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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).

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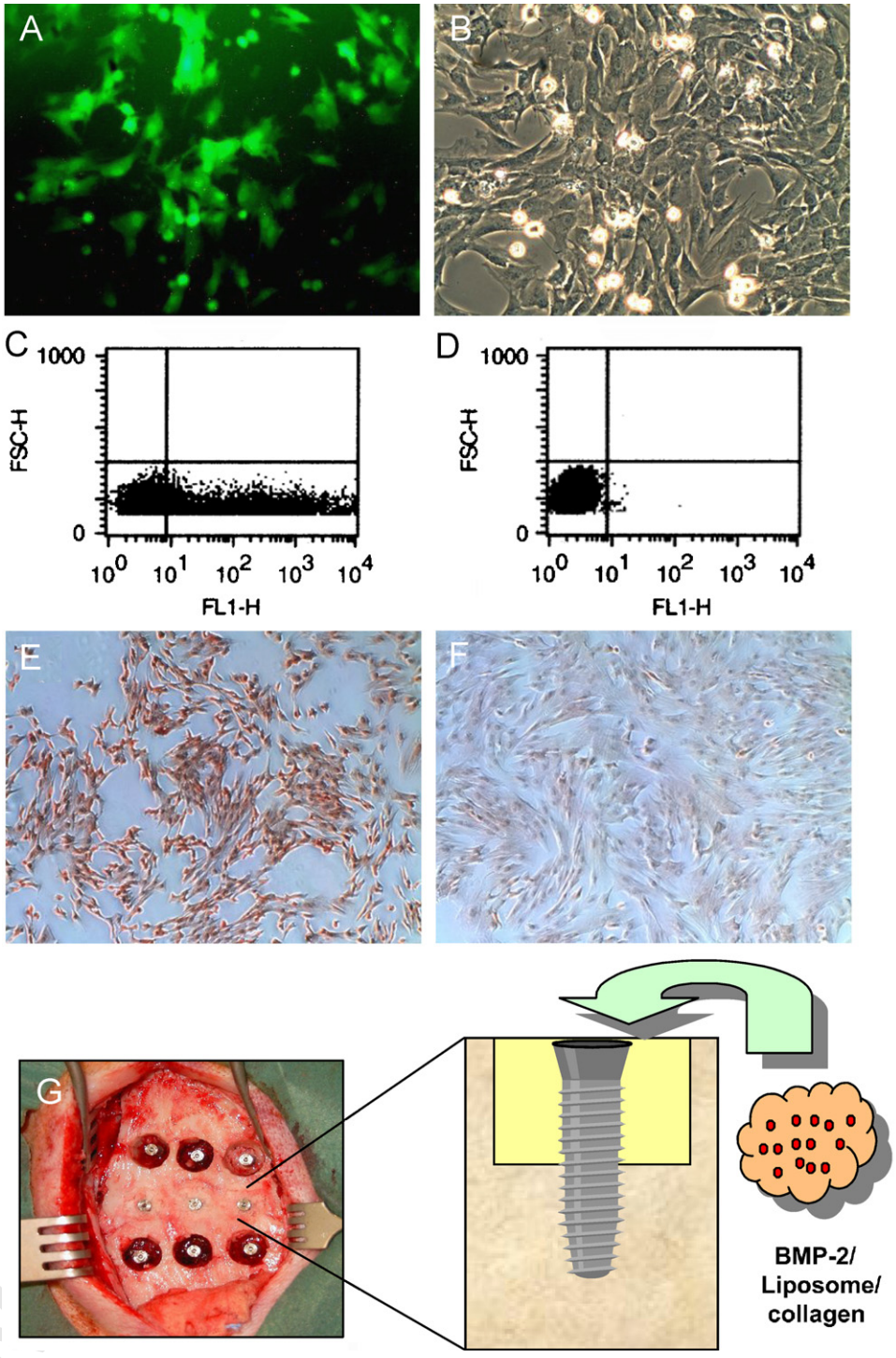

Fig. 1. In vitro gene transfer efficacy and in vivo gene delivery. Primary pig BMSC (1.5×10^5 cells) were treated with Metafectene–p*CMV*GFP complexes (1 μg DNA/4 μl of the lipid) and observed under the fluorescent microscope (A and B in bright field) and 55% of pig BMSCs were detected as GFP-positive cells, using FACS analysis, 3 days after transfection (C); the negative control is shown in (D). Most cells clearly showed BMP-2 staining on day 3 after transfection with p*CMV*BMP-2 plasmid (E) compared with endogenous BMP-2 levels observed in untransfected cells (control, shown in (F)) after immunohistochemical staining. Nine peri-implant bone defects were created on a pig calvariae (G). After creation of the bone defect (10 mm in diameter, 7 mm in depth), an implant (3.5×14 mm) was inserted in the centre of each bone defect. Half of the implant (7 mm in height) was submerged below the bottom of the bone defect for stability, followed by liposome-mediated BMP-2 gene delivery with a collagen carrier. Magnification: 100 × (A, B) and 50 × (E, F).

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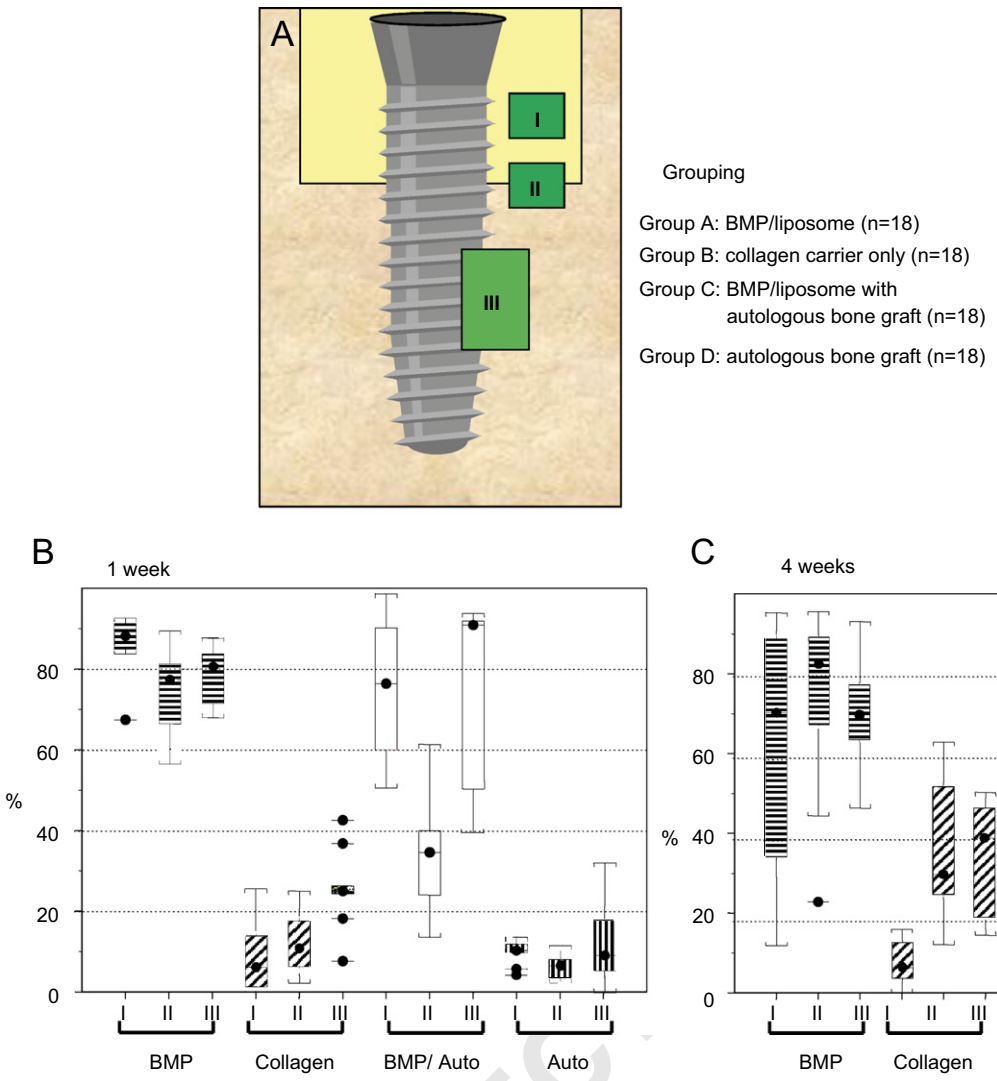

Fig. 2. In vivo efficacy of BMP-2 gene delivery. For evaluation of the efficiency of in vivo gene delivery, a peri-implant bone defect was divided into 3 regions of interests (ROIs), as shown in (A). The in vivo transfection rate was measured by calculating the proportion of BMP-2-positive cells among the total counterstained cell population at each ROI I 1 week (B) and 4 weeks (C) after surgery, in all groups. In the first week, the BMP-2-producing cells in both liposomal BMP-2 groups, with/without autologous bone graft, were significantly higher than those for the control groups irrespective of the region of interest (B). Although the proportion of BMP-2-positive cells in the control groups increased at week 4, they did not reach the level of BMP-2 expression observed in the liposomal groups at the same time point. The high BMP-2 expression in the liposomal groups at week 4 is most likely due to endogenous BMP-2 released by non-transfected cells stimulated through a paracrine signalling pathway by the transfected cells (C).

2.4. Biopsy harvest and preparation of the specimens

The animals were sacrificed on days 7 and 28. The pigs were sedated by an intramuscular injection of azaperone (1 mg/kg) and midazolam (1 mg/kg) in the neck. They were then euthanized by an intravascular injection of 20% pentobarbital solution into an ear vein until cardiac arrest occurred. The skull caps of the sacrificed animals were removed and immediately frozen at −80 °C. The preparation procedure was followed as described in previous studies [27–30]. Briefly, the individual bone defects were separated using a standard cutting system (Exakt Apparatebau GmbH, Norderstedt, Germany). Immersion fixation was carried out using 1.4% paraformaldehyde at 4 °C. The specimens were dehydrated in an ascending alcohol series at room temperature in a dehydration unit (Shandon Citadel 1000, Shandon GmbH, Frankfurt, Germany). Technovit 9100® (Heraeus Kulzer, Kulzer Division, Werheim, Germany) was used for embedding.

2.5. Microradiography

The undecalcified resin embedded sections were reduced to 150–180 μm using a grinding unit (Exakt Apparatebau GmbH, Norderstedt). Subsequently, the samples were irradiated in the Faxitron® cabinet X-ray unit using 11 kV tube voltages and 2.5 mA for 6 min. The developed X-rays (Kodak, Stuttgart, Germany) were scanned with an Epson scanner at 1200 dpi and 12-bit greyscale and stored in Tiff-format. Mineralization ratio was analysed in the defect area using Bioquant Osteo software V7.10.10 (BIOQUANT Image Analysis Corporation, Nashville, USA) in all groups on day 28.

2.6. Immunohistochemistry

The embedded samples were cut into 5 μm-sections using a microtome (Leica microsystems, Heidelberg, Germany), and endogenous peroxidase

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was blocked by incubation with 3% hydrogen peroxide for 15 min. Serum-free blocking agent (DAKO, Hamburg, Germany) was used to prevent non-specific protein binding. The sample was then allowed to react with a rabbit monoclonal antibody against rhBMP-2 (Santa Cruz Biotechnology, Santa Cruz, California, USA) for 1 h at room temperature. Following careful washing with PBS, first a biotinylated anti-rabbit goat IgG (DAKO, Hamburg, Germany) and then a streptavidin–HRP complex (DAKO, Hamburg, Germany) were applied, which was detected by 3-amino-9-ethylcarbazole (AEC; DAKO, Hamburg, Germany). The procedure was completed by hematoxylin–eosin counterstaining. All samples were accompanied by a negative control.

The peri-implant bone area was divided into three different regions of interest (ROI) as shown in Fig. 2A. The ROI I was confined to the central area of the peri-implant bone defect, and ROI II was designated along the margin of the bone defect. The bone–implant interface area without a peri-implant bone defect was labelled ROI III. Each ROI was selected at least 3 times per sample by different individuals and the BMP-2-positive cells were counted in each ROI using a counting grid. The proportion of BMP-2-positive cells among the total counterstained cell population was analysed in each ROI in the specimens from all groups on days 7 and 28 after surgery.

3. Results

To identify the transfection efficiency of the liposomal vector, pig BMSC were transfected with p*CMV*GFP in vitro. Three days after transfection, approximately half of the cells were detectable under a fluorescent microscope (Fig. 1A) and 55% of the pig BMSCs were GFP-positive on the FACS Calibur flow cytometer (Fig. 1C). Transfection efficiency of vector into pig BMSC was confirmed by immunohistochemistry 3 days after transfection in vitro with p*CMV*BMP-2 plasmid. Most of cells clearly showed BMP-2 staining compared with the endogenous BMP-2 production observed in untransfected control cells (Fig. 1E and F). Furthermore, the in vivo transfection rate was measured by calculating the number of BMP-2 positive cells among the total counterstained cell population in each ROI. In the first week, the BMP-2-producing cells in both liposomal BMP-2 groups with/without autologous bone graft were significantly higher than those for the control groups, irrespective of the ROI (Fig. 2B). Although the number of BMP-2-positive cells in the control groups increased at week 4, the level of expression did not reach the BMP-2 expression levels observed in the liposomal groups at the same time point (Fig. 2C). BMP-2 was detected in high quantity of cells immigrating into the centre of the defect (ROI I) in liposomal group A 1 week after transfection (Fig. 3A) compared with the control group (Fig. 3B). Irrespective of the ROI, abundant numbers of both immigrating cells and trabecular cells lining the marginal bone surrounding the bony defects cells in ROI II and III were BMP-2-positive (Fig. 3C and E) compared with those in group B (collagen group) (p-value = 0.001) (Fig. 3D and F).

New bone matrix with dense trabecular patterns formed in the defect area without achieving complete contact de novo bone formation to the implant surface in the centre of the defect in group A (liposomal group) at week 4, as observed under light microscopy (Fig. 4A and E in a high magnification) and microradiography (Fig. 4C). Most of the bone defect area around ROI II was filled with newly formed bone matrix in group A (Fig. 4G), while in group B (collagen group) new bone formation with a thin, loose trabecular pattern was observed only around the border of the defect (Fig. 4B, D, H). Loose fibrous tissue filled the defect centre with no bone matrix detectable at ROI I 4 weeks after surgery (Fig. 4F). The bone mineralization ratio in the defect at 4 weeks in group A was significantly higher than that in group B (p-value = 0.016), but there was no gross difference in the mineralization ratio in the defect area between groups C (autologous bone graft with liposomal vector) and D (autologous bone only) 4 weeks after grafting (Fig. 4I). Interestingly, at the early stages of wound healing after grafting, there was a clear difference in the bone matrix reorganization of the particulated bone chips between these 2 groups (Fig. 5). One week after bone grafting, new bone trabeculae were emerging directly from the particulated bone chips. New bone matrix had already begun to be organized at ROI I and II in group C (bone graft/liposomal vector) (Fig. 5A and C at a higher magnification of the dotted rectangular area in A). At ROI I in group C (autologous/liposomal group), most of the cells in the trabecular-lining were still tightly bound to the surface of the bone chips and showed positive BMP-2 staining (Fig. 5E and G at a higher magnification (1000 ×) of the rectangular area indicated in E) at week 1 in immunohistochemical staining. In the centre of the defect, a newly formed bone matrix was found without the intervention of fibrous tissue directly on the implant surface (Fig. 5I at a higher magnification (100 ×) of the linear rectangular area indicated in Fig. 5A). Active BMP-2 production was detectable on the newly organized bone matrix in the same area, indicating rapid bone remodelling of the grafted bone via liposomal gene delivery (Fig. 5K) in group C (autologous/liposomal group) at week 1 in immunohistochemical staining. Particulated bone was still scattered diffusely in the defect, and new bone matrix organization with a trabecular pattern from grafted bone chips was not detectable at week 1 in group D (autologous bone graft) (Fig. 5B and D at a higher magnification (200 ×) of dotted rectangular area in B). BMP-2-producing cells lining the bone chips were rarely found (Fig. 5F and H at a higher magnification (1000 ×) of the rectangular area indicated in F). Scattered bone particles without matrix organization were also commonly observed around the implant surface, without BMP-2 activation, at week 1 in autologous bone graft group D (Fig. 5J and L).

4. Discussion

Non-viral gene delivery is easy to use and safer than viral vector systems, but has a relatively low efficiency of target gene delivery [14,17]. Most gene therapy applications use viral vectors because they have evolved specific machinery to deliver DNA into cells [14]. Previous studies on bone regeneration have reported successful results using viral

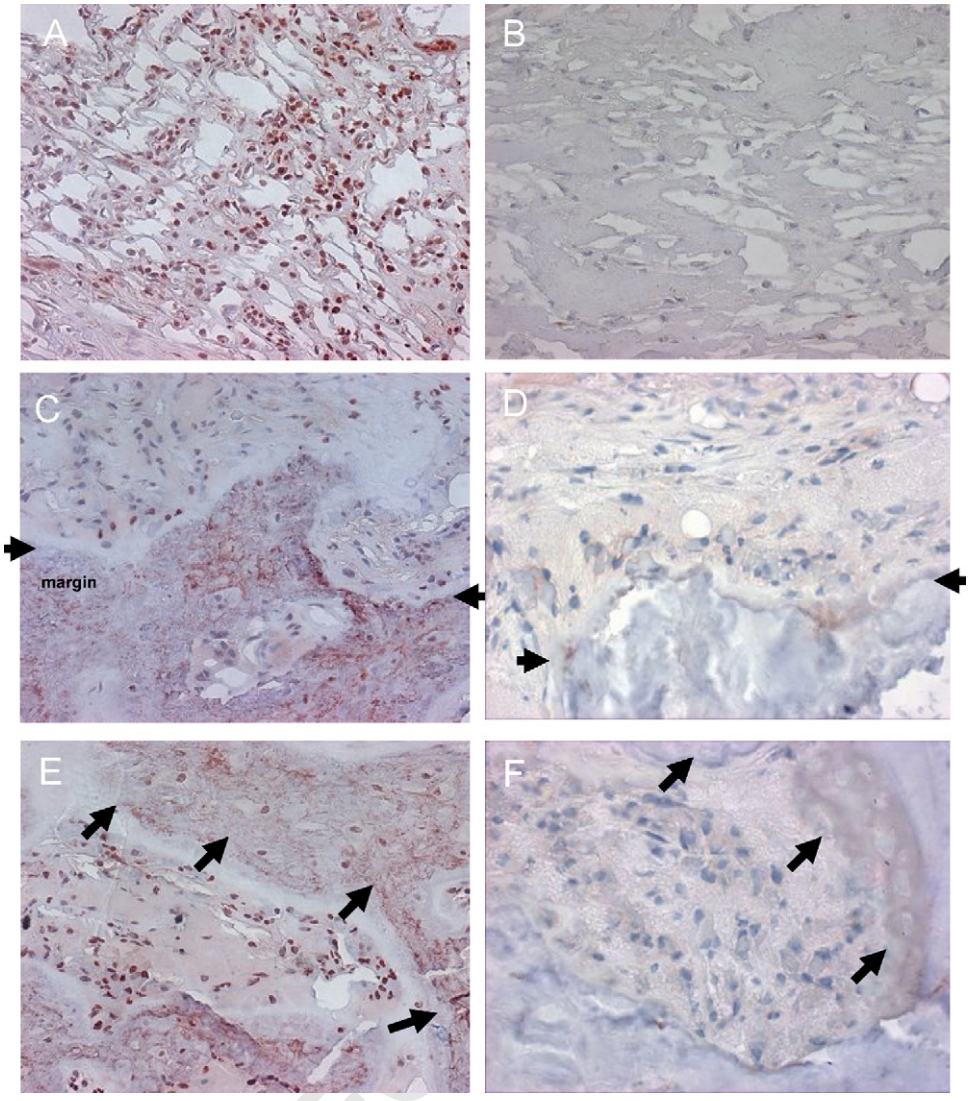

Fig. 3. Detection of BMP-2-producing cells in vivo by immunocytochemistry. After 1 week, BMP-2 production at each ROI was compared between the liposomal group (A, C, E) and collagen-only group (B, D, F). BMP-2 was detected in high quantity of cells immigrating into the centre of the defect (ROI I) in liposomal group A 1 week after transfection (A) compared with the control group (B). Abundant numbers of both immigrating cells and trabecular cells lining the marginal bone (arrows in C–F) surrounding the bony defects cells in ROI II (C) and III (E) were BMP-2-positive (shown in red) compared with those in group B (collagen group) (D, F). Magnification: 320× (A, B) and 400× (C–F).

vectors, such as adenoviruses or retroviruses, carryingBMP-2 cDNA [1–6]. But, recent studies have shown that ex vivo transgene-activating cell transplantation using non-viral gene transfer can effectively induce bone and cartilage regeneration in animal models [8,15,20]. Although there is no clear-cut explanation for the favourable results obtained in vivo with the less efficient non-viral gene transfer compared with viral vector systems, 2 factors seem to be important. First, short-term transient expression of transgenes, such as BMP, by target cells continued for more than 1 week, irrespective of the vector used, and appears to be sufficient to induce successful bone or cartilage repair in some animal models, although viral vector systems provide more potent induction of tissue repair. This assumption is supported by the previous successful results of rhBMP-2 application achieved with a single-dose administration [11–13]. BMP protein that is only present for a few days may be important for recruiting a sufficient number of the required cells or initiating differentiation cascades [13]. In this study, direct gene delivery using liposomal vectors efficiently introduced BMP-2 into cells immigrating into the defects during initial wound healing. Abundant BMP-2-producing cells were observed throughout the defect area 1 week after gene delivery, and BMP-2 secretion was maintained for 4 weeks. Considering that the in vitro transgene expression decreased after 1 week in our previous study [8], the maintenance of BMP production after 4 weeks is likely to be due to endogenous BMP released by non-transfected cells that was stimulated through a paracrine pathway. In

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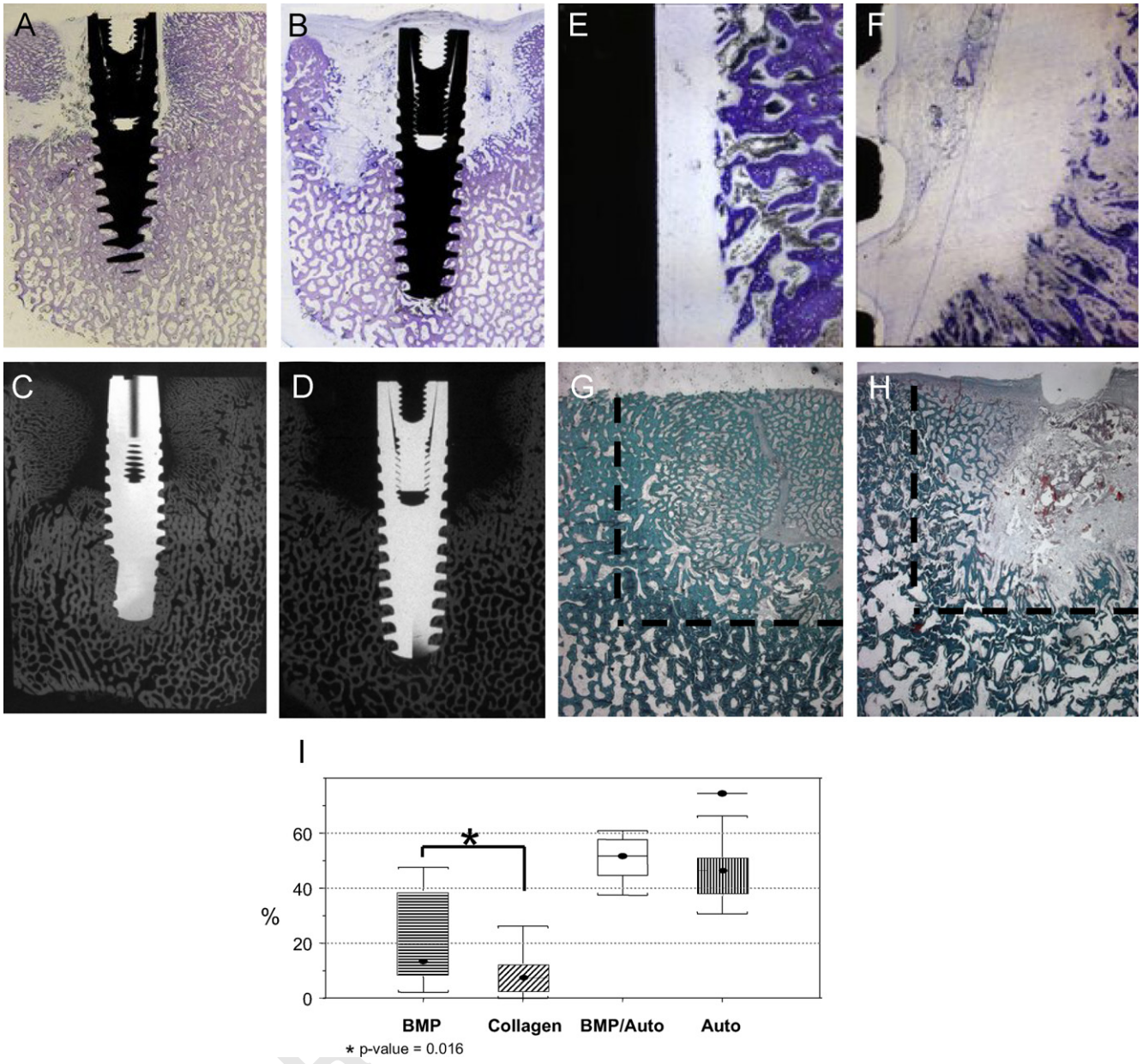

Fig. 4. Bone regeneration and mineralization ratio in the bone defects. Four weeks after gene transfer in the liposomal group (A, C, E, G), new bone matrix with a dense trabecular pattern formed in the bone defect area, but did not achieve complete osseointegration to the implant surface located in the centre of the bone defect, as shown by toluidine blue staining (A and E at a higher magnification) and microradiography (C). Most of the bone defect area around ROI II was filled with newly formed bone matrix in the liposomal group (G). In the collagen group (B, D, F, H), new bone formation with a thin, loose trabecular pattern was observed only around the border of the bone defect (B, D, H), and loose fibrous tissue filled the centre of the defect without bone matrix detectable at ROI I 4 weeks after surgery (F at a higher magnification in B). The bone mineralization ratio in the total bone defect area at week 4 in group A was significantly higher than that in group B, but there was no gross difference in the mineralization ratio between the autologous bone graft with liposomal vector and autologous bone groups in the bone defect areas 4 weeks after bone graft (I). Toluidine blue staining: A, B, E and F; microradiography: C, D; Trichrome-Goldner staining: G, H. Dotted lines in G, H: bone defect margins.

this study, bone regeneration in the bony defects was significantly enhanced in the liposomal group. This result implies that direct gene delivery using liposomal vectors might also be an efficient means to initiate transgene expression from neighbouring cells during early stage in wound healing. Second, an ex vivo cell-mediated approach may have additional advantages apart from the efficient delivery of the chosen vector system. Target cells can be properly selected ex vivo according to the objective of the gene delivery, and can be transfected in vitro under optimized conditions and transplanted at the best time to achieve the desired effect. Transplanted cells themselves could be used as a massive source of mobilized osteoprogenitor cells in the defect where they may immediately

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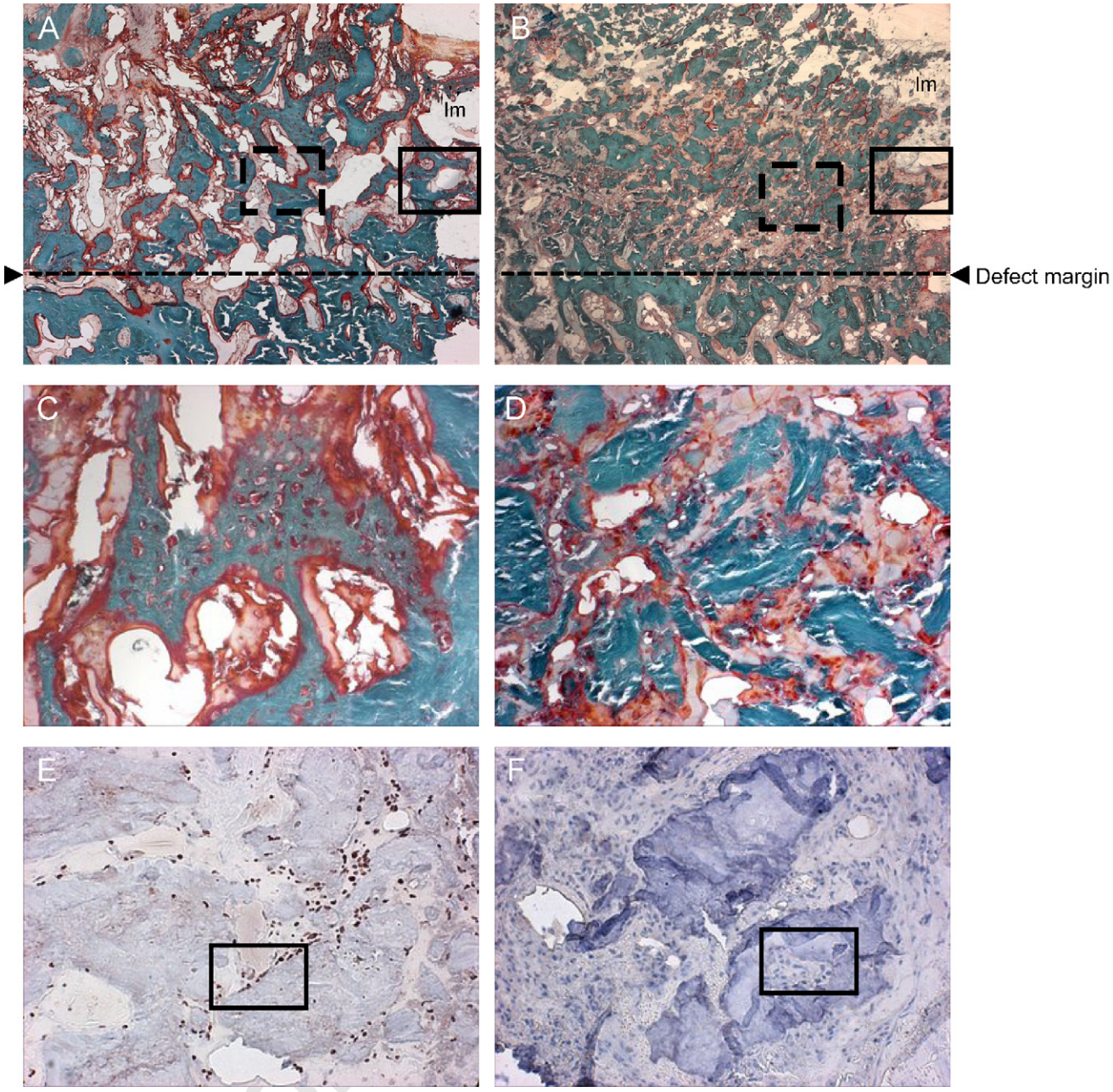

Fig. 5. Rapid new bone matrix organization in a combined treatment of autologous bone graft/liposomal gene delivery. In the early stages of wound healing after bone graft, there was a clear difference in the bone matrix reorganization of particulated bone chips between the autologous bone graft/liposomal group (A, C, E, G, I, K) and the autologous bone graft group (B, D, F, H, J, L). One week after bone graft, new bone trabeculae were emerging directly from the particulated bone chips, and new bone matrix had already begun to be organized at ROI I and II above the defect margin in the bone graft/liposomal group (A and C at a higher magnification of dotted rectangular area in A). At ROI I, most of the cells in the trabecular-lining seemed to be osteoblasts, and were still tightly bound to the bone chip surface. These cells stained positively for BMP-2 after immunohistochemical staining of the autologous/liposomal group at week 1 (E and G at a higher magnification of the rectangular area indicated in E) in immunohistochemical staining. Directly on the implant surface in the centre of the bone defect, a newly formed bone matrix was often found without the intervention of fibrous tissue in between, suggesting osseointegration directly on the implant surface in the centre of the defect (I at a higher magnification of the linear rectangular area indicated in A). Active BMP-2 production (arrows in K) was detectable in the newly organized bone matrix in the same area (I) in the autologous/liposomal group at week 1 in immunohistochemical staining. Meanwhile, particulated bone was scattered diffusely without new bone matrix organization in the bone defect (B and D at a higher magnification of dotted rectangular area in B), as well as around the implant surface (J at a higher magnification of the linear rectangular area indicated in B), at week 1 in the autologous bone graft only group. BMP-2-producing cells lining the bone chips were rarely found in both areas (F, H and L). Im: implant surface area; Trichrome-Goldner staining: A–D, I and J; immunohistochemistry: E–H, K, and L. Magnification: 25× (A, B), 200× (C–F), 1000× (G, H) and 100× (I–L).

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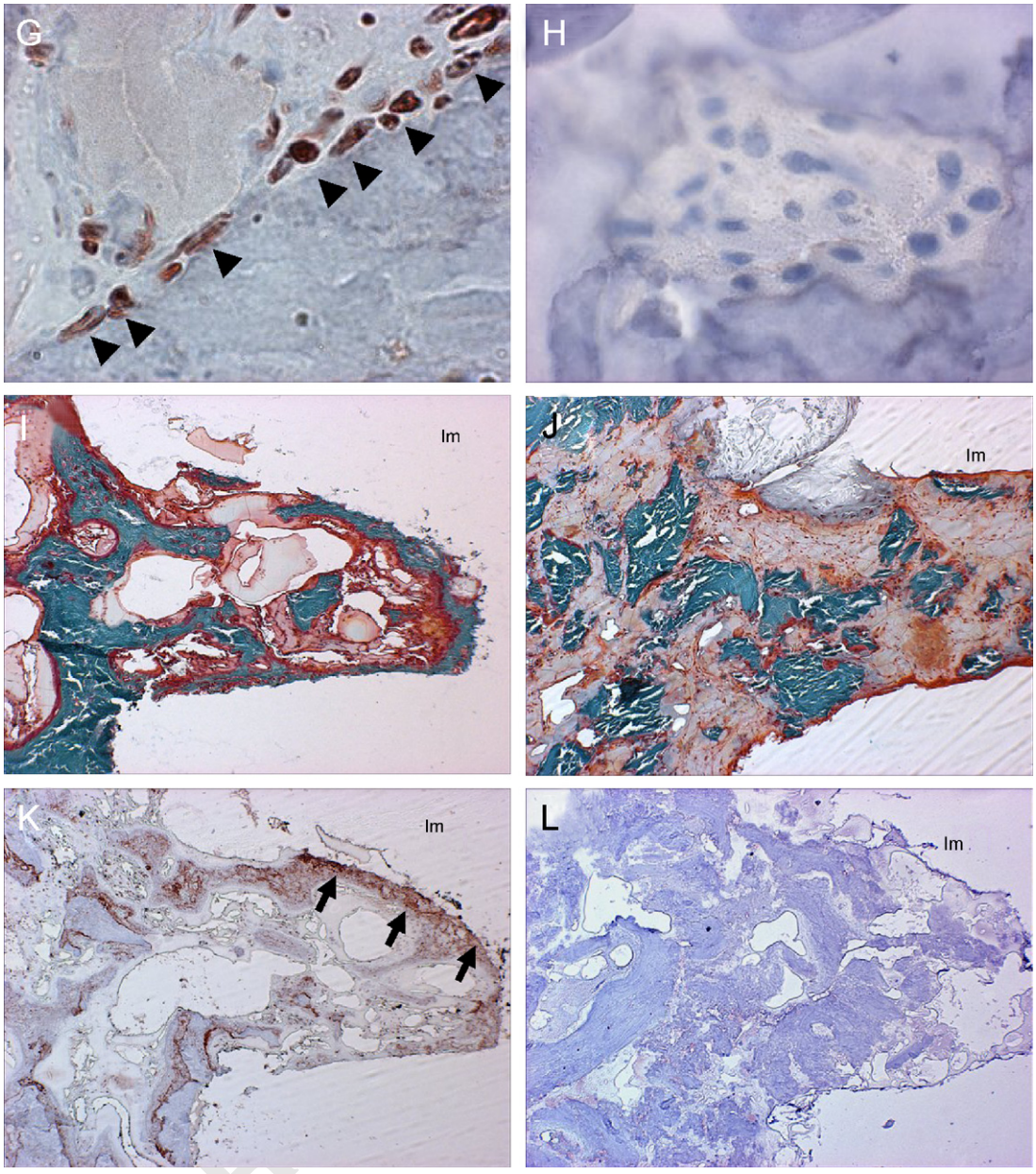

Fig. 5. (*Continued*)

affect neighbouring cells via paracrine mechanisms. This synergistic effect of target cell transplantation with gene transfer might offer advantages comparable to those previously reported for a combination of bone marrow transplantation and application of rhBMP-2 [31].

One difficulty of direct gene delivery without ex vivo cell intervention is the inability to select and efficiently transfect target cells, as is possible under in vitro condition. The direct application of non-viral vector systems in bone tissue engineering could not ensure that a sufficient number of stem/progenitor cells are available at the right place and time of gene delivery, which might be an important requirement for efficient gene delivery. Our result showed that liposomal vectors carrying the BMP-2 gene did not induce complete bone healing and osseointegration in the centre of the defect (ROI I in the experiment) compared with the complete healing observed in ROI II. This may be due to an insufficient number of target cells, e.g. stem cells or osteogenic progenitors, mobilized to the centre of the defect at the time of direct gene delivery. Poor transfection efficiency must not be the reason for incomplete healing of the central portion of the defect because most of the cells immigrating into the centre of defect were successfully transfected in our result. This finding is likely to be consistent with the result of another study using non-viral vectors with a porous hydroxyapatite carrier that showed that efficient gene delivery was mainly achieved in mobilized cells surrounding the carrier [32]. The combined

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use of an autologous bone graft with a liposomal gene delivery in our study also seems to support this assumption that initial cell mobilization is an important requirement for efficient direct vector application. Particulated bone chips provided abundant quantity of bone-related cells in the centre of the bone defect as target cells of gene delivery. Cells derived from autologous bone grafted to the defect could easily be transfected and achieve osseointegration to the implant surface in the centre of defect at an early stage of wound healing.

Furthermore, transfection efficiency should take into consideration not only the number of cells transfected, but also the amount of protein released. Because the in vivo ratio of BMP-2-producing cells was quite similar to the in vitro transfection rate of BMSC, the amount of BMP-2 protein produced by each transfected cell might be an important factor determining the bone regeneration potential. In a previous study on ectopic bone formation in rats, it was shown that the point of time at which bone formation occurred was dependent on the amount of rhBMP-2 present at the injection site [10]. The different amounts of BMP-2 protein released from transfected cells according to the efficacy of the gene delivery system is also likely to be responsible for the different capabilities of the transgene-activating cells leading to bone formation in vivo.

In our study, liposomal vector applied to the surface of the implant seemed to work favourably. Previous studies proposed that the use of coated implants with incorporated active ingredients could release drugs locally, and thereby obtain a high concentration of the drug in the area of interest without systemic side effects. Bessho et al. [21] showed that the use of BMP–atelopeptide type-I collagen mixture is an effective method to obtain a greater bond strength at the bone–implant interface within a shorter time period than titanium implants without BMP. In our pilot study, liposomal vector delivering a reporter gene (of GFP) coated on the implant surface induced cells in contact with the surface to produce GFP protein after 3 days [25]. Our result on the bone–implant surface showed a convincing accordance with that of our pilot study. A significant difference in the number of BMP-2 producing cells was observed between the liposomal group and the control group at the bone–implant interface.

Liposomal vector transfer worked not only on the implant surface, but also in the bony defect filled with collagen carrier.

Collagen has received increasing attention recently due to its excellent biocompatibility, degradation into physiological end-products, and suitable interaction with cells and other macromolecules. The favourable influence of collagen on cellular infiltration and wound healing is well known [14,27,30,33]. The combination of rhBMP-2 with a collagen sponge matrix has proven to be a very promising therapeutic aid in a variety of applications [11,12,24]. Whether a collagen sponge as a carrier for gene transfer could allow better cell viability and easy access between mobilized cells and the liposomal vector should be verified in further investigations.

One of most interesting findings was that most of the trabecular-lining cells showed strong BMP-2-positive staining when we treated autologous bone with the liposomal vector. These findings indicate that not only migrating cells, but also existing trabecular-lining cells in the particulated bone are accessible for transfection. Augmented autologous bone in the bone defect seemed to start reorganizing the new bone trabeculae, indicating that autologous bone graft combined with BMP-2 gene delivery can accelerate bone remodelling in grafted bone.

In the past few decades, bone tissue engineering has focused on 4 requirements for bone regeneration: (1) morphogenic signals, i.e. growth and differentiation factors, (2) host cells that will respond to the signal, i.e. are capable of differentiating into osteoblasts, (3) a biomaterial carrier of this signal that can deliver the morphogenetic signal to specific sites and serve as a (degradable) scaffold for the growth of the responsive host cells, and (4) a viable, well vascularized host bed. For several decades, the 'gold standard' in bone-defect management has been autologous bone grafting. However, its use is limited when the defect volume exceeds the volume of accessible autologous graft material. Therefore, there is a growing need to provide alternatives to traditional bone grafting [29]. Our results suggest that a combined approach of autologous bone graft, to supply available cells and matrix, combined with delivery of a gene providing a morphogenetic signal has a synergistic effect on rapid bone remodelling. This combined approach may provide a new therapeutic modality for cell and gene therapy. Further investigation on various combined therapies involving both transferred cells and vector systems in large bony defects will be necessary to obtain concrete evidence for this approach.

5. Conclusions

BMP-2 gene delivery using a liposomal vector in the chosen model efficiently transfected immigrating cells as well as neighbouring cells, and enhanced bone regeneration in the defect. BMP-2 gene delivery combined with bone graft could induce a rapid osseointegration of the bone–implant interface at an early stage of wound healing.

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