Ultrastructural Characterization of Tissue Response to Sintered Carbonate Apatite in Rabbit Bone

Yasutoku KOGAYA, Masahiro HASEGAWA, Atsumasa UCHIDA and Yutaka DOI

1Department of Oral Anatomy, Division of Oral Structure, Function and Development, Asahi University School of Dentistry, 1851 Hozumi, Mizuho City, Gifu 501-0296, Japan
2Department of Orthopaedic Surgery, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie 514-8507, Japan
3Dental Materials Science, Division of Oral Functional Science and Rehabilitation, Asahi University School of Dentistry, 1851 Hozumi, Mizuho City, Gifu 501-0296, Japan

Corresponding author, Yutaka Doi E-mail: ytkdoi@dent.asahi-u.ac.jp

Received May 8, 2006/Accepted May 31, 2006

Our previous in vivo and in vitro studies revealed excellent tissue biocompatibility and osteoconductivity of porous sintered carbonate apatite (CA). The present study focused on the ultrastructural details of cells involved in the degradation of CA and new bone formation. Electron microscopy indicated that multinucleated giant cells (MNGCs) were actively involved in CA resorption. MNGCs extended their irregular cytoplasmic protrusions deeply into the interstitial spaces between CA particles. Endophagosomes were formed by encircling partially dissolved or intact CA crystals via the development of pseudopodia-like cytoplasmic protrusions, the configuration of which was somewhat different from that of the typical ruffled border of bone-resorbing osteoclasts. Subsequently, most CA particles in MNGCs were irregular in shape, suggesting that acidic degradation of CA occurred mainly intracellularly. Mononuclear cells, such as macrophage-like and/or fibroblast-like cells, also took up and degraded some CA. Growth of very thin needle-like crystals was observed in close association with CA. Osteoblasts directly faced the CA and secreted osteoid matrix. At the CA-bone interface, an electron-dense and homogeneous thin layer free of collagen fibers was sometimes observed, suggesting an involvement in CA-bone bonding.

Key words : Carbonate apatite, Bone, Multinucleated giant cells

INTRODUCTION

Synthetic degradable biomaterials are applied to bone defects as substrates to guide bone regeneration. Various types of calcium phosphate such as hydroxyapatite, β-tricalcium phosphate, and/or calcite are widely used as synthetic bone replacement materials due to their biocompatibility and osteoconductivity. The porosity, pore size, and interconnectivity of such grafts influence the extent of new bone formation. In addition, their resorption properties are influenced by particle size, crystallinity, and composition. Sintered carbonate apatite (CA) has finer particles with a greater specific surface area than hydroxyapatite, and has several features similar to bone apatite, such as the inclusion of carbonate and high acid solubility.

In a study of noncarbonated and carbonated apatite implants, Ellies et al. demonstrated an increase in new bone formation with increasing carbonate content. Hasegawa et al. revealed that newly formed bone was placed in direct contact with CA, and that tartrate-resistant acid phosphatase (TRAP)-positive multinucleated giant cells (MNGCs) resorbed and degraded CA. Thus, CA is an effective bioresorbable bone substitute. The ultrastructural details of cells involved in the degradation of CA and new bone formation, however, remain largely unexplored. In the present study, the ultrastructural features of the MNGCs, osteoblasts, and other cells related to bone regeneration processes were investigated with transmission electron microscopy.

MATERIALS AND METHODS

CA, a carbonate-containing hydroxyapatite with a chemical composition and crystallinity similar to those of bone, was synthesized as previously described. Five mature female Japanese white rabbits weighing between 3.0 and 4.0 kg were used. A cylindrical defect (4 mm in diameter) was created at the level of the distal metaphysis of femur. Bone debris was carefully removed by saline irrigation, and a sintered porous CA block was packed into the defect. The wound was then closed in layers. All rabbits were allowed free activity in the cage without immobilization.

At six months after implantation, the rabbits were anesthetized with intravenous pentobarbital sodium (Nembutal, 20 mg/kg of body weight, Abbott Laboratories, Chicago, IL), and then perfused through the left ventricle and into the aorta with Ringer’s solution followed by 2% glutaraldehyde/4% paraformaldehyde in 0.1 M cacodylate buffer containing 0.05% CaCl2 (pH 7.4) for 15 minutes. The distal femur was removed, immersed in the same fixative...
for an additional six hours, and post-fixed with 2% osmium tetroxide in cacodylate buffer (pH 7.4) for two hours. The specimens were then dehydrated and embedded in Taab 812 resin (Taab, London, UK). Ultra-thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with electron microscopy (JEM 1200EX). All animal experiments were performed in accordance with the Guidelines for animal experimentation of the Animal Research Committee, Asahi University.

RESULTS

Our previous studies\(^9,10\) at light microscopic level indicated that bone growth around the CA occurred at one month after the operation, bone ingrowth into the pores of CA by three months, and that CA was partially surrounded by newly-formed bone after six months. MNGCs were observed at each stage mentioned above. Leveraging on the findings of our previous studies\(^9,10\), the present study sought to investigate the ultrastructural details of CA resorption and new bone formation at six months after the operation.

At light microscopic level, there was no capsule formation typical of a foreign body reaction. Connective tissue cells were regularly localized adjacent to the interface of CA (Fig. 1a). On the surface of CA masses, there was simultaneous new bone matrix formation by osteoblasts and CA resorption by MNGCs (Figs. 1b and c).

CA crystals in the CA block before implantation appeared rhombic in shape. There were interstitial spaces between the CA particles, where there were electron-lucent membranous structures, probably representing Epon resin (Fig. 2).

Electron microscopy indicated that not only MNGCs (which are rich in mitochondria and small vacuoles), but also mononuclear cells such as macrophage-like and fibroblast-like cells, were involved in CA resorption and degradation (Figs. 3 and 4). Endophagosomes were formed by encircling the CA

---

Fig. 1  (a) Light micrograph showing that the implanted carbonate apatite (CA) was surrounded by bone tissue (B). Bar=100 \( \mu \)m. (b) A higher magnified micrograph of the region indicated by the outlined area 1 in Fig. 1a. Note the multinucleated giant cell (arrow) facing the surface of CA. Bar=20 \( \mu \)m. (c) A higher magnified micrograph of the region indicated by the outlined area 2 in Fig. 1a. Osteoblasts (OB) secreting osteoid matrix (OS) adjacent to CA (stained with toluidine blue). Bar=20 \( \mu \)m.
Fig. 2  Electron micrograph of carbonate apatite block before grafting (embedded in Epon resin). Note the hexagonal-shaped CA crystals and electron-lucent membranous structures (asterisk), probably representing Epon resin. Bar = 500 nm.

Fig. 3  (a) Electron micrograph of multinucleated giant cell (MNGC) resorbing carbonate apatite (CA) particles. Ruffled border, large vacuoles, and clear zone as generally seen in active osteoclasts are not observed in the MNGC. Bar = 2 μm. (b) A higher magnification of CA crystals in phagosomes (arrow) of MNGCs. Bar = 500 nm. (c) MNGCs taking up CA particles. Note that endophagosomes are formed by encircling the CA particles via development of pseudopodia-like cytoplasmic protrusions and that some cytoplasmic protrusions extend deeply into the interstitial spaces between CA particles. Bar = 500 nm. (d) Small vacuoles (arrow) are often observed in MNGCs. Bar = 200 nm. (e) Golgi apparatus in the sub-nuclear zone. M: mitochondria. Bar = 200 nm. (f) After phagocytosis of CA particles by MNGCs, CA crystals become irregular in shape (arrow), suggesting acidic intracellular degradation. Bar = 500 nm.
Carbonate apatite particles are also taken up by mononuclear cells, macrophage-like (Fig. 4a) and fibroblast-like (Fig. 4b) cells. Some particles in the vacuoles of these cells have high electron density and intact shape, but some are electron-lucent and irregular in shape. Bar = 2 μm.

Multinucleated giant cell resorbing carbonate apatite particles and osteoblasts (OB) secreting initial bone matrix. Bar = 1 μm. (b) Osteoblast adjacent to CA particles is characterized by well-developed rough endoplasmic reticulum. Bar = 1 μm. (c) Unmineralized new bone matrix, osteoid (OS), is formed adjacent to the CA by osteoblasts. Note an electron dense layer (arrow) at the interface between OS and CA. Bar = 500 nm. (d) CA particles are embedded in mineralized bone (B). OC indicates future osteocyte. Bar = 2 μm. (e) Homogenous, moderately electron-dense substances (asterisk) are sometimes observed between CA particles. Note the growth of very thin needle-like crystals (arrows) closely associated with CA. Bar = 500 nm. (f) CA particles are completely embedded in heavily mineralized bone (B). Bar = 500 nm.
particles via the development of pseudopodia-like cytoplasmic protrusions (Figs. 3a-c), the configuration of which was different from that of the typical ruffled border of bone-resorbing osteoclasts. The cytoplasmic protrusions facing CA were irregular in shape and some extended deeply into the interstitial spaces between CA particles (Figs. 3a-c). MNGCs did not form a clear zone, a cytoplasmic area representing the adhering plasma membrane (Fig. 3a). Vacuoles in the MNGCs were smaller compared with those of osteoclasts resorbing bone (Fig. 3d). Rough endoplasmic reticulum and Golgi apparatus were also observed, but were poorly developed (Fig. 3e). After phagocytosis, most CA particles were irregular in shape, suggesting intracellular acidic degradation (Fig. 3f). Some particles in phagosomes of macrophage-like and fibroblast-like cells were observed as electron-lucent materials irregular in shape, indicating that acidic degradation of CA also occurred intracellularly (Figs. 3f, 4a and b).

Osteoblasts with well-developed rough endoplasmic reticulum were observed adjacent to the CA particles and sometimes in close vicinity to the MNGCs (Figs. 5a and b). Osteoblasts secreted new bone matrix directly facing the CA remnants, and the CA particles were eventually embedded within the newly formed mineralized bone (Figs. 5c, d and f). An electron-dense layer free of collagen fibers at the interface between CA and newly formed unmineralized bone (Figs. 5c, d and f). A moderately electron-dense fine granular substance was present in some interstitial spaces between CA particles, and the growth of very thin needle-like crystals was closely associated with CA (Fig. 5e).

**DISCUSSION**

Although this study indicated that MNGCs extended pseudopodia-like cytoplasmic protrusions deeply into the interstitial spaces between CA particles — which rendered the block porous, the configuration was somewhat different from that of the typical ruffled border of bone-resorbing osteoclasts. Osteoclast-mediated degradation of hydroxyapatite implanted into sheep bone was achieved by simultaneous resorption and phagocytosis\(^\text{15}\). For MNGCs, the change of their morphological features depends on the substance being resorbed. For example, chondroclasts do not form ruffled borders or a clear zone\(^\text{12}\). In bone resorption, the dissolution of hydroxyapatite and then degradation of the organic matrix are performed by osteoclasts\(^\text{15}\). The ruffled border membrane is formed by the rapid fusion of acidic intracellular vesicles\(^\text{14}\) and composed of two different domains: the lateral area where the exocytotic vesicles are found, and the central area where endocytosis occurs\(^\text{14}\).

The cytoplasmic protrusions of MNGCs which were observed adjacent to the CA seemed to be cytoplasmic processes rather than the specialized ruffled border. The findings that the shape of the CA adjacent to the MNGCs or immediately after being taken up by the MNGCs was almost intact or only partially degraded indicated that the extracellular dissolution of CA particles did not occur actively and that the resorption might be due to phagocytosis rather than the more specialized mechanism being used by osteoclasts. In contrast, the shape of CA in the phagosomes of macrophage-like cells and/or MNGCs was sometimes irregular (crescent-shaped) and electron-lucent, suggesting that the central portion of the CA crystals was intracellularly dissolved by a specific acid.

Hasegawa *et al.*\(^\text{9}\) demonstrated a TRAP-positive reaction for the MNGCs. Recently, Halleen *et al.*\(^\text{15}\) revealed that TRACP, localized in transcytotic vesicles in resorbing osteoclasts, can generate highly destructive reactive oxygen species capable of destroying organic extracellular matrices such as collagen and other proteins. As CA included no organic materials, TRACP in the MNGCs might have a role in the degradation of cell debris that was often observed in the interstitial spaces between the CA particles (data not shown). Mononuclear cells such as macrophage-like and fibroblast-like cells were also involved in the resorption and degradation of CA, because CA particles were observed in the vacuoles of these cells. In initial orthodontic root resorption, the surface layers of mineralized cementum are removed by mononucleated non-clast cells\(^\text{16}\).

Membrane-bound matrix vesicles play a key role in the initial phase of biological mineralization. Cell debris — with structures similar to matrix vesicles — found in the interstitial spaces between CA particles might be related to tissue mineralization. Needle-like crystals grew in association with homogeneous, moderately electron-dense materials and/or CA particles. Although the physicochemical properties of the needle-like crystals were not known, they might represent the re-crystallization of mineral elements dissolved in the interstitial spaces between the CA particles. Homogenous electron-lucent materials, representing Epon resin, were observed in the interstitial spaces between CA particles before implantation. In the interstitial spaces between CA particles after implantation, granular materials rather than homogenous materials were observed, probably stemming from the accumulation of tissue fluid.

Osteoblasts facing the CA were sometimes observed to be close to MNGCs and which secreted new bone matrices, including collagen fibrils. An electron-dense layer free of collagen fibers, which was interposed between the bone matrix and CA and which was probably secreted by osteoblasts, might have a role in bonding the CA with the newly formed bone.
matrix. De Bruijn et al.\(^{17}\) demonstrated that an electron-dense layer with a thickness of 20 to 60 nm was regularly present at the bone-HA interface, which contained both organic and inorganic material and was rich in glycosaminoglycans. The electron-dense layer observed in the present study seemed to be a structure similar to that described above.

In conclusion, our study indicated that: (1) porous CA blocks implanted into rabbit bone were resorbed mainly by MNGCs, but that mononuclear cells such as macrophage-like and fibroblast-like cells were also involved in CA resorption; (2) osteoblasts, facing the outer surface of the CA remnants, secreted new bone matrices; and (3) some CA particles were embedded in heavily mineralized bone.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-aid for Scientific Research (B) (Nos. 12470428 and 16390570) from the Japan Society for the Promotion of Science, and in part by Research Project “Biomimetic Materials Processing” (No. JSPS-RFTF 99R13101), Research for the Future (RFTF) Program, also from the Japan Society for the Promotion of Science.

REFERENCES