Histological Evaluation of Apatite Cement Containing Atelocollagen

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INTRODUCTION

Apatite cement (AC), which consists of an equimolar mixture of tetracalcium phosphate (TTCP; Ca₄(PO₄)₂) and dicalcium phosphate anhydrous (DCPA; CaHPO₄) or dicalcium phosphate dihydrate (DCPD; CaHPO₄·2H₂O), has generated much interest since AC sets to form hydroxyapatite (AP) under ambient conditions⁴. Although conventional AC (c-AC) has some shortcomings—such as a long setting time of 30-60 minutes, recently improvised apatite cements have expanded their clinical applications in the fields of orthopedic, plastic and reconstructive, and oral and maxillofacial surgery. For example, fast-setting calcium phosphate cement (fs-AC) is able to set within five minutes⁵,⁶, while anti-washout type FSAC (aw-AC: formerly known as non-decay type FSCPC or nd-FSCPC) is stable and sets even when the paste is immersed in serum immediately after mixing⁷,⁸. It is reported that aw-AC is formed by controlling two independent processes which occur when the paste is in contact with liquid: one is the formation of AP which is the key step in the cement setting reaction, and the other is the penetration of fluid into the cement paste, which induces wash-out. For the fabrication of aw-AC, the AP property is achieved using fs-AC as the base cement. With fs-AC, it accelerates the formation of AP, i.e., the setting reaction of AC, through the adding of neutral sodium hydrogen phosphate in the liquid phase. The anti-washout property, on the other hand, is achieved by adding a viscous gel into the liquid phase. Various viscous gels such as sodium alginate, chitosan, and collagen are reported to be effective in reducing liquid penetration into cement paste. Prepared in this way, aw-AC is thought to be more useful than AC, especially in surgical procedures where complete hemostasis is sometimes very difficult.

In terms of viscous gels, collagen is known to accelerate cementum regeneration and osteoconduction when it is used as a coating material on calcium phosphate ceramics, such as AP and tricalcium phosphate⁹,¹⁰. As osteoconductive properties are vital to the repair of bony defects, we hypothesized that composites of AC and collagen might produce even more useful biomaterials with higher osteoconductivity. Besides, collagen—which is a primary component of the organic matter that constitutes bone—is known to play important roles in the formation of bone and contributing to its mechanical properties. If collagen does form a network structure in AC with firm bonding to the apatite crystals formed by AC, then mechanical strength might be increased too. Thus, AC-collagen composite might indeed be a much sought-after biomaterial since bone is a natural composite primarily composed of apatite crystals and collagen. Further, the physical properties of collagen may enhance the appeal of AC. Collagen functions as a viscous substance, and could help to improve the manipulation and placement of AC in surgical
MATERIALS AND METHODS

Preparation of AC (ate)
The powder phase of AC (ate), an equimolar mixture of tetracalcium phosphate (TTCP; Ca$_4$(PO$_4$)$_2$O) and dicalcium phosphate anhydrous (DCPA; CaHPO$_4$), was prepared as described previously. A neutral sodium hydrogen phosphate (pH 7.4) was mixed with 0.2 mol/l Na$_2$HPO$_4$ and 0.2 mol/l NaHPO$_4$ so that the solution had pH 7.4 at 37°C. The solution had approximately Na$_2$H$_2$PO$_4$ as its formula, and this formula would be used in this paper for simplicity sake. An aqueous 2% atelocollagen solution (Atelocollagen, taken from calf skin corium, Koken, Tokyo, Japan) containing 0.2 mol/l Na$_2$H$_2$PO$_4$ was used as the liquid phase of AC (ate). As for c-AC, distilled water was used as its liquid phase. Powder and liquid phases were mixed with a spatula at a powder to liquid ratio (P/L ratio) of 3:5.

Soft tissue experiment
Ten-week-old male rats of the Wistar strain, obtained commercially (Charles River, Japan) and fed with standard pellets and water ad libitum, were used for the implantation study.

For AC implantation, the abdomen of the rat was shaved, washed, and disinfected with iodine. Four longitudinal incisions of about 1 cm were made through the full thickness of the skin. Subsequently, lateral to the incisions, subcutaneous pockets were created by blunt dissection with scissors. Each experimental material was mixed with the liquid phase at a powder to liquid ratio (P/L ratio) of 3:5 on glass using a spatula for 20 seconds, and then packed in a cylindrical mold so that the paste samples were 4.7 mm in diameter and 8 mm in height. The mold was made by cutting the front portion of a 1-cm$^3$ plastic syringe (Terumo, Tokyo, Japan). A force of approximately 0.5 MPa was applied to pack the cement paste into the molds. After smoothing the surface of the cement on the open side, the syringe piston was pushed to randomly insert the paste into each subcutaneous pocket. These procedures were done quickly so that the cement was implanted within one minute after mixing. Finally, the wounds were carefully closed.

At one week after surgery, the rats were killed with a lethal dose of sodium pentobarbital (Nembutal®, Abbott Co., Chicago, IL). After soft X-ray photographs were obtained to record the AC shape in each rat, the implant materials, including all surrounding tissues, were removed and fixed in 10% neutral buffered formalin. The samples were demineralized in 10% EDTA at 4°C for a month, and then embedded in methyl methacrylate (OsteoResin®, Wako Co., Osaka, Japan). After polymerization, serial sections of 4 μm thickness were cut using a rotary microtome (RM 2065, Leica Co., Nussloch, Germany). The sections were stained with hematoxylin-eosin and investigated by light microscopy. Five specimens implanted independently were used for histological evaluation.

X-ray diffraction analysis
Compositions of the cements were analyzed using powder X-ray diffraction (XRD)$^{[9][13][14][27]}$. After the specimens were removed from the subcutaneous pockets at one week after implantation, they were immediately immersed in liquid N$_2$. They were then lyophilized and freeze-dried (Automatic Freeze-Dryer 10-010, Virtis Co., Gardiner, NY). In this manner, the conversion of AC into AP was stopped at a specific time. Freeze-dried samples were ground into fine powders and characterized by XRD. XRD patterns of the specimens were recorded with a vertical-mounted diffractometer system (ADG-301, Toshiba, Tokyo, Japan) using Ni-filtered CuK$_\alpha$ radiation ($\lambda=0.1540$ nm) generated at 30 kV and 16 mA. The samples were first scanned from 3 to 60° 2θ (where θ is the Bragg angle) to determine the reaction products in continuous mode (1.0° 2θ/min, time constant of 2 seconds) on a strip chart recorder and digital recorder (Thermodec E, Eto Denki, Tokyo, Japan). Five specimens implanted independently were used for XRD analysis.

Bone tissue experiment
The rats were anesthetized by intraperitoneal injection of Nembutal®. The legs were shaved and infiltration anesthesia with 0.6 mL of 2% lidocaine-epinephrine solution (Xylocaine®, Fujisawa Pharmaceutical Co., Osaka, Japan) was applied around the medial end of the tibia to arrest bleeding from bone marrow and control early postoperative pain. When the medial end of the tibia was exposed, a 4×6 mm bone cavity was formed with a dental
round bur. The powder phases of AC were mixed with the liquid phases at a powder to liquid (P/L) ratio of 3.5 on glass using a spatula for 20 seconds. The cement paste thus prepared was packed with a dental cement condenser in the cavity within one minute after mixing. At the end of each experimental period, the animals were sacrificed by lethal injection of sodium pentobarbital. Tibiae containing c-AC or AC (ate) were removed from four rats each at two, four, and eight weeks after surgery. The specimens, each comprising the tibia with surrounding tissue, were fixed in 10% neutral buffered formalin, dehydrated in graded ethanol, and embedded in polyester resin (Rigolac®; Oken Co., Tokyo, Japan). Thin sections of about 200 μm were cut with a low-speed diamond saw. They were then hand-ground to approximately 70 μm. The sections were stained with 5% toluidine blue and investigated by light microscopy.

RESULTS

Soft tissue experiment

Figure 1 shows the appearance of a rat abdomen at one week after surgery, where the specimens of c-AC (left side) and AC (ate) (right side) had been implanted. A large swelling was observed around the c-AC specimen, whereas no inflammatory response was observed around AC (ate).

Figure 2 shows a histological specimen of subcutaneous tissue surrounding the c-AC implant at one week after implantation. The c-AC implant was surrounded by thick granulation tissue containing many macrophages and foreign body giant cells, as well as moderate inflammatory cell infiltration consisting of lymphocytes and plasma cells. It should also be mentioned that many of these cells were seen around small particles of cement which had become scattered in the cutaneous tissue during the implantation procedure.

Figure 3 shows a histological specimen of subcutaneous tissue surrounding an AC (ate) implant at one week after implantation. The AC (ate) specimen was surrounded by thin fibrous tissue with a slight inflammatory response. Few macrophages and foreign body giant cells were observed in the connective tissue adjacent to the cement implant.

Figure 4 shows the XRD patterns of a specimen removed from a rat at one week after implantation, together with those of the powder phase of AC and poorly crystallized AP for comparison. Formation of AP and unreacted TTCP were observed in c-AC and AC (ate) specimens. Moreover, unreacted DCPA was not found in both AC.

Bone tissue experiment

No new bone formation was observed along the cement at the edge of the pre-existing cortical bone regardless of AC type, i.e., c-AC or AC (ate), at two weeks after surgery (data not shown).

Figure 5 shows the transverse sections of c-AC and AC (ate) at four weeks after surgery. At four weeks, new bone formation was observed along the cement at the edge of the pre-existing cortical bone in both c-AC and AC (ate). However, in the case of AC (ate), more abundant and thicker new bone had formed along the cement in the bone marrow when compared with c-AC.

Fig. 1 Appearance of a rat abdomen at one week after surgery, at the sites where c-AC (left side) and AC (ate) (right side) were implanted. Severe swelling was observed around c-AC. Fluctuation was induced in the swelling by palpation. The cylindrical shape of AC (ate) could be observed through the skin.

Fig. 2 Photomicrograph of subcutaneous tissue surrounding a c-AC implant at one week after surgery. A large vesicle (V), the wall of which consisted of thick vascular granulation tissue (G), has formed subcutaneously. (Original magnification × 52; hematoxylin-eosin stain)
Fig. 3 Photomicrograph of subcutaneous tissue surrounding an AC (ate) implant (C) at one week after surgery. The AC (ate) implant was covered by thin fibrous tissue which included a few giant cells and presented slight inflammatory cell infiltration. (Original magnification ×52; hematoxylin-eosin stain)

Fig. 4 Powder X-ray diffraction patterns of c-AC and AC (ate) removed from a rat at one week after implantation. The powder phases of calcium phosphate cement and poorly crystallized AP are shown for comparison (TTCP: tetracalcium phosphate; DCPA: dicalcium phosphate anhydrous).

Fig. 5 Transverse sections of rat tibia containing c-AC and AC (ate) at 4 weeks after implantation. Elongated new bone (arrows) has formed along the cement from the edge of the cortical bone. (C: cement; original magnification ×15; toluidine blue stain)

Fig. 6 Transverse sections of rat tibia containing c-AC and AC (ate) at 8 weeks after implantation. In the case of AC (ate) at 8 weeks, the cement was almost completely surrounded by mature bone (arrows) extending from both edges of the pre-existing cortical bone. (C: cement; original magnification ×15; toluidine blue stain)
Figure 6 shows the transverse sections of c-AC and AC (ate) at eight weeks after surgery. In the case of AC (ate) at eight weeks, the cement was almost completely surrounded by mature bone extending from both edges of the pre-existing cortical bone. New bone covered most of the cement surface and many activated osteoblasts were found in the new bone. On the other hand, c-AC was not entirely surrounded by elongated mature bone at eight weeks after surgery. It should also be noted that no inflammatory response was observed in the bone marrow (data not shown).

DISCUSSION
Results obtained in this study clearly demonstrated that AC (ate) elicited an excellent tissue response, whereas c-AC caused inflammation. In this study, c-AC was found to cause a severe inflammatory response at one week after surgery when the cement paste, not the set mass, was implanted subcutaneously in rats.

One of the key differences between c-AC and AC (ate) was the mechanical strength at the initial stage. c-AC took a long time to develop mechanical strength, whereas AC (ate) exhibited some mechanical strength from the initial stage. When a cement paste is implanted subcutaneously, the paste is subjected to pressure from the covering skin. In the case of c-AC, the paste crumbled—since it had no mechanical strength to resist the pressure at this initial stage. In contrast, AC (ate) had a much higher mechanical strength at the initial stage, and was thus able to maintain its original shape at implantation while setting. Crumbling of the c-AC paste could present a serious problem, even when the cement is inserted into subcutaneous tissue areas which are considered to be subjected to low mechanical stress.

With the crumbling of c-AC, inflammatory reactions might occur depending on the size of AP particles. When the shape of c-AC was not retained, small particles or AP powder would form rather than an AP mass. Tissue response is dependent on the type, form, or surface character of a biomaterial. In addition, it has been reported that tissue reaction to materials also differs depending on particle size. Brandwood et al. reported that carbon particles up to 20 μm in diameter were phagocytosed, whereas larger particles were not phagocytosed but became surrounded by aggregations of macrophages, some of which migrated onto the particle surfaces. Although there is no report concerning the effect of particle size on soft tissue response to bioactive materials such as AP, small AP particles could cause an inflammatory response. In the present study, we did not examine the cause of the severe inflammatory reaction in detail. It might arise from micro pH increase and decrease, particle size of AP, or other factors associated with the crumbling of c-AC.

AC (ate) showed a better soft tissue response than c-AC at one week after implantation. Furthermore, we observed the promotion of bone formation when compared with c-AC, at least within our experimental periods. In terms of physical properties, atelocollagen incorporation into AC offered the added advantage of adhesive properties. The paste became viscous like an adhesive, especially when atelocollagen was incorporated into the powder phase, such that it became difficult to wipe the paste off the glass plate. These properties indicated that this cement would adhere to the bone defect surface. In the surgical reconstruction of a bone defect, filling of the bone defect without leaving a space between the existing bone and the bone substitute is essential; otherwise, soft tissue will invade the space and prevent new bone growth. In this respect, c-AC is clearly superior to sintered AP, and AC-atelocollagen composite is superior to c-AC.

Collagen is a main component of the organic matter that constitutes bone. This structural protein participates in the assembly of various kinds of macromolecules in the extracellular matrix. In particular, type I collagen is an attractive molecule for manufacturing biomaterials due to its favorable biological properties. It has been reported that type I collagen enhances the adhesion and proliferation of osteoblasts. Moreover, it promotes various processes in the differentiation of osteoblasts, such as alkaline phosphatase activity, expression of noncollagenous extracellular matrix proteins (osteocalcin, osteopontin, and osteonectin), and deposition of minerals into the matrix; it also supports the maintenance of their phenotype in vitro. It was probable that AC (ate) might exhibit these properties, which meant promoting the adhesion of osteoblasts to AC and bone formation along AC surface. Collagen could also serve as a matrix in which the particles of AC or AP are anchored. In particular, composites composed of AP and collagen show great promise as biomaterials, since bone is a natural composite primarily composed of these two phases. Based on the results obtained, it was thought that AC (ate) led to the promotion of bone formation.

In conclusion, it was found that AC (ate) showed an excellent tissue response when compared with c-AC. When c-AC was implanted subcutaneously in rats immediately after mixing, it failed to set and triggered a severe inflammatory response. Therefore, c-AC should be used when its setting reaction can be assured. On the other hand, AC (ate) possessed excellent biomechanical properties, and thus could be expected to be a compatible substitute
for bone. We recommended the use of AC (ate) for surgical applications, namely in the fields of orthopedic, plastic and reconstructive surgery, and oral and maxillofacial surgery.

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REFERENCES

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