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Original research

BMP-2 gene transfer under various conditions with *in vivo* electroporation and bone induction

Hiromitsu Yamamoto[a], Mariko Kawai[b,*], Noriko Shiotsu[c], Minori Watanabe[c], Yasuhiro Yoshida[d], Kazuomi Suzuki[d], Hiroki Maruyama[e], Jun-ichi Miyazaki[f], Mika Ikegame[b], Kazuhisa Bessho[a], Toshio Yamamoto[b]

[a] *Department of Oral and Maxillofacial Surgery, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Sakyo-ku, Kyoto 606-850, Japan*
[b] *Department of Oral Morphology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8525, Japan*
[c] *Dental School, Okayama University, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8525, Japan*
[d] *Department of Biomaterials, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8525, Japan*
[e] *Department of Clinical Nephroscience, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan*
[f] *Division of Stem Cell Regulation Research, Osaka University Medical School, 2-2 Yamadaoka, Suita city, Osaka 565-0871, Japan*

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ABSTRACT

In our previous study, we successfully induced bone formation in rat skeletal muscle using a gene-transfer system, in which a non-viral BMP-2 expression vector was applied to the muscle by *in vivo* electroporation at 100 V. With the ultimate goal of applying this method to maxillofacial bone regeneration therapy, we sought to establish a safer system in which the gene is transferred into the target area with a lower voltage, but with the same efficiency. The *LacZ* or *BMP-2* gene was transferred using *in vivo* electroporation under various conditions: 25–100 V, 50–200-ms loading time, and 8–128 pulses. The gene-transfer efficiency or bone induction was quantified by measuring β-galactosidase or alkaline phosphatase (ALP) activity and calcium content. Histological, immunohistochemical, and X-ray analyses were also used to examine the effect of the gene transfer. When the voltage for *in vivo* electroporation was lowered, the gene transfer efficiency was also reduced. However, by increasing the loading time or number of pulses, it was possible to improve the gene-transfer efficiency to the same level attained at 100 V, and *in vivo* electroporation under the lower voltage conditions successfully induced new bone. We have established a safe and efficient gene-transfer system for bone regeneration therapy using a non-viral BMP gene expression vector and *in vivo* electroporation.

1. Introduction

Therapies for the safe, targeted delivery of bone morphogenetic protein (BMP) [1,2], a biologically active substance that induces bone formation, are anticipated for clinical use. We have focused on the method of delivery of the BMP gene into a target area. Gene therapy generally uses a viral vector, such as adenovirus [3,4], which can be toxic or induce an immune response [5,6]. Non-viral vectors are safer, but the gene-transfer efficiency of non-viral plasmid vectors is lower than that of viral vectors [7]. For our gene-delivery system, we constructed a non-viral plasmid vector that expresses BMP-2 (pCAGGS-BMP-2) and used *in vivo* electroporation to promote its transfer into rat skeletal muscle [8,9], where ectopic bone formation is easy to evaluate. *In vivo* electroporation is an attractive method because it has high gene-transfer efficiency and is easy, safe, and inexpensive, requiring only a plasmid and a simple device [10–13].

The gene-transfer efficiency of *in vivo* electroporation is affected by various factors, such as the electric voltage, loading time, number of pulses, and type of electrode [11,14,15]. Reported *in vivo* electroporation techniques use standardized parameters, either low voltage and long duration, or high voltage and short duration [11,16]. Although efficient gene transfer is possible with an electric potential of 100 V (low voltage and long duration), we sought to improve the safety of our *in vivo* electroporation method for actual clinical use.

Our final goal is to apply our gene transfer system to bone regeneration therapy in oral and maxillofacial regions. When we applied our system to the rat intraoral region at a level of 100 V, the pulse shock to the target area was relatively strong. Although no electrical injury was observed on the oral mucosa of the rats, each

* Corresponding author. Tel.: +81 86 235 6632; fax: +81 86 235 6634.
E-mail address: marikoka@md.okayama-u.ac.jp (M. Kawai).

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pulse shock at this voltage caused strong head shaking. In clinical practice using electrical stimulation to head region, for psychiatric disorders, some patients lose consciousness when they are treated by electroconvulsive therapy (ECT) [17] at a level of 100 V without general anesthesia. Today, to prevent on muscle convulsion, modified electroconvulsive therapy (m-ECT) with muscle relaxant under general management by anesthesiologists is performed [18]. Although it is successful to take away muscle convulsion, some complications still remained as memory impairments [19]. We concluded that it was too difficult to apply our *in vivo* electropolation system at a level of 100 V for oral and maxillofacial regions like ECT or m-ECT. We need more simple and safe therapy without anesthesia and muscle relaxant for future trials of intraoral bone regeneration.

We reasoned that an appropriate lower voltage would be similar to that of a transcutaneous electrical neural stimulator such as the myomonitor, which is a medical instrument used to treat temporomandibular joint disorder [20], and which uses pulses under 50 V without any anesthesia. In this study, we therefore optimized the parameters for *in vivo* electroporation using voltages below 100 V to achieve efficient gene transfer and bone regeneration.

2. Materials and methods

2.1. Preparation of BMP-2 and LacZ expression vectors

The pCAGGS-BMP-2 and pCAGGS-LacZ expression vectors were described previously [9,10], and were prepared using a Qiagen EndoFree Plasmid Giga Kit (Qiagen GmbH). The plasmids were grown in *Escherichia coli*, DH5α.

2.2. Gene transfer by transcutaneous in vivo electroporation

Nine-week-old male Wister rats (each treatment group $N = 6$) were anesthetized by an intraperitoneal injection of pentobarbital sodium (5.0 mg/100 g of body weight), and the fur on the target area of the leg was removed with clippers. Plate electrodes were coated with keratin cream (Fukuda Denshi) and attached to the skin at the target site transcutaneously. In some cases the skin at the center of the target area was incised with a #11 scalpel, and then held back with forceps so electrodes could be attached directly to the gastrocnemius muscle. The accuracy of the applied electric current was confirmed by measuring the resistance between the electrodes, which were placed opposite one another at the middle of the gastrocnemius muscle. A total of 25 μg plasmid DNA was injected with a 28-gauge needle into the center of the muscle between the electrodes. Electroporation was performed immediately after the injection, using an electroporator. The treatment was initially applied using 8 electrical pulses at 100 V for 50 ms. After these conditions were set as the control, the voltage was reduced to 50 or 25 V with various loading times (50–200 ms), and numbers of pulses (8–128 pulses).

2.3. Measurement of β-galactosidase production

Twenty-four hours after the transfer of the LacZ expression vector, 0.1 g of the gene-transferred muscle was removed and homogenized at 12,000 rpm/min with 5000 μl of the Beta-Glo® Reagent from the Beta Glo Assay System (Promega). This substrate is cleaved by β-galactosidase to form luciferin and galactose. The luciferin was then processed in a firefly luciferase reaction to generate light. After centrifugation, the supernatant was collected and 100 μl was transferred into individual wells of a 96-well plate, in duplicate. Absorbance (OD 420 nm) was measured with a luminometer, and the Relative Light Units (RLU) for each electroporation configuration was determined as the value compared to that at 100 V, 50 ms, and 8 pulses, which was defined as the standard.

2.4. Histological analysis

Twenty-four hours after transfer of the LacZ expression vector, the gene-transferred region was isolated and fixed with 4% paraformaldehyde. Samples were embedded in paraffin wax, cut into 5-μm serial sections, and stained with Hematoxylin-eosin (H-E). For the BMP gene-transfer groups ($N = 1$ for each treatment group), the target region was excised 20 days after the gene transfer, embedded in paraffin wax, sectioned, and stained with H-E.

2.5. Immunohistochemical analysis

The above-described *LacZ*-gene-transfected samples were deparaffinized, rehydrated, and treated with 3% H_2O_2 to block endogenous peroxidase activity. The sections were then incubated with an anti-β-galactosidase antibody (1:400) (Promega) and an HRP-labeled anti-mouse antibody. The labeling reaction was visualized with diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin. In the group receiving *BMP-2* gene transfer, muscle sections were taken 10 days after the transfer and were stained with an anti-human BMP-2 antibody (1:400) (Sigma) and an HRP-labeled anti-mouse antibody. Immunohistochemical analysis was performed using the same procedure as for the *LacZ*-gene-transfected samples.

2.6. X-ray analysis

Twenty days after gene transfer by *in vivo* electroporation, the gene-transferred region was surgically removed and analyzed by soft X-ray imaging.

2.7. Biochemical analysis

After soft X-rays were taken, the same samples ($N = 5$) were homogenized at 12,000 rpm/min. After centrifugation, the supernatant was collected and the alkaline phosphatase activity (IU/mg protein) was measured using the 4-nitrophenylphosphate method. In addition, the calcium content of the rest of the sample, except the

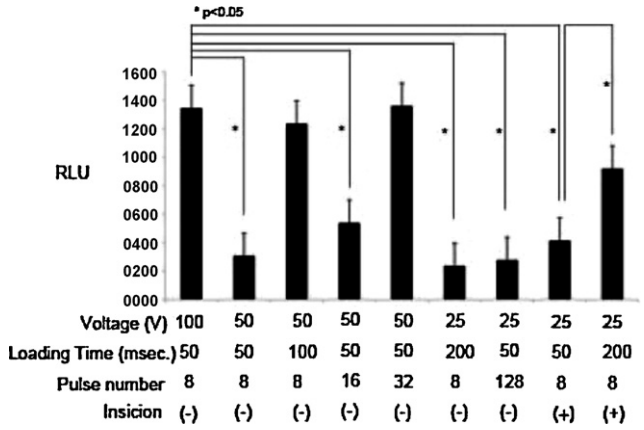

Fig. 1. Gene-transfer efficiency at 50 V and 25 V β-galactosidase production was determined using a substrate that is cleaved by β-galactosidase to generate galactose and luciferin, which was and measured by a luminometer. The Relative Light Unit (RLU) was the value compared to that obtained at 100 V, 50 ms, and 8 pulses, which was defined as the gene-transfer efficiency under standard conditions. The gene-transfer efficiency using 50 V or 25 V and various loading times or pulse numbers is shown. The gene-transfer efficiency when electrodes were placed in direct contact with the target tissue after skin incision is also shown. Each group $N = 5$, significance level 0.05, $^*p < 0.05$, error bars show the standard error of the mean.