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Journal of Cellular Biochemistry 110:1094–1100 (2010)

Journal of Cellular Biochemistry

VEGF Receptor Binding Peptide-Linked High Mobility Box Group-1 Box A as a Targeting Gene Carrier for Hypoxic Endothelial Cells

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ABSTRACT

High mobility group box-1 (HMGB-1) is a nuclear protein that can bind to and condense plasmid DNA. In this study, we developed a recombinant VEGF receptor binding peptide (VRBP) linked to HMGB-1 box A (VRBP-HMGB1A) as a targeting gene carrier to hypoxic endothelial cells. Hypoxic endothelial cells in ischemic tissues of solid tumors are important targets for gene therapy. A recombinant VRBP-HMGB1A expression vector, pET21a-VRBP-HMGB1A was constructed. VRBP-HMGB1A was over-expressed in BL21 strain and purified by nickel-chelate affinity chromatography. Complex formation between VRBP-HMGB1A and pCMV-Luc was confirmed by gel retardation assay. pCMV-Luc was retarded completely at a 2/1 weight ratio (peptide/plasmid). For transfection assays, calf pulmonary artery endothelial (CPAE) cells were incubated under hypoxia for 24 h, prior to transfection to induce the VEGF receptors on the cells. VRBP-HMGB1A/pCMV-Luc complexes were transfected to hypoxic CPAE cells. The highest transfection efficiency was at a 30/1 weight ratio (peptide/plasmid). In addition, VRBP-HMGB1A had higher efficiency than poly-L-lysine (PLL) specifically in hypoxic CPAE cells, However, VRBP-HMGB1A had lower efficiency than PLL in 293, H9C2, and normoxic CPAE cells. In MTT assay, VRBP-HMGB1A was less toxic than PLL to cells. In conclusion, VRBP-HMGB1A is a potential gene carrier for targeting hypoxic endothelial cells and thus, may be useful for cancer gene therapy. J. Cell. Biochem. 110: 1094–1100, 2010.

KEY WORDS: ENDOTHELIAL CELLS; GENE DELIVERY; HIGH MOBILITY GROUP BOX-1; HYPOXIA; PLASMID

Non-viral vectors have been developed as alternatives to viral vectors for gene therapy, due to their non-immunogenecity and high biocompatibility [Kang et al., 2005]. However, cytotoxicity and low delivery efficiency have limited the clinical application of non-viral vectors. Various non-viral carriers including cationic liposomes, polymers, and peptides have been investigated to improve cytotoxicity and delivery efficiency [Han et al., 2000; Kang et al., 2005]. One non-viral gene delivery approach is to use endogenous nuclear proteins such as histones as gene carriers [Kaouass et al., 2006]. Histones 1B and 2 have been evaluated in terms of cytotoxicity and transfection efficiency [Balicki et al., 2000, 2002; Puebla et al., 2003]. Another nuclear protein, high mobility group box (HMGB) protein, also has several advantages as a gene carrier. HMGB proteins bind strongly to DNA [Thomas, 2001; Andersson et al., 2002; Wu et al., 2003; Stott et al., 2006], and have their own nuclear localization signals (NLSs) for nuclear trafficking [Sloots and Wels, 2005]. Thus, the HMGB protein/DNA complex translocates easily into the nucleus after cellular entry, due to the NLS. In previous reports, it was shown that HMGB-1 delivered DNA to cells more efficiently than poly-L-lysine (PLL) [Kim et al., 2008a,b; Han et al., 2009]. HMGB-1 is composed of two homologous, but distinct, HMG boxes (box A and B), and an acidic C-terminal tail [Yoshioka et al., 1999; Thomas, 2001; Li et al., 2003]. Each HMG box is about 75 amino acids in length and binds non-specifically to the minor groove of DNA. The acidic C-terminal tail has many negatively charged amino acids, which decreases the electrostatic interaction between HMGB-1 and DNA [Kim et al., 2008b]. In addition, HMGB-1 Box B has pro-inflammatory activity [Li et al., 2003]. Therefore, the acidic C-terminal tail and box B should be deleted to make HMGB-1 safe for use in gene therapy. In a previous report, we evaluated HMGB-1 box A (HMGB-1A), with both box B and the acidic C-terminal tail eliminated, as a gene carrier [Kim et al., 2008a]. HMGB-1A itself had lower transfection efficiency than PLL. However, in combination with the cell penetrating peptide TAT,

Grant sponsor: National Research Foundation of Korea (Ministry of Education, Science and Technology); Grant numbers: 20090073844, 20090081874.

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Received 6 February 2010; Accepted 19 March 2010 • DOI 10.1002/jcb.22621 • © 2010 Wiley-Liss, Inc.
Published online 19 May 2010 in Wiley InterScience (www.interscience.wiley.com).

TAT-HMGB-1A delivered plasmid more efficiently than PLL into cells without toxicity [Kim et al., 2008a]. Furthermore, with a targeting ligand, artery wall binding peptide (ABP), TAT-HMGB1-ABP had higher transfection efficiency specifically with smooth muscle cells [Han et al., 2009].

The VEGF receptor is an important target for cancer gene therapy. For rapid growth, cancer cells usually require a higher level of nutrients and blood. However, the formation of new blood vessels, or angiogenesis, in tumor tissues does not provide enough nutrients and blood to meet this requirement, resulting in chronic hypoxia within the tumor [Hemmerlein et al., 2001]. Therefore, angiogenesis is a critical step in tumor growth. Angiogenesis provides nutrition for tumor growth and a route for metastasis [Yancopoulos et al., 2000; Gale et al., 2002; Carmeliet, 2005]. Endothelial cells play an essential role in angiogenesis. Under hypoxic conditions, angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are induced and activate endothelial cells [Komi et al., 2007]. Therefore, endothelial cells in the tumor hypoxic region are important in the treatment of solid tumors. Indeed, it has been reported that angiogenesis inhibitors can reduce tumor growth by suppressing endothelial cells [Folkman and Kalluri, 2004]. Also, hypoxic endothelial cells are found in various ischemic diseases such as ischemic heart disease and limb ischemia [Semenza et al., 2000; Vincent et al., 2000]. The VEGF receptor is highly expressed in hypoxic endothelial cells and may be useful for targeting hypoxic endothelial cells in tumors or ischemic tissues [Brogi et al., 1996].

El-Mousawi et al. [2003] screened a peptide phage-display library with Flt-1 and identified ASSSYPLIHWRPWAR as a VEGF receptor binding peptide (VRBP). In this study, VRBP-linked HMGB-1A was produced for development of a hypoxic endothelial cell targeting gene carrier. VRBP-HMGB1A expression vector was constructed and the over-expression and purification conditions for VRBP-HMGB1A were optimized. Physical characterization was performed with VRBP-HMGB1A/plasmid complex. Transfection and MTT assays were performed to evaluate VRBP-HMGB1A as a targeting gene carrier. PLL is a widely used gene carrier. In this research, PLL with a molecular weight of 10 kDa was used as a control, as it was a peptide carrier with similar molecular weight as HMGB1A. Our results suggest that VRBP-HMGB1A delivers plasmid specifically into hypoxic endothelial cells.

MATERIALS AND METHODS

CLONING OF pET21A-VRBP-HMGB1A

pET21a-HMGB-1A was previously constructed [Kim et al., 2008a]. The VRBP cDNA was prepared by annealing the synthesized oligonucleotides, 5′-CTAGCTAGCTCATCGTACCCACTAATACACTGGAGACCATGGGCAAGACTCGAGGAATTCC-3′ and 5′-GGAATTCCTCGAGTCTTGCCCATGGTCTCCCAGTTTAGTGGGTACGATGAGCTAGCTAG-3′. The VRBP cDNA was inserted upstream of the HMGB-1A cDNA in pET21a-HMGB-1A at the *Nhe*I and *Eco*RI sites. A 6× histidine repeat was located at the C-terminus of VRBP-HMGB1A for purification by nickel-chelate affinity chromatography. The construction pET21a-VRBP-HMGB1A was confirmed by direct sequencing.

EXPRESSION OF VRBP-HMGB1A PROTEIN

Escherichia coli BL21 strain carrying the pET21a-VRBP-HMGB1A was grown to OD_{600} of 0.8 at 37°C in LB medium containing 50 μg/ml ampicillin. The expression of VRBP-HMGB1A was induced by the addition of 0.5 mM IPTG at 37°C for 6 h. Cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C. The bacterial pellets were 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 (5 × 20 s bursts) with 5 min of cooling on ice between bursts. To separate the soluble protein fraction from the cell debris, bacterial cell lysates were cleared by centrifugation at 15,000 rpm for 30 min, and the peptide was purified from the supernatant.

PURIFICATION OF VRBP-HMGB1A

The presence of the C-terminal stretch of 6× histidines in VRBP-HMGB1A confers a high affinity to nickel ion. VRBP-HMGB1A was purified by nickel-chelate affinity chromatography. The supernatant from the lysed *E. coli* cells containing VRBP-HMGB1A was loaded onto a nickel (Probond resin; Invitrogen, Carlsbad, CA) column pre-equilibrated with an equilibration buffer [50 mM NaH_2PO_4 (pH 8.0) containing 300 mM NaCl, and 10 mM imidazole]. After unbound proteins were removed by washing with wash buffer [50 mM NaH_2PO_4 (pH 8.0) containing 300 mM NaCl, and 100 mM imidazole], VRBP-HMGB1A was eluted by a step gradient of imidazole (125, 150, 175, 200, and 225 mM) in 50 mM NaH_2PO_4 (pH 8.0) containing 300 mM NaCl, at a rate of 1 ml/min. The purified VRBP-HMGB1A was 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 (Spectra/Por dialysis membrane, MWCO; Millipore, Billerica, CA) at 4°C overnight. The purified VRBP-HMGB1A was stored at −80°C until use.

GEL RETARDATION ASSAY

VRBP-HMGB1A/pCMV-Luc complexes were prepared at various weight ratios by mixing 1 μg pCMV-Luc with increasing amounts of purified VRBP-HMGB1A in PBS and incubated for 20 min at room temperature. After incubation, the samples were electrophoresed through a 1% agarose gel in the presence of ethidium bromide. The plasmid was visualized using a UV transilluminator.

PARTICLE SIZE AND ZETA POTENTIAL

The hydrodynamic diameters and zeta potential values of VRBP-HMGB1A/pCMV-Luc complexes were determined by the Zetasizer Nano ZS system (Malvern Instruments, Worcestershire, UK) as described previously [Kim et al., 2008a].

CELL CULTURE AND IN VITRO TRANSFECTION

Calf pulmonary aorta endothelial (CPAE) cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 15% FBS, and human embryonic kidney (HEK) 293 and H9C2 cells were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO_2. The cells were seeded in 12-well

plates at a density of 2.0×10^4 cells per well at 37°C. After 24 h, the cells were incubated at the desired concentration of oxygen (normoxia, 20% oxygen; hypoxia, 1% oxygen) for 20 h. VRBP-HMGB1A/pCMV-Luc complexes were prepared by mixing 1 μg pCMV-Luc with increasing amounts of VRBP-HMGB1A. As a control, PLL/pCMV-Luc complex was prepared at a weight ratio of 2/1 (peptide/plasmid), which is optimal condition for in vitro transfection [Lee et al., 2001]. Peptide/pCMV-Luc complex was added to each well. The cells were incubated under hypoxia or normoxia for an additional 24 h.

LUCIFERASE ASSAY

The transfected cells were washed with PBS and then lysed in 100 μl 1× lysis buffer (Promega, Madison, WI) for 20 min at room temperature. The lysates were cleared by centrifugation and total protein assays were carried out using a BCA protein assay kit (Pierce, Iselin, NJ). Luciferase activity was measured at room temperature for 30 s in relative light units (RLU) using a 96-well plate luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The final values of luciferase are reported in terms of RLU/mg protein.

CYTOTOXICITY ASSAY

Cytotoxicity of free VRBP-HMGB1A or VRBP-HMGB1A/pDNA complex was evaluated with the MTT test using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide reagent. CPAE cells were seeded in 24-well plates at a density of 1.0×10^4 cells per well. After 1 day, the cells were exposed to hypoxic conditions in a hypoxia chamber containing 1% O_2 at 37°C for 24 h. The cells were transfected with 0.5 μg pCMV-Luc per well using VRBP-HMGB1A and PLL. VRBP-HMGB1A/pCMV-Luc and PLL/pCMV-Luc complexes were prepared at 30/1 and 2/1 weight ratios, respectively. Free VRBP-HMGB1A or PLL was added to the cells at various weight ratios. The cells were then incubated for an additional 24 h at 37°C. After incubation, 100 μl of 2 mg/ml MTT solution in 1× PBS was added to each well. The plates were incubated for an additional 4 h at 37°C. The MTT-containing media was removed and 750 μl of DMSO was added to dissolve the formazan crystals formed by the live cells. Absorbance was measured at 570 nm using a microplate reader.

STATISTICAL ANALYSIS

The one-way analysis of variance (ANOVA) was used for comparisons involving more than two groups. The comparisons with two groups were made by Student's *t*-test. *P*-value <0.05 was thought to be statistically significant.

RESULTS

DESIGN, EXPRESSION, AND PURIFICATION OF VRBP-HMGB1A

Wild-type HMGB-1 has three domains, box A, box B, and an acidic c-tail (Fig. 1A) [Thomas, 2001]. In a previous study, box B was excluded from the design of the HMGB-1 based gene carrier, as box B has a pro-inflammatory effect [Kim et al., 2008a]. In addition, the acidic C-terminal tail was eliminated, as it contained a number of negative charges that may interfere with the charge interaction between the peptide and DNA [Kim et al., 2008a]. To produce a hypoxic endothelial cell-targeting carrier, VRBP was linked to HMGB-1A (Fig. 1A). For purification of the peptide, a 6× his-tag was located at the C-terminus of box A. The VRBP-HMGB1A expression vector, pET21a-VRBP-HMGB1A was constructed and confirmed by sequencing (Fig. 1B). pET21a-VRBP-HMGB1A was transformed to the BL21 strain and VRBP-HMGB1A was over-expressed. VRBP-HMGB1A was purified by nickel-chelate affinity chromatography. The calculated molecular weight of VRBP-HMGB1A was 12 kDa. In SDS–PAGE analysis, a 12-kDa VRBP-HMGB1A peptide was enriched after the affinity chromatography (Fig. 2).

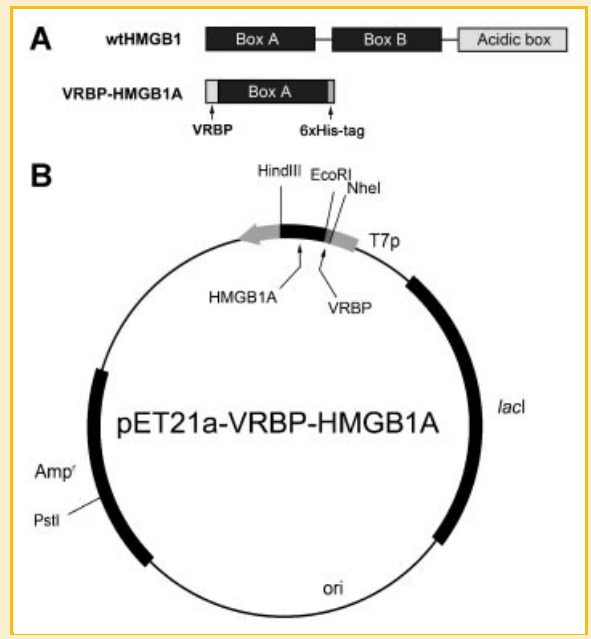

Fig. 1. Schematic diagrams of VRBP-HMGB1A and expression plasmids. A: Diagrams of HMGB-1 and VRBP-HMGB1A and (B) the expression plasmid, pET21a-VRBP-HMGB1A.

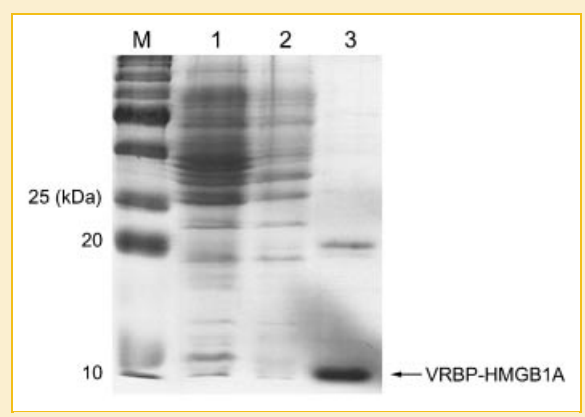

Fig. 2. SDS–PAGE analysis of the purified VRBP-HMGB1A. VRBP-HMGB1A was purified and analyzed by SDS–PAGE: lane 1, crude lysates from bacteria without induction; lane 2, crude lysates from bacteria after IPTG induction; lane 3, purified VRBP-HMGB1A. M indicates molecular weight markers.

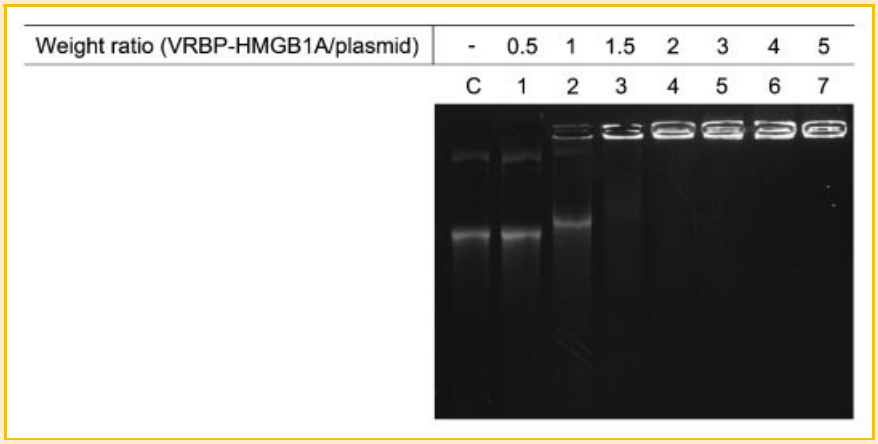

Fig. 3. Gel retardation assay VRBP-HMGB1A/pCMV-Luc complex was analyzed using 1% agarose gel electrophoresis. The complete retardation of pCMV-Luc was achieved at and above a 2/1 weight ratio of VRBP-HMGB1A and plasmid.

PHYSICAL CHARACTERIZATION OF VRBP-HMGB1A/PLASMID COMPLEX

A gel retardation assay was performed to confirm the complex formation of the purified VRBP-HMGB1A with plasmid. In the gel retardation assay, a fixed amount of pCMV-Luc was mixed with increasing amount of VRBP-HMGB1A. As negative charges of pCMV-Luc were neutralized by positive charges of VRBP-HMGB1A in VRBP-HMGB1A/plasmid complex, the electrophoretic mobility of pCMV-Luc in a gel was changed by the addition of VRBP-HMGB1A. As a result, the mobility of pCMV-Luc decreased as the amount of VRBP-HMGB1A increased (Fig. 3). The plasmid band was completely retarded at or above a 2/1 weight ratio (peptide/plasmid), suggesting complex formation between pCMV-Luc and VRBP-HMGB1A (Fig. 3).

The particle size of the VRBP-HMGB1A/plasmid complex was measured by dynamic light scattering at various ratios. The particle size ranged from 205 to 442.6 nm and decreased with increasing amount of VRBP-HMGB1A (Fig. 4A). Using the same instrument, the zeta potential of the complex was determined. The surface charge of the VRBP-HMGB1A/pCMV-Luc complex was positive at all the tested ratios (Fig. 4B).

IN VITRO TRANSFECTION

The transfection efficiency of VRBP-HMGB1A into hypoxic endothelial cells was analyzed by in vitro transfection assay. CPAE cells were incubated under hypoxia for 24 h before transfection to induce the VEGF receptor. The VRBP-HMGB1A/pCMV-Luc complexes were prepared at various weight ratios. The complexes were added to the cells and the cells were incubated under hypoxia for an additional 24 h. As a result, VRBP-HMGB1A showed the highest transgene expression at a 30/1 weight ratio (peptide/plasmid; Fig. 5).

To determine the specificity of VRBP-HMGB1A for hypoxic endothelial cells, transfection assays into normoxic or hypoxic CPAE cells were performed using VRBP-HMGB1A and PLL. VRBP-HMGB1A had about 7.9 times higher transfection efficiency than PLL in hypoxic CPAE cells (Fig. 6A). However, the transfection efficiency of VRBP-HMGB1A to normoxic CPAE cells was lower

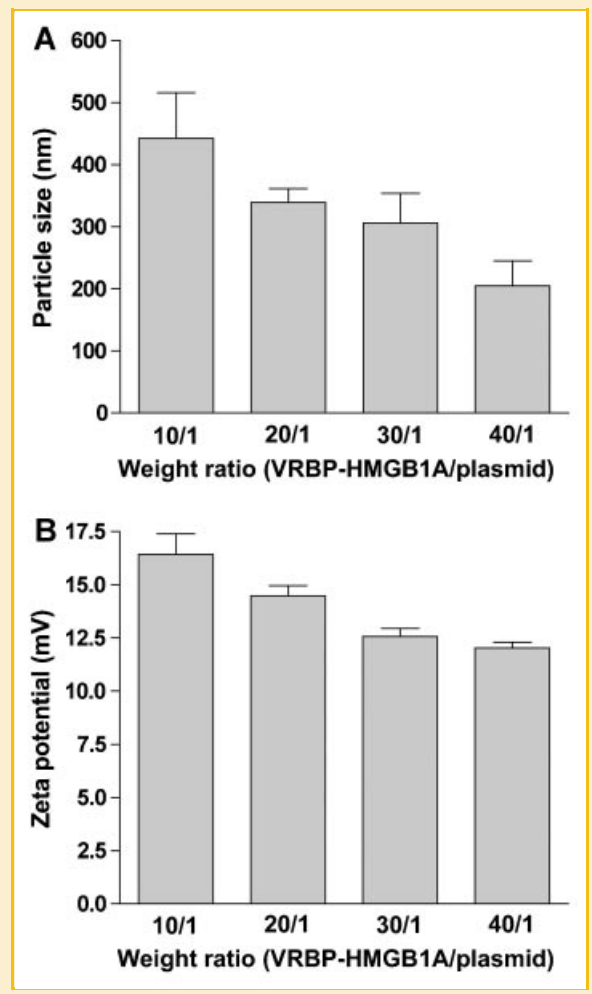

Fig. 4. A: Particle size and (B) zeta potential of VRBP-HMGB1A/plasmid complex. Particle size and zeta potential of VRBP-HMGB1A/pCMV-Luc complexes were measured at various weight ratios. The data are expressed as mean values (± standard deviation) of quadruplicate experiments.

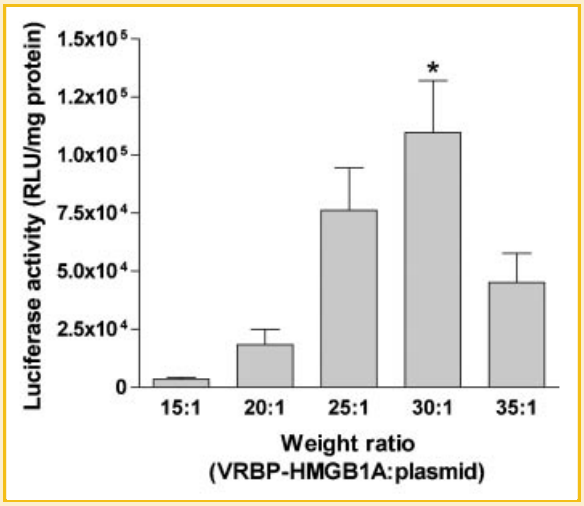

Fig. 5. Transfection efficiency VRBP-HMGB1A depending on weight ratio. VRBP-HMGB1A/plasmid complexes were prepared at various weight ratios and transfected to hypoxic CPAE cells. Transfection efficiency was measured by Luciferase assay. The data are expressed as mean values (± standard deviation) of quadruplicate experiments. $^{*}P < 0.05$ as compared with 15:1, 20:1, and 35:1, but no statistical significance as compared with 25:1.

than that of PLL (Fig. 6A). In addition, transfection assays showed that VRBP-HMGB1A had lower transfection efficiency than PLL in 293 and H9C2 cells regardless of oxygen concentration (Fig. 6B,C). Taken together, these results demonstrate that VRBP-HMGB1A has higher transfection efficiency than PLL, specifically in hypoxic endothelial cells.

CYTOTOXICITY OF VRBP-HMGB1A

To examine the cytotoxicity of VRBP-HMGB1A, an MTT assay was performed. VRBP-HMGB1A/pCMV-Luc or PLL/pCMV-Luc complex was transfected into the cells. After 24 h, the cell viability was measured by MTT assay. The results show that the VRBP-HMGB1A/pCMV-Luc complex is less toxic to CPAE cells than the PLL/pDNA complex (Fig. 7).

DISCUSSION

Targeting the delivery of therapeutic DNA to hypoxic endothelial cells is useful for the development of safe and efficient cancer gene therapy. VRBP was previously reported to bind specifically to Flt-1 (VEGF receptor 2) [El-Mousawi et al., 2003]. In this research, we linked VRBP to HMGB-1A to produce a gene carrier with a targeting ligand to hypoxic endothelial cells. We then demonstrated the delivery of plasmid to hypoxic endothelial cells using the VRBP-HMGB1A.

VRBP-HMGB1A is composed of three parts, VRBP, HMGB-1A, and a 6× his-tag (Fig. 1A). The DNA binding part is HMGB-1A. HMGB-1A has 18 positive and 10 negative amino acids. The net charge of HMGB-1A is +8. Compared with PLL of a similar molecular weight, the charge density of HMGB-1A was much lower than that of PLL. This low charge density may be the main cause for

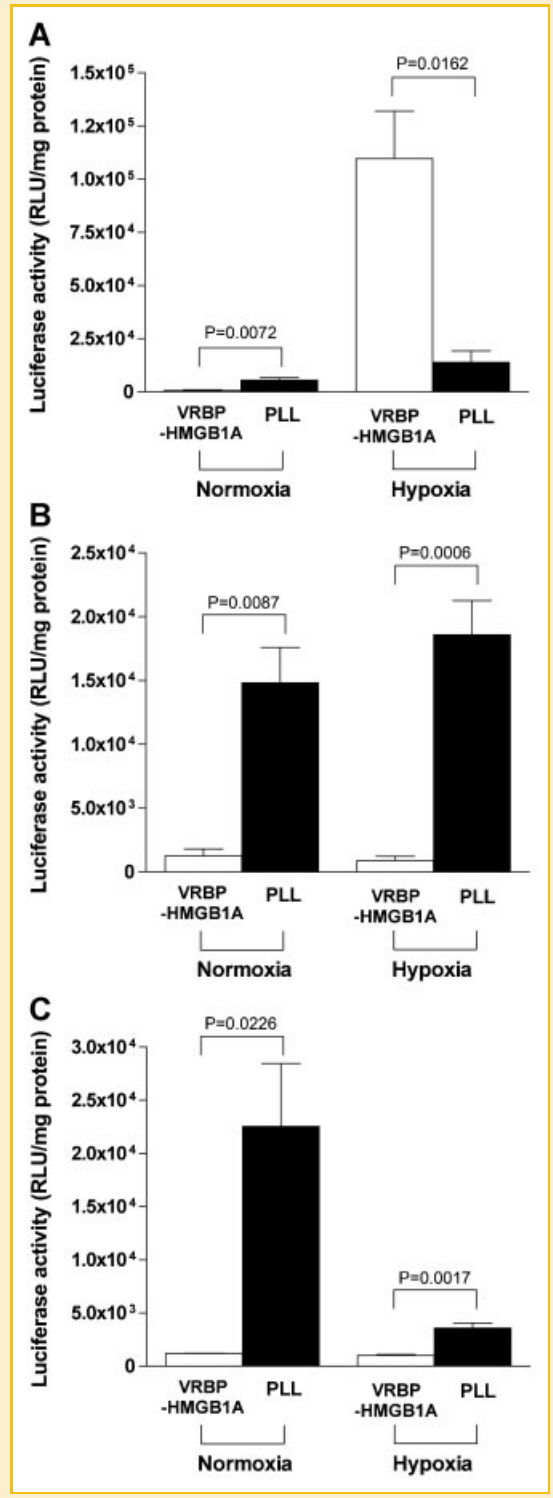

Fig. 6. Transfection efficiency of VRBP-HMGB1 into various types of cells. VRBP-HMGB1A/plasmid complexes were prepared at a 30/1 weight ratio and transfected into normoxic or hypoxic CPAE (A), 293 (B), and H9C2 (C) cells. PLL/plasmid complex was transfected into the cells as a control. The transfection efficiency was measured by luciferase assay. The data are expressed as mean values (± standard deviation) of quadruplicate experiments.

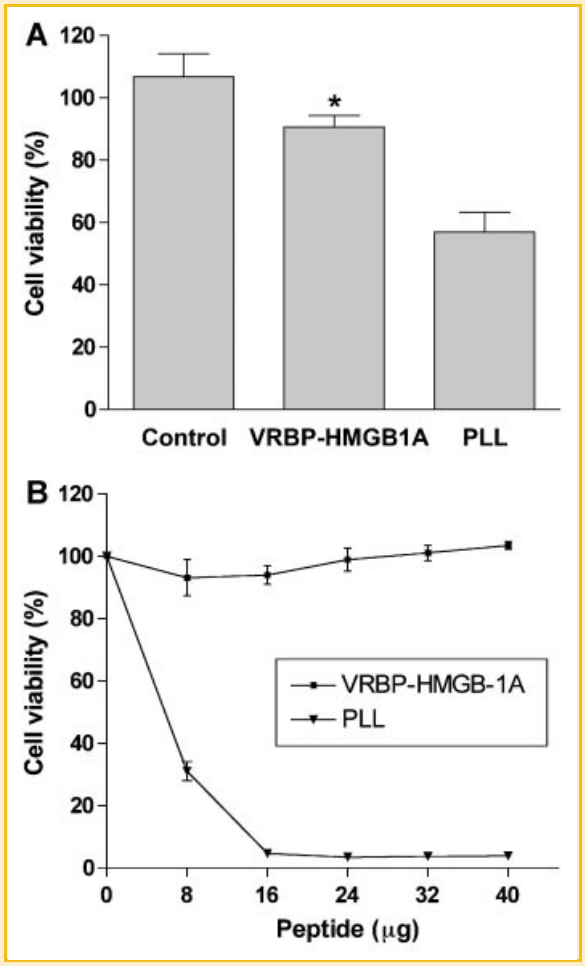

Fig. 7. Cytotoxicity of VRBP-HMGB1A and PLL. A: Cytotoxicity of VRBP-HMGB1A/plasmid complex. VRBP-HMGB1A/pCMV-Luc or PLL/pCMV-Luc was transfected into CPAE cells. After transfection, the cell viability was measured by MTT assay. The data are expressed as mean values (± standard deviation) of quadruplicate experiments. $^{*}P < 0.01$ as compared with PLL, but no statistical significance as compared with control. B: Cytotoxicity of free VRBP-HMGB1A. Various amounts of free VRBP-HMGB1A and PLL were added to CPAE cells. After the addition, the cell viability was measured by MTT assay. The data are expressed as mean values (± standard deviation) of quadruplicate experiments.

the low toxicity of HMGB-1A. A previous report showed that the high charge density of a cationic gene carrier might induce cellular toxicity [Benns et al., 2001]. For example, it was shown that polyethylenimine (PEI), a cationic gene carrier with a high charge density, aggregates on the surface of the cellular membrane and induces rupture of the membrane [Fischer et al., 1999]. Therefore, the low charge density of HMGB-1A may help to reduce cytotoxicity. Although HMGB-1A has a low charge density, it stably binds to plasmid [Kim et al., 2008a]. A previous report showed that the interaction between the HMG box and double-stranded DNA was facilitated by hydrophobic as well as charge interactions [Stott et al., 2006]. HMGB-1A has several hydrophobic amino acids and the hydrophobic amino acids interact with a hydrophobic base stack of double-stranded DNA. Therefore, the hydrophobic interaction may further stabilize the HMGB-1A/plasmid complex, compared with a simple cationic peptide/DNA complex.

VRBP is located at the N-terminal region of the HMGB-1A peptide. VRBP increases the transfection efficiency with hypoxic endothelial cells (Fig. 4). At the other end of the HMGB-1A peptide, a 6× his-tag is attached. The 6× his-tag was not eliminated after purification by nickel-chelate affinity chromatography, as the 6× his stretch may help to increase transfection efficiency, due to its proton buffering effect. The imidazole group of histidine has a pK_a of 6.0 and is protonated under the acid conditions of the endosome. Poly-histidine or histidine-conjugated polymer has a proton buffering effect in the endosome, which facilitates endosomal escape [Benns et al., 2000; Fajac et al., 2000].

VEGF receptor binding peptides (VRBPs) were previously screened by El-Mousawi et al.[2003. In the present study, the VRBP cDNA was linked to the HMGB-1A cDNA. The fusion protein of VRBP and HMGB-1A specifically delivered DNA to hypoxic endothelial cells, suggesting that VRBP of the fusion protein binds to the VEGF receptor. In addition, the binding of VRBP to the VEGF receptor might interfere with normal binding of VEGF to the receptors, and thus have an anti-angiogenic effect [El-Mousawi et al., 2003]. Therefore, it will be interesting to determine whether VRBP-HMGB1A, like VRBP, has an inhibitory effect on the VEGF receptor.

HMGB-1A is a domain of an endogenous nuclear protein. Modification of HMGB-1A with a targeting ligand, VRBP, increases transfection efficiency specifically when targeting hypoxic endothelial cells. With its non-toxicity and specificity, VRBP-HMGB1A may be useful in developing a clinically applicable non-toxic gene carrier to target hypoxic endothelial cells.

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