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A novel synthetic peptide vector system for optimal gene delivery to bone marrow stromal cells

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Abstract: A 23-amino acid, bifunctional, integrin-targeted synthetic peptide was evaluated for *ex vivo* gene delivery to rabbit bone marrow stromal cells (BMSCs). The peptide $(K)_{16}$GRGDSPC consists of an amino terminal domain of 16 lysines for electrostatic binding of DNA, and a 7-amino acid integrin-binding domain at the carboxyl terminal. PcDNA3-EGFP plasmids were transfected into BMSCs by $(K)_{16}$GRGDSPC and the positive cells gave out a bright green fluorescence. High levels of gene delivery of pcDNA3-TGF-β1 plasmids were obtained with 2 to 4 μg/ml DNA concentration, with $(K)_{16}$GRGDSPC at an optimal peptide: DNA w/w ratio of 3 : 1, with a required exposure time of more than 4 h but shorter than 24 h for BMSC exposure to the peptide/DNA complexes with completely absent serum in the initial stage; with 100 μM chloroquine and at least 8 h exposure for BMSC exposure to chloroquine; with a fusogenic peptide at an optimal $(K)_{16}$GRGDSPC/DNA/fusogenic peptide w/w ratio of 3 : 1 : 5; and with Lipofectamine 2000 at an optimal $(K)_{16}$GRGDSPC/DNA/Lipofectamine 2000 w/w ratio of 3 : 1 : 2 at a constant DNA concentration of 2 μg/ml. Chloroquine, the fusogenic peptide and Lipofectamine 2000 all significantly promoted gene delivery, but chloroquine was more effective than the fusogenic peptide and had obvious synergistic effects with Lipofectamine 2000. Under optimal conditions, TGF-β1 gene was transfected into BMSCs without observable toxicity, and the stable expression was examined by RT-PCR and Western blot analysis. The stable transgenic cells showed obvious bands. This novel synthetic peptide, providing a new way for the use of polylysine and RGD motif in DNA vector system, is potentially well suited to *ex vivo* gene delivery to BMSCs for experimental and clinical applications in the field of bone tissue engineering. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: non-viral vector; bone marrow stromal cells; optimization; bone tissue engineering

INTRODUCTION

For gene therapy to be successful, the vector system must be efficient in delivering an exogenous gene into the cells and in expressing the gene product. In addition, gene expression should be prolonged and the vector non-toxic. Viral vectors are still considered to be the most efficient gene transfer systems but the efficiency of liposome-based non-viral vectors is continuously improving. In addition, such non-viral systems provoke less host inflammatory and immune responses and are relatively easy to prepare in large quantities. Receptor-mediated molecular conjugates are also used to deliver plasmid DNA and consist mainly of receptor-binding ligand conjugated to a DNA-binding moiety, usually the high-molecular-weight oligo-L-lysine.

Knowledge of the mechanisms of entry of the adenovirus into cells by interaction with integrins and the coxsackievirus and adenovirus receptor inspired us to develop peptide-based gene transfer vectors that mimic the adenovirus. We have developed a 23-amino acid linear bifunctional synthetic peptide as a DNA vector, which consists of an integrin-targeted domain containing an arginine-glycine-aspartic (RGD) acid tripeptide motif and a DNA-binding moiety consisting of a short stretch of 16 lysine residues. The oligopeptide can form condensed complexes with DNA, and the peptide/DNA complexes can be internalized into cells mediated by cell surface integrin receptors via a phagocytic mechanism. The formation of condensed peptide/DNA complexes leads to resistance to nuclease degradation. This novel peptide probably represents an optimal approach to the use of polylysine and RGD motif in DNA vectors targeting the integrin. Here, we show that targeted gene delivery into rabbit bone marrow stromal cells (BMSCs) can be achieved with the novel vector. This has potential implications for the use of *ex vivo* gene delivery for experimental and clinical applications in the field of bone tissue engineering.

MATERIALS AND METHODS

Peptides

The synthetic peptide vector is a 23-amino acid linear bifunctional peptide [NH_2-(lys)$_{16}$-Gly-Arg-Gly-Asp-Ser-Pro-Cys-CO_2H] ($(K)_{16}$GRGDSPC) consisting of a 16-lysine chain at the amino terminal for electrostatic binding of DNA, and the 7-amino acid integrin-binding domain at the carboxyl terminal. Peptide synthesis was performed on an Applied

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Biosystems, Inc. (ABI, Foster City, CA) solid-phase batch peptide synthesizer (model 431A). The peptide $(K)_{16}$GRGDSPC was synthesized on a small scale using the standard fluorenylmethoxycarbonyl (Fmoc)-amino acid-coupling protocol but with sequential benzoic anhydride capping steps during the generation of the *N*-terminal hexadeca-L-lysine sequence. Once the synthesis was concluded, the peptide was cleaved from the resin using a trifluoroacetic acid (TFA) solution containing the following scavenger: phenol (7%, w/v), thioanisole (4%, v/v) and ethanedithiol (2%, v/v). The peptide was precipitated using ice-cold methyl *t*-butyl ether (MTBE) and pelleted by centrifugation. The pellet was then washed repeatedly with MTBE and dried *in vacuo*. This dried pellet was dissolved in a minimum volume of aqueous TFA (0.1%, v/v), and then applied to a gel-filtration column. The column was eluted with aqueous TFA (0.1%, v/v) at a flow rate of 0.5 ml/min, and the salt-free peptide-containing fractions were then combined and freeze-dried, giving the crude peptide. This crude peptide (10 mg) was dissolved in aqueous acetic acid (7%, v/v) and the solution buffered to pH 6.0 with an aqueous ammonia solution. Dimethyl sulfoxide was added [20% (v/v) final concentration] and the solution stirred at ambient temperature for 24 h. Following a five-fold dilution in water, the crude peptide was applied to a reversed-phase C_{18} high-pressure liquid chromatography (HPLC) column (6 μm particle size) previously equilibrated with aqueous TFA (0.1%, v/v) and then eluted with a linear gradient of TFA (0.1%, v/v) in acetonitrile. Fractions were collected at a flow rate of 5 ml/min and the eluant was monitored at 223 nm. Fractions containing the peptide were combined and freeze-dried to give a lyophilized powder (9.5 mg). Then by matrix-assisted laser desorption/ionization-time of flight (MALDITOF) mass spectrometry, an $[M + H]^+$ molecular ion was observed at m/z 2741.3, which entirely accorded with the expected calculated mass from the empirical formula: m/z 2741.3.

The fusogenic peptide (based on the sequence of amino terminal 20-amino acids of the influenza virus haemagglutinin) [NH_2-Gly-Leu-Phe-Glu-Ala-Leu-Leu-Glu-Leu-Leu-Glu-Ser-Leu-Trp-Glu-Leu-Leu-Leu-Glu-Ala-CO_2H] was synthesized and purified as above. The purity of the fusogenic peptide was 96%.

The peptides were supplied as dry power, and were stored desiccated at −20 °C. Quantities of 1–2 mg were dissolved at 1 mg/ml in phosphate buffered saline [(PBS)), pH 7.3] and kept under stirring overnight at room temperature. They were then frozen in small aliquots and stored at −20 °C.

Reporter Genes

The plasmid pcDNA3.1-EGFP encoding green fluorescent protein (GFP) was purchased from Jingsai Biotechnology Corporation (Wuhan, China) and the plasmid pcDNA3.1-TGF-β1 encoding transforming growth factor β1 was constructed in our laboratory [1]. These two types of DNAs were prepared from overnight bacterial cultures by alkaline lysis and purified using a P10 000 column under endotoxcin-free conditions. The purified DNA was diluted in sterile PBS water to 1mg/ml and frozen in small aliquots at −20 °C.

Preparation of Bone Marrow Stromal Cells

Rabbit-derived BMSCs were obtained as described previously [2], and the third-passage BMSCs were used in these studies. BMSCs in the exponential phase of growth were harvested and diluted to a concentration of 5×10^4/ml with complete DMEM medium without addition of any serum.

Preparation of Vector/DNA Complexes and Optimization of Factors Affecting Gene Transfection Efficiency

Peptide $(K)_{16}$GRGDSPC/EGFP DNA complexes were prepared at a constant peptide/DNA weight for weight (w/w) ratio of 3 : 1 using 5 μg of DNA in each well of the 24-well culture plate. The required amounts of peptide and plasmid DNA were diluted in 50 μl of serum-free DMEM at room temperature. After gentle vortexing for 2 min, the peptide solution was added drop-wise to the plasmid DNA solution. The mixed solution was gently vortexed for a further 5 min and diluted to a final volume of 1.0 ml and then was allowed to stand at room temperature for 30 min to allow peptide/DNA complex formation, before adding to the pre-cultured 3rd generation BMSCs.

Peptide $(K)_{16}$GRGDSPC/TGF-β1 DNA complexes were prepared as above, but at the constant 3 : 1 peptide/DNA w/w ratio the plasmid DNA were added to give various final concentrations ranging from 1 to 16 μg/ml. The various complexes were separately added to the cells after a 5-min vortexing and diluting to a final volume of 1.0 ml and a further 30 min standing for vector/DNA complexes formation at room temperature. Optimal DNA concentration was determined by following enzyme-linked immunosorbent assays (ELISA) for TGF-β1. Furthermore, at the constant optimal DNA concentration, which was determined in the preceding step, appropriate amounts of peptide were added to give a series of peptide/DNA w/w ratio from 1 : 1 to 7 : 1 and the optimal peptide/DNA w/w ratio was determined. Then optimal exposure time was determined when time of the cells exposed to the prepared optimal peptide/TGF-β1 DNA complexes ranged from 0.5 to 24 h. Subsequently, the effects of serum on the receptor-mediated gene delivery was examined when fetal bovine serum was added to give various serum concentrations of 1 to 20% in the initial 4 h of gene delivery. And then the effects of endosomal buffer chloroquine on the gene delivery were evaluated. In the preceding steps, chloroquine was freshly prepared from the powder to 1.0 mM in DMEM, filtered with 0.22 μm micropore filter film and the appropriate volume was added to each well to reach a constant final concentration of 100 μM in the first 24 h of gene delivery. But in this step, optimal peptide/DNA complexes were prepared and chloroquine solution was added into each well to give various final concentrations of 50 μM to 200 μM. Once the optimal chloroquine concentration was determined, and keeping various exposure times of 1–20 h of the cells to the chloroquine solution, the optimal exposure time was also determined.

As for the involved fusogenic peptide, the appropriate volume of the stock solution of 1 mg/ml of fusogenic peptide in PBS was added to the optimal peptide $(K)_{16}$GRGDSPC/TGF-β1 DNA complexes at the end of the 30-min standing incubation at room temperature, to give a final concentration of 5–30 μg/ml. The $(K)_{16}$GRGDSPC/TGF-β1 DNA complexes plus the fusogenic peptide mixtures with various $(K)_{16}$GRGDSPC/TGF-β1 DNA/fusogenic peptide w/w ratios were allowed to stand at room temperature for a further 10 min before adding to the cells.

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For complex formation involving both the peptide $(K)_{16}$GRGDSPC and Lipofectamine 2000, the peptide $(K)_{16}$GRGDSPC/TGF-β1 DNA complexes were prepared using the optimal amount of DNA, and the optimal peptide/DNA w/w ratio per 0.9 ml and Lipofectamine 2000 were prepared separately with various amounts per 0.1 ml. The peptide/DNA complexes were mixed with the Lipofectamine 2000 solution after they were allowed to stand for 30 min. The mixtures were gently vortexed for 2 min and allowed to stand for a further 10 min before adding to the cells. In these experiments, chloroquine was added to give the optimal concentration and the optimal Lipofectamine 2000 alone recommended by manufacture was used as control.

Transfection of BMSCs

The cultured BMSCs were washed thrice with serum-free complete DMEM medium and the medium was removed before the addition of 1.0 ml of the vector/DNA complexes to each well of a 24-well culture plate. Except for the specially mentioned steps, chloroquine was added to give an optimal final concentration, and gene transfection was performed as per the following protocol: The cells were incubated with the vector/DNA complexes for 4 h at 37 °C in 95% air and 5% CO_2, then an equal volume of complete medium containing 20% fetal bovine serum was added and appropriate volume of chloroquine was added again to give the optimal concentration. After 24 h of culture, the medium was replaced with 1 ml of complete culture medium containing 10% fetal bovine serum. For detection of reporter gene expression, the cells were harvested after a further 48 h culture (i.e. 72 h after gene transfer).

Reporter Gene Assays

Gene expressions of EGFP and TGF-β1 plasmid DNA were assayed. The experiments were repeated on six separate occasions. The empty vector pcDNA3.1 was transfected into BMSCs as a control.

Detection of EGFP gene expression. The transfected cells were washed thrice with PBS at 37 °C and then the gene expression was observed under a fluorescent microscope. The number of positive cells giving out green fluorescence was counted from five random fields of view under ×100 magnification. The total number of cells in these five fields was also counted and the percentage of positively transfected cells was calculated.

Detection of TGF-β1 gene expression. TGF-β1 gene expression was detected by using an ELISA kit according to the manufacture's instructions. When the cells were cultured for 72 h after gene transfer, the supernatants were aspirated respectively from the wells and the concentrations of secreted TGF-β1 protein were assayed. Subsequently, the optimal transfection conditions were screened out and the effects of the involved chloroquine, fusogenic peptide and Lipofectamine 2000 were evaluated. Then the transfected cells under optimal transfection conditions continued to culture with replaced complete DMEM medium containing 10% fetal bovine serum and antibiotic G418 at an initial concentration of 600 μg/ml. About a week later most of the cells were killed by G418 because the plasmids containing the anti-G418 marker gene (neo) failed to integrate into cell chromosomes. The surviving cells were selected with changed DMEM medium containing a decreased G418 of 200 μg/ml. When selected by G418 for about two weeks, positive clones were formed and then the stable transgenic cells were transferred from the wells to a 100-ml cell culture flask and were grown to a large scale. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot test were performed to detect TGF-β1 gene expression. The PCR primer pairs (P1: 5′-TCTGGTACCTGAACCCGTGTTG-3′; P2: 5′-TTCGGTACCTTGCTGTACTGCGT-3′) were designed to amplify a desired 582 bp fragment from the TGF-β1 cDNA. Western blot analysis was also performed to detect the expression of TGF-β1 protein as previous described [3].

RESULTS

Gene Expression of EGFP Plasmid DNA

Figure 1 demonstrates that under a fluorescent microscope, the transient transfected BMSCs of the treated group gave out a bright green fluorescence, whereas the untransfected cells were dark and invisible. The transient transfection efficiency was calculated to be 32.6 ± 5.1%. This direct viewing of EGFP expression indicated that our designed novel peptide $(K)_{16}$GRGDSPC was able to mediate the exogenous gene into BMSCs and the exogenous gene could effectively express in the host cells.

Gene Expression of TGF-β1 Plasmid DNA

Influences of physical properties of peptide (K)$_{16}$GRGDSPC/TGF-β1 DNA complexes and serum on integrin-mediated gene transfer. TGF-β1 plasmid DNA were transfected into BMSCs by the vector of the peptide $(K)_{16}$GRGDSPC for 72 h, and the expression levels of TGF-β1 were measured by ELISA kits. The results given in Figure 2(a) demonstrate that at 1 μg/ml of DNA or lower, there was very little gene transfer. Optimal gene transfer occurred with 2–4 μg/ml of

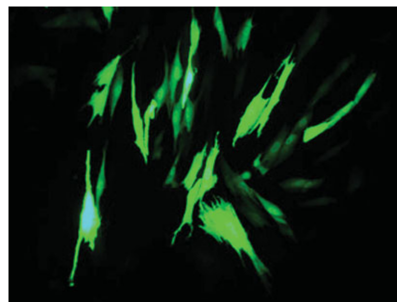

Figure 1 Visualization of exogenous EGFP gene delivery to bone marrow stromal cells mediated with a novel RGD-containing peptide vector. In the treated group, the positive cells (transient transfection cells) gave out a bright green fluorescence and the negative cells (untransfected cells) were invisible under a fluorescent microscope, whereas the control group gave out no green fluorescence.

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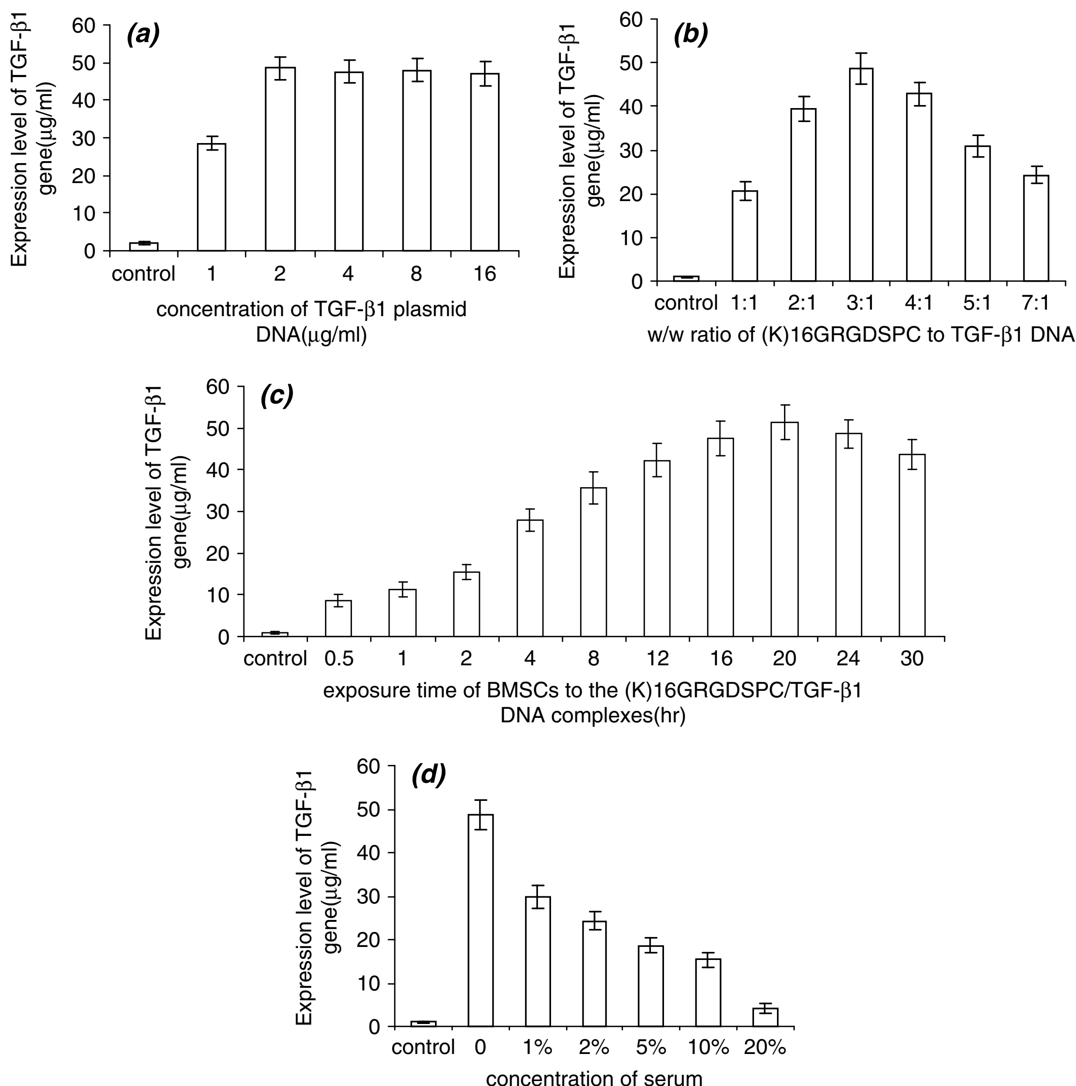

Figure 2 Influences of DNA concentration (a), peptide $(K)_{16}$GRGDSPC/TGF-β1 DNA w/w ratio (b), exposure time (c) and serum (d) on integrin-mediated gene transfer.

DNA. Higher DNA concentrations (up to 16 μg/ml) did not result in a better gene transfer. The results given in Figure 2(b) demonstrate that when the peptide $(K)_{16}$GRGDSPC/TGF-β1 DNA w/w ratio was 3:1 with a constant amount of 2 μg of DNA per well, optimal gene transfer was obtained. The results given in Figure 2(c) demonstrates that it was adverse to the gene expression when the time of the cells exposing to the peptide/DNA complexes was shorter than 4 h or longer than 24 h. The serum also showed great effects on the gene transfer. It can be seen in Figure 2(d) that even when the serum concentration was just 1% in the transfection complex solution in the initial 4 h of the gene transfer, gene transfer significantly decreased. When serum concentration increased to 20%, almost no gene transfer was observed.

Influences of endosomal disrupting agents on integrin-mediated gene transfer. It has been demonstrated that assistance with the escaping of peptide/DNA complexes from the endocytic vesicle to avoid degradation in the lysosomes was very crucial for obtaining a high gene transfer efficiency [4,5]. Two acid-dependent endocytic escape agents, chloroquine and a fusogenic peptide, were evaluated for gene transfer into BMSCs with the vector of peptide $(K)_{16}$GRGDSPC.

Figure 3(a) demonstrates that the peptide $(K)_{16}$GRGDSPC/TGF-β1 DNA complexes provided high-level gene delivery to BMSCs when supplemented with 100 μM chloroquine. Higher chloroquine concentrations did not result in desired higher gene transfer efficiency; it was even less effective probably because of chloroquine toxicity. Figure 3(b) demonstrates that chloroquine was indispensable and essential for the gene transfer, especially in the middle and late stages of the transfer. Figure 3(c) demonstrates that the time of exposure of the cells to chloroquine was also an obviously influencing factor on the gene transfer: it should

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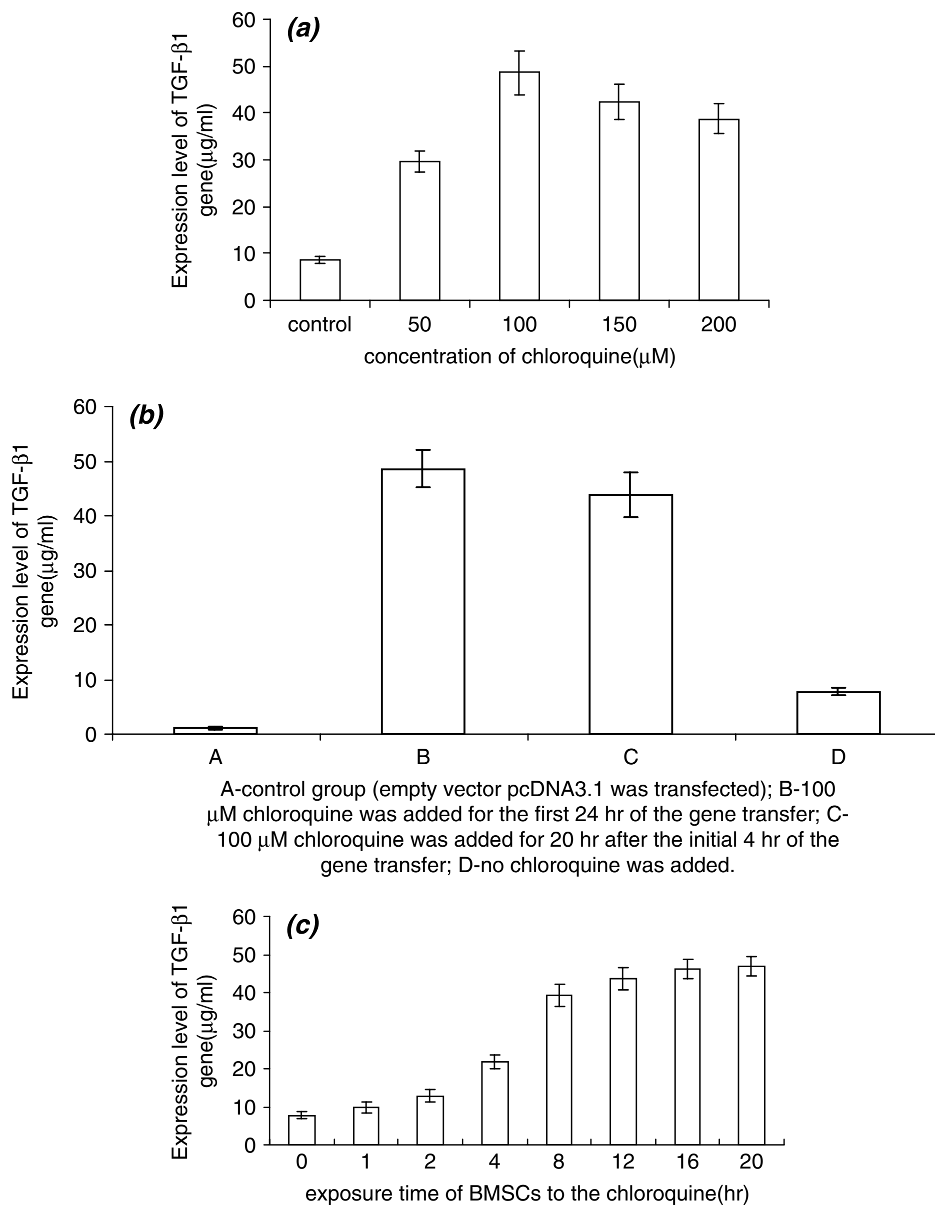

Figure 3 Influences of chloroquine on integrin-mediated gene transfer: (a) effect of concentration; (b) effect of addition under different conditions; (c) effect of exposure time.

be kept longer than 8 h. This result backed up the previous conclusion that chloroquine did not play an important part in the early stage of gene transfer, but it was very crucial in the middle and late stages of the transfer.

The appropriate volume of the stock solution of 1 mg/ml of fusogenic peptide in PBS was added to the peptide $(K)_{16}$GRGDSPC/TGF-β1 DNA complexes that were prepared at the optimal DNA concentration of 2 μg/ml and the optimal peptide/DNA w/w ratio of 3 : 1 at the end of the 30-min standing incubation at room temperature, to give a concentration of 3–30 μg/ml. The results given in Figure 4 demonstrate that the addition of this fusogenic peptide to $(K)_{16}$GRGDSPC/DNA complexes enhanced exogenous

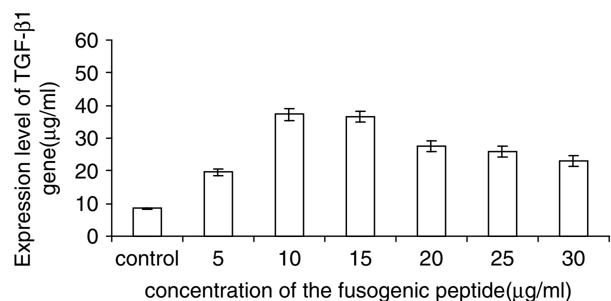

Figure 4 Influences of a fusogenic peptide on integrin-mediated gene transfer.

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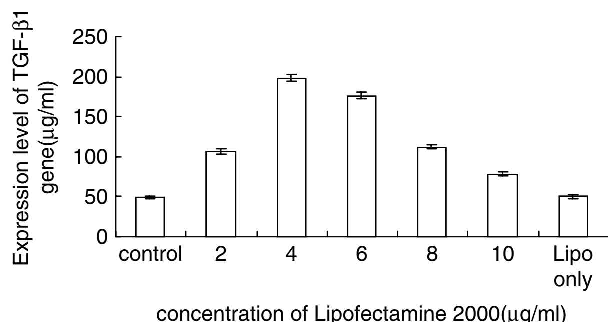

Figure 5 Influences of Lipofectamine 2000 on integrin-mediated gene transfer.

TGF-β1 gene transfer into BMSCs. The optimal concentration of 10 µg/ml of the fusogenic peptide corresponded to a $(K)_{16}$GRGDSPC/DNA/fusogenic peptide w/w ratio of 3:1:5 (i.e. 6:2 : 10 µg). The maximum level of TGF-β1 gene expression with the fusogenic peptide approached just 75% of that with the optimal chloroquine concentration of 100 µM.

The influence of Lipofectamine 2000. The results given in Figure 5 demonstrate that the addition of Lipofectamine 2000 significantly enhanced the gene transfer mediated by the non-viral vector of $(K)_{16}$GRGDSPC. The optimal effect was seen at $(K)_{16}$GRGDSPC/DNA/Lipofectamine 2000 w/w ratio of 3:1:2 (i.e. 6:2 : 4 µg). At this optimal ratio, TGF-β1 gene expression level was approximately 4 times as effective as the optimal chloroquine concentration and more than 5 times as effective as the optimal fusogenic peptide concentration with peptide $(K)_{16}$GRGDSPC. It was approximately 4 times as effective as Lipofectamine 2000 alone at the manufacturer-recommended Lipofectamine 2000/DNA w/w ratio of 6:1 using the same amount of TGF-β1 DNA. The transfection efficiency by the peptide $(K)_{16}$GRGDSPC under standard conditions (vector/DNA w/w ratio of 3:1, DNA concentration of 2 µg/ml and chloroquine concentration of 100 µM) was also shown to be equal to that by the lipid vector Lipofectamine 2000 alone.

Stable Expression of TGF-β1 Gene in BMSCs

BMSCs were transfected with TGF-β1 plasmid DNA under optimal transfection conditions. The cells were routinely incubated and selected with the antibiotic G418. The positive clones formed after about two weeks of the gene transfer (as shown in Figure 6). When they grew to a large scale, the stable transgenic cells were harvested with 0.25% trypsin and RT-PCR and Western Blot analysis were performed. After electrophoresis in a 1.5% agarose gel slice, TGF-β1 cDNA amplification products displayed a bright band, which accorded with desired fragment length (582 bp). The control group did not display any obvious band, which empty plasmid pcDNA3.1 was transferred into by the novel peptide vector and resulted in similar anti-G418 clones (as shown in Figure 7). Western blot analysis showed very similar results; i.e. a bright brown band was displayed in the transgenic group, which indicated that a large amount of TGF-β1 protein expressed in the stable transgenic BMSCs (as shown in Figure 8(a)), whereas there was no obvious band in the control group (as shown in Figure 8(b)).

Figure 6 Stable transgenic BMSCs clones.

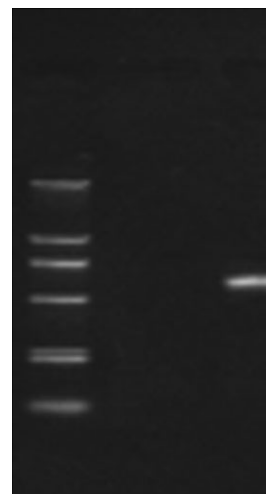

Figure 7 RT-PCR results: transgenic cells showed a bright band, whereas cells of control group showed none.

DISCUSSIONS

That ligands conjugated with cationic polymers to construct different types of novel composite gene delivery vectors is a very attractive topic in the research on

Figure 8 Western blot analysis results: transgenic cells displayed a bright brown band, whereas the control group displayed none.

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improving non-viral vectors. These receptor-mediated targeted gene delivery vector systems are bifunctional vehicles. On one hand, the ligands bind specially to their specific cell surface receptors with high-affinity; on the other, the cationic polymers package the plasmid DNA through electrostatic interactions between the positively charged polymers and the negatively charged phosphate backbones of the DNA. They can form highly condensed structures that allow internalization and protection of the packaged DNA from the effects of nucleases. Many different ligands such as asialoglycoprotein [6], tranferrin [7], lactose [8], mannose [9], monoclonal antibody fragments [10], insulin [11], nerve growth factor [12], vasoactive intestinal peptide [13], neurotensin [14], secretin [15] and folate [16] have been employed for targeting, while DNA-binding moiety usually uses high-molecular-weight poly-L-lysine, but occasionally polyethyleneimine, protamine, histone, non-histone and cyclodextrin polymers [17–20]. One obvious advantage of these non-viral gene delivery systems is their lack of packaging-related size constraints that limit the insert size of viral vectors [21]. Our project group designed a novel ligand-cationic polymer sequence that was not seen in publications. We chose an RGD-containing seven-peptide GRGDSPC as ligand and conjugated it to a DNA-binding moiety consisting of 16 lysine residues to construct a novel gene delivery system. The seven-peptide ligand had high affinities for integrin receptors of cell surfaces via the special RGD tripeptide motif. One important reason for choosing GRGDSPC as the ligand was that the GRGDSPC peptide had much higher affinities with BMSCs than RGD, GRGD and RGDS peptides [22]. The polylysine chain [$(K)_{16}$] electrostatically interacted with exogenous plasmid DNA and packaged the DNA and GRGDSPC peptide integrated with integrin receptors of seed cell surfaces via the special RGD tripeptide motif. Peptide $(K)_{16}$GRGDSPC/plasmid DNA complexes were effectively mediated into seed cells through a receptor-mediated internalization mechanism. The relatively small size of the oligo-L-lysine chain and the relatively small molecular weight of the composite vector probably avoided the problem of complement activation that was inherent in larger ligand–DNA constructs [23]. In addition, it was also considered that cysteine (2-amino-3-thiopropionic acid) had sulfhydryl group that was convenient for conjugation of the peptide with cross-linker agents. The peptide's conjugation with the heterobifunctional cross-linker sulfo-LC-SPDP and the following conjugation with biomimic matrix materials are very crucial steps for our next bone defect restoration studies in bone tissue engineering.

This RGD-containing peptide vector was synthesized with the poly-L-lysine domain $(K)_{16}$ attaching to the amino terminal rather than at the carboxyl terminal of the peptide. When $(K)_{16}$ was inserted at the carboxyl terminal, the protective radical groups of $(K)_{16}$ produced *t*-butyloxycarbonyl, which had hydrophobic interactions, and restrained $(K)_{16}$ from combination with RGD sequence. This change in position of the poly-L-lysine domain made the peptide hydrophilic and made peptide synthesis easier and with better yields. So, our designed peptide $(K)_{16}$GRGDSPC was synthesized from the carboxyl terminal to the amino terminal. It resulted in a satisfactory output of the desired peptide in which the GRGDSPC domain was synthesized in advance of the poly-L-lysine chain.

In contrast to the approximately 100% gene transfer efficiency of a viral vector, this novel peptide was less effective; it was also less effective than other non-viral vectors. But its advantages over viral vectors, such as ease of synthesis, low immunogenicity, low cytotoxity and high safety and absence of package-related size restrictions, are of great value for gene delivery to BMSCs, which are regarded as the most suitable seed cells for bone tissue engineering studies. Visualized gene transfer was exhibited by transferring the EGFP reporter gene. Fluorescence given out by GFP had no species-specificity and did not interfere with the adhesion, proliferation and differentiation functions of host cells [24]. When the EGFP gene was transfected into the cells for 24 h, the cells gave out green fluorescence *in situ*, which could be directly observed under a fluorescence microscope, by flow cytometry or using a laser scanning confocal microscope (LCSM). The green-fluorescence protein continuously and stably expressed in host cells and reached its maximum after 72 h of the gene transfer [25]. In our studies we observed that EGFP DNA-transfected BMSCs gave out bright green fluorescence under LCSM, whereas the cells of the control group did not give out any visible fluorescence. The results directly demonstrated that an exogenous gene could be transferred into BMSCs by our novel vector of peptide $(K)_{16}$GRGDSPC and the gene could effectively express in BMSCs.

TGF-β1 was a key growth factor for regulating BMSCs to differentiate into osteoblasts. TGF-β1 gene was potentially used as an ideal target gene for bone defect restoration in bone tissue engineering, which was transfected into seed cells by the peptide vector to produce tissue-engineered bones. For the vector of peptide $(K)_{16}$GRGDSPC, it was very essential to screen out the optimal transfection conditions and keep the transfection system at the optimal situation to obtain a high gene transfer efficiency. To realize their potentials, strategies were evolved for target cell binding, cellular internalization, endocytic exit, and so on. We sought to establish the parameters that would guide us in the use of this novel targeted non-viral vector system in the next bone tissue engineering studies *in vitro* and *in vivo*.

In our studies, we found that the vector of peptide $(K)_{16}$GRGDSPC presented optimal gene transfer when the concentration of TGF-β1 DNA was 2–4 μg/ml

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and the peptide/DNA w/w ratio was 3 : 1. Higher DNA concentration or w/w ratio did not yield the desired better gene expression. The two parameters affected the degree of saturation of the binding sites of the targeted cell surface. As a result, the rate of endocytosis of vector/DNA complexes and ultimately the expression level of target gene were affected. It was therefore important to use optimally effective concentration of the vector/DNA complexes. However, unnecessarily high concentrations would result in wastage of expensive reagents and increase the risk of unexpected side effects. As shown in Figure 2(a), optimal transfection efficiency was obtained in the range of 2–4 μg/ml of DNA. At higher concentrations of 8–16 μg/ml of DNA, there was no corresponding increase of gene expression possibly because enough vector/DNA complexes saturated the specific integrin receptor of cell surfaces and more complexes did not obviously promote the rate of endocytosis. But there was also no tendency to be reduced. Also, under routine microscopy the cells always appeared to grow well, suggesting that there was no obvious cytotoxicity at these high DNA concentrations. In addition, Figure 2(b) showed that transfection efficiency was related to the w/w ratio of peptide $(K)_{16}$GRGDSPC to DNA. The optimal gene transfer was obtained at the w/w ratio of 3 : 1 using a constant amount of DNA, and a lower or higher ratio resulted in a decrease in transfection efficiency. When the w/w ratio was too low, DNA was relatively excessive and not all of it could be packaged into peptide vector, and then the formation of the vector/DNA complexes was affected. When the w/w ratio was too high, excessive peptide $(K)_{16}$GRGDSPC would competitively inhibit the specific adhesion of the formed vector/DNA complexes to the integrin receptors of the cell surfaces, which would significantly decrease the transfection efficiency. So, the density and the total number of integrin receptors of the cell surfaces determined the most appropriate amount of vector/DNA complexes. Sufficient time of exposure of the cells to the vector/DNA complexes was needed for optimal gene delivery as shown in Figure 2(c). Though saturation of target sites occurred within only 5 min of exposure to vector/DNA complexes [26], BMSCs in this study were exposed to the vector/DNA complexes for at least half an hour. As shown in Figure 2(c), an incubation time of less than 4 h presented very poor gene transfer in spite of the continuous presence of chloroquine. These results suggested that the vector/DNA at the cell surfaces at shorter incubation times in the transfection medium contributed little to the DNA, which entered the nucleus for gene expression. This might be because the vector/DNA complexes at the cell surfaces were rapidly lost after removal of the transfection medium, or inactivated by the serum present in the normal culture medium. In any case, prolonged exposure of more than 4 h of the BMSCs to vector/DNA complexes was needed for effective gene transfer. However, too long exposure of more than 20 h had an adverse effect and gene transfer tended to be reduced. Probably, the peptide $(K)_{16}$GRGDSPC vector to some extent had a little cytotoxicity and affected cell growth and the gene transfer. Long-term absence of serum is another possible reason.

Serum has been reported to interfere with several non-viral DNA vector systems, particularly those relying on electrostatic interactions for the binding of the vector to DNA [27]. In the preceding section, we observed that 10% serum did not interfere with gene transfer when serum was added to the transfection medium after 4 h of the gene transfer. The possible reason was that after 4 h of the gene transfer, peptide $(K)_{16}$GRGDSPC/DNA complexes were already tightly bound to the cell surfaces and were sufficiently endocytosed into cytoplasm. But it was clearly demonstrated in Figure 2(d) that serum was a crucial influencing factor on the gene transfer, especially in the initial stage. In the presence of just 1% serum in the transfection medium in the initial 4 h of the gene transfer, there was a drastic reduction of transfection efficiency by approximately 50%. Gene transfer was almost completely inhibited by 20% serum.

Chloroquine, as an endosomal disrupting agent, was reported to have strong facilitative effects on peptide vector system [28]. Chloroquine was uncharged at neutral pH environments and transfects the lipid bilayers of cell membranes freely. Once it entered the acidic compartments such as endosomes and lysosomes, chloroquine became protonated and was not able to transfect the membranes any longer. This caused osmotic swelling of endocytic vesicles and then inhibited lysosomal enzymes by buffering the contents of these vesicles. The importance of chloroquine was emphasized by the fact that there was almost little transfection efficiency when no chloroquine was added to the transfection medium in the course of gene transfer. The results given in Figure 3(a) demonstrated that the optimal transfection efficiency was obtained with addition of 100 μM final concentration chloroquine. Higher concentrations resulted in a reduction of transfection efficiency because of its inevitable cytotoxicity. Also, it was demonstrated that chloroquine was not essential in the initial stage of the gene transfer. The time course from cell attachment to the presence of endocytosed macromolecules in the lysosomes was reported to be between 30 min and a few hours [29], which was consistent with our observations in this study that the requirement for chloroquine was not immediate but essential, especially in the middle and late stage of gene transfer.

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Fusogenic peptide was another type of endosomal disrupting agent. Our fusogenic peptide (NH_2-Gly-Leu-Phe-Glu-Ala-Leu-Leu-Glu-Leu-Leu-Glu-Ser-Leu-Trp-Glu-Leu-Leu-Leu-Glu-Ala-CO_2H) was based on the sequence of amino terminal amino acids of the influenza virus haemagglutinin. The amino acid residues were non-polar, except for five glutamate residues. The γ-carboxyl groups of the glutamate residues also became protonated and non-polar when the pH values in the endocytic compartment fell. The protonation of all five glutamates resulted in a hydrophobic stretch of amino acids, which then inserted into and disrupted the lipid bilayers. So, the fusogenic peptide was used to promote gene transfer. The optimal transfection was obtained when the fusogenic peptide concentration was 10 µg/ml corresponding to a $(K)_{16}$GRGDSPC/DNA/fusogenic peptide w/w ratio of 3:1:5 (i.e. 6:2 : 10 µg), and the maximum level of TGF-β1 gene expression approached just 75% of that obtained with the optimal chloroquine concentration of 100 µM. The fusogenic peptide was presumed to interact by electrostatic forces with the positively charged $(K)_{16}$GRGDSPC/DNA complexes. This could diminish the cell surface charge, and consequently favour aggregation and increase in size of the $(K)_{16}$GRGDSPC/DNA complexes. The change of physical property of the complexes was considered to promote the gene transfer.

Cationic lipids have been reported to enhance gene delivery mediated by viral or non-viral vectors [30,31], presumably because of their membrane-disruptive properties. Lipofectamine 2000 as a commercial cationic lipid is widely used in gene delivery. Optimal effects were observed with $(K)_{16}$GRGDSPC/DNA/Lipofectamine 2000 w/w ratio of 3:1:2 (i.e. 6:2 : 4 µg). At this optimal w/w ratio, TGF-β1 gene expression level was approximately 4 times as effective as the optimal chloroquine concentration and more than 5 times as effective as optimal fusogenic peptide concentrations with $(K)_{16}$GRGDSPC. It was also approximately 4 times as effective as Lipofectamine 2000 alone at manufacturer-recommended Lipofectamine 2000/DNA w/w ratio of 6:1 using a same amount of TGF-β1 plasmid DNA. These results indicated an obvious synergism. The transfection efficiency given by the peptide $(K)_{16}$GRGDSPC under standard conditions was shown to be equal to that by Lipofectamine 2000 alone. This meant that the transfection efficiency given by the novel peptide was satisfactory and the peptide had as promising an application perspective as the commercial lipid vector. In addition, in recent years, gramicidin (GRMN), amphiphilic gelling agents, specific bactericide, diphtheria venom, pseudomonal toxin transmembrane domain, histidine and rhinovirus-related synthetic peptide have been found to be able to buffer lysosomal enzyme and enhance receptor-mediated gene transfection efficiency [32–35]. These reagents can be considered to add to the peptide $(K)_{16}$GRGDSPC vector system. Their promoting effects need further study.

Finally, stable TGF-β1 gene expression in BMSCs was examined in our studies. The results from RT-PCR and Western blot analysis further demonstrated our previous conclusions that exogenous TGF-β1 gene could be effectively transfected into BMSCs by our novel peptide vector $(K)_{16}$GRGDSPC and the optimal transfection efficiency was relatively satisfactory despite it being much lower than viral vectors. Non-viral DNA vector systems rarely incorporate nuclear localization signals, and this could be the basis of the poor transfection efficiency reported for BMSCs and other cultures. In the case of the peptide $(K)_{16}$GRGDSPC, these considerations led Collins *et al.* to study gene delivery to the corneal endothelium of rabbit corneas early in the development of their RGD-containing oligopeptide vector system, because these cells rarely underwent mitosis [23]. The ability of oligopeptide to transfect the corneal endothelium with high efficiency [4], as well as the results reported here with post-mitotic BMSCs, suggested that nuclear translocation of the transfected DNA was not a key problem with this vector system. In our studies, the positive clones of BMSCs formed after about 2 week of continuous G418 selection and the transgenic cells grew to a large scale after at least 7–10 days culture, and then the stable gene expression was examined. This time course lasted for at least 3–4 weeks, which implied that TGF-β1 gene could continuously express in BMSCs for at least 3–4 weeks. This was very favourable for BMSCs to differentiate into osteoblasts under the control of TGF-β1.

In conclusion, we designed and synthesized the novel peptide $(K)_{16}$GRGDSPC, which was found to be an effective non-viral vector to mediate exogenous genes into BMSCs. The peptide vector is very promising in producing marked effects in the gene-activated bone matrix research to follow in the field of bone tissue engineering.

This novel integrin-targeted non-viral DNA vector system, consisting entirely of a synthetic peptide, probably represents an optimal approach to the use of polylysine and RGD motif in DNA vectors targeting the integrin and is ideally well suited to *ex vivo* gene delivery to BMSCs for experimental and clinical applications in the field of bone tissue engineering.

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