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Regeneration of Calvarial Defects with *Escherichia coli*-Derived rhBMP-2 Adsorbed in PLGA Membrane

Mitsuaki Ono[a] Wataru Sonoyama[a] Kazuki Nema[a] Emilio Satoshi Hara[a] Yasutaka Oida[a] Hai Thanh Pham[a] Katushi Yamamoto[b] Kazuo Hirota[b] Kazushige Sugama[c] Walter Sebald[d] Takuo Kuboki[a]

[a]Department of Oral Rehabilitation and Regenerative Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, [b]GC Corp., Tokyo, and [c]Osteopharma Inc., Osaka, Japan; [d]PCII, Biozentrum, Universität Würzburg, Würzburg, Germany

Key Words

Escherichia coli-derived recombinant human bone morphogenetic protein-2 · Polylactide-co-glycolide · Ectopic bone formation · Bone regeneration

Abstract

Objective: *Escherichia coli*-derived recombinant human bone morphogenetic protein-2 (E-BMP-2) has been shown to be as effective as mammalian cell-derived BMP-2. However, several in vitro and in vivo experiments are still necessary to validate the effectiveness of E-BMP-2 due to the difference in synthesis process, mainly related to protein nonglycosylation. The objective of this study was to investigate whether biodegradable polylactide-co-glycolide (PLGA) membrane is a suitable carrier for E-BMP-2 delivery for bone regeneration of critical-sized defects in rat calvaria. ***Materials and Methods:*** First, the osteoinductive effect of E-BMP-2 was confirmed in vitro in mouse bone marrow stromal cells by analysis of osteocalcin mRNA levels, and calcium deposition was detected by alizarin red staining. Before in vivo experiments, the release profile of E-BMP-2 from PLGA membranes was determined by ELISA. E-BMP-2 (0, 1, 5 and 10 μg/μl) was applied for ectopic and orthotopic bone formation and was analyzed by X-ray, micro-CT and histology. ***Results:*** Release-profile testing showed that PLGA membrane could retain 94% of the initially applied E-BMP-2. Ectopic bone formation assay revealed that combination of E-BMP-2/PLGA membrane strongly induced bone formation. Stronger osteoinductivity with complete repair of critical-sized defects was observed only with PLGA membranes adsorbed with 5 and 10 μg/μl of E-BMP-2, whereas no bone formation was observed in the groups that received no membrane or 0-μg/μl dose of E-BMP-2. ***Conclusion:*** PLGA membrane was shown to be a suitable carrier for sustained release of E-BMP-2, and the E-BMP-2/PLGA membrane combination was demonstrated to be efficient in bone regeneration in a model of critical-sized defects. © 2014 S. Karger AG, Basel

Abbreviations used in this paper

BMP-2	bone morphogenetic protein-2
C-BMP-2	BMP-2 from Chinese hamster ovary cells
DW	distilled water
E-BMP-2	*Escherichia coli*-derived rhBMP-2
HE	hematoxylin and eosin
mBMSCs	mouse bone marrow stromal cells
NRO	normalized radio-opacity
PLGA	polylactide-co-glycolide
rhBMP-2	recombinant human BMP-2
RUNX2	runt-related transcription factor 2

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Dr. Takuo Kuboki
Department of Oral Rehabilitation and Regenerative Medicine
Okayama University Graduate School Medicine, Dentistry and Pharmaceutical Sciences
2-5-1 Shikata-cho, Okayama 700-8525 (Japan)
E-Mail kuboki@md.okayama-u.ac.jp

Introduction

Reconstruction of bone defects still remains one of the major clinical issues in orthopedics and dentistry. Most classical techniques are based on the transplantation of homologous bone tissue [Giannoudis et al., 2005], which, however, presents some disadvantages related to potential immunological reactions and viral or bacterial transmission to the host. Tissue engineering offers more attractive potential solutions for repair of bone defects. Basically, three methods can be applied for bone tissue engineering, namely cell therapy, gene therapy and cytokine therapy. Cell therapy involves the transplantation of in vitro treated/induced preosteoblasts or osteoblasts [Kitoh et al., 2004]. This method demands excessive cost and time to culture the necessary amount of cells for transplantation. In addition, transplanted cells should be from the original donor so as to avoid potential risks of graft-versus-host disease. Gene therapy involves the transduction of expression vectors of a target gene (e.g. BMP-2) to cells in vitro or in vivo; however, this approach faces some public hesitation. And finally, cytokine therapy is considered the most useful and promising technique and it involves the implantation of cytokines or growth factors at the desired site.

Bone morphogenetic protein-2 (BMP-2), which is a member of the transforming growth factor β (TGF-β) superfamily of polypeptide, is widely known as a potent osteogenic agent that induces differentiation of mesenchymal cells toward osteogenic lineage in vitro and bone formation in vivo [Wozney et al., 1988; Vladimirov and Dimitrov, 2004; Reddi and Reddi, 2009]. Recombinant human BMP-2 (rhBMP-2) is currently approved by the American Food and Drug Administration and is available for clinical application [Friedlaender et al., 2001; Govender et al., 2002; Burkus et al., 2003; Glassman et al., 2007; 2008]. However, high doses in the milligram range are typically required to obtain satisfactory bone regenerative results in the clinical setting [Boyne, 2001; Herford and Boyne, 2008] and the current system to isolate and purify rhBMP-2 from Chinese hamster ovary cells (i.e. C-BMP-2) only enables low protein yields.

In order to overcome such hindrances, our research group has succeeded to produce rhBMP-2 in an *Escherichia coli* production system, which is particularly attractive for biotechnology because of its ability to grow rapidly and at high density on inexpensive substrates [Ruppert et al., 1996; Kubler et al., 1998]. A previous report has already demonstrated the similar effectiveness of *E. coli*-derived rhBMP-2 (E-BMP-2) compared to C-BMP-2 in inducing the gene expression of osteogenic marker genes *Runx2* and *Ocn* in a mouse bone marrow-derived ST2 cell line [Yano et al., 2009]. Moreover, E-BMP-2 has also been shown to induce ectopic new bone formation in vivo [Yano et al., 2009]. However, the main difference between E-BMP-2 and C-BMP-2 is that E-BMP-2 does not undergo glycosylation, a critical step in protein folding. Consequently, differences may exist in its 3-dimensional structure as well as in its functional bioactivity. Therefore, the function of the E-BMP-2 should be evaluated in vitro and preclinical studies before its full application in humans.

In order to quantitatively and qualitatively predict the efficacy of the E-BMP-2, additional in vivo experiments are still required, especially in terms of its ability to interact with carriers (biomaterials). Biomaterials have been regarded as important aids for tissue engineering and sustained drug delivery. A number of previous reports have demonstrated the notable role of biomaterials in potentiating the osteogenic effect of rh-BMP-2. For instance, enhanced bone formation has been reported when rhBMP-2 was applied in combination with hydrogels or collagen sponge [Yamamoto et al., 2003; Lee et al., 2010]. These materials not only directly interact with rhBMP-2, but also gradually release the growth factor at the target location [Geiger et al., 2003; Yamamoto et al., 2003]. On the other hand, a previous report showed adipose tissue formation in the area of the defect treated with E-BMP-2 adsorbed in block-type macroporous biphasic calcium phosphate [Park et al., 2011]. Therefore, selection of the appropriate biomaterial for delivery of rhBMP-2 is also another crucial factor to obtain successful results.

Among the many different types of biomaterials currently available to repair bone defects, biodegradable polylactide-co-glycolide (PLGA) membranes are the most commonly used synthetic polymers to deliver BMPs [Issa et al., 2008] and are extensively used in the dental field for guided bone regeneration due to particular properties that serve as a barrier to prevent high-proliferating soft tissues from invading the bone defect and, in the meantime, to support for slow-growing osteoblasts to form new hard tissue at the alveolar ridge. Therefore, in order to further validate the effectiveness of E-BMP-2 for application in such conditions, we herein tested the capability of E-BMP-2 to regenerate critical-sized defects in the calvaria of rats in combination with biodegradable PLGA membranes as a carrier for the controlled release of E-BMP-2 at the target site. We first verified the efficiency of E-BMP-2 incorporation in PLGA

membranes and further demonstrated a complete regeneration of critical-sized defects in rat calvaria at 16 weeks after surgery.

Materials and Methods

Materials

E-BMP-2 was prepared using methods reported previously [Yano et al., 2009]. A biodegradable PLGA membrane (GC membrane®) was provided by the GC Corporation.

Cells and Cell Culture

Mouse bone marrow stromal cells (mBMSCs) were isolated as described previously and cultured in alpha-minimal essential medium (a-MEM, Invitrogen, Carlsbad, Calif., USA) containing 20% fetal bovine serum (FBS, Invitrogen), 2 mM of glutamine, 100 U/ml of penicillin, 100 mg/ml of streptomycin sulfate, 10 nM of dexamethasone and 55 mM of 2-mercaptoethanol (normal medium, Invitrogen). The mBMSCs at the 2nd passage were used for the experiments. The mBMSCs were cultured with normal medium and after achieving confluency, the medium was substituted with mineralization medium supplemented with 100 mM of ascorbic acid 2-phosphate (Wako Pure Chemical Industries, Osaka, Japan) and 2 mM of beta-glycerophosphate (Sigma, St. Louis, Mo., USA) for 5 and 21 days. E-BMP-2 (100 ng/ml) was added to osteogenic medium in the experimental group. Calcium deposits were detected by staining with 2% alizarin red S (pH 4.2, Sigma).

Real-Time Reverse Transcription Polymerase Chain Reaction Analysis

Total cellular RNA was extracted using RNeasy (Qiagen, Gaithersburg, Md., USA) according to the manufacturer's protocol and cDNA was synthesized by reverse transcription of RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, Calif., USA). Primers were designed using Beacon Software (Bio-Rad) with parameters set for use with real-time RT-PCR and for a Tm of 62 ± 3°C with an amplicon size of 75–200 bp. Real-time RT-PCR was performed to quantify the expression of mRNA using an iQ5 (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). For each primer set, melting curves were performed to ensure that a single peak was produced, and the products were further analyzed by gel electrophoresis. All real-time RT-PCR reactions were normalized to the levels of ribosomal protein S29 (s29) mRNA. Primers for RT-PCR were as follows: *S29* sense: 5′-GGAGTCACCCACGGAAGTTCG-3′, *S29* antisense: 5′-GGAAGCAGCTGGCGGCACATG-3′, *Ocn* sense: 5′-CCAAGCAGGAGGGCAATAAGGTAG-3′, *Ocn* antisense: 5′CTCGTCACAAGCAGGGTCAAGC-3′.

Preparation and Release Testing of E-BMP-2-Adsorbed PLGA Membrane

To prepare the E-BMP-2-adsorbed PLGA membranes, the membranes were incubated in 100 µl of distilled water (DW) containing 1 µg/µl of E-BMP-2, at 4°C for 24 h. Subsequently, excess of E-BMP-2 solution was removed by gently tapping, before proceeding to release testing. The total amount of protein adsorbed onto the membrane was calculated by simple subtraction between the initial and final amount of E-BMP-2 solution in the tube. In other words, if 88 µl of solution was remaining in the tube after incubation, we considered that 12 µl (i.e. 12 µg) of E-BMP-2 was adsorbed onto the membrane.

For analysis of the release profile of E-BMP-2 from the membranes, these were immediately placed into 1 ml of phosphate-buffered saline (PBS) solution and incubated at 37°C. Supernatant was then periodically sampled to measure the concentration of E-BMP-2 released from the membranes by enzyme-linked immunosorbent assay (ELISA, R&D, Minneapolis, Minn., USA). The same volume of fresh PBS was added to maintain equal volumes of the suspensions (i.e. 1 ml). Supernatant samples were collected at 0, 5, 15, 30, 60, 120 and 180 min. The release profile was calculated in percentage in relation to the total amount of adsorbed E-BMP-2.

Animal Experiments

The animals were treated according to the Guidelines and under the Ethical Committee approval for Animal Research of the Okayama University Dental School (OKU-2010433) as well as according to the principles of the Declaration of Helsinki. Twelve-week-old female Wistar rats (CLEA Japan Inc., Tokyo, Japan) were utilized for ectopic and orthotopic bone formation experiments. Before surgery, general anesthesia was induced by initial inhalation of isoflurane (Isoflu: Dainippon Sumitomo Pharma Co., Osaka, Japan), followed by intraperitoneal injection of a mixture of xylazine (8 mg/kg; Bayer, Tokyo, Japan) and ketamine (80 mg/kg; Sankyo, Tokyo, Japan).

Assay of Ectopic Bone Formation

The dorsal regions were shaved and two incisions were made with blunt scissors. The skin was then retracted away from the underlying tissue, creating completely separated subcutaneous pockets for implantation of E-BMP-2-treated (1 µg/µl) or DW-treated membranes (control group). Three weeks after implantation, all animals were deeply anesthetized and underwent perfusion fixation with 0.1 M phosphate buffer and 4 % paraformaldehyde (PFA, Merck, Darmstadt, Germany).

Assay of Orthotopic Bone Formation

The head was shaved and an incision was made to expose the calvaria. Under irrigation with saline solution, a 6.5-mm diameter defect was created by means of a trephine burr (SLAKIT, Neobiotech, Seoul, Korea) and bone fragments were thereafter removed. After bleeding cessation, PLGA membranes (10×10 mm^2) treated with 1, 5 or 10 µg/µl of E-BMP-2 were inserted onto the defect. Control groups were those with no-membrane or DW (0 µg/µl). Thereafter, the pericranium and skin were sutured separately. Four weeks and 16 weeks after implantation, all animals were deeply anesthetized and underwent perfusion fixation with 4% PFA/0.1 M phosphate buffer.

X-Ray and Microcomputed Tomographic Analysis

All samples were radiographed with a soft X-ray source, mFX-1000 (FUJIFILM, Tokyo, Japan) at 20 KV, 100 mA and 5 s. For evaluation of bone regeneration, the density of radio-opacity was measured with image analysis software (Image J, JAVA-based free software). The radio-opacity of the regenerated bone (6.5 mm diameter) and the original bone surrounding the defect were measured with three diagonal straight lines (length: 8.0 mm) drawn at an equal angle. The mean normalized rate of the radio-opacity (NRO) was calculated by dividing the mean radio-opacity of the defect by that of the normal bone of a rat of the same age without the operation.

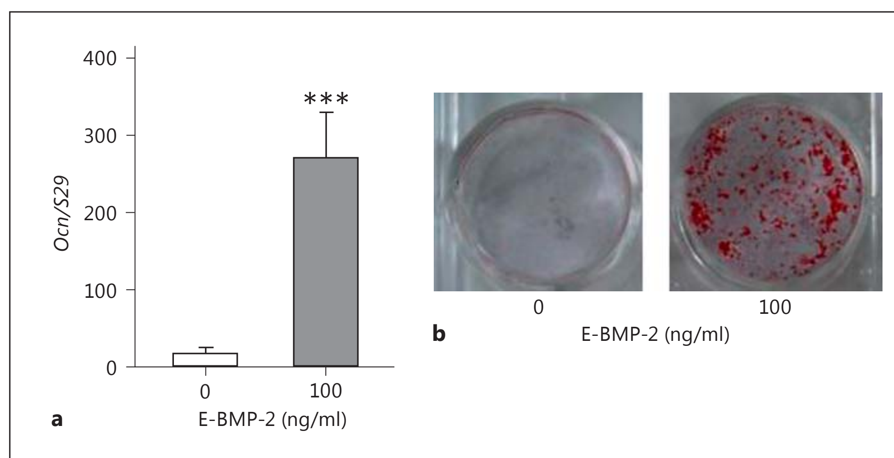

Fig. 1. Osteogenic effect of E-BMP-2 on mBMSCs. **a** The mRNA expression levels of *Ocn* were measured by real-time RT-PCR 5 days after treatment with E-BMP-2, and normalized to that of S29 ribosomal protein. *** $p < 0.001$ (unpaired t test), compared to control group. **b** Alizarin red S staining of Ca^{2+} deposition of mBMSCs cultured in osteogenic induction medium with or without E-BMP-2 for 21 days.

Subsequently, samples were scanned and reconstructed with 10.7-μm isotropic voxels on a micro-CT analysis system (SkyScan 1174, SkyScan, Aartselaar, Belgium). The digital data were further elaborated by reconstruction and volumetric softwares (NRecon, CTAn, CTvol and CTVox, SkyScan).

Histological and Immunohistochemical Analysis

After fixation, samples were decalcified with 10% ethylenediamine tetraacetic acid (EDTA, Nacalai tesque, Kyoto, Japan) for 1 week. Samples were then dehydrated through a gradient of ethanol and finally embedded in paraffin. Paraffinized samples were cut in sections of 5 μm thickness, mounted on glass slides, deparaffinized and stained with hematoxylin and eosin (HE) and then observed under a light microscope.

For immunohistochemical analysis, deparaffinized sections were treated with trypsin for antigen retrieval, blocked with goat serum, incubated with first antibody against Runt-related transcription factor 2 (RUNX2, Calbiochem, Beeston, Nottingham, UK) overnight and then proceeded to second antibody incubation followed by signal development using the 3-amino-9-ethylcarbazole color development system (Invitrogen-Zymed, Carlsbad, Calif., USA).

Statistical Analysis

The unpaired Student t test or one-way factorial analysis of variance (ANOVA) followed by the Tukey test was used for the statistical analysis (Prism 5, GraphPad Software, Inc., La Jolla, Calif., USA). p values <0.05 were considered to be statistically significant. All statistical data were presented as the mean ± SD.

Results

Effect of E-BMP-2 on Osteoblastic Differentiation of Mouse Bone Marrow Cultures

To confirm the osteogenic function of E-BMP-2 in an ex vivo culture of mBMSCs, we first determined the mRNA expression of *Ocn* in mBMSCs cultured in osteogenic differentiation media by real-time RT-PCR. E-BMP-2 induced a significant increase in the mRNA expression of *Ocn* by almost 300-fold compared to control group (fig. 1a). This result was in accordance with a notable calcium accumulation detected by alizarin red S staining in the E-BMP-2-treated cells (fig. 1b).

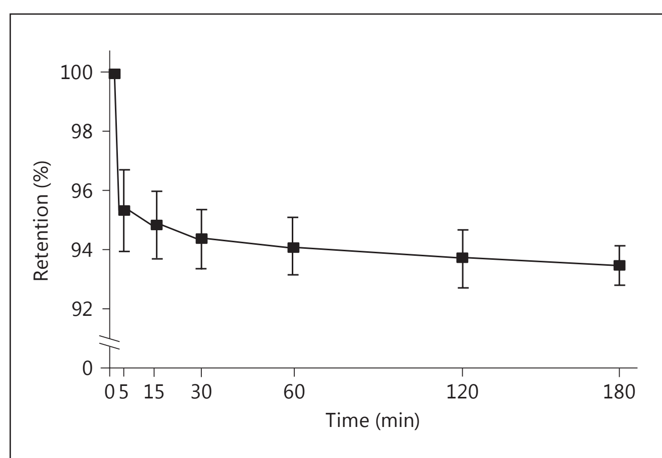

Fig. 2. Investigation of the in vitro release profile of E-BMP-2 from PLGA membranes. Graph shows the release rate of E-BMP-2 from the PLGA membranes up to 180 min. Ninety-four percent of the initially adsorbed E-BMP-2 still remained in the membrane.

In vitro Release of E-BMP-2 from PLGA Membranes

A previous report showed that PLGA-unbound rhBMP-2 is released during the initial 72–96 h, but there was no substantial release of rhBMP-2 for up to 1 week, suggesting that the bound protein is released slowly upon degradation of the polymer [Duggirala et al., 1996]. Based

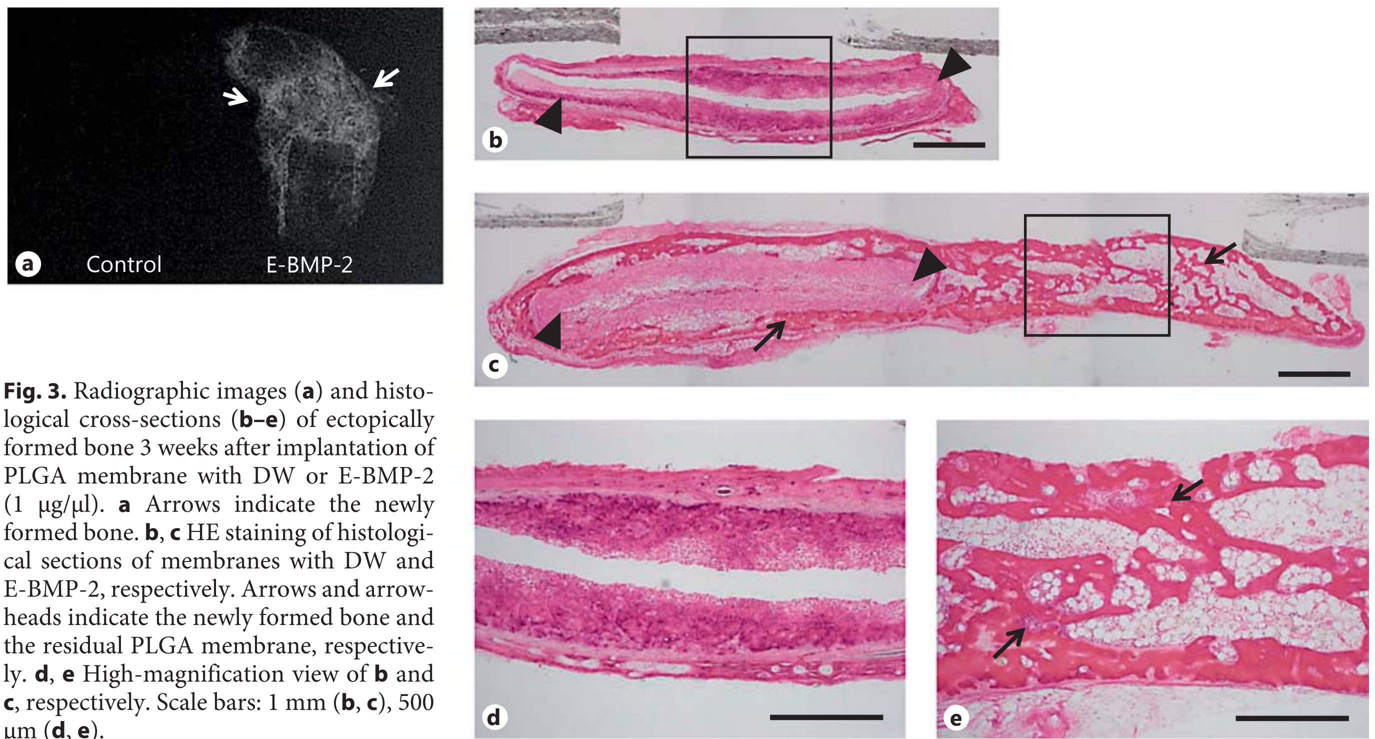

Fig. 3. Radiographic images (**a**) and histological cross-sections (**b–e**) of ectopically formed bone 3 weeks after implantation of PLGA membrane with DW or E-BMP-2 (1 μg/μl). **a** Arrows indicate the newly formed bone. **b**, **c** HE staining of histological sections of membranes with DW and E-BMP-2, respectively. Arrows and arrowheads indicate the newly formed bone and the residual PLGA membrane, respectively. **d**, **e** High-magnification view of **b** and **c**, respectively. Scale bars: 1 mm (**b**, **c**), 500 μm (**d**, **e**).

on this data, we investigated the release profile of E-BMP-2 from PLGA membranes mainly in the initial period, in order to evaluate the amount of free protein that is released. As shown in figure 2, approximately 5% of the initially incorporated E-BMP-2 was released from PLGA membranes within the initial 5 min, but no substantial release was observed thereafter. After a wash with PBS for 180 min, the amount of remained E-BMP-2 adsorbed on the PLGA membranes was 94%.

Ectopic Bone-Inducing Capacity of E-BMP-2

Next, to confirm the effect of E-BMP-2-treated membrane on ectopic bone formation, E-BMP-2-treated or DW-treated PLGA membranes were implanted into the back subcutis of rat and after 3 weeks, membranes were analyzed by soft X-ray (fig. 3a) and histological sectioning (fig. 3b–e). E-BMP-2-treated membrane showed a notable radio-opacity (fig. 3a), indicating the formation of ectopic bone. Staining of histological sections also confirmed formation of bone in the E-BMP-2-treated group (fig. 3c, e), whereas a fibrous connective tissue was observed around the DW-treated membrane (fig. 3b, d), which was undetectable through X-ray analysis (fig. 3a).

Evaluation of Orthotopic Bone Formation

To investigate the effect of BMP-2-treated membrane on orthotopic bone formation, E-BMP-2-treated PLGA membranes were implanted onto the full-thickness critical-sized calvarial defects for 4 and 16 weeks. Based on the radiographic findings, the membranes treated with 5 μg/μl of E-BMP-2 showed a stronger osteoinductivity than either the 1- or 10-μg/μl dose groups (fig. 4a, c). A quantitative analysis of radio-opacity of the newly formed bone confirmed the above visual radiographic findings (fig. 4b, d). Three-dimensional micro-CT images at 4 weeks after implantation showed that the calvarial bone defect was not completely restored in any dose group (fig. 4a). However, histological findings showed a new bone formation around PLGA membranes in the 5- and 10-μg/μl dose groups, whereas no obvious bone formation was observed in the no-membrane and 0-μg/μl dose group. It is of note that residual PLGA membranes could still be observed in all groups after 4 weeks (fig. 5). In addition, immunohistochemical analysis showed that most of the cells lining the bone surface were positive for RUNX2, which indicates the osteoblast commitment of the cells (fig. 6b, c).

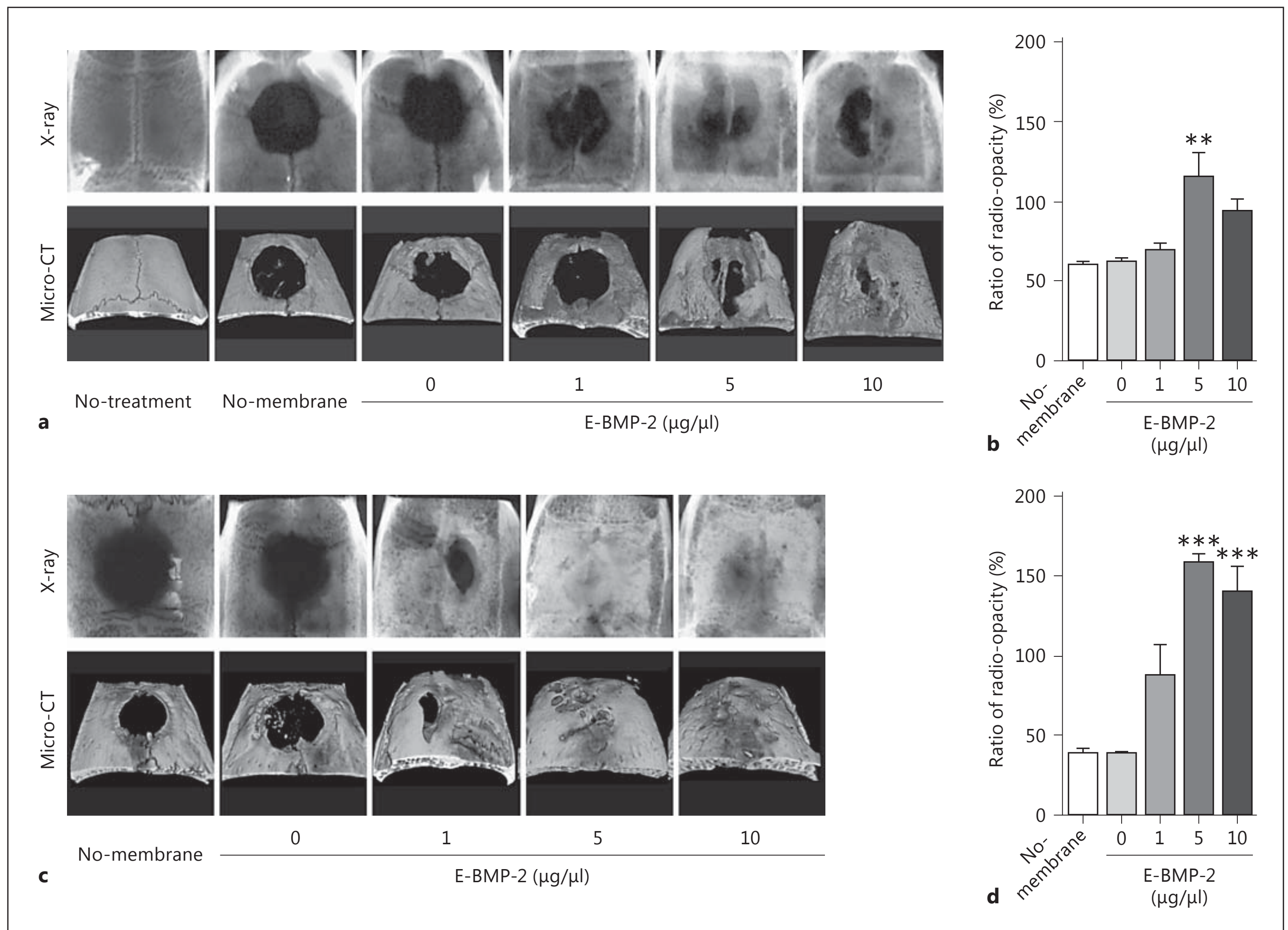

Fig. 4. Radiographic and 3-dimensional micro-CT images of the rat calvarial bone defects at 4 (**a**) and 16 (**c**) weeks after surgery. **b**, **d** The mean normalized radio-opacity (NRO) of the defects against the peripheral original bone. **a**, **b** At 4 weeks after implantation, the defects of calvarial bone were not completely restored in any dose group. The mean NRO of the 5-μg/μl group was significantly higher than that of the no-membrane (only defect) group. **c**, **d** At 16 weeks, the defects were completely restored in the groups treated with 5 and 10 μg/μl E-BMP-2 doses, which was in accordance with the increased NRO (**d**). ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA/Tukey test), compared to respective defect (no-membrane) group.

At 16 weeks after the operation, bone regeneration was significantly enhanced in the 5- and 10-μg/μl dose groups compared to the no-membrane group (fig. 4c, d). Based on the histological findings, PLGA membranes were absorbed completely in all dose groups of E-BMP-2, and the calvarial defects that received 5 and 10 μg/μl of E-BMP-2 were completely restored. In contrast, no bone formation was observed in the no-membrane and 0-μg/μl dose group of E-BMP-2 (fig. 7).

Discussion

Our research group has succeeded in producing and purifying rhBMP-2 in an *E. coli* production system which enables production of the protein on a large scale and is therefore it is supposed to have a great economic impact not only on the research field but also on the clinical setting. The biochemical properties and osteoinducing capacity of E-BMP-2 have been previously demonstrated to be similar to those of C-BMP-2 [Yano et al., 2009].

In this study, we focused on the interaction of E-BMP-2 with the PLGA membrane in ectopic and ortho-

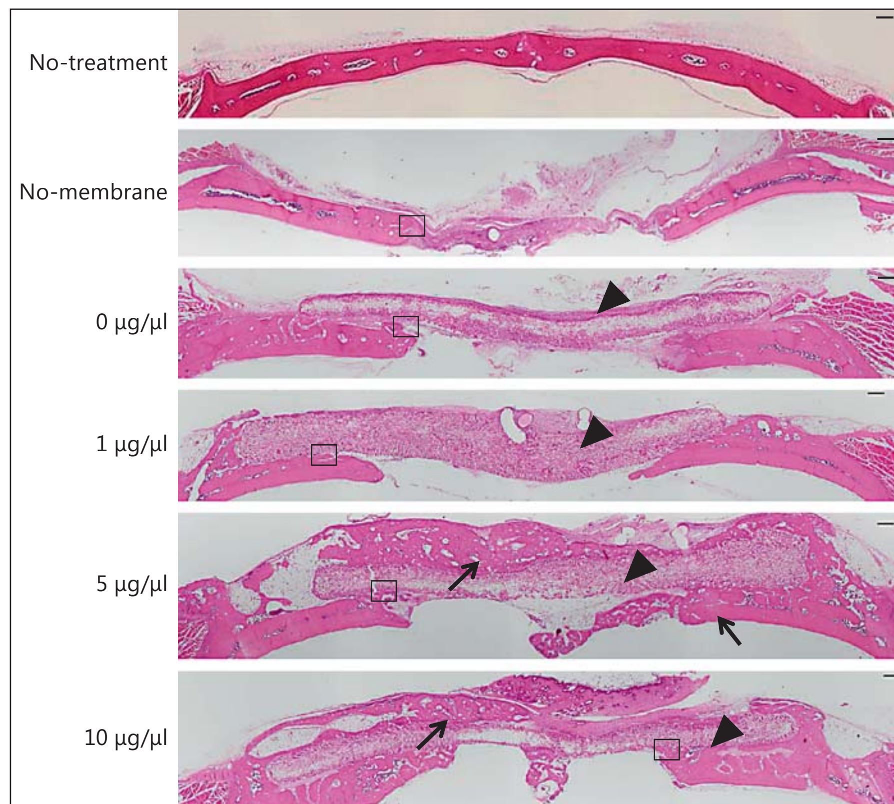

Fig. 5. Histological appearance of the rat calvarial defects at 4 weeks after implantation of the PLGA membrane with E-BMP-2. Bone regeneration was observed in the calvarial defect in the 5- and 10-μg/μl dose group. In contrast, no obvious bone formation was observed in either the no-membrane or 0-μg/μl dose group. Arrows and arrowheads indicate the newly formed bone and the residual PLGA membrane, respectively. Scale bar: 300 μm. Squares indicate the areas shown in figure 6 in high-magnification images.

topic bone formation. Such biomaterials/carriers are a major component in tissue engineering as it potentiates the effects of a particular protein or cytokine. A previous report using E-BMP-2 with block-type macroporous biphasic calcium phosphate showed that residual material remained unresorbed even after a long healing period, which could then limit or inhibit the formation of new bone [Kim et al., 2012]. Moreover, an adipose tissue was formed in the area of the defect treated with rhBMP-2 and biphasic calcium phosphate, and the newly formed bone presented characteristics similar to bone marrow, i.e. a porous bony tissue [Park et al., 2011]. However, in most cases involving reconstruction of large bone defects, a cortical bone is preferred instead of a porous bone. Therefore, selection of the adequate material/carrier is crucial for optimization of the osteoinductive effects of E-BMP-2 towards cortical bone formation.

Our results showed that a biodegradable PLGA membrane is a suitable carrier for delivery of E-BMP-2, and for cortical-like bone formation. The E-BMP-2 release test showed that the amount of adsorbed E-BMP-2 remaining on the membranes was 94% (fig. 2). In ectopic bone formation assay, both X-ray and histologic images showed bone formation not only around the PLGA membrane but also in areas relatively distant from the membrane itself (fig. 3). Although the possibility that E-BMP-2 could be released to distant areas cannot be discarded, the space that remained between the dissected soft tissues in the subcutis at the proximity of the membrane could be another factor allowing for new bone formation at more distant sites.

According to the manufacturer, PLGA membrane presents a complete degradation after 16 weeks; therefore, we expected to observe the full effect of E-BMP-2 at this time point. Indeed, the experiments of the calvarial defect model showed that E-BMP-2 slightly induced bone formation around the residual PLGA membrane in the 5-μg/μl dose group at 4 weeks after implantation (fig. 4a, fig. 5). After 16 weeks, implanted PLGA membranes were completely resorbed and bone formation was dramatically induced, with a complete restoration of the calvarial defects (fig. 4c, fig. 7). These data suggest that E-BMP-2 could be trapped by the PLGA membrane and then slowly released in situ as the PLGA membrane was gradually degraded. Therefore, the PLGA membrane could be a strong candidate as an ideal carrier for

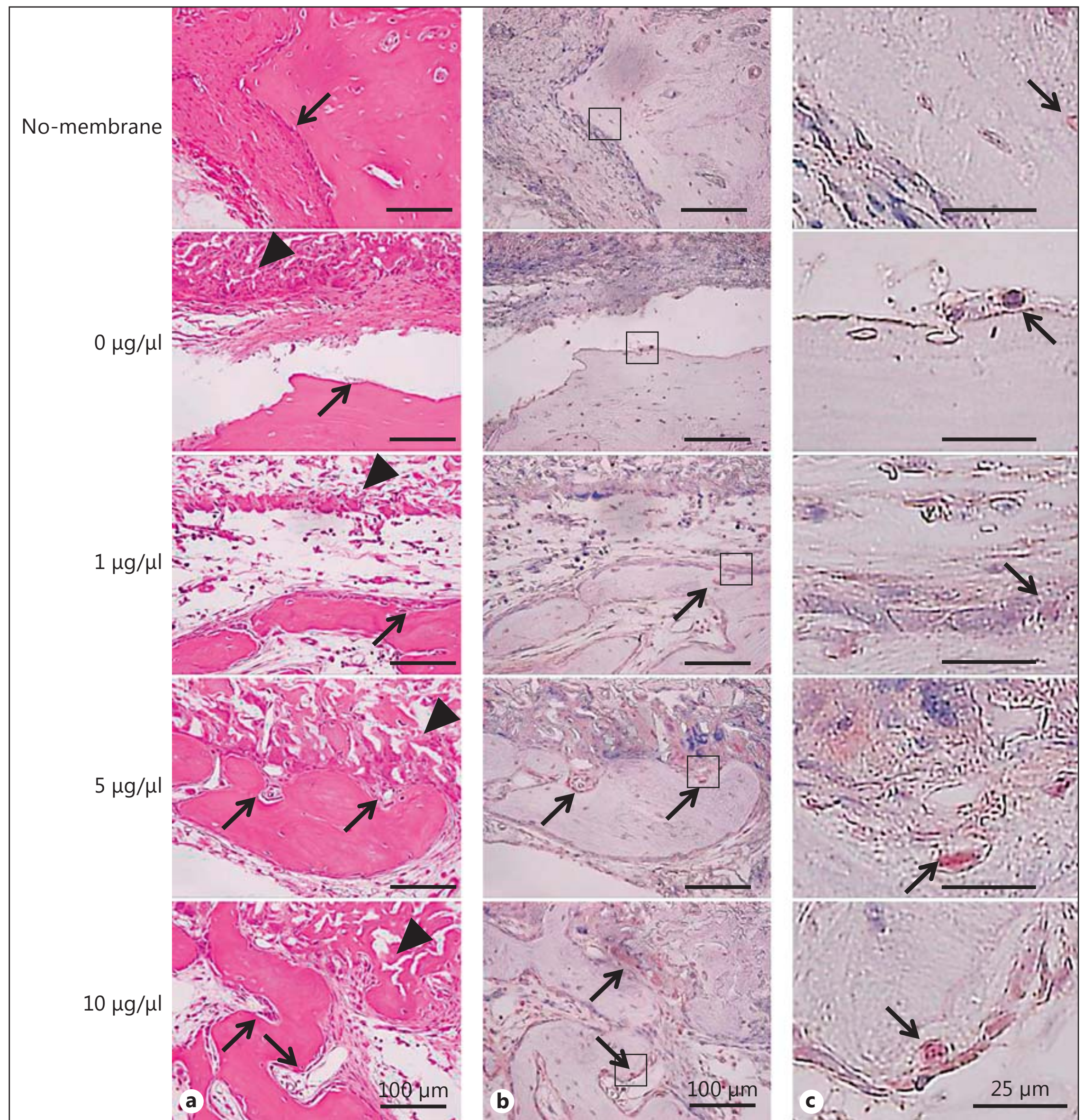

Fig. 6. Higher magnification of histological and immunohistological analysis of the rat calvarial defects at 4 weeks after implantation of the PLGA membrane with E-BMP-2. **a** HE staining. **b**, **c** Immunohistochemical analysis against RUNX2. Arrows show osteoblast cells lining the surface of bone. Arrowheads show PLGA membrane. Squares indicate the areas shown in (**c**) in high-magnification images. Note the stronger signal intensity in the lining cells in the groups treated with E-BMP-2.

the delivery of E-BMP-2 in cases of clinical transplantation as well as for guided bone regeneration. Interestingly, a high dose of 10 µg/µl did not induce more bone formation than 5 µg/µl (fig. 5, 7). Apparently, a 10-µg/µl dose induced the formation of cancellous-like bone, whereas 5 µg/µl induced the formation of more radio-opaque cortical-like bone. Although a deeper investigation of the mechanisms behind these findings is underway, we anticipated the need to establish an optimal dosage of E-BMP-2 in humans.

In conclusion, our study showed that E-BMP-2 in combination with PLGA membrane efficiently induced bone formation both in ectopic and calvarial defect models and that the PLGA membrane is a suitable carrier for

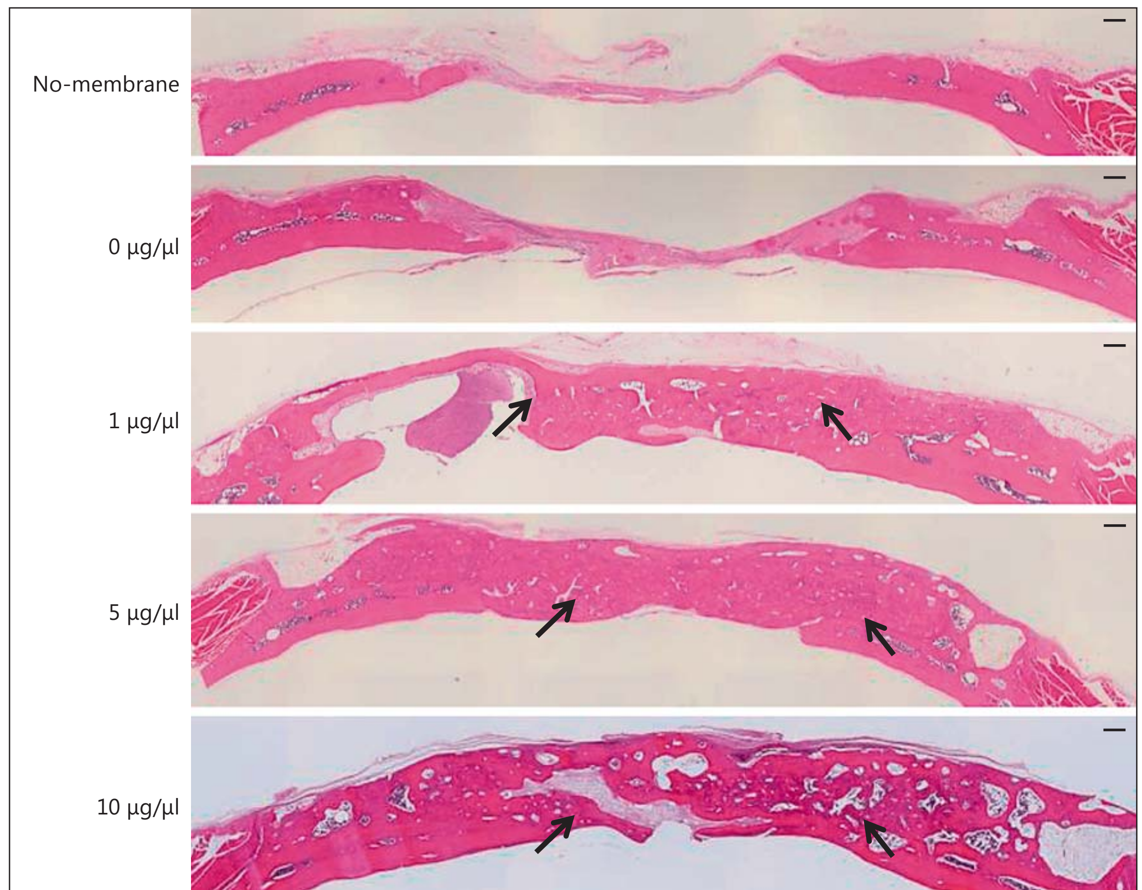

Fig. 7. Histological appearance of the rat calvarial defects at 16 weeks after implantation of the PLGA membrane with E-BMP-2. In the 1-, 5- and 10-µg/µl dose groups, bone regeneration was clearly observed in the whole area of the calvarial defect. In contrast, no obvious bone formation was observed in either the no-membrane or 0-µg/µl dose group. Arrows indicate the newly formed bone. Scale bar: 300 µm.

the gradual release of E-BMP-2 and for inducing cortical-like bone formation. Future preclinical studies using large animals should be performed to predict safety and efficacy before the treatment eventually becomes available for human subjects.

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