

Immunolocalization of bone morphogenetic protein 2 during the early healing events after guided bone regeneration

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Objective. The objective of this study was to evaluate the immunolocalization of bone morphogenetic protein 2 (BMP-2) after autogenous block grafting covered or not with an e-PTFE membrane.

Study Design. Forty-eight rats were divided into 2 groups, autogenous block graft (B) and autogenous block graft + e-PTFE membrane (MB), and were evaluated by immunohistochemistry at baseline and 3, 7, 14, 21, and 45 days.

Results. The largest number of positive cells in the recipient bed was observed after 3 days in both groups. At the graft border, the largest number of positive cells was seen after 7 days in group B and after 14 days in group MB. The highest proportion of staining in the graft was observed after 3 days in group B and after 21 days in group MB.

Conclusions. High proportions of stain were related to intense revascularization and osteogenesis. Except for the interface, BMP-2 staining occurred later in group MB than in group B in all structures analyzed. (Oral Surg Oral Med Oral Pathol Oral Radiol 2012;113:533-541)

Autogenous bone has been recognized as the gold standard for bone grafting, providing osteogenic cells, extracellular matrix, and molecular signals for the induction of bone differentiation.^{1,2} The use of this type of graft combined with a membrane for guided bone regeneration (GBR) has yielded positive and predictable clinical results.³⁻⁵ The advent of new techniques, such as immunohistochemistry and in situ hybridization, has permitted the study of osteogenesis and bone repair at cellular and molecular levels.⁶

Bone morphogenetic proteins (BMPs), which regulate cellular proliferation, differentiation, and extracellular matrix production, were first described by

Marshall Urist in 1965,⁷ and are the best known and most researched of the musculoskeletal growth factors.⁸

Bone morphogenetic protein 2 (BMP-2) plays an important role in the cascade of cellular events that regulate bone formation and repair, inducing the differentiation and proliferation of mesenchymal cells and the synthesis of extracellular matrix.⁹⁻¹³ BMPs are able to induce the differentiation of undifferentiated cells around blood vessels into osteoblasts and chondrocytes and thus regulate bone formation.¹²⁻¹⁴ Before that phase of cytodifferentiation is complete, cells aggregate and proliferate in sites of condensation. At that moment, the concentration gradients of BMP are increased in these sites of condensation. This characteristic justifies the term morphogenetic protein.¹⁵

Concerning exogenous BMP-2, Springer et al.¹⁶ demonstrated in rat mandibles that bone apposition of the experimental versus control sides was not statistically significantly different when one of the growth factors was applied alone (rhBMP-2; basic fibroblast growth factor [bFGF]). The application of bFGF and the application of rhBMP-2 alone resulted in predictable bone generation in the irradiated mandible with the bone apposition being equal to that of the nonirradiated side. The application of both growth factors together or none at all after irradiation results in significantly reduced bone apposition. When an antagonist of BMP-2, noggin protein, was applied in 2-month-old minipigs, the quantity and quality of spontaneous bone regenerates was not altered. No disruption of subsequent cranial development was seen.¹⁷

Using an experimental rat model for the evaluation of the early stages of bone repair, Jardim et al.¹⁸ demonstrated that autogenous bone grafts combined with an

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expanded polytetrafluoroethylene (e-PTFE) membrane result in more intense formation of new bone than autogenous grafts alone. These significant differences in the repair process were observed as early as 21 days after surgery. In a complementary study, De Marco et al.¹⁹ observed that revascularization was correlated with the results obtained in the study of Jardini et al.¹⁸ Revascularization was more intense and extensive in the group receiving the autogenous bone graft alone when compared with the group treated with the membrane-covered graft across all experimental periods. Thus, revascularization was directly related to new bone formation around the graft, as well as to graft replacement with new bone or even to graft resorption when grafts without a membrane were used. In the same rat model, Nascimento et al.²⁰ evaluated the influence of osteopenia in autogenous bone graft healing with or without an e-PTFE membrane in rats. Osteopenia did not influence bone graft repair.

Studies have demonstrated the temporal and spatial localization of BMP-2 during fracture healing^{14,21-24} and during distraction osteogenesis.²⁵⁻²⁷ Data regarding the role of BMP-2 in guided bone regeneration (GBR) or autogenous bone block grafts are scarce. Therefore, the objective of the present study was to investigate the immunolocalization of BMP-2 in autogenous bone grafts covered or not with an e-PTFE membrane.

MATERIAL AND METHODS

The study protocol was approved by the Ethics Committee on Animal Experimentation of the Institute of Biomedical Sciences, University of São Paulo (No. 073/03).

Forty-eight male Wistar rats weighing 250 to 300 g were selected and divided into 2 groups: autogenous bone block graft covered with an e-PTFE membrane (WL Gore and Associates, Flagstaff, AZ) (group MB) (Figure 1) and autogenous block graft (group B) (Figure 2).

The experimental model has been described in detail by Jardini et al.¹⁸ and De Marco et al.¹⁹ Briefly, parietal bone served as the bone graft donor site, whereas the region of the mandibular angle was the recipient area.

Animals were humanely killed at baseline and 3, 7, 14, 21, and 45 days after surgery. Four animals per group were used for each experimental period. The animals were anesthetized and the hemimandible, including the operated area, was fixed in 10% buffered formalin for a maximum period of 48 hours. Each specimen was recorded and then decalcified in 10% EDTA, pH 7.8, in a microwave oven.

The sections were submitted to immunohistochemistry. Specimens in which the primary antibody was replaced with bovine serum albumin and fetal bovine

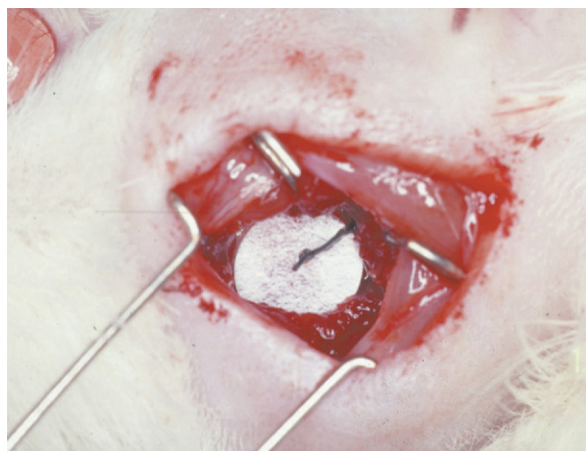


Fig. 1. Group MB—operated situs.

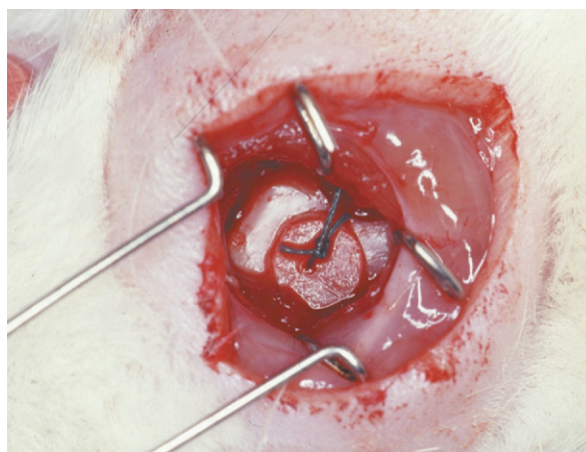


Fig. 2. Group B—operated situs.

serum in Tris buffer were used as negative controls. Human fetal (12 weeks) bone (rib and femur) was used as positive control. The sections (3 μ m) were dewaxed, rehydrated, and submitted to antigen retrieval in 0.5% pepsin, pH 1.8, for 30 minutes at 37°C. The specimens were then treated with 6% hydrogen peroxide/methanol solution (1:1) for 30 minutes to block endogenous peroxidase activity. Immunohistochemistry was performed using the Dako Autostainer (Dako Corp., Carpinteria, CA). Briefly, the sections were incubated with the primary anti-BMP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and diluted 1:10 for 30 minutes. Next, the sections were incubated with the streptavidin-biotin complex (LSAB Peroxidase K0690 kit, Dako), followed by incubation with the Dako Liquid DAB-Plus substrate-chromogen system (code K3468) for 3 minutes. The sections were then counterstained with Mayer's hematoxylin. Negative controls were treated as described previously with a solution of 1% bovine serum albumin in Tris-HCl, pH 7.4 replacing the primary antibody.

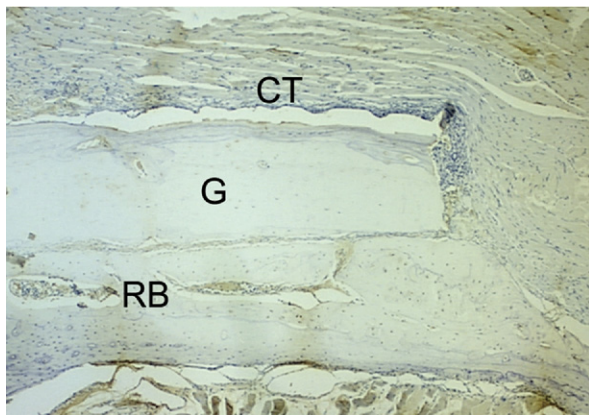


Fig. 3. Group B specimen 45 days. Photomicrograph showing graft (G), receptor bed (RB), and connective tissue (CT) (original magnification $\times 40$).

Qualitative and quantitative analysis were performed. For descriptive analysis, all slides were examined under an Axioscope 2 Plus light microscope (Carl Zeiss, Jena, Germany) at magnifications ranging from $\times 250$ to $\times 630$. Slides showing intracytoplasmic staining without a background reaction were selected for cell counting. In each slide, images of consecutive areas of the following structures were captured at $\times 400$ magnification: graft border, graft, connective tissue above the graft, graft-host interface, and recipient bed. The images were captured using an Axioscope 2 Plus microscope (Carl Zeiss) coupled to a digital camera (SV Micro—Sound and Vision, Boston, MA). This system was connected to a computer and the Axio Vision 3.0 program (Carl Zeiss) was used for analysis.

Quantitative analysis was performed by a calibrated examiner who obtained an intraclass coefficient of correlation of 0.993 (0.989 to 0.996) at P less than .001.

RESULTS

No clinical complications were observed after surgery and none of the animals were lost during the experiment.

A problem with some specimens and the immunohistochemistry technique was noted in this study. Many sections were lost from the glass microscope slide. Willbold and Witte³² describe an optimized and standardized embedding and cutting technique using Technovit 9100 (Heraeus Kulzer, Germany). With this technique, it is possible to perform enzyme histochemistry, immunohistochemistry, a great variety of classical histologic stains, and even in situ hybridization, because this new polymerization system, also based on methyl methacrylate, polymerizes in the absence of oxygen and at low temperatures, so the DNA or most of the proteins are not denatured.

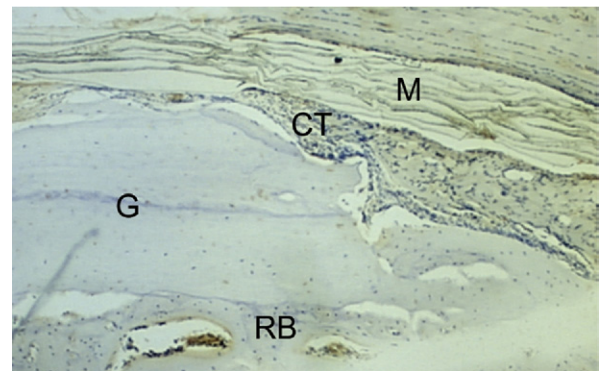


Fig. 4. Group MB specimen 45 days. Photomicrograph showing membrane (M), graft (G), receptor bed (RB), and connective tissue (CT) (original magnification $\times 40$).

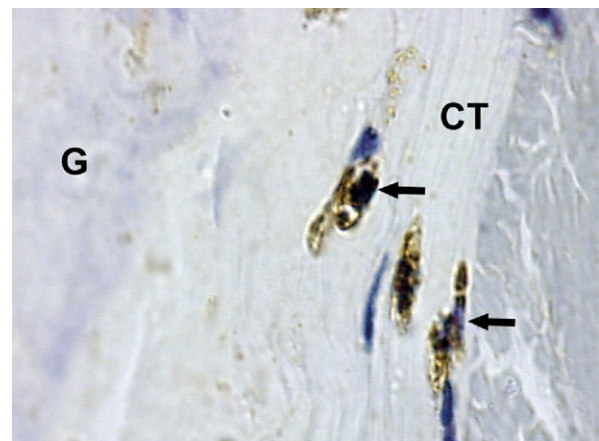


Fig. 5. Group B specimen 7 days. Photomicrograph showing connective tissue (CT) above the graft (G). Osteoprogenitor cells staining for BMP-2 (arrows) (original magnification $\times 630$).

Descriptive analysis

At baseline, both groups (B and MB) presented discontinuity and blood clotting in all structures analyzed. No baseline intracytoplasmic BMP-2 staining was observed in either group. At the end of the experiment, 45 days, all the structures were integrated in both groups B (Figure 3) and MB (Figure 4).

Immunolocalization of BMP-2 in autogenous bone block grafts

At 3 days, formation of new bone started in the inner cortical zone of the recipient bed, the region most distant from the graft. BMP-2 staining was seen in osteocytes and osteoblasts of the recipient bed. No staining was observed in the other structures. At 7 days, BMP-2 immunostaining was seen in osteocytes and osteoblasts of the recipient bed and in osteoprogenitor cells located at the graft-host interface, in connective

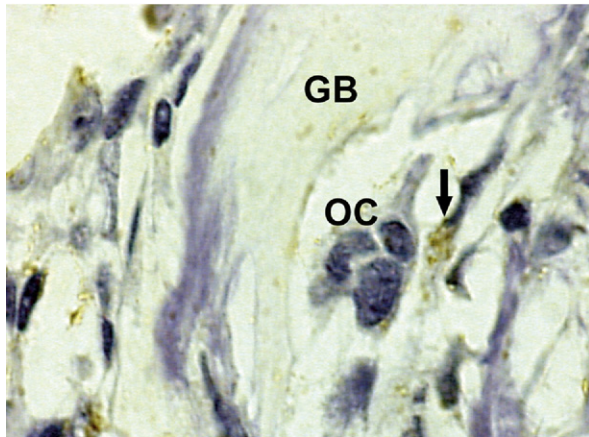


Fig. 6. Group B specimen 7 days. Photomicrograph showing osteoprogenitor cell staining for BMP-2 (arrow) on the graft board (GB) beside an osteoclast (OC) (original magnification $\times 630$).

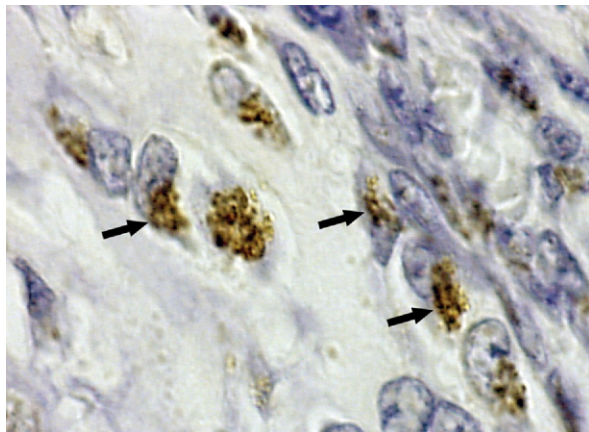


Fig. 7. Group B specimen 14 days. Photomicrograph showing osteoprogenitor cells staining for BMP-2 on the graft board (arrows) (original magnification $\times 630$).

tissue above the graft (Figure 5) and at the graft border, a region characterized by the onset of bone remodeling (Figure 6). Fourteen days after surgery, BMP-2-positive cells were detected in the region of the graft border (Figure 7) and in woven bone edges between the recipient bed and graft. At 21 days, when bone graft integration with the recipient bed became evident, BMP-2-positive cells were observed in immature trabecular bone (interface) (Figure 8). Immunostaining was seen in regions close to the perforation both in the recipient and at the interface (Figure 9). Forty-five days after surgery, the graft was integrated with the recipient bed. Intracytoplasmic staining was observed in perivascular cells located at the graft border (Figure 10). BMP-2 staining was also seen in the recipient bed, graft, and connective tissue above the graft.

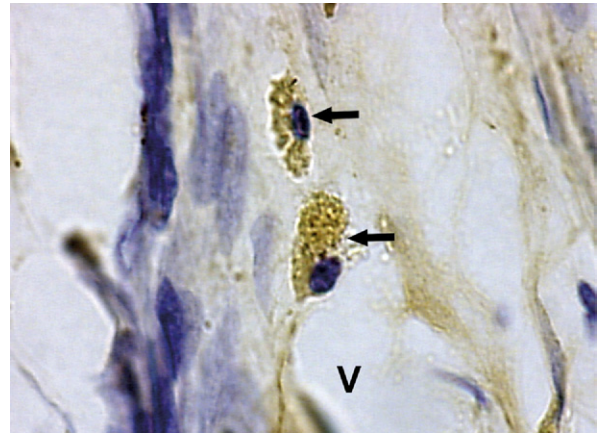


Fig. 8. Group B specimen 21 days. Photomicrograph showing perivascular cells staining for BMP-2 (arrows) on the interface (original magnification $\times 630$). V, blood vessel.

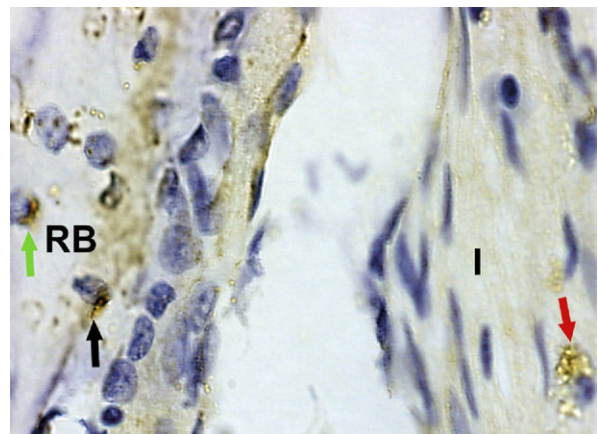


Fig. 9. Group B specimen 21 days. Photomicrograph showing osteocytes (green arrow), osteoblast (black arrow), and osteoprogenitor cell (red arrow) staining for BMP-2 on the receptor bed (RB) and on the interface (I) (original magnification $\times 400$).

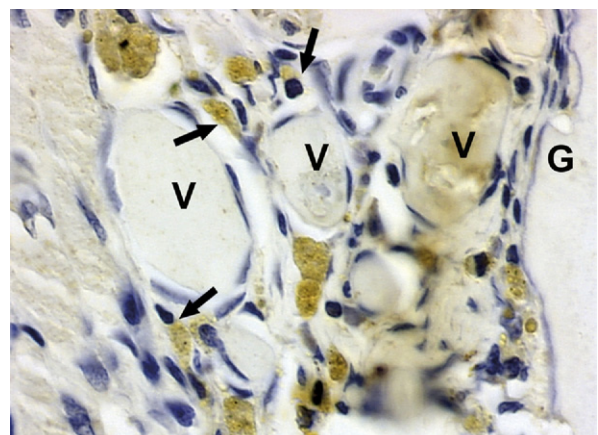


Fig. 10. Group B specimen 45 days. Photomicrograph showing the graft board with perivascular osteoprogenitor cells staining for BMP-2 (arrows) (original magnification $\times 400$). G, graft; V, blood vessels.

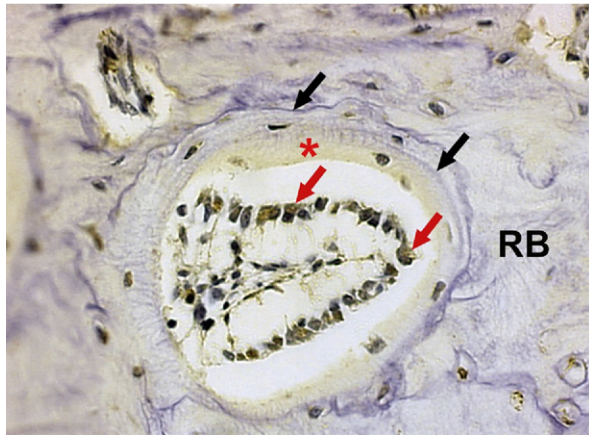


Fig. 11. Group MB specimen 3 days. Photomicrograph showing woven bone (*) and reversion lines (black arrows) on the receptor bed (RB). Osteoblasts staining for BMP-2 (red arrows) (original magnification $\times 200$).

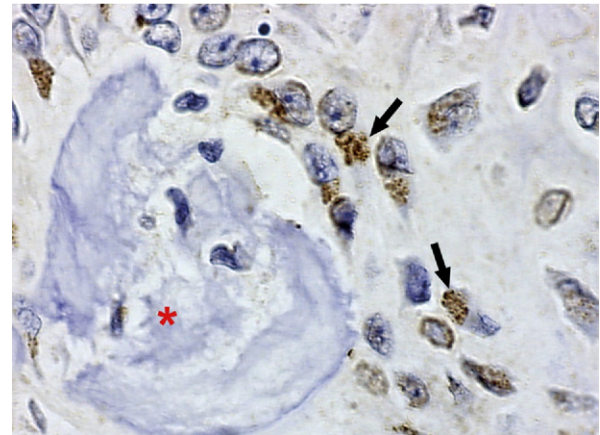


Fig. 13. Group MB specimen 7 days. Photomicrograph showing the graft board with osteoblasts staining for BMP-2 (arrows) and bone remodeling area (*) (original magnification $\times 400$).

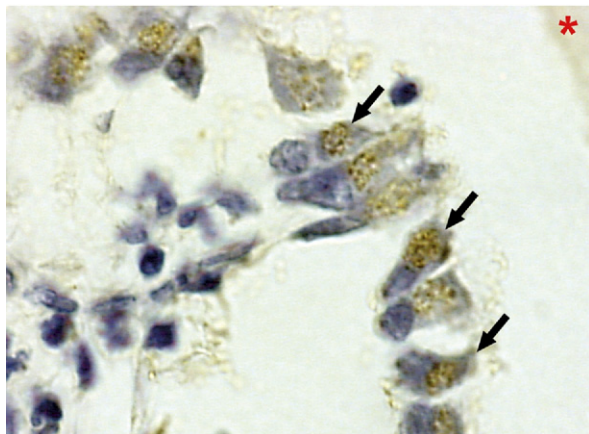


Fig. 12. Group MB specimen 3 days. Photomicrograph showing osteoblasts (arrows) staining for BMP-2 depositing woven bone (*) (original magnification $\times 630$).

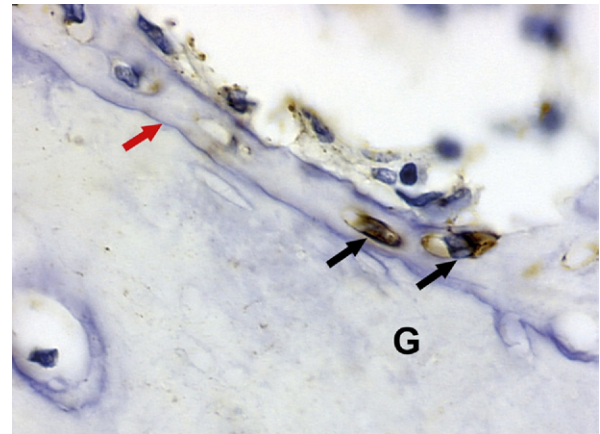


Fig. 14. Group MB specimen 21 days. Photomicrograph showing substitution area on the graft (G). Osteocytes recently imprisoned in the new matrix (black arrows) and reversion line (red arrow) (original magnification $\times 400$).

Immunolocalization of BMP-2 in autogenous bone block grafts covered with an e-PTFE membrane

At 3 days, intracytoplasmic BMP-2 staining was seen in the inner cortical zone of the recipient bed. Deposition of osteoid in resorption canals was observed in this region of bone remodeling (Figure 11). BMP-2 staining was seen in osteocytes and osteoblasts in this region (Figure 12). Seven days after surgery, staining was seen in osteocytes of the recipient bed and in osteoprogenitor cells of young connective tissue found at the graft-host interface. BMP-2-positive cells were observed at the graft border (Figure 13) and in connective tissue above the graft; regions located beneath the membrane. Osteocytes recently trapped inside the graft were also positive. At 14 days, intracytoplasmic staining was

observed in areas of marked osteoblastic activity, such as the interface and graft border.

Twenty-one days after surgery, integration of the graft with the recipient bed and a large area of new bone formation at the graft border were observed. This period was characterized by extensive replacement of destructured bone matrix with new matrix, indicating bone remodeling in the graft. Intracytoplasmic staining was observed inside the graft and at the graft borders; regions characterized by the presence of osteoid and reversal lines (Figure 14). Staining of osteoprogenitor cells was seen in connective tissue above the graft, even close to the membrane (Figure 15). BMP-2-positive cells were observed in the recipient bed and at the graft-host interface (Figure 16). Forty-five days after surgery, when the structures were completely inte-

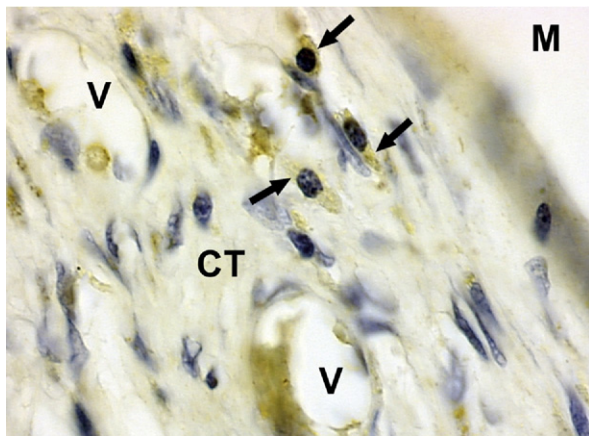


Fig. 15. Group MB specimen 21 days. Photomicrograph showing connective tissue (CT) above the graft. Osteoprogenitor cells staining for BMP-2 (arrows) (original magnification $\times 400$). V, blood vessels; M, membrane space.

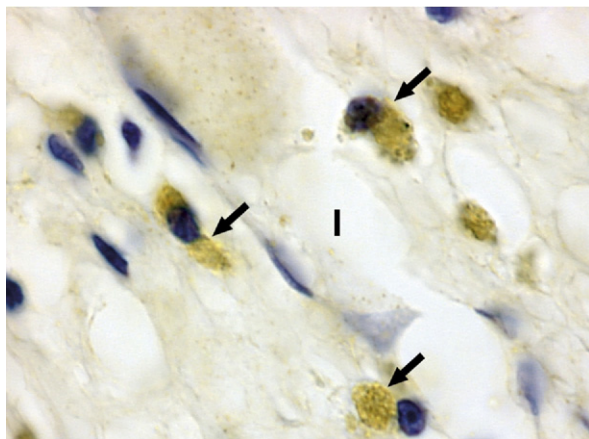


Fig. 16. Group MB specimen 21 days. Photomicrograph showing osteoprogenitor cells staining for BMP-2 (arrows) on the interface (I) (original magnification $\times 630$).

grated and the graft was well remodeled, staining was seen in the recipient bed, graft, and integration area (interface), as well as at the graft border and in connective tissue above the graft.

Quantitative analysis

The number of BMP-2-positive cells in the different structures (graft border, recipient bed, graft, interface, and connective tissue above the graft) was compared between the B and MB groups.

At the graft border, the largest number of positive cells was observed after 7 days in group B and after 14 days in group MB (Figure 17, A and B). In the graft, the largest number of positive cells was observed after 3 days in group B and after 21 days in group MB, although the mean number was similar across all experimental periods (Figure 17, A and B). In connective

tissue above the graft, the largest number of positive cells was seen after 7 days in group B and after 21 days in group MB (Figure 17, A and B). The largest number of positive cells at the graft-host interface was observed after 21 days in group B and after 7 days in MB (Figure 17, A and B). In the recipient bed, the largest number of positive cells was noted after 3 days in both groups (B and MB) (Figure 17, A and B). The number of positive cells was always lower at 45 days in all structures.

For all structures analyzed, no significant difference in the number of BMP-2-positive cells was observed between groups (B \times MB) at any time point. In addition, no difference in BMP-2 immunolocalization was observed over time in group B or group MB.

DISCUSSION

The present study defines and characterizes the presence, localization, and chronology of BMP-2 expression during wound healing after GBR using an onlay autogenous graft covered or not with an e-PTFE membrane. Immunolocalization of BMP-2 was evaluated in the B and MB groups at different times after surgery and in different structures. Overall analysis according to structure showed a higher proportion of staining at the graft border, at the graft-host interface, in connective tissue above the graft, and in the recipient bed, irrespective of the group studied. Lower proportions were observed in the graft. Analyzing the same structure, differences in staining were observed between time points and between groups.

In the recipient bed, the largest number of positive cells was observed after 3 days in groups B and MB. No difference in BMP-2 staining in the recipient bed was observed between groups during this early stage of repair. During this phase, the periosteum reacts to stimuli received during surgery and undifferentiated mesenchymal cells or preosteoblasts proliferate. Vascular buds are present in the more superficial region of the recipient bed, which start to orient themselves in the direction of the graft.¹⁹ Studies on fracture healing reported cytoplasmic staining in proliferating osteogenic cells during the early stages after fracture creation.^{14,21,22,24} Studies investigating distraction osteogenesis^{25,26} observed expression of BMP-2, -4, and -7 immediately after surgery, even before the onset of the distraction process. These data point to the expression of BMP-2 during the early stage of bone healing.

The largest number of positive cells was detected at the graft border on day 7 in group B and on day 14 in group MB, with the observation of positive staining in osteoblasts and osteoprogenitor cells in areas of bone remodeling and of a large quantity of newly formed bone. A slight decrease in the proportion of BMP-2 staining was observed after 14 days in both groups, but

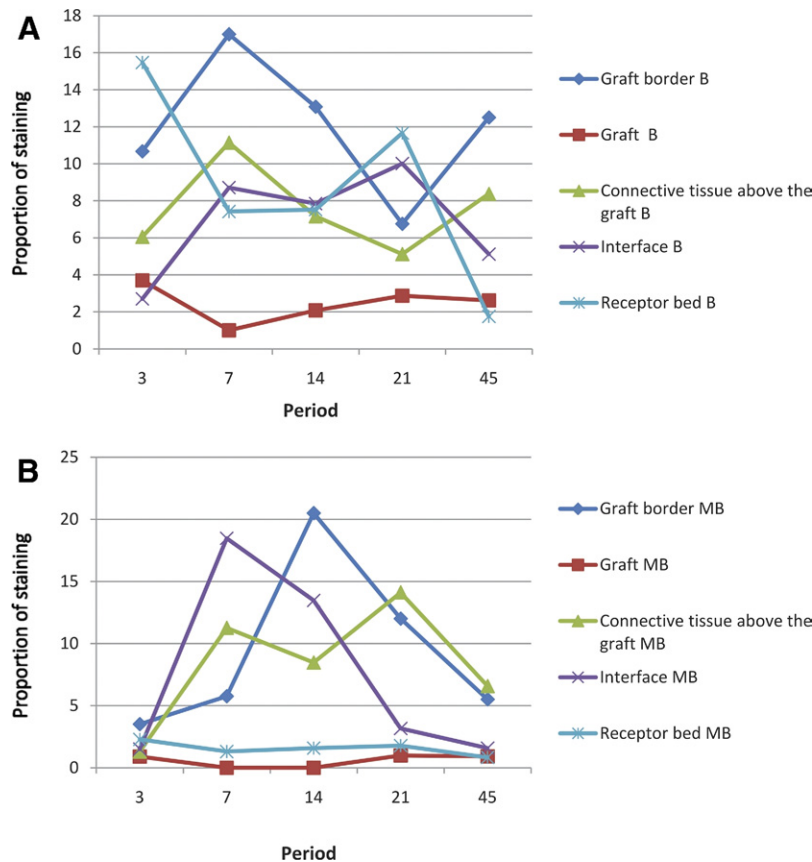


Fig. 17. **A**, Proportion of BMP-2 staining in group B at different time points after surgery. **B**, Proportion of BMP-2 staining in group MB at different time points after surgery.

positive cells were detected in this area of intense osteogenic activity even after 21 and 45 days.

In connective tissue above the graft, the highest proportion of BMP-2 staining was observed in osteoprogenitor cells after 7 days in group B and after 21 days in group MB. However, a slight decrease in the proportions of staining was observed between 7 and 45 days, with BMP-2-positive cells still being detected at the end of the experiment. In agreement with these findings, several investigators reported the expression of BMP-2 in immature bone cells,^{24,26} multinucleated cells (preosteoclasts),^{22,23} and osteoid²⁸ during bone repair 7 to 28 days after surgery. Onishi et al²² suggested that BMP-2 and BMP-4 are involved in the differentiation and maturation of osteoclasts; however, in contrast with Onishi et al.,²² no staining of osteoclasts was observed in the present study.

Bone resorption and remodeling activity and the onset of osteogenesis were observed at the graft border and in the upper portion of the graft in group B between 7 and 14 days. The BMP-2 staining observed in group B at the graft border and in connective tissue above the graft is intimately related to the formation of a vascular plexus at the graft borders on day 7 and to the penetration

of vessels into the graft, permitting mesenchymal and osteoprogenitor cells to arrive at the site and to act on the remodeling process. The same event was observed in group MB between 14 and 21 days. Thus, graft replacement occurs earlier in group B than in group MB.¹⁸⁻²⁰

At the graft-host interface, the largest number of positive cells was observed between 7 and 21 days in groups B and MB, respectively, with positive osteoprogenitor cells being detected in areas of woven bone edges between the recipient bed and graft. The graft, bed, and interface practically formed a single structure as early as day 21 after surgery.¹⁸ Newly formed blood vessels penetrating almost throughout the graft were observed during this period.¹⁹

In group B, the highest proportion of BMP-2 staining at the graft-host interface was observed at 21 days (later than in group MB), whereas in the other structures analyzed, the highest proportion was observed during earlier stages. Thus, BMP-2 immunostaining generally occurs slightly later in group MB. This difference in the predominant expression of BMP-2 and the time when it occurs, as well as the difference between groups, might be related to the more intense revascularization in group B.¹⁹

BMP-2 staining was seen in the graft, with the largest number of positive cells being observed after 3 days in group B and after 21 days in group MB. However, in the 2 groups the proportion of staining was lower when compared with that observed for the other structures. In group MB, the membrane-covered graft did not come into contact with surrounding connective tissue because the diameter of the membrane exceeded the borders of the graft, a fact that resulted in cell-mediated repair and in a newly formed vascular network originating from the recipient bed. Therefore, in group MB, revascularization of the graft occurred from the recipient bed and the weak staining for BMP-2 might be explained by this slow revascularization and graft replacement.^{18,19}

Marked proportions of staining were observed in the region of the graft border, connective tissue above the graft, interface, and recipient bed between 21 and 45 days, suggesting that BMP-2-positive cells are still present at 45 days. These results agree with those reported by Alam et al.,²⁹ who evaluated bone regeneration after implantation of 3 different types of bone graft. New bone formation was observed in the group receiving the autogenous bone graft after 4 weeks, but the number of BMP-2-positive cells was reduced. Similar findings have been reported by Si et al.,¹⁴ Rauch et al.,²⁵ and Spector et al.²³ Our results also demonstrated a higher proportion of BMP-2-positive cells between 3 and 21 days and a lower number of positive cells at subsequent time points.

The MB group presented higher proportions of staining during later periods of the early stage of healing in all structures when compared with group B, except for the recipient bed, in which a higher proportion of staining was observed after 3 days in both groups, and the graft-host interface, at which a higher proportion was observed in group B after 21 days. Taken together, these results indicate that areas showing a higher proportion of staining are sites of more intense osteogenic activity.

All of these observations are intimately related to the process of graft revascularization described by De Marco et al.¹⁹ In that study, revascularization was more intense in group B and was accompanied by the preservation of structures and greater gain in bone volume as demonstrated by Jardini et al.¹⁸ Revascularization was also observed in group MB, but the process was slower.

In the present study, BMP-2 antibody staining was observed in osteoprogenitor cells, osteoblasts, and osteocytes located in areas of osteogenesis close to blood vessels. McCullough et al.³⁰ observed staining of perivascular chondrocytes and suggested that diffusion factors released by perivascular cells might be involved in the differentiation and apoptosis of adjacent chon-

drocytes. These authors, as well as Onishi et al.²² and Spector et al.,²³ found that BMP staining in osteoclasts is variable, ranging from intense to moderate. BMPs present in osteoclast cytoplasm may simply be the result of phagocytosis of BMP-containing bone matrix. However, it has been suggested that osteoclasts synthesize BMPs. The expression of BMPs by osteoclasts at resorption sites during bone remodeling represents an important mechanism of intercellular communication that leads to the recruitment and differentiation of osteoblasts.³¹ The expression of BMP-2 in osteoclastlike multinucleated cells also suggests that BMPs are involved in the differentiation and maturation of osteoclasts.²²

CONCLUSIONS

Intracytoplasmic staining of BMP-2 was consistently observed in areas of revascularization and osteogenesis. Intracytoplasmic BMP-2 staining was seen in osteoprogenitor cells, osteoblasts, and osteocytes, irrespective of the period or structure analyzed. The highest proportions of staining were observed at the graft border and graft-host interface, in connective tissue above the graft, and in the recipient bed, whereas the lowest proportion was detected in the graft. Except for the interface, BMP-2 staining occurred later in group MB than in group B in all structures analyzed.

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