Resorption analysis of deproteinized cancellous bovine bone

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Commercially available deproteinized cancellous bovine bone (DPBB) has been indicated as non-absorbable bone filling materials. Stoichiometric hydroxyapatite (HA) was not resorbed by osteoclasts while calcium-deficient and carbonate-rich apatite converted from octacalcium (OCP hydrolysate) was resorbed by osteoclasts. We analyzed the chemical composition of DPBB and compared the tissue reactions around two materials after implantation into mouse bone marrow. X-ray diffraction analysis and Fourier transform infrared spectroscopy showed that DPBB was a carbonate-rich apatite. Micro-CT analysis indicated the massive bone formation on both materials at 2 weeks, then gradually resorbed. At 12 weeks, osteoclasts were directly attached to both materials. The ultrastructure of ruffled borders on DPBB was identical to osteoclasts resorbing normal bone while ruffled border on OCP hydrolysate showed irregular shape. These results indicated that DPBB was the absorbed material and that the structure of ruffled border of osteoclasts might be regulated by the size or orientation of HA.

Keywords: Deproteinized cancellous bovine bone, Octacalcium phosphate, Osteoclast, Transmission electron microscopy

INTRODUCTION

In oral and maxillofacial surgery, bone grafting is needed to recover bone defects due to malformation, cysts, or tumors. For dental implant installation, sinus lifting has been selected to augment bone volume in the case of highly atrophic alveolar ridge, particularly in the maxillary molar region. Autogenous bone grafting has been performed in many of these cases as “gold standard” for the long time. However, another operation field must be required to get bone grafts, which increases the risk of morbidity. Various biomaterials and/or growth factors have been investigated as alternative clinical methods for many years.

Deproteinized cancellous bovine bone (DPBB) has been reported as a non-absorbed bone filling material and is used for dental implants and other treatments. Stoichiometric ceramic hydroxyapatite (HA) is also used as a bone filling material for various surgical treatments. Previous studies showed that the grafted bone was absorbed by osteoclasts, while osteoclasts could not absorb the chemically equivalent HA.

HA in bone and tooth is a calcium-deficient and carbonate-containing HA. Octacalcium phosphate is the precursor of HA and convert to calcium-deficient and carbonate-containing HA during hydrolysis in vivo. We previously indicated that the octacalcium phosphate (OCP) implanted into mouse calvaria converted to the calcium-deficient and carbonate-containing HA. We also indicated that this converted HA was resorbed by active osteoclasts. These results strongly suggest that the resorption by osteoclasts might be dependent on the chemical composition of HA. Recent studies indicated the existence of osteoclasts on the implanted DPBB. However, there are few reports on the precise structure of osteoclasts on DPBB.

Therefore, in this study, we first analyzed the chemical composition of DPBB and then, compared the tissue reaction of DPBB and OCP after implantation into mouse bone marrow.

MATERIALS AND METHODS

Characterization of DPBB

X-ray diffraction analysis (XDA) and Fourier transform infrared spectroscopy (FTIS) were used to characterize DPBB (particle size 0.5–1 mm, Bio-Oss, Geistlich, Wolhusen, Switzerland). Powder XRD patterns of the synthetics were obtained by a scanning step with Cu Ka X-rays on a Rigaku Electrical (Tokyo, Japan), RAD-2B diffractometer at 40 kV, 20 mA. FTIR spectra of the synthetics were obtained using a diffuse reflectance attachment to a Jasco FT/IR 350 (Jasco, Tokyo, Japan).

Approximately 500 spectral scans were usually conducted over the range of 4,000–400 cm⁻¹ with a resolution of 4 cm⁻¹. The calcium and phosphorus contents of the synthetics were determined with an atomic absorption spectrophotometer and colorimetry and acid phosphate was determined using a previously reported procedure after pyrolysis of the solid at 600°C for 24 h under atmospheric conditions.

Procedure of the material implantation into bone marrow

Male ICR mice (8 weeks old) were obtained from Sankyo Laboratories (Tokyo, Japan) and maintained under specific pathogen-free conditions. A total of 45 mice...
RESULTS

Characteristics of DPBB
XDA analysis indicated the diffraction pattern of DPBB. Diffraction angle showed a peak at 26, 29, 32–35, 41, 46–53 degrees (Fig. 1a), which was approximately the same as HA. FTIR analysis indicated that the infrared
absorption spectrum (IR spectrum) showed a peak in 560–600, 870, 960, 1,030–1,130, and 1,410–1,460 cm\(^{-1}\). Thus, 560–600, 960 and 1,030–1,130 cm\(^{-1}\) were the same as the peak of the IR spectrum of the HA, while 870 and 1,410–1,460 cm\(^{-1}\) showed the peak of carbonic acid, which indicated that DPBB was the calcium-deficient and carbonate apatite.

**Micro-CT analysis**
At 2 weeks after the surgery, trabecular bones were formed in the bone marrow space without the implantation of OCP hydrolysate or DPBB (Fig. 2a), which decreased with the progress of time (Figs. 2b, c). At 12 weeks, most trabecular bones disappeared and the burr hole formed by the drill was completely recovered by the cortical bone (Fig. 2c).

In the case of OCP hydrolysate or DPBB implanted group, the bone formation could not be clearly detected at 2 weeks after the surgery (Figs. 2d, g). At 4 weeks, newly formed bone was detected on the implanted materials (Figs. 2e, h). The volume of newly formed bone showed a tendency to decrease 12 weeks later (Figs. 2f, i).

**Histological and histochemical analysis of bone formation and resorption on the implanted materials**
In the control group without implantation, trabecular bone was formed in the bone marrow space, which was resorbed by osteoclast at 2 weeks after the surgery (Figs. 3a, d). At 4 weeks, bone volume of the trabecular bone in the bone marrow tended to decrease by active osteoclast resorption (Figs. 3b, e). At 12 weeks, most of trabecular bones were disappeared in the bone marrow space (Figs. 3c, f).

In the case of OCP hydrolysate implanted group, most of the implanted OCP hydrolysate was covered by the newly formed bone at 2 weeks after the surgery (Fig. 4a). Many TRAP-positive osteoclasts were attached to the newly formed bone surface and resorbed bone (Fig. 4d).
In the DPBB implanted group, active bone formation was detected at 2 weeks after the surgery (Fig. 5a). However, some areas of DPBB were still not covered by bone and TRAP positive osteoclasts attached to newly formed bone and DPBB (Fig. 5d). At 4 and 12 weeks, active bone formation could not be detected, and TRAP positive osteoclasts attached to bone and DPBB as described by the results at 2 weeks after the surgery (Figs. 5b, c, e, f).

**Ultrastructural analysis of osteoclast on DPBB and OCP hydrolysate**

Multinucleated osteoclasts were directly attached on the surface of DPBB and OCP hydrolysate (Figs. 6a, b). Osteoclasts attached to both materials and showed the well-developed clear zones and ruffled borders, which indicated the active resorption of DPBB and OCP hydrolysate (Figs. 6a, b). However, the ultrastructure of ruffled border of osteoclast were quite different between the two groups. Osteoclasts attached to OCP hydrolysate developed the elongated processes and showed large spaces between each process (Fig. 6c). Ruffled border of osteoclast attached to DPBB consisted of finger-type and plate-type processes (Fig. 6d), which were the commonly detected structures of osteoclasts resorbing normal bone.

Decalcified DPBB were comprised of crystal ghosts of HA (Fig. 6b). However, DPBB under the ruffled border was filled with granular materials (Figs. 6b, d).

**DISCUSSION**

Bone marrow contains mesenchymal stem cells and osteoblast precursor cells, and also is the hematopoietic organ. In this study, massive bone formation was detected in all groups until 2 weeks after the surgery. Subsequently, newly formed bone in the bone marrow was rapidly resorbed by osteoclasts to probably ensure the hematopoietic space. Therefore, bone marrow is the
suitable site for the analysis of active bone formation and resorption. We histologically examined the bone marrow reaction by OCP hydrolysate or DPBB implantation.

One of the requirements as the bone filling material is the rapid bone induction on the material and subsequent replacement of the recipient’s own bone by the resorption of the material. The bone induction on the OCP hydrolysate was faster than that on the DPBB. We previously indicated the rapid osteoinduction on OCP, which might result by the upregulation of calcium concentration around the OCP microenvironment and subsequent induction of BMPs by macrophages. As OCP hydrolysate is a calcium-deficient HA prepared through OCP hydrolysis and the chemical characteristics is almost similar to DPBB, the bone formation progressed equally between OCP hydrolysate and DPBB.

After 4 weeks, active osteoclastic resorption of bone and the implanted materials was detected. Interestingly, the ultrastructure of ruffled border of osteoclast was quite different depending on the implanted materials. Ruffled border of osteoclasts facing OCP hydrolysate showed irregular shape of cell processes, whereas ruffled border facing DPBB consisted of finger-type and plate-type processes. Domon and Wakita clearly described the ultrastructure of ruffled border of in vivo osteoclasts.

The crystal size and the orientation of HA was also different between the two materials. OCP hydrolysate is formed by the conversion from OCP, which are plate-like structures with several μm length and submicron in width. The length and width of HA in bone was much smaller than OCP hydrolysate.

Osteoclasts attached to DPBB had well-developed ruffled border. However, typical Howship lacunae could not be detected. DPBB just beneath the ruffled border of osteoclasts consisted of fine granular materials different from typical crystal ghosts, which indicated the resorption of DPBB by osteoclasts. However, the resorption activity of osteoclasts was much lower than those facing normal bone. This suggests that the structure of ruffled border might be regulated by the size and the orientation of HAs in the mineralized tissues.

It has been reported that bone matrix participates in the differentiation and the activity of osteoclasts. Henriksen et al. also indicated that the age of the bone plays an important role in controlling osteoclast-mediated resorption, with significantly higher levels of osteoclast differentiation and resorption on aged bones when compared to young bones. DPBB is the product from burnt up bone. Therefore, the material which adhered to the surface of HA might be comprised of serum proteins. Although the details of absorbed materials on HA are still uncertain, the resorption activity of osteoclasts attached to DPBB and OCP hydrolysate might be regulated not by HA but some substitute adhering to HA.

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