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Expression of MMP-2, 9 and 13 in newly formed bone after sinus augmentation using inorganic bovine bone in human

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Background and Objective: The aim of the present study was to analyse the expression of MMP-2, MMP-9 and MMP-13 in newly formed bone following maxillary sinus augmentation using inorganic bovine bone substitute, because these MMPs play a major role in bone remodeling and bone resorption.

Material and Methods: Deproteinized bovine bone (Bio-Oss®) was used to fill cavities after elevating the sinus mucosa. Twenty patients with edentulous posterior maxilla were treated with 20 sinus-augmentation procedures using a two-stage technique. Forty-nine Straumann® endosseous implants were used to complete the implant-prosthetic rehabilitation. One cylinder-shaped bone biopsy from each patient was taken from the augmented maxillary region using trephine burs at the second stage of surgery, 8 months after grafting. A biopsy was also taken as a control from the upper molar region from six different patients who did not undergo the sinus procedure. All biopsies were subjected to biochemical analysis and staining for TRAP.

Results: No implant losses or failures occurred. The large number of TRAP-positive multinucleated osteoclasts in resorption lacunae indicated that the resorption was very active in all grafts, in contrast with the control group. Zymography and western blot analysis demonstrated a significantly increased expression of MMP-2, MMP-9 and MMP-13 in the newly formed bone compared with controls ($p < 0.05$).

Conclusion: The quantity of osteoclastic cells and the increased expression of proteolytic enzymes suggest that 8 months after grafting, inorganic bovine bone is slowly resorbing and is the site of important remodeling of the newly formed bone by means of resorption and synthesis.

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The placement of implants in highly atrophic maxillae continues to be one of the major challenges in implant dentistry. Sinus floor elevation is one of the preferred options to resolve this problem. In addition to autogenous bone, which is considered as the gold standard, various bone-grafting materials have been used in sinus augmentation, including freeze-dried bone allografts (1), xenografts (2) and hydroxyapatite (3). Among the bone

substitutes, inorganic bovine bone has been recommended by several authors (4).

Bio-Oss® (Geistlich Biomaterials, Wolhusen, Switzerland) is a bone substitute that has been used and evaluated for bone-augmentation purposes. It meets all European and US standards for safety regarding disease transmission (5). Histological studies of animal specimens showed that it has bone-conductive properties (6,7).

The role of MMPs during bone development has been studied extensively (8). These enzymes are expressed by osteoclasts and osteoblasts, and they play an important role in bone remodeling and resorption (9–11).

Bone remodeling requires the recruitment of osteoclastic precursors, their differentiation and their adhesion to bone matrix. The use of protease inhibitors in the different phases of bone resorption shows that MMPs are involved in the resorption cycle (12). The presence of an acidic pH within the resorption gap enclosed between the active osteoclast and the bone matrix is the major barrier to the direct action of these endopeptidases during the resorption phase. The role of MMPs is thus centered on the adhesion and migration of osteoclasts during bone remodeling (13).

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have similar substrates but are primarily expressed by osteoblasts and osteoclasts, respectively. MMP-2 is expressed only at the first stages of bone formation (14) and its loss reduces the connectivity density of the trabeculae (15).

In contrast, the expression of MMP-9 is regarded as a marker of osteoclastic activity (16). In autogenous bone grafts, a close relationship exists between MMP-9 expression and bone graft resorption (17).

MMP-13 is involved in normal remodeling of bone during adult skeletal repair and it plays a direct role in the initial stages of extracellular matrix degradation (18). MMP-13 is associated with alveolar bone resorption and is one of the most active MMPs during the adhesion of the osteoclast to the mineralized extracellular matrix (19).

These reports have led us to analyze the expression of MMP-2, MMP-9 and MMP-13 after maxillary sinus augmentation using inorganic bovine bone substitute. The expression of these MMPs might help us to understand the rate and the effectiveness of bone development and bone remodeling.

Material and methods

Patients

This study reports 20 grafted sinuses in 20 patients [10 women and 10 men; mean age 58.5 years (range, 40–74 years)] who had to undergo sinus floor elevation because of a lack of bone in which to place endosseous implants. The inclusion criterion was to have < 3 mm of residual bone in the posterior maxilla (Fig. 1A). The preoperative planning consisted of both clinical examination of the maxilla and panoramic radiographic assessment. Five of the 20 patients smoked more than 10 cigarettes/d; therefore, smoking was not an exclusion criterion in the present investigation. All patients were in good overall health with no significant diseases, and none was on chronic drug therapy. Preoperatively, patients were informed about the surgical procedures, asked for their full cooperation during treatment and signed a written informed consent. The study was approved by the Ethical Committee of St Joseph University in Beirut.

Surgical technique

In all 20 maxillary sinus floor grafts, the same surgical approach was used under local anesthesia, as described previously (20–22). The incision was made at the middle of the alveolar crest, and a mucoperiosteal flap was elevated, exposing the lateral wall of the sinus. A bony window was outlined using a piezoelectric device. The lateral window was pushed inwards and upwards, creating a new horizontal sinus floor. The grafting material (Bio-Oss®) was hydrated with a saline solution and was gently packed into the sinus until it

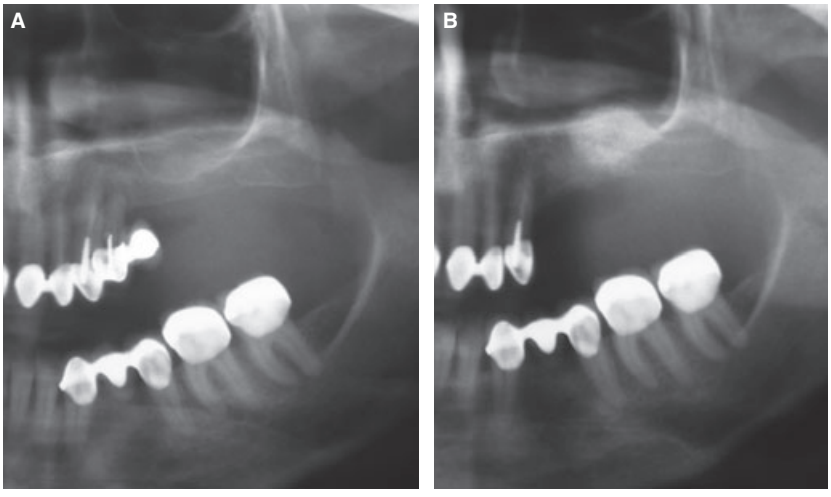

Fig. 1. Panoramic radiography. (A) The vertical bone supply in the posterior region of the maxilla is insufficient for implant placement. (B) Eight months after augmentation of the maxillary sinus floor using inorganic bovine bone, the new height of bone is adequate for implant placement.

filled the entire space created by the membrane elevation. No membranes were used. The flap was sutured tension-free using interrupted 4-0 silk. The second stage of the surgery was performed 8 months after the initial grafting procedure (Fig. 1B). A new flap was raised to expose the alveolar ridge, and 49 Straumann® implants were placed.

One horizontal bony core was harvested, for histological examination, from the lateral window area of each patient using a 3-mm-diameter trephine bur under abundant saline irrigation (group P, $n = 20$). At the end of the surgery, the mucosa was sutured with 4-0 silk. Amoxicillin (1 g twice daily for 6 d) and analgesics (as needed) were prescribed. One biopsy was also taken as a control from the upper molar region before implant placement in six different patients who did not receive a sinus lift procedure. Three months elapsed before the implants were prosthetically loaded (group C, $n = 6$).

Biochemical studies

Each of the 26 bone biopsies was cut and reduced to 50 ± 5 mg under sterile conditions.

We have used a plate containing 32 wells, and each biopsy sample ($n = 26$) was placed alone in a well and maintained for 72 h in 3 mL of Dulbecco's modified Eagle's medium containing 1.5 mM Ca^{2+} and supplemented with 0.2% lactalbumin hydrolysate (Sigma, St Louis, MO, USA), 2 mM L-glutamine, 100 U/mL of penicillin and 100 μg/mL of streptomycin (Boehringer, Mannheim, Germany), at 37°C in an atmosphere of 95% air and 5% CO_2. Cell viability was evaluated by determination of lactate dehydrogenase activity in conditioned medium. To ensure that the liberation of proteolytic enzymes in our culture medium was not secondary to tissue necrosis, the culture medium contained 1.5 mM Ca^{2+}, as recommended previously (23). After 72 h of culture, all proteinase levels were quantified on a tissue weight basis.

Zymographic analysis

Aliquots of supernatant from the biopsies were diluted 50:50 with sodium dodecyl sulfate (SDS) nonreducing sample buffer. Gelatinolytic activities attributed to gelatinases A and B (MMP-2 and MMP-9, respectively) were identified by gelatin zymography of the diluted biopsy supernatants after electrophoresis on an SDS–polyacrylamide gel containing 1 mg/mL of type I porcine gelatine, as previously described (24). The location of gelatinolytic activity was detected as clear bands on a background of uniform staining. The use of HT1080 supernatant as an external standard in each gel allowed the comparison of gelatinolytic activities on different gels.

Western blotting analysis of MMP-2, MMP-9 and MMP-13

Samples were mixed with a nonreducing sample buffer. As for zymographic analysis, gels were run in Laemmli buffer conditions at 80 V for 15 min and then at 160 V for 90 min. Proteins were transferred onto a polyvinylidene difluoride (PVDF; Millipore, Bedford, MA, USA) membrane at 80 V for 1 h. Nonspecific binding sites were blocked by incubation of the membrane for 1 h in phosphate buffered saline (PBS) containing 5% nonfat milk and 0.05% Tween-20 and then the membrane was washed in PBS (four times, for 15 min each wash). The membrane was incubated overnight at 4°C with a monoclonal primary antibody directed against human MMP-2, MMP-9 and MMP-13 (diluted 1:500 in PBS; Oncogene-Merck Eurolab, Val de Fontenay, France), washed, then incubated with an appropriate horseradish peroxidase (HRP) secondary antibody (diluted 1:1000 in PBS; Sigma). Peroxidase activity was detected by chemiluminescence (covalight: Ab-Cys). A Kodak Biomax MR film was used for revelation. Multiple exposures were examined to ensure that the results analyzed reflected those produced in the linear range of the film.

Quantification of zymogram lysis bands and blots by image analysis

The average surface of bands obtained after PAGE and/or membrane blotting was determined semi-automatically, following their contour with a calibrated electronic slide, as described previously (25). The bands were then observed using a CFR 126 video camera; black and white images were converted into 256 different grey levels using Sophretec image memory, transferred to a BFM microcomputer and finally analyzed using software for mathematical morphology.

For zymographic analyses, gelatinase activities were expressed as pixels (S) × grey level of samples/pixels (S) × grey level of HT1080. The HT1080 was used as a quantitative standard for several zymograms. Quantitative analysis is the average gelatinolytic activity of all the samples (six controls and 20 patients). For western blotting analyses, the quantities of gelatinases or collagenases were expressed as dot pixels (S) × (255 − grey level) (26). Comparison was made between the controls and the patients in one western blot (the most representative) for each MMP investigated.

TRAP

After dehydration, the biopsies were embedded, without demineralization, using methyl methacrylate (Merck, Darmstadt, Germany), polymerized at −20°C for 48 h and then sectioned using a Polycut E microtome (Leica, Wetzlar, Germany). Serial sections of 4 μm thickness were obtained. The biopsies were processed for enzyme analysis to detect TRAP, which is secreted by osteoclasts during bone resorption and this secretion is directly correlated with the resorptive behavior. TRAP was detected using hexazotized pararosanilin and naphthol ASTR phosphate (Sigma). Nonosteoclastic acid phosphatase activity was inhibited by 50 mM tartric acid added to the substrate solution.

Results

None of the 20 patients treated by surgery showed postoperative sinus complications (e.g. sinus congestion, graft infection and/or poor wound healing), and all implants were clinically integrated.

On the outer surface of the particles, it was possible to observe the presence

of many active osteoclasts that were in the process of actively resorbing bone (TRAP+) (Fig. 2A), in contrast to the control group where the number of osteoclasts was very small (Fig. 2B).

The quantitative and qualitative analysis of MMP-2, MMP-9 and MMP-13 secreted from bone explants after 72 h of organ culture (zymographic and western blotting analysis) showed that the intensities of the 72-kDa (pro-MMP-2) and the 66-kDa (active MMP-2) bands were strong. The expression of these two forms of MMP-2 was greatly increased in the conditioned medium with the biopsies of bone substitute when compared with controls ($p < 0.05$) (Fig. 3A).

Gelatinolytic bands migrating with an apparent molecular mass of 88 kDa (active MMP-9) were detectable in the conditioned medium with the biopsies of bone substitute but a band corresponding to the pro-gelatinase B 92 kDa (pro-MMP-9) was not. However, pro-MMP-9 and active MMP-9 were undetectable in samples from all the control patients (Fig. 3B).

Following SDS–PAGE and western blotting of proteins secreted into the conditioned medium of bone explants from the grafted areas and from the control patients, MMP-2, MMP-9 and MMP-13 were detected as bands with approximate molecular mass values of 70 kDa for MMP-2, 90 kDa for MMP-9 and 50 kDa for MMP-13 (Fig. 4A).

The amounts of MMP-2, MMP-9 and MMP-13 were increased in the conditioned culture medium for the newly formed bone compared with the control donors ($p < 0.05$) (Fig. 4B).

Discussion

Maxillary sinus lift procedures are designed to promote the formation of sufficient vertical bone in the maxillary sinus before placement of dental implants (1). The success or failure of treatment with osseointegrated implants in edentulous patients depends on the volume and quality of the newly formed bone in the augmented space (27).

Clinically, the high osteoconductive property of Bio-Oss® has been widely demonstrated (28). Numerous investigators have recognized that Bio-Oss® is an appropriate synthetic material for the treatment of osseous defects and maxillary sinus augmentation (29). The present study confirms the pre-existing data because all patients treated with Bio-Oss® implants after maxillary sinus augmentation procedures had a great clinical response. The use of Bio-Oss® alone showed a 100% implant success rate after 12 months of loading. This is in agreement with Froum *et al.* (30), who found similar implant success rates when bovine bone was used with or without autogenous bone.

Histologically, the present study was able to show the increase of resorption lacunae and active osteoclasts in bone grafts compared with controls. This increased number of osteoclasts suggests that bone remodeling in these grafts is higher than in normal mature bone. This may be a result of the remodeling of the newly formed bone or the resorption of the grafted material, or both simultaneously.

In the literature, the resorption of bovine bone substitute material has been the subject of controversy. Resorption of Bio-Oss® has been described in animal experiments on rabbits and dogs (6,31). The duration of resorption *in vivo* has been reported to be between 2 and 3 years (30). Tadjoedin *et al.* (32) reported a decrease in Bio-Oss® mass by 10% per year as a result of osteoclast activity. Schlickewei and Paul (33) described the resorption of Bio-Oss® as physiologic remodeling, requiring a time interval of 1–5 years in the case of human bone and 6–12 months in the case of rodents.

On the other hand, Ewers *et al.* (34) showed that Bio-Oss® particles were still present after 4.5 years without any evident signs of resorption.

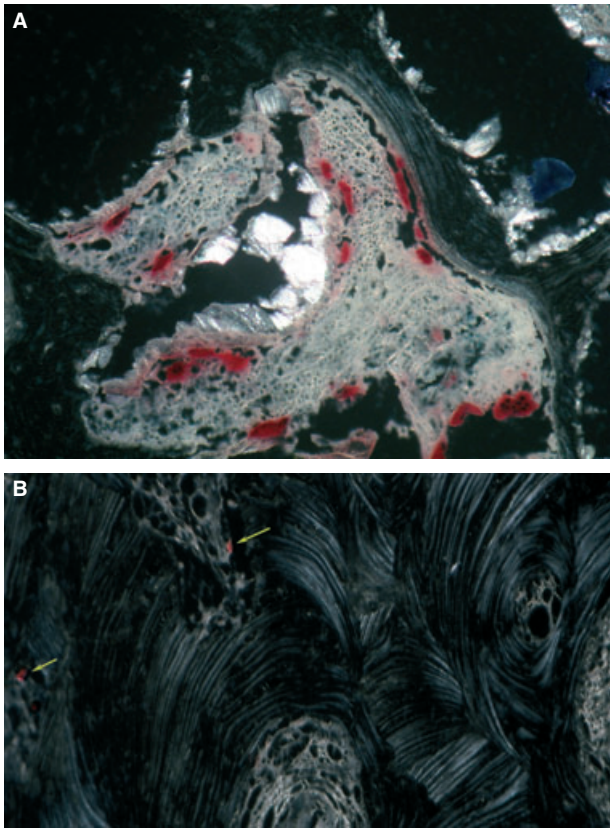

Fig. 2. Enzyme analysis (TRAP). (A) Histological studies show an increased number and activity of TRAP-positive-stained osteoclasts in biopsies taken from the grafted area, in contrast with the control patients (B) where the numbers and activities of osteoclasts are reduced. These osteoclasts are in close contact with the newly regenerated bone and Bio-Oss® particles. Magnification ×52.

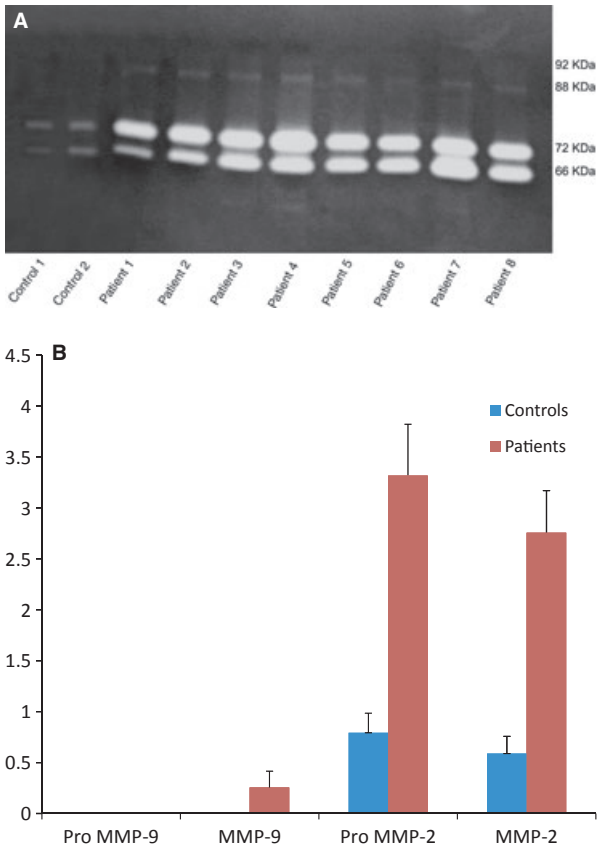

Fig. 3. (A) Gelatinolytic activities detected on gelatin zymograms. With Control 1 and Control 2, only two lysis bands are visible, at 72 kDa (progelatinase A) and at 66 kDa (activated gelatinase A). For all the biopsies taken from the grafted areas (Patient 1 to Patient 7), two lysis bands are clearly visible at 72 and 66 kDa, and a lysis band at the limit of detection appears at 88 kDa (activated gelatinase B). (B) Quantification of gelatinases in the grafted area of patients and controls after zymographic analysis. Ordinate: gelatinase activity; surface of the lysis band in pixels (S) × grey level of samples/pixels (S) × grey level of HT1080. Abscissa: controls and patients grafted with bovine bone substitute. 92 kDa, pro-MMP-9; 88 kDa, activated MMP-9; 72 kDa, pro-MMP-2; 66 kDa, activated MMP-2. In controls, no gelatinolytic activities were detected at 92 kDa (pro-MMP-9) and 88 kDa (MMP-9), and low gelatinolytic activities appeared at 72 kDa (pro-MMP-2) and 66 kDa (MMP-2). In patients, significantly more active MMP-2 and pro-MMP-2 were present compared with controls ($p < 0.05$), and a gelatinolytic activity at the limit of detection appears at 88 kDa (pro-MMP-9). The active forms of MMP-9 were not detected.

In this study, the increased number of TRAP-positive osteoclasts around the deproteinized bone particles and the penetration of bone into the graft material suggest that deproteinized bone may be slowly resorbed concomitantly with the remodeling of the newly formed bone.

MMPs are a family of proteolytic enzymes expressed by both osteoclasts and osteoblasts (35), including collagenases, gelatinases A and B, and stromelysins.

MMP-13, also called collagenase-3, is mainly expressed in bone and cartilage under physiological conditions. Several studies have shown MMP-13 involvement in bone tissue remodeling and osteoblastic cell differentiation (36). Indeed, MMP-13$^{-/-}$ mice show defects in growth plate cartilage and disturbed ossification (9). Collagenase-3 expresses a weak gelatinolytic activity that can be revealed by gelatin zymography (37). However, with a molecular mass of around 55 kDa, it is difficult to differentiate between the three human collagenases (MMP-1, MMP-8 and MMP-13) by zymographic analysis. Therefore, to avoid confusion and errors in interpretation, and for more specificity, we preferred to study MMP-13 expression by western blotting.

Western blotting revealed an increased expression of MMP-13 in culture media for the experimental group compared with the control group. This increase contributes to the reshaping of the newly formed bone because MMP-13 is involved in physiologic bone remodeling (18). Also, MMP-13 promotes the remodeling of newly deposited trabecular bone during long-bone development (9).

MMP-13 is expressed by osteoblasts but not by osteoclasts (38). This expression is induced by factors initiating bone resorption, in particular parathyroid hormone and prostaglandin E2 (39). Inhibition of MMP-13 results in the absence of osteoclast adhesion to the bone matrix (40). In this study, MMP-13 present in new bone matrix may be required for the initial degradation of the extracellular matrix components, allowing the timely remodeling of bone (18).

Gelatinase B (MMP-9) belongs to a two-member family, gelatinase A and gelatinase B. Denatured collagen (gelatine) is their preferential substrate but they also degrade type IV and type V collagen, as well as elastin (41). The strong affinity of MMP-9 for denatured collagen allows them to maintain the collagenic digestion initiated by the collagenases (42). MMP-9 is mainly produced by polynuclear neutrophils, but is also produced by many other cells, such as macrophages and osteoclasts once they are activated. Its precise role during the resorption has not yet been established. However, it seems probable that it plays a major role in the adhesive and invasive potential of osteoclasts by digesting denatured collagen within the bone matrix immediately after collagenase-3 (which is secreted by the osteoblasts during the activation of the resorption) achieves its function (43). It has been suggested that gelatinase B is necessary for the migration of TRAP-positive pre-osteoclasts from the periosteum and is a major component of the mechanism that determines where and when the osteoclasts will attack bone (44). The

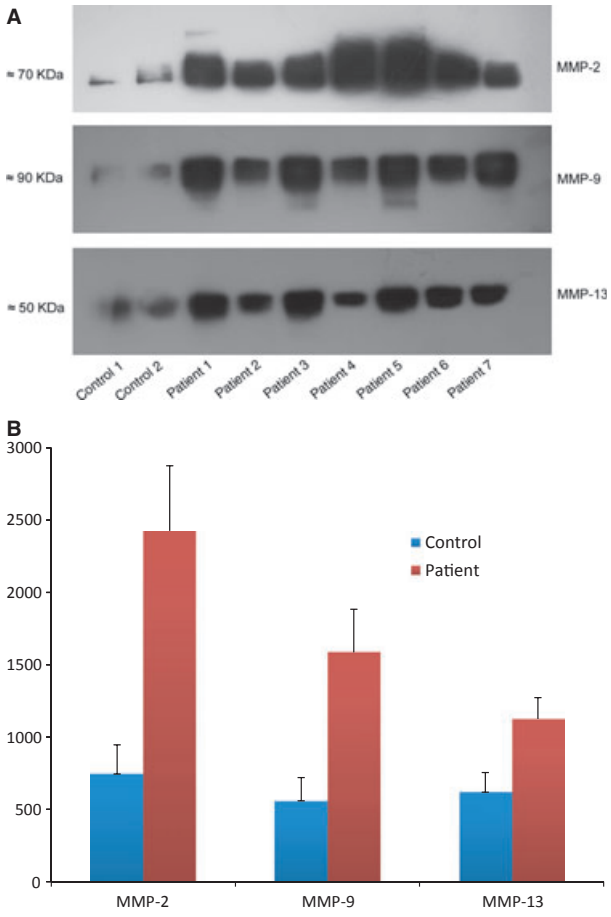

Fig. 4. (A) Western blotting analysis: MMP-2, MMP-9 and MMP-13 were detected in the culture media of bone biopsies taken from the grafted areas (Patient 1 to Patient 7) and those taken from the control donors (Control 1 and Control 2). (B) Quantification of western blot analysis: MMP-2, MMP-9 and MMP-13 were expressed as: (ordinate) S = Surface of bands (pixels) × (255 − grey level). The amounts of MMP-2, MMP-9 and MMP-13 were increased for all the patients grafted compared with the control donors ($p < 0.05$).

increased synthesis of MMP-9 and MMP-13 indicates that resorption is active in all bone biopsies taken from the grafted areas.

On the contrary, MMP-2 (gelatinase A) is the only MMP that is produced constitutively by chondrocytes, osteoblasts and fibroblasts (45). In a study of collagenase expression during mouse embryogenesis, Mattot *et al.* (14) found that MMP-2 was expressed only at the onset of bone formation, indicating a role in bone synthesis. MMP-2 has a compositional influence on the biomechanical properties of bone (46), and the loss of MMP-2 was found to weaken bone. This effect was associated with a decrease in mineralization density of the tissue (15).

As an increased synthesis of MMP-2 is related to bone formation (47), this suggests that at 8 months the grafted site is still subject to bone synthesis.

Conclusion

The results of our study demonstrate that at 8 months, the deproteinized bovine bone mineral Bio-Oss® appears to be highly biocompatible. The large quantity of TRAP-stained osteoclasts around the deproteinized bone particles, and the increased expression of MMP-2, MMP-9 and MMP-13 in the culture conditioned medium compared with controls indicate that the newly formed bone remains the site of important remodeling by means of resorption and apposition.

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