ARTICLE
Journal of Cellular Biochemistry 107:163–170 (2009)

Journal of Cellular Biochemistry

# A High Mobility Group B-1 Box A Peptide Combined With an Artery Wall Binding Peptide Targets Delivery of Nucleic Acids to Smooth Muscle Cells

Jee Seung Han, Kyunghwa Kim, and Minhyung Lee*

*Department of Bioengineering, College of Engineering, Hanyang University, Seoul 133-791, Republic of Korea*

## ABSTRACT

The TAT-high mobility group box-1 A box peptide (TAT-HMGB1A) has been reported previously to be able to deliver DNA into cells without cytotoxicity. In this study, an artery wall smooth muscle cell-targeting carrier was developed using TAT-HMGB1A combined with an artery wall binding peptide (ABP). For the production of ABP linked TAT-HMGB1A (TAT-HMGB1A-ABP), pET15b-TAT-HMGB1A-ABP was constructed by inserting the ABP cDNA into pET15b-TAT-HMGB1A. TAT-HMGB1A-ABP was expressed in *E. coli* and purified by Nickel chelate chromatography. Gel retardation assays showed that TAT-HMGB1A-ABP formed a complex with the plasmid at or above a 5:1 weight ratio (peptide:plasmid). At a 20:1 weight ratio, the zeta-potential was ~25 mV and the particle size was ~120 nm. TAT-HMGB1A-ABP had the highest transfection efficiency in A7R5 smooth muscle cells at a weight ratio of 20:1. TAT-HMGB1A-ABP exhibited higher transfection efficiency in A7R5 cells than PLL or TAT-HMGB1A, while TAT-HMGB1A-ABP had lower transfection efficiencies in Hep3B hepatoma, 293 kidney, NIH3T3 fibroblast, and Raw264.7 macrophage cells compared with PLL. Together, these results suggest that the ABP moiety of the peptide increased transfection efficiency specifically in smooth muscle cells. In a competition assay, the transfection efficiency of TAT-HMGB1A-ABP in A7R5 cells was reduced by the addition of free ABP. MTT assays showed that TAT-HMGB1A-ABP did not produce any cytotoxicity in A7R5 cells. Therefore, TAT-HMGB1A-ABP may be useful for a targeting gene delivery to smooth muscle cells.J. Cell. Biochem. J. Cell. Biochem. 107: 163–170, 2009. 

**KEY WORDS:** ARTERY WALL BINDING PEPTIDE; HIGH MOBILITY GROUP BOX-1; GENE DELIVERY; SMOOTH MUSCLE CELLS; TRANSFECTION

Gene therapy is a method intended for the treatment of diseases by intracellular delivery of therapeutically relevant genes. Since the first clinical trial was conducted, gene therapy has been developed as a promising strategy for the treatment of hard-to-cure diseases [Edelstein et al., 2004]. Gene carriers can be classified into two groups, namely, viral and non-viral vectors [Kang et al., 2005]. Non-viral vectors have lower delivery efficiency than viral vectors, but considering the advantages of non-viral vectors, such as no immunogenicity and low toxicity, non-viral vectors may be useful carriers for gene therapy to treat diseases that require low levels of therapeutic proteins and transient gene expression.

Endogenous DNA-binding proteins including histones and high mobility group (HMG) proteins have been used for non-viral gene delivery carriers [Kaneda et al., 1989; Bottger et al., 1990; Ellison et al., 1996; Isaka et al., 1998; Balicki et al., 2000, 2002; Puebla et al., 2003; Weng et al., 2004; Kim et al., 2008c]. One of HMG proteins, high mobility group box-1 (HMGB1) has been investigated as a gene carrier. HMGB1 is a DNA binding protein and participates in maintaining nucleosome structure, regulating gene transcription, and modulating the activity of steroid hormone receptors [Zlatanova and van Holde, 1998; Grigorov et al., 2001; Lildballe et al., 2008]. In addition, in terms of extracellular space, HMGB1 acts as a pro-inflammatory cytokine, and is involved in sepsis, rheumatoid arthritis, stroke, and myocardial infarction [Andersson et al., 2002; Li et al., 2003; Kim et al., 2006, 2008, 2008a]. Recently, it was suggested that HMGB1 might be an angiogenic factor in tumors [Logsdon et al., 2007; Zhang et al., 2008]. HMGB1 has a dipolar structure and is subdivided into two homologous HMG boxes, A and B, each box of about 75 amino acids in length. Additionally, it contains an acidic C-terminal tail [Li et al., 2003]. HMGB1 binds non-specifically to the minor groove of DNA, and forms a stable complex in solution [Thomas, 2001; Andersson et al., 2002; Stott et al., 2006]. The N-terminus contains heparin-binding motifs, whereas the B-box confers pro-inflammatory activity to the protein

Grant sponsor: Ministry of Education, Science and Technology in Korea; Grant number: F104AA010002-08A0101-00200.

*Correspondence to: Minhyung Lee, PhD, Department of Bioengineering, College of Engineering, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791, Republic of Korea. E-mail: minhyung@hanyang.ac.kr

Received 28 October 2008; Accepted 27 January 2009 • DOI 10.1002/jcb.22112 • 2009 Wiley-Liss, Inc.
Published online 13 March 2009 in Wiley InterScience (www.interscience.wiley.com).

and is involved in binding to the receptor for advanced glycation end products (RAGE) [Logsdon et al., 2007; Ramasamy et al., 2008].

To utilize the DNA binding property of HMGB1 for gene delivery, recombinant HMGB1 peptides have been produced and evaluated as gene carriers [Kim et al., 2008b,c]. Both HMGB1 boxes A and B have a high content of basic amino acids and their positive charges contribute to charge interactions with DNA. The acidic C-terminal tail, however, decreases the affinity of HMGB1 peptide for DNA due to its high level of negative charge [Kim et al., 2008c]. In addition, the HMGB1 Box B has pro-inflammatory activity [Li et al., 2003]. With respect to gene delivery, to exploit only the useful aspects of HMGB1, a truncated derivative was produced in which the box B and the acidic C-terminal tail were eliminated [Kim et al., 2008b]. Additionally, an HIV-TAT peptide was attached to the truncated HMGB1 to enhance its entry into cells [Kim et al., 2008b].

Artery walls are an important target organ for the treatment of cardiovascular disease by non-viral gene therapy [Affleck et al., 2001]. It is well known that low density lipoprotein (LDL) can be taken up by many different types of cells, including vascular smooth muscle cells [Krauss, 1995; Sawamura et al., 1997; Goldberg et al., 1998; Havel, 1998; Scanu, 1998]. Apolipoprotein B-100 (apo B-100) is a major protein component of LDL and contains many receptor-binding domains such as the LDL receptor-binding domain, artery wall cell-binding domain, and heparin-binding domain. Shih et al. [1990] demonstrated that a synthetic peptide containing amino acid residues 1,000–1,016 of apo B-100 (Arg-Ala-Leu-Val-Asp-Thr-Leu-Lys-Phe-Val-Thr-Gln-Ala-Glu-Gly-Ala-Lys) comprises the arterial wall-binding domain.

In this study, the arterial wall binding peptide (ABP) was linked to TAT-HMGB1A in order to increase the transfection efficiency of the peptide and to make it selective to artery wall cells. ABP linked to TAT-HMGB1A (TAT-HMGB1A-ABP) was produced and purified by recombinant DNA techniques. TAT-HMGB1A-ABP was evaluated as a gene carrier of smooth muscle cells, and our results suggest that TAT-HMGB1A-ABP is a non-toxic gene carrier, and may be useful for gene delivery targeting to smooth muscle cells.

## MATERIALS AND METHODS

### CLONING OF TAT-HMGB1A-ABP CONSTRUCTS

The pET21a-TAT-HMGB1A plasmid was constructed previously [Kim et al., 2008b]. To eliminate the stop codon at the end of the TAT-HMGB1A cDNA, the TAT-HMGB1A cDNA was amplified by PCR using specific primers and inserted into a pET15b vector (Novagen, Madison, WI). The sequences of the primers were as follows: Forward primer, 5′-CCGGAATTCATGGGCAAAGGAGAT-CCTAAG-3′, reverse primer, 5′-CCCAAGCTTGATGTAGGTTTTCA-TTTCTCTTTC-3′. The ABP cDNA was synthesized chemically and inserted downstream of the TAT-HMGB1A cDNA in the pET15b plasmid. The sequence of the cDNA coding ABP was as follows: upstream DNA 5′-AGAGCCTTGGTGGATACCCTGAAGTTTGTAACT-CAAGCAGAAGGTGCGAAG-3′. A 6× histidine repeat was located at the N-terminus of TAT-HMGB1A-ABP to allow for purification by nickel chelate affinity chromatography. The construction of pET15b-TAT-HMGB1A-ABP was confirmed by direct sequencing.

### EXPRESSION OF THE TAT-HMGB1A-ABP FUSION PROTEIN

pET15b-TAT-HMGB1A-ABP was transformed into the *E. coli* BL21 strain for the expression of TAT-HMGB1A-ABP. Bacterial cultures were incubated in LB medium containing 50 μg/ml ampicillin at 37°C and 220 rpm until the culture reached an $OD_{600}$ of 0.8, at which time the expression of TAT-HMGB1A-ABP was induced by the addition of 0.5 mM IPTG at 37°C for 6 h. After harvesting the bacteria by centrifugation the bacterial pellet was resuspended in ice-cold cell lysis buffer (50 mM $NaH_2PO_4$, pH 8.0) containing 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF. The bacteria were lysed by sonication (3 × 30 s bursts) with 5 min of cooling on ice between bursts.

### PURIFICATION OF TAT-HMGB1A-ABP

The presence of the 6 histidines repeat in the N-terminus of the protein conferred a high affinity to nickel ions, thereby allowing the TAT-HMGB1A-ABP peptide to be purified by nickel-chelate affinity chromatography in a single step procedure. To separate the soluble protein fraction from the cell debris, bacterial cell lysates were cleared by centrifugation at 15,000 rpm for 30 min. The supernatant from the lysed *E. coli* cells containing the TAT-HMGB1A-ABP was loaded onto a nickel (Probond resin, Invitrogen, Carlsbad, CA) column (Glass Econo Column, Bio-Rad, Hercules, CA) pre-equilibrated with equilibration buffer (50 mM $NaH_2PO_4$ (pH 8.0) containing 300 mM NaCl, and 20 mM imidazole). After unbound proteins were removed by washing with equilibration buffer, the fusion protein was eluted via a stepwise gradient of imidazole (100, 150, 200, 250, 300 mM) in 50 mM $NaH_2PO_4$ (pH 8.0) containing 300 mM NaCl at a rate of 1 ml/min. Each eluted fraction was assayed with a BCA assay kit (Pierce, Rockford, IL) and analyzed by electrophoresis on a 12% (w/v) sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE). The purified protein fractions were pooled and dialyzed against phosphate buffered saline (PBS) containing 20% glycerol and 0.15 mM PMSF using a membrane with a molecular weight cut-off of 6,000–8,000 Da (Spectra/Por dialysis membrane, MWCO, Millipore, 6,000–8,000 Billerica, CA) at 4°C overnight and stored at −80°C until use.

### GEL RETARDATION ASSAY

DNA condensation of peptides was confirmed by a gel retardation assay with pCMV-Luc. pCMV-Luc is a luciferase reporter plasmid, in which luciferae expression is regulated by the cytomegalovirus (CMV) immediate early promoter/enhancer and the SV40 polyadenylation signal. Peptide/DNA complexes were prepared by mixing 0.5 μg pCMV-Luc with increasing amounts of purified TAT-HMGB1A-ABP in PBS, followed by incubation for 30 min at room temperature. The complexes were analyzed by 1% agarose gel electrophoresis in the presence of ethidium bromide. DNA was visualized using a UV transilluminator.

### PARTICLE SIZE AND ZETA POTENTIAL

The hydrodynamic diameters and zeta potential values of the peptide/plasmid complexes were determined with a Zetasizer Nano ZS system (Malvern Instruments, Worcestershire, UK) as described previously [Kim et al., 2008c].

## CELL CULTURE AND IN VITRO TRANSFECTION

A7R5 rat smooth muscle, Hep3B human hepatoma, 293 human embryonic kidney, NIH3T3 mouse fibroblast, and Raw264.7 rat macrophage cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% FBS at 37°C in a 5% $CO_2$ atmosphere. For the transfection assay, cells were seeded at a density of $2.5 \times 10^4$ cells/well in a 6-well plate and grown for 48 h at 37°C. TAT-HMGB1A-ABP/plasmid complexes were prepared by mixing 2 μg of pCMV-Luc with increasing amounts of peptide. Poly-L-lysine (PLL)/pCMV-Luc and TAT-HMGB1A/pCMV-Luc complexes were prepared at 2:1 and 20:1 weight ratios, respectively [Lee et al., 2001; Kim et al., 2008b], and the peptide/pCMV-Luc complexes were added to each well. For the competition assay, free ABP was added to the designated wells at various concentrations. The cells were then incubated for 4 h at 37°C in a 5% $CO_2$ incubator in serum free medium. After 4 h, the growth medium was replaced with fresh DMEM containing 10% FBS. The cells were incubated for an additional 24 h at 37°C before being harvested for analysis.

## LUCIFERASE ASSAY

After removal of the growth medium, the cells were washed with 1 ml of PBS, and 200 μl of reporter lysis buffer (Promega, Madison, WI) was subsequently added to each well. After a 20 min incubation period at room temperature, cells were harvested and transferred to microcentrifuge tubes. The lysates were cleared by centrifugation at 13,000 rpm for 3 min. Luciferase activity in relative light units (RLUs) was measured at room temperature for 60 s using a 96-well plate luminometer (Berthold Detection System GmbH, Pforzheim, Germany). Protein concentrations of the extracts were determined using a BCA assay kit (Pierce). The final values of luciferase activity were calculated as RLU/mg total protein.

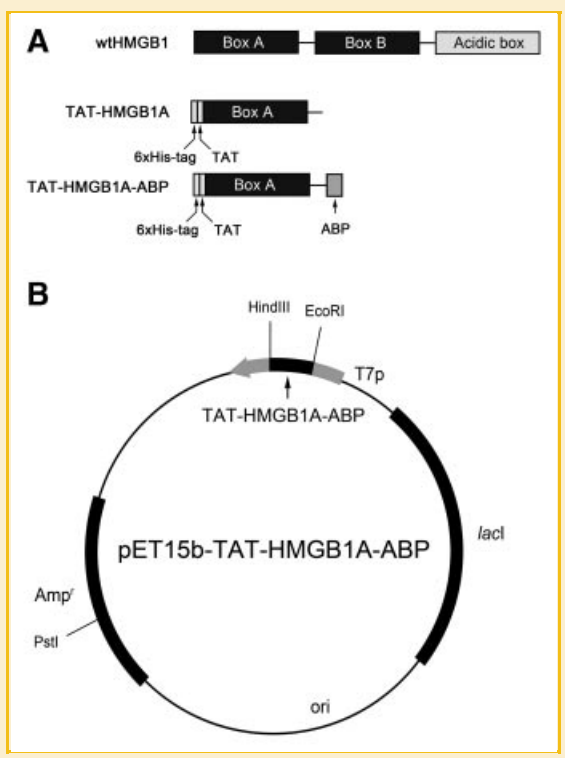


Fig. 1. Structures of HMGB1s and the expression plasmid. A: The structures of wtHMGB1, TAT-HMGB1A, and TAT-HMGB1A-ABP, (B) the expression plasmid, pET15b-TAT-HMGB1A-ABP.

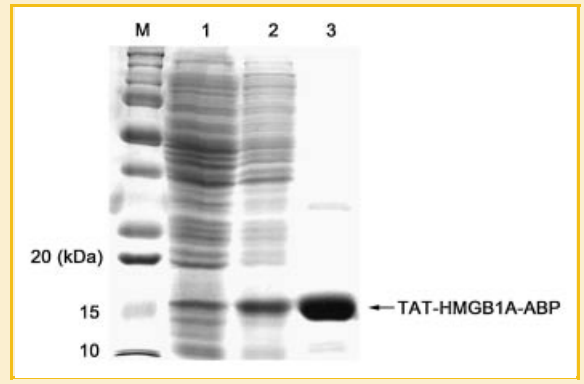


Fig. 2. SDS–PAGE. TAT-HMGB1A-ABP was purified in a single step procedure by nickel-chelate affinity chromatography. The purified peptide was analyzed by SDS–PAGE. Lane 1, crude lysate from bacteria without induction; lane 2, crude lysate from bacteria after IPTG induction; lane 3, purified TAT-HMGB1A-ABP. M indicates molecular weight markers.

## CYTOTOXICITY ASSAY

Evaluation of cytotoxicity was performed using the MTT assay. Briefly, A7R5 cells were seeded at a density of $6 \times 10^3$ cells/well in 24-well microassay plates (Falcon Co., Becton Dickenson, Franklin Lakes, NJ) and transfected after 24 h. The peptides were added to the cells and incubated for 4 h at 37°C in serum free medium; PLL was used as a control in both the transfection and cytotoxicity assays. Cells were incubated for an additional 24 h at 37°C in medium containing 10% FBS. At the end of the transfection experiments, 100 μl of 2 mg/ml MTT reagent in 1 × PBS was added to the wells, and the plates were incubated for an additional 4 h at 37°C. Afterwards, the MTT-containing medium was removed and 750 μl

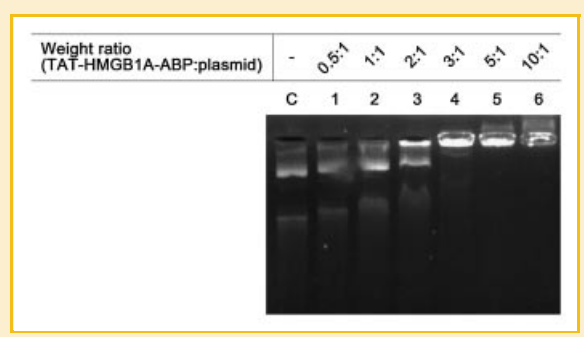


Fig. 3. Gel retardation assay. TAT-HMGB1A-ABP/plasmid complexes were analyzed with 1% agarose gel electrophoresis. Complete retardation of the plasmid was achieved at and above a 5:1 weight ratio of the TAT-HMGB1A-ABP/pDNA complex.

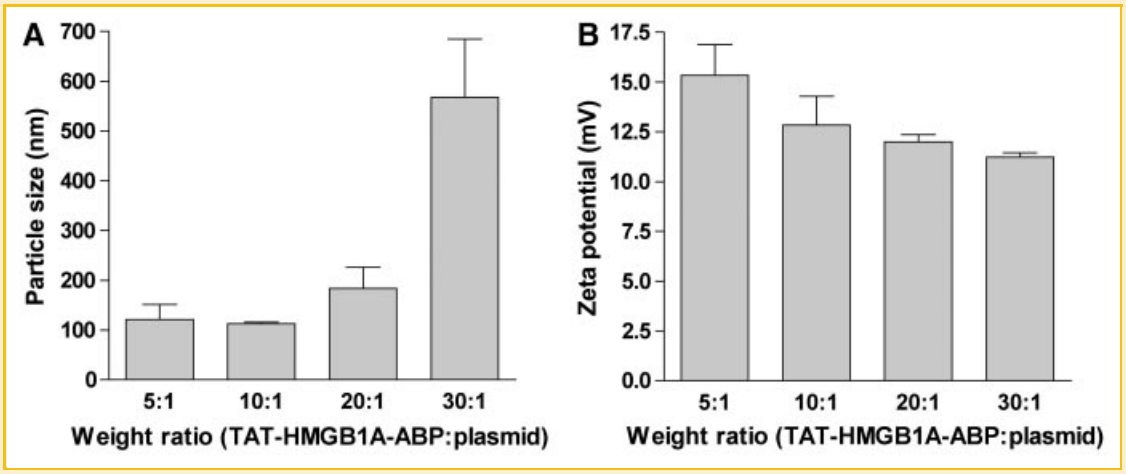


Fig. 4. A: Particle size and (B) Zeta potential of TAT-HMGB1A-ABP/plasmid complex. Particle size and zeta potential of TAT-HMGB1A-ABP/plasmid complex were measured at various weight ratios. Data is expressed as the mean ± standard deviation of triplicate experiments.

of DMSO was added to dissolve the formazan crystals formed by the live cells. The cytotoxicity of free TAT-HMGB1A-ABP and PLL was evaluated by measuring the absorbance at 570 nm. Specifically, cell viability (%) was calculated according to the following equation: cell viability $(\%) = (OD_{570(sample)}/OD_{570(control)}) \times 100$, where $OD_{570(sample)}$ represents the measurement from the wells treated with protein and $OD_{570(control)}$ represents the measurement from the wells treated with PBS buffer only.

### STATISTICAL ANALYSIS

The comparison of luciferase activity or VEGF concentration was made by Student's *t*-test. *P*-value under 0.05 was thought to be statistically significant.

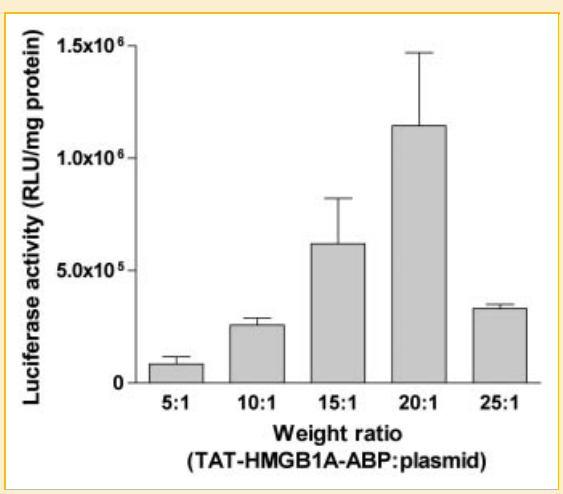


Fig. 5. Transfection efficiency of different weight ratios of TAT-HMGB1A-ABP. TAT-HMGB1A-ABP/plasmid complexes were prepared at various weight ratios and transfected into A7R5 cells. Transfection efficiencies were measured by a luciferase assay. Data is expressed as the mean ± standard deviation of quadruplicate experiments.

## RESULTS

### DESIGN, EXPRESSION, AND PURIFICATION OF THE RECOMBINANT TAT-HMGB1A-ABP PEPTIDE

To increase the transfection efficiency of TAT-HMGB1A into smooth muscle cells, the ABP domain of apo B-100 was linked to TAT-HMGB1A (Fig. 1A). To construct the TAT-HMGB1A-ABP expression plasmid, the cDNA encoding the ABP of apo B-100 was chemically synthesized and inserted downstream of TAT-HMGB1A in pET15b-TAT-HMGB1A (Fig. 1B). TAT-HMGB1A-ABP was designed such that it contained a 6× histidine repeat at the N-terminus of the peptide to allow for purification using nickel chelate affinity chromatography. The acidic tail of wild-type HMGB1 (wtHMGB1) is very negatively charged, and interfered with the charge-based interaction between the DNA and the peptide [Kim et al., 2008c]. In addition, the Box B of wtHMGB1 has a pro-inflammatory effect [Li et al., 2003]. For these reasons, the acidic tail and Box B of wtHMGB1 were not included in TAT-HMGB1A-ABP. The construction of the plasmid was confirmed by DNA sequencing. The expression of TAT-HMGB1A-ABP was induced by the addition of IPTG. After lysis, the cell lysate was subjected to nickel chelate affinity chromatography, and the resulting purified TAT-HMGB1A-ABP was analyzed by SDS–PAGE. A major band was detected that had an apparent molecular mass that matched that of the sequence predicted weight, thereby confirming the purification of the peptide (Fig. 2, lane 3).

### PHYSICAL CHARACTERIZATION OF TAT-HMGB1A-ABP/PLASMID COMPLEXES

Formation of TAT-HMGB1A-ABP/plasmid complexes was confirmed by a gel retardation assay (Fig. 3). Briefly, pCMV-Luc was incubated with increasing amounts of TAT-HMGB1A-ABP. The resulting complex was completely retarded at a 5:1 weight ratio (TAT-HMGB1A-ABP:plasmid). The particle sizes of TAT-HMGB1-ABP/plasmid complexes were measured by dynamic light scattering. The results showed that TAT-HMGB1A-ABP/plasmid complexes had

a particle size of ~120 nm (Fig. 4A), which was relatively stable up to a 20:1 weight ratio. However, the size increased abruptly at a 30:1 weight ratio, suggesting the aggregation of complexes. It was previously reported that the complex with particle size of around 100 nm is easily taken up by cells via endocytosis [Han et al., 2000; Lee and Kim, 2002]. Thus, the particle size of TAT-HMGB1A-ABP/plasmid complex may be good for endocytosis. The surface charge of the complexes was around 15 mV at a 5:1 charge ratio, and did not change significantly with higher weight ratios. The positive charge of the complex is useful for interaction of the complex with negatively charged cell surface and increase transfection efficiency [Kang et al., 2005; Kim et al., 2005].

## IN VITRO TRANSFECTION

Transfection efficiency is dependent on the ratio between peptide and DNA [Kim et al., 2008b,c]. To optimize the transcription conditions, in vitro transfection assays were performed with TAT-HMGB1-ABP/plasmid complexes prepared at various weight ratios. TAT-HMGB1-ABP/plasmid complexes were transfected into A7R5 smooth muscle cells, and the results showed that the highest luciferase expression was obtained at a 20:1 weight ratio (TAT-HMGB1-ABP/plasmid) (Fig. 5). Under these conditions, the transfection efficiency of TAT-HMGB1-ABP was compared with that of both PLL and TAT-HMGB1A. In the transfection assay of A7R5 cells, TAT-HMGB1A-ABP had higher transfection efficiency than PLL and TAT-HMGB1A (Fig. 6A). However, the transfection efficiency of TAT-HMGB1A-ABP was much lower than that of PLL in Hep3B hepatoma cells (Fig. 6B), 293 kidney cells (Fig. 6C), NIH3T3 fibroblast (Fig. 6D), and Raw264.7 macrophage cells (Fig. 6E). The transfection efficiency of TAT-HMGB1A-ABP in Hep3B, 293, NIH3T3, or Raw264.7 cells was similar to that of TAT-HMGB1A, indicating that the ABP moiety of TAT-HMGB1A-ABP may have indeed increased the transfection efficiency specifically to smooth muscle cells. To confirm this supposed interaction of ABP with smooth muscle cells, a competition assay with free ABP was performed. The chemically synthesized free ABP was added to the transfection mixtures, and the transfection efficiency of TAT-HMGB1A-ABP was measured. As a result, the transfection of TAT-HMGB1A-ABP decreased with the addition of free ABP at a concentration of 300 μg/ml (Fig. 7). Therefore, the higher transfection efficiency of TAT-HMGB1A-ABP to A7R5 cells was mediated by a specific interaction of ABP with the cells.

## CYTOTOXICITY OF TAT-HMGB1A-ABP

To evaluate the cytotoxicity of TAT-HMGB1A-ABP, an MTT assay was performed where A7R5 cells were incubated with increasing amounts of TAT-HMGB1A-ABP or PLL for 24 h. The results showed that PLL was toxic to A7R5 cells at or above a concentration of 8 μg/ml (Fig. 8A). TAT-HMGB1A-ABP did not produce toxicity at any of the concentrations tested (Fig. 8A). A7R5 cells were also incubated

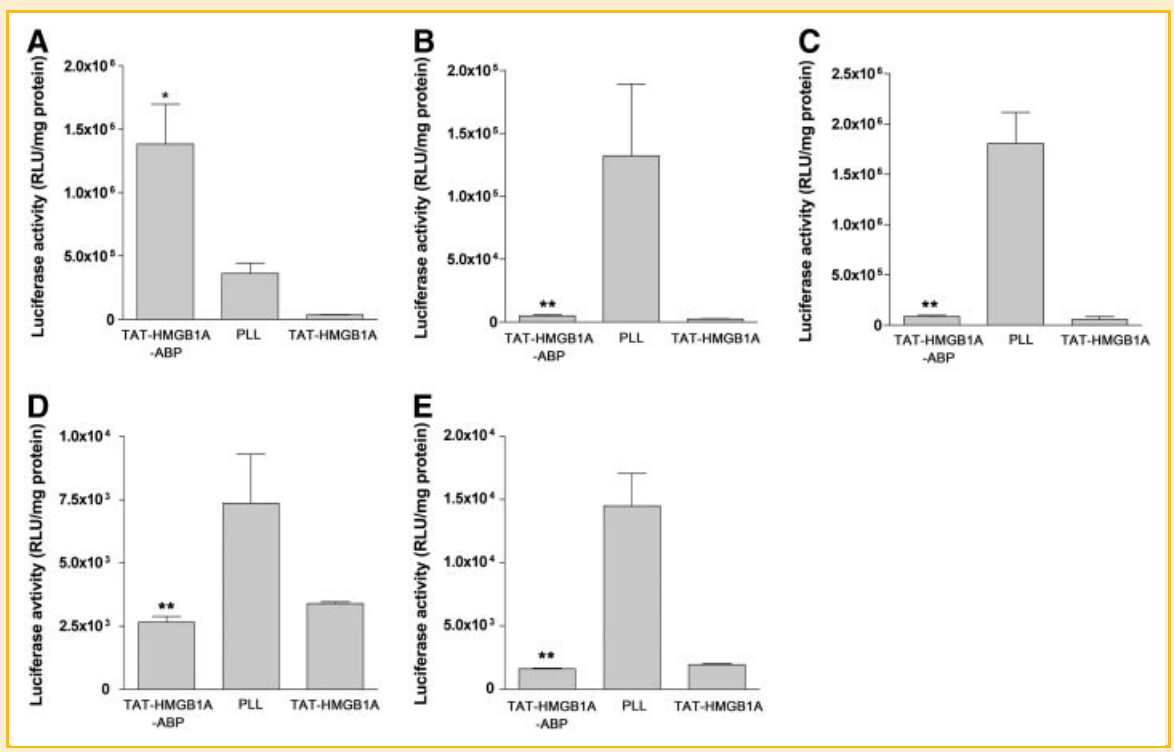


Fig. 6. Transfection efficiency of TAT-HMGB1-ABP in various cells. The TAT-HMGB1A-ABP/plasmid complex was transfected into A7R5 (A), Hep3B (B), 293 (C), NIH3T3 (D), and Raw264.7 (E) cells. Transfection efficiencies were measured by a luciferase assay. The transfection efficiency of TAT-HMGB1-ABP was compared with that of PLL or TAT-HMGB1A. Data is expressed as the mean ± standard deviation of quadruplicate experiments. $^{*}P < 0.01$ as compared with PLL and TAT-HMGB1A. $^{**}P < 0.01$ as compared with PLL.

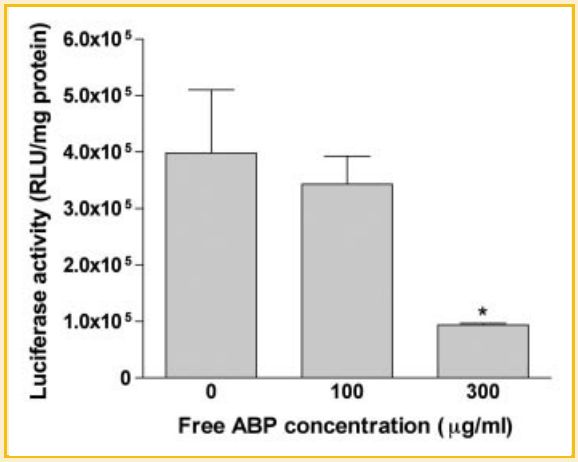


Fig. 7. Competition assay with free ABP. The TAT-HMGB1A-ABP/plasmid complex was transfected into A7R5 cells in the absence or presence of free ABP, and transfection efficiencies were measured by a luciferase assay. Data is expressed as the mean ± standard deviation of quadruplicate experiments. *$P < 0.01$ compared with 0 or 100 μg/ml ABP.

with TAT-HMGB1A-ABP/plasmid or PLL/plasmid complex (Fig. 8B). TAT-HMGB1A-ABP/plasmid complex showed less toxicity than PLL/plasmid. Therefore, TAT-HMGB1A-ABP may be a safer carrier than PLL in gene delivery to smooth muscle cells.

## DISCUSSION

In the present study we linked the HMGB1A peptide with the ABP peptide and showed that the resulting fusion peptide has a potential use as a gene carrier for targeting smooth muscle cells. In a previous report we showed that recombinant TAT-HMGB1A formed stable complexes with DNA and could mediate delivery of genes to cells [Kim et al., 2008b]. Furthermore, the recombinant TAT-HMGB1A peptide did not appear to exert any toxicity. Unfortunately, the advantages of HMGB1A are hampered by its low transfection efficiency. Indeed, in 293 cells, TAT-HMGB1A has a much lower transfection efficiency compared with that of PLL [Kim et al., 2008b]. Therefore, to improve the transfection efficiency of TAT-HMGB1A to smooth muscle cells, the ABP domain of apo B-100 was linked to TAT-HMGB1A. In vitro transfection assays showed that the ABP linked peptide did indeed increase the transfection efficiency in a manner that was selective to smooth muscle cells.

The HMGB1 peptide is a nuclear protein and is a component of chromatin structure. In the nucleus, HMGB1 plays a role in gene regulation as well as providing a scaffold for DNA compaction. Since HMGB1 is an endogenously expressed protein, it is likely that HMGB1A has low cytotoxicity and immunogenecity. Indeed, in our MTT assays, TAT-HMGB1A-ABP had no detectable cytotoxicity. It has been previously reported that the toxicity of a gene carrier is mainly due to the high charge density of the carrier [Fischer et al., 1999]. While TAT-HMGB1A-ABP had high content of basic amino acids, which contributed to the overall charge interaction of the

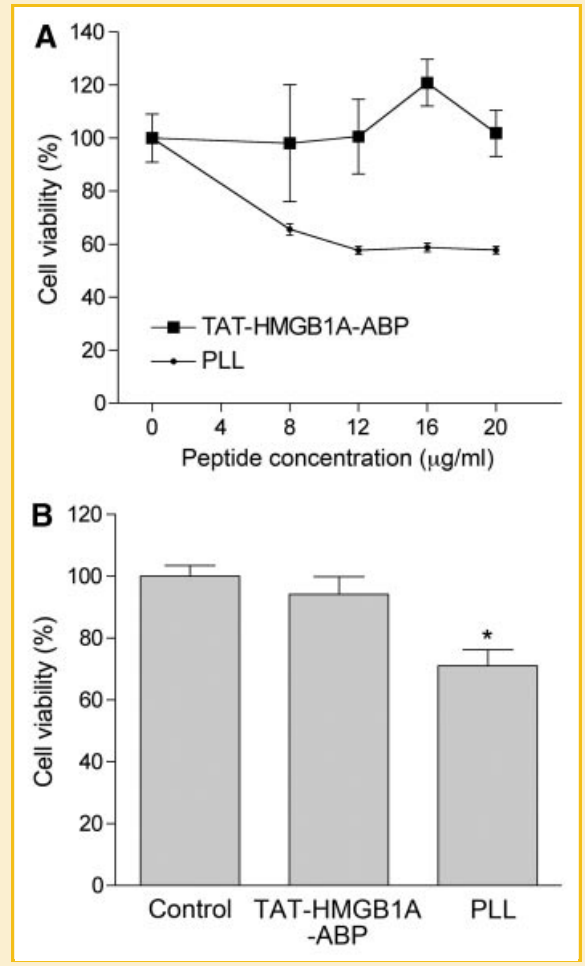


Fig. 8. Cytotoxicity of TAT-HMGB1A-ABP and PLL. A7R5 cells were incubated with peptides (A) or peptide/DNA complexes (B). After the indicated incubation period, cell viability was measured by the MTT assay. Data is expressed as the mean ± standard deviation of pentaplicates experiments. *$P < 0.05$ as compared with PLL.

peptide plasmid complex, its charge density may not be as high as other cationic peptides such as PLL, since TAT-HMGB1A-ABP also contains acidic amino acids that may effectively cancelled out some of the positive charges of the basic amino acids. Due to the relatively low charge density of TAT-HMGB1A-ABP, the cytotoxicity of the peptide was much lower than PLL. The low charge density of TAT-HMGB1A-ABP may not only have reduced cytotoxicity, but it may have also decreased the stability of the peptide/DNA complex; however, previous reports have shown that HMGB1 can stably bind to DNA [Wu et al., 2003; Stott et al., 2006; Kim et al., 2008b,c]. In the present study, TAT-HMGB1A-ABP stably bound to plasmid DNA with a particle size of around 120 nm. This stable complex formation may have been due to hydrophobic interactions as well as charge interactions between the peptide and DNA. Specifically, HMGB1 has hydrophobic amino acids such as phenylalanine, which may interact with hydrophobic regions of base stacks of the DNA core, further reinforcing the stability of the peptide/DNA complex [Stott et al., 2006]. TAT peptide has high contents of arginines, which may

increase charge density and toxicity of HMGB1A peptide. However, TAT-HMGB1A-ABP did not show any toxicity in the range of amount in this research.

In an X-ray crystallography study of a HMG peptide and DNA, the HMG peptide was found to bind double stranded DNA in a well-defined structure [Stott et al., 2006]. Therefore, it is likely that the well-defined structure of the peptide/DNA complex may not have allowed further condensation when the extra TAT-HMGB1A-ABP component was added. The extra TAT-HMGB1A-ABP may stack on the surface of the TAT-HMGB1A-ABP/DNA complex without further condensation at higher weight ratios. We speculate that the ability to increase particle size with no significant change of surface charge with increasing weight ratios (Fig. 4B) may have been due to this phenomenon, although further study is required to identify the specific mechanism responsible for this observation.

In this research, we have shown that TAT-HMGB1A-ABP has higher transfection efficiency than PLL and is specific to smooth muscle cells, confirming the role of the targeting ligand, ABP. This result shows that specific peptide linked TAT-HMGB1A can deliver DNA to specific type of cells. Using recombinant technology as described here, TAT-HMGB1A can be easily modified with targeting peptides to generate gene carriers for other types of cells. HMGB1 is also an angiogenic factor and therefore, the elimination of box B might not be necessary for ischemic disease gene therapy [Schlueter et al., 2005; Kitahara et al., 2008]. Together, the evidence presented here suggests that HMGB1 peptides can and should be modified depending on their specific application and disease target. The results here have demonstrated the value of TAT-HMGB1A-ABP in vitro and it would be interesting to test the peptide in gene therapy in vivo.

Artery wall targeting gene delivery has been shown to be useful for ischemic disease gene therapy [Affleck et al., 2001]. In this study, TAT-HMGB1A-ABP exhibited higher transfection efficiency than PLL and was specific to smooth muscle cells. The competition assay with free ABP confirmed the higher transfection efficiency and suggested that the uptake may be mediated by an interaction of ABP with a specific receptor. In addition, TAT-HMGB1A-ABP did not produce any cytotoxic effects in smooth muscle cells. Therefore, TAT-HMGB1A-ABP has the potential to be useful as a gene carrier for targeting artery wall smooth muscle cells.

## ACKNOWLEDGMENTS

This work was financially supported by the Ministry of Education, Science and Technology (F104AA010002-08A0101-00200) in Korea.

## REFERENCES

Affleck DG, Yu L, Bull DA, Bailey SH, Kim SW. 2001. Augmentation of myocardial transfection using TerplexDNA: A novel gene delivery system. Gene Ther 8:349–353.

Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ. 2002. HMGB1 as a DNA-binding cytokine. J Leukoc Biol 72:1084–1091.

Balicki D, Reisfeld RA, Pertl U, Beutler E, Lode HN. 2000. Histone H2A-mediated transient cytokine gene delivery induces efficient antitumor responses in murine neuroblastoma. Proc Natl Acad Sci USA 97:11500–11504.

Balicki D, Putnam CD, Scaria PV, Beutler E. 2002. Structure and function correlation in histone H2A peptide-mediated gene transfer. Proc Natl Acad Sci USA 99:7467–7471.

Bottger M, Platzer M, Kiessling U, Strauss M. 1990. Transfection by DNA-nuclear protein HMG1 complexes: Raising of efficiency and role of DNA topology. Arch Geschwulstforsch 60:265–270.

Edelstein ML, Abedi MR, Wixon J, Edelstein RM. 2004. Gene therapy clinical trials worldwide 1989–2004—An overview. J Gene Med 6:597–602.

Ellison KE, Bishopric NH, Webster KA, Morishita R, Gibbons GH, Kaneda Y, Sato B, Dzau VJ. 1996. Fusigenic liposome-mediated DNA transfer into cardiac myocytes. J Mol Cell Cardiol 28:1385–1399.

Fischer D, Bieber T, Li Y, Elsasser HP, Kissel T. 1999. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: Effect of molecular weight on transfection efficiency and cytotoxicity. Pharm Res 16:1273–1279.

Goldberg IJ, Wagner WD, Pang L, Paka L, Curtiss LK, DeLozier JA, Shelness GS, Young CS, Pillarisetti S. 1998. The NH2-terminal region of apolipoprotein B is sufficient for lipoprotein association with glycosaminoglycans. J Biol Chem 273:35355–35361.

Grigorov I, Milosavljevic T, Cvetkovic I, Petrovic M. 2001. HMG-1 as regulatory trans-acting protein in the acute phase-induced expression of the rat liver haptoglobin gene. Gen Physiol Biophys 20:401–412.

Han S, Mahato RI, Sung YK, Kim SW. 2000. Development of biomaterials for gene therapy. Mol Ther 2:302–317.

Havel RJ. 1998. Receptor and non-receptor mediated uptake of chylomicron remnants by the liver. Atherosclerosis 141 (Suppl 1): S1–S7.

Isaka Y, Akagi Y, Kaneda Y, Imai E. 1998. The HVJ liposome method. Exp Nephrol 6:144–147.

Kaneda Y, Iwai K, Uchida T. 1989. Introduction and expression of the human insulin gene in adult rat liver. J Biol Chem 264:12126–12129.

Kang HC, Lee M, Bae YH. 2005. Polymeric gene carriers. Crit Rev Eukaryot Gene Expr 15:317–342.

Kim YH, Park JH, Lee M, Kim YH, Park TG, Kim SW. 2005. Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. J Control Release 103:209–219.

Kim JB, Sig Choi J, Yu YM, Nam K, Piao CS, Kim SW, Lee MH, Han PL, Park JS, Lee JK. 2006. HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J Neurosci 26:6413–6421.

Kim JB, Lim CM, Yu YM, Lee JK. 2008a. Induction and subcellular localization of high-mobility group box-1 (HMGB1) in the postischemic rat brain. J Neurosci Res 86:1125–1131.

Kim K, Han JS, Kim HA, Lee M. 2008b. Expression, purification and characterization of TAT-high mobility group box-1A peptide as a carrier of nucleic acids. Biotechnol Lett 30:1331–1337.

Kim K, Han JS, Park JH, Ko KS, Lee M. 2008c. Expression and characterization of a recombinant high mobility group box 1 AB peptide with a 6-histidine tag for delivery of nucleic acids. Enzyme Microb Technol 43:410–416.

Kitahara T, Takeishi Y, Harada M, Niizeki T, Suzuki S, Sasaki T, Ishino M, Bilim O, Nakajima O, Kubota I. 2008. High-mobility group box 1 restores cardiac function after myocardial infarction in transgenic mice. Cardiovasc Res 80:40–46.

Krauss RM. 1995. Dense low density lipoproteins and coronary artery disease. Am J Cardiol 75:53B–57B.

Lee M, Kim SW. 2002. Polymeric gene carriers. Pharm News 9:407–415.

Lee M, Nah JW, Kwon Y, Koh JJ, Ko KS, Kim SW. 2001. Water-soluble and low molecular weight chitosan-based plasmid DNA delivery. Pharm Res 18:427–431.

Li J, Kokkola R, Tabibzadeh S, Yang R, Ochani M, Qiang X, Harris HE, Czura CJ, Wang H, Ulloa L, Warren HS, Moldawer LL, Fink MP, Andersson U, Tracey KJ, Yang H. 2003. Structural basis for the proinflammatory cytokine activity of high mobility group box 1. Mol Med 9:37–45.

Lildballe DL, Pedersen DS, Kalamajka R, Emmersen J, Houben A, Grasser KD. 2008. The Expression Level of the Chromatin-Associated HMGB1 Protein Influences Growth, Stress Tolerance, and Transcriptome in Arabidopsis. J Mol Biol 384:9–21.

Logsdon CD, Fuentes MK, Huang EH, Arumugam T. 2007. RAGE and RAGE ligands in cancer. Curr Mol Med 7:777–789.

Puebla I, Esseghir S, Mortlock A, Brown A, Crisanti A, Low W. 2003. A recombinant H1 histone-based system for efficient delivery of nucleic acids. J Biotechnol 105:215–226.

Ramasamy R, Yan SF, Schmidt AM. 2008. Stopping the primal RAGE reaction in myocardial infarction: Capturing adaptive responses to heal the heart? Circulation 117:3165–3167.

Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T, Miwa S, Katsura Y, Kita T, Masaki T. 1997. An endothelial receptor for oxidized low-density lipoprotein. Nature 386:73–77.

Scanu AM. 1998. Atherothrombogenicity of lipoprotein(a): The debate. Am J Cardiol 82:26Q–33Q.

Schlueter C, Weber H, Meyer B, Rogalla P, Roser K, Hauke S, Bullerdiek J. 2005. Angiogenetic signaling through hypoxia: HMGB1: An angiogenetic switch molecule. Am J Pathol 166:1259–1263.

Shih IL, Lees RS, Chang MY, Lees AM. 1990. Focal accumulation of an apolipoprotein B-based synthetic oligopeptide in the healing rabbit arterial wall. Proc Natl Acad Sci USA 87:1436–1440.

Stott K, Tang GS, Lee KB, Thomas JO. 2006. Structure of a complex of tandem HMG boxes and DNA. J Mol Biol 360:90–104.

Thomas JO. 2001. HMG1 and 2: Architectural DNA-binding proteins. Biochem Soc Trans 29:395–401.

Weng L, Liu D, Li Y, Cao S, Feng Y. 2004. An archaeal histone-like protein as an efficient DNA carrier in gene transfer. Biochim Biophys Acta 1702:209–216.

Wu Q, Zhang W, Pwee KH, Kumar PP. 2003. Rice HMGB1 protein recognizes DNA structures and bends DNA efficiently. Arch Biochem Biophys 411:105–111.

Zhang CL, Shu MG, Qi HW, Li LW. 2008. Inhibition of tumor angiogenesis by HMGB1 A box peptide. Med Hypotheses 70:343–345.

Zlatanova J, van Holde K. 1998. Linker histones versus HMG1/2: A struggle for dominance? Bioessays 20:584–588.