Immunohistochemical study of rat parietal bone defect repair with β-tricalcium phosphate and carbonate apatite

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Abstract: In order to examine effects of β-tricalcium phosphate (TCP) or carbonate apatite particles (CAP) in bone repair, we studied bone healing processes histopathologically and immunohistochemically in rat parietal bone defects filled with TCP or CAP. Without TCP or CAP filling, the bone repair was carried out by osteoblasts derived from the edge of the bone defect but did not occur at the center area of the defect. However, with TCP or CAP filling, conductive bone formation occurred along the surfaces of both particles, indicating that those ceramics definitely induced osteoblastic differentiation, which was confirmed by immunohistochemical expressions of such osteoblastic differentiation markers as BMP2/4, Runx2 and Osterix. The expressions of BMP2/4 preceding those of the other osteoblastic markers seemed to be essential for the conductive bone formation by calcium phosphate ceramics. Osteoblastic cells on these particles were derived from resting osteoblasts on the remaining bone surface. Type I collagen, dentin matrix protein 1, and osteocalcin were also expressed in osteoblastic cells as well as in the bone matrix, suggesting that these molecules participated in both bone formation and regulation of osteoblastic differentiation.

Key words: calcium phosphate, osteoconductivity, immunohistochemistry

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Introduction

Bone repair using calcium phosphate ceramics has been widely adopted in the fields of oral surgery and orthopedic surgery. In particular, β-tricalcium phosphate (TCP) is frequently used as a ceramic material (1-4) due to the expedienity of its preparation and its superior osteoconductive and osteoinductive properties (5-6). Nowadays, dental implants are still under development, and primary efforts have been made in developing biocompatible ceramic materials which will adhere to the surface of artificial tooth roots made of titanium. Doi et al. have developed a new biocompatible ceramic material, carbonate apatite particles (CAP), which succeed in adhering to titanium (7-8). The objective of such research has been the osseo-integration of artificial tooth roots to the jawbone through physical and organic bonds via the osteoconductive properties of calcium phosphate ceramics. However, the osteoconductive properties of various ceramics, which are necessary for the future application of ceramics in clinical fields, have not been fully elucidated.

In order to clarify the osteoconductive properties of TCP and CAP, immunohistochemical expressions of factors related to osteoblastic differentiation were examined in the bone repair process of rat parietal bone defects filled with TCP or CAP.

Materials and methods

TCP and CAP preparation

1) TCP preparation: At a 1:2 molar ratio, CaCO₃ and CaHPO₄·2H₂O were mixed in acetone solution. After air drying, the mixture was sintered at 1,100°C for 2 hrs and crushed, and a sieve was used to collect TCP granules with particle sizes ranging from 300 to 500 μm (1).

2) CAP preparation: To a 1.2 M Na₂HPO₄ solution containing 6 M Na₂CO₃, 2 M Ca(NO₃)₂ solution was added dropwise over a period of 2 hrs at 100°C, after which CAP was synthesized at pH 9.0 for 3 days. Precipitates were washed using distilled water, followed by filtering and
drying. After air-drying, precipitates were sintered at 750°C for 2 hrs and crushed, and a sieve was used to collect CAP ranging from 300 to 500 μm in size (7).

Fig. 1 shows the surface structures of TCP (a) and CAP (b), as observed by scanning electron microscopy. Both TCP and CAP possessed macro- and micro-pores.

Animals and experiments
The present study was performed after obtaining approval from the Animal Study Ethics Committee of Asahi University (08-008). The study was conducted using parietal bone defects in 8-week-old male Wistar rats under general anesthesia induced by intraperitoneal administration of 0.2 ml of 50 mg/ml pentobarbital sodium (Abbott Laboratories, Abbott Park, IL, USA). After incision of the skin and periosteum to expose the parietal bone surface, two circular critical bone defects were created with a trephine bur (4.3 mm in external diameter). One side of the defect was treated with TCP or CAP, while the other side was left alone as control. Approximately 10 mg of TCP or CAP prepared in the above manner was carefully filled into bone defects using a dental instrument. To avoid bacterial infections, cefpirome sulfate (Shionogi Co., Ltd., Osaka, Japan) was intramuscularly injected at doses of 60 to 80 mg/kg/day for 3 days. Rats were then fed a normal diet during the experimental period.

Tissue sample collection
At 3 and 5 days, and at 1, 2, 4 and 8 weeks after surgery, 3 rats from the TCP and CAP groups with controls (a total of 36 rats) were sacrificed by perfusion of 10% neutral buffered formalin under general anesthesia. The parietal bone was then removed and fixed in 10% neutral buffered formalin overnight at 4°C. During the present study, none of the rats died of infection or exhibited brain damage due to particle filling.

Table 1. Primary antisera used in this study

<table>
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<tr>
<th>antibody against</th>
<th>host</th>
<th>clonality and subclass</th>
<th>concentration (μg/ml)</th>
<th>antigen retrieval</th>
<th>tissue</th>
<th>3d</th>
<th>5d</th>
<th>1w</th>
<th>2w</th>
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1) Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
2) Abcam Inc., Cambridge, MA, USA
3) Takara Bio Inc., Otsu, Shiga, Japan

*M, mesenchymal tissue; O, osteoid tissue or osteoblastic cells and/or osteocytes; +, positive expression; -, no positive expression
d, days after operation; w, weeks after operation
Specimen preparation and analysis

Fixed samples were demineralized with 10% neutral buffered EDTA for 3 weeks and were then embedded into paraffin, and 4-μm sliced sections were prepared. Each section was subjected to hematoxylin-eosin (HE) staining in order to observe the bone repair process and to evaluate and compare with the osteoconductive properties of TCP and CAP in these processes.

For analysis of protein distribution, immunohistochemical stainings summarized in Table 1 were performed by using primary antibodies against basic fibroblast growth factor (bFGF), FGF receptor (FGFR-2, -3, -4, -5, -6: FGFR), platelet-derived growth factor BB (PDGF), PDGF receptor (PDGFR), bone morphogenetic proteins 2/4 (BMP2/4), runt-related transcription factor 2 (Runx2), Osterix, type I collagen, dentin matrix protein-1 (DMP1), and osteocalcin. Sections were deparaffinized with xylene, and were immersed into descending grades of ethanol from 100% to 80%, and 0.3% H2O2 included methanol was used to block endogenous peroxidase activities. For antigen retrieval in the case of bFGF, PDGFR and BMP2/4, heat treatment was performed in an autoclave for 2.5 min at 121℃, and in the case of FGFR and type I collagen, 0.1% (W/V) trypsin treatment was carried out for 30 min at 37℃. After blocking with 1% BSA, each primary antibody was allowed to react overnight at 4℃, and horseradish peroxidase-labeled secondary antibodies (Nichirei Biosciences Inc., Tokyo, Japan) were used for detection of the primary antibodies. For visualization of reactive products, 3,3’-diaminobenzidine tetrahydrochloride was used as a chromogen substrate, and followed by counterstaining with hematoxylin.

Results

Normal structure of parietal bone

Rat parietal bone was composed of two compact bone layers, the upper and lower plates. One to three layers of osteoblastic cells were thought to be resting osteoblasts covering the surface of the upper plate, and loose connective tissues covered the outermost layer. The surface of the lower plate was covered with a single layer or two layers of resting osteoblastic cells, on which short, fine fibers were arranged orthogonally. Small medullar cavities containing marrow cells were sporadically present in the fused region. Trabecular bone was also covered by resting osteoblastic cells (data not shown).

Repair process of parietal bone defect

1) Control group: At day 3, the defects were filled with plasma components and necrotic tissue fragments, and a negligible number of mesenchymal cells appeared in the defects. At day 5, spindle-shaped osteoblastic cells with eosinophilic extracellular matrix, which were thought to be derived from covering the outer- and inner-plates tissues, appeared on the defect edge of the parietal bone cut surface (Fig. 2a, CS). Granulation tissue cells proliferated and had filled the defects by day 5. At the same time, the osteoblastic cells formed osteoid tissue islands toward the center of the defect (Fig. 2a). As this bone formation advanced, granulation tissue had become fibrous at 2 weeks. At 4 weeks, bone formation activities slowed down and were apparently terminated by 8 weeks. However, the defects were never completely repaired with newly formed bone trabeculae (Fig. 2b).

2) TCP and CAP groups: After day 3, TCP and/or CAP particles were in the necrotic tissue due to hemorrhage and were surrounded by granulation tissue after day 5. At the same time, spindle-shaped cells differentiated on the surface of particles (Fig. 2c). Some of these differentiated into osteoblastic cells at 1 week, and they began to form conductive osteoid tissue around many, but not all particles, and conductive osteoid tissue formation and calcification culminated after 1 week and 2 weeks. Although some TCP or CAP particle surfaces were surrounded by cells differentiating into spindle-shaped cells 2 weeks later, these cells did not show osteoid formation and calcification (Fig. 2d).

Fig. 2. Histological findings of bone repair process in the absence (a, b) and presence of TCP (c, d) at day 5 (a, c), 8 weeks (b), and 2 weeks (d) after bone defect formation. Hematoxylin and eosin (HE) stain, (a, d) x 100, (b) x 40, (c) x 200. In the control group, at day 5 after the parietal bone defect was made, there was a dense proliferation of fibroblastic cells scattering small osteoid islands, and osteoblastic cells (arrow heads) were aligned on the edge of the cut surface of parietal bone defect (CS) (a). Bone repair was still not completed even after 8 weeks, (arrow) (b). In contrast, spindle-shaped cells were densely packed around TCP particles (asterisks) at day 5 (c). Conductive bone formation was seen at 2 weeks around the TCP particles (asterisks) with some areas of incomplete osteoid formation (arrowheads) (d).
2d).

3) Immunohistochemical findings in TCP and CAP groups: bFGF and PDGF, and their receptors were identically expressed in mesenchymal cells in the granulation tissue of defects around TCP or CAP particles. The ratio of cells expressing these molecules peaked between day 5 and week 2. However, bFGF- and PDGF-expressing cells in the granulation tissue decreased markedly in number, except for osteoblastic cells in the osteoconductive region, while their receptors were expressed in fibroblasts in the granulation tissue after 2 to 8 weeks (Fig. 3a). Some mesenchymal cells which adhered to TCP or CAP particles expressed BMP2/4 after day 3 (Fig. 3b). After day 5 to week 2, BMP2/4-expressing cells on the particle surface increased in number, and the cells apparently differentiated into osteoblastic cells which formed osteoid tissues around particles (Fig. 3c). However, BMP2/4 expression was restricted to osteoblastic cells in the osteoconductive region after 4 weeks. After day 3 to 5, Runx2, as an activated transcriptional factor for osteoblastic differentiation, was expressed in the nuclei of mesenchymal cells surrounding TCP or CAP particles (Fig. 3d), and its expression was shifted and restricted in only osteoblastic cells of newly-formed osteoid tissues or new bone trabeculae after 1 to 2 weeks (Fig. 3e). After 4 weeks, Runx2-expressing cells decreased markedly in number, and Runx2 was not expressed in any cell types after 8 weeks. Apparent osteoblasts and osteocytes on conductive osteoid tissues and/or new bone trabeculae around TCP and CAP particles continued to express Osterix, a transcriptional factor, after 1 to 2 weeks (Fig. 3f). Type I collagen was expressed in numerous mesenchymal cells around TCP and CAP particles after day 5 (Fig. 3g). DMP-1 was not expressed in osteoblastic cells on the surface of TCP and CAP particles after 3 days to 1 week, although it was localized in the osteocytes within matured osteoid tissues or in newly-formed bone trabeculae from 2 to 8 weeks (Fig. 3h). Osteocalcin was expressed in mesenchymal cells on TCP and CAP particles, and it was localized in conductive bone matrices after 1 to 2 weeks (Fig. 3i). Although osteocalcin continued to be localized in the bone matrix, osteocalcin-expressing cells were limited to resting osteoblastic cells after 4 weeks. These results are summarized in Table 1.

![Fig. 3. Immunohistochemical findings of bone repair processes with TCP or CAP. Immunoperoxidase stain for PDGF (a), BMP2/4 (b-c), Runx2 (d-e), Osterix (f), type I collagen (g), DMP1 (h), and osteocalcin (i) at day 3 (b), day 5 (d), week 1 (a, g, i), week 2 (c, e, f, h) after bone defect formation, hematoxylin counterstain. (a-g, i) × 200, (h) × 100. Asterisks, TCP or CAP particles. PDGF was expressed in osteoblastic cells and mesenchymal cells forming conductive new bone around CAP particles at 1 week (a). BMP2/4 were positive in fibroblastic cells on the surface of TCP particles at day 3 (b). BMP2/4 were expressed in osteoblastic cells on the surface of TCP particles as well as in osteocytes at 2 weeks (c). Runx2 was expressed in mesenchymal cells differentiating to osteoblasts on the surface of TCP particles as well as in osteoblasts in the defect edge of the parietal bone cut surface at day 5 (d). Runx2 was also demonstrated in osteoblasts or osteocytes in conductive new bone formation at 2 weeks (e). Osterix was positive in osteoblasts forming conductive bone matrices at 2 weeks (f). Type I collagen was labeled in fibrous bundles as well as in mesenchymal cells around CAP particles at 1 week (g). DMP1 was positive in osteoblasts in their conductive bone formation as well as in osteocytes in mature bone matrix at 2 weeks (h). Osteocalcin was strongly localized in activated osteoblasts as well as in the conductive bone matrix at 1 week (i).]
Discussion

Previous reports concerning osteoinductivity and osteoconductivity have focused on the following: 1) type of calcium phosphate possessing osteoconductive properties; 2) relationship between the surface structure of calcium phosphate crystals and osteoinductive or osteoconductive properties; and 3) differences in osteoinductive or osteoconductive properties among animal species. With regard to the first point, studies have shown that hydroxyapatite and TCP have good osteoconductive properties (8). With regard to the second point, the rate of bone formation on the surface structures with macro- and micro-pores is higher than that for smooth structures (9-10). With regard to the third point, numerous calcium phosphate ceramics are known to have osteoinductive activities in humans, pigs, sheep, rabbits, rats, monkeys and baboons, although these properties are more evident in dogs and pigs than in sheep, rabbits, and rats (11-14). In the present study, the parietal bone defects in rats without any artificial bioceramics were not completely reconstituted by new bone formation, and the osteoconductivity in the parietal bone defects embedded with TCP or CAP particles was also incomplete. These findings suggest that the granulation tissue proliferating in the defect lacked the potential for osteoblastic cell differentiation activity.

The immunohistochemical findings that BMP2/4, Runx2, and Osterix were not expressed in the defect granulation tissues support this suggestion. However, osteoblastic cells, which express those osteoblast differentiating factors (15-20), proliferated and were differentiated on the surface of TCP and/or CAP particles, and only these osteoblastic cells formed conductive osteoid tissues around the particles. Such osteoblastic cells were limited around TCP or CAP particles, indicating that TCP and CAP have abilities to induce osteoblastic cells, and that the process of osteoblastic differentiation is initiated by the expression of BMP2/4 in cells adhering to TCP or CAP particle surfaces. Although the present study could not clarify the derivation of such osteoblasts on particle surfaces, one possibility strongly suggested was that the osteoblastic cells on the TCP and CAP particles close to the edges of bone defect surfaces were derived from the resting osteoblastic cells on the bone surface. This suggestion can be supported by the finding that there were no cells with the potential to be differentiated into osteoblasts in the granulation tissue without any filled bioceramics or the center area of the parietal bone defect where TCP or CAP particles were filled. The growth factors, bFGF and PDGF, and their receptors are known to function in inducing osteoblastic differentiation in mesenchymal cells (21-22). Therefore, the expression of these ligands and receptors by numerous cells is thought to play an important role in promoting mesenchymal cell proliferation as well as inducing osteoblastic cell differentiation on the surface of TCP or CAP particles filled in the bone defects.

The most important finding in this study was that adhesion of BMP2/4-expressing cells to calcium phosphate ceramics was the starting point for the expressions of a series of transcription factors for osteoblastic differentiation, such as Runx2 and Osterix. Yuan et al. (23) also investigated the adhesion of BMP2 to phosphate calcium ceramics, reporting that this adhesion was essential for conductive bone formation. The initiation of BMP2 adhered to particles in the granulation tissue after wounding was considered to be a trigger of osteoblastic differentiation from resting osteoblasts located close to the particles. Thus, osteoconductivity as shown in osteoid production by cells on the surface of calcium phosphate might have an essential role for tissue regeneration and repair as scaffold in tissue engineering. Moreover, such calcium phosphate bioceramics as TCP and CAP with potentials to induce osteoblastic cell differentiation are considered to be candidates for important biomimetic materials in tissue engineering for wound healing.

Regarding the expression of extracellular matrix (ECM) components, the present study showed that type I collagen and non-collagenous proteins, DMP1 and osteocalcin, were expressed by both osteoblastic cells on the particles as well as by undifferentiated mesenchymal cells in the granulation tissue (24). Type I collagen has been shown to contribute to regulating osteoblastic survival and differentiation. DMP1 is a phosphoprotein that was first isolated from rat dentin (25), while its existence in bone matrix has also been noted (26-28). DMP1 regulates osteoblastic differentiation and facilitates hydroxyapatite formation, as well (29). From the timing and cell types of DMP1 expression obtained in the present study, DMP1 appears to be correlated with osteoblastic differentiation. Osteocalcin is the most common non-collagenous protein in the bone matrix and is known to bind with calcium via G-carboxyglutamic acid (30). Although its functions have not been fully elucidated, osteocalcin is believed to be involved in bone calcification by binding to hydroxyapatite (31) and in regulating osteoblastic differentiation (32). In the present study, osteocalcin was highly expressed in both osteoblasts and cells in granulation tissues. Taken together with the results from the present study, ECM absorbed to the surface of TCP or CAP particles apparently participates in both the formation of bone matrices and mineralization as well as in the regulation of osteoblastic differentiation.

Finally, we would like to emphasize that TCP and CAP possess osteoconductive bioproperties without histotoxicity, and that as a result, they may be usable in clinical settings, although there were slight chronological and quantitative differences in their osteoconductivity. Kondo et al. (33) reported that highly-purified TCP exhibited the highest osteoconductive properties in dog dorsal muscles, emphasizing the importance of its chemical purity. In addition, biomimetic studies have shown that surface energy on ceramic surfaces might be different according to cell type, and adhesion of tissue fluid may be involved in osteoconductivity (34-35). Slight differences in osteoconductive properties between TCP and CAP may also be due to other factors including biocompatibility differences recognized by macrophages, which result in foreign body granulation surrounding these particles (36). Thus further investigations are warranted before these chemical compounds can safely
be applied to dental practices.

References


