

Full Paper

A New Technique With Calcium Phosphate Precipitate Enhances Efficiency of In Vivo Plasmid DNA Gene Transfer

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Abstract. In vivo gene transfer with plasmid vector has been applied experimentally and clinically; however, the low level of gene transfer efficiency with plasmid vector is a problem. We speculated that the combination of calcium phosphate precipitate (CaP) and plasmid vector could solve this problem because CaP stabilizes plasmid DNA. In the present study, we used a plasmid expression vector encoding enhanced green fluorescent protein and combined the vector with CaP. Then, this combination was mixed with bovine type I atelocollagen. After incubating this mixture in phosphate-buffered saline, the amount of the plasmid DNA in the supernatant was low when the plasmid DNA was combined with CaP. Furthermore, the plasmid DNA, which was combined with CaP, was stable in DNase digestion in vitro. The plasmid vector with or without CaP, together with the atelocollagen, was transplanted subcutaneously or injected in the bone marrow of the femurs of rats. Then, the fluorescence was observed under a confocal laser scanning microscope and the fluorescence intensity in the tissue homogenates was measured. In these animal experiments, the fluorescence was extensive when the plasmid DNA was combined with CaP. These results indicate that our formula, collagen/CaP/DNA, appeared efficient for in vivo gene transfer.

Keywords: calcium phosphate, plasmid, gene transfer, atelocollagen, enhanced green fluorescent protein

Introduction

Gene therapy, in which a therapeutic gene is transferred to cells, is potentially promising and various gene transfer systems have been developed and tried experimentally and clinically. Currently, in vivo gene transfer with expression plasmid vector is one of the emerging remedies because gene transfer with plasmid vector is simple and clinically safe compared to the other transfer systems with virus vectors. Indeed, a solution containing an expression plasmid vector encoding one of the angiogenic proteins, such as VEGF, FGF, and HGF, is injected to the diseased areas of the patients to induce the formation of blood vessels (1–11). Furthermore, expression plasmid vector encoding bone-inducing pro-

tein, for example BMP-4, was combined with collagen and transplanted to the segmental bone defect of experimental animals, consequently regenerating bone at the defective area (12). However, large amounts of plasmid vectors have been used in these studies, suggesting the low efficiency of gene transfer.

On the other hand, calcium phosphate (CaP) precipitate, in which plasmid vector is incorporated, has been used for in vitro gene transfer (13–22). Since CaP precipitate stabilizes nucleic acid (14, 15), we speculated that CaP precipitate would be also useful for in vivo gene transfer. In the present study, we combined CaP precipitate with expression plasmid vector encoding enhanced green fluorescent protein (EGFP), mixed it with collagen, and then transplanted or injected it into rats. We examined whether this formula enhances gene transfer efficiency or not.

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Materials and Methods

The protocol for our study was approved by the Recombinant DNA Experiment Committee and the Animal Experiment Committee of our institution. All the experimental processes followed the institutional guidelines for the care and manipulation of laboratory animals.

EGFP-coding plasmid vector

pEGFP-N1, EGFP-coding plasmid vector, was purchased from CLONTECH Laboratories, Inc., Palo Alto, CA, USA. The plasmid vector was harbored in the JM109 strain of *E. coli* for amplification. The transfected JM109 was amplified in LB medium. The plasmid encoding pEGFP-N1 was purified with a plasmid purification kit (Quiagen, Valencia, CA, USA) and dissolved in water at a concentration of 1.0 mg/ml.

Freeze-dried pellets and gels containing plasmid vectors

The following four solutions were prepared. pEGFP-N1 was dissolved in phosphate-buffered saline (PBS) at a concentration of 0.1 $\mu\text{g}/\mu\text{l}$, and 200 μl of this solution was mixed with equal amount of 2% bovine atelocollagen (Atelocollagen Implant; KOKEN Co., Ltd., Tokyo) (pEGFP-collagen). Twenty micrograms of pEGFP-N1 was precipitated in 200 μl of CaP solution (CaPhosTM Mammalian Transfection Kit; CLONTECH Laboratories, Inc.) according to the manufacturer's instructions, and then 200 μl of 2% atelocollagen was added and mixed (pEGFP-CaP-collagen). Twenty micrograms of pEGFP-N1 was dissolved in 400 μl of PBS (pEGFP). Partially similar to "pEGFP-CaP-collagen", but 200 μl of PBS was added instead of atelocollagen (pEGFP-CaP). These solutions were separately transferred to 1.5-ml plastic tubes and lyophilized. A pellet, which contained 20 μg of pEGFP-N1, was formed in each tube.

We prepared collagen gels containing pEGFP-N1. Forty micrograms of pEGFP-N1 was dissolved in 20 μl of PBS and mixed with 20 μl of 2% atelocollagen (pEGFP-collagen Gel). Similar to the above "pEGFP-CaP-collagen", 40 μg of pEGFP-N1 was precipitated in 20 μl of CaP solution and mixed with 20 μl of 2% atelocollagen (pEGFP-CaP-collagen Gel).

In vivo gene transfer

Male Wistar rats, 6-week-old, were used. Under Nembutal anesthesia, each pellet containing pEGFP-N1 was transplanted subcutaneously on the calvaria of each animal after incision. Furthermore, 40 μl of "pEGFP-collagen Gel" or "pEGFP-CaP-collagen Gel" was directly injected into the bone marrow cavity from the

proximal end of the tibia with an 18G needle. At 1 and 2 weeks after the operation, the animals were sacrificed under chloroform anesthesia. The skin of the transplanted area was examined with a confocal laser scanning microscope (CLSM) (LSM 510; Zeiss, Jena, Germany) at 488 nm excitation and 509 nm emission. Then, the trimmed samples were homogenized in sonication buffer (50 mM NaH_2PO_4 , 10 mM Tris-HCl, 200 mM NaCl, pH 8.0) and their fluorescence was measured with a spectrofluorometer (FP-777; JASCO, Tokyo) at the same wavelength. The tibias were longitudinally cut and bone marrow cavities were scanned with a confocal laser scanning microscope.

Plasmid release

The pellets of the above "pEGFP-collagen" or "pEGFP-CaP-collagen" were separately transferred to 1.5-ml tubes. After addition of 1.0 ml of PBS, the tubes were incubated with vigorously shaking for 1 h at 37°C. After centrifugation, the amount of the plasmid DNA in the supernatant was measured with a spectrophotometer (DU-64; Beckman, Fullerton, CA, USA). The data was displayed as percentage of the total plasmid amount in the collagen pellet.

Plasmid stability

To clarify the stability of the plasmid conjugated with CaP, the plasmid was subjected to DNA digestion. Ten micrograms of pEGFP-N1 was dissolved in 200 μl Hepes-buffered saline, followed by addition of 12.4 μl of 2 M CaP solution to obtain precipitates according to the manufacturer's protocol. Then the supernatant was removed after short centrifugation. For digestion, the precipitate was suspended in 100 μl of 5 mM magnesium chloride solution, and then 2 μg (1 $\mu\text{g}/\mu\text{l}$) of DNase was added and the solution was incubated at 37°C for 1 h. After incubation, the solution was treated with 0.25 M EDTA at pH 8.1 for 24 h and dialyzed against distilled water at 4°C overnight. Ten microliters of the solution was analyzed by 1% agarose gel electrophoresis. In parallel, the same amount of the plasmid DNA without CaP conjugation was similarly processed.

Results

EGFP expression from pEGFP-N1 plasmid on calvaria was examined under the CLSM. At 1 week, no fluorescence was detected in any group. At 2 weeks, fluorescence was observed in the pEGFP-collagen and pEGFP-CaP-collagen groups; however, neither the pEGFP group nor pEGFP-CaP group produced fluorescence. Notably, the fluorescence of the pEGFP-CaP-collagen group was much extensive compared to that

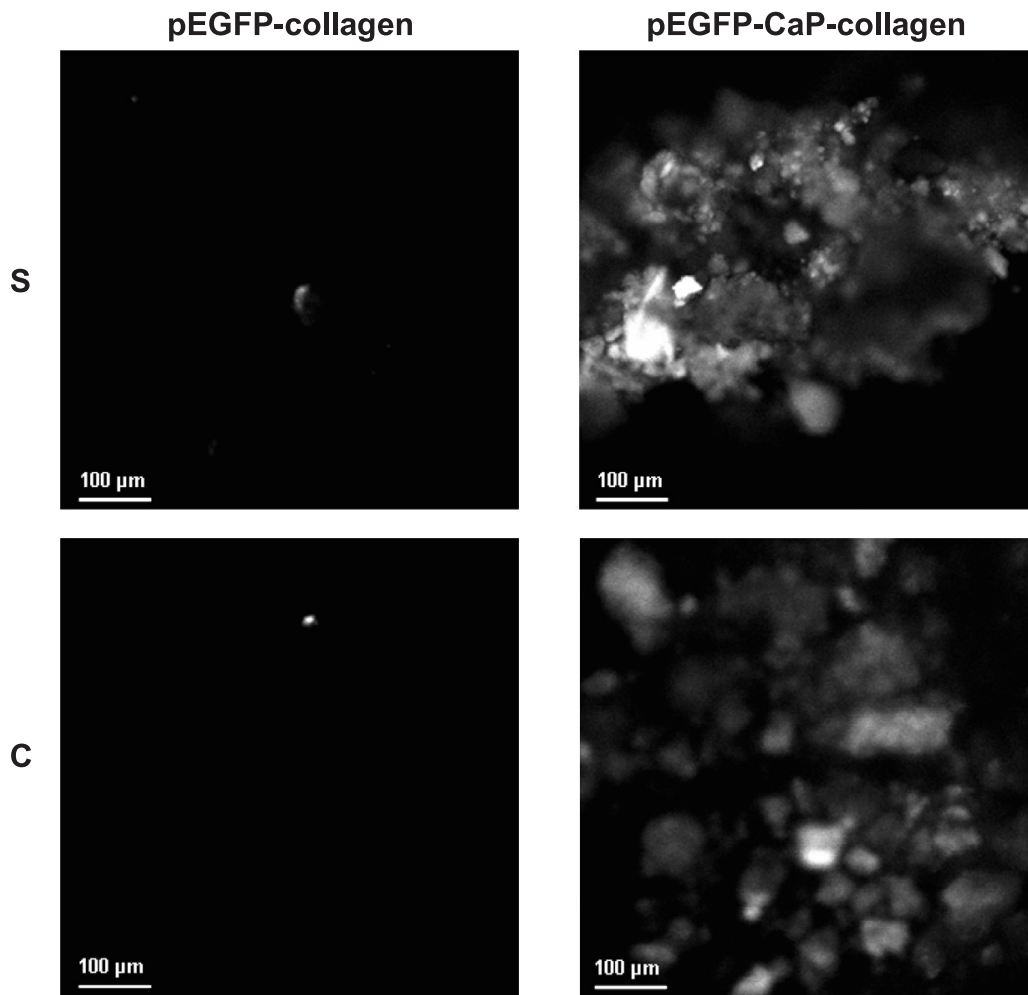


Fig. 1. Fluorescence images after subcutaneous transplantation of pEGFP. The pellets of pEGFP-collagen or pEGFP-CaP-collagen, which contained 20 μ g of pEGFP, were transplanted into a subcutaneous region of the rat heads. Two weeks after the operation, EGFP expression on the skin side (S) and the calvarial side (C) was examined under a confocal laser scanning microscope.

of the pEGFP-collagen (Fig. 1). Corresponding to the CLSM images, fluorescence measurement of the tissue homogenates revealed intense fluorescence of the pEGFP-CaP-collagen group (Fig. 2).

After the injection of pEGFP-N1 plasmid into the bone marrow, the fluorescence was not observed at 1 week. At 2 weeks after, fluorescence was detected, and the bone marrow where pEGFP-CaP-collagen Gel was injected was more fluorescent than the one injected with pEGFP-collagen Gel (Fig. 3).

As shown in Fig. 4, the incubation for 1 h in PBS released pEGFP-N1 from the pellets of both pEGFP-collagen and pEGFP-CaP-collagen. However, the former released more plasmid DNA than the latter. Longer incubation for 24 h or up to a week did not change the concentration of the released plasmid DNA in the solution of each group (data not shown). The ratio,

the absorbance of 260 nm wavelength to the absorbance of 280 nm wavelength, represents protein contamination. This ratio was more than 1.8 in our measurement. Thus, the contamination of protein (collagen) was negligible.

After the DNase digestion, the electrophoresis showed the open and closed circle bands of pEGFP-N1 in the sample of CaP precipitated plasmid (lane 2 of Fig. 5) as shown in intact pEGFP-N1 (lane 3 of Fig. 5), whereas the sample of the plasmid solution without CaP conjugation produced no band (lane 4 of Fig. 5).

Discussion

Generally, since both plasmid DNA and the outer cellular surface are negatively charged, intracellular entry of plasmid DNA is extremely difficult even if

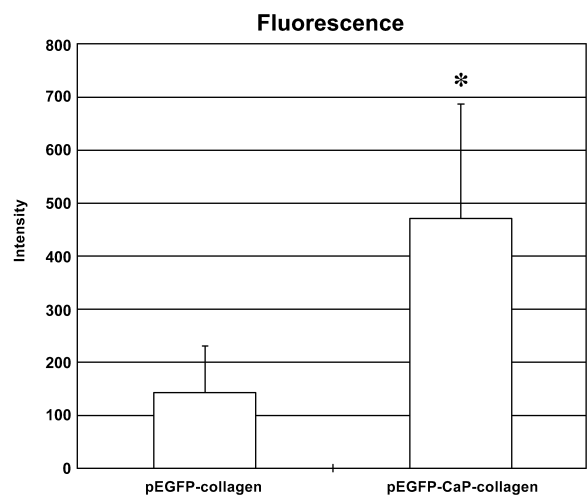


Fig. 2. Florescence intensity after subcutaneous transplantation of pEGFP. The pellet of pEGFP-collagen or pEGFP-CaP-collagen, which contained 20 μ g of pEGFP, was transplanted into a subcutaneous region of the rat head. Two weeks after the operation, the tissue of the transplanted area was homogenized in the buffer and the fluorecence was measured with a spectrofluorometer. Values are expressed as the mean \pm S.D. (n = 3). *Significant difference from the pEGFP-collagen group ($P<0.05$).

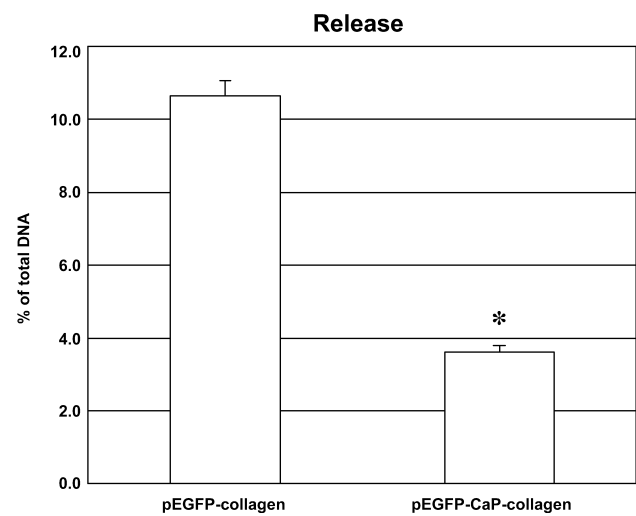


Fig. 4. pEGFP release from the pellets containing pEGFP. The pellets of pEGFP-collagen or pEGFP-CaP-collagen were incubated in PBS with shaking and the released pEGFP in PBS was measured with a spectrophotometer after 1-h incubation. Values are expressed as the mean \pm S.D. (n = 3) as percentage of the total plasmid DNA in the initial pellet. *Significant difference from pEGFP-collagen group ($P<0.05$).

plasmid DNA can reach the cell surface. Therefore, plasmid DNA must be manipulated for efficient gene transfer. Such modifications that are widely used for transfection rely on CaP precipitation, electroporation, and lipofection. Furthermore, ballistic gene transfer is another method of gene transfer, in which small particles carrying plasmid DNA are shot into cells (23 – 29). The

ballistic technique and electroporation are less dependent on cellular state and force plasmid DNA to penetrate into cells, whereas CaP precipitation and lipofection depend on cellular endocytosis.

Lipofection with liposomes of synthetic cationic lipid containing plasmid DNA is remarkably effective compared to the calcium phosphate method (30). However,

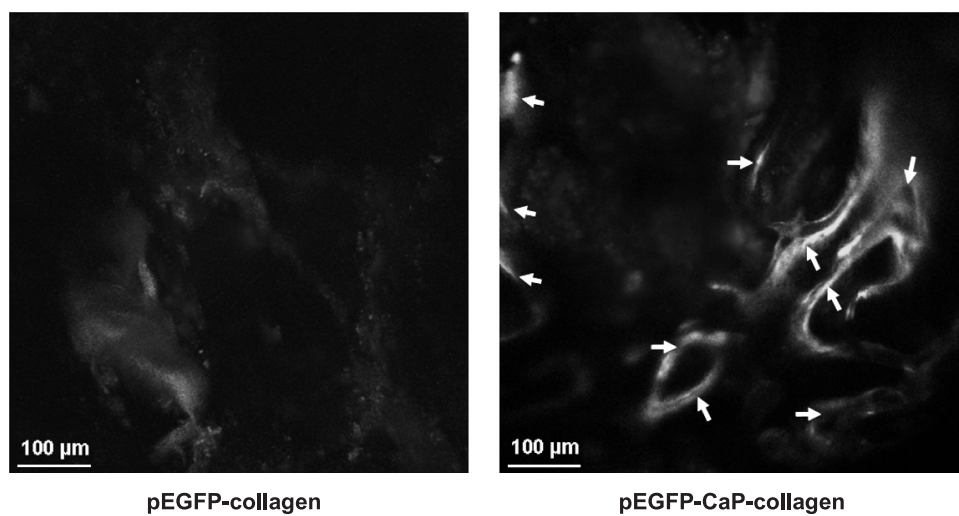


Fig. 3. Florescence images after subcutaneous transplantation of pEGFP. The gel of pEGFP-collagen Gel or pEGFP-CaP-collagen Gel, which contained 40 μ g pEGFP, were injected into the bone marrow cavity of the rat tibia. Two weeks after the injection, the tibia was longitudinally cut, and EGFP expression in the bone marrow was examined under a confocal laser scanning microscope. Arrows indicate the EGFP expressing cells around the trabeculae, which include osteoblasts.

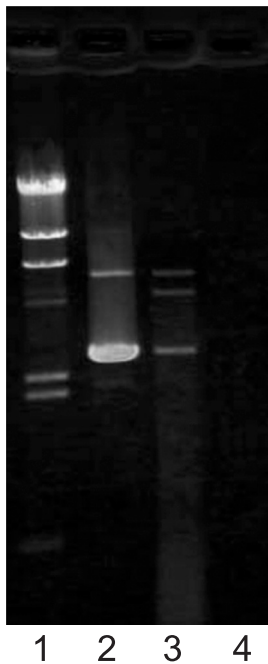


Fig. 5. Sensitivity to DNAase of pEGFP with or without CaP. pEGFP was precipitated with CaP and incubated with DNase. After incubation, CaP precipitates were demineralized with EDTA, dialyzed, and applied for agarose gel electrophoresis. Lane 1: DNA markers (Lamda DNA/Hind III), Lane 2: Intact pEGFP, Lane 3: pEGFP digested with DNase after CaP precipitation, Lane 4: pEGFP digested with DNase.

notably, lipofection has been frequently applied *in vitro* but not *in vivo*. Since lipofection requires strict conditions including correct cell density and components of the culture medium, it is difficult to apply this method *in vivo*. Although other gene transfer systems, such as the gene gun and electroporation, are also advantageous in transfection, these methods need special apparatuses. Furthermore, they are not proper for gene transfection to the inner region of the body unless the transfection site is surgically exposed.

Dr. Bonadio and his collaborators proposed the “gene activated matrix” for *in vivo* gene transfer of plasmid DNA (31–33). In the gene activated matrix, plasmid DNA is incorporated into atelocollagen and this combination is transplanted into the tissue defect. Atelocollagen holds plasmid DNA and acts as a scaffold for the cells. Indeed, fibroblastic cells from the surrounding tissue migrate in the gene activated matrix, and they incorporate plasmid DNA expressing the gene encoded in the plasmid DNA. Consequently, the protein, which stimulates tissue regeneration, is released. This method is unique and advantageous as an *in vivo* gene transfer method because of the following reasons: Firstly, it is suitable for gene transfer to the inner region of the body. Secondly, this method is simple without requiring

any special apparatus. Indeed, biodegradable matrices including genes have been applied to regeneration of various tissues experimentally (31–35). However, a large amount of plasmid DNA (>1 mg) has been used, which suggests low efficiency of gene transfer in this method.

Calcium precipitation of plasmid DNA with CaP is a common and conventional method of gene transfer for mammalian cells *in vitro*. In this method, plasmid DNA forms a tight complex with CaP, and DNA in this complex is resistant to nucleases in the serum of the culture medium (14, 15). In the present study, we used plasmid DNA encoding EGFP and combined this plasmid DNA with CaP. Then, these were further incorporated into the atelocollagen and transplanted or injected in the animals. The present results demonstrated that our formula, which consists of CaP, plasmid DNA (20 or 40 μ g), and atelocollagen, produced clear fluorescence compared to the combination without CaP. Furthermore, in our recent preliminary study, the combination with atelocollagen, CaP and expression plasmid vector encoding human BMP-2 gene (20 μ g), which was similar to the formula of the present study, induced ectopic bone when transplanted subcutaneously in rats. However, the formula without CaP did not induce ectopic bone (36). Thus, gene activated matrix in combination with CaP could increase gene transfer efficiency *in vivo*.

In vitro incubation in PBS solution showed that our formula with CaP releases less plasmid DNA than the formula without CaP, which is similar to the previous gene activated matrix. Since plasmid DNA forms a tight complex with CaP, it is likely that our formula also keeps plasmid DNA *in vivo* at the transplanted or injected site. Furthermore, the results of the present DNase digestion experiment revealed that plasmid DNA in the CaP complex is resistant to DNase, confirming the previous studies in which plasmid DNA in the CaP complex is resistant to serum DNase (14, 15). These phenomena would contribute to the high efficiency of gene transfer with our formula.

Fundamentally, it is likely that the cells migrating into the collagen sponge were mainly fibroblastic cells. These cells could be regarded as the targets of the plasmid, and the plasmid was taken in by endocytosis. Subsequently, these cells expressed the encoded protein (31). The duration of the gene expression in this gene transfer system would depend on the site of the application and the size of the gene-containing collagen-and-CaP matrix, which presumably influence the period of the matrix degradation and the duration of the gene expression. When pEGFP-CaP-collagen was transplanted subcutaneously, strong fluorescence was detected at 2 weeks and also at 4 weeks afterwards (data not

shown). In another experiment, we transplanted the matrix of the expression vector encoding human BMP-2 into the rat bone defect and examined human BMP-2 expression with RT-PCR, in which we could distinguish human BMP-2 expression from rat's own BMP expression. We confirmed that human BMP-2 expression continued for 8 weeks after transplantation (manuscript in preparation). Although we did not check the cessation of the gene expression, gene transfer with plasmid expression vector will theoretically decline with time.

Clinical trials of gene transfer with plasmid vectors encoding angiogenic proteins, such as FGF, VEGF, and HGF, have already been started. In these clinical trials, solution containing these plasmid vectors are injected. Although the safety of the plasmid DNA administration has been speculated, decrease in plasmid DNA dose is obviously ideal. Utilizing our formula, it would be possible to reduce the dose of plasmid DNA in these clinical trials. Notably, our formula is simple and it does not require a special device and reagent. Furthermore, after freeze-drying our formula would be stable in a refrigerator. These points are advantageous compared to the other gene transfer systems including electroporation, lipofection and ballistic methods. Although further studies are required, the present study indicates that our formula in combination with CaP, plasmid DNA, and atelocollagen would be useful in experimental and therapeutic fields employing plasmid DNA transfer.

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