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Regeneration with Biomaterial Bone Substitute Combined with Autogenous PRP, Bone and β-TCP

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Histological and immunohistochemical findings are presented from two patients using composite biomaterial graft in combination with autogenous cancellous bone, platelet-rich plasma (PRP) and β-tricalcium phosphate (β-TCP). A composite biomaterial graft was constructed and implants were installed using a staged approach into a 59-year-old man with a large alveolar bone defect in the right mandibular first molar (Patient 1) and a 51-year-old woman with an upper left horizontal impacted canine (Patient 2). Six months later, core biopsies of the grafted areas from both patients were histologically and immunohistochemically stained for osteocalcin, platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1) and CD68.

Wound healing and the handling of graft material are improved by PRP. The histological evidence demonstrated that new bone was generated in both patients. Neither inflammatory cells nor necrosis were evident in the graft areas. Osteoid with osteoblasts and osseous granulation tissue were mainly evident in Patient 1, but mature lamina bone had generated in Patient 2, suggesting that bone regeneration depends on the type of defect and condition of the blood supply. Immunohistochemically, osteocalcin and TGF- β 1 were localized in the cells (osteoblasts) around osteoid and PDGF was detected in the osseous granulation tissues of Patient 1. To some extent, resolved β-TCP particles were surrounded by CD68 positive macrophages.

Key words : PRP, β-TCP, PDGF, TGF-β1, Osteocalcin, CD68

INTRODUCTION

Among the many types of biomaterials used for bone grafting, autogenous cancellous bone is considered to be the best, as it provides scaffolding for osteoconduction, growth factors for osteoinduction and progenitor stem cells for osteogenesis[1,2]. However, availability is limited by procurement morbidity and high cost, presenting significant disadvantages.

Allografts and xenografts lack the osteogenetic properties of autogenous bone, and they introduce potential for both transferring disease and triggering a host immune response[3]. Synthetic bone grafts such as hydroxyapatite or tricalcium phosphate, while good platforms for osteoconduction, lack intrinsic properties of osteoinduction and osteogenesis[4,5]. Whereas second-generation biomaterials were designed to be either resorbable or bioactive, the next generation combines these properties to develop materials that once implanted, will help the body heal itself[5].

A composite graft that combines a synthetic scaffold with autogenous osteoprogenitor cells and growth factors in a procedure with low morbidity, could potentially deliver the benefits of autogenous bone grafts without the disadvantages. Preclinical studies support the concept that platelets possess growth factors that stimulate and enhance wound healing, including osseous regeneration[5~16]. Marx et al.[7] have shown a 40% decrease in the time taken for autogenous bone grafts to heal when platelet-rich plasma (PRP) is incorporated into the site. Their results, together with the current case series, suggest that PRP may allow earlier implant placement and loading. They also reported that a monoclonal anti-

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body assessment of cancellous cellular marrow grafts revealed cells that can respond to growth factors through membrane receptors. The additional amounts of growth factors obtained by adding platelet-rich plasma to grafts increased the radiographic maturation rate by 1.62- to 2.16- fold that of grafts without PRP[9]. Histomorphometry also showed that bone density was greater in grafts with than without PRP. The clinical outcomes following the incorporation of PRP gel into ablative surgical procedures of the maxillofacial region, mandibular reconstruction, repair of alveolar clefts and implant placement have been favorable. Platelets produce and release many growth and differentiation factors that are critical for the stimulation and regulation of wound healing, including platelet derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1)[10], vascular endothelial growth factor (VEGF) and insulin-like growth factor (I-LGF).[8]

The present study immunohistochemically evaluates the effects on bone regeneration of a composite biomaterial substitute consisting of autogenous cancellous bone, β-TCP and autogenous PRP in human alveolar bone.

MATERIALS AND METHODS

Bone grafts

The composite biomaterial graft consisted of a mixture of autogenous cancellous bone, 500-1000 mesh porous β-tricalcium phoshate (β-TCP) particles (Bioresorb, Oraltronics) and autogenous PRP (0.5 g/0.4 ml). Cancellous bone was collected from the implant preparation of two patients : one was a 59-year old man who presented with severe chronic periodontitis and the other was a 51-year-old woman who had lost the #11 tooth before operation. Autogenous PRP and thrombin were concentrated by ultracentrifugation using a Labofuge 300 (HERAUS) and a disposable syringe system 30 minutes before surgery to minimize risk of disease transmission and contamination. Both the patients were contended to be the subjects and that data obtained with their bone and PRP would be presented in public in the Journal. The composite biomaterial was implanted using a staged approach.

Preparation of bone regeneration tissue

Core biopsies obtained 6 months after surgery by a modification of the implant site preparation protocol using trephine, were histologically evaluated. Bone fragments were immediately fixed in 3.7% paraformaldehyde-0.1 M phosphate buffer (PB ; pH 7.4) for 24 h at room temperature. After fixation and dehydration through a graded series of ethanol solutions, the fragments were embedded in paraffin. Serial sections (4 μm) were then cut and mounted on glass slides.

Light and Immunohistochemical microscopy

Osteocalcin, PDGF, TGF-β1 and cluster differentiation (CD) 68 were visualized by immunohistochemical staining[16]. The primary antibodies were rabbit polyclonal antiserum against PDGF, TGF-β1 (Santa Cruz Biotechnology. Inc. CA. USA), as well as mouse monoclonal antibodies to osteocalcin (Takara Biomedicals, Otsu, Japan) and to human macrophage lysosome membrane (CD 68) (DAKO A/S, Glostrup, Denmark). Secondary antibodies were included in an indirect peroxidase polymer labeled immunostaining system (ENVISION+, DAKO, Kyoto, Japan).

Immunostaining

Specimens were deparaffinized in xylene and rehydrated through a descending series of ethanol solutions, stained with hematoxylin and eosin, and then immunohistochemically stained[16]. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes followed by 1% bovine serum albumin (BSA) for 30 min, then the sections were incubated with the primary antibodies for 2 h at room temperature. Sequential incubations for 30 min with peroxidase-labeled ENVISION + goat anti-rabbit IgG were followed by color development in 1.0 mg/ml 3'-diaminobenzidine (DAKO, Kyoto, Japan) in phosphate buffered saline (PBS ; pH 7.4) containing 0.3% hydrogen peroxide for 2-5 minutes. The sections were then counterstained with hematoxylin and dehydrated through an ascending series of ethanol solutions and xylene. All primary antibodies were diluted in 10 mM PBS containing 0.1% BSA and each step was followed by a thorough rinse with PBS. The primary antibody was replaced with non-immune rabbit/mouse IgG in negative control sections. The histological findings were analyzed by mi-

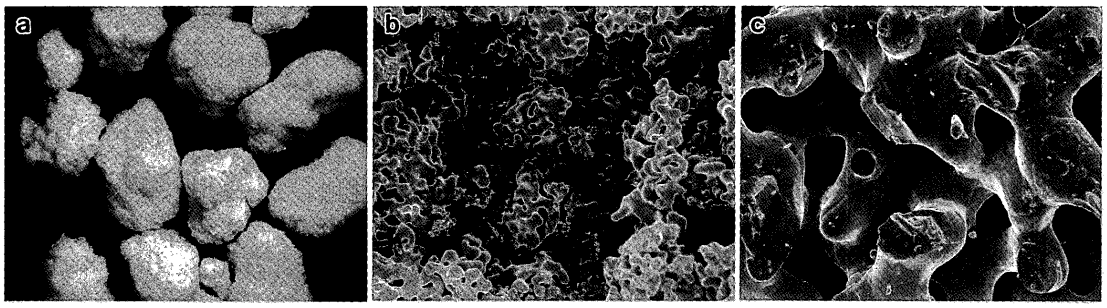

Fig. 1 Photomicrograph (a) of and SEM images of β-TCP at lower (b) and higher (c) magnifications.

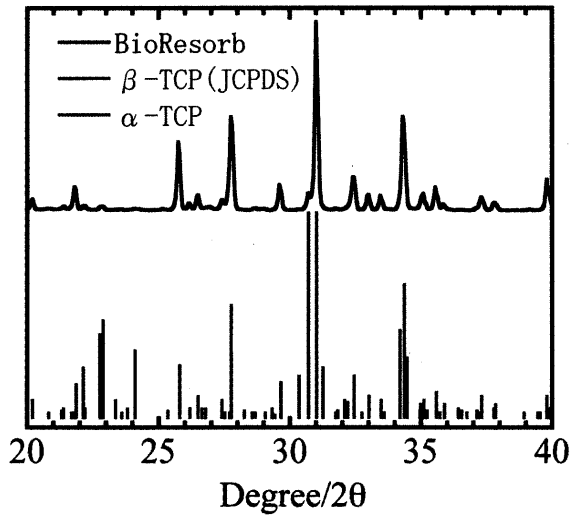

Fig. 2 X-ray diffraction pattern of β-TCP. Note that some α-TCP is present in addition to β-TCP.

croscopy (Nikon ECLIPSE E1000, Nikon, Tokyo, Japan).

RESULTS

Porous calcium phosphate material

Figure 1 shows a photomicrograph (Fig. 1a) and SEM images (Fig. 1b and c) of β-TCP. In the SEM images, interconnecting pores ranging in size from 20 to 50 μm are evident between microsections. The surfaces of the particles, however, are relatively very smooth. Figure 2 shows an X-ray diffraction pattern of β-TCP. The X-ray diffraction pattern suggested that some α-TCP was present in addition to β-TCP.

Case 1

A 59-year-old man presented with severe chronic periodontitis and a large alveolar bone defect in the

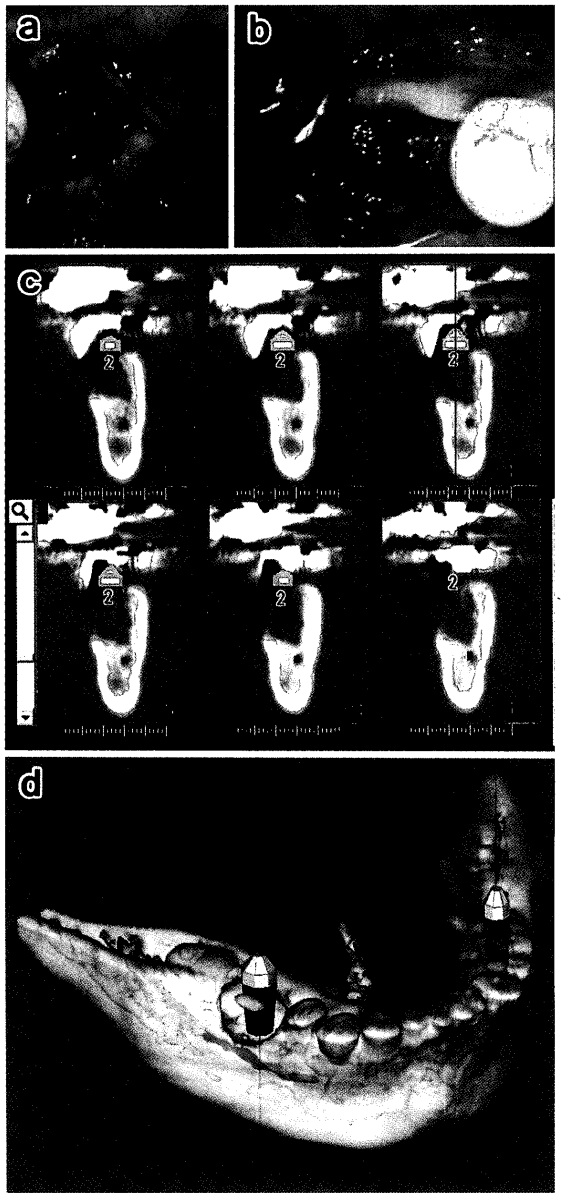

Fig. 3 Photographs before operation (a) and 6 months after operation (b), CT (c) and 3D (d) images of the defect from Patient 1 before operation. Severe chronic periodontitis and a large alveolar defect are evident in the right mandibular first molar.

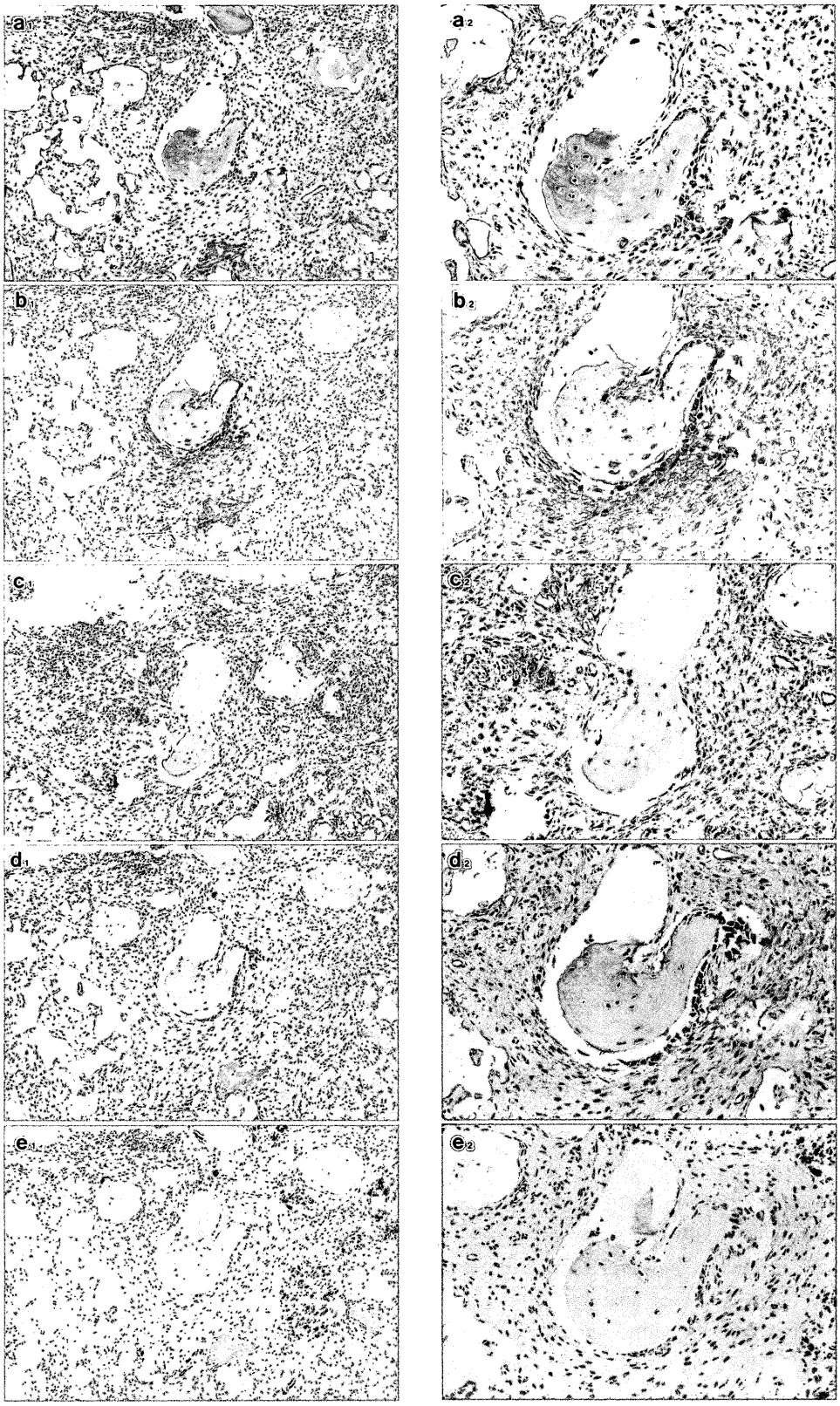

Fig. 4 HE stained specimen (original magnification ×10 (a_1) and 25 (a_2)), osteocalcin positive tissue specimen (original magnification ×10 (b_1) and 25 (b_2)), PDGF positive tissue specimen (original magnification ×10 (c_1) and 25(c_2)), TGF-β positive tissue specimen (original magnification ×10 (d_1) and 25(d_2)) and CD68 positive tissue specimen of the defect from Patient 1 (original magnification ×10 (e_1) and 25 (e_2)). In Fig. 4a, osteocalcin is immunohistochemically localized in osteoblast and in cells around osteoid.

right mandibular first molar (Fig. 3). A histological analysis indicated no evidence of inflammatory cells and necrosis in the graft area. Bone that had regenerated in the graft area was mainly osteoid (Fig. 4a). Osteocalcin (Fig. 4b) and TGF-β1 (Fig. 4d) were immunohistochemically localized in osteocytes and in cells around osteoid. In addition, PDGF was also stained in the osseous granulation tissue and spindle fibrous tissues but not in regenerated bone and osteoid (Fig. 4c). Cells were CD68 positive in osseous granulation tissue especially around small, but rarely located around larger β-TCP particles (Fig. 4e).

Case 2

A 51-year-old woman had lost the #11 tooth. The #23 tooth was horizontally impacted and the root of #21 was absorbed. The #21 and #23 teeth were extracted and the biomaterial was grafted onto the one wall defect. (Fig. 5). Histological analyses found no evidence of inflammatory cells and necrosis in the graft area. Bone had regenerated in the graft area, lamina bone was defective and no β-TCP particles remained (Fig. 6a). Osteocalcin (Fig. 6b) and TGF-β1 (Fig. 6c) tissues were positive in the regenerated bone.

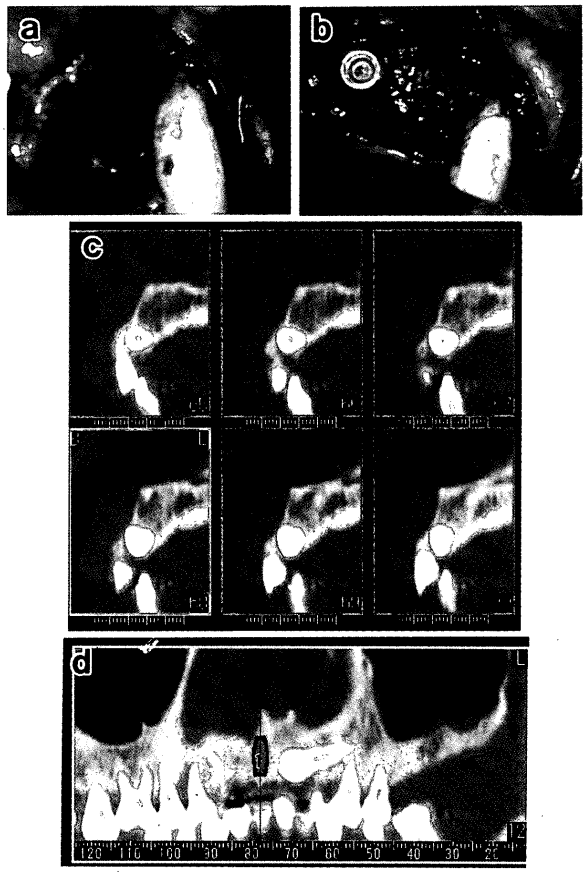

Fig. 5 Photographs before operation (a) and 6 months after operation (b), CT image (c) and panoramic view (d) of the defect from Patient 2.

DISCUSSION

Patient morbidity during graft procurement is eliminated and disease transmission and immunogenic reactions are no longer a concern when grafting syn-

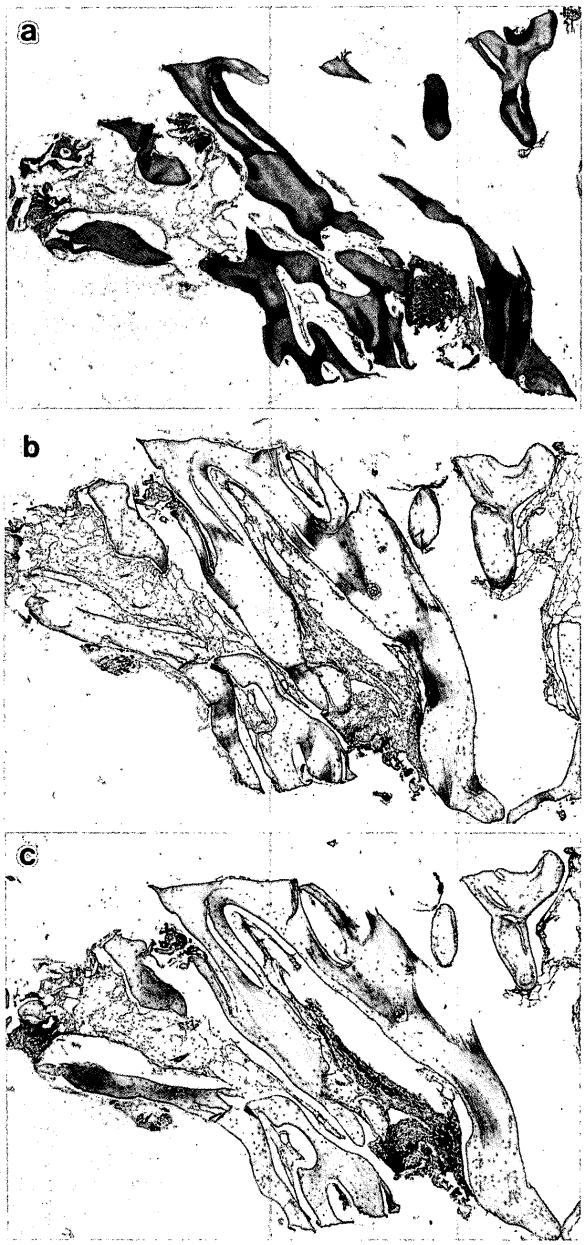

Fig. 6 HE stained specimen (a, original magnification ×10), osteocalcin positive tissue specimen (b, original magnification ×10) and TGF-β positive tissue specimen of the defect from Patient 2 (c, original magnification ×10)

thetic biomaterials[4~6,11]. Synthetic bone grafts such as β-TCP, while good platforms for osteoconduction, lack any intrinsic properties of osteoinduction and osteogenesis[4,6]. Autogenous cancellous bone from the implant site was used for osteogenesis and PRP[12~16] included for osteoinduction in the present study. Although β-TCP has interconnection porosity, the particle surface appeared smooth in SEM images. Interconnection may be an advantage in holding PRP, but smoothness may render PRP retention difficult, thus failing to slowly and continually release PRP.

New bone had formed in both patients by 6 months after surgery, although it consisted mainly of osteoid and osseous granulation tissue in Patient 1 (Fig. 4) and mainly mature lamina bone in Patient 2 (Fig. 6). Regeneration was obviously faster in Patient 2 than in Patient 1, indicating that the rate of regeneration seems to depend on the type of defect, with respect to the blood supply for example. When the implant is loaded, the healing period should be considered according to the conditions of the graft site.

The immunohistochemical findings in the graft area of Patient 1 revealed osteoblasts producing osteocalcin and TGF-β1 (Fig 4a and d),indicating early bone regeneration and osseous granular tissue and spindle fibroblast cells expressed PDGF. Since growth factors derived from platelets directly influence wounds for less than 5 days, the PDGF found in Patient 1 (Fig. 4c) was probably derived from blood or fibroblasts[10]. Six months after surgery, β-TCP particles remained at the graft site of Patient 1 (Fig. 4a). The immunohistochemical findings demonstrated PDGF osseous granulation tissue and spindle fibroblast cells. Monoclonal antibody (CD68) staining revealed macrophages only around smaller β-TCP particles (Fig. 4e), suggesting that the PDGF in the granulation tissue was derived from platelets, and not from macrophages. The β-TCP particles were suspended in the extracellular fluid and eventually disintegrated into smaller particles. Phagocytosis by macrophages preferentially occurred at smaller β-TCP particles that remained and seemed to function as a scaffold.

Platelet-rich plasma includes PDGF, the TGF-β family, VEGF and I-LGF. These factors contribute to wound healing, vascular formation and guide macrophages. The direct influence of growth factors derived from active platelets in wounds is less than 5 days. Marx et al.[7,8] showed that healing and bone regeneration activity can be extended by two mechanisms. The first is the increase and activation of bone marrow stem cells into osteoblasts, which themselves secrete TGF-β. The second and more dominant mechanism seems to be the chemotaxis and activation of macrophages that replace the platelets as the primary source of growth factors after three days. Macrophages are attracted to grafts by the actions of PDGF and when the oxygen gradient created between the graft dead space and adjacent normal tissue is above 20 mmHg[9]. As the influence of PDGF fades, macrophage-derived growth factors become predominant. However, macrophage-derived growth factors and angiogenic factors may actually be identical to PDGF, but synthesized by macrophages instead of by platelets[7].

Bone marrow stem cells secrete TGF-β to continue self-stimulated bone regeneration as an autocrine response as indicated by the presence of continued TGF-β activities arising from marrow cells. By 4 weeks, the revascularized graft eliminates the oxygen gradient required to maintain macrophage activity. Thus macrophages leave the area, as they no longer required by a graft that is now self-sustaining even though immature, with woven osteoid, rather than mature lamellar bone.

The present study found PDGF in osteoid and in new lamellar bone and TGF-β in and around regenerated bone. The actual maturation of the graft from disorganized woven bone into mature lamellar bone with haversian systems may involve a third and final group of growth factors that are not present in PRP. In this regard, adding other growth factors such as rhBMP-2 to the composite biomaterial graft would be essential to accelerate the formation of mature bone.

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自家骨，PRPおよβ-TCPからなる骨補填材による骨再生

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キーワード：PRP，β-TCP，PDGF，TGF-β1，Osteocalcine，CD68

β-リン酸三カルシウム(β-TCP)，自家骨ならびに多血小板血漿(PRP)からなる混合生体骨補填材をヒト2人に埋入し，その有用性を組織的ならびに免疫組織化学的に評価した．被験者一人は59歳の男性で下顎右側第一大臼歯部に歯冠大の辺縁性歯根膜炎による骨吸収が認められ，もう一人の被験者は51歳の女性で左側上顎埋伏犬歯を有していた．6ヵ月後，両被験者より採取した組織を組織学的ならびにオステオカルシン，血小板由来成長因子(PDGF)，形質転換促進因子β1(TGF-β1)およびCD68について免疫組織化学的染色を行った．

PRPにより創傷治癒ならびに補填材の操作性の向上が認められた．組織学的に両被験者共に新生骨の再生が認められ，骨補填部位では炎症性細胞ならびに壊死組織は認められなかった．被験者1では主として骨芽細胞と骨性肉芽組織を含む類骨が認められたが，被験者2では成熟した層板骨が認められ，骨の再生は欠損形態と血液供給の状態に依存することが示唆された．免疫組織化学的な所見では被験者1においてオステオカルシンならびにTGF-βが類骨周辺の細胞(骨芽細胞)に局在していることが認められた．PDGFは骨性肉芽組織に認められ，吸収されたβ-TCP小顆粒の周辺にはCD68陽性のマクロファージの存在が確認できた．

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