Biochemical and Biophysical Research Communications 447 (2014) 383–387

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Polyethyleneimine-coating enhances adenoviral transduction of mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 20 March 2014
Available online 12 April 2014

Keywords:
Mesenchymal stem cells
Adenovirus vectors
Polyethyleneimine
Gene transduction

ABSTRACT

Mesenchymal stem cells (MSCs) are non-hematopoietic cells with multi-lineage potential, which makes them attractive targets for regenerative medicine applications. Efficient gene transfer into MSCs is essential for basic research in developmental biology and for therapeutic applications involving gene-modification in regenerative medicine. Adenovirus vectors (Advs) can efficiently and transiently introduce an exogenous gene into many cell types via their primary receptors, the coxsackievirus and adenovirus receptors (CARs), but not into MSCs, which lack CAR expression. To overcome this problem, an Adv coated with cationic polymer polyethyleneimine (PEI) was developed. In this study, we demonstrated that PEI coating with an optimal ratio can enhance adenoviral transduction of MSCs without cytotoxicity. We also investigated the physicochemical properties and internalization mechanisms of the PEI-coated Adv. These results could help to evaluate the potentiality of the PEI-coated Adv as a prototype vector for efficient and safe transduction into MSCs.

1. Introduction

Mesenchymal stem cells (MSCs) have generated a great amount of enthusiasm over the past decade as a novel therapeutic paradigm for a variety of diseases [1]. The clinical potential of MSCs is mainly attributed to four important biological properties: the ability to home to sites of inflammation following tissue injury when injected intravenously; the ability to differentiate into various cell types; the ability to secrete multiple bioactive molecules capable of stimulating the recovery of injured cells and inhibiting inflammation and the ability to perform immunomodulatory functions. However, the therapeutic success of MSC therapy has been limited because of its low efficiency, and the genetic modification of MSCs might be a promising option for improving their therapeutic potential.

Adenovirus vectors (Adv) are among the most frequently used vectors in gene transduction because of many useful features, such as high transduction efficiency, the ease of producing high-titer stocks and the low risk of gene mutation [2,3]. Advs can efficiently and transiently introduce exogenous genes into many cell types via their primary receptors, the coxsackievirus and adenovirus receptors (CARs) [4]. However, gene transfer with Advs is less efficient in MSCs because of the scarcity of CARs on their cell surfaces. For this reason, the application of Advs as gene transfer vectors for MSC transduction has been limited.

Previously, we generated a cancer-specific gene therapy system using an Adv conjugated to polyethylene glycol [5–9]. Other groups have also reported that the physical coating or chemical conjugation of Advs as an alternative approach for adenoviral gene therapy. This gave us the idea to modify an Adv with other polymers to overcome the obstacle of MSC transduction [10–14]. In this study, we considered coating an Adv with the cationic polymer polyethyleneimine (PEI). PEI is one of the most widely used cationic polymers that can coat a gene-expressing plasmid

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http://dx.doi.org/10.1016/j.bbrc.2014.03.142
0006-291X/

directly and is often considered the gold standard of gene transfection [15]. We hypothesized that a PEI-coated Adv could bypass CAR-mediated endocytosis and preserve the properties of the Adv after entering into MSCs, thus yielding efficient nuclear translocation and high transgene expression.

In this study, we coated an Advs with different coating ratios of PEI and compared their transgene expression and cytotoxicity in MSCs. We also investigated their physicochemical properties, internalization mechanisms and antibody evasion ability. This is the first report to demonstrate the potential of PEI coating in Adv-mediated gene transfer into MSCs.

2. Materials and methods

2.1. Mice and cell lines

Three-week-old male SD rats (50–60 g) and five-week-old female Balb/c mice were used in this study. All of the animal experimental procedures were performed in accordance with the guidelines for the welfare of animals of Chinese Academy of Medical Sciences and Zhejiang University.

MSCs were isolated from the bone shaft of femurs of three-week-old male SD rats. Briefly, both ends of rat femurs were cut away from the epiphysis, and the bone marrow was flushed out using a syringe (21-gauge needle) with 1 ml of DMEM supplemented with 10% FBS, L-glutamine, penicillin (50 U/ml), and streptomycin (50 U/ml). The cell suspension was placed into two 25-cm^2 flasks and cultured at 37 °C in 5% CO_2. The medium was changed on day 4 of the culture and every 3 days thereafter. After sub-confluence was reached, the cells were detached from the flask using 0.25%trypsin/0.02%EDTA. Third or fourth-passage cells at sub-confluence were used for all experiments.

2.2. Vectors

The E1/E3-deleted Adv type 5 expressing firefly luciferase, which is under the control of the cytomegalovirus promoter, was constructed with an improved in vitro ligation method, as previously reported [16,5,17,18]. Adv was amplified in 293 cells using established methods [19] and was purified by cesium chloride step-gradient ultracentrifugation. The virus particles (vp) and biological titer were determined via spectrophotometry [20] and the Adeno-X Rapid Titer protocol (Clontech Laboratories, Mountain View, CA, USA), respectively. The ratio of the particle-to-biological titer was between 10 and 30.

2.3. Preparation of PEI-coated Adv

Polyethyleneimine (branched 25 kDa PEI, Sigma) stock concentrations of 4 mg/mL were used in the experiments. An Adv solution (2×10^9 vp/ml) was incubated with the PEI stock at different concentrations at room temperature for 15 min. Particle size and zeta potential were measured using a Malvern Zetasizer (Zetasizer 3000 176 HSa, Malvern). Morphology was observed under a transmission electron microscope (TEM, H-9500, Hitachi).

2.4. In vitro gene transduction

MSCs (2×10^4 cells/well) were seeded into 48-well plates. The following day, each well was treated with 2×10^8 vp (10^4 vp/cell) of luciferase-encoding Adv (approximately 300–10^3 MOI) or PEI-coated Adv. After 24 h, luciferase activity was determined using the luciferase assay system (Promega, Madison, WI, USA) in accordance with the manufacturers' instructions, and the amount of protein was measured with the BCA assay. Luciferase activity was calculated as relative light units (RLU)/mg protein.

2.5. Cytotoxicity

MSCs (2×10^4 cells/well) were seeded into 48-well plates. The following day, each well was treated with 2×10^8 vp (10^4 vp/cell) of luciferase-encoding Adv (approximately 300–10^3 MOI) or PEI-coated Adv. After 24 h, cell viability was determined using the MTT assay system (MTT, Sigma) in accordance with the general protocol.

2.6. Endocytosis-dependency of cellular uptake

MSCs (2×10^4 cells/well) were seeded into separate 48-well plates. The following day, the cells were pretreated with or without endocytosis inhibitor 2.5 mM amiloride HCl hydrate (Sigma–Aldrich, USA), 6.65 mg/mL methyl-β-cyclodextrin (MBCD) (Kaiyang Bio Co., Shanghai, China) or 10 μg/mL chlorpromazine (CPZ) (Kaiyang Bio Co., Shanghai, China) for 1 h. Each well was treated with 2×10^8 vp (10^4 vp/cell) of luciferase-encoding Adv or PEI-coated Adv. After 24 h of cultivation, luciferase activity was determined using the luciferase assay system.

2.7. Anti-adenovirus serum

Balb/c mice were intravenously injected with luciferase-encoding Adv (10^9 vp/mouse) or PBS. After 28-days, blood was collected, and serum was separated. MSCs (2×10^4 cells/well) were seeded into separate 48-well plates. The following day, the cells were pretreated with 10 μl serum from mice injected with PBS or 10 μl serum containing 15 μg/ml anti-adenovirus antibody from mice injected with Adv for 1 h. Each well was treated with 2×10^8 vp (10^4 vp/cell) of luciferase-encoding Adv or PEI-coated Adv. After 24 h of cultivation, luciferase activity was determined using the luciferase assay system.

2.8. Statistical analysis

All results were expressed as the mean ± standard deviation. Differences were compared using Student's *t*-test.

3. Results and discussion

3.1. Construction of PEI-coated Advs

At first, to find the optimal coating ratio, we constructed PEI-coated Advs using different concentrations of PEI stock solution. First, 2×10^9 vp/mL Adv was simply mixed with 2, 4, 8, 16, 32, 64 or 128 μg/mL PEI at room temperature for 15 min (we named the resulting Advs PEI-Adv2, PEI-Adv4, PEI-Adv8, PEI-Adv16, PEI-Adv32, PEI-Adv64 and PEI-Adv128, respectively). The molar ratios of PEI to Adv were 2.4×10^4, 4.8×10^4, 9.6×10^4, 1.9×10^5, 3.8×10^5, 7.7×10^5 and 1.5×10^5, respectively (Table 1). PEI, as a cationic polymer, was attracted to the surface of Adv, which is an anionic particle.

3.2. Transgene expression and cytotoxicity of PEI-coated Advs

Next, we compared the transgene expression caused by luciferase-encoding Adv and PEI-coated Advs with different PEI ratios in MSCs derived from the bone marrow of SD rats. Luciferase expression with PEI-Adv2 to PEI-Adv128 was 23, 19, 15, 16, 4, 1.4 and 0.0038-fold respectively, compared with that from uncoated Adv (Fig. 1). Luciferase expression with the PEI-coated Advs decreased

Table 1
Construction of PEI-coated Adv.

Name of PEI-coated Adv	Conc of Adv (vp/mL)	Conc of PEI (μg/mL)	Molar ratio (PEI/Adv)
PEI-Adv2	2×10^9	2	2.4×10^4
PEI-Adv4		4	4.8×10^4
PEI-Adv8		8	9.6×10^4
PEI-Adv16		16	1.9×10^5
PEI-Adv32		32	3.8×10^5
PEI-Adv64		64	7.7×10^5
PEI-Adv128		128	1.5×10^5

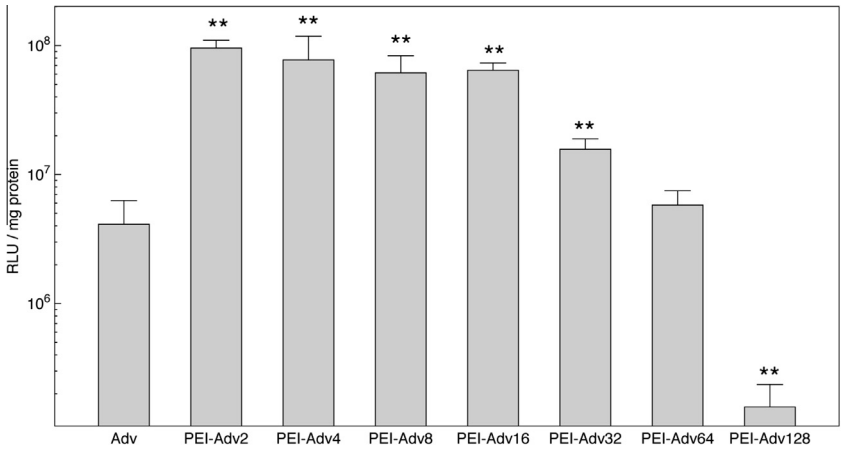

Fig. 1. In vitro transgene expression with PEI-coated Adv. MSCs (2×10^4 cells/well) were transduced with the indicated luciferase-encoding Adv or PEI-Advs. After culturing for 24 h, luciferase expression was measured ($n = 6$). All data are represented as the means ± SD ($^{**}P < 0.01$ compared with Adv group).

with increasing concentration of PEI, in a dose-dependent manner. At lower concentrations, PEI coating enhanced the expression of Adv. however, at concentration of 128 μg/mL, PEI coating significantly reduced the expression of Adv, which is perhaps due to the cytotoxicity of PEI at high concentration. Therefore, we next evaluated the cytotoxicity of Adv and PEI-coated Advs in MSCs compared with untreated cells. An MTT assay was performed 24 h after transduction of Adv or PEI-coated Advs into MSCs. In accordance with the result for transgene expression, the decreased viability of MSCs treated with PEI-Advs correlated with increasing concentrations of PEI (Fig. 2). For PEI-Adv8 and PEI-Advs with higher PEI concentrations, the viability of MSCs was lower than 95% and was significantly lower than that of untreated MSCs. The viability of MSCs treated with PEI-Adv128 even decreased to 82.56%, which could be one reason why luciferase expression with PEI-Adv128 was reduced significantly. Another reason for the low efficiency of luciferase expression with PEI-Adv128 might be the aggregation of PEI-coated Advs caused by an abundunce of cationic PEI. Finally, 2 μg/mL PEI was chosen as the optimal concentration for 2×10^9 vp/mL Adv (i.e., the molar ratio of PEI to Adv was 2.4×10^4) because this facilitated the highest transgene expression and lowest cytotoxicity observed with PEI coating. PEI-Adv2 was used in the following studies and is hereafter referred to as PEI-Adv.

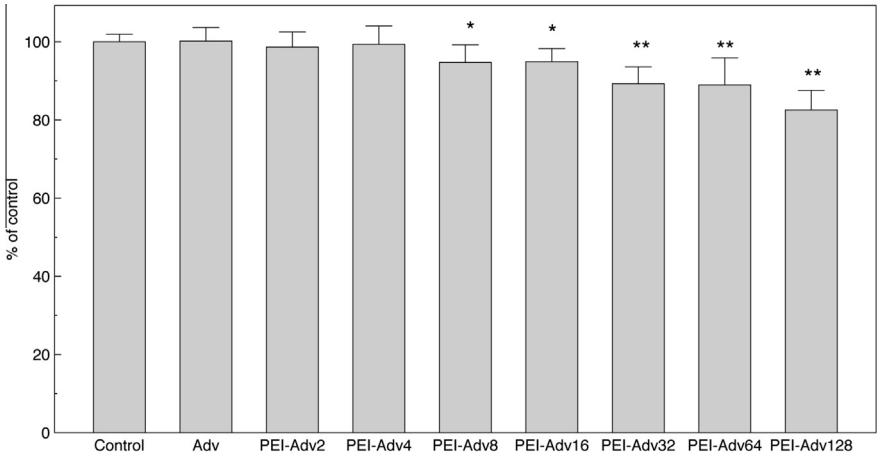

Fig. 2. Cell viability of MSCs treated with PEI-coated Adv. MSCs (2×10^4 cells/well) were transduced with the indicated Adv or PEI-Advs. After culturing for 24 h, cell viability was measured ($n = 6$). All data are represented as the means ± SD ($^{*}P < 0.05$, $^{**}P < 0.01$ compared with the untreated control group).

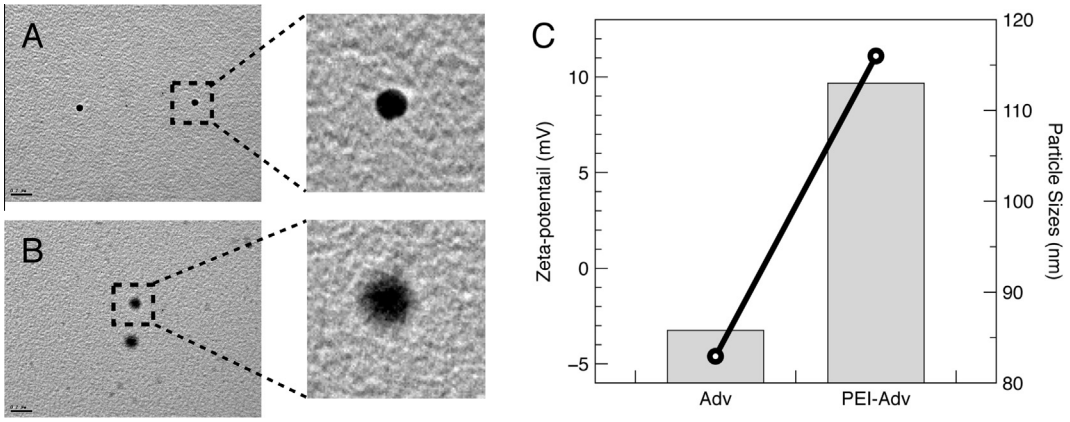

Fig. 3. Physicochemical properties of PEI-coated Adv. (A, B) Morphology of Adv (A) and PEI-Adv (B) with TEM observation. Left: 30,000×, Right: 600,000×. (C) Zeta-potential and particle size of Adv and PEI-Adv. Left: zeta-potential. Right: particle size.

3.3. Physicochemical properties of PEI-Adv

After optimization of PEI-Adv, we investigated why PEI coating could enhance adenoviral transduction in MSCs. First, we evaluated the physicochemical differences between Adv and PEI-Adv. With the help of TEM, we observed that PEI-Adv (Fig. 3B) looks slightly larger than Adv (Fig. 3A). This observation was confirmed after the particle sizes were measured with a zetasizer (Fig. 3C right *y*-axis). The particle size of Adv was 85.8 ± 12.25 nm, while the particle size of PEI-Adv was 113.0 ± 12.30 nm. Moreover, Adv particles were clear whereas PEI-Adv particles were blurry under TEM observation. No matter how we focused on a particle of PEI-Adv, it could not be imaged more clearly, and looked likely to be atomized. These results proved that PEI coated adenoviral particles and formed a hydration layer surrounding each adenoviral particle. In addition to morphology, we also evaluated the differences in zeta-potential. PEI enhanced the surface charge of Adv from −4.6 mV to 11.1 mV. This transformed an anionic adenoviral particle into a cationic particle. Because cell membrane is anionic, coating with cationic PEI could help Adv to enter the membrane of MSCs actively and bypass the requirement for the primary receptor CAR to be present on cell surfaces.

3.4. Internalization mechanisms of PEI-Adv

Next, to further investigate how PEI altered the pathway of adenoviral internalization into MSCs, we analyzed the endocytosis of PEI-Adv in the presence of endocytosis inhibitors (Fig. 4A). The transduction of Adv was inhibited by the clathrin-mediated endocytosis inhibitor CPZ and the lipid-raft inhibitor MBCD, but was not inhibited by the macropinocytosis-mediated endocytosis inhibitor amiloride. However, PEI-Adv transduction was only inhibited by MBCD. MBCD can selectively extract cholesterol to organize sphingolipid rafts, which is related to caveolae-mediated and macropinocytosis-mediated endocytosis [21]. The transduction of unmodified Adv and PEI-Adv were both inhibited by MBCD, but were not inhibited completely by amiloride. This result indicated that both Adv and PEI-Adv were allowed to enter into the cells through caveolae-mediated endocytosis. In contrast, the clathrin-mediated endocytosis inhibitor CPZ, which primarily affects recep-

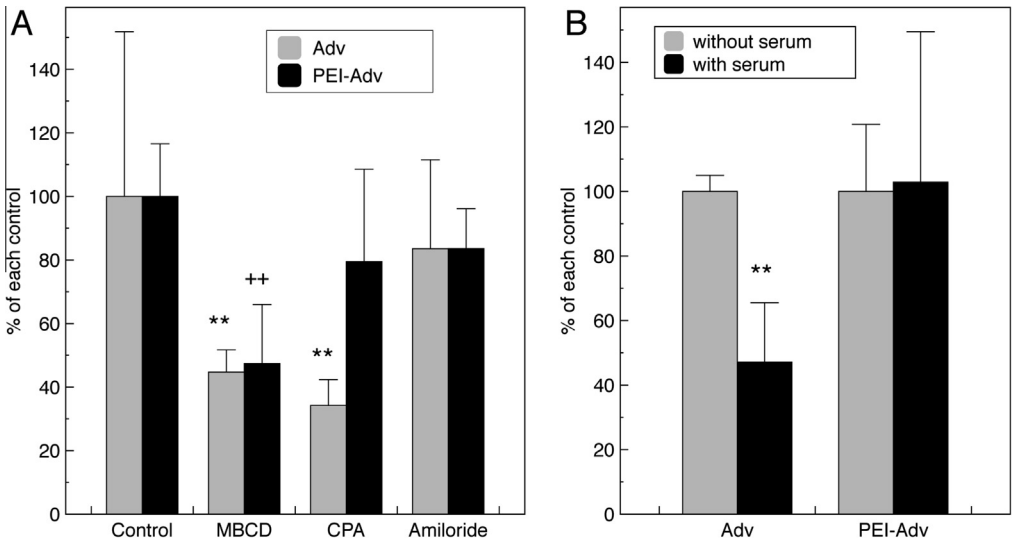

Fig. 4. Internalization mechanisms of PEI-coated Adv. (A) Endocytosis-dependency of cellular uptake of PEI-coated Adv. MSCs (2×10^4 cells/well) were pretreated with or without amiloride, MBCD, or CPZ for 1 h. The MSCs were then transduced with luciferase-encoding Adv or PEI-Adv. After culturing for 24 h, luciferase expression was measured ($n = 6$). All data are represented as the means ± SD (** or $^{++}P < 0.01$ compared with each control group). (B) Transgene expression with PEI-Adv in the presence of anti-adenovirus serum. MSCs (2×10^4 cells/well) were pretreated with anti-adenovirus serum or control serum for 1 h. The MSCs were then transduced with luciferase-encoding Adv or PEI-Adv. After culturing for 24 h, luciferase expression was measured ($n = 6$). All data are represented as the means ± SD ($^{**}P < 0.01$ compared with the control serum group).

tor-mediated endocytosis [22], could only inhibit Adv transduction. These results suggested that PEI coating helped Adv to enter MSCs without the requirement for CAR receptors. After PEI coating, low expression of the CAR receptors of MSCs could not limit the internalization of Adv. These results could explain the enhanced transgene expression with PEI-Adv.

To further demonstrate the effectiveness of PEI coating in enhancing adenoviral transduction, we evaluated transgene expression with PEI-Adv in the presence of anti-adenovirus serum. The serum was collected from Balb/c mice intravenously injected with Adv for 28 days. The concentration of the anti-adenovirus antibody in the serum was 15 μg/ml. In the presence of serum containing anti-adenovirus antibody, luciferase expression with Adv was decreased to 47% compared with the control serum (Fig. 4B). In contrast, luciferase expression with PEI-Adv did not change in the presence or absence of anti-adenovirus serum. These results indicated that PEI could tightly coat Adv, thus shielding it from the anti-adenovirus antibody. Therefore, we confirmed that PEI coated Adv as a whole until entering into MSCs and made it possible for Adv to be attracted to and endocytosed by MSCs, which lack the CAR receptors that are necessary for Adv transduction.

4. Conclusions

PEI could coat Adv to form a cationic particle and could enhance adenoviral transduction into MSCs by bypassing CAR receptors to enter MSCs. These results help to determine the potential of PEI-coated Adv as a prototype vector with suitable efficacy and safety for transduction into MSCs. With enhanced gene transduction, MSCs may integrate into targeted tissues more quickly, differentiate into specified cell types more precisely, secrete specific bioactive molecules capable of inhibiting inflammation more rapidly and perform immunomodulatory functions more efficiently. In conclusion, PEI-coated adenoviral transduction makes MSCs more attractive in clinical therapy and fundamental research.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgments

This study was supported in part by grants from the National Natural Science Foundation of China (81101719); the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry ([2012] 1707); and the National Natural Science Foundation of China (81273441).

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