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Matrix Metalloproteinase-9 Regulates Graft Bone Resorption

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ABSTRACT

Objective: To explore the relationship between bone resorption and matrix metalloproteinase–9 (MMP-9) expression in autogenous and allogenic bone grafts.

Materials and Methods: A total of 18 critical-size (10 × 15 mm) defects were created in rabbit mandibles bilaterally. Three groups of six defects each were grafted with autogenous endochondral (EC) bone, autogenous intramembranous (IM) bone, and allogenic IM bone. Three months later, the defects were retrieved for quantitative analysis on the basis of histology, immunohistochemistry, and in situ hybridization.

Results: A close relationship existed between MMP-9 expression and graft bone resorption. The parallel between MMP-9 expression and graft bone resorption suggested that bone resorption was accomplished in part by increased MMP-9 production evident in preosteoclasts and osteoclasts.

Conclusions: MMP-9 plays an important role in graft bone resorption. Autogenous EC bone grafts express higher levels of MMP-9 leading to more resorption than autogenous or allogenic IM bone grafts.

KEY WORDS: MMP-9, Bone resorption, Autogenous EC bone, Autogenous IM bone, Allogenic IM bone.

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INTRODUCTION Return to TOC

Bone resorption is a critical step for the integration of graft bone with host bone as well as new bone formed in bony defects. The main cell responsible for this process is the osteoclast. Resorption of bone consists of multiple steps, including osteoclast recruitment, osteoclast differentiation, activation of osteoclast function, degradation of bone matrix proteins, and bone mineral mobilization.[1]

Degradation of the organic matrix in bone depends on the activity of two major classes of proteolytic enzymes: the cysteine proteinase (CPs) family and the matrix metalloproteinase (MMPs) family.[2,3] Both CPs and MMPs participate in the degradation of the collagenous bone matrix within the osteoclastic resorption lacuna.[2]

Furthermore, a sequential action of enzymes was proposed in osteoclastic degradation of calvarial bone: when a low pH is created to

dissolve the mineral, CPs digest part of the bone matrix at a such low pH, and MMPs digest the rest of the bone matrix when the pH increases somewhat.[3] MMPs is a family of at least 23 highly homologous Zn^{++}-endopeptidases that collectively cleave most if not all the constituents of the extracellular matrix.[4] Among them, matrix metalloproteinase–9 (MMP-9), also known as gelatinase B, is a key factor involved in bone resorption.[5,6] In the mouse embryo, MMP-9 is mainly expressed in osteoclasts.[5] Tezuka et al[6] reported that MMP-9 was secreted by isolated osteoclasts, and the expression of MMP-9 in vivo was also observed in osteoclasts in metacarpal bones of newborn rabbits by in situ hybridization. Furthermore, detection of MMP-9 expression in the intramembranous (IM) bones of the craniofacial region reveals that MMP-9 may play a key role in osteoclastic bone resorption.[7]

Availability of a broad, well-rounded alveolar ridge is essential for the success of orthodontic tooth movement. However, alveolar bony defects can be created after extraction for orthodontic treatment, and collapse of the alveolar ridge, eg, after trauma, poses a problem for implant placement and orthodontic treatment. Furthermore, patients with cleft lip and palate often require a secondary bone graft procedure to allow for eruption of anterior teeth and orthodontic treatment.

Therefore, bone grafting is essential to restore form and function of the alveolar ridge. Autogenous bone graft is hard to harvest for a large defect.[8] Fresh-frozen allogenic bone grafts commercially available from bone banks have been used to treat deficient bone with some success.[9] However, resorption of structural bone grafts is a problem in both autogenous and allogenic bone grafts. Limited understanding of the basic biologic principles of resorption of bone graft and subsequently the integration process has made it difficult to improve the success rate of bone graft surgery.

Therefore, we examined the localization and the expression of the MMP-9 gene at messenger RNA (mRNA) and protein level in autogenous endochondral (EC) and IM bone grafts and in allogenic IM bone grafts at the rabbit mandibular defects. The aim of this study was to examine the level of expression of MMP-9 three months after grafting and to relate that to the resorption of different types of bone grafts.

MATERIALS AND METHODS Return to TOC

Animals and materials

Eighteen adult (5–12 months old) New Zealand White rabbits that weighed 3.5–4 kg were used. The project was approved by the Committee for the Use of Living Animals in Teaching and Research at the University of Hong Kong (No. 368-99). Three rabbits were used for preparation of fresh-frozen allogenic IM bone graft harvested from bilateral mandibles, and six rabbits were used for autogenous EC and IM bone grafts using the same procedure as previous one.[10] The remaining nine rabbits were divided into three groups. Two defects per rabbit were created in the bilateral mandibles, making a total of 18 defects. Three groups of six defects each were grafted with autogenous EC bone, autogenous IM bone, and fresh-frozen allogenic IM bone.

Surgical procedure

The surgical procedures are the same as we described previously.[10] In brief, after being anesthetized, nine rabbits underwent a central incision in hemicervical regions. A critical-size (10 × 15 mm) ostectomy, which did not interrupt the mandibular continuity, was created bilaterally in mandibular body. Two bone grafts were implanted in the mandibular defects bilaterally in each rabbit. Holes were drilled to allow for fixation of the bone grafts with stainless steel wires. In the allogenic bone graft group, the fresh-frozen allogenic IM bone was placed into the defect and fixed. In the autogenous EC and IM bone graft groups, IM bone was placed in one side, whereas EC bone in another side of mandible. Three months after surgery, the animals were sacrificed.

Histological preparation

The defect areas, including the surrounding tissues, were harvested for histological preparation. Tissues were decalcified and stained with periodic acid-Schiff (PAS) reaction technique. Enzyme histochemical staining for tartrate-resistant acid phosphatase (TRAP) was performed using a commercial kit (Sigma Ronkonkoma, NY).

Immunohistochemistry

Affinity-purified goat polyclonal anti–MMP-9 antibody C-20 (Santa Cruz Biotechnology, Inc, Calif) was used. Negative controls were obtained in parallel, avoiding the first antibody. Standard avidin-biotin complex (DAKO, Wiesentheid, Germany) methodology was applied, and antibody binding was observed using diaminobezidine (Sigma).

In situ hybridization

The murine MMP-9 complementary DNA (323 bp) was kindly provided by Dr D. Rice (Kings College, London, UK). The plasmids were linearized using appropriate restriction enzymes. The single-stranded antisense and sense probes were synthesized in the presence of digoxigenin-11-uridine-5"-triphosphate using SP6 RNA polymerase (Roche, Nutley, NJ). In situ hybridization was performed on wax

sections.[11] The hybridization temperature was 52°C, and the incubation time was 18 hours. Posthybridization wash was carried out under high-stringency conditions to remove unbound riboprobes. Localization of hybridized transcripts in the specimen was observed using alkaline phosphatase–conjugated antidigoxingenin Fab fragments (Roche) and NBT/BCIP (Roche) as the chromogen.

Quantitative analysis

Quantitative analysis of graft bone resorption represented by the percentage of graft bone resorption shown with PAS staining, and quantitative analysis of the areas of MMP-9 gene signal among the graft bone zone shown with immunohistochemistry and in situ hybridization, were carried out on serial sections of defects. The 10 × 15–mm defect was divided into five regions spaced 1500 μm apart.

From among 20 sections in each region, six sections were randomly taken, giving a total of 30 sections from each defect. These sections were stained with PAS, immunohistochemistry and in situ hybridization, respectively. Ungrafted autogenous IM and EC bone fragments and allogenic IM bone fragment for templates were processed in same serial sections for PAS staining and quantified for the amount of original graft bone. Thus, the percentage of graft bone resorption was generated from the difference of areas between ungrafted and grafted bone. Quantitative analysis was performed using a computer-assisted image analyzing system (Leica Q5501W) with Leica Q-win Pro.software (Version 2.2).

Statistical methods

Data were analyzed by statistical computer software (Graphpad Instat, V.2.04a). The one-way analysis of variance method was used to compare sections drawn from the five regions in each defect. The arithmetic mean, standard deviation , and 95% confidence intervals were calculated for each experimental group. The means of each group were analyzed with the Bonferroni multiple comparisons test. The critical level of statistical significance chosen was $P < .05$.

The size of the method error in digitizing the area of new bone was calculated by the formula $(\pm (\Sigma d^2/2n)^{1/2})$, where d was the difference between the two registrations of a pair and n was the number of double registrations. Ten sections were drawn at random and digitized. The method error of the image analysis did not exceed 0.029 mm^2, which was insignificant compared with the results. Hypothesis testing indicated no significant difference among the duplicate intraobserver ($P = .134$) and interobserver registrations ($P = .339$) of the 10 randomly drawn sections.

RESULTS Return to TOC

Immunolocalization of MMP-9

A little space of positive staining was observed in the allogenic IM bone group (Figure 1a). Intense positive staining was localized in the bone marrow within the graft bone in the autogenous IM bone group (Figure 1b). Numerous and intense positive staining was observed among the graft bone area in the autogenous EC bone group (Figure 1c). An adjacent section stained without MMP-9 primary antibody was used as a negative control (Figure 1d. Immunoreactive MMP-9 expression was mainly localized in the cytoplasm of multinucleated osteoclasts (Figure 1e) and mononuclear preosteoclasts (Figure 1f).

Expression of MMP-9 mRNA

Slightly blue signal of mRNA was localized on the surface of allogenic bone in allogenic IM bone group (Figure 2a). An adjacent section stained with TRAP recognized only mature osteoclasts involved in the bone-resorption sites (Figure 2b). Many positive signals of mRNA were shown among graft bone area in autogenous IM bone group (Figure 2c). Abundant and intensive purple/blue signals of mRNA were present around the surface of graft bone and in the bone marrow nearby in autogenous EC bone group (Figure 2d). Higher magnification showed that MMP-9 mRNA expression was mainly localized in the cytoplasm of active multinucleated osteoclasts and some mononuclear preosteoclasts in the marrow space (Figure 2e). All control sections hybridized with the sense probe were negative (Figure 2f). MMP-9 mRNA expression was associated with multinucleated osteoclasts covering the surface of graft bone and mononuclear preosteoclasts near the bone-resorbing compartment.

Quantitative analysis

Figure 3 shows a close correlation between the expression of MMP-9 and bone resorption. In the graph, the level of MMP-9 was directly correlated by graft bone resorption in three experimental groups for a total of 540 tissue sections, digitized in Leica Q-win system. The percentage of autogenous EC bone graft resorption was 138% more than that of autogenous IM bone (P ;lt .01) and 206% more than that of allogenic IM bone (P ;lt .01). The percentage of autogenous IM bone graft resorption was 28% more than that of allogenic IM bone ($P > .05$). The area of MMP-9–positive immunohistochemical staining in defects grafted with autogenous EC bone was 145% more than that with autogenous IM bone (P ;lt .01) and 222% more than that with allogenic IM bone (P ;lt .01). The area of positive immunohistochemical staining in defects grafted with autogenous IM bone was 31% more than that with allogenic IM bone ($P > .05$). The positive area of MMP-9 mRNA signals in defects grafted with autogenous EC bone was 151% more than that with autogenous IM bone (P ;lt .01) and 217% more than that with allogenic IM bone (P ;lt .01). The area of mRNA signals in defects grafted with autogenous IM bone was 27% more than that

with allogenic IM bone ($P > .05$).

DISCUSSION Return to TOC

Fresh autogenous bone of EC or IM origin is used clinically[8] as well as experimentally[12] to regenerate bone in osseous defects. However, clinical practice and literature concur that bone grafts from the calvaria and facial sites, which are IM bone, have a superior volumetric maintenance and survival over the grafts from rib, tibia, or iliac crest, which are EC bone.[13,14]

In this study, we confirmed the above results by revealing the different percentage of graft bone resorption between the two different origins of bone grafts when grafted into mandibular defects for 3 months. The percentage of autogenous EC bone graft resorption in defects grafted with was 138% more than that of autogenous IM bone ($P < .01$) (Figure 3).

Interestingly, the amount of MMP-9 expression in the grafted EC bone was found to be significantly higher than that expressed by IM bone grafts (protein 145% and mRNA 151%; $P < .01$) (Figure 3), pointing to a close correlation between graft resorption and levels of expression of MMP-9. It is well-known that expression of MMP-9 leads to bone resorption. It cleaves various components of the extracellular matrix, such as collagens IV and V, proteoglycans, elastin, and denatured collagen (gelatin).[6] It was concluded that MMP-9 is one of the major proteases constitutively produced by osteoclasts and may be involved in degradation of bone matrix proteins during osteoclastic bone resorption.[6] Therefore, the higher levels of expression of MMP-9 by the EC bone grafts 3 months after grafting could be a contributing factor to the loss of volume of bone grafts from EC origin over bone grafts from IM origin. Because EC bone grafts heal through the EC ossification route in which cartilage intermediate stage is formed first and later is replaced by bone,[15] it is conceivable that the level of expression of MMP-9 is maintained at a higher level 3 months after grafting because the cartilage matrix has to be vascularized to trigger the onset of EC ossification.

The invasion of new blood vessels requires removal of the cartilage matrix followed by migration of new blood vessels into the hypertrophic cartilage matrix.[16] These blood vessels deliver mesenchymal cells and osteoprogenitor cells, which engage in osteogenesis that later replaces the cartilage. This process requires the expression of MMP-9 for the breakdown of the matrix to allow for the invasion of the new blood vessels, and that could explain the high level of MMP-9 expressed by the grafted EC bone. Such a high level of MMP-9 eventually leads to graft bone resorption and that explains why EC bone grafts undergo more resorption.

On the other hand, IM bone grafts expressed much less MMP-9 three months after grafting (Figure 3), which could be because of the fact that IM bone grafts heal directly through bone bypassing a cartilage intermediate stage.[15] Also, the fact that IM bone grafts revascularize earlier than EC bone grafts.[17] Such higher levels of MMP-9 required for the breakdown of the matrix to allow earlier revascularization must have been reached soon after grafting. The levels expressed at 3 months after grafting in the IM bone graft could be lower. Furthermore, the levels of MMP-9 detected 3 months after grafting could be expressed by the osteoclasts for the purpose of integration of the grafted bone with the newly formed bone, which is a process that continues to occur until the grafted bone is replaced by new bone.

Allogenic IM bone grafts were found to undergo much less resorption than autogenous EC bone grafts (206%) and slightly less than autogenous IM bone grafts (28%). This is because of the fact that osteoclasts-expressing MMP-9 are absent from the allogenic bone (Figures 1a and 2a) but actively engaged in resorption in autogenous bone (Figures 1c and 2d). Because there are signs of resorption of the allogenic bone grafts, one must wonder in the absence of such osteoclasts in the allogenic bone, where the MMP-9 comes from and how such a process of resorption of allogenic bone is carried out? In this study, we found that mononuclear cells present in perivascular sites of new blood vessels recruited from the host tissues expressed MMP-9 (Figure 1f). This MMP-9 could be the factor needed for the resorption of the allogenic bone graft to allow for the invasion of new blood vessels into the grafted allogenic bone and help with its integration with the newly formed bone as well as the host bone.

The above-mentioned data clearly show that allogenic bone showed less resorption and subsequently better volume maintenance compared with autogenous EC bone grafts. These data have to be considered in light of the ability of the bone graft to integrate and amalgamate with the host bone. Without the required resorption for the blood vessels to invade the allogenic bone graft, the chances of integration are minimal. The reason is that the invading blood vessels recruited from the host bone carry the osteoprogenitor cells required for the process of osteogenesis. As these blood vessels invade the allogenic bone graft, they deliver the osteoprogenitor cells, which will differentiate into bone-making cells and start forming bone using the allogenic bone as a scaffold.[10] Although both IM autogenous and allogenic bone grafts show similar levels of resorption or volume maintenance, the autogenous bone graft was able to integrate and fuse with the host bone and participate in the process of osteogenesis through bone induction,[18] whereas the allogenic bone failed to resorb, which led to failure of blood vessel invasion within 3 months after grafting, and it only contributed to the process of osteogenesis through acting as a scaffold.

CONCLUSIONS Return to TOC

- MMP-9 plays an important role in graft bone resorption.
- Autogenous EC bone grafts expressed higher levels of MMP-9, leading to resorption and subsequently volume loss, whereas IM bone grafts expressed less MMP-9 and subsequently showed better volume maintenance.
- In defects in the oral cavity, such as alveolar clefts, cleft palate, or narrow alveolar ridge, it would be more appropriate to use bone grafts of IM origin whenever possible.

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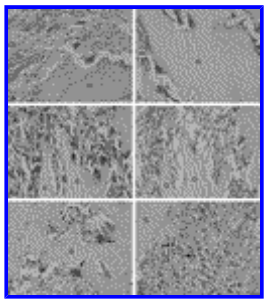
FIGURES Return to TOC



Click on thumbnail for full-sized image.

FIGURE 1. Immunohistochemical analysis of matrix metalloproteinase–9 (MMP-9) in the resorption pits of bone grafts. (a) A little space of brown staining on the surface of allogenic intramembranous (IM) bone graft. (b) Intense brown staining localized in the bone marrow within autogenous IM bone graft. (c) Numerous and intense brown staining among autogenous endochondral bone graft. (d) An adjacent section stained without MMP-9 primary antibody as negative control. (e) Immunoreactive MMP-9 expression was mainly localized in the cytoplasm of multinucleated osteoclasts (arrow) (f) and mononuclear preosteoclasts (arrow). G, graft bone. Note: regions expressing brown color are positive for MMP-9 expression

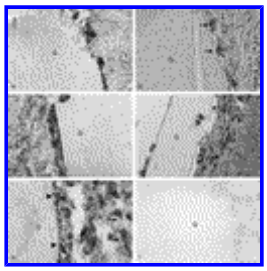


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FIGURE 2. In situ hybridization analysis for matrix metalloproteinase–9 (MMP-9) messenger RNA (mRNA) expression in the resorption of bone grafts. (a) Slightly blue signal of mRNA localized on the surface of allogenic bone graft. (b) An adjacent section stained with tartrate-resistant acid phosphatase recognized only mature osteoclasts (arrow) involved in the bone resorption sites. (c) Many blue signals of mRNA among autogenous intramembranous bone graft. (d) Abundant and intensive blue signals of mRNA around the surface of autogenous endochondral bone graft and in the bone marrow nearby. (e) Higher magnification showed that MMP-9 mRNA expression was mainly localized in the cytoplasm of active multinucleated osteoclasts (arrowhead) and some mononuclear preosteoclasts (arrow) in the marrow space. (f) The negative control section hybridized with the sense probe. G, graft bone. Scale bar: 10 μm. Note: regions expressing blue signal are positive for gene expression

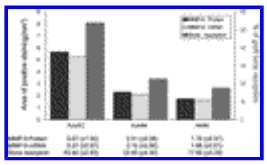

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FIGURE 3. Quantitative analysis of bone resorption and matrix metalloproteinase–9 expression (protein and messenger RNA) in the resorption of three different bone grafts

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