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Original paper

Osteoclast recruitment and polymorphism during the healing process in dental implant surgery

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Abstract

Objectives: To demonstrate the role of plasma rich in growth factors (PRGF) in osteoclast recruitment and function during bone remodeling and healing of alveolar crest bone defect in dental implant surgery.

Method and materials: The histological examination was performed on bone carrots harvested after the implant surgery, in a patient with advanced alveolar bone atrophy in the posterior maxilla. Sinus floor elevation and bone augmentation were performed, associated with local application of PRGF. The samples were histologically examined using the Goldner's trichrome method.

Results: Microscopically, the particular aspects of new bone formation and remodeling were revealed: polymorphic bone trabeculae characterized by the association of woven, lamellar and necrotic bone. A prominent feature was the presence of numerous osteoclasts and osteoclast precursors on the surface of necrotic bone fragments, suggesting the intense bone resorption. Fewer macrophages were found on the bone trabeculae consisting of woven and lamellar bone, where remodeling was moderate.

Conclusions: Bone remodeling and healing at the site of implant surgery induced new bone formation and bone resorption by osteoclasts. Osteoclasts exhibited significant polymorphism and were associated with numerous osteoclast precursors. Local application of PRGF could influence bone remodeling by maintaining the balance between bone resorption and bone formation.

Keywords : alveolar crest bone defect, bone remodeling, osteoclasts, osteoclast precursors, plasma rich in growth factors PRGF.

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Introduction

Nowadays, the use of dental implants is a common practice as tooth loss is a frequent problem occurring as a result of disease or trauma (POPA & al [1]). Dental implant surgery often requires grafting procedures in order to increase the bone quantity and quality at the recipient bed and to promote the optimal implant osseointegration and stability (F.S. SALMEN & al. [2]).

In dental implant surgery, free autologous bone grafts are the most widely used (N. VENKATARAMAN & al [3]). A frequent complication that occurs after autologous grafting is necrosis by insufficient blood supply (I.H. KALFAS [4]). In order to minimize graft necrosis and to improve the healing and integration process, several methods have been proposed, including the use of free vascularized or pedicle grafts (G.K.B. SÀNDOR & al. [5]). New biotechnologies, consisting in the association of bone grafts with various preparations such as platelet-rich plasma (PRP) and plasma rich in growth factors (PRGF) were proposed for stimulating and accelerating the healing process (E. ANITUA [6], F. MOLINA-MIÑANO & al [7]).

After the surgical trauma, a hemorrhage is formed around the graft; the mediators released from the recipient bed and from the blood act as chemo-attractants for the mesenchymal stem cells, inflammatory cells, and phagocytes. Depending on the local stimuli, the mesenchymal cells proliferate and differentiate into endothelial cells, fibroblasts or osteoblasts, resulting in the formation of blood vessels, connective tissue and bone; on the other hand, inflammatory cells and phagocytes play a role in graft incorporation, bone resorption, and the resulting remodeling process (I.H. KALFAS [4]).

Therefore, remodeling implicates not only the bone forming osteoblasts, but also the active osteoclasts that progressively eliminate the primary woven bone and allow the deposition of the bone lamellae leading to secondary lamellar bone formation during the healing process (VENKATARAMAN & al [3], I.H. KALFAS [4], S.N. KHAN & al. [8]).

The key cellular elements are the osteoclasts, multinucleated cells capable of bone resorption and essentially implicated in bone tissue homeostasis and remodeling (T. MARTIN & al. [9]).

Osteoclast formation and functions are regulated by systemic mediators such as: parathyroid hormone, Interleukin 1 (IL-1), tumor necrosis factor (TNF), transforming growth factor (TGF), monocyte colony stimulating factor (M-CSF), and 1,25-dihydroxyvitamin D3 (S.A. HIENZ & al. [10]); additionally, there are several cytokines released in the local microenvironment by the stromal cells in the bone marrow that control the osteoclastogenesis: interleukins, TNF-α, TGF-β, kinins and thrombin (R GRUBER [11]); the osteoclast is also capable to secrete cytokines that regulate its function (M.P. YAVROPOULOU & al. [12], T.J. MARTIN [13]).

From their formation site (bone marrow), mononucleated precursors circulate in the blood stream and upon arrival to the target site, they fuse to form the multinucleated osteoclast; alternatively, the mononuclear precursors may fuse with the local osteoclasts or even pre-existing multinucleated osteoclasts may fuse to form very large cells (T.J. MARTIN [13], G.R. MUNDY & G.D. ROODMAN [14], A.J. KAHN & D.J. SIMMONS [15], D.G. WALKER [16], G. GOTHLIN & J.L.E. ERICSSON [17], P.J. NIJWEIDE & al. [18]).

Numerous animal and clinical studies have investigated the effect of PRP and PRGF in combination with autologous bone and other types of graft materials, taking into consideration the following criteria: bone radiographic density, morphometric parameters of bone tissue, and the rapidity of healing. However, the results were controversial: Marx et al. reported increased bone density after using PRP with autologous bone for mandibular reconstructions, but Aghaloo et al. did not find significant radiographic or morphometric differences upon using PRP in bone lesions in rabbits (R.E. MARX & al. [19], T.L. AGHALOO & al. [20]). Molina et al. reported a higher amount of neo-formed bone in defects filled with autologous bone plus PRGF, but without significant differences compared with the controls. In vitro investigations confirmed platelet contribution in osteoclastogenesis and osseous resorption, but an exact mechanism is yet to be proposed (F. MOLINA-MIÑANO & al [7], C. FIORAVANTI & al. [21]).

Aim: Our study aimed at demonstrating the role of PRGF in bone remodeling by investigating the histological features during healing of alveolar crest bone defect.

Materials and Methods

The histological study was performed on bone carrots harvested from the alveolar crest 17 month after the initiation of the implant surgery, in a patient with advanced alveolar bone atrophy in the left posterior maxilla. The patient was clinically healthy and had no co-morbidities. Written and informed consent of the patient was obtained for tissue harvesting and histological examination.

Firstly, the sinus floor elevation and bone augmentation were performed, using autologous bone and bovine xenograft associated with PRGF; implants were inserted 6 months later. The outcome was not favorable due to an infectious complication involving left maxillary sinusitis and vestibular abscess after 3 months. Therefore, the implants and the necrotic infected bone were removed; the sinus was curetted and grafted with blood clot, fibrin clot and PRGF. The new implants were inserted 8 months later.

The harvested bone carrots were processed for histological examination; firstly, it was fixed in 10% buffered formalin for 7 days; then the sample was decalcified with 5% trichloroacetic acid, dehydrated with graded ethanol solutions (70°, 95°, absolute), clarified with n-buthanol and embedded in paraffin; finally, the tissue was sectioned at 5 µm and stained using the Goldner's trichrome technique. The slides were examined under an Olympus BX41 light microscope; images were taken with a digital camera and processed with Adobe Photoshop CS2 software.

Results and Discussions

The histological examination revealed characteristic features of new bone formation and remodeling; the new bone occupied the entire intervention area and displayed various morphological features, according to the location and the stage of development.

In the superficial area, woven bone had a dense structure, corresponding to the cortical bone on the surface of the alveolar crest. In the profound areas, the new bone had the structure similar to cancellous bone, with trabeculae of various shape and size delimiting areoles (Fig. 1a, 1b, 2a). In the areoles, next to the trabeculae, there was zonal chronic inflammatory infiltrate containing mononuclear cells, without tendency of acutization (Fig. 1a, 1b). The most significant feature was the polymorphism of the trabeculae due to the coexistence of woven bone, lamellar bone, as well as necrotic bone (Fig. 1a, 1b, 2a).

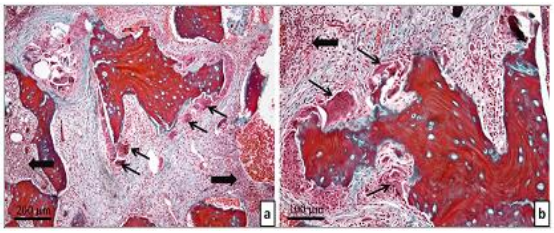

Figure 1. Bone trabeculae in various stages of remodeling. (a) and (b) trabeculae consisting of woven bone and lamellar bone, surrounded by osteoclasts (thin arrows); in the areoles, chronic inflammatory infiltrate associated with the trabeculae (thick arrows).

It could be easily estimated that the new bone had been initially primary woven bone, which still composed the main mass of the trabeculae, but by remodeling, it has been progressively replaced by secondary lamellar bone. However, in some areas necrotic woven bone could be identified; it was either associated with larger trabeculae (Fig. 2a), or formed isolated smaller fragments (Fig. 2b, 3b).

Larger bone trabeculae consisted of woven bone and lamellar bone in various proportions. Primary woven bone contained a large number of isodiametric osteocytes and lower mineral content in the matrix; lamellar bone contained irregularly arranged bone lamellae, and fewer flattened osteocytes embedded into the calcified matrix; small osteoblasts and the osteoid layer bordered the trabeculae (Fig. 2a). Bone resorption was moderate, and isolated osteoclasts or few osteoclast precursors were identified on the surface of the trabeculae (Fig. 1a, 1b).

Smaller bone fragments exhibited various degrees of necrosis and/or resorption; (Fig. 2b, 3b, 3c). Necrotic bone had fissures detaching smaller fragments from the main trabeculla; it contained dischromic bone matrix and degenerating osteocytes or empty osteocytic lacunae; osteoblasts were absent on the surface (Fig. 2b, 3b). Isolated or grouped giant multinucleated cells (osteoclasts) and numerous osteoclast precursors were present in the vicinity of these fragments (Fig. 2b, 3b, 3c).

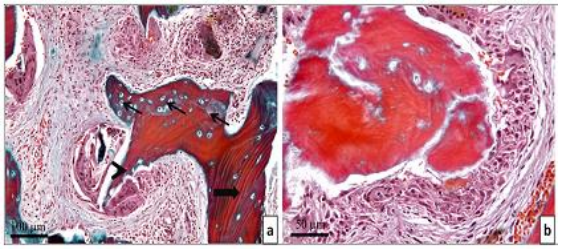

Figure 2. Bone trabecullae in various stages of necrosis and remodeling. (a) Bone trabeculla consisting of woven bone (thin arrows), lamellar bone (thick arrow) and necrotic bone covered by large osteoclasts (arrowhead) (b) Necrotic woven bone associated with the larger trabecula and surrounded by osteoclasts and osteoclast precursors.

Osteoclasts exhibited significant polymorphism regarding the shape, size, aspect of the cytoplasm and number of nuclei (Fig. 3). Osteoclasts' morphology seemed to be related to the formation stage and the functional state. Cells in early stages were smaller and contained three to six nuclei; these osteoclasts were either isolated in the areoles, or attached to the surface of small bone fragments (Fig. 3a). Mature active osteoclasts were larger and contained numerous nuclei, according to their size; their number depended on the type of bone tissue they were associated with. Active osteoclasts were fewer and rested in deep resorption lacunae on the surface of woven bone, and practically absent on the lamellar bone (Fig. 3b); by contrast, active osteoclasts were very numerous and completely surrounded smaller necrotic bone fragments, suggesting that the bone resorption was highly active (Fig. 2b, 3c). Resting and degenerating osteoclasts showed signs of apoptosis: condensed or vacuolated cytoplasm and shrunk irregular nuclei; these cells were in the proximity of intensely resorbed bone fragments or debris (fig. 3d).

Both early and active osteoclasts were associated with precursor cells that showed obvious tendency to fuse with one another (Fig. 2a, 3b). The osteoclast precursors were irregularly shaped, with many cytoplasmic processes, contained acidophilic cytoplasm and a single, round, euchromatic nucleus. The precursors located in the proximity and around bone fragments were recruited in variable number, according to the intensity of the bone resorption (Fig. 2a, 3b). Very numerous osteoclast precursors and active osteoclasts were associated with grouped small bone fragments, remnants of larger necrotic trabeculae that were intensely resorbed (Fig. 3c, 3d). In moderate numbers, mononuclear osteoclast precursors and osteoclasts were present inside the fibrous capsule organized around the small necrotic bone fragments (Fig. 4a, 4b).

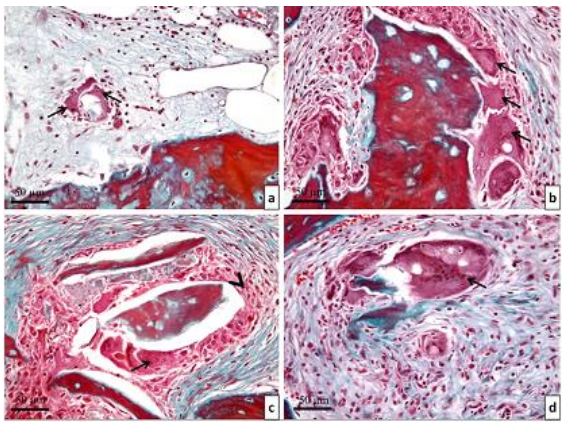

Figure 3. Polymorphic osteoclasts and osteoclast precursors. (a). Small osteoclasts isolated in the connective tissue within the areoles (arrows); (b). Active osteoclasts on the surface of necrotic bone with multiple euchromatic nuclei and abundant acidophilic cytoplasm (arrows); (c). Active osteoclasts (arrows) and numerous precursors with increased tendency to fuse (arrowhead). (d). Apoptotic osteoclasts with pyknotic nuclei and dark cytoplasm (arrow). (Goldner's trichrome)

Necrotic bone fragments were completely sequestered by osteoclasts and numerous precursor cells; at the periphery, a connective tissue capsule was formed, thus resulting in the organization of a structure similar to an encapsulated granuloma. (Fig. 4a, 4 b)

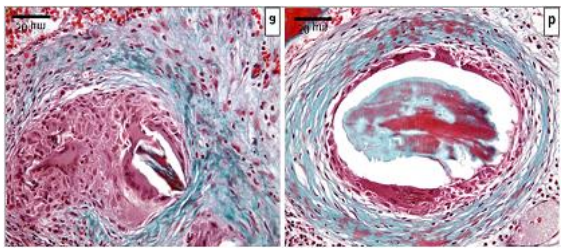

Figure 4. Encapsulated granulomas. (a) The connective tissue in the proximity of the necrotic bone showing tendency to condense and to enclose numerous osteoclasts and osteoclast precursors; (b) A thick capsule completely isolating the necrotic bone fragment and the osteoclasts on the bone surface (Goldner's trichrome)

Any surgical intervention in bone induces a complex tissue response due to the release of cytokines

such as: bone morphogenetic protein 2 (BMP-2), platelet derived growth factors (PDGF), TGF and vascular endothelial growth factor (VEGF), that activate osteogenesis and promote the regeneration of the traumatized bone (D. GERARD & al. [22], G. WEIBRICH & al. [23]). The healing process occurs in two steps: the initial woven bone is rapidly formed by osteoblasts to fill in the defect; but this immature bone has a low mineral content and a disorganized structure, thus its biomechanical properties are poor. In the next step, the woven bone is replaced by mature lamellar bone, due to coupled osteoclastic-osteoblastic activity – the remodeling process. The repair process will proceed until all the bone defect is filled with lamellar bone (N. VENKATARAMAN & al [3]).

Frequently, however, bone healing may be incomplete or delayed, depending on the influence of factors such as: nutrition (blood supply), mechanical forces (pressure or instability), and competition from the surrounding tissues (the ingrowth of the soft tissue in the bone defect) (F.S. SALMEN & al. [2], I.H. KALFAS [4], G.K.B. SÀNDOR & al. [5]).

Besides these factors, some other particular aspects are significant for healing of an autologous bone graft. When introducing a graft in a bone defect, the healing process may be influenced by the graft itself in two ways: passively, because the graft acts as a mechanical barrier, and actively, by the interaction between the graft surface and the molecules released from the graft and the biological environment. For example, an autologous graft may enhance the healing process due to the presence of the viable cells and bone inductive agents in the graft, while an allograft could have a negative effect, by eliciting an immunological reaction (F.S. SALMEN & al. [2], R.A. HOROWITZ & al. [24]). Moreover, even though the vascular supply at the recipient bed is a prerequisite, the survival of the osteocytes present within the bone graft seems to greatly influence the outcome, since these cells may be implicated in the repair process (N. VENKATARAMAN & al. [3]).

The repair process is different for the compact bone which consists of densely packed concentric bone lamellae, compared with the cancellous bone, which is porous and formed by trabeculae enclosing the marrow. Due to the medullary component, the bone graft in the spongy bone is in close relationship with numerous cells and blood vessels in the bone marrow, and thus the healing occurs more rapidly than in the compact bone (T. BAUER [25]).

However, in our case, the healing process was heterogeneous, and the evolution depended on the vascularization. In zones with deficient blood supply, the woven bone underwent necrosis and elicited intense osteoclastic activity. In bone fragments consisting of necrotic bone matrix and degenerating osteocytes, no osteoblasts were present on the surface and osteoid formation was not initiated. Instead, the necrotic bone had a chemotaxis effect and induced the massive recruitment of osteoclast precursors and their differentiation into active osteoclasts, leading to intense bone resorption. By contrast, in zones with appropriate nutrition, the remodeling of the woven bone into lamellar bone was favorable; trabeculae consisted of an association of woven and lamellar bone, and the bone tissue underwent remodeling by balanced apposition and resorption.

These particular aspects explain the presence of both necrotic bone fragments and trabeculae consisting of newly formed woven and lamellar bone.

In our case, the healing process was favorable because in cancellous bone, that occupied most of the bone defect, regeneration is sustained by several local mechanisms. Due to the increased surface, more mesenchymal cells populate the bone surface and differentiate into osteoblasts (M. MOGA & al. [26]); the osteocytes in the bone matrix are closer to the periphery and their nutrition is better, and the vascular ingrowth was demonstrated to occur 30% faster than in compact bone. Osteoblasts lining the surface secrete osteoid which mineralizes to form the immature bone. In the final remodeling stage, the immature newly formed bone and the necrotic bone are resorbed by osteoclasts and replaced by mature lamellar bone; with time, the cancellous bone fills the entire bone defect, and the healing is complete (I.H. KALFAS [4], S.N. KHAN & al. [8], A.J. KAHN & D.J. SIMMONS [15], S.S. JENSEN & al. [27]).

Bone remodeling is essential for tissue homeostasis and regeneration of bone defects. Osteoclasts function and their differentiation from precursors play a key role in bone remodeling due to

their resorptive activity. Osteoclast precursors derive from the mononuclear hemopoietic cells: granulocyte/ macrophage progenitor cells that give rise to granulocyte and monocyte cell lineages (T.J. MARTIN [13], T. NAGASAWA & al. [28]). Monocytes are the common origin of several other types of multinucleated cells that have phagocytic activity, including Langhans giant cells, Touton giant cells and multinucleated giant cells in epulis (W.G. BRODBECK & al. [29], A.B. BOȘCA & al. [30]).

Osteoclast formation is a complex process encompassing several stages: proliferation and recruitment of progenitor cells; differentiation of progenitor cells; fusion of the mononuclear osteoclast precursors in order to form adult osteoclasts (T.J. MARTIN [31]).

Osteoclast differentiation and maturation is further regulated by the RANK-RANKL signaling mechanism. RANK (Receptor activator of nuclear factor kappa B) is a receptor molecule expressed on the surface of the osteoclasts that interacts with RANK ligand molecule (RANKL) produced and expressed on the stromal cells and osteoblasts' surface. Alternatively, immunocompetent cells, such as activated T lymphocytes can secrete RANKL molecules; thus, during inflammation, the osteoclast-mediated bone resorption is initiated (S.A. HIENZ & al. [10], T. NAGASAWA & al. [28], B.F. BOYCE & L. XING [31]).

In the bone tissue, the osteoclast binds to the bone surface by a mechanism that implicates the integrin αvβ3, the dominant osteoclast integrin and the marker of the osteoclast phenotype. This integrin is not present in macrophage precursors, but is induced in osteoclasts by RANKL. Integrin αvβ3 enables the osteoclast to recognize a tripeptide sequence (Arg-Gly-Asp) that is present in the macromolecules in the bone matrix: osteopontin, fibronectin, vitronectin and fibrinogen (W.G. BRODBECK & J.M. ANDERSON [29]).

In the present case, the local mediators were supplemented with growth factors present in the PRGF: TGF-β1, VEGF (Vascular endothelial growth factor) and IGF (Insulin-like growth factor); these proteins modulate cell proliferation, differentiation and migration (chemotaxis) (F. MOLINA-MIÑANO & al [7], R. FARINA & al. [32]). PRGF has the advantage of being an autologous product, it is a source of multiple growth factors and it is reabsorbed in several days after initiating local regeneration (E. ANITUA & al. [33]). Addition of growth factors such as TGF-β and IGF to the autologous graft stimulates the differentiation of cell lines implicated in bone regeneration and of the woven bone to lamellar bone (N. VENKATARAMAN & al [3], K.M. LACCI & A. DARDIK [34], J. ALSOUSOU & al. [35]).

Our results indicate that the recruitment of osteoclast precursors was induced by two mechanisms: the direct action of the chemotactic factors in PRGF and the indirect stimulation of osteoclasts, which secrete cytokines that control the migration and differentiation of the precursors. Moreover, the presence of numerous osteoclasts suggests an intense osteolytic activity necessary for removal of necrotic bone and the remodeling of woven bone in the intervention area.

The large number of mononucleated osteoclast precursors associated with multinucleated osteoclasts is explained by the life cycle of the osteoclasts. In vivo osteoclasts have an up to 2-week lifespan and a half time of 6 to 10 days (C. GRAY & al. [36]); in order to achieve an appropriate bone resorption, osteoclasts have to be renewed constantly.

Osteoclast precursors recruited in the proximity of the necrotic bone fragments formed groups close to active osteoclasts, suggesting that they were differentiating and fusing to form new osteoclasts, thus promoting the bone resorption and remodeling. A particular aspect was the presence of osteoclast precursors and active osteoclasts inside the granulomas sequestering necrotic bone fragments.

Conclusions

PRGF controls the resorption of the necrotic bone by osteoclasts, and new bone formation associated with bone trabeculae of whose viability was preserved.

The osteoclasts polymorphism is consistent with the formation stage and the functional state; moreover, their association with numerous osteoclast precursors is correlated with the intensity of bone remodeling.

Therefore, the association of osteoinductive agents, such as PRGF, sustains the remodeling process by maintaining the balance between bone resorption and bone formation.

The outcome of bone healing depends on the extent to which the circumstances in the environment

in the bone defect allow the new bone formation and remodeling in order to promote regeneration.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article, and there was no financial support that could have influenced the outcomes. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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All authors have equal contribution to this article.

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