Enhanced Bone Augmentation by Controlled Release of Recombinant Human Bone Morphogenetic Protein-2 from Bioabsorbable Membranes

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Background: The present study was undertaken to determine the effect of recombinant human bone morphogenetic protein-2 (rhBMP-2)-loaded biodegradable membranes on bone augmentation in a rabbit calvarial model.

Methods: Five μg of rhBMP-2 was loaded into a stiff hemispherical dome membrane made of poly(L-lactide) and tricalcium phosphate (PLLA/TCP). The release kinetics of rhBMP-2 from the membrane were determined in vitro using a human BMP-2 immunoassay. Twelve rhBMP-2–loaded dome membranes (test group) and 12 control dome membranes (control group) were placed on the partial-thickness calvarial defects of 24 rabbits. The animals were sacrificed at 4 and 8 weeks, and undecalcified ground sections were prepared. Newly formed bone area and height were measured histomorphometrically and calculated by percentage ratio to the total submembranous space area and height below the dome.

Results: In vitro release results demonstrated that rhBMP-2 was released consistently over a 4-week period following a high initial burst release on the first day. At both 4 and 8 weeks, histomorphometric analysis revealed that the test group showed significantly higher newly formed bone heights and areas than the control group ($P < 0.01$). In the control group, new bone height was 36.3% of the dome height and the new bone area reached 8.2% of the submembranous space area at 8 weeks, while the test group reached 87.3% and 35.4%, respectively.

Conclusion: These results suggest that the use of rhBMP-2–loaded PLLA/TCP membranes can result in additional bone augmentation, which is due to the osteoinductive properties of rhBMP-2 released from the membrane during healing. *J Periodontol 2003;74:865-872.*

KEY WORDS

Animal studies; bone, craniofacial; L-polylactide; membranes, bioabsorbable; osteogenesis; proteins, bone morphogenetic; tricalcium phosphate.

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The availability of sufficient alveolar bone volume is a prerequisite for the proper placement of dental implants. In such situations, alveolar bone augmentation based on the guided bone regeneration (GBR) concept can be performed either before or in conjunction with implant placement. The principles of GBR have been successfully applied to augment resorbed alveolar crests both in experimental animal studies[1,2] and in humans.[3-6]

In contrast, unsuccessful bone regeneration has resulted from membrane collapse and from the subsequent lack of space maintenance for bone ingrowth.[7-9] In an attempt to resolve these problems, titanium-reinforced membranes and membrane-supporting devices such as mini-screws or pins and graft materials have been investigated.[10-12] While GBR membranes are used to prevent the influx of mucogingival connective tissue into a defect site, they do not enhance osteoblast proliferation, migration, or bone matrix synthesis. The regenerative potential remains dependent on the native osteogenic potential of the sites concerned.[12-14]

Bone morphogenetic protein (BMP) was first noted by Urist[15] in 1965 and has since been investigated for its bone-inducing properties. Recently, advances in recombinant DNA technology have allowed the cloning and characterization of several BMPs, and have yielded quan-

tities of purified recombinant proteins for therapeutic evaluation.[16-18] Of these, recombinant human bone morphogenetic protein-2 (rhBMP-2) has been found to have a high osteoinductive capacity.[16]

The ability of rhBMP-2 to stimulate bone formation has been demonstrated in various preclinical models, including ridge augmentation,[19-21] subantral augmentation,[22-24] and peri-implantitis defect reconstruction,[25] as well as in humans.[26,27] Meanwhile, various studies have demonstrated that the host-tissue response is dose- and carrier-dependent.[28,29] It should be noted that a successful carrier system must enable a therapeutic level of the protein to act as a differentiation factor.

Our group has reported promising results[30-34] on drug-loaded biodegradable membranes for GBR. In previous studies, poly(L-lactide) (PLLA)-based membrane materials controlled the release of tetracycline and platelet-derived growth factor, which resulted in gains in bone regeneration.

The present study was designed to determine the effect of an rhBMP-2–loaded poly(L-lactide)/tricalcium phosphate (PLLA/TCP) membrane on bone augmentation in rabbit calvarial bone.

MATERIALS AND METHODS

Membrane Fabrication

Hemispherically shaped PLLA/TCP membranes were fabricated using methods previously described.[34] Five g of PLLA (molecular weight 370,000)[§] was dissolved in 3 ml of methylene chloride mixed with 1.5 ml of ethyl acetate, and then TCP[‖] was added to a 50:50 (w/w) ratio on polymer weight. PLLA/TCP solutions were cast on a hemispherical metal mold and dried under vacuum for 24 hours to remove residual solvent. The dome membrane had a height of 4 mm, a base diameter of 8 mm, and a thickness of 0.5 mm. A horizontal 4 mm rim extended from the base of the dome. Fifty µl of rhBMP-2 solution (100 µg of rhBMP-2[¶] dissolved 1 ml of phosphate buffered saline) was soaked into the inner concave face of the dome membrane (5 µg of rhBMP-2/membrane). At least 30 minutes after the rhBMP-2 solution had completely soaked into the membrane, the membrane was freeze-dried and kept at –20°C until required.

In Vitro rhBMP-2 Release

The release kinetics of rhBMP-2 from membranes were determined in vitro. Four membranes were used for the in vitro release experiment. Each membrane was immersed in a glass vial containing a phosphate buffer at pH 7.4 as a releasing medium (5 ml). The sealed vials were placed in a shaking water bath at 37°C and shaken at 15 rpm. At predetermined time intervals over a 4-week period, samples were withdrawn from the vials, which were replenished with fresh medium. The concentration of rhBMP-2 released into the samples was assayed using a human BMP-2 immunoassay kit.[#] The mean values of 4 samples were determined.

Surgical Procedures in Rabbit Calvaria

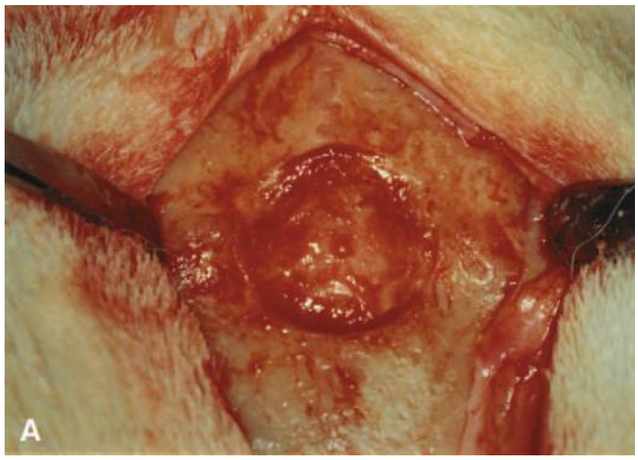
Twenty-four male New Zealand white rabbits (weighing 2.5 to 3.0 kg) were used in this study. The animals were kept in standard cages and fed *ad libitum* with a standard laboratory diet and water. The animals enrolled in this research were cared for and processed in accordance with the Seoul National University Guidelines for the care and use of laboratory animals.

Rabbits were anesthetized with pentobarbital** (0.3 mg/kg). The top of the head in each rabbit was carefully shaved, disinfected with iodine and 5% chlorhexidine digluconate, and after wiping, anesthetized with 2% lidocaine solution.[††] A midline incision from the nasofrontal area to the external occipital protuberance was made, and a skin-periosteal flap was raised to expose the calvarial surface on both sides of the midline. The external cortical plate was then indented circularly using a trephine bur,[‡‡] and demarcated external cortical bone was removed using a round bur. Care was taken to irrigate sufficiently and to avoid involvement of the internal cortical plate and the brain. Twelve dome-shaped PLLA/TCP (control) and 12 rhBMP-2–loaded dome-shaped PLLA/TCP (test) membranes were randomly allocated to 24 rabbits. The dome-shaped membranes were properly fitted over the circular calvarial defects and then tightly fixed to the defect rim using a membrane fixation pin[§§] (Fig. 1). The periosteum was then sutured over the membrane using resorbable suture material, and the cutaneous flap was adapted and sutured with silk. Immediately after surgery, the animals were given antibiotics by intramuscular injection.

Specimen Preparation

Four weeks later, 6 control and 6 test animals were sacrificed by an overdose of pentobarbital. Eight weeks after surgery, the remaining 6 control and 6 test animals were sacrificed. The calvarial bone with membranes was dissected and fixed with neutral buffered formalin. The samples were then rinsed with water, dehydrated, and embedded in super low-viscosity embedding medium.[‖‖] Undecalcified ground sections were prepared.[¶¶] The embedded specimens were mounted on acrylic glass slabs and cut in the vertical plane to encompass the entire membrane and sur-

§ Purac Biochem BV, Gorinchem, The Netherlands.
‖ Shimaku's Pure Chemical, Osaka, Japan.
¶ R & D System Inc., Minneapolis, MN.
# Quantukine BMP-2, R & D System Inc.
** Nembutal, Abbott Laboratories, North Chicago, IL.
†† Xylocaine, Astra, Södertält, Sweden.
‡‡ 3i-Implant Innovations Inc., Palm Beach Gardens, FL.
§§ Frios pin, Friatec, Mannheim, Germany.
‖‖ Polysciences Inc., Warrington, PA.
¶¶ Exakt Cutting and Grinding System, Exakt, Hamburg, Germany.



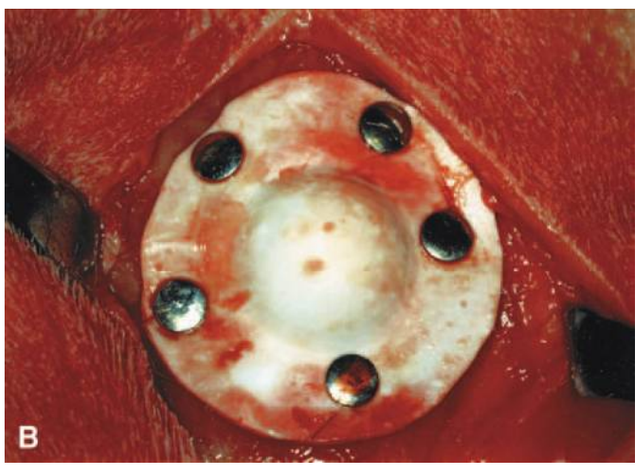


Figure 1.
Partial-thickness bone defect in rabbit calvaria ***(A)*** *and hemispherical PLLA/TCP dome membrane* ***(B)****, fixed to the bone by pins.*

rounding tissues using a diamond saw. The sections were then ground and polished to a final thickness of 30 µm. Final thin sections were stained## and examined under a light microscope.

Histomorphometric Analysis

The most central section was chosen in each animal for the histomorphometric measurement of new bone in the space below the dome. After conventional microscopic examination, computer-assisted histomorphometric measurements of newly formed bone were obtained using an automated image analysis system*** coupled with a video camera on a light microscope. Sections were analyzed under 10× magnification. Newly formed bone height and area were determined and expressed as a percentage of the total height and area of the space below the dome. All specimens were examined and measured 3 times in random order by one researcher, and the means determined.

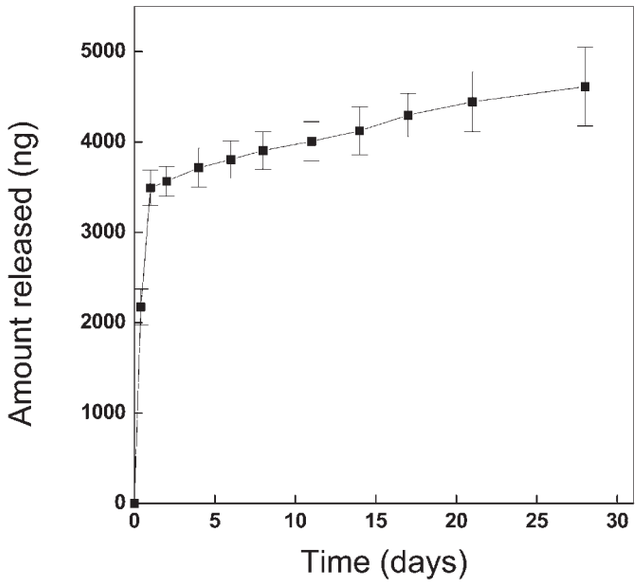


Figure 2.
Cumulative release of rhBMP-2 from PLLA/TCP membranes in vitro. The value at each period is expressed as the mean of 4 samples, and the error bars represent standard deviations.

Statistical Analysis

The unpaired *t* test was used to identify differences between groups and differences over time within groups. The level of significance was set at $\alpha = 0.01$.

RESULTS

Release Kinetics of rhBMP-2 In Vitro

The cumulative release kinetics of rhBMP-2 from the membranes are shown in Figure 2. The amount of rhBMP-2 released was determined over 4 weeks. rhBMP-2 release from the membrane occurred in two phases: an initial immediate phase for the first day and a second phase thereafter. During the initial phase, 2.2 µg and 3.5 µg of rhBMP-2 were released into the medium at 12 and 24 hours, respectively. Thus, approximately 70% of the rhBMP-2 was released during the first day. Following this initial burst, rhBMP-2 was consistently released at a rate of 7 to 10 ng/day for up to 4 weeks.

Bone Augmentation Under Dome Membranes

In both the test and control groups, the surgical sites healed uneventfully with no signs of infection or membrane exposure. No other signs of inflammation or other adverse reactions were observed. All membranes integrated excellently into the surrounding tissue and maintained their original dome shape during the entire course of the study.

Multiple stain kit, Polysciences Inc.
*** Image Access Application, Bildanalys System, Stockholm, Sweden.

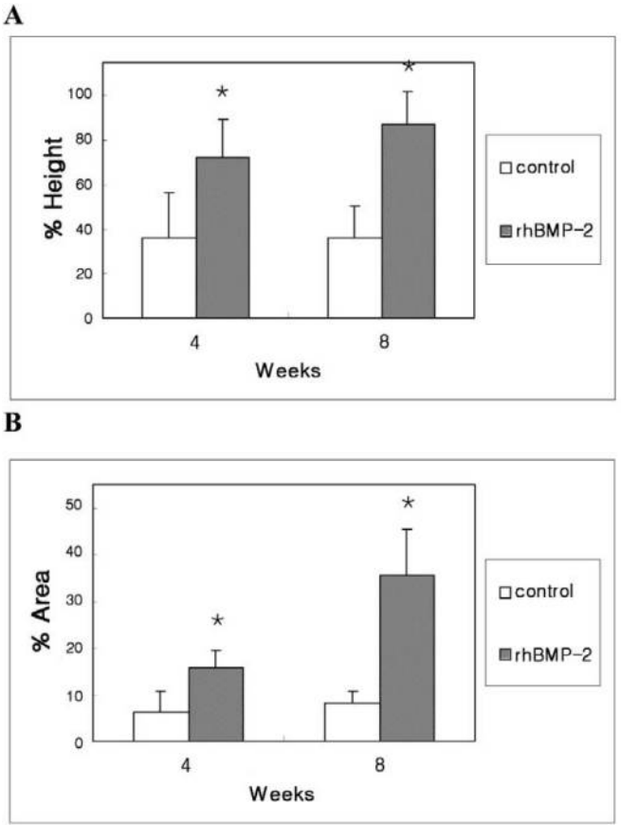


Figure 3.
*New bone height **(A)** and area **(B)** in relation to the total submembranous space available. Values are means of 6 specimens in each group at each period; error bars represent standard deviations. *Statistical significance (P <0.01) between control and test group in each time period.*

Figure 3 illustrates the new bone height and area in relation to the total submembranous space available. At 4 and 8 weeks, the test group showed significantly more bone height and area than the control group (P <0.01). In the control group, bone height and area did not differ significantly at 4 and 8 weeks (P >0.05), whereas in the test group, bone height and area were significantly greater at 8 weeks (P <0.01).

In 4-week specimens, the original dome shape and texture of the membrane was well conserved in both the control and test groups. In the control group, most specimens demonstrated minimal amounts of new bone over the former wound area, while most of the space appeared empty or was, at best, occupied by soft tissue; bone formation did not extend much above the former calvarial surface (Fig. 4). In the control specimens, the upper margin of the augmented bone reached a height of 36.1 ± 20.3% (range, 12.8% to 65.2%) of the submembranous hemispherical space height and occupied 6.1 ± 4.6% (range, 1.1% to 20%) of the available space area. In the test group, bone augmentation advanced significantly from the former calvarial surface. The newly formed bone tended to grow along the inner surface of the membrane (Fig. 5). The maximal height of the new bone reached 72.4 ± 17.4% (range, 44.8% to 93.5%), and the bone occupied 15.9 ± 3.7% (range, 12.0% to 21.8%) of the total area.

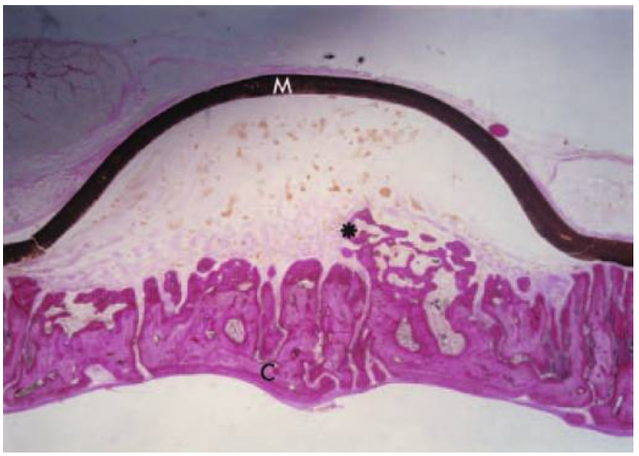


Figure 4.
*Control membrane at 4 weeks. New bone formation was minimal and amounted to coverage of the former wound area only, while most of the space appeared to be empty or, at best, occupied by soft tissue. M: membrane; C: calvarial bone; *new bone. (Undecalcified ground section; multiple stain; original magnification ×3.125.)*

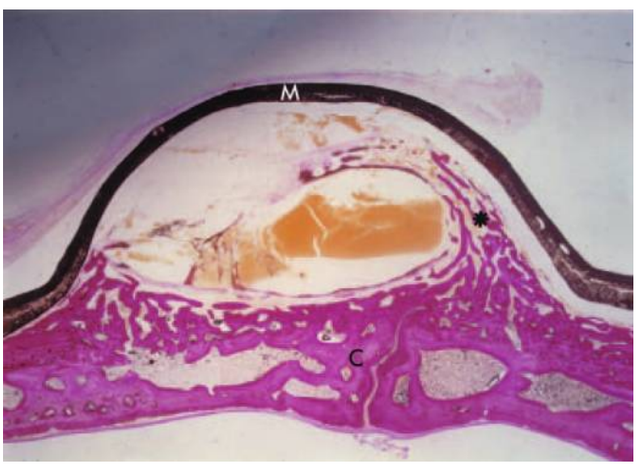


Figure 5.
*Test membrane at 4 weeks. A significant amount of new bone grew from the defect, and tended to advance along the inner surface of the membrane. M: membrane; C: calvarial bone; *new bone. (Undecalcified ground section; multiple stain; original magnification ×3.125.)*

In the 8-week specimens, the original dome shape of the membrane was well conserved, but marginal erosion of the membrane was demonstrated in both groups. In the control group, the new bone height and area were unchanged versus the 4-week specimen, and the new bone remained minimal and localized just

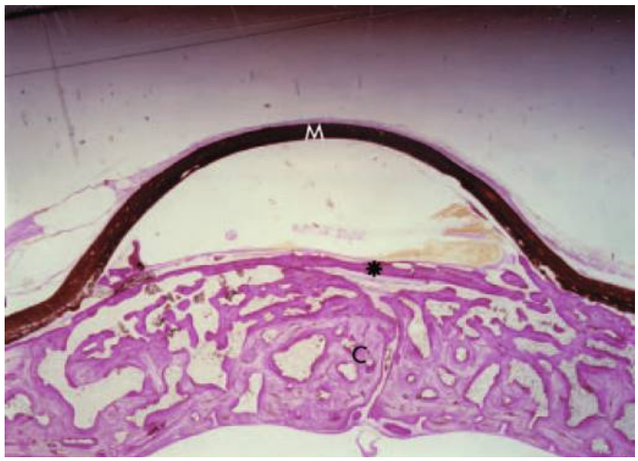


Figure 6.
*Control specimen at 8 weeks. The newly formed bone was localized just above the former calvarial surface. The available space below the membrane was still empty or occupied mainly by soft tissue. M: membrane; C: calvarial bone; *new bone. (Undecalcified ground section; multiple stain; original magnification ×3.125.)*

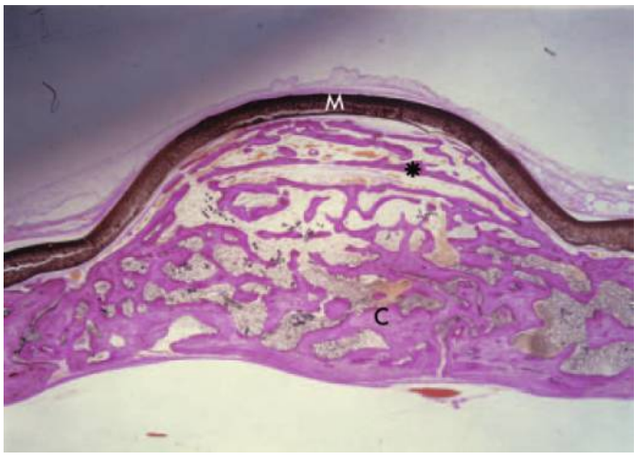


Figure 7.
*Test specimen at 8 weeks. The entire submembraneous space was filled by newly formed bone. M: membrane; C: calvarial bone; *new bone. (Undecalcified ground section; multiple stain; original magnification ×3.125.)*

above the former calvarial surface (Fig. 6). Most of the space below the membrane was still occupied by soft tissue. The upper margin of the new bone reached a height of 36.3 ± 13.8% (range, 18.5% to 52.2%) of the space below the dome, and augmented bone occupied 8.2 ± 2.6% (range, 5.0% to 11%) of the available space. In the test group, bone augmentation under the membrane was noteworthy (Fig. 7). Most of the specimens showed new bone extending to the top of the available space under the membrane. Compared to the 4-week specimens, the bone filling the space under the membrane was much more dense and mature. New bone formation resulted in a trabecular pattern. The intertrabecular space contained fibrous connective tissue and bone marrow rich in blood vessels (Fig. 8). The frontline of the augmented bone reached 87.3 ± 14.5% (range, 62.1% to 98.7%) in height and occupied 35.4 ± 10.0% (range, 28.1% to 49.7%) of the total area.

In both groups, there was no evidence of endochondral ossification as a process of bone formation at either 4 or 8 weeks. In all specimens, the membranes retained their gross hemispherical frame structures throughout the entire observation period. However, microscopically, most of the 8-week specimens in both groups revealed membrane degradation without any significant inflammation or giant cell type reaction. Some newly formed bone infiltrated into, and was deposited onto, the eroded membrane surface (Fig. 9).

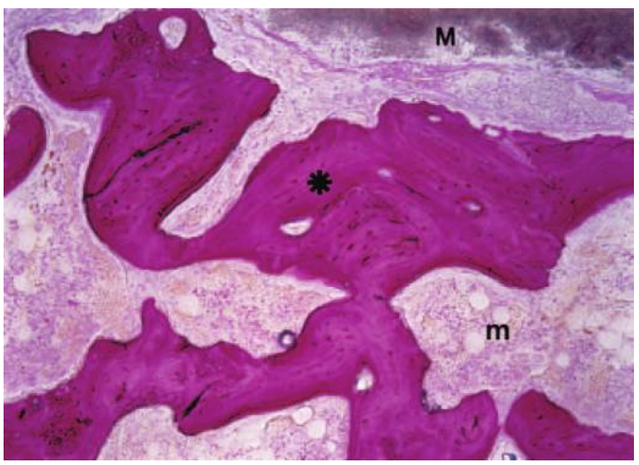


Figure 8.
*Test specimen at 8 weeks. Newly formed bone exhibited a trabecular pattern. The space between trabecula contained fibrous connective tissue and bone marrow rich in blood vessels. M: membrane; *new bone; m: marrow. (Undecalcified ground section; multiple stain; original magnification ×25.)*

DISCUSSION

This study evaluated rhBMP-2–induced bone augmentation using PLLA/TCP membrane carriers. Significantly enhanced bone formation was observed in rhBMP-2–loaded PLLA/TCP membrane-treated sites compared to control membranes over the 8-week study period. The level of bone augmentation within the submembranous space, as measured by height and area, was significantly higher for the rhBMP-2 membranes than for the controls. Dome-shaped PLLA/TCP membranes maintained their shapes against soft tissue compression to allow new bone formation within the space they defined. At 8 weeks, the augmented bone height of the rhBMP-2–loaded membrane group reached approximately 90% of the space defined by the membrane.

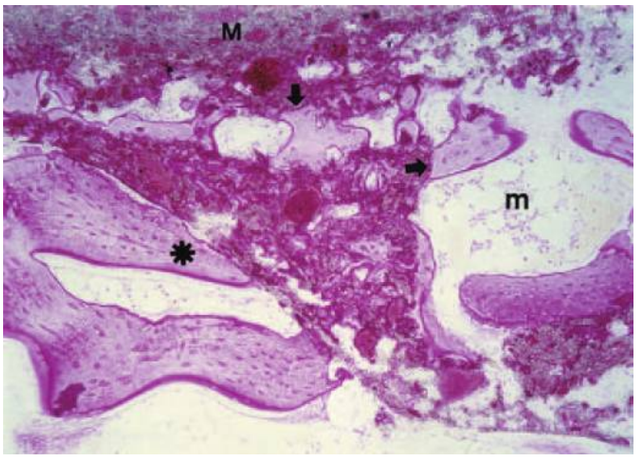


Figure 9.
*Test specimen at 8 weeks. The specimen showed surface erosion of the membrane. Some neoformed bone infiltrated into, and was deposited onto, the eroded surface (arrows). M: membrane; *new bone; m: marrow. (Undecalcified ground section; multiple stain; original magnification ×25.)*

The findings of this study are consistent with the mode of action of rhBMP-2, which is a differentiation factor that stimulates precursor cells to become mature bone-forming cells, and which stimulates osseous tissue formation. Extensive evidence supports the role of BMPs as regulators of bone induction, maintenance, and repair.[35-37] It has been proposed that a primary action of BMPs is to differentiate mesenchymal precursor cells into cartilage- and bone-forming cells. Because of this mode of action, rhBMP-2 heals bony defects in a manner differing from that of other growth factors, which affect already differentiated or committed bone-forming cells present in bone. rhBMP-2 thus affects precursor cells, presumably cells from the marrow environment and the soft tissue surrounding the defect site, to infiltrate the defect area and to differentiate into cartilage and bone cells.[38] Bone marrow stromal cells and perivascular mesenchymal cells offer an important source of pluripotential progenitors capable of differentiating into both osteoblasts and chondroblasts under the appropriate conditions.[37,39,40]

Kostopoulos et al.[41,42] demonstrated the importance of intact periosteum in guided bone regeneration. They suggested that a secluded space created by an occlusive barrier adjacent to existing bone or periosteum may be filled out with bone tissue and that the periosteum left covering bone might play an important role in regeneration. In the present study, the external cortical plate of calvarial bone was removed to expose the bone marrow, and a secluded space was created by a membrane between the inner surface of periosteum and the calvarial bone. Because bone marrow is a major source of preosteoblastic cells as mentioned above, cells from the bone marrow are capable of bone formation without periosteum. In addition to the effect of membrane exclusion, rhBMP-2 released from the membrane might significantly affect cells from the marrow environment to differentiate bone-forming cells in the test group. As a result, the test group showed significantly higher bone augmentation than the control group.

The use of a carrier appears to be essential for the delivery, retention, and release of BMPs at a defect site. A successful carrier system must enable vascular and cellular invasion and allow the BMP to act as a differentiation factor. The carrier should also be reproducible, absorbable, non-immunogenic, moldable, and space-providing to define the contours of resulting bone.[43] Numerous biomaterials, including collagen,[19-28] decalcified bone matrix,[44-46] deproteinized bovine bone mineral,[28] hyaluronan,[47] hydroxyapatite,[36,48] and various poly(α-hydroxy acids)[28,49-51] have been used as candidate carriers for BMPs. However, these materials still suffer from problems such as poor space-providing capacity and slow absorption. Therefore, the search for alternative carriers continues. The PLLA/TCP membrane used in this study possesses several therapeutic advantages. It is biocompatible, moldable, absorbable, and is able to provide available space for bone regeneration and even augmentation due to its stiffness. In addition, it has the properties required of a biodegradable barrier membrane and of a carrier for bone-inducing agents. These biophysical characteristics have already been tested and discussed in our previous study.[34]

Another important carrier function is to maintain the growth/differentiation factor at the site of implantation, to enhance its local concentration, and to minimize its systemic concentration.[29] The release kinetics of rhBMP-2 from several carriers in a rat ectopic assay have been reported.[52,53] Winn et al.[52] analyzed the pharmacokinetics of rhBMP-2 absorbed on type I collagen, poly(D, L-lactide), and deproteinized bovine bone. They found that rhBMP-2 release consisted of an initial burst effect with a half-life of less than 10 minutes, which appeared to be carrier independent, and a secondary release characterized by a half-life of 1 to 10 days, which was carrier dependent. Uludag et al.[53] also reported on the two-phase release of rhBMP-2, which they described as a highly variable initial release burst during the first few hours of implantation, followed by a more gradual second release phase. Even though the release kinetics in vitro as determined by the present study were somewhat different from those of the aforementioned rat ectopic assay in vivo, the release of rhBMP-2 from the PLLA/TCP membrane showed a similar two-phase release pattern. In the present in vitro assay, approximately 70% of the initially loaded rhBMP-2 was released in one day, and a subsequent gradual consistent release was observed over

28 days. Sykaras et al.[54] reported on this constant and prolonged release of rhBMP-2. Morphogenetically, it is essential that rhBMP-2 is biologically active during the late phase of the inflammatory reaction, which peaks 2 to 3 days after surgery. The correct timing of BMP action with respect to wound healing events may be more important than delayed release; however, host-dependent variations in the healing process necessitate the extended presence of BMP to ensure its availability when needed. For this reason, Sykaras et al.[54] suggested that the controlled application of rhBMP-2 allowed a constant and prolonged release that renders it effective over time and helps to form a chemotactic gradient necessary for cell response.

In summary, the PLLA/TCP membranes evaluated in the present study demonstrated a sustained-release controlling potential of rhBMP-2 and significantly enhanced bone augmentation over time. These results suggest that rhBMP-2–loaded PLLA/TCP membranes can allow additional bone augmentation, probably because of the osteoinductive properties of rhBMP-2 released from the membrane during healing.

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