RGD Conjugation to Polyethyleneimine Does Not Improve DNA Delivery to Bone Marrow Stromal Cells

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Bone marrow stromal cells (BMSC) modified with therapeutic genes are being actively pursued for gene therapy protocols. To develop safe and effective nonviral methods for BMSC modification, the cationic polymer polyethyleneimine (PEI) has been utilized to condense plasmid DNA for intracellular delivery. This study was conducted to explore the feasibility of increasing the PEI's effectiveness by coupling integrin-binding arginine-glycine-aspartic acid (RGD) peptides to the polymer. BMSC from rats were isolated and expanded in culture for gene transfer studies. In contrast to our expectations, RGD-conjugated PEI did not exhibit an enhanced binding to BMSC. This was the case where the peptides were conjugated to PEI by short, disulfide linkages or long poly(ethylene glycol) linkages. Using a reporter gene for the enhanced green fluorescent protein, the transfection efficiency of RGD-conjugated PEI was also lower than the delivery by the native PEI, which exhibited equivalent transfection efficiency to that of an adenovirus. We conclude that native PEI was sufficient for the transformation of BMSC and that coupling of the integrin-binding RGD-peptides did not improve the effectiveness of this polymer for BMSC transfection.

Introduction

Bone marrow stromal cells (BMSC) are actively pursued for cell-based gene therapy protocols.[1] Pluripotent BMSC can be readily obtained from a patient's bone marrow, can be expanded in vitro, and are capable of differentiating into a variety of adherent and nonadherent cells.[2,3] Genetic modification of BMSC has been primarily achieved by using viral vectors such as retroviruses, lentiviruses, or adenoviruses.[4,5] The viral vectors offer highly efficient means to express exogenous proteins, but their utility in clinics was recently discouraged due to safety concerns, such as the immunogenicity of viral particles and haphazard integration of viral genes into the host genome. Polycationic polymers offer an alternative to viral vectors for cellular modifications.[6] The positively charged polymers can interact electrostatically with negatively charged deoxyribonucleic acid (DNA) molecules and facilitate the passage of DNA through the cell membrane, which is negatively charged under physiological conditions and impedes the uptake of naked DNA. Polymers are safe alternatives to viral-based delivery vehicles since they do not integrate into the cellular genome. Among polymers explored as gene carriers, polyethyleneimine (PEI) has received the most attention[7,8] due to its strong binding to DNA molecules. A significant drawback of native PEI is its nonspecific interaction with anionic molecules found on cell surfaces and in solution. A high specificity toward cells is desirable to prevent competitive binding of polymers to serum proteins and to facilitate DNA uptake into the cells. Polymers with a high cell-binding capacity will require minimal concentration for effective gene transfer, ultimately reducing polymer toxicity on target cells.[9]

One possible avenue to design PEI-based vectors with BMSC-specific interactions is to incorporate moieties capable of interacting with cell-surface integrins into the polymers. Although the primary function of integrins is the regulation of cell adhesion to arginine-glycine-aspartic acid (RGD)-containing extracellular matrix proteins, integrins are utilized by viruses to facilitate their intracellular penetration by an endocytosis pathway.[10] Combining RGD-peptides with PEI was initially reported by Erbacher et al., who obtained an enhanced transfection efficiency in immortal (HeLa) cells as a result of CYGG**RGD**TP conjugation to PEI.[11] However, grafting a control peptide, CYGG**RGE**TP, where the integrin-binding RGD sequence was replaced with nonbinding RGE, also improved the transfection efficiency of PEI under some conditions,[11] suggesting a beneficial effect of the modification per se rather than improved cellular uptake via the integrin pathway. Kunath et al. linked a linear **RGD**C-peptide to PEI and demonstrated better binding with **RGD**C-PEI conjugates to an $\alpha_v\beta_3$-expressing Mewo cell line, as compared to unmodified PEI.[12] A beneficial effect of improved cell binding on transfection was obtained with formulations containing certain polymer/DNA (so-called N/P) ratios, but not all.

We are interested in exploring the potential of RGD-modified PEI for the delivery of exogenous genes to clinically useful BMSC. Toward this end, we recently reported on the preparation and characterization of RGD-PEI conjugates with a controlled number of peptide substitutions.[13] The physicochemical properties of the RGD-PEI conjugates were reported, but no studies were conducted on their capability to deliver functional genes to BMSC. In this study, RGD-PEI conjugates were used to deliver a reporter plasmid (i.e., plasmid expressing the enhanced

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green fluorescent protein, pEGFP) to BMSC. The BMSC were isolated from rats for the purposes of this study using previously reported established techniques.[14] Here, we show that PEI exhibited a high affinity to BMSC, and it was actively internalized by the cells. Conjugation of a RGD-peptide to the PEI, however, did not lead to increased cellular uptake of the polymers; on the contrary, it resulted in reduced cellular binding and correspondingly reduced expression of the delivered pEGFP.

Materials and Methods

Materials. Branched polyethyleneimine (25 kDa), Hanks' Balanced Salt Solution (HBSS), and trypsin/EDTA were purchased from Sigma (St. Louis, MO). *N*-Succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) was obtained from Molecular Biosciences (Boulder, CO) and *N*-hydroxy-succinimide-poly(ethylene glycol)-maleimide (NHS-PEG-MAL; 2.3 kDa) was from Nektar Therapeutics (Huntsville, AL). HPLC-grade acetonitrile, trifluoroacetic acid (99.8%), and water were purchased from Fisher Scientific (Fairlawn, NJ). Glycine-arginine-glycine-aspartic acid-serine-proline-cysteine peptide (GRGDSPC; >98% purity) was purchased from American Peptide Company (Sunnyvale, CA). Dialysis tubing with a MW cutoff of 12−14 kDa was purchased from Spectrum Laboratories (Gardena, CA). Dulbecco's Modified Eagle Medium (high glucose with L-glutamine) was from GIBCO (Grand Island, NY). Lipofectamine 2000 was from Invitrogen Corporation (Carlsbad, CA), and fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrencewille, GA). Tissue culture treated 24-well plates were purchased from Corning (Acton, MA), and 48-well plates were from BD Biosciences (Bedford, MA). Fluorescein isothiocyanate (FITC) was purchased from PIERCE (Rockford, IL). An adenovirus expressing the GFP was prepared as described before.[15]

Peptide Conjugation to PEI. Methods for the conjugation of peptides to PEI were described in detail elsewhere.[13] In a typical reaction, 1 mL of PEI solution (6.7 mg/mL in distilled/dionized H_2O (ddH_2O); 20 mM -NH_2 content) was mixed with 0.2 mL of SPDP solution in absolute ethanol to give the desired SPDP concentrations. The solution was diluted to 3 mL with borate buffer (pH = 8.4) containing 0.25 M NaCl. After 3 h at room temperature, the reaction was stopped by dialysis against 0.15 M NaCl solution for 2 days. The extent of SPDP derivatization was then determined by spectroscopy, as described before.[13] The derivatized PEI was then mixed with 0.1 mg/mL GRGDSPC solution in 0.1 M phosphate buffer (pH = 7.4) and incubated at room temperature for 3 h, after which the solutions were dialyzed against 0.15 M NaCl. The extent of peptide conjugation was analyzed on a VYDAC C-18 reverse-phase high pressure liquid chromatography (RP-HPLC) column, as described previously.[13] The peptide content (mM) and polymer content (mM) of conjugate solutions were used to calculate the number of GRGDSPC substituted per PEI.

In one series of studies, FITC-labeled PEI was reacted with NHS-PEG-MAL (0−0.9 mM) for 2 h and dialyzed overnight against 0.15 M NaCl. The polymers were then incubated with 1.2 mol of excess of GRGDSPC or cysteine as a control for 2 h (mol excess over NHS-PEG-MAL) and extensively dialyzed at room temperature to remove the unreacted peptide and cysteine.

Preparation of pEGFP Plasmid. pEGFP (pEGFP-N2 from BD Biosciences) is a 4.7 kb plasmid that contains an EGFP and a kanamycin resistance gene. The plasmid was replicated in the kanamycin resistant DH5-α *Escherichia coli* strain grown in Luria−Bertani medium. The plasmid DNA was purified using a Qiagen Plasmid Giga Kit according to the manufacturer's protocol. The concentration and purity of the plasmid were determined by UV spectroscopy as recommended by the manufacturer. The plasmid preparation was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8.0) at a concentration of 0.4 mg/mL.

Preparation of Polymer/pEGFP Complexes. Equal volumes of pEGFP and polymer solutions were prepared separately in a 150 mM NaCl solution. The concentrations of solutions were calculated such that the DNA amount would be 1 μg/well for the transfection experiments performed in 24-well plates and 4 μg/well for the experiments performed in 6-well plates. It was previously determined that the N/P ratio of 2:1 was optimal for effective transfection of BMSC and the PEI/DNA complexes were prepared at this ratio, unless stated otherwise. The solutions were mixed in microcentrifuge tubes, and the mixtures were vortexed for 1 min, followed by incubation at room temperature for 20 min to allow for polymer/pEGFP complex formation.

Polymer Binding to BMSC and C2C12 Cells. The isolation/expansion of the BMSC[14] and the culture of C2C12 cells[16] were described before. The cells were seeded in multiwell plates after being expanded in 25 cm^2 T-flasks. Both unmodified and RGD-modified PEI was labeled with FITC (1 mM) according to manufacturer's directions for cell-binding studies. When BMSC reached confluence in 48-well plates, the medium was removed, and cells were rinsed with HBSS (×2). A total of 200 μL of DMEM was added into each well followed by the addition of 20 μL of FITC-labeled polymer solution. Cells were incubated with the polymers for a given period of time (see figure caption) under standard tissue culture conditions. The contents of the wells were then removed, and the wells were rinsed with HBSS (×2) to remove the unbound polymers. Fluorescence associated with BMSC was measured with a plate reader (λ_{abs} = 485 nm, λ_{em} = 525 nm; Thermo Labsystems, Waltham, MA). The initial polymer concentrations added to the well, and the final concentrations of the polymer remaining in the well (polymer bound to the cells) were calculated based on standard curves generated with the original FITC-labeled polymer solutions and expressed in units of μg/mL. In some experiments, the cells were preincubated with free GRGDSPC (50 μg/mL) for 30 min, before the binding of the polymers to the cells was determined as stated previously.

For visualization studies using the fluorescent microscope, BMSC were seeded on sterile cover glasses at 30−50% confluence and allowed to attach for 1 day. The cells were then incubated with FITC-labeled PEI for 3 h at either 4 or 37 °C and then fixed with 70% ethanol for direct observation under the microscope.

Transfection Studies. EGFP expression was monitored by a fluorescence plate reader and flow cytometry using a fluorescence activated cell sorter (FACS) to determine the transfection efficacy in BMSC. The trypsinized BMSC were seeded in 24-well plates for the transfection experiments analyzed by the fluorescence plate reader. The cells were allowed to grow until ∼50% confluence after which the basic medium (DMEM + 10% FBS) in the wells was replaced with 400 μL of serum-free DMEM. Polymer/DNA complexes were prepared as described previously, and 100 μL of complex solution was added into each well and incubated for 5 h. The cells were then washed with HBSS, supplemented with 1 mL of fresh medium, and incubated for another 24 h. Transfection efficiency of the polymers was compared to the transfection efficiency of an adenoviral vector expressing the GFP and the commercial transfection agent Lipofectamine. A total of 30 μL of adenovirus (multiplicity of infection: 100) was added in each well, and the same procedure was followed in the transfection experiments with the polymers. In the case of Lipofectamine, 10 μL of Lipofectamine was mixed with 1 μg of pEGFP in 90 μL of DMEM, and the transfection was carried out in the same manner. To measure GFP transfection by fluorescence spectroscopy, cells were washed with HBSS (×2), 200 μL of HBSS was added to the wells, and the fluorescence emission was determined (λ_{abs} = 485 nm, λ_{em} = 525 nm).

For the transfection experiments performed to quantify the GFP-positive cells by FACS analysis, the trypsinized BMSC were seeded in 6-well plates. The cells were allowed to grow until ∼50% confluence after which the basic medium (DMEM + 10% FBS) in the wells was replaced with 1 mL of fresh basic medium. Polymer/DNA complexes were prepared as described previously, and 200 μL of complex solution was added into each well and incubated for 24 h. For the FACS analysis, polymers were tested at two different N/P ratios (2:1 and 10:1) and compared to the adenoviral vector that was added at a concentration

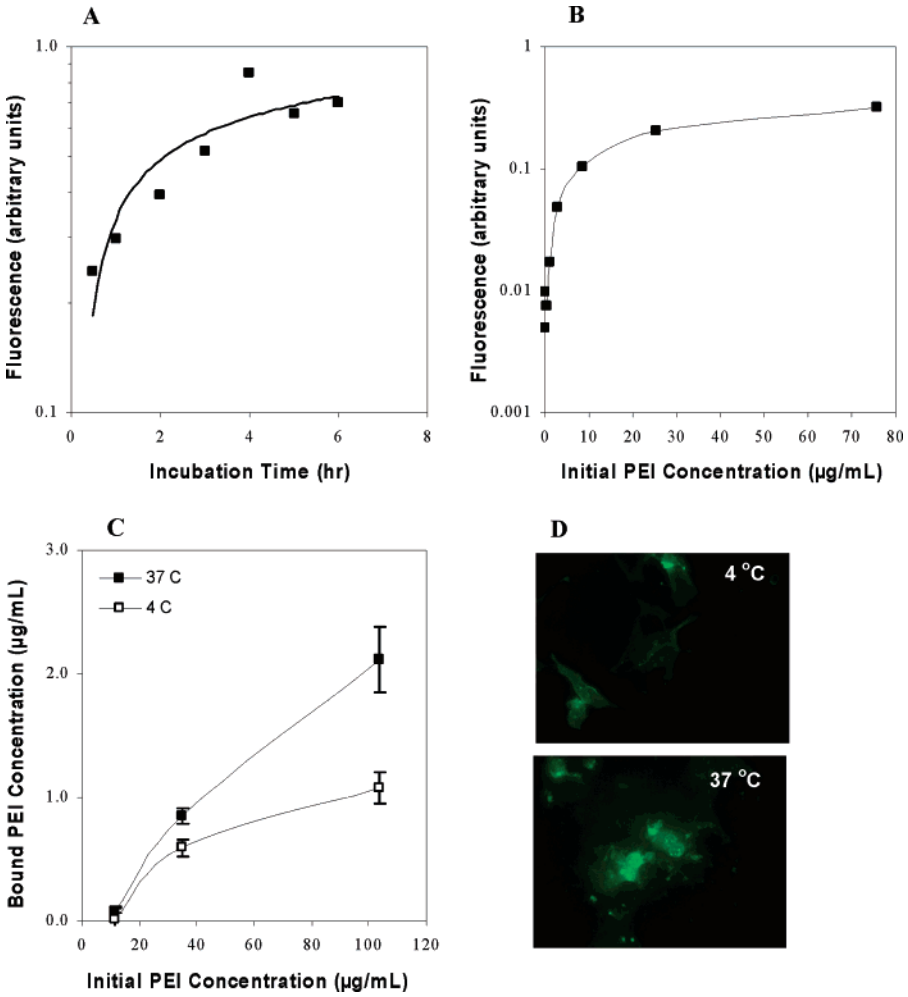

Figure 1. Binding of FITC-labeled PEI to BMSC as a function of incubation time (A), polymer concentration in culture (panel B; 2 h incubation), and incubation temperature (panel C; 4 and 37 °C for 2 h incubation). The results in panels A and B were shown as the amount of fluorescence measured, whereas in panel C the bound fluorescence was converted into PEI concentration by using a standard curve of FITC-labeled PEI. (D) Microscopic images of cells incubated with FITC-labeled PEI at 4 and 37 °C. Nuclear localization of the fluorescent polymer was evident in most cells incubated at 37 °C but not at 4 °C.

of 40 μL/well. For the FACS analysis, BMSC was washed with HBSS ($\times$2), trypsinized, and resuspended in HBSS with 3.7% formalin. A Becton Dickinson FACScan Analytic Flow Cytometer with a fixed 488 nm argon laser and three-color capabilities was used to count the GFP positive BMSC with 10 000 events per sample.

Statistical Analysis. Where indicated, the data are summarized as the mean $\pm$ standard deviation (SD) of triplicate measurements (unless indicated), and an unpaired Student's *t*-test was used to assess statistical differences ($p < 0.05$) between the group means.

Results

PEI Binding to BMSC. The effects of incubation time, polymer concentration, and temperature on the binding of FITC-labeled PEI were first investigated. The PEI binding was increased with incubation time until a plateau was reached after 4 h (Figure 1A). Using an incubation time of 2 h, the binding of PEI to BMSC was found to be dependent on the polymer concentration added into the wells (Figure 1B). Maximal binding was observed at $\sim$30 μg/mL of PEI, after which the binding of PEI reached a plateau. To investigate the effect of temperature on PEI binding, FITC-labeled PEI was incubated with BMSC at 37 and 4 °C, where active (i.e., energy dependent) cellular transport was abolished. There was a reduction in PEI binding at 4 °C (Figure 1C), indicating an active uptake of PEI into the BMSC at 37 °C. To ensure that the difference was not due to temperature-dependent differences in binding per se, ethanol-fixed cells were used to assess binding of FITC-labeled PEI at 37 and 4 °C, and a similar binding was observed at the two temperatures using the fixed BMSC (not shown). Finally, FITC-labeled PEI was incubated with BMSC at 37 and at 4 °C, and the uptake was observed with a fluorescent microscope after 3 h. Consistent with reduced binding observed in Figure 1C, cell staining was reduced at 4 °C with no clear nuclear staining at this temperature (Figure 1D). BMSC incubated at 37 °C, on the other hand, exhibited a stronger uptake of PEI and gave significant nuclear staining (>70% at 37 °C vs $\sim$10% at 4 °C; manuscript in preparation).

Effect of PEI Modifications on BMSC and C2C12 Binding. The desired conjugates were obtained as described previously in a two-step reaction, whereby PEI was first modified with SPDP to introduce thiol-reactive dithiopyridines, followed by the addition of a GRGDSPC peptide for reaction between the cysteine thiols of the peptide and the dithiopyridine groups on PEI.[11] To assess the effect of SPDP modification, SPDP-modified PEI with a dithiopyridine content of 0$-$0.225 mM was prepared. The binding of the modified PEI to BMSC was assessed after 1 h (Figure 2A) and 5 h (Figure 2B) of incubation. There was no apparent difference in cell binding between the unmodified PEI and PEI modified with different concentrations

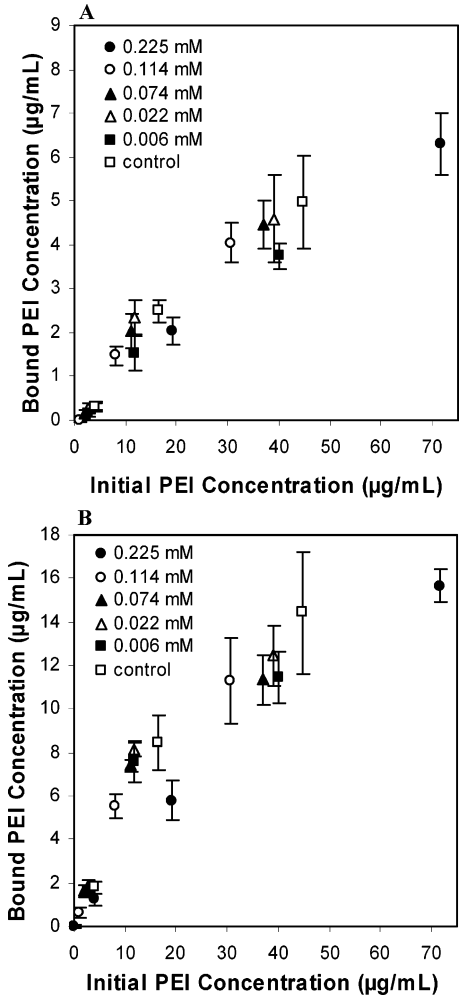

Figure 2. Effect of SPDP modification on PEI binding to BMSC (mean ± SD) after 1 h (A) and 5 h (B) of incubation. Each symbol represents a single polymer preparation that was reacted with varying concentrations of SPDP (0−10 mM SPDP), and the final dithiopyridine concentrations were determined. The final concentrations of dithiopyridine in the FITC-labeled PEI ranged between 0.006 and 0.225 mM. The cell binding of each preparation was tested over the polymer concentrations indicated in the horizontal axis. As compared to native PEI (control; open squares), SPDP modification did not affect cell binding at both assessment points.

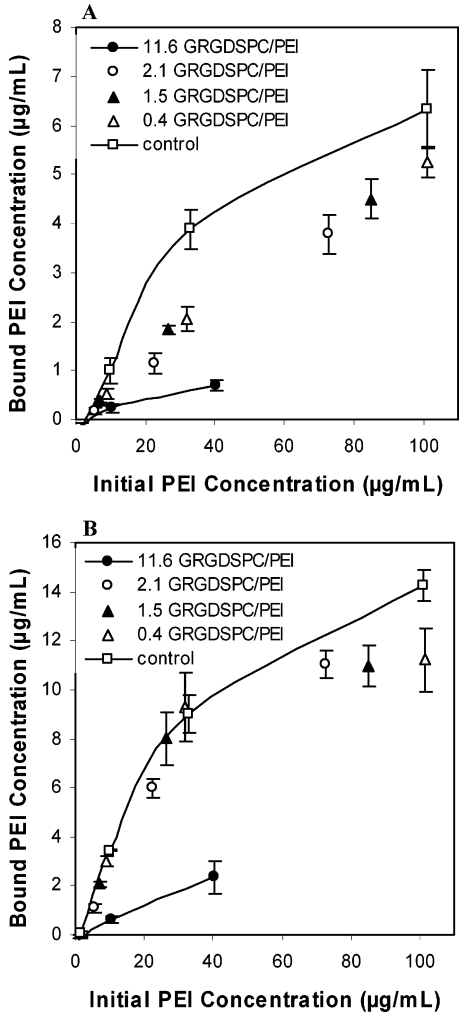

Figure 3. Effect of GRGDSPC substitution on PEI binding to BMSC (mean ± SD) after 1 h (A) and 5 h (B) of incubation. The SPDP-modified PEI polymers from Figure 2 (except the PEI that was least modified; i.e., 0.006 mM dithiopyridine concentration) were used to obtain peptide substitutions between 11.6 and 0.4 GRGDSPC/PEI, and their binding was assessed over the polymer concentrations indicated in the horizontal axis. As compared to native PEI (control; open squares with solid line), the GRGDSPC substitution did not affect the cell binding at lower substitutions (<2.1 peptides/PEI) but decreased cell binding at the highest substitution (11.6 peptides/PEI; solid circles with solid line).

of SPDP, irrespective of the incubation time. Next, the effect of GRGDSPC modification of PEI on cell binding was investigated (Figure 3). The conjugates used in this study had 11.6−0.4 GRGDSPC/PEI, whereas the control PEI (i.e., PEI incubated with GRGDSPC without SPDP and dialyzed) had 0.07 GRGDSPC/PEI (i.e., reflecting the fraction of undialyzed free peptide content). All conjugates with ≤2.1 GRGDSPC/PEI gave a similar binding pattern at 1 h (Figure 3A) and 5 h (Figure 3B), which was equivalent to that of native PEI. The conjugate with the highest degree of GRGDSPC substitution (11.6) exhibited a significantly lower binding than PEI. The lower uptake of this sample was most evident after 5 h of incubation.

An alternative cell type, C2C12 cells were also used to compare the binding of PEI to that of GRGDSPC-PEI conjugates. The C2C12 cells were previously utilized in our lab for preparing RGD-grafted polymeric cell-culture surfaces[16] since they attach strongly to RGD-grafted surfaces. Similar to the results with BMSC, the GRGDSPC-PEI conjugates also did not offer an advantage in binding to the C2C12 cells (Figure 4).

Finally, the binding of GRGDPSC-PEI conjugates to BMSC was investigated in the presence of free GRGDSPC to determine the relative contribution of the GRGDSPC moiety to overall cell binding. The peptide concentration used was 10-fold higher than the peptide content of the polymers. No significant effect of the peptide on polymer binding was observed with either the native PEI or the two GRGDSPC-PEI preparations with 16.6 and 9.1 peptides/polymer (not shown).

Cell Binding of PEG-PEI. The beneficial effect of GRGDSPC on PEG-modified PEI was then explored. The heterofunctional PEG was attached to the FITC-labeled PEI via the terminal succinimide ester, and its maleimide terminal was reacted with either GRGDSPC or cysteine as a control. Using an NHS-PEG-MAL concentration of 0−0.9 mM to modify PEI, the PEI attachment to BMSC was reduced as a function of PEG concentration used to modify the PEI (Figure 5A). The PEI modified with 0.9 mM PEG gave a binding similar to the background. The BMSC binding of PEG-PEIs reacted with the GRGDSPC was not any better than the corresponding PEG-PEIs (Figure 5B).

Transfection by Modified Polymers. The relative efficiencies of PEI and GRGDSPC-PEI conjugates for EGFP expression

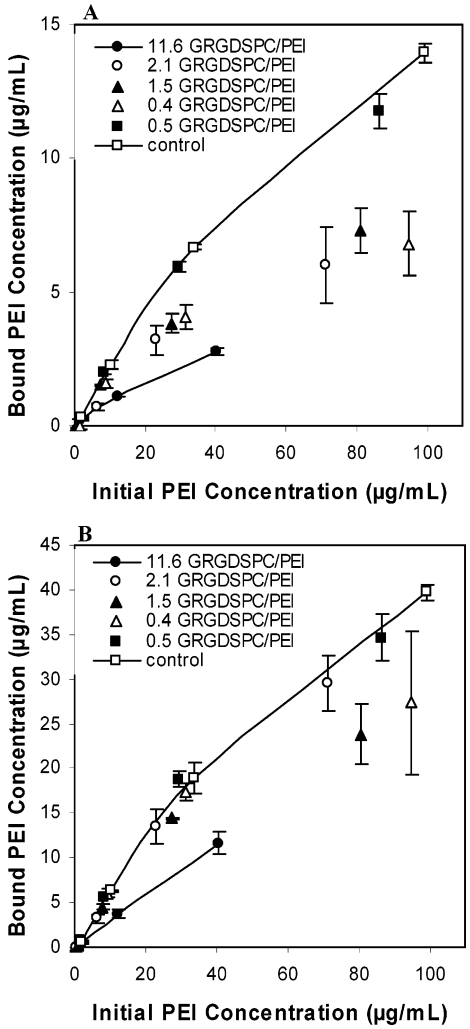

Figure 4. Effect of GRGDSPC substitution on PEI binding to C2C12 cells (mean ± SD) after 1 h (A) and 5 h (B) of incubation. The SPDP-modified PEI polymers from Figure 2 were used to obtain peptide substitutions between 11.6 and 0.4 GRGDSPC/PEI, and their binding was assessed over the polymer concentrations indicated in the horizontal axis. As compared to native PEI (control; open squares with solid line), GRGDSPC substitution did not increase cell binding, which is most evident at the highest substitution (11.6 peptides/PEI; solid circles with solid line).

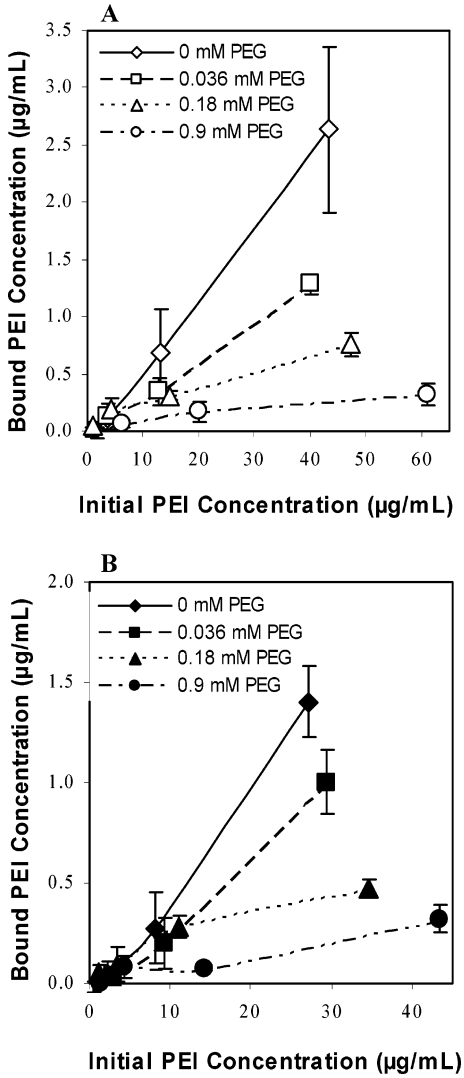

Figure 5. Effect of GRGDSPC substitution on BMSC binding of PEG-modified PEI (mean ± SD). The PEI polymers, first reacted with NHS-PEG-MAL (0−0.9 mM) and then with either cysteine (A) or GRGD-SPC (B), were used for cell binding. Each polymer was incubated with the cells for 5 h at various concentrations. Note the reduction in BMSC binding as a function of NHS-PEG-MAL concentration used for PEI modification (A). Substituting GRGDSPC to the PEG-modified PEI did not improve the BMSC binding (B).

were evaluated with a fluorescence plate reader and FACS analysis. Two reference samples were a GFP-expressing adenovirus and a commercially available lipid formulation (Lipotectamine). Using the fluorescence plate reader (Figure 6A), a significant GFP expression was observed with the adenovirus, but Lipofectamine was not effective for the EGFP expression in BMSC. The transfection efficiency by unmodified PEI was equivalent to that of the adenovirus. Conjugating GRGDSPC to the PEI, however, reduced the transfection efficiency as compared to PEI ($p < 0.05$). There was no significant difference in transfection efficiency between the negative controls (i.e., untreated BMSC and BMSC exposed to pEGFP alone) and the GRGDSPC-PEI-based delivery ($p > 0.05$). FACS analysis (Figure 6B) indicated the adenovirus to be the most effective as well, with approximately 14% of the cells being GFP positive. PEI was less effective, giving roughly 10% transfected cells. PEI conjugates with GRGDSPC also gave reduced transfection efficiency, with no obvious benefit at high concentrations and a slight reduction at lower concentrations ($p < 0.001$).

Discussion

Integrins are a class of transmembrane glycoproteins that play important roles in cell adhesion, cell−extracellular matrix interactions, and intracellular signaling pathways. These cell adhesion receptors have been widely studied, and integrins were found to be the receptors that many viruses and bacteria use to bind to cell surfaces.[17] The common sequence found in the functional domains of integrin-binding proteins, RGD, can mimic natural ligands such as fibronectin, vitronectin, laminins, and collagen in short peptide forms. These RGD-bearing short peptides can bind to most known integrins.[18,19] The BMSC from rats express a range of integrins[20] and bind to RGD-immobilized matrixes avidly.[21,22] Our previous data indicated improved BMSC binding to RGD-grafted polymer surfaces (4-fold better vs ungrafted surfaces),[23] suggesting the presence of integrins on BMSC capable of binding to GRGDSPC-grafted surfaces. Accordingly, we expected the RGD-PEI conjugates to be superior to PEI in transfecting BMSC with pEGFP. This was shown not to be the case in this study. Our studies indicated

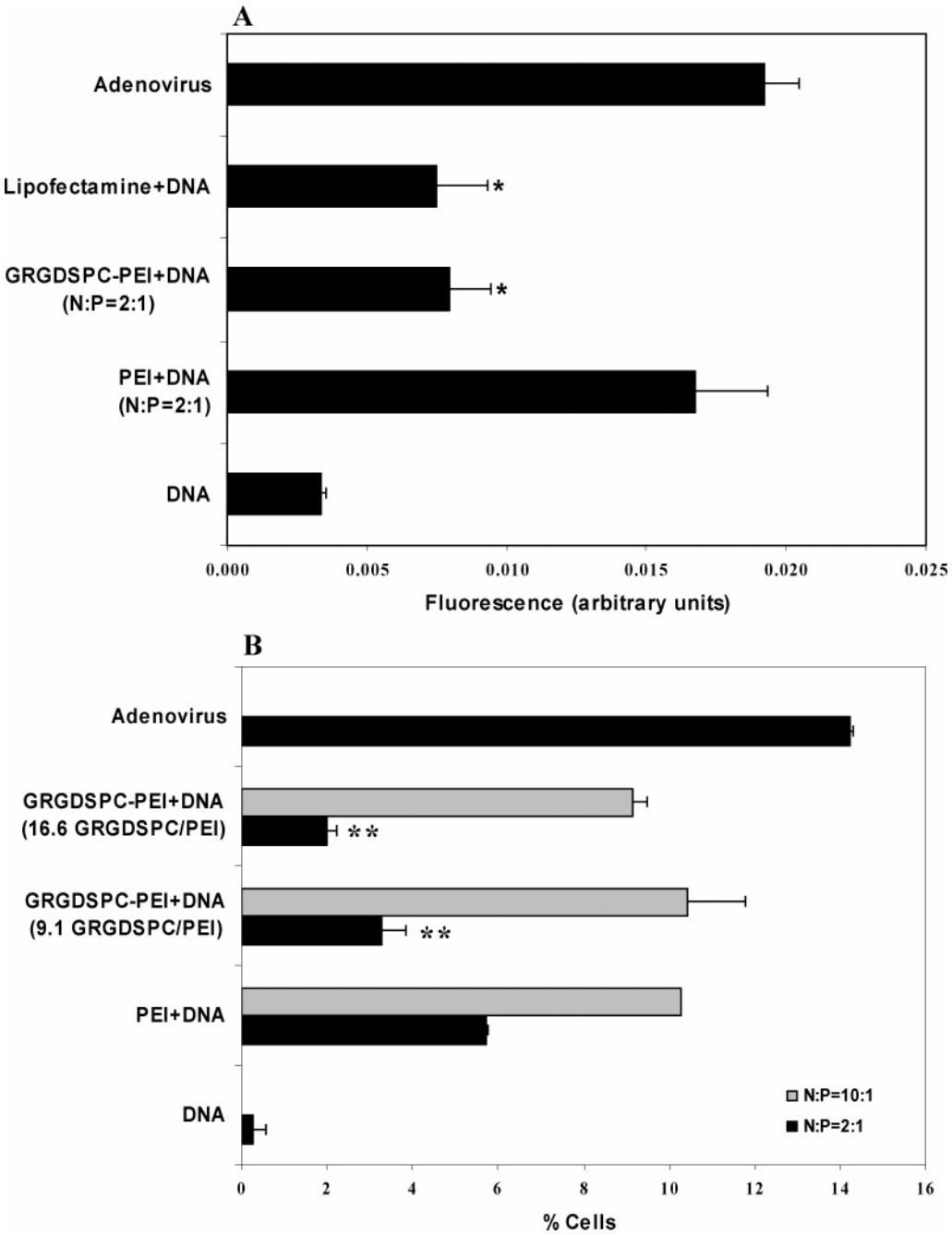

Figure 6. Relative transfection efficiency of an adenoviral vector, lipofectamine, native PEI, and GRGDSPC-PEI (11.6 peptides/PEI) (A). The polymers were mixed with pEGFP at a N/P ratio of 2 (20 min complexation) and incubated with BMSC for 5 h. After removing the medium and further 24 h incubation, the extent of fluorescence from a plate reader, after subtracting the background (i.e., BMSC without any treatment) fluorescence, was used as a measure of successful transfection. The adenoviral vector and the native PEI gave the highest GFP transfections, and GRGDSPC-conjugated PEI resulted in significantly less transfection (*: $p < 0.05$ as compared to unmodified PEI). Relative transfection efficiency of an adenoviral vector, PEI, and GRGDSPC-PEI as determined by flow cytometry (B). The results (mean $\pm$ SD) are expressed as the percentage of cells positive for EGFP fluorescence. The polymers were mixed with the pEGFP at N/P ratios of 2 and 10 (20 min complexation) and incubated with BMSC for 24 h. The EGFP expression was analyzed after 24 h of incubation with the DNA/polymer complexes. Among the polymers, no difference in EGFP expression was evident at the high N/P ratio, but GRGDSPC-linked PEIs gave a significantly reduced level of expression at the lower N/P ratio of 2 (**: $p < 0.001$).

that native PEI was actively transported by the BMSC, as evident by the temperature-dependent accumulation of the polymer intracellularly. Free GRGDSPC peptide did not influence the binding of PEI, as well as that of GRGDSPC-PEI; this was indicative of a binding mechanism that was mostly independent of integrins for all polymers. Presumably, the highly cationic nature of the polymers was sufficient for significant binding to the cell surfaces via nonspecific interactions. The fact that the initial SPDP modification of PEI did not compromise cell binding was indicative of the possibility of modifying some polymer amines without losing the necessary cell binding. The presence of the peptide on the polymers, however, reduced cell binding for such SPDP-modified polymers. One reason for this might be increased steric hindrance of PEI binding to the cell surface due to the presence of a lengthy peptide on PEI. Another reason might be the negative charge of aspartic acid in the peptide since cellular surfaces are anionic under physiological conditions. Consistent with this, Erbacher et al. showed that RGD grafting reduced the zeta potential of PEI/plasmid complexes as a function of RGD density, giving a neutral charge

at N/P ratios used for transfection studies, in contrast to the positive charge of the PEI/plasmid polyplexes.[11] This change might have contributed to the better efficiency of RGD-PEI conjugates in these studies. Another group has also reported a reduced transfection by RGD-PEI conjugates in A549 lung adenocarcinoma cells,[12] similar to the results with BMSC in this study. Therefore, PEI seems to be sufficient for some cell types, and conjugating the RGD-peptide does not improve and sometimes reduces its ability for effective DNA delivery.

The transfection experiments with the GRGDSPC conjugates were also consistent with the binding effect of the peptide. The BMSC are generally regarded as difficult cells to transfect since they are not immortal, and the relatively low transfection efficiencies observed in this study (<15%) were consistent with this expectation. The positive control in the transfection experiments (i.e., the adenoviral delivery) yielded a similar efficiency to that of PEI mediated pEGFP delivery, both in the amount of EGFP expressed (Figure 6A) as well as the percentage of cells expressing the EGFP (Figure 6B). The GRGDSPC attachment to PEI was detrimental based on both of these parameters, except when the polymer concentration was increased, which resulted in overriding the inhibitory effect of the peptide conjugation. It is possible that GRGDSPC conjugation might have also affected the size of polyplexes formed with pEGFP since the presence of the peptide was known to reduce plasmid binding.[12] This might have changed the polyplex sizes so formed, and contributed to the decreased transfection efficiency, in addition to reduced binding of the polymers to cells per se.

Our results indicated RGD substitution not to be beneficial in increasing the uptake of PEG-modified PEIs either. PEG-substituted PEI is an attractive option for in vivo delivery of DNA/PEI polyplexes since PEG modification was expected to reduce nonspecific interactions of polyplexes with serum proteins and nontarget cells.[24] Conjugating PEG to PEI was demonstrated to reduce the transfection efficiency due to reduced cell binding.[12,25] Incorporating RGD-peptides to such conjugates has been attempted to compensate for this problem and to improve their transfection efficiency; however, linking RGDC to PEI-PEG did not recover the lost transfection efficiency in $\alpha_v\beta_3$-integrin expressing Mewo cells.[12] The large size of PEG (~25 kDa) in that study was considered a likely reason for the lack of RGD effect. Utilizing a smaller PEG (~3.4 kDa) between the PEI and the RGD-peptide led to a beneficial effect of RGD conjugation on cell binding in the hands of another group.[25] However, cell binding of PEI-PEG-RGD conjugates was not as strong as the unmodified PEI, and the conjugates were found to be equivalent in gene delivery when compared to PEI in siRNA delivery to endothelial and N2 tumor cells. Although an even smaller PEG linker (2.3 kDa) was used in our studies, conjugating GRGDSPC to PEI did not recover the lost cell-binding capability of the native PEI.

Our studies focused on linear RGD-peptides, and it is likely that RGD-peptides with improved affinity might still offer a benefit in cell delivery. A cyclic RGD-peptide (-C-DCRGDCF-C-) was conjugated to PEI using a PEG linker for transfection of endothelial cells.[26,27] The effectiveness of the conjugate to bind to cells was evaluated by investigating its ability to inhibit cell attachment; the binding of the RGD conjugate (when the PEG linker was ~1 kDa but not more than 5 kDa) was similar to that of free cyclic RGD. Although the relative effectiveness of the conjugate to bind to cells with respect to PEI was not reported, the transfection efficiency of the PEI-RGD conjugate was improved over that of PEI when the cells expressed RGD-binding $\alpha_v\beta_3/\alpha_v\beta_5$ integrins. In the absence of integrin expression, PEI-RGD conjugates gave a lower transfection efficiency as compared to the unmodified PEI.

The cell toxicity of the polymers used in this study (data not shown) directly correlated with the cell-binding results. The PEI polymers whose cell binding was not altered with a particular modification (SPDP) also displayed no apparent changes in toxicity toward the BMSC. Polymers with reduced cell binding, either by PEG or high GRGDSPC engraftment, exhibited reduced toxicity on BMSC as well. Collectively, a positive correlation between cell binding and toxicity was evident, attesting to the predominant mechanism of toxicity with the highly cationic polymer PEI.

In conclusion, BMSC were successfully transfected with a GFP-expressing adenovirus as well as a PEI/pEGFP polyplex. Grafting an RGD-peptide to PEI, which was expected to better mimic an adenoviral binding mechanism to BMSC, did not lead to better transfection and, in fact, reduced the customary transfection ability of the PEI. The underlying basis for this observation was the reduced cell binding of the peptide-modified PEI. In addition, GRGDSPC grafting was not able to compensate for the reduced cell binding of the PEG modified PEI polyplexes. Further efforts to enhance the transfection ability of PEI could be directed toward the design of RGD mimics with improved binding to BMSC integrins or by application of other cell-surface binding peptides that do not display a negative effect on the binding of PEI to BMSC.

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