DNA nanoparticles encapsulated in 3D tissue-engineered scaffolds enhance osteogenic differentiation of mesenchymal stem cells

Hossein Hosseinkhani [1 *], Mohsen Hosseinkhani [2], Nathan P. Gabrielson [3], Daniel W. Pack [3 4], Ali Khademhosseini [5 6], Hisatoshi Kobayashi [7 8]

[1]International Center for Young Scientists (ICYS), National Institute for Materials Science (NIMS), Tsukuba, Ibaraki 305-0044, Japan

[2]Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University Hospital, Kyoto 606-8507, Japan

[3]Department of Chemical and Biomolecular Engineering, University of Illinois, Urbana, Illinois 61801

[4]Department of Bioengineering, University of Illinois, Urbana, Illinois 61801

[5]Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology (MIT), Cambridge, Massachusetts 02139

[6]Department of Medicine, Center for Biomedical Engineering, Brigham and Women's Hospital, Harvard Medical School, Cambridge, Massachusetts 02139

[7]Biomaterials Center, National Institute for Materials Science, Tsukuba, Ibaraki 305-0044, Japan

[8]Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo 113-8549, Japan

email: Hossein Hosseinkhani (hossein.hosseinkhani@nims.go.jp)

[*]Correspondence to Hossein Hosseinkhani, International Center for Young Scientists (ICYS), National Institute for Materials Science (NIMS), Tsukuba, Ibaraki 305-0044, Japan

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KEYWORDS

DNA nanoparticles • plasmid DNA • enhanced gene expression • scaffold • osteogenic differentiation

ABSTRACT

In this study, we enhanced the expression of a plasmid DNA in mesenchymal stem cells (MSC) by the combination of three-dimensional (3D) tissue-engineered scaffold and nonviral gene carrier. To function as an enhanced delivery of plasmid DNA, acetic anhydride was reacted with polyethylenimine (PEI) to acetylate 80% of the primary and 20% of the secondary amines (PEI-Ac_{80}). This acetylated PEI has been demonstrated to show enhanced gene-delivery efficiency over unmodified PEI. Collagen sponges reinforced by incorporating of poly(glycolic acid) (PGA) fibers were used as the scaffold material. DNA nanoparticles formed through simple mixing of plasmid DNA encoding bone morphogenetic protein-2 (BMP-2) and PEI-Ac_{80} solutions were encapsulated within these scaffolds. MSC were seeded into each scaffold and cultured for several weeks. Within these scaffolds, the level of BMP-2 expression by transfected MSC was significantly enhanced compared to MSC transfected by DNA nanoparticles in solution (in 2D tissue culture plates). Homogeneous bone formation was histologically observed throughout the sponges seeded with transfected MSC by using DNA nanoparticles after subcutaneous implantation into the back of rats. The level of alkaline phosphatase activity and osteocalcin content at the implanted sites of sponges seeded with transfected MSC by using DNA nanoparticles were significantly higher when compared with those seeded with other agents. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 2007

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ARTICLE TEXT

INTRODUCTION

The major aim of gene therapy is to deliver a gene into cells to enable functional repair of desired tissues for a variety of diseases. Recently, a number of DNA delivery systems have been investigated to improve the efficacy of gene transfection.[1-3] The two major approaches for gene delivery are based on viral-mediated and nonviral-mediated gene transfections.[4] However, considering the immunological and safety issues of viral vectors, the development of nonviral vector systems is of particular interest and therapeutic potential. There are several advantages in using nonviral vectors including the lower toxicity, lower immune responses, and decrease chance of integration of the viral genes into the genome of the cells. Therefore, gene delivery with nonviral vectors may be safer despite significantly lower expression efficiency.

For successful tissue regeneration, the renewable source of cells, such as stem cells, is required. In particular, mesenchymal stem cells (MSC) have been widely investigated to use either by themselves or combination with the scaffold for their applications to regenerative medicine. An emerging research approach with MSC is the combinational therapy of MSC with exogenous gene expression. The cells can not only function as the carrier vehicle to target genes to the site of action, but also positively participate in the process of tissue repair. Therefore, several studies have investigated therapy via tissue regeneration by genetically engineered MSC. Over the last decade, gene therapy has captured the scientific and public interest with the promise to deliver genes and proteins to specific tissues or to replace deficient host cell populations. Gene modification is preferred over addition of growth factor to the cell as, typically,[1] the half-life of the selected growth factor is short[2]; a single administration is usually not sufficient for a biological effect[3]; the quantities required are prohibitively expensive; and[4] continuous protein production increases the likelihood that a desired outcome will be achieved. In planning gene-therapy strategies for tissue engineering, one must consider two major avenues: direct gene delivery *in vivo* using viral or nonviral vectors or *in vitro* cell mediated gene therapy. In both cases, the aim is to deliver a therapeutic gene of a growth factor or cytokine, into the target tissue. Better understanding of molecular mechanism of MSC differentiation will eventually allow us to properly manipulate MSC both *in vivo* and *in vitro* for the regeneration of tissues and organs. Nolta et al.[5] and Bartholomew et al.[6] used retroviral vectors to genetically engineer human bone marrow stromal cells to express interleukin-3 (IL-3), green fluorescent protein (GFP), and human erythropoietin. These cells were transplanted into immunodeficient mice or were loaded into immunoisolatory devices (IIDs) and surgically implanted into either autologous or allogeneic baboon recipients for various therapeutic applications. Successful bone regeneration by MSC genetically engineered by retrovirus or adenovirus vector carrying human BMP-2, -4, or -7 genes has also been reported.[7-12] In addition, collagen matrices have been used for gene delivery and bone tissue formation.[13-16] For example, Fang et al.[15] and Bonadio et al.[16] have used collagen matrices to deliver DNA for the induction of bone formation *in vivo*.[15][16]

Recently, a system using genetically engineered urothelial cells was developed for *in vivo* gene therapy.[17] Cells seeded onto 3D polymer scaffolds were genetically modified, and formed an organlike structure with stable expression of the transgene *in vivo*. This paradigm provided a proof of principle for the use of tissue-engineering techniques to improve methods of gene transduction. The 3D transgene cell construct can be potentially used as therapeutic cell-based gene delivery or as an *in vitro* model system for the testing of genetic manipulations to study the effects of gene expression on tissue development.

The objective of this study was to investigate the effect of 3D-culture system on gene transfection of MSC and *in vivo* bone formation. The gene-delivery vector used was produced by reacting acetic anhydride with the primary and secondary amine groups of polyethylenimine (PEI), generating an acetylated PEI that was capable to from DNA nanoparticles when mixed with plasmid DNA. In recent publications, Pack and coworkers reported that a new class of polycations was capable to condense DNA and transfecting genes to various cell lines with relatively high efficiency.[18][19] This new class of polycations has been shown to transfect HEK293, MDA-MB-231, and C2C12 cell lines with efficiencies 30- to 60-fold higher than unmodified PEI.[19] The acetylated PEI materials have been reported in two previous publications.[18][19] Acetylation of PEI decreases cytoxicity, compared to unmodified PEI, in several cell lines as reported by Gabrielson et al.[18] Collagen sponge reinforced by incorporation of poly(glycolic acid) (PGA) fibers was selected as the cell scaffold and was used to evaluate effect of the 3D-culture system and cell scaffold type on the transfection efficiency of DNA nanoparticles (composed of PEI-Ac_{80} and plasmid DNA encoding BMP-2) on MSC. The osteoinduction activity of the scaffolds seeded with transfected MSC was compared with other control agents. We studied bone regeneration by subcutaneous implantation of scaffolds in the back of rats in terms of histological and biochemical examinations.

MATERIALS AND METHODS

Materials

An aqueous solution of type I collagen, prepared from porcine tendon by pepsin treatment (3 mg/mL, pH 3.0) in HCl, was kindly supplied by Nitta Gelatin, Osaka, Japan. The nonwoven fabric prepared from PGA fiber of 20 μm in diameter (0.5 mm thickness, 200-210 g/m^2) was obtained from Gunze, Kyoto, Japan. Branched PEI with an average molecular weight of 25 kDa and acetic anhydride were purchased from Sigma Chemical (St. Louis, MO) and used without further purification. All solvent and reagents were of analytical grade and were used as received.

Preparation of plasmid DNA

A pBacBH2 was used in this study. The pBacBH2 is the expression vector consisting of coding sequence of hBMP-2 inserted at the downstream into polyhedrin promoter of baculovirus (pBacBH2, 1230 bp). The nucleotide sequence surrounding the site of recombination including the initiation codon of the construct (pBacBH2) is shown in Figure 1. Construction source of pBacBH2 has been reported in detail previously.[20] The plasmid DNA was amplified in a transformant of *Escherichia coli* bacteria and isolated from the bacteria by Qiagen Maxi kit-25 (Qiagen K.K., Tokyo, Japan). The absorbance ratio at the wavelength of 260-280 nm to assess the purity of plasmid DNA was measured to be between 1.8 and 2.0.

Figure 1. Structure of the recombinant transfer vector, pBacBH2. A BamHI

fragment containing hBMP-2 cDNA (318-1547:1230 bp) was inserted into the baculovirus transfer vector pBlueBacII downstream of polyhedrin promoter including the initiation codon.
[Normal View 25K | Magnified View 65K]

Acetylation and characterization of PEI

Branched 25 kDa PEI (0.5 g) was transferred to a 20-mL scintillation vial and dissolved in 3 mL of freshly distilled methanol. Sufficient acetic anhydride to achieve the desired degree of acetylation was added and the vial was sealed. The reaction was carried out at 60° C with stirring for 4 h. The reaction was quenched with double-distilled water (0.5 mL). The solvent was removed under reduced pressure. The remaining solution was purified by gel filtration chromatography (PD-10 columns, GE Healthcare, Uppsala, Sweden) and eluted with doubled distilled water. The purified polymers were then concentrated and stored at -80°C. To determine the extent of acetylation, the polymer was dissolved in D_2O and ^1H NMR spectra were acquired on a Varian Unity 400 with a 5-mm probe. The extent of primary and secondary amine acetylation was determined by peak integration using the following formula:

$$\text{degree of primary amine acetylation (\%)} = \left(\frac{E_{bb}}{4} \times \frac{3}{M'} \times N'\right)^{-1} \times 100\%$$

$$\text{degree of secondary amine acetylation (\%)} = \left(\frac{E_{bb}}{4} \times \frac{3}{M''} \times N''\right)^{-1} \times 100\%$$

where M' is the integration of δ 1.7-1.75 peaks (R-NHCOC$\mathbf{H}_3$, acetylated primary amines), M'' is the integration of δ 1.8-1.85 peaks (R_2-NCOC$\mathbf{H}_3$, acetylated secondary amines), and E_{bb} is the integration of δ 2.3-2.8 peaks ([C$\mathbf{H}_2$C$\mathbf{H}_2$N]$_x$[C$\mathbf{H}_2$C$\mathbf{H}_2$NH]$_y$[C$\mathbf{H}_2$C$\mathbf{H}_2$NH_2]$_z$, ethylene backbone). N' and N'' are mole fractions of primary and secondary amines of the PEI starting material, respectively.

Preparation of DNA nanoparticles

DNA nanoparticles were prepared at room temperature by dissolving the desired amount of DNA in 150 m*M* NaCl, 20 m*M* PIPES, pH 7.2, and subsequently adding an equal volume of polymer solution, similarly dissolved, to achieve the desired polymer/DNA weight ratio of 3:1. The combined solution was gently agitated at 4°C and allowed to incubate for 30 min to form DNA nanoparticles.

Electrophoresis of DNA nanoparticles

Formation of DNA nanoparticles was confirmed by electrophoresis. A solution of DNA (pGL3, Promega, Madison, WI) at 1 μg/10 μL) was prepared in 150 m*M* NaCl, 20 m*M* PIPES at pH 7.2. Appropriate amounts of each polymer dissolved in double-distilled water were added to 10 μL of the DNA solution to achieve the desired polymer/DNA weight ratio. Polyplexes were incubated at 4°C for 30 min, after which loading dye was added and 10 μL of the solution was run on a 0.75% agarose gel (70 V, 70 min). DNA was visualized with ethidium bromide.

Measurement of DNA nanoparticles size

DNA nanoparticles were formed with pGL3 in 150 m*M* NaCl, 20 m*M* PIPES, pH 7.2 at the optimum weight ratio for each of the modified and unmodified polymers. Following incubation at 4°C, the polyplexes were subjected to size measurement on a 90Plus Partical Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY). Five scans were performed for each sample.

Preparation and culture of MSC

MSC were isolated from the bone shaft of femurs of 3-week-old male Fischer F344 rats according to the technique reported by Lennon et al.[21] Briefly, both the ends of rat femurs were cut away from the epiphysis and the bone marrow was flushed out by a syringe (21-gauge needle) with 1 mL of Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 15% fetal calf serum (FCS) and 50 U/mL penicillin and streptomycin. The cell suspension (5 mL) was placed into two T-25 flasks (Iwaki Glass, Funabashi, Chiba, Japan) and cultured at 37°C in a 95% air-5% CO_2 atmosphere. The nonadhered cells and impurities were removed from flasks each time during the exchange of the medium. The medium was changed on the fourth day of culture and every 3 days thereafter. When the cells of the first passage became subconfluent, usually 7-10 days after seeding, the cells were detached from the flask by treatment for 5 min at 37°C with PBS solution of 0.25 wt % trypsin and 0.02 wt % ethylenediaminetetraacetic acid (EDTA). Cells were normally subcultured at a density of 2×10^4 cells/cm^2. The cells of second-passage at subconfluence were used for all the experiments.

Fabrication of collagen sponge incorporating PGA fiber

Collagen sponges incorporating different amounts of PGA fiber were fabricated by the conventional freeze-drying method, followed by cross-linking of combined dehydrothermal, glutaraldehyde, and UV. Briefly, the PGA-nonwoven fabric was immersed in acetone for 1 h to remove oils and fats, and rinsed three times by DDW at 25°C for 10 min. The PGA-nonwoven fabric was loosened with a tweezers to obtain the component of PGA fibers. PGA fibers loosened at weights of 1.5, 3, 6, and 12 mg were homogeneously placed into a polystyrene mold (Costar, 24 well; Corning Life Sciences, New York), and then 0.75 mL of collagen solution was poured into each mold. The resulting collagen solution was frozen at -20°C and freeze-dried to obtain a collagen sponge incorporating PGA fiber. The freeze-dried sponge was dehydrothermally cross-linked at 140°C for 12 h under 0.1-torr vacuum conditions and additionally underwent chemical cross-linking with 0.4 wt % of glutaraldehyde solution in 0.2 vol % acetic acid for 24 h at 4°C. Following DDW

washing, the sponge was freeze-dried and additionally underwent UV cross-linking for 1 h at 100 V in a FUNA-UV-LINKER FS-1500 (Funakoshi, Japan), and then sterilized by ethylene oxide gas at 40°C. A similar preparation procedure was performed only for collagen solution to obtain collagen sponge without PGA fiber incorporation. This crosslinking procedure is the same as that for preparation of artificial dermis reported previously.[22] Our previous study demonstrated that 12 mg of PGA fiber incorporation (collagen/PGA weight mixing ratio of 0.2) was the best amount to fabricate a sponge with higher resistance to compression and *in vitro* cells proliferation.[23] On the basis of the result, 12 mg of PGA fiber incorporation was used in this study.

Mechanical measurement

The compression test was done for dried collagen sponge with or without PGA fiber incorporation at room temperature on an autograph machine (AGS-5D, Shimadzu, Kyoto, Japan) at a cross-head speed of 0.5 mm/min. The compression modulus was calculated from the slope of the initial linear portion of the stress-strain curve. The experiment was performed for five sponge samples independently.

Cell seeding into collagen sponge scaffolds

MSC were seeded into the scaffolds of collagen sponges with PGA fiber incorporation according to an agitated seeding method to uniformly seed the cells into the porous 3D-cell scaffold.[24] Briefly, 0.5 mL of cell suspension (1×10^6 cells/mL) and the scaffold were placed in 12-mL tubes (Iwaki Glass, Funabashi, Japan) on an orbital shaker (Bellco Glass, Vineland, NJ) and agitated at 37°C at 300 rpm for 6 h. The MSC-seeded scaffolds were thoroughly washed with PBS to exclude nonadherent cells and subjected to the following experiments.

In vitro transfection of MSC by DNA nanoparticles

2D- and 3D-static methods were used to culture MSC for their plasmid DNA transfection. Briefly, 0.4 mL of DNA nanoparticles solution was incorporated into the dried scaffolds of collagen sponges with PGA fiber incorporation by simple solution dropping, followed by leaving overnight at 25°C in a clean bench. Next, MSC (1×10^6 cells/mL) were seeded into the scaffolds according to the procedure mentioned earlier. As controls, 0.4 mL of plasmid DNA solution and culture medium containing MSC (1×10^6 cells/mL) were incorporated into the scaffolds similarly.

In the first method (2D), the MSC suspension (1×10^6 cells/mL) was plated into each well of 24-well culture plates (Code 3800-6100, Iwaki brand, Scitech Div. Asahi Techno Glass, Chiba, Japan) and cultured at 37°C in a 95% air-5% CO_2 atmosphere to reach cell confluency for about 70%. The DNA nanoparticles solution was then added to each well and incubated for 4 h and washed. Then, the cells were incubated for additional 2 days, washed twice with 1 mL of PBS, and lysed by 100 μL of a lysis buffer (0.1*M* Tris-HCl, 2 m*M* EDTA, 0.1% Triton X-100). The cell lysate was kept at -80°C to measure the transfection level and protein amount. In the second method (3D), the scaffolds incorporated with the DNA nanoparticles were placed in each well of 24-well culture plates and then the MSC suspension (1×10^6 cells/mL) was inoculated into the scaffolds, followed by incubation for 2 days at 37°C in a 95% air-5% CO_2 atmosphere. A similar *in vitro* transfection was done for unmodified 25-kDa PEI.

To measure the level of gene transfection of MSC cultured by the 3D method, the scaffolds collected were washed three times with PBS, cut down with a scissors, and homogenized in the lysis buffer (0.1*M* Tris-HCl, 2 m*M* EDTA, 0.1% Triton X-100). The sample lysate (2 mL) was centrifuged at 12,000 rpm for 5 min at 4°C, and the supernatant was carefully collected and kept in the ice. To measure the expression level of BMP-2 gene, 50 μL of the supernatant was collected and the BMP-2 protein was determined by a human BMP-2 ELISA Kit (AN'ALYZA, Human BMP-2 Immunoassay System, TECHNE, MN). Each experiment was carried out three times independently. The protein concentration of the lysate was also assayed by the Lowry kit (Lot. No. L8900, Nakalai Tesque, Kyoto, Japan). Briefly, 50 μL of cell lysate was mixed with 1 mL of the kit test solution, followed by leaving for 10 min at 25°C. After addition 0.1 mL of 1*N* phenol aqueous solution, the solution mixture was incubated for 30 min at 25°C and the absorbance was determined at the wavelength of 750 nm. The protein concentration was calculated based on the calibration curve prepared by the use of standard albumin solutions.

In vivo experiment

All procedures were performed in accordance to the specifications of Guideline for Animal Experiments of National Institute for Materials Science (NIMS). Wistar male rats, age 6 weeks were anesthetized by intraperitoneal injection (3.0 mg/100 g body weight) of chloral hydrate (Wako Pure Chemical Industries, Osaka, Japan) shortly after superficially induced anesthesia by ether inhalation. On each side of the back of a rat, three small incisions were made and extended subcutaneously with forceps. Three pieces of collagen sponge with PGA fiber incorporation seeded with transfected MSC by 3D-culture method were implanted subcutaneously into the three sites on the right side of the back and three counterparts from the control group into the left side. The wounds were then closed with 4-0 silk sutures. As controls, scaffolds incorporated with PBS, naked plasmid DNA, or that containing the MSC was used. Each experiment was done for six rats independently unless mentioned otherwise.

Soft X-ray observation

After 3 weeks, rats were sacrificed with cervical dislocation, and the skin tissue including the scaffold-implanted site was taken out and radiographically examined by soft X-rays (Hitex HX-100, Hitachi, Japan) at 54 KVP and 2.5mA for 20 s.

Assessment of the bone mineral density

The bone mineral density (BMD) of new bone formed was measured using Dual Energy X-ray Absorptometry (DEXA) utilizing a bone mineral analyzer (Dichroma Scan 600, Aloka, Tokyo, Japan) at 4 weeks after treatment with collagen sponge. The instrument was calibrated with a phantom of known mineral content. Each scan was performed at a speed of 20 mm s^{-1} and the scanning length was 1 mm. DEXA measurement was performed for six samples per each experimental group and the region of interest (ROI) for each sample was 1×1 cm^2.

Histological analysis

The implants were harvested for histological analysis 3 weeks after implantation. For each time point, scaffolds from either group were obtained for histological analysis. Once they were removed from the subcutaneous sites on the back of rats, the scaffolds were fixed in 10 wt % neutral buffered formalin solution, dehydrated in sequentially increasing ethanol solutions to 100 vol % ethanol, immersed in xylene, and embedded in paraffin. The skin tissues were cross-sectioned to 5-μm thickness with a Tissue-Tek (OCT compound, Miles) and stained with Mayer's hematoxylin-eosin (H-E) solution. These specimens were observed on Olympus AX-80 fluorescence microscope equipped with Olympus DP50 digital camera (KS Olympus, Tokyo, Japan).

In vitro and *In vivo* osteogenic evaluation

To analyze the osteogenic differentiation of MSC, the intracellular alkaline phosphatase (ALP) activity and bone osteocalcin (OCN) content were determined. ALP activity was determined by the *p*-nitrophenylphosphate (*p*NPP) hydrolysis method using the ALP Assay Kit (Lot. No. TJ791, Wako Pure Chemical Industries, Osaka, Japan). According to the procedure mentioned earlier, the DNA nanoparticles were incorporated into collagen sponges with PGA fiber incorporation. The scaffolds were then seeded with MSC and the MSC transfection experiment was performed. The scaffolds seeded with transfected MSC were then implanted subcutaneously into back of rat for *in vivo* ectopic bone evaluation. In *in vitro* experiments, the scaffolds collected were washed thrice with PBS, cut down with a scissors, and in the *in vivo* experiments, the skin tissue including the scaffold-implanted site was taken out after 1, 2, 3, and 4 weeks. The tissues obtained were freeze-dried and crushed. The scaffolds and the crushed tissues (5 mg) were homogenized in the lysis buffer (0.2% IGEPAL CA-630, 10 m*M* Tris-HCL, 1 m*M* $MgCl_2$, pH 7.5). The sample lysate (2 mL) was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was assayed for ALP activity, using *p*-nitrophenyl-phosphate as substrate. To each well of 96-well culture plates (well area = 28.26 mm^2, Code 3526, Corning, NY), an aliquot (2.5 μL) of supernatant was added to 25 μL of 56 m*M* 2-amino-2-methyl-1,3-propanediol (pH 9.8) containing 10 mL *p*-nitrophenyl-phosphate with 1 m*M* $MgCl_2$, and the mixture was incubated at 37°C for 30 min. Then, 250 μL of 0.02*N* NaOH was added to the wells to stop the reaction before absorption at 405 nm and was measured with a spectrophotometer. ALP was determined as millimoles of *p*-nitrophenyl released per scaffold after 30-min incubation.

To determine the OCN content of cells, the scaffolds collected and the crushed tissue (5 mg) were washed thrice with PBS and then treated with 1 mL of 40 vol % formic acid for 10 days at 4°C under vortex mixing to decalcify. After the decalcification process, the cell extraction was applied to a Sephadex™ G-25 column (PD-10, Amersham Pharmacia Biotech, Sweden) for gel filtration. The resulting solution was freeze-dried and subjected to an OCN rat enzyme-linked immunosorbent assay (ELISA) (rat osteocalcin ELISA system, Amersham Bioscience, Tokyo, Japan).

Statistical analysis

All the data were statistically analyzed to express the mean ± the standard deviation (SD) of the mean. Student's *t* test was performed and $p < 0.05$ was accepted to be significant.

RESULTS

Preparation and characterization of acetylated PEI and DNA nanoparticles

Acetylated PEI was prepared by reacting acetic anhydride and PEI (Fig. 2). The extent of acetylation was determined by ^1H NMR. The reaction yielded a modified PEI with 80% of its primary amines and 20% of its secondary amines acetylated.

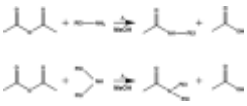


Figure 2. Synthesis of acetylated PEI. Primary amines of PEI react with acetic anhydride to form secondary amines and secondary amines react with acetic anhydride to generate tertiary amines.
[Normal View 14K | Magnified View 30K]

Figure 3 shows the electrophoretic patterns for DNA nanoparticles at different polymer:DNA weight ratios. PEI-Ac_{80} was able to condense plasmid DNA and prevented its migration through an agarose gel with the addition of 0.6 μg of polymer per 1 μg of DNA, well below the optimum transfection weight ratio of 3 μg of polymer per 1 μg of DNA. Unmodified PEI is able to condense plasmid DNA with the addition of as little as 0.1 μg of polymer per 1 μg of DNA (data not shown), as indicated in previous study by Pack et al.[25]

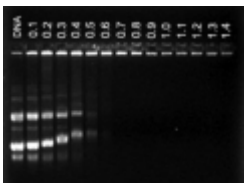
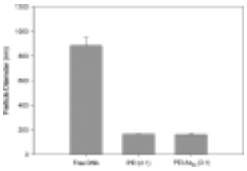


Figure 3. Electrophoretic patterns of DNA nanoparticles formed with DNA nanoparticles formed with PEI-Ac_{80} at various weight ratios. The polymer to DNA weight ratio used to form each complex is given above the corresponding lane.
[Normal View 18K | Magnified View 52K]

Figure 4 shows the apparent molecular size of naked plasmid DNA and DNA nanoparticles as determined by dynamic light scattering. The formation of DNA nanoparticles with PEI-Ac_{80} caused the apparent molecular size of plasmid DNA to be reduced from 881.2 ± 71.8 nm to about 161.2 ± 1.8 nm. The size of DNA nanoparticles formed with acetylated PEI was comparable to those formed with unmodified PEI.

Figure 4. Apparent molecular size of free plasmid DNA, DNA nanoparticles formed with PEI-Ac_{80} prepared at the indicated optimum transfection weight ratios, shown in parentheses.



[Normal View 10K | Magnified View 23K]

Morphology of collagen sponges incorporating PGA fiber and mechanical property

Figure 5(A-C) shows the appearance of PGA fibers component to be incorporated and collagen sponges with or without PGA fiber incorporation. SEM photographs of cross-sections of collagen sponges with or without incorporating PGA fiber are shown in Figure 5 (D) and (E). Irrespective of the fiber amount incorporated, by direct observation on SEM results, we found that every collagen sponge possessed an interconnected porous structure with an average pore size of 180 μm, and their intrastructural appearance was similar. Although some PGA fibers were exposed in the pores of PGA-incorporated sponges, the internal structure was similar to that of sponges without PGA fiber incorporation.

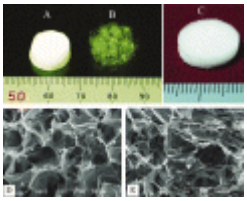


Figure 5. Light microscopic photographs of a collagen sponge without PGA fiber incorporation (A), a frame structure of PGA fiber incorporated (B), a collagen sponge incorporating PGA fiber at a collagen/PGA weight ratios of 0.2 (C), and cross-sectional SEM photographs of a collagen sponge without PGA fiber incorporation (D) and collagen sponge incorporating PGA fiber at collagen/PGA weight ratios of 0.2 (E). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
[Normal View 71K | Magnified View 278K]

Figure 6 shows the compression modulus of collagen sponge with or without PGA fiber incorporation. Incorporation of PGA fiber enabled collagen sponge to increase the compression modulus. Collagen sponge with the highest amount of PGA fiber incorporated exhibited a much higher modulus.

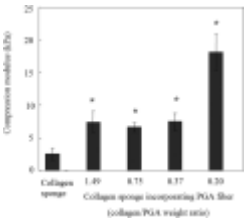


Figure 6. Compression modulus of collagen sponges with or without PGA fiber incorporation. *$p < 0.05$; significant against the compression modulus of collagen sponge.
[Normal View 17K | Magnified View 39K]

In vitro transfection of plasmid DNA into MSC

Figure 7 shows *in vitro* BMP-2 expression of MSC cultured by 2D and 3D methods 2 days after transfection with naked plasmid DNA-BMP-2, DNA nanoparticles formed with PEI-Ac_{80} or unmodified PEI. The optimal polymer/DNA weight mixing ratio for PEI-Ac_{80} and unmodified PEI was found to be 3:1 and 2:1, respectively (data are not shown). The highest level of BMP-2 expression was observed for the dose of plasmid DNA and the transfection time to be 0.01 μg/μL and 2 days after transfection (data are not shown). 3D-culture method enhanced the level of BMP-2 expression when compared with the 2D culture method. Acetylated PEI significantly enhanced the level of BMP-2 expression when compared with unmodified PEI. However, naked plasmid DNA-BMP-2 impregnated into scaffold did not enhance the level of gene expression for 3D-culture method and the expression level was similar to that of 2D culture method.

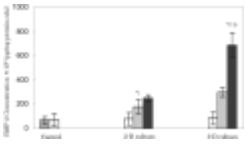


Figure 7. *In vitro* BMP-2 expression of MSC cultured by the 2D- and 3D-culture methods 2 days after transfection with naked plasmid DNA-BMP-2 (□), DNA nanoparticles formed with unmodified PEI ( ), and DNA nanoparticles formed with PEI-Ac_{80} (▪) incorporated into collagen sponges with PGA fiber incorporation at a collagen/PGA fiber weight ratio of 0.2. MSC were cultured by 2D culture method (▨) and 3D-culture method in collagen sponges with PGA fiber incorporation (▩) without any treatment as a control group. The dose of plasmid DNA was 0.01 μg/μL. *$p < 0.05$; significant against the expression level of control group. †$p < 0.05$; significant against the expression level of 2D group at the corresponding group. ‡$p < 0.05$; significant against the expression level of naked plasmid DNA group at the corresponding group. §$p < 0.05$; significant against the expression level of DNA nanoparticles formed with unmodified PEI at the corresponding group.
[Normal View 10K | Magnified View 24K]

Osteogenic differentiation of MSC transfected with DNA nanoparticles by 2D- and 3D-culture methods

Figure 8(A) and (B) shows the time course of the ALP activity and OCN content of MSC cultured by 3D-culture method after transfection with naked plasmid DNA-BMP-2, DNA nanoparticles formed with PEI-Ac_{80} or unmodified PEI. Significantly, higher ALP activity was detected for MSC transfected by the 3D method than the 2D method (ALP activity data for 2D method are not shown). In the 3D-culturing group, the ALP activity increased with time for the initial 2 weeks and thereafter leveled off, although the level was still high compared with that of 2D system. Irrespective of the culture method, the ALP activity of MSC transfected with the DNA

nanoparticles formed with PEI-Ac_{80} was always high when compared with that of unmodified PEI and naked plasmid DNA-BMP-2. The 3D-culture method exhibited the highest OCN content when compared with 2D culture method.

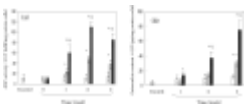


Figure 8. Time-course of ALP activity (A) and OCN contents (B) of MSC cultured by 3D-culture method after transfection with naked plasmid DNA-BMP-2 (□), DNA nanoparticles formed with unmodified PEI. ( ), and DNA nanoparticles formed with PEI-Ac_{80} (▪) incorporated into collagen sponges with PGA fiber incorporation at a collagen/PGA fiber weight ratio of 0.2. MSC were cultured by -D culture method in collagen sponges with PGA fiber incorporation without any treatment as a control group. The dose of plasmid DNA was 0.01 μg/μL. *$p < 0.05$; significant against the ALP activity or the OCN contents of control group. †$p < 0.05$; significant against the ALP activity or the osteocalcin contents of naked plasmid DNA group at the corresponding week. ‡$p < 0.05$; significant against the ALP activity or the osteocalcin contents of DNA nanoparticles formed with unmodified PEI group at the corresponding week.
[Normal View 17K | Magnified View 47K]

In vivo experiment

Figure 9 shows H-E-stained cross-sections and soft X-ray photographs of the subcutaneous tissues 3 weeks after implantation of collagen sponges with PGA fiber incorporation seeded with PBS, MSC, naked plasmid DNA-BMP-2, DNA nanoparticles formed with PEI, DNA nanoparticles formed with PEI-Ac_{80}, and transfected MSC by using DNA nanoparticles formed with PEI-Ac_{80} or unmodified PEI in 3D-culture method. As is apparent, H-E staining demonstrated that both collagen sponges with PGA fiber incorporation seeded with transfected MSC by using DNA nanoparticles formed with PEI-Ac_{80} or unmodified PEI-induced bone formation in the subcutaneous tissue, although the extent of bone formation was higher in the sponges seeded with transfected MSC by using DNA nanoparticles formed with PEI-Ac_{80}. Apparently, the edge of implants and the subcutaneous tissue became radiopaque strongly on the X-ray film when MSC transfected by DNA nanoparticles formed with PEI-Ac_{80} or unmodified PEI, whereas no radiopaque area was found at the scaffolds and skin when implanted with PBS, naked plasmid DNA-BMP-2, DNA nanoparticles formed with PEI, DNA nanoparticles formed with PEI-Ac_{80}, and MSC. The radiopaque area was larger for MSC transfected by DNA nanoparticles formed with PEI-Ac_{80} than DNA nanoparticles formed with unmodified PEI.

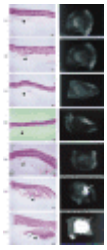


Figure 9. Histological cross-sections and soft X-ray photographs of ectopically formed bone 3 weeks after implantation of collagen sponges with PGA fiber incorporation at collagen/PGA fiber weight ratio of 0.2 impregnated with PBS (A), MSC (B), naked plasmid DNA (C), DNA nanoparticles formed with PEI (D), DNA nanoparticles formed with PEI-Ac_{80} (E), transfected MSC by 3D method by DNA nanoparticles formed with unmodified PEI (F), and transfected MSC by 3D method by DNA nanoparticles formed with PEI-Ac_{80} (G). The dose of plasmid DNA was 0.01 μg/μL. Each specimen subjected to H-E staining. Arrows and arrowheads indicate the newly formed bone and the residual collagen sponge, respectively. The scale bar measures 1 mm in full cross section. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
[Normal View 98K | Magnified View 427K]

Figure 10 shows the results of BMD of the subcutaneous tissues 3 weeks after implantation of collagen sponges with PGA fiber incorporation seeded with PBS, MSC, naked plasmid DNA-BMP-2, DNA nanoparticles formed with PEI, DNA nanoparticles formed with PEI-Ac_{80}, and transfected MSC by using DNA nanoparticles formed with PEI-Ac_{80} or unmodified PEI in 3D-culture method. Significantly, higher amount of BMD was observed when MSC transfected by DNA nanoparticles formed with PEI-Ac_{80}.

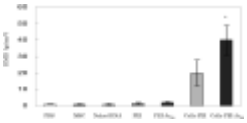


Figure 10. Bone mineral density (BMD) of tissues around the implanted site of rats 3 weeks after implantation of collagen sponges with PGA fiber incorporation at collagen/PGA fiber weight ratio of 0.2 impregnated with PBS (□), MSC ( ), naked plasmid DNA ( ), DNA nanoparticles formed with PEI ( ), DNA nanoparticles formed with PEI-Ac_{80} ( ), transfected MSC by 3D method by DNA nanoparticles formed with unmodified PEI ( ), and transfected MSC by 3D method by DNA nanoparticles formed with PEI-Ac_{80} (▪). The dose of plasmid DNA was 0.01 μg/μL. *$p < 0.05$; significant against BMD of unmodified PEI group.
[Normal View 10K | Magnified View 21K]

Figure 11(A) and (B) shows time course of the ALP activity and OCN content of subcutaneous tissues around the implanted site of collagen sponges with PGA fiber incorporation seeded with PBS, MSC, naked plasmid DNA-BMP-2, and transfected MSC by using DNA nanoparticles formed with PEI-Ac_{80} or unmodified PEI 1, 2, 3, and 4 weeks after implantation of scaffolds, respectively. Significantly, higher ALP activity was detected for both collagen sponges with PGA fiber incorporation seeded with transfected MSC by using DNA nanoparticles formed with PEI-Ac_{80} or unmodified PEI, although the extent of ALP activity was higher in the sponges

seeded with transfected MSC by using DNA nanoparticles formed with PEI-Ac_{80}. In the 3D-culturing group, the ALP activity increased with time for the initial 3 weeks, and thereafter leveled off, although the level was still high when compared with that of other groups. DNA nanoparticles formed with PEI-Ac_{80} exhibited the highest OCN content among all the groups.

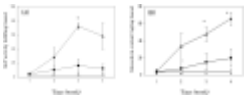


Figure 11. Time course of ALP activity (A) and osteocalcin contents (B) of tissues around the implanted site of collagen sponges with PGA fiber incorporation at collagen/PGA fiber weight ratio of 0.2 impregnated with PBS (○), MSC (□), naked plasmid DNA (△), transfected MSC by 3D method by DNA nanoparticles formed with unmodified PEI (▪), and transfected MSC by 3D method by DNA nanoparticles formed with PEI-Ac_{80} (▲). The dose of plasmid DNA was 0.01 μg/μL. *$p < 0.05$; significant against the ALP activity of DNA nanoparticles formed with unmodified PEI at the corresponding week.
[Normal View 16K | Magnified View 43K]

DISCUSSION

The goal of our research is to enhance the level of gene expression by combination of a 3D tissue-engineered scaffold and nonviral gene carrier for different cells including MSC. Therapeutically, important genes are used for *in vitro* pharmacological, physiological, and developmental researches as well as *in vivo* cell-based gene-therapy applications. Gene transfection with 3D tissue constructs is a new field and few studies have been published on the *in vitro* transfection of genes into 3D tissue constructs.[26][27] We indicated that the gene-expression level of BMP-2 plasmid DNA was enhanced not only by the DNA nanoparticles formed with PEI-Ac_{80}, but also by combinationally using the incorporation into collagen sponge reinforced with PGA fiber as a 3D-culture method.

It is now recognized that one of the tissue-engineering strategies is preceded by the following three principal steps: the proliferation of cells, the seeding of cells into a suitable scaffold, and the maintenance of the differentiation phenotype of the engineered tissues. The scaffolding materials should be biocompatible, biodegradable, and osteoinductive to accept the attachment and migration of osteoblasts. Among many materials currently used as cell scaffolds, collagen has been widely used because of its cell compatibility and its processability into various shapes. The drawback of collagen sponge as a scaffold for cell proliferation and differentiation is its poor mechanical strength. To overcome the inherent nature of sponge, the combination with other materials has been attempted.[28] In addition, the materials to be combined should be bioabsorbable. From the viewpoint of clinical application, it is preferable to select a material that has been clinically used. Several biodegradable synthetic polymers, such as PGA and its copolymers with L-lactic acid, DL-lactic acid, and ε-caprolactone, have been fabricated into scaffolds of nonwoven fabric and sponge shapes for tissue engineering. The mechanical resistance of the scaffolds to compression is acceptable in a practical sense for tissue-engineering applications, because of their hydrophobic nature. However, the cell attachment to the surface of synthetic polymer scaffolds is poor compared to that of collagen. PGA has approved by U.S. Food and Drug Administration (FDA) for human clinical applications. As it is shown in Figure 6, the incorporation of PGA fiber enabled collagen sponges to increase the resistance to compression. The *in vitro* culture experiment revealed that the number of MSC attached increased with the incorporation of PGA fiber to a significantly high extent when compared with that of the original collagen sponge.[24]

The efficiency of transfection with expression vector has been shown to be related to cell-cycle activity.[29] Attachment of cells to substrata is a major factor controlling their structure, function, and cell cycle.[30] At first, cells attach to the surface of fibers throughout the polymer scaffold, then spread, and proliferate. The 3D fibrous matrices can provide greater available surface area for cell attachment and spreading than 2D surfaces (i.e., tissue culture plate). Moreover, the 3D scaffold surface affects cell adhesion, spreading and proliferation, and controls the spatial arrangement of cells and their transmission of biochemical and mechanical signals. Cell adhesion, spreading, and cytoskeletal reorganization initiate signaling cascades that govern gene expression.[31] It has been demonstrated that hematopoietic cell adhesion to the cell matrix might govern expression of transgene.[32] Our results indicate that, in 2D culture, the initial rate of cell growth was higher, but their proliferation was not maintained once the cells reached confluence.[24] However, the cells growth in 3D matrices continued for longer time periods than those of 2D matrices.[24] The higher the cell growth, the better the gene transfection for the cells.[33] It has been found that effective transfection of multicellular hepatocyte spheroids required the process of spheroid formation.[34]

Currently, the combinational strategy of tissue-engineering principles with gene therapy involves the introduction of foreign genes into cell grown *in vitro*, selection for stable tranfectants, and seeding the modified cells into polymer scaffolds.[35-37] This process is slow and requires considerable time to establish stable transfectants. It is possible that the enhanced cell proliferation also accelerates gene transfection. Xie et al.[33] indicated that cells transfected with β-galactosidase gene marker grown in polymer scaffolds retain the ability to express marker genes even under transient conditions for longer than cells grown on 2D surfaces and transfected with similar plasmids. Thus, establishing a tissuelike construct on polymer scaffold, followed by stable or transient expression plasmid transfection, may offer a superior method to the conventional *in vitro* gene transfection and then application of cells directly into a host or seeding them into scaffolds.

The proposed technique of gene transfer to 3D cell-scaffold constructs is based on the use of 3D fibrous scaffold to guide cell organization in conjunction with gene manipulation. In comparison with 2D culture method, the environment of cells maintained in the 3D culture scaffold is closer to that *in vivo*, which can affect the gene expression and behavior of cells.[38] The level of gene expression was enhanced in 3D-culture method for the DNA nanoparticles formed with PEI-Ac_{80} incorporated into collagen sponge reinforced with PGA fiber (Fig. 7). This can be explained from the viewpoint of cell attachment and mechanical strength of collagen sponge. Our previous results demonstrated that when MSC were seeded into the collagen sponge with or without PGA fiber incorporation, larger number of MSC attached in the collagen sponge incorporating with PGA fiber than the collagen sponge without PGA fiber incorporation.[24]

Taken together, the level of gene expression was enhanced by DNA nanoparticles formed with PEI-Ac_{80}, incorporation of DNA nanoparticles into the collagen sponge reinforced with PGA fiber, and 3D-culture method. It is highly conceivable that the electrostatic interaction between the cell membrane of negative charge and the DNA nanoparticles with a positive charge is a driving force to attach the DNA nanoparticles to cells, which are an initially important step for gene transfection. When the DNA nanoparticles formed with PEI-Ac_{80} solution was added into the MSC, the level of gene expression was low (Fig. 7). This may be explained by the fact that larger volume of DNA nanoparticles reduced the surface area necessary for the attachment of the DNA nanoparticles to the surface of cells, resulting in reduced internalization of the DNA nanoparticles into the cells. It may be hypothesized that the incorporation of DNA nanoparticles into collagen sponge reinforced with PGA fiber provided lower dose of plasmid DNA and larger surface area both of which enhance the internalization of the DNA nanoparticles into the cells. On the other hand, since plasmid DNA is a large, negatively charged molecule up to 1 μm in length, it is impossible to make the plasmid DNA to internalize into cells following the attachment onto the cell membrane of negative charge.[39] When the plasmid DNA is ionically mixed with a positively charged nonviral vector, it is well recognized that the molecular size decreases by its condensation. It is likely that the condensed, positively charged DNA nanoparticles can electrostatically interact with the cell membrane for internalization.

The soft X-ray observation and histological study clearly reveals that collagen sponge with PGA fiber incorporation seeded with transfected MSC by using DNA nanoparticles formed with PEI-Ac_{80} or unmodified PEI-induced bone formation homogeneously throughout the scaffolds [Fig. 8(F,G)], although the extent of bone formation was higher in the sponges seeded with transfected MSC by using DNA nanoparticles formed with PEI-Ac_{80}. These results are in good agreement with the results of *in vitro* MSC transfection (Fig. 7). As is shown in Figure 7, 3D-culture method significantly enhanced the level of gene expression when compared with 2D culture method when DNA nanoparticles formed with PEI-Ac_{80} was used. Therefore, as we expected that when scaffold seeded with transfected MSC and implanted subcutaneously into the back of rats, higher bone formation were observed compared to other groups.

ALP is an ectoenzyme, produced by osteoblasts, that is likely to be involved in the degradation of inorganic pyrophosphate to provide a sufficient local concentration of phosphate or inorganic pyrophosphate for mineralization to proceed. Therefore, ALP is a useful marker for the osteoblast activity. OCN, also known as bone Gla protein, is a highly conserved noncollagenous protein that contains three γ-carboxyglutamic acid residues that allow it to bind calcium. Although the function of OCN is not quite clear, it is well recognized that only osteoblasts or cells with osteoblastic nature produce OCN. OCN is already known to play an important role in the process of ossification for bone formation. Like ALP, OCN is also selected as a marker of osteogenic differentiation.[40] The results of *in vitro* and *in vivo* study (Figs. 8 and 11) indicate that the ALP activity increased rapidly and saturated at 3 weeks, while the temporal changes in the OCN content increased steadily with time, which was in good agreement with the course of bone formation in the scaffolds. The 3D-culture method significantly increased both the ALP and OCN levels of MSC compared with 2D culture method. It is conceivable that the promoted cell differentiation is due to the enhanced gene expression.

Collagen sponges have been used for the delivery of plasmid DNA for tissue engineering.[15][16] However, naked plasmid DNA delivery is limited to the period of application because of low stability *in vivo* and degradation in the body. Prior to encapsulation, DNA may be condensed with cationic polymers to decrease particle size, protect DNA from degradation, promote interaction with cell membranes, and facilitate endosomal release via the proton sponge effect.[41] Another important issue is that naked plasmid DNA is not able to transfect cells *in vitro* to create genetically engineered stem cells. Transplantation of engineered stem cells for *in vivo* tissue-engineering applications is an attractive technology when nonviral vector is applied. As shown in the Results section of this study, the modified PEI was enabled to transfect stem cells significantly higher than naked DNA when 3D-culture method was employed. Similar studies by Mooney and coworkers indicated that PEI was enabled to condense plasmid DNA encoding for bone morphogenetic protein-4 (BMP-4) and induced significantly bone formation when incorporated in poly(lactic-*co*-glycolic acid) (PLGA) scaffolds after implanted into a cranial critical-sized defect.[42] As discussed earlier, our previous study indicated that acetylation of PEI not only reduced the toxcicity of PEI but also increased transfection efficiency 20-to 60-fold over unmodified PEI in different cell lines. The combinational technology of transfected stem cells with tissue-engineered scaffolds is challenging for the next generation of tissue regeneration using nonviral gene-delivery systems.

CONCLUSION

The level of gene expression *in vitro* was enhanced by a combination technology of DNA nanoparticles formed with PEI-Ac_{80}, incorporation of DNA nanoparticles into the collagen sponge reinforced with PGA fiber, and 3D-culture method. The present findings will provide attractive combinational strategy of tissue-engineering principles with gene therapy for tissue regeneration.

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