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The effect on bone regeneration of a liposomal vector to deliver BMP-2 gene to bone grafts in peri-implant bone defects

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Abstracts

Successful bone–implant osseointegration in large peri-implant bone defects is often difficult, even through autologous bone grafting. Recently, cell-mediated regional gene therapy was introduced to deliver potent morphogens or growth factors in regenerative medicine. We applied liposomal vectors carrying bone morphogenetic protein (BMP)-2 cDNA directly into freshly created peri-implant bone defects on pig calvariae, with or without autologous bone graft. The BMP-2 gene was efficiently introduced into immigrating cells and trabecular cells lining the marginal bone surrounding the bony defect. After 1 week, abundant BMP-2 protein was detected throughout the peri-implant bone defect by immunohistochemistry. At 4 weeks, BMP-producing cells were still present in the defect and peri-implant area, which significantly enhanced new bone formation, compared with the control groups. Interestingly within a week of BMP-2 gene delivery with bone grafts, most osteoblastic cells lining the grafted bone chips also produced BMP-2. Particulated bone was immediately reorganized into newly formed trabecular bone. Grafted bone without BMP-2 gene delivery was still scattered and new bone matrix formation was not detected until 4 weeks after bone grafting. In conclusion, direct application of the BMP-2 gene using a liposomal vector enhanced bone regeneration in a bony defect and gene delivery combined with bone graft could induce a rapid osseointegration of the bone–implant interface at earlier stage.

Keywords: Bone morphogenetic protein (BMP); Gene transfer; Liposome; Bone graft; Dental implant; De novo bone formation

1. Introduction

Recently, regional gene therapy approaches have been reported for bone and cartilage regeneration in animal defect models [1–9]. Bone morphogenetic protein (BMP) transgenes have often been used for bone repair and successful results have been reported in various animal experiments [1–5,8]. BMP-2 is a potent osseoinductive factor [10] shown to induce osteogenic differentiation of mesenchymal cells, and further administration of recombinant BMP-2 protein in vivo is known to induce orthotopic and ectopic de novo bone formation [11,12]. Although encouraging results have been achieved with BMP-2 and other recombinant BMPs (rhBMPs) in animal experiments and clinical applications, several problems such as high cost, relatively high protein doses from several micrograms up to milligrams, as well as a short protein half-life are obstacles that still have to be overcome [13].

Various approaches using gene transfer to deliver potent morphogens or growth factors have been investigated in regenerative medicine [6–8,14]. Until now, in vivo gene delivery in gene therapy approaches have commonly used viral vector systems such as adenoviruses or retroviruses. Recently, non-viral gene delivery systems were introduced as another useful vector for the purpose of tissue repair in animal models [15,16]. Although less efficient than the viral methods, non-viral gene transfer with liposomal vectors may offer several advantages over viruses, e.g. ease of preparation and application, and fewer immunological and safety problems [14,17–19]. Most non-viral approaches are restricted to transient gene expression. However, transgene

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expression using liposomal gene delivery can persist from a few days to weeks, and was sufficient for inducing bone and cartilage regeneration in animal defect models [8,15,20]. We have previously reported successful bone repair via cell-mediated gene transfer by means of BMP-2-producing cell transplantation in rat critical-sized bone defects using a liposomal vector [8]. In this study, we performed direct BMP-2 gene delivery without ex vivo cell intervention into peri-implant bone defects in pig calvarium to investigate whether a liposomal vector system can efficiently introduce BMP-2 gene to cells immigrating into a bony defect during de novo bone formation.

In reconstructive dental and orthopaedic surgery, several investigations have reported that coating the implant surfaces with cytokines or growth factors improves bone healing in the area adjacent to the bone–implant interface [21–24]. A greater bond strength at the bone–implant interface was achieved using a rhBMP–collagen mixture adjacent to titanium implants, and occurred in a shorter time period than titanium implants without rhBMP [21,24]. In our pilot study, a reporter gene (green fluorescent protein, GFP)/liposome complex coated on a titanium surface successfully delivered the reporter gene into immigrating cells surrounding the titanium implant surface [25].

In large peri-implant bone defects, conventional autologous bone graft is the so-called gold standard in transplantation surgery [26]. Even through autologous bone grafting, it is often hard to accomplish successful bone–implant osseointegration. Fibrous healing can result from the initial failure of osteogenic activation producing new bone matrix directly on the implant surface during the early stages of wound healing. In this study, we investigated whether liposomal vectors can access the cells of the trabecular-lining in autologous particulated bone chips and induce osseous integration on the implant surfaces, and whether this has a synergistic effect on the surrounding autologous bone grafts.

2. Materials and methods

2.1. In vitro experiments

Eight adult pigs (18 months old) were used for the in vivo animal study. The research project was approved by the Animal Research Committee for animal research of the government of Midfrankonia (approval no. 621.2531.31-14-01, Ansbach, Germany). Before performing in vivo experiments, the efficiency of liposomal gene delivery was evaluated in vitro. Primary bone marrow stromal cells (BMSC) were isolated under sterile conditions from aspirated bone marrow from the tibia of the animals. The collected bone marrow was filtered through a 70 μm-pore filter, then transferred to 100 mm culture vessels with alpha-medium (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum, L-glutamine (0.35 mg/ml), and penicillin/streptomycin (100 IU/ml). The next day, non-attached cells were carefully washed off and the culture medium was replaced on the 4th day. When the cells were 80% confluent, they were trypsinized and used for the experiments. For BMP-2 gene delivery, a p*CMV*BMP-2 plasmid containing human BMP-2 cDNA (donated by Dr. G. Gross, Gesellschaft für Biochemische Forschung, Braunschweig, Germany) under the control of the CMV promoter was constructed as previously described [25]. For liposome-mediated transfection, the plasmid p*CMV*GFP-C1 (Clonetech, California, USA) containing GFP cDNA or p*CMV*BMP-2 was mixed with Metafectene (Biontex, Munich, Germany; 4 μl per μg of DNA) according to the manufacturer's protocol. The transfection rate was analysed with p*CMV*GFP-transfected cells on the FACS Calibur flow cytometer (BD Biosciences, Heidelberg, Germany). Transfection efficiency using p*CMV*BMP-2 was analysed by immunohistochemical staining with untransfected cells as a control. For immunohistochemical staining, the cells were fixed with 4% paraformaldehyde for 10 min, blocked with protein blocking solution (DAKO, Hamburg, Germany), and incubated with anti-human BMP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) for 1 h at room temperature. Following careful washing with phosphate-buffered saline (PBS), first a biotinylated anti-mouse rabbit IgG (DAKO, Hamburg, Germany) and then a streptavidin–HRP complex (DAKO, Hamburg, Germany) were applied. BMP-2 protein was detected by treating the cells with 3-amino-9-ethylcarbazole (AEC; DAKO, Hamburg, Germany).

2.2. Creation of peri-implant bone defects

All surgical procedures were performed using intravenous anesthesia with Midazolam and Ketamin HCL (Ketavet®, Ratiopharm, Ulm, Germany) under standard monitoring conditions. The local anesthesia was supplemented by local administration of 4% Articain® containing epinephrine (1:100,000) (Ultracain-DS forte, Hoechst GmbH, Frankfurt am Main, Germany) in the forehead region. Nine peri-implant bone defects were created on a pig calvariae (Os frontale). A perioperative antibiotic was administered 1 h preoperatively, and for 2 days postoperatively (Streptomycin, 0.5 g/d, Gruenenthal, Stolberg, Germany). An incision was first made in the skin and the periosteum of the skull to gain access to the neurocranium. Using a trephine drill (1 × 1 cm, Roland Schmid, Fuerth, Germany), bony defects (10 mm in diameter, 7 mm in depth) were created (Fig. 1G). The defects were positioned at least 10 mm apart to avoid biological interactions. After cleansing the inside of the bony defects with saline solution, implants (3.5 × 14 mm, Ankylos, Friadent GmbH, Mannheim) were inserted in the centre of each defect, and half of the implant (7 mm in height) was submerged below the bottom of the defect for stability.

2.3. In vivo gene delivery

Both experimental and control groups were included. The two experimental groups included animals with liposomal BMP-2 gene delivery with ($n = 18$, group C) or without ($n = 18$, group A) autologous bone grafting. Two control groups consisted of animals treated with collagen carrier only ($n = 18$, group B), or autologous bone grafting without vector delivery ($n = 18$, group D) (Fig. 2A). Twelve micrograms of the p*CMV*BMP-2 plasmid and 60 μl of liposome (Biontex, Munich, Germany) were each diluted with 200 μl DMEM (Invitrogen, Karlsruhe, Germany), combined, and left at room temperature for 15 min. After incubation of the DNA/liposome complex, the complex was gently pipetted up and down 3–4 times and the p*CMV*BMP-2/liposome complex was delivered onto the implant surface and the peri-implant defect filled with 5 cm^2 collagen sponge (Lyostypt® Braun-Melsungen AG OPM, Melsungen, Germany). For gene delivery with autologous bone graft, the autologous bone harvested from the defects that were created was used after crushing the bone in a bone mill (Quetin Dental Products, Leimen, Germany) to obtain standardized particle sizes. The bone chips were mixed with 472 μl p*CMV*BMP-2/liposome complex and immediately transferred into peri-implant bone defects. After filling the defects with delivery materials, the superficial surfaces of all defects were covered with a fibrin sealant (Tisseel, Baxter, Deerfield, USA). In all groups, the periosteum and skin over the defects was sutured in two layers (Vicryl® 3.0; Vicryl® 1.0; Ethicon GmbH & Co KG, Norderstedt, Germany).