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Feasibility of BMP-2 Gene Therapy Using an Ultra-Fine Needle

Kenji Osawa, Yasunori Okubo, Kazumasa Nakao,
Noriaki Koyama and Kazuhisa Bessho
Department of Oral and Maxillofacial Surgery, Graduate School of Medicine, Kyoto University
Japan

1. Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β superfamily and have the unique ability to control the differentiation of mesenchymal stem cells into osteoblastic cells [1]. Bone morphogenetic protein-2 (BMP-2), an osteoinductive member of the BMP family, plays an important role in bone growth and regeneration [2], and the clinical applications of recombinant human BMP-2 (rhBMP-2) are being investigated [3,4]. However, a large quantity of the recombinant protein and carrier are necessary, and the carriers often have problems with antigenicity, biocompatibility, biodegradability, and infection. An alternative, more efficient approach, gene transfer, may be able to target specific cells with specific promoters, and appropriate vectors to attain sustained gene expression. BMP-2 gene transfer with adenovirus have been investigated extensively [5-7]. Although the adenovirus vector is very efficient, potential toxicity and immunogenicity may limit its clinical application [8]. Furthermore, its therapeutic application would require efficient and reliable manufacture of viral vectors that are free of helper viruses and a reduction in immunogenicity. On the other hand, nonviral methods are safe and do not require immunosuppression for successful gene delivery, but suffer from lower transfection efficiencies. DNA injection followed by application of electric fields (electroporation) has been more effective for introducing DNA into muscle tissue than the use of simple intramuscler DNA injection [9]. Although this method should have the highest potential for clinical application, there is a concern that the electric pulse causes tissue damage. In addition, this method requires special equipment, and optimization of the parameters is necessary. Recently, ultrasound-enhanced gene transfer (sonoporation) has been investigated [10]. We recently reported osteoinduction by microbubble enhanced transcutaneous sonoporation of BMP-2 plasmid DNA [11]. Although this method seems to be safer than electroporation, it also requires special equipment and it is necessary to optimize the parameter of ultrasound. In this chapter, we report the human BMP-2 gene transfer using an ultra-fine needle and describe the feasibility of BMP-2 gene therapy using this new apparatus.

2. Materials and methods

To obtain human BMP-2 cDNA, a polymerase chain reaction (PCR) was performed using a pUC BMP-2 plasmid [12] and the following primers: 5′-AGA GAG AG *GAATTC* G TCG

ACC ATG GTG GCC GGG ACC CGC T (ATG, initial codon) and human BMP-2 reverse primer, 5′-AGA GAG AG *GAATTC* CTA GCG ACA CCC ACA ACC CTC CAC AA (CTA, stop codon). Both primers had EcoRI recognition sites (italicized). The PCR protocol consisted of 25 cycles of 15 sec at 98ºC, 2 sec at 65ºC, and 30 sec at 74ºC with KOD DNA polymerase (Toyobo, Osaka, Japan). The PCR product was digested using EcoRI and ligated into the EcoRI-digested cloning site of the pCAGGS expression vector, which contains CAG (cytomegalovirus immediate-early enhancer/chicken β-actin hybrid) promoter [13], to yield pCAGGS-BMP-2. The 1197-bp insert sequence was confirmed by DNA sequencing. As a control, pCAGGS-lacZ, which causes the cytoplasmic expression of E.coli β-galactosidase [13], was used. The plasmid vectors were grown in Escherichia coli DH5α and prepared with a Qiagen Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. To verify the identity and purity of the plasmid vectors, agarose-gel electrophoresis was performed after restriction endonuclease digestion. The plasmid DNA concentration was determined using a UV/visible spectrophotometer (DU-530, Beckman, Fullerton, CA).

To determine the effect of the ultra-fine needle transfection on mammalian cells, C3H10T1/2 (passage 9-10), a mouse fibroblastic cell line, was obtained from RIKEN Cell Bank (Tsukuba, Japan). The cells were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin, streptomycin and ampicillin (PSA). The cell cultures were grown at 37˚C in a humidified atmosphere of 95% air-5% CO2. Once confluent, the cells were reseeded into 35-mm glass bottom dishes and incubated 24 hours. Then the medium is replaced with Hank's balanced salt solution (HBSS) supplemented with the plasmid DNA (0.1, 0.2, and 0.3 mg/ml) to be delivered. The cells cultured on a dish were set on a stage and pierced the plasma membrane with the apparatus.

In the present study, we used the ultra-fine needle transfection apparatus SU100 (Olympus, Tokyo, JAPAN) attached to an inverted confocal microscope (IX81, Olympus, Tokyo, Japan). This apparatus was attached to an inverted microscope. A target cell was placed under the needle by the x-y stage controller. To pierce the cell membrane, the needle was lowered vertically by z-stage controller. The needle tip stayed inside the cell for one second. Then, cells were washed with fresh culture medium a few times, followed by incubation in a CO2.

First, to optimize the amount of the plasmid DNA in the medium, the lacZ gene, which causes the cytoplasmic expression of E.coli β-galactosidase, was transferred using pCAGGS-lacZ. As many as 100 cells were transfected under various concentration of the lacZ-encoding plasmid. On day 2 after transfection, the cells were fixed for 5 min in 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS) at room temperature. They were subsequently washed with PBS and stained for 2 hours at 37ºC in 5-Bromo-4chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining solution containing 1 mg/ml X-gal, 2 mM MgCl2, 5 mM K3Fe(CN)6 and 5 mM K4Fe(CN)6◦3H2O in PBS (pH 7.4). Experiments were performed in triplicate. Cells expressing ß-galactosidase were counted and the results were presented as the mean and standard deviation Difference in the β-galactosidase activity was assessed by analysis of variance.

Next, BMP-2 gene transfer was performed using pCAGGS-BMP-2 and pCAGGS-LacZ as a control. The cells were harvested one day after transfection. RNA was isolated from the cells using a Qiagen RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Human BMP-2 mRNA and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA were detected by reverse transcription-polimerase chain reaction (RT-PCR) using the following primers: human BMP-2 forward, 5′-TCTGACTGACCGCGTTACTC-3′; human BMP-2 reverse, 5′-

TCTCTGTTTCAGGGCCGAACA-3' ; GAPDH forward, 5'-ACTCCACTCACGGCAAATTC-3'; and GAPDH reverse, 5'-CCTTCCACAATGCCAAAGTT-3'. The human BMP-2 forward primer was designed to hybridize with the sequence immediately downstream of the transcriptional start site of the CAG promoter to ensure that the PCR products were not contaminated by plasmid DNA and genomic DNA. The PCR products were analyzed by 2% agarose gel electrophoresis to detect the 285-bp human BMP-2 mRNA and 682-bp GAPDH mRNA. In addition, to determine early osteoblastic differentiation, alkaline phosphatase (ALP) staining was performed using a Sigma diagnostic ALP kit (Sigma, St.Louis, MO) on 7 days after transfection. Moreover, to confirm the terminal differentiation of osteoblast, von Kossa staining, which stains phosphates and the carbonates deposited in mineralized tissue, was performed on 21 days after transfection. For von Kossa staining, cells in dishes were fixed with 4% paraformaldehyde and were then soaked in 0.1M $AgNO_3$ solution for 15 min. After exposure to ultraviolet light at least 5 min, the dishes were washed with PBS.

3. Results

On day 2 after lacZ gene transfer, we found that X-gal positive cells were present in all of the groups performed transfection (Figure 1). In 0.1 mg/ml group, transfection efficiency reached 40.2±22.4%. In addition, 0.2 mg/ml group, the transfection efficiency significantly enhanced ($p<0.05$ versus 0.1mg/ml group) and reached 71.2±16.8%. In 0.3mg/ml group, moreover, transfection efficiency significantly ($p<0.01$ versus 0.1mg/ml group) and reached 100%. It was suggested that when 0.3 mg/ml of plasmid DNA was used, gene transfer was performed most efficiently.

According to the results of lacZ gene transfer, we performed transfection with BMP-2-encoding plasmid DNA at the concentration of 0.3 mg/ml. We detected human BMP-2 mRNA expression by RT-PCR one day after transfection (Figure 2). On day 7 after transfection, ALP-positive cells were found (Figure 3). Furthermore, on day 21 after gene transfer, von Kossa positive areas were also found (Figure 4).

4. Discussion

We have demonstrated the transfer of the human BMP-2 gene to mouse fibroblastic cells by cell membrane perforation with an ultra-fine needle, and have shown that it caused the expression of human BMP-2 mRNA one day after transfection. On day 7 after transfection, we saw an increase in ALP activity. On day 21 after transfection, moreover, calcification was seen. It is known that rhBMP-2 can induce the differentiation of non-osteogenic cell lines into osteoblastic cells, indicating that the BMP-2 gene could be transfected into C3H10T1/2 cells with an ultra-fine needle to induce the differentiation of fibroblast into osteoblast.

Our previous studies demonstrated that implantation of rhBMP-2 with a carrier matrix [14], *in vivo* adenovirus-mediated gene transfer [15], and *in vivo* plasmid DNA-mediated gene transfer, such as electroporation [16], sonoporation [11], or repeat plasmid injection [17], could cause osteoinduction. However, the above techniques showed some limitations in practical usage. The purification of rhBMP-2 was laborious and expensive, and carrier matrices created problems with antigenicity, biocompatibility, biodegradability, and infection. In addition, surgical procedures were required in order to implant a carrier matrix. The direct delivery of human BMP-2 genes using adenoviral vectors caused an immune response that needed to be systemically or locally suppressed. Non-viral gene

transfer using naked plasmid DNA is a useful technique to reduce the side effect of gene therapy. However, the low transfection efficiency is a major obstacle to the clinical application. Numerous attempts have been made to overcome the relatively low transfection efficiency, including the application of electric pulses and / or ultrasonic devices. Although these techniques are efficient to some degree, using a large amount of plasmid DNA is needed to enhanced the effect [11, 17]. In addition, direct injection of naked plasmid DNA into the animal or human body is associated with toxicity and immunogenicity [18]. However, this technique is also limited due to the low transfection efficiency. It is necessary to repeat the transfection procedure to raise the efficiency [11, 17].

There are two general strategies in BMP-2 gene therapy: BMP-2-encoding vector is directly delivered to the body (*in vivo*) or genetically transduced cells by BMP-2-encoding vector are transplanted (*ex vivo*). In the case of the *in vivo* approach, it is difficult to control the specific cell population transduced genetically [19] and significant antibody response to the vector can be caused [20]. Concerning this issue, an *ex vivo* approach has the ability to overcome these problems. Previous studies showed that transfer of BMP-2-encoding vector into a mesenchymal cell line stimulated the osteogenic pathway via autocrine and paracrine mechanisms *in vitro* [21] and *in vivo* [22], and that cell mediated BMP-2 gene transfer using the stably transfected mesenchymal progenitor cell line C3H10T1/2 resulted in enhanced bone defect repair in a mice nonunion fracture model [23]. Another previous *ex vivo* gene transfer experiment suggested that neighbouring cells including periosteal cells and non-transfected mesenchymal stem cells were affected by paracrine mechanisms, and BMP-2-producing cells may themselves differentiate into osteoprogenitor cells in an autocrine activity [24]. In this study, we demonstrated efficient gene transfer without using chemical reagent and / or physical energy. This technique was simple to pierce the plasma membrane and resulted in extremely high efficiency, with the efficiency reaching to 100%. Thus, human BMP-2 gene transfer using an ultra-fine needle may be a feasible method for the *ex vivo* gene therapy of musculoskeletal disorders. However this technique is low-throughput. Future improvement of this apparatus in order to raise the throughput is indicated. In addition, injection of transfected cells is necessary and can cause damage to the target tissue. Injection of transfected cells with naked plasmid DNA into target tissue causes various degrees of damage and inflammatory reaction, depending on factors such as the needle size, the nature of solutions. One of the purposes for local growth factor application by gene delivery is production of growth factor at a defined site, thereby avoiding potentially negative effects on other organs and immune reactions. However, after direct injection, extensive spread of the vector DNA was observed. In the liver and in the spleen, reporter gene expression could be detected histochemically in several cells. In both of these organs as well as the lung, vector DNA was detected by nested PCR in all animals examined at 3 days and at 14 days after direct intraarticular injection. This indicated a broad systemic distribution of viral particles. Nevertheless, it was reported that no external DNA could be detected in the lung, the liver, or the spleen of most animals that have received cells infected *ex vivo* [20].

Our results suggest that BMP-2 gene transfer using an ultra-fine needle may allow gene delivery to be used for bone regeneration. The response to the procedure could be monitored with clinical examinations (e.g., X-ray). These findings showed that transfection using an ultra-fine needle with BMP-2-encoding plasmid caused the expression of the human BMP-2 gene in transfected cells, which demonstrated a feasibility of initiating the cascade of events to enhance bone induction. This study has suggested the possibility of the

clinical application of gene therapy using an ultra-fine needle. Furthermore, the clinical application of BMP-2 gene therapy is consequently facilitated.

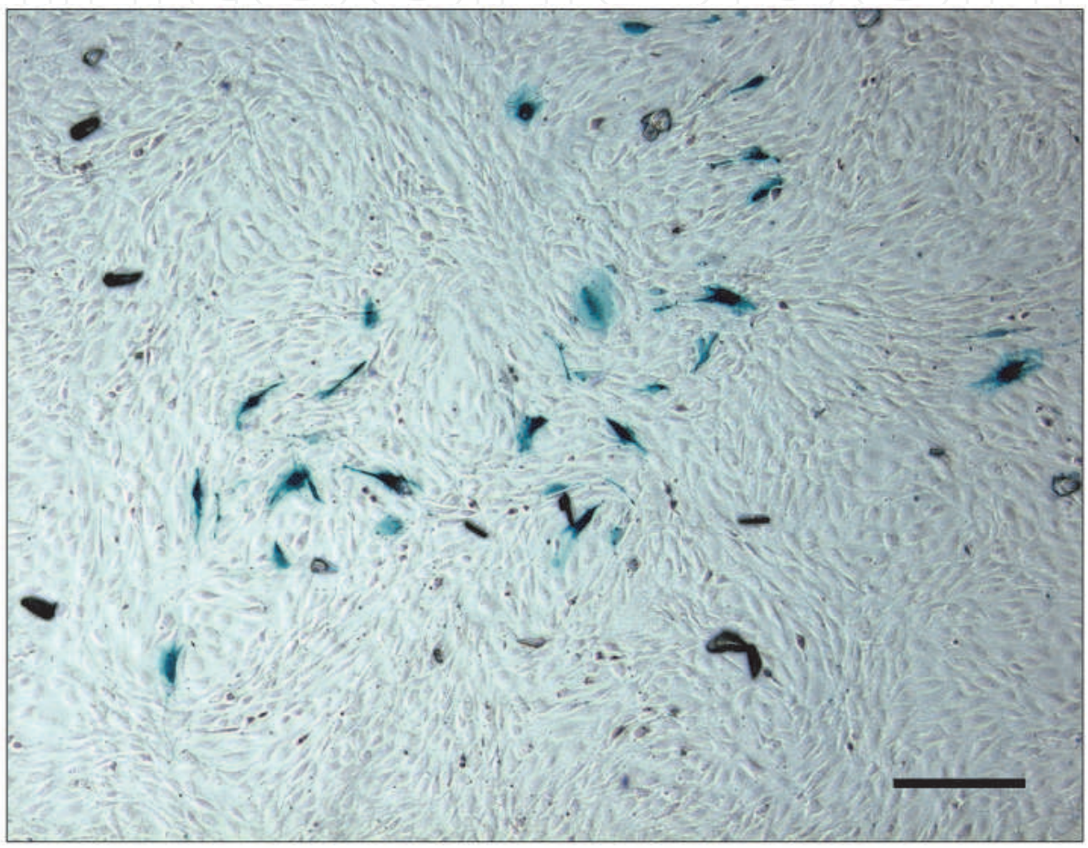

Fig. 1. Beta-galactosidase expression in C3H10T1/2 cells using an ultra-fine needle apparatus. X-gal staining was performed two days after transfection. Blue-stained cells were counted to examine the transfection efficiency and the maximum efficiency reached to 100%. Scale bar: 250 μm.

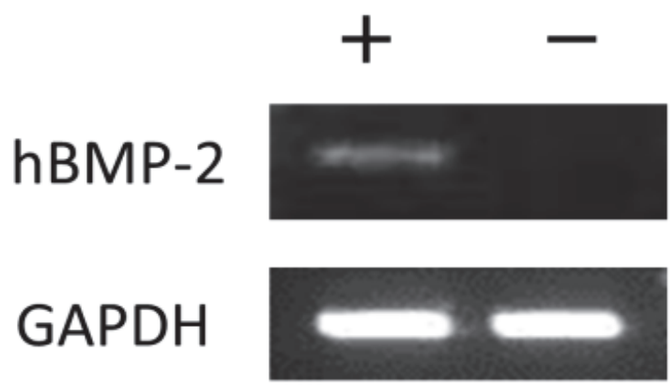

Fig. 2. Expression of the human BMP-2 transgene following the ultra-fine needle transfection was detected. RT-PCR was performed a day after transfection.

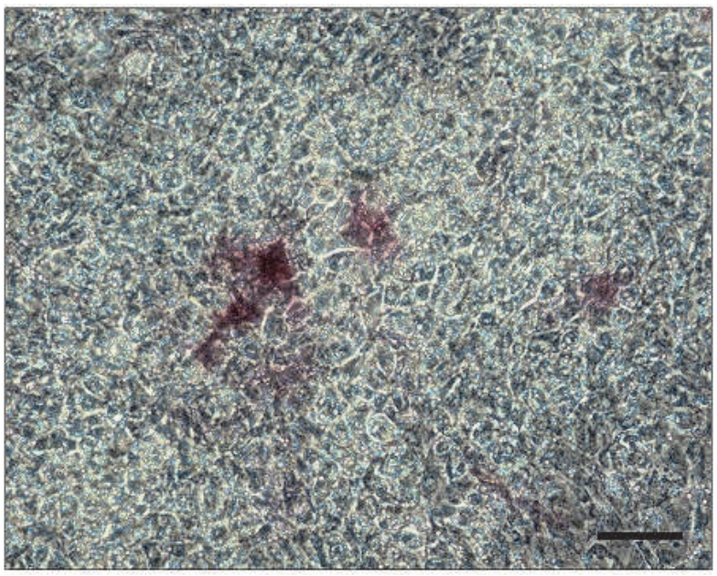

Fig. 3. ALP activity was observed in the region of the transfected cells. ALP staining was performed 7days after transfection. Skale bar: 100 μm

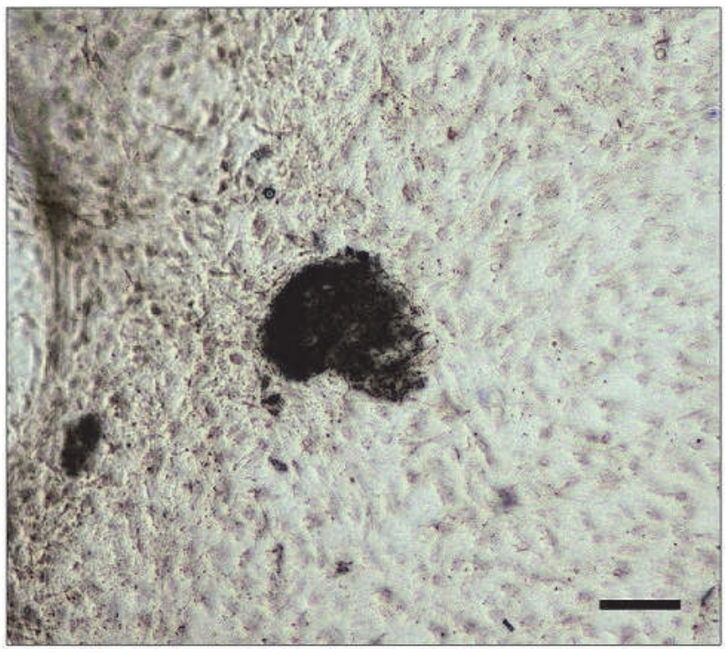

Fig. 4. Von Kossa staining was performed 21 days after transfection. Calcium deposition was confirmed in the region of the transfected cells. Skale ber: 100μm

5. References

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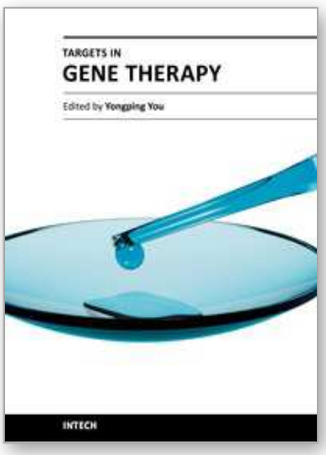

Targets in Gene Therapy

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

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