of rHMGB-1A was measured by MultiTox-Glo cytotoxicity assay. The results showed that rHMGB-1A was as toxic as siRNA with an 80% cell viability (Fig. 7B). However, the viability of the cells treated with PEI and PLL was around 20%, suggesting that rHMGB-1A was much less toxic than PEI and PLL. rHMGB-1A originated from an endogenous protein with only a His tag modification, thus was not expected to be toxic. The results suggest that rHMGB-1A is a safer siRNA carrier than PEI or PLL.

DELIVERY OF ANTI-TNF-α siRNA WITH rHMGB-1A

Our results showed that rHMGB-1A reduced TNF-α secretion in wtHMGB-1 or LPS activated cells (Fig. 2). rHMGB-1A has also been reported to have an anti-inflammatory effect previously [Andersson et al., 2002; Qiu et al., 2008]. Therefore, rHMGB-1A may be useful in anti-inflammatory therapy, and delivering siRNAs against pro-inflammatory cytokines may enhance the effect. To confirm this synergy between siRNA and rHMGB-1A, TNF-α siRNA was complexed with rHMGB-1A and delivered in Raw 267.4 cells. After LPS activation, the TNF-α level was measured by ELISA. The control scrRNA/rHMGB-1A complex reduced the TNF-α level (Fig. 8A), and The TNF-α siRNA/rHMGB-1A complex reduced the TNF-α level even further (Fig. 8A). The TNF-α mRNA level in Raw 264.7 cells were measured by RT-PCR. The results suggest that the TNF-α mRNA level was decreased by delivery of TNF-α siRNA/rHMGB-1A complex (Fig. 8B). However, scrRNA/rHMGB-1A complex did not show this effect (Fig. 8B). Raw 264.7 cells were treated by rHMGB-1A to identify the effect of rHMGB-1A on TNF-α secretion (Fig. 8C). The TNF-α level was slightly increased by the treatment of 50 μg rHMGB-1A. However, the increase was insignificant and may be nonspecific. Instead, Raw 264.7 cells were activated with wtHMGB-1A and the effect of TNF-α siRNA/rHMGB-1A complex was

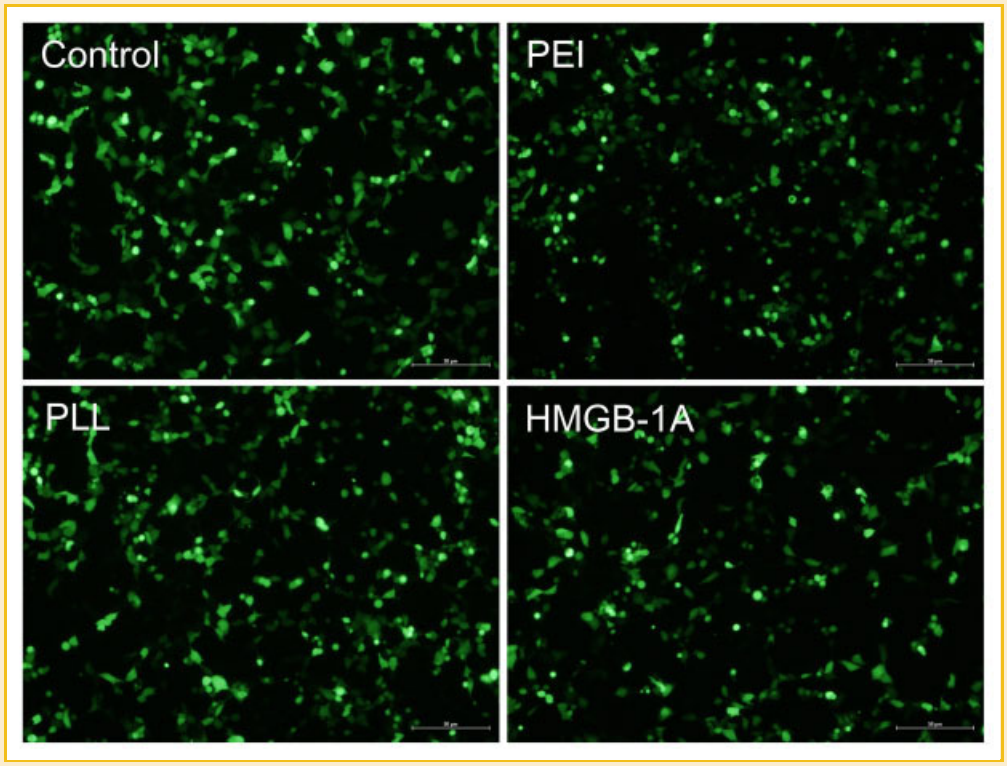

Fig. 6. GFP silencing efficiency of siRNA/rHMGB-1A complex. GFP siRNA/carrier complexes were prepared as described in the Materials and Methods section. GFP siRNA/carrier complexes were added to GFP expressing cells. After 24 h, GFP expression was verified by fluorescence microscopy.

measured by ELISA (Fig. 8D). The results confirmed that the TNF-α siRNA/rHMGB-1A complex reduced the TNF-α level further, compared with the scrRNA/rHMGB-1A complex (Fig. 8D). These results confirmed that the TNF-α siRNA and rHMGB-1A had a synergistic effect in reducing TNF-α.

DISCUSSION

HMGB-1 is a nuclear protein that contributes to chromatin structure in the nucleus [Thomas, 2001]. In normal cells, HMGB-1 is involved in transcriptional regulation as an architectural transcription factor

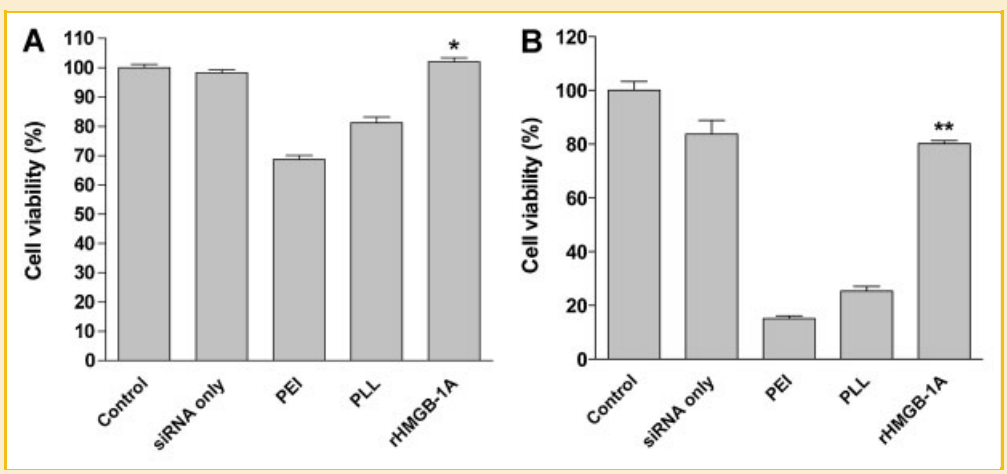

Fig. 7. siRNA/rHMGB-1A complex cytotoxicity. siRNA/carrier complexes were prepared as described in the Materials and Methods section. HEK 293 cells were incubated with siRNA/carrier complexes for 4 h. After incubation, the media were replaced with fresh DMEM. After an additional 48 h, cell viability was measured by (A) MTT assay and (B) MultiTox-Glo cytotoxicity assay. The data are expressed as the mean values (±standard deviation) of quadruplicate experiments. $^{*}P < 0.01$ as compared with PEI and PLL, but no significance as compared with control and siRNA only. $^{**}P < 0.01$ as compared with control, PEI, and PLL, but no significance as compared with siRNA only.

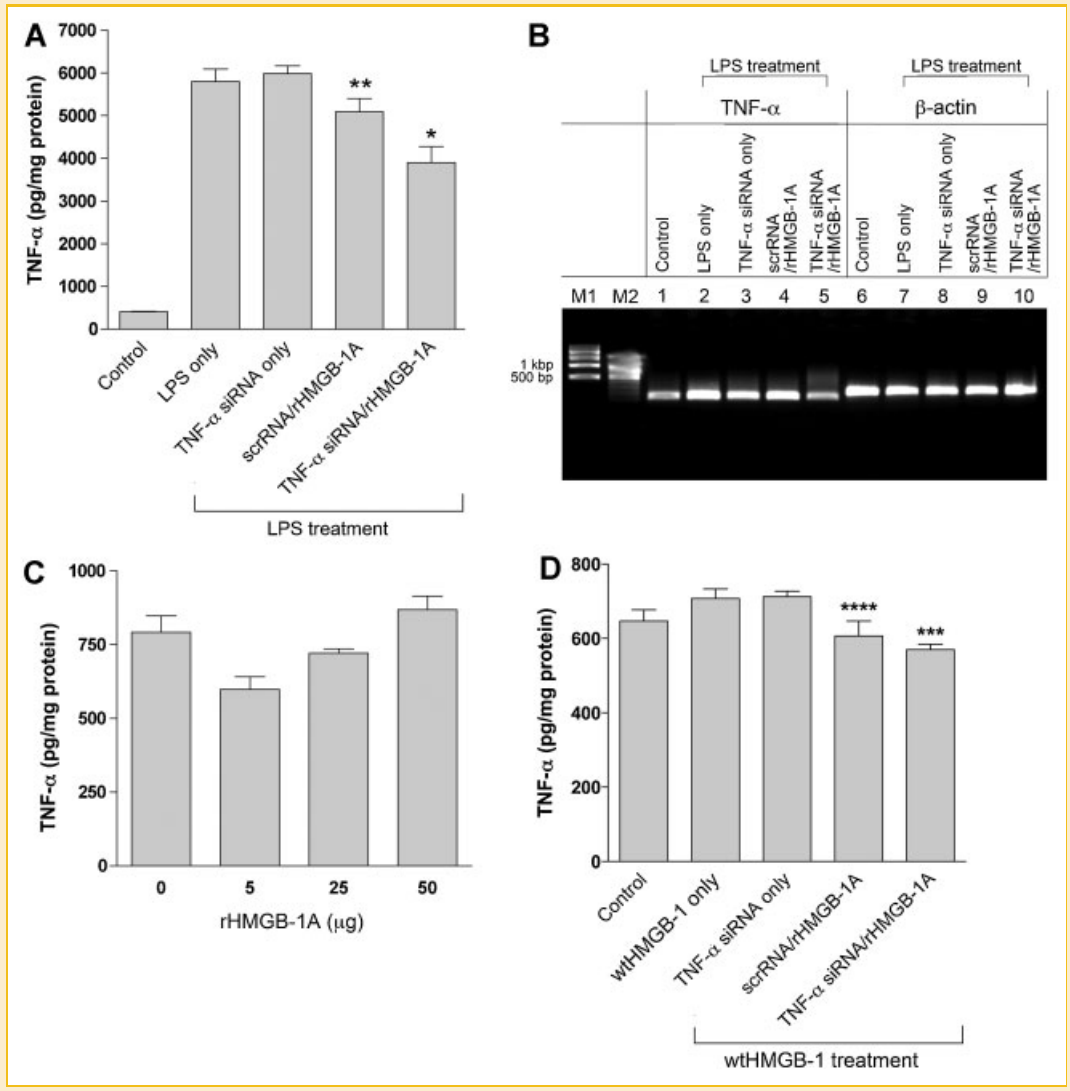

Fig. 8. Reduction of TNF-α by TNF-α siRNA/rHMGB-1A complex. TNF-α siRNA/carrier complexes were prepared as described in the Materials and Methods section. A: TNF-α siRNA/carrier complexes were added to LPS activated Raw 264.7 cells. After 24 h, the TNF-α protein level was measured by ELISA. The ELISA data are expressed as the mean values (±standard deviation) of quadruplicate experiments. $^{*}P < 0.05$ as compared with control, LPS only, TNF-α siRNA only, and scrRNA/rHMGB-1A. $^{**}P < 0.05$ as compared with control and TNF-α siRNA only, but no significance as compared with LPS only. B: The TNF-α mRNA level in LPS-activated Raw 264.7 cells was measured by RT-PCR. C: Raw 264.7 cells were incubated with rHMGB-1A. After 24 h, the TNF-α protein level was measured by ELISA. D: Raw 264.7 cells were activated with wtHMGB-1. TNF-α siRNA/carrier complexes were added to HMGB-1A-activated Raw 264.7 cells. After 24 h, the TNF-α protein level was measured by ELISA. The $^{***}P < 0.05$ as compared with control, wtHMGB-1 only, TNF-α siRNA only, and scrRNA/rHMGB-1A. $^{****}P < 0.05$ as compared with control, wtHMGB-1 only, and TNF-α siRNA only.

[Banerjee and Kundu, 2003; Grasser, 2003; Liu et al., 2009; Lv et al., 2009]. However, when released from necrotic cells, HMGB-1 is a pro-inflammatory cytokine [Scaffidi et al., 2002]. In this study, rHMGB-1A was produced and evaluated as an anti-inflammatory cytokine and siRNA carrier.

A gel retardation assay showed that rHMGB-1A complexed with siRNA, though siRNA was not completely retarded, even at 1:10 weight ratio (siRNA:rHMGB-1A). HMG box binds short double-stranded DNA by hydrophobic and charge interactions [Stott et al., 2006]. The HMG box peptide/DNA complex had regular tertiary structure with a large DNA bend by X-ray crystallography. Two molecules of HMG box peptide bind to 25 bp double strand DNA, though the negative charges on the DNA may be not completely neutralized at this ratio. Extra rHMGB-1A in the binding mixture may not disrupt the regular structure of the DNA/rHMGB-1A complex, since hydrophobic and charge interactions stabilize the binding. Thus, excess rHMGB-1A likely does not increase the amount of rHMGB-1A per DNA molecule. The structure of the siRNA/rHMGB-1A complex may be similar to the DNA/rHMGB-1A complex. Indeed, the siRNA/rHMGB-1A complex at a 1:10 weight ratio seemed to have negative charge in the gel retardation assay (Fig. 3), suggesting that excess rHMGB-1A did not neutralize the siRNA. Therefore, it is unlikely that more rHMGB-1A induces tighter or smaller complex between siRNA and rHMGB-1A. Cationic polymers, such as PEI, bind siRNA mainly by charge interaction. Therefore, more cationic polymers may make a tighter

siRNA/polymer complex, suggesting that the siRNA/rHMGB-1A complex is less tight than the siRNA/PEI complex. In heparin competition assay, rHMGB-1A releases siRNA at a lower heparin concentration than PEI. PEI has been suggested to bind siRNA too tightly and not release it in the cytoplasm [Malek et al., 2009]. In the current study, rHMGB-1A was more efficient than PEI at gene silencing in Raw 264.7 cells, perhaps due to more efficient siRNA release from rHMGB-1A in the cytoplasm.

In the TNF-α ELISA, rHMGB-1A effectively reduced the TNF-α level in Raw 264.7 macrophage cells activated by wtHMGB-1, showing that rHMGB-1A is a wtHMGB-1A antagonist. Interestingly, rHMGB-1A also decreased the TNF-α level in LPS activated macrophage cells. LPS bind TLRs on the macrophage surface and induces wtHMGB-1 secretion [Kawahara et al., 2009]. In this experiment, increased HMGB-1 by LPS may be antagonized by rHMGB-1A, reducing TNF-α. In the previous reports, it was proved that antibody against wtHMGB-1, ethyl pyruvate, heat shock proteins, and sodium butyrate inhibited the expression of wtHMGB-1 [Agnello et al., 2002; Ulloa et al., 2002; Quivy and Van Lint, 2004]. Compared with these HMGB-1 inhibitors, HMGB-1A has an advantage as an siRNA carrier as well as a wtHMGB-1 antagonist. Therefore, HMGB-1A may be useful for inflammatory diseases as a delivery carrier of various siRNAs with anti-inflammatory effect.

rHMGB-1A is an antagonist of the pro-inflammatory cytokine HMGB-1 and an efficient siRNA carrier. In addition, rHMGB-1A was not toxic to cells. Combining rHMGB-1A and therapeutic siRNA may be useful in gene therapy for various inflammatory diseases.

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