Biotechnology and Bioprocess Engineering 17: 160-167 (2012)
DOI 10.1007/s12257-011-0423-x

RESEARCH PAPER

Selective Gene Delivery to Cancer Cells Secreting Matrix Metalloproteinases Using a Gelatin/Polyethylenimine/DNA Complex

Jiang Ge, Sang-Hyun Min, Dong Min Kim, Dong Chul Lee, Kyung Chan Park, and Young Il Yeom

Received: 1 September 2011 / Revised: 1 November 2011 / Accepted: 2 November 2011

Abstract We developed a gene delivery strategy targeting metastatic tumors by exploiting the specific matrix metalloproteinases (MMPs) secreting properties of metastatic tumor cells. A ternary polyplex has been formed by coating polyethylenimine/DNA (PD) complex with an excessive amount of negatively charged gelatin B (GPD-B). We show that GPD-B's gene delivery activity could be targeted to cancer cells *via* the MMP-mediated proteolytic process, while GPD-A, made from positively charged gelatin A, was not successful in exhibiting such activity. The 1,10-Phenanthroline, an MMP2 inhibitor, abrogated the MMP-dependent transfection activity of GPD-B. GPD-B carried much less positive surface charges than PD, and thus exhibited significantly reduced interactions with erythrocytes. However, MMP2 elevated the positiveness in GPD-B's surface charge and, thus, its interaction with erythrocytes. These results suggest that the anionic gelatin coating may confer improved stabilities on GPD-B in the surrounding medium, while MMP2-mediated disintegration of the gelatin coat enhances the gene delivery to metastatic cancer cells *via* increasing the likelihood of local charge-mediated interactions between the polyplex and cancer cell membrane.

Keywords: gelatin, MMP, metastatic tumor, targeting, polyethylenimine (PEI)

Jiang Ge, Sang-Hyun Min, Dong Min Kim, Dong Chul Lee, Kyung Chan Park, Young Il Yeom*
Medical Genomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea
Tel: +82-42-860-4109; Fax: +82-42-879-8119
E-mail: yeomyi@kribb.re.kr

Jiang Ge
Bioengineering College, Dalian University, Dalian 116-622, China

1. Introduction

Efficient and specific delivery of therapeutic genes to target cells is a fundamental requirement in gene therapy. Although a variety of viral and non-viral gene delivery vectors have been reported so far, the majority of them lack either target cell specificity and/or suffer from a poor gene transfer efficiency *in vivo* [1-4]. Recently, many efforts have been focused on the targeting of gene delivery using the elements that either interact with specific cell-surface proteins or are specifically cleaved by enzymes expressed by target cells [5-10].

The cationic polymer, polyethylenimine (PEI) has been shown to mediate an efficient gene transfer to a variety of cell types, even without the addition of any cell-binding ligands or endosomolytic moiety [1,11]. In addition to its DNA condensing property, PEI possesses a very effective endosomolytic activity due to its strong buffering capacity at the acidic endosomal pH [1]. These properties have drawn the attention of several researchers, who have considered PEI as an attractive option for use as a DNA-binding core in designing more advanced vectors that contain receptor-binding domains or other molecules that can facilitate the cellular entry process of polyplexes [10-12].

Matrix metalloproteinases (MMPs) belong to a family of structurally related endopeptidases that mediate the degradation of different protein components of extracellular matrix and basement membrane, and play an important role in tumor invasion and angiogenesis [13]. MMP levels are often found to be elevated in invasive or metastatic tumors [14,15]. Therefore, there has been considerable interest in exploiting MMPs as the targets for novel therapeutic agents [5,6].

Previously, it was reported that active gelatinase A

(MMP2) destroys collagens including, types I-XI, elastin, and gelatin [16]. Gelatin is a denatured, biodegradable protein obtained by acid or alkaline hydrolysis of collagen, and can exist either as a positively (gelatin A) or negatively (gelatin B) charged form depending on the method of collagen hydrolysis. In the past, gelatins have been used in designing of different gene delivery systems [17-20]. We reasoned that coating a positively charged DNA-carrier complex with the negatively charged gelatin would reduce the nonspecific interaction of the complex with serum components, while its degradation by MMPs nearby the metastatic tumor cells would allow the positively charged core part to be exposed for interaction with cell membrane, thereby providing a tool for tumor-targeted gene delivery. In the present work, we have formulated ternary gene delivery complexes containing DNA, PEI, and gelatin, and further evaluated their feasibility as a tumor-specific gene delivery system by examining their transfection property and dynamic behaviors in the presence or absence of MMP2.

2. Materials and Methods

2.1. Materials

Gelatin types A and B, tris (hydroxymethyl) aminomethane, boric acid, ethylenediaminetetraacetic acid (EDTA), and ethidium bromide were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) and trypsin-EDTA (0.25%) were purchased from Hyclone (Logan, UT, USA). Iscove's modified Dulbecco's medium (IMDM) and Dulbecco's phosphate-buffered saline (PBS) were purchased from GIBCO BRL (NY, USA) and polyethylenimine (branched, M_r ~25,000) was from Aldrich (Milwaukee, WI, USA). Luciferase assay reagent was obtained from Promega (Madison, WI, USA). The reporter plasmids were pCMV-LUC and pEGFP-N1 (Clontech, CA, USA).

2.2. Cell culture

The C3 tumor cell line, derived from murine fibroblasts of C57BL/6 by HPV infection [21], was grown in IMDM supplemented with 8% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 55 mM 2-mercaptoethanol. The cervical cancer cell line HeLa was purchased from ATCC (Manassas, Va, USA) and grown in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. All the cells were maintained at 37°C in a humidified, 5% CO_2 incubator.

2.3. Preparation of plasmid and DNA-carrier complexes

One microgram of plasmid DNA was mixed with 1.8 μL of 20 mM PEI (N/P ratio of 12) and incubated for 10 min in a 1.5 mL microcentrifuge tube at room temperature. To prepare the PD complex, an appropriate amount of distilled water and 3 μL of NaCl were added to the DNA/PEI mixture to bring the volume of the final complex to 100 μL. In the case of the GPD polyplex, preincubated DNA and PEI mixture were added with various amounts of gelatin, and the polyplex was incubated for another 10 min at room temperature. The weight ratio of gelatin to DNA ranged from 0.018 to 18.

2.4. Measurement of particle size and zeta potential of DNA-carrier complexes

The surface charge of DNA-carrier complexes was determined by electrophoretic light scattering (ELS-8000; Otsuka Electronics, Japan) measurement. DNA-carrier complexes were prepared at a DNA concentration of 10 μg/mL in 500 μL of saline (150 mM NaCl in water), and every individual sample was measured in duplicates for 2 min at room temperature.

2.5. *In vitro* transfection

Typically, C3 cells (3×10^4) and HeLa cells (5×10^4) were seeded in each well of 24-well plates at 70 to 80% confluence 24 h prior to transfection. Immediately before transfection, cells were replaced with 0.4 mL of fresh medium with MMP2 (10 μg/mL) or 1,10-phenanthroline (PH; 10 μg/mL), and then each well received 100 μL of freshly prepared PD or GPD polyplex containing 1 μg of DNA. After 6 h, the medium was refreshed with complete media, and the cells were incubated for 24 h at 37°C in 5% CO_2. Finally, the cells were washed twice with PBS, harvested by trypsinization, resuspended in 250 mM Tris-HCl (pH 7.8)/1 mM DTT, and lysed by means of three freeze-thaw cycles. The lysates were centrifuged at 12,000 rpm for 20 min at 4°C to pellet debris, and the luciferase activity in the supernatant was measured using a luminometer (Biolumat LB 9500; Berthold, Germany). For this, an aliquot of the supernatant (20 μL out of total 40 μL) was mixed with 350 μL of 25 mM glycylglycine (pH 7.8)/2 mM ATP/10 mM $MgSO_4$, and the bioluminescence was measured for 30 sec in an integration mode after the automatic injection of 100 mL of D-luciferin substrate (0.03% in 25 mM glycylglycine, pH 7.8).

2.6. Gelatin zymography

The MMP activity was characterized using zymography [22]. Conditioned media normalized for cell numbers were concentrated using a Centricon apparatus (Millipore, USA). A 15-μL aliquot of each sample was mixed with an equal volume of Laemmli's 2 × sodium dodecyl sulfate (SDS) sample buffer in the absence of a reducing agent and incubated for 30 min at room temperature. The samples

were electrophoresed on 10% polyacrylamide gels copolymerized with 1mg/ml gelatin (Invitrogen, CA, USA). After electrophoresis, the gels were soaked in zymogram renaturing buffer containing 2.5% Triton X-100 for 30 min to remove SDS, and then incubated at 37°C overnight in zymogram developing buffer. Pre-stained molecular mass standards in the range of 250 ~ 4 kDa were used for reference. After incubation, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 and destained with Gel-Clear destaining solution (Invitrogen). Gelatinolytic activities were detected as transparent bands against a background of gelatin stained with Coomassie Brilliant Blue. Conditioned media were obtained from the 24-h cultures of HeLa cells in serum-free DMEM and of C3 cells in serum-free IMDM. Positive control for MMP was used as previously reported [22].

2.7. Erythrocytes aggregation assay

Fresh blood was collected from C57/BL6 mice and immediately mixed with sodium citrate to a final concentration of 25 mM. Erythrocytes were washed thrice on ice with cold Ringer solution. Two hundred microliters of washed erythrocyte suspension was mixed with PD or GPD-B polyplex with or without MMP2 treatment (10 μg/mL; 6 h) and incubated for 1 h at 37°C in a 24-well plate (Nunc, Rochester, NY, USA).

3. Results and Discussion

We intended to formulate a non-viral gene delivery complex carrying a targeting capability for metastatic tumors as well as the stability in the surrounding medium. We searched for an agent that, when added to the positively charged core complex, can increase its environmental stability by neutralizing the surface charge, while undergoing disintegration from the core complex near the target tumor cells. Gelatins, the degradation products of collagen, were chosen for this purpose as they can be further digested by the metastatic tumor-associated enzymes, MMPs (MT1-MMP, MMP-2, and MMP-9), and exist either in positively charged (gelatin A) or negatively charged (gelatin B) forms depending on the method of collagen hydrolysis [17,20].

To follow our hypothesis, we initially examined whether electrically neutral DNA-carrier complexes could be formulated by coating a positively charged DNA-PEI (PD) complex with gelatins. For this, we primarily prepared a positively charged PD complex by allowing the self-assembly of the two macromolecules at an N/P ratio of 12, and then coated it with either positively charged gelatin A or negatively charged gelatin B. Measurement of the surface charge of the complex by using an electrophoretic light scattering analyzer showed that the PD complex had a zeta potential of +18.3 mV (Fig. 1). Addition of increasing amount of gelatin A to the PD complex led to a slight increase in the surface charge of the resulting gelatin/PEI/DNA complex (GPD-A). The ternary complex appeared to have reached the maximum possible positiviness in the surface charge when 0.18 μg of gelatin A was added. On the other hand, the surface charge of the ternary complex formed by coating of PD with gelatin B (GPD-B) decreased gradually by the addition of increasing amount of gelatin, reaching a value of +5.25 mV when 18 μg of gelatin B was used (Fig. 1).

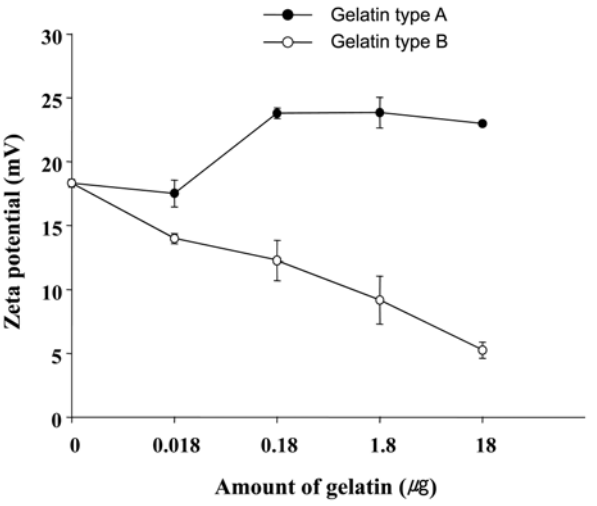

Fig. 1. Zeta potential of PEI/DNA (PD) complexes coated with different amounts of gelatin A or B. The PD complex containing 5 ìg of luciferase reporter DNA and 9 μL of 20 mM PEI (N/P = 12) was mixed with the indicated amounts of gelatin A or B, and the zeta potential of the resulting complexes was measured by dynamic light scattering at room temperature (mean ± SD, n = 2).

Subsequently, the gene transfer behavior of GPD-A and -B towards the cells expressing different levels of MMP2 was examined. The level of MMP2 protein expressed by tumor cells was estimated in the culture supernatant by gelatin zymography. As shown in Fig. 2A, C3 cells expressed MMP2 at a relatively high level, while HeLa cells expressed the same at a very low level. We compared the effects of different gelatin coatings on the gene transfer behavior of PD complex (N/P = 12) in these two cell lines. The transfection efficiency of GPD-A complexes was not significantly altered by the gelatin A coating in both C3 and HeLa cells when compared with that of uncoated PD complex (Fig. 2B). In contrast, the transfection efficiency of GPD-B gradually decreased with increasing amount of gelatin B in HeLa cells, while it remained virtually unchanged in C3 cells (Fig. 2C). The transfection efficiency of the GPD-B complex coated with 18 μg of gelatin B was reduced by 5 ~ 10 folds when compared to that of PD

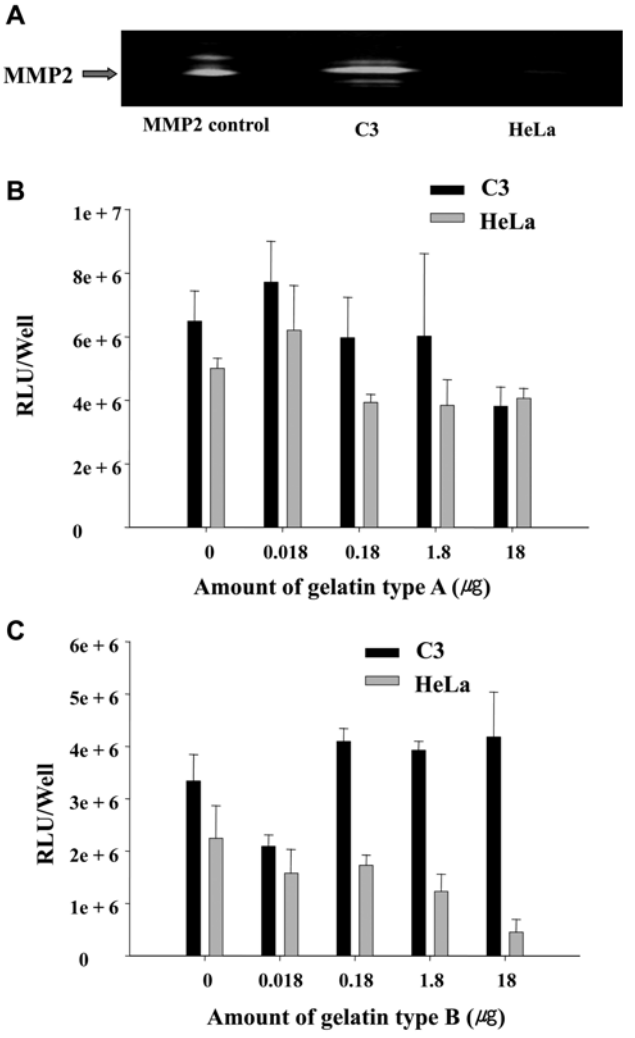

Fig. 2. The effect of gelatin coating on the transfection efficiency of PEI/DNA complex in C3 and HeLa cells. (A) The amount of MMP2 secreted into the culture medium of C3 and HeLa cells was determined by gelatin/plasminogen zymography. (B ~ C) The PD complex containing 1 μg of luciferase reporter DNA and 1.8 ìL of 20 mM PEI (N/P = 12) was mixed with the indicated amounts of gelatin A (B) or B (C), and the mixtures were transfected into C3 and HeLa cells, as described in Materials and Methods. The cells were subsequently incubated for 48 h, and the luciferase activity was measured in the cell lysates (mean ± SD, n = 3).

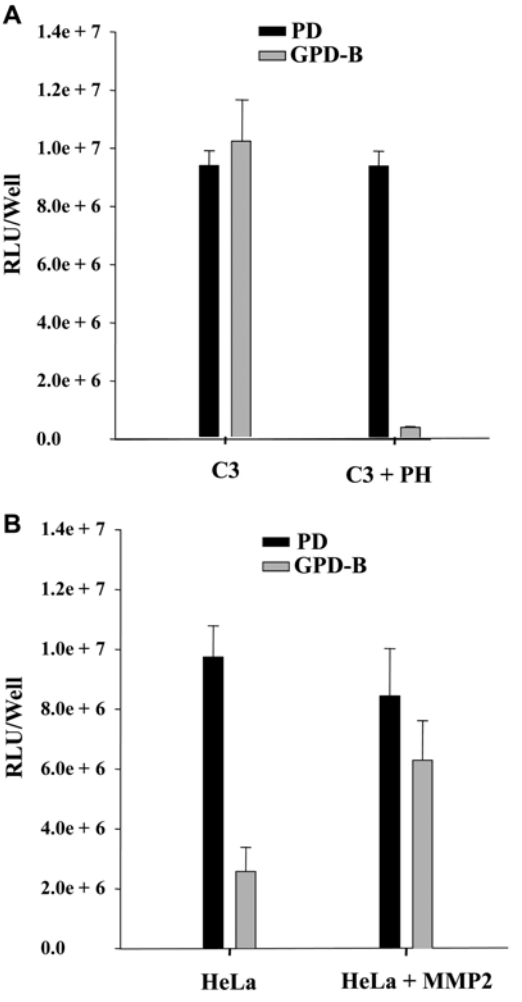

Fig. 3. The effect of MMP2 on the transfection efficiency of GPD-B complexes. Transfection efficiencies were determined in C3 cells with or without PH treatment (A), or in HeLa cells with or without MMP2 treatment (B). The cells were treated with PH and MMP2 immediately before transfection and subsequently transfected with PD (PEI:DNA = 12:1, N/P ratio) or GPD-B (gelatin B:DNA = 18:1, w/w ratio) polyplex. After 6 h, the medium was replaced with fresh one, and the cells were additionally incubated for another 24 h.

complex in HeLa cells (Fig. 2C). Thus, it appears that the transfection efficiency of GPD-B but not of GPD-A is positively related to the cellular expression level of MMP. These results suggest the possibility that the transfection capacity of GPD-B complex was disrupted by the negatively charged gelatin B coating in the MMP-negative HeLa cells but could be re-activated in response to MMP expression in C3 cells.

We further characterized GPD-B complex for the MMP dependence of its gene delivery behavior by modulating the MMP2 activity in C3 and HeLa cells. As shown in Fig. 3A, the transfection efficiency of GPD-B complex was similar to that of PD complex in C3 cells, but decreased dramatically upon treatment with the MMP2 inhibitor, 1,10-phenanthroline (PH). On the other hand, the transfection efficiency of GPD-B was much lower than that of PD in HeLa cells, but restored significantly upon MMP2 treatment (Fig. 3B). These data indicate that the inactive GPD-B was activated in HeLa cells by the addition of exogenous MMP2 protein, whereas in C3 cells it remained inactive by the addition of an MMP2 inhibitor.

Fig. 4 additionally shows the MMP dependence of the transfection of GPD-B complex through microscopic examination. Cells were transfected with PD or GPD-B com-

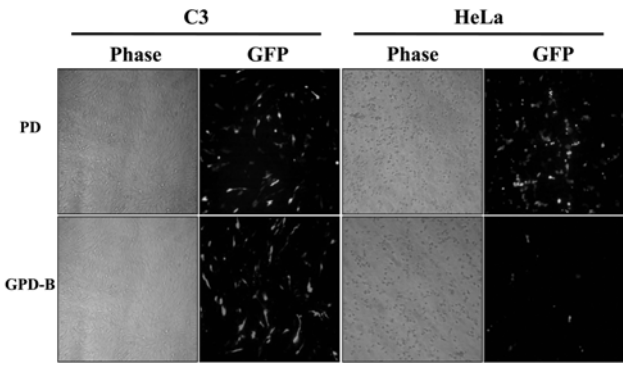

Fig. 4. Microscopic comparison of the transfection efficiency of PD and GPD-B complexes in C3 (left) and HeLa cells (right). The transfection of PD (12:1, N/P) or GPD-B (gelatin B:DNA = 18:1, w/w ratio) complex containing 1 μg of GFP reporter DNA was carried out as described in Materials and Methods. Twenty-four hours after the transfection, cells were imaged by phase-contrast and fluorescence microscopy.

plex carrying the pEGFP-N1 plasmid DNA encoding green fluorescence protein (GFP) and microscopically examined for GFP expression. It was observed that PD complex mediated similar levels of GFP expression in both HeLa and C3 cells. However, transfection with GPD-B complex resulted in much higher frequency of GFP expression in C3 cells than in HeLa cells. This result is consistent with the above finding that the transfection by GPD-B complex occurs in an MMP2-dependent manner.

The above results suggest that the MMP2-dependency of GPD-B-mediated transfection might be attributed to the changes in the surface charge of GPD-B complex, caused by the exposure of core positive charge through the degradation of gelatin coating by MMP. Thus, we measured the zeta potential of GPD-B in the presence or absence of MMP2 *via* electrophoretic light scattering analyses. As shown in Fig. 5A, the zeta potential of the MMP2-treated GPD-B complex was 2 ~ 3 mV higher than that of untreated GPD complex. The changes in the surface charge of GPD complex are shown in more detail in the zeta-potential distribution map (Fig. 5B). After treatment with MMP2 (10 μg/mL) for 6 h, the main zeta-potential distribution peak of MMP2-treated GPD-B shifted towards the positive direction (from 5.08 to 8.48 mV). In contrast,

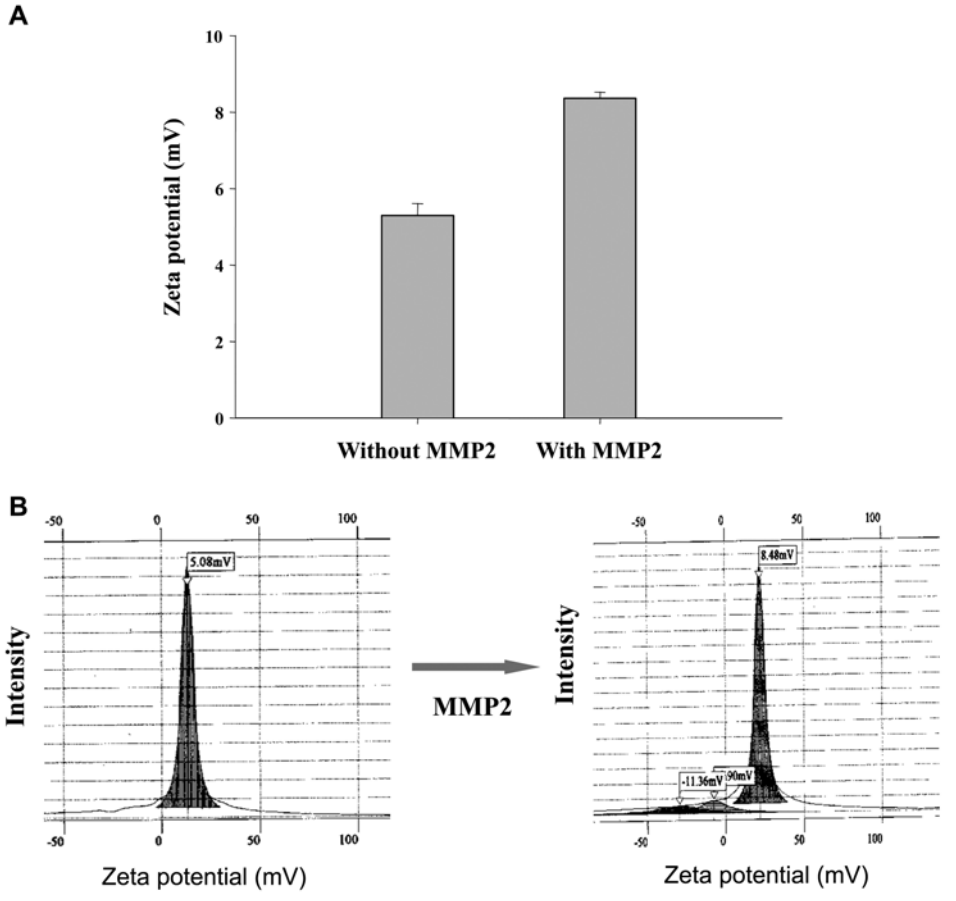

Fig. 5. Changes in the surface charge of GPD-B complex caused by MMP2 treatment. GPD-B polyplex (PEI:DNA = 12:1, N/P ratio; gelatin B:DNA = 18:1, w/w ratio) was treated with MMP2 at 37°C for 6 h, and the surface charge of the complex was measured as described in Materials and Methods. (A) Histogram of surface charge (mean ± S.D., n = 3). (B) Zeta-potential distribution map.

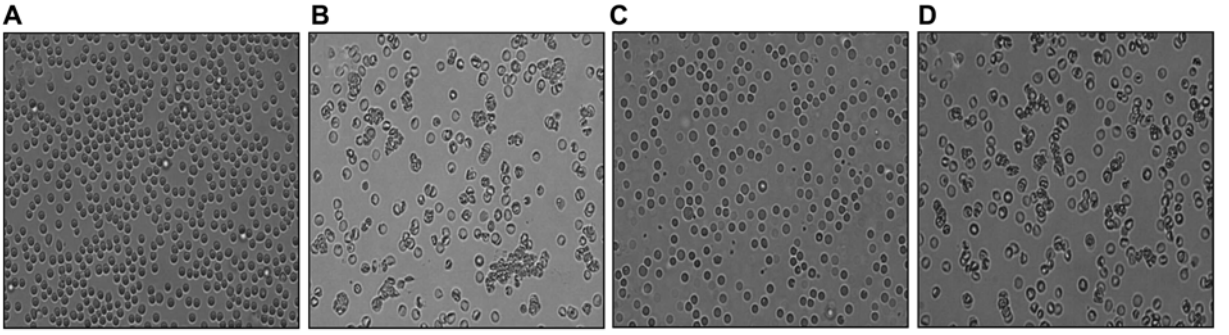

Fig. 6. The effect of MMP2 on the interaction of the GPD-B complex with erythrocytes. Erythrocytes prepared from C57/BL6 mice were mixed with the respective polyplexes, incubated for 1 h at room temperature, and observed under a microscope. (A) Untreated erythrocytes as control; (B ~ D) erythrocytes treated with PD (PEI:DNA = 12:1, N/P ratio) (B), GPD-B (gelatin B:DNA = 18:1, w/w ratio) (C), or MMP2-treated GPD-B complexes (D).

several lower peaks shifted towards the opposite direction. These may be the peaks formed by the products obtained after gelatin degradation by MMP2. These results indicate that MMP2 led to degradation of the gelatin coating from GPD-B, thus exposing the positive charge of the core complex for interaction with the negatively charged components on target cell membrane.

Shielding the positive surface charge of polyplexes has recently been shown to reduce the aggregation of polyplexes caused by non-specific interactions with plasma components and erythrocytes [12,23-25]. We performed an erythrocyte aggregation assay in order to investigate the interaction of the GPD-B polyplex with erythrocytes [12]. Fresh mouse erythrocytes were incubated with PD, GPD-B, or GPD-B that has been treated with MMP2. It was observed that PD induced an extensive aggregation of

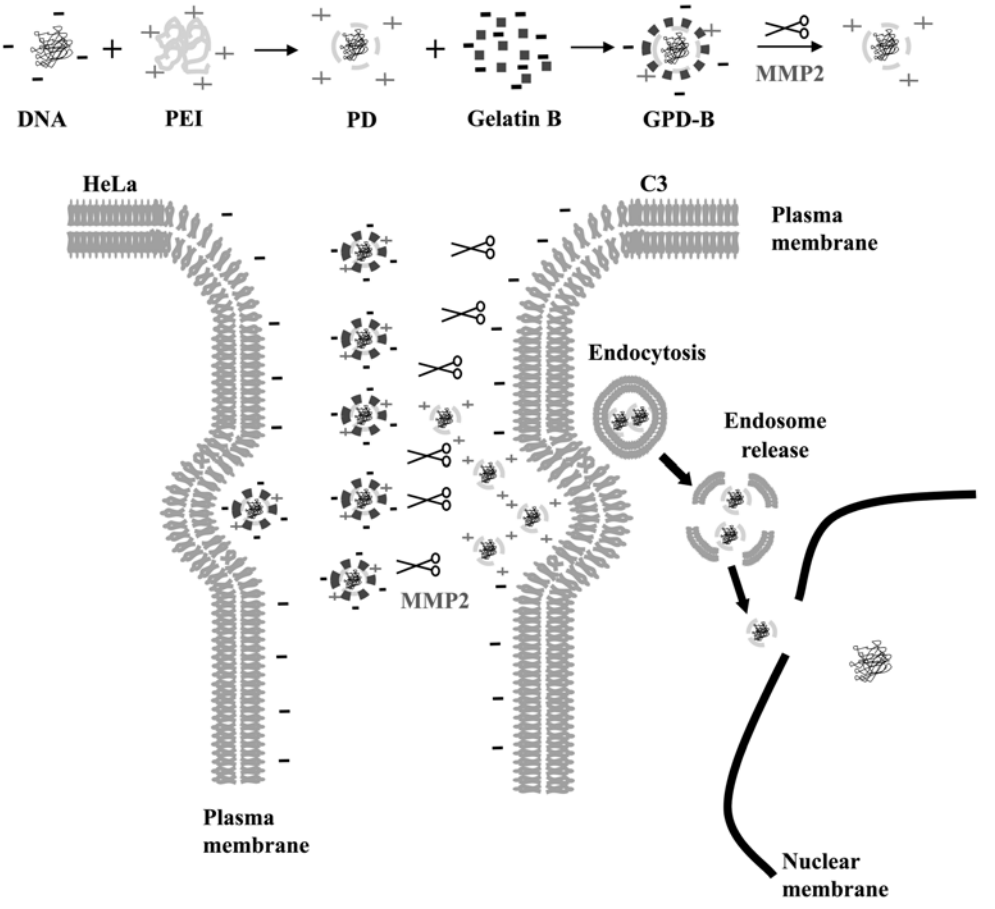

Fig. 7. Suggested scheme of the gene delivery process involving the GPD-B polyplexes.

erythrocytes (compare Figs. 6B with 6A), while GPD-B (gelatin/DNA (w/w) = 18) induced only a minimal level of aggregation (Fig. 6C). However, GPD-B induced a significant level of aggregation in the presence of MMP2 (Fig. 6D). These data indicate that the increase in the surface positive charge of GPD-B complex, caused by MMP2-mediated degradation of gelatin coating, enhanced the interaction between polyplex and the anionic cell membrane of erythrocytes.

In this study, we prepared a ternary polyplex consisting of a positively charged PD (PEI-DNA) complex coated with cationic or anionic gelatin. We confirmed that the anionic gelatin B coating could decrease the surface charge of the complex and mediate a specific transfection into the MMP-secreting C3 cells. However, the cationic gelatin A-coated GPD failed to show the metastatic tumor-specific gene delivery. From these results, it was inferred that the selective transfection of MMP-secreting tumor cells by GPD-B was due to changes in the surface charge of the complex caused by the removal of anionic gelatin coat *via* MMP-mediated proteolytic degradation (Fig. 7). This hypothesis was supported by the differences in the zeta potential of GPD-B complex before and after the MMP2 treatment. The increased surface charge of the resultant GPD-B could enhance the interaction of the polyplex with anionic cellular membrane, as revealed by the erythrocyte aggregation assay. From the results, it was apparent that the GPD complex could be efficiently transfected into the MMP-positive cells, likely through the typical PEI-mediated gene delivery procedure.

The PEI is a stable cationic gene carrier, which has DNA-condensing property and a very effective endosomolytic activity due to its strong buffering capacity at the acidic endosomal pH [1]. These characteristics make it one of the most efficient non-viral vectors available till date [1,11,26]. However, the use of PEI-mediated gene delivery *in vivo* is severely limited due to lowering of transfection efficiency in the presence of serum and by the non-specific interaction with unwanted cells [23]. Cationic gene carriers, in general, are associated with these problems, thus undermining their efficacy and usage. Shielding of the positive charge of cationic gene carriers by introducing hydrophilic polymer or anionic polymers is known to provide a steric stabilization and reduction in the non-specificity of vectors [12,23,27-29]. In our experiment, coating of the PD complex with the negatively charged gelatin significantly lowered the positive surface charge and reduced non-specific interactions with the cell. Moreover, the gelatin coating enabled selective gene delivery to MMP-secreting cells. Since gelatin is a biodegradable, naturally occurring protein that is both inexpensive and simple to manipulate, formulation of the gelatin-based GPD type complexes can be an attractive approach for developing a gene delivery tool, targeting the metastatic tumor cells.

4. Conclusion

We described the formulation of a novel ternary gene delivery system formed by coating the PEI/DNA complex with an excessive amount of gelatin. The ternary complex made from negatively charged gelatin B coating showed an MMP-dependent activity profile, a decreased positive charge on its surface, and reduced erythrocyte aggregation, while the one made from positively charged gelatin A was not successful in exhibiting any such activity. Our results suggest that coating of the positively charged core complex (PEI/DNA) with negatively charged gelatin can improve the stability of the resulting complex in body fluid; while the MMP2-mediated disintegration of gelatin coating can expose the positive charge of the core complex to facilitate charge-mediated interactions between the polyplex and target cell membrane with further enhancement in the tumor specificity of gene delivery system.

Acknowledgement

This work was supported by the KRIBB Research Initiative Program and the Grant-in-Aid for the 21C Frontier Human Genome Functional Analysis Project from the Korea Ministry of Science and Technology.

Nomenclature

PEI : Polyethylenimine
MMP : Matrix metalloproteinase
GPD : Gelatin/polyethylenimine/DNA
GPD-B : GPD complex made from negatively charged gelatin B
GPD-A : GPD complex made from positively charged gelatin A

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