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Transfer of the bone morphogenetic protein 4 gene into rat periodontal ligament by *in vivo* electroporation

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ABSTRACT

Objective: Regulation of alveolar bone metabolism is required in clinical dentistry. The aim of the present study was to establish a method for gene transfer into the periodontal ligament (PDL) by *in vivo* electroporation with a plasmid vector and to investigate the effects of *BMP-4* transfer into the PDL.
Design: Plasmids containing mouse *BMP-4* cDNA (pCAGGS-BMP4) were transfected into cultured rat PDL cells by *in vitro* electroporation, and BMP-4 production and secretion were detected by immunocytochemistry and western blotting. Next, pCAGGS-BMP4 was injected into the PDL of rats, and electroporation was performed *in vivo*, using original paired-needle electrodes. BMP-4 expression was examined by immunohistochemical staining 3, 7, 14, 21, and 28 days after electroporation. Control groups were injected with pCAGGS by electroporation, injected with pCAGGS-BMP4 without electroporation, or subjected to neither injection nor electroporation.
Results: *In vitro*-transfected rat PDL cells exhibited production and secretion of the mature-form BMP-4. After *in vivo* electroporation of pCAGGS-BMP4, site-specific BMP-4 expression peaked on day 3, gradually decreased until day 14, and was absent by day 21. We observed no unfavorable effects such as inflammation, degeneration, or necrosis.
Conclusions: Gene transfer by electroporation with plasmid DNA vectors has several advantages over other methods, including the non-viral vector, non-immunogenic effects, site-specific expression, simplicity, cost-effectiveness, and limited histological side effects. Our results indicate that the method is useful for gene therapy targeting the periodontal tissue, which regulates alveolar bone remodeling.

1. Introduction

Periodontal tissue reacts to stimuli by active remodeling *via* the expression of specific molecules involved in periodontal tissue repair, regeneration, and maintenance (Lekic & McCulloch, 1996). Thus, the artificial modulation of periodontal ligament (PDL) molecules could make it possible to increase alveolar bone mass and promote the maturation and calcification of newly formed bone. In orthodontics, enhanced alveolar bone formation is important to control the anchorage value during tooth movement for a more predictable outcome and to prevent relapse. Bone formation is also required after orthognathic and bone graft surgery. Furthermore, alveolar bone loss associated with

Abbreviations: ALP, alkaline phosphatase; BMD, bone mineral density; BMP, bone morphogenetic protein; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PDL, periodontal ligament.

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periodontitis and insufficient bone support for dental implants require bone formation.

PDL cells are characterized by various protein markers such as collagen type III, osteopontin, bone morphogenetic proteins (BMPs), osteocalcin, and bone sialoprotein (Kramer, Nares, Kramer, Grogan, & Kaiser, 2004). Of the many proteins associated with PDL cells, BMP-4, a member of the transforming growth factor-β superfamily, is particularly important for bone growth and remodeling (Leong & Brickell, 1996; Rosen & Wozney, 2002) and is induced by bone fractures (Nakase et al., 1994), mechanical stress (Sato et al., 1999), and tensile stress (Ikegame et al., 2001). Many studies have examined ectopic bone formation (Kawai et al., 2003; Kishimoto, Watanabe, Nakamura, & Kokubun, 2002; Kotajima, Kishimoto, Watanuki, Hatori, & Kokubun, 2006; Kramer et al., 2004; Luk et al., 2003) and BMP-mediated bone formation in cases of femoral (Rose et al., 2003; Rundle et al., 2003) or alveolar bone loss (Jin, Anusaksathien, Webb, Rutherford, & Giannobile, 2003; King, King, & Hughes, 1998; Rajshankar, McCulloch, Tenenbaum, & Lekic, 1998; Wikesjö et al., 1999). BMP-4 also plays an important role in the differentiation and patterning of hard and soft periodontal tissues after birth (Kim et al., 2007). However, the effects of BMP-4 on the PDL remain unclear.

Exogenous BMP-4 stimulates osteopontin, *BMP-2*, and *Cbfa1* mRNA expression in human PDL cells (Xu et al., 2004), and BMP-4-positive cells are found in the PDL area after tooth transplantation and in osteoblast-like cells on the newly formed alveolar bone surface (Hosoya et al., 2008). Therefore, the potential use of BMPs to induce *de novo* bone formation is promising (Rosen & Wozney, 2002). However, protein-based treatment requires a large amount of highly refined recombinant BMP (Wang et al., 1990), a carrier that gradually releases BMPs or a scaffold for osteogenic cells (Reddi, 2000; Wozney et al., 1988), *e.g.*, the collagen sponge in the alveolar bone loss model (King et al., 1998; Rajshankar et al., 1998; Wikesjö et al., 1999), and an invasive surgical procedure.

Gene transfer has proven useful for the regulation of gene expression and protein synthesis in biological research and in gene therapy and may be an alternative method for BMP introduction. Viral expression vectors have attracted a great deal of interest for gene transfer, both directly and indirectly (*via* transplanted cultured cells treated *ex vivo* with a viral vector), including genes encoding BMPs (Bloquel, Fabre, Bureau, & Scherman, 2004; Kishimoto et al., 2002), but these vectors are limited by their long expression period, non-localized infection area, and potential to induce immunological reactions (Wilson, 2009). Thus, the development of a suitable method for BMP gene transfer in a clinical setting is urgently needed.

One potential strategy is gene transfer by electroporation, which has been successfully adopted by developmental biologists to misexpress genes of interest (Funahashi et al., 1999; Nakamura, Sato, & Suzuki-Hirano, 2008; Odani, Ito, & Nakamura, 2008) and has recently been applied *in vivo* (Bloquel et al., 2004). In previous reports, the electroporation of BMP-4 expression plasmids into mouse muscle tissue induced ectopic bone formation (Kishimoto et al., 2002; Miyazaki & Miyazaki, 2008). The use of plasmid vectors for electroporation ensures transient, but ubiquitous expression, without risk of infection. Therefore, in the present study, we established an *in vivo* electroporation method to transfer plasmid vectors into the PDL and to investigate the effects of PDL transfection with *BMP-4*.

2. Materials and methods

2.1. Plasmid DNA

The 1.6-kb mouse *BMP-4* cDNA (Jones, Lyons, & Hogan, 1991) was kindly provided by Dr. Brigid L. M. Hogan, and the expression vector pCAGGS (Fig. 1a) (Niwa, Yamamura, & Miyazaki, 1991) was provided by Dr. Junichi Miyazaki. Mouse *BMP-4* cDNA was inserted into the multiple cloning site of pCAGGS to form pCAGGS-BMP4, and the pCAGGS-GFP vector (containing the green fluorescent protein [*GFP*] gene), pCAGGS-lacZ vector (containing the *lacZ* gene), and pCAGGS mock vector were used as control plasmids. All of the plasmids were transferred to *Escherichia coli* DH5α (Toyobo, Osaka, Japan), extracted using the Qiagen EndoFree Plasmid Maxi Kit (Valencia, CA, USA), and eluted in TE buffer (10 mM Tris [pH 8.0] and 1 mM EDTA). The concentration and purity of the plasmids were measured using a spectrophotometer (GeneQuant Pro; Amersham Biosciences Co., Piscataway, NJ, USA) and adjusted to 2.0 μg/μL.

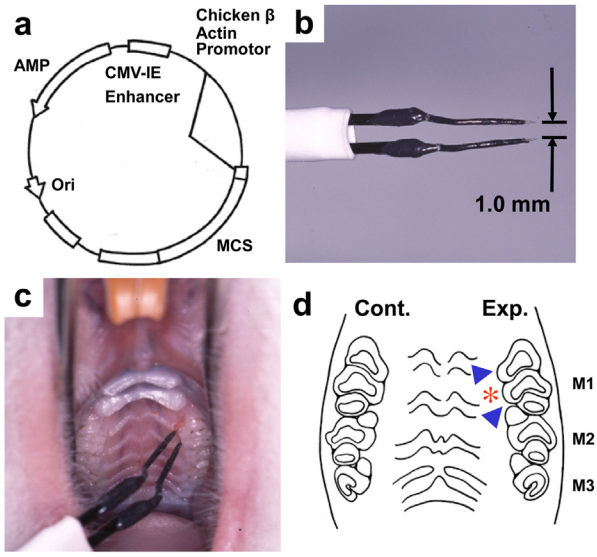

Fig. 1. Structure of the pCAGGS plasmid vector and method for *in vivo* gene transfer into the periodontal ligament (PDL). (a) Structure of the pCAGGS plasmid vector possessing a CAG promoter (enhancer of the cytomegalovirus and chicken β-actin promoter). Mouse *BMP-4*, *GFP*, or *lacZ* was inserted into the multiple cloning site (MCS). (b) The needle-type electrodes consisted of a pair of stainless steel wires. (c) Electrodes inserted into the palatal PDL of the rat upper first molar. (d) Illustration of c. Red asterisk, plasmid injection site; blue arrowhead, electrode insertion site; M1, upper first molar; M2, upper second molar; M3, upper third molar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. In vitro *electroporation*

Rat PDL cells (mixed populated cell line established by Kubota et al.) (Kubota, Chiba, Obinata, Ueda, & Mitani, 2004) were cultured until confluent in 12-well plates at 33 °C in α-modified minimal essential medium (Wako, Osaka, Japan) supplemented with 10% (v/v) fetal bovine serum (Biocell Laboratories, Inc., Compton, CA, USA), 100 U/mL penicillin-G, and 100 μg/mL streptomycin sulfate (Wako) in a 5% CO_2 atmosphere. Thereafter, the cells were incubated at 37 °C, and the culture media was changed every 3 days.

For electroporation, cells were taken from culture wells and suspended in electroporation buffer (25% Opti-MEM1 and 75% cytosalts: 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4 [pH 7.6], and 5 mM $MgCl_2$) (van den Hoff, Moorman, & Lamers, 1992). The plasmid (10, 20, or 30 μg) was added to 600 μL of cell suspension by adjusting the volume of the plasmid solution (pCAGGS-BMP4, 2.0 μg/μL), and 30 μg of pCAGGS-GFP plasmid was used as a negative control. Electric pulses were generated using a CUY21 *in vitro* electroporator (Nepa Gene, Ichikawa, Japan) and applied to 4-