Bone formation following implantation of fibrous calcium compounds (β-$Ca(PO_3)_2$, $CaCO_3$(aragonite)) into bone marrow

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Bone formation around three types of fibrous calcium-containing crystals has been examined histologically using rats. The implanted materials are (i) calcium metaphosphate (β-$Ca(PO_3)_2$) fibers having aspect ratios of 15–80 with 2–20 μm in diameter, (ii) β-$Ca(PO_3)_2$ fibers surface-modified using dilute NaOH and (iii) calcium carbonate ($CaCO_3$; aragonite phase) whiskers having aspect ratios of 15–40 with 0.5–3 μm in diameter. β-$Ca(PO_3)_2$ fibers show a mechanically high strength with a low modulus of elasticity, and the surface-modified fibers have a thin layer consisting of a calcium orthophosphate phase. $CaCO_3$ whiskers were used for comparison reasons. The materials were implanted for 4, 8, and 12 weeks into bone defects created in the bone marrow of rat tibiae. Cancellous bone formation was observed around β-$Ca(PO_3)_2$ fibers, the surface-modified fibers and $CaCO_3$ whiskers after implantation for 12, 4 and 4 weeks, respectively. $CaCO_3$ whiskers were scarcely observed after 12 weeks for resorbing. The calcium phosphate fibrous materials show combined advantages of mechanically high strength for toughening a matrix phase and biological activities; thus, these materials may prove to be useful for novel applications in the biomedical field.

Introduction

Calcium phosphate hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HA) and related calcium phosphate ceramics are attractive materials for bone and tooth implants due to their high biological compatibility and safety properties. A large number of calcium phosphate materials have been proposed as artificial bone grafts. New bone is directly formed on implanted calcium phosphate ceramics such as sintered HA and sintered β-tricalcium phosphate (β-$Ca_3(PO_4)_2$; β-TCP) [1, 2]. These materials show excellent biocompatibility and osteoconductivity and have already been used as bone substitutes in some important clinical applications. However, since the ceramics have a higher brittleness and modulus of elasticity than natural bone [3, 4], much attention is now being given to high-strength and high-toughness biomaterials with the modulus matching bone tissue.

Unidirectionally crystallized $CaO–P_2O_5$ glass–ceramics are produced by reheating glass rods under a temperature gradient around the glass transition temperature (T_g) [5]. The glass–ceramics containing crystalline β-$Ca(PO_3)_2$ fibers show a high bending strength (≈ 640 MPa), low Young's modulus (≈ 85 GPa) and a fracture behavior similar to that of natural bone. The results imply that crystalline β-$Ca(PO_3)_2$ fibers have high strength and flexibility. In our earlier work, β-$Ca(PO_3)_2$ fibers were extracted from the crystallized products of calcium ultlaphosphate glasses [6]. The fracture strength and toughness of some materials could be improved by embedding needle-like crystals and/or fibers into the matrix phase. The fracture toughness of HA ceramic has been reported to be improved by introducing various fibers [7–9]. Composite biomaterials having a mechanically good compatibility

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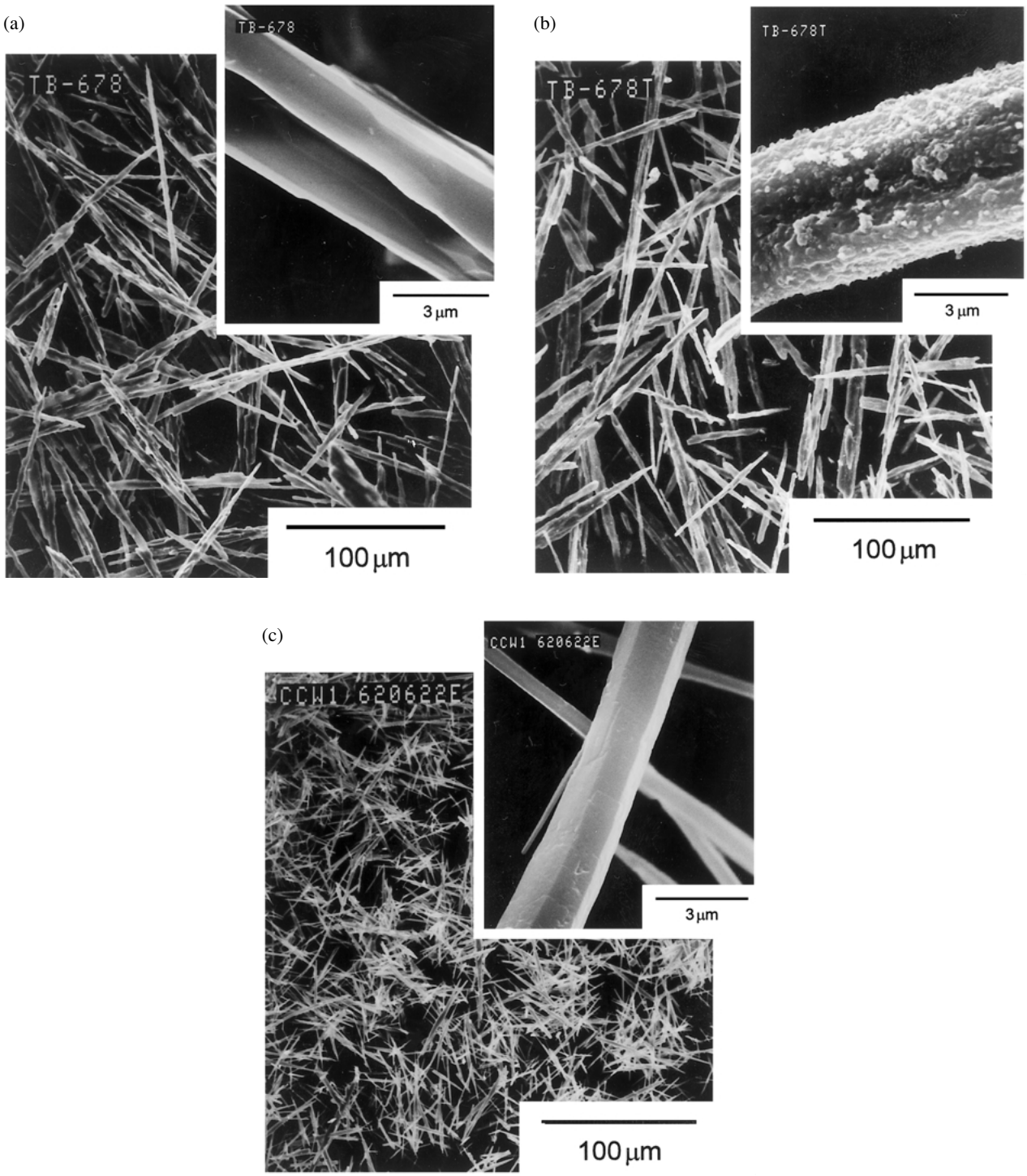


Figure 1 SEM photographs of the fibrous materials tested before implantation. (a) β-$Ca(PO_3)_2$ fibers (CPF), (b) CPF treated with NaOH aq. (CPF-t) and (c) $CaCO_3$ (aragonite phase) whiskers (CCW).

with bone can be obtained by introducing the high-strength and flexible β-$Ca(PO_3)_2$ fibers into polymers or ceramics for bone replacement [10, 11].

Fine fibrous materials are feared to be biohazardous due to their morphology [12, 13]. Preliminary, qualitative study on biocompatibility and safety was performed in the present work for use of β-$Ca(PO_3)_2$ fibers as biomaterials. The fibers were implanted into bone marrow of rat tibiae for histological observations. And also, the fibers surface-modified into the orthophosphate layer and $CaCO_3$ whiskers were implanted into the bone defect sites for comparison with the original β-$Ca(PO_3)_2$ fibers.

Materials and methods

Materials tested

Three kinds of fibrous materials have been prepared for the present study: (i) β-$Ca(PO_3)_2$ fibers (denoted as CPF), (ii) surface treated β-$Ca(PO_3)_2$ fibers with dilute NaOH (denoted as CPF-t) and (iii) $CaCO_3$ whiskers (denoted as CCW).

Preparation of CPF

CPF was prepared by following the method developed in our previous work [6]. The crystallized product consisting of fibrous β-$Ca(PO_3)_2$ crystals having a

glassy phase as the matrix was obtained by heating the ultraphosphate glass ($46CaO \cdot 54P_2O_5$ in molar percentage) at 600 °C for 30 h. CPF was extracted from the crystallized product by aqueous leaching at 80 °C for 70 h and subsequently it was fractionized by sieving. The obtained CPF has aspect ratios of 15–80 with diameter of 2–20 μm, as shown in Fig. 1(a).

Preparation of CPF-t

Upon treatment of CPF with NaOH aqueous solution, a thin calcium orthophosphate layer can be formed on the surface [11, 14]. Since the calcium orthophosphates such as HA and β-TCP show a good biocompatibility, CPF-t having a calcium orthophosphate layer may show a high biocompatiblity with bone tissue. When the CPF-t was soaked in simulated body fluid at 37 °C, a new calcium phosphate phase was formed biomimetically on its surface [14]. CPF-t was prepared by soaking 15 g of CPF in 300 ml of 0.2 mol/l NaOH aqueous solution at 70 °C for 8 h. The obtained CPF-t was examined by X-ray diffractometry (XRD) and laser Raman spectroscopy. Although only β-$Ca(PO_3)_2$ crystals were identified by XRD analysis, Raman bands in the spectrum of CPF-t due to PO_4 groups were observed with those due to PO_3 groups, as reported by Kasuga *et al.* and Fujino *et al.* [11, 12]. Fig. 1(b) shows scanning electron micrographs (SEM photos) of CPF-t. The photos indicate that the morphology of the fiber is almost the same as that of CPF except the surface of the fiber. The strength of the surface-modified fibers having a thin layer consisting of a calcium orthophosphate phase is not degraded in comparison with the original CPF [11, 14]. CPF-t is also a high-strength fiber.

Preparation of CCW

A bone graft substitute derived from coral consisting predominantly of $CaCO_3$ (aragonite phase) is biodegradable and has a great potentiality in use for grafting some bone defects [15]. Tissue reactions with the needle-shaped crystals has not been reported so far. CCW has been prepared by the method mentioned in Ota *et al.* [16], which was a modification from Ota *et al.* [17]. CCW was formed by adding a $Ca(OH)_2$ aqueous solution into distilled water (DW) into which CO_2 gas was continuously blown at 80 °C. Fig. 1(c) shows SEM photos of CCW. The obtained CCW has aspect ratios of 15–40 with diameter of 0.5–3 μm. XRD analysis showed that the needle-shaped $CaCO_3$ consists of aragonite phase. It has already been reported that the needle-shaped $CaCO_3$ is a single crystal, i.e. a so-called whisker [17].

Methods

Before implantation, each of the three needle-shaped materials were sterilized conventionally with ethylene oxide gas. A mixture consisting of each material and saline as vehicle (1 g CPF/5 ml saline, 1 g CPF-t/3 ml saline, 1 g CCW/5 ml saline) was implanted into the bone marrow of tibiae of SD (Crj : CD) SPF female rats weighing 266–320 g (16 months old). The rats were anesthetized with sodium pentobarbital, which was injected into the inter peritonei at a dose of 0.6 mg/kg body weight. The front surface of both tibiae was exposed by means of a skin, intermembrane muscular and periosteum incision. A hole-shaped defect was created using a bone cutter. Each hole was ≈ 2 mm in diameter with ≥ 1 mm depth and extended to the bone marrow. The materials containing vehicle or only saline were inserted into the bone marrow through the hole using a pair of tweezers or syringe. After the insertion, the hole was closed with a surgery adhesive and the skin was sutured.

Thirty-six rats were used for the experiment and they were divided into three groups with 12 rats. Each experimental group consists of four materials (CPF, CPF-t, CCW, saline) and three implantation times (4, 8, 12 weeks). Both legs of each rat were used. Three rats (6 legs) were examined for each implantation.

After implantation for 4, 8 and 12 weeks, the rats were anesthetized with ethyl ether to take blood samples from the abdominal artery. Hematological values (red blood cell count, white blood cell count, hemoglobin, hematocrite, platelet, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration) were measured by an auto Coulter counter. After blood collection, both tibiae were removed from the rats by exsanguination. All the segments of the tibiae containing the implant materials were fixed in a 10% neutral buffered formaldehyde solution for one to three days and subsequently stained with villanueva bone stain for more than three days. The stained tibiae were embedded in methyl methacrylate (MMA) resin after dehydratation in alcohol. Thin sections (≈ 4 μm in thickness) were prepared from the polymer embedded specimens by slicing with a rotary microtome. The implanted materials and bone tissues around them were observed with optical microscope.

Results and discussion

Fig. 2(a)–(g) shows the histological micrographs after implanting CPF, CPF-t, CCW and vehicle for four weeks. CPF and CPF-t shown by arrows in Fig. 2(a) and (c) were surrounded by fibroblasts. A mussa consisting of CPF or CPF-t and fibroblasts was formed at the implantation site. Formation of osteoids and osteoblasts were observed widely on the cancellous bone touched with the mussa. As shown by arrows in Fig. 2(b), CPF embedded in the osteoids were observed. CPF-t which was contacted directly with bone were observed as shown in Fig. 2(d), suggesting that the calcium orthophosphate layer on CPF may improve the compatibility with bone tissue. On the other hand, fibroblasts were not found around CCW as shown by arrows in Fig. 2(e). The cancellous bone including CCW were found everywhere as shown in Fig. 2(f). When the vehicle was inserted into the hole, the spherical and translucent nuclea cells have been mainly infiltrated as shown in Fig. 2(g).

Eight weeks postoperatively, CPF enclosed with the hyaline material (Fig. 3(a)) and CPF-t which was contacted directly with bone (Fig. 3(b)) were observed in the implanted sites. Bone, osteoid and hyaline material including CPF or CPF-t were more frequently observed in the specimens than those after four weeks. Amount of

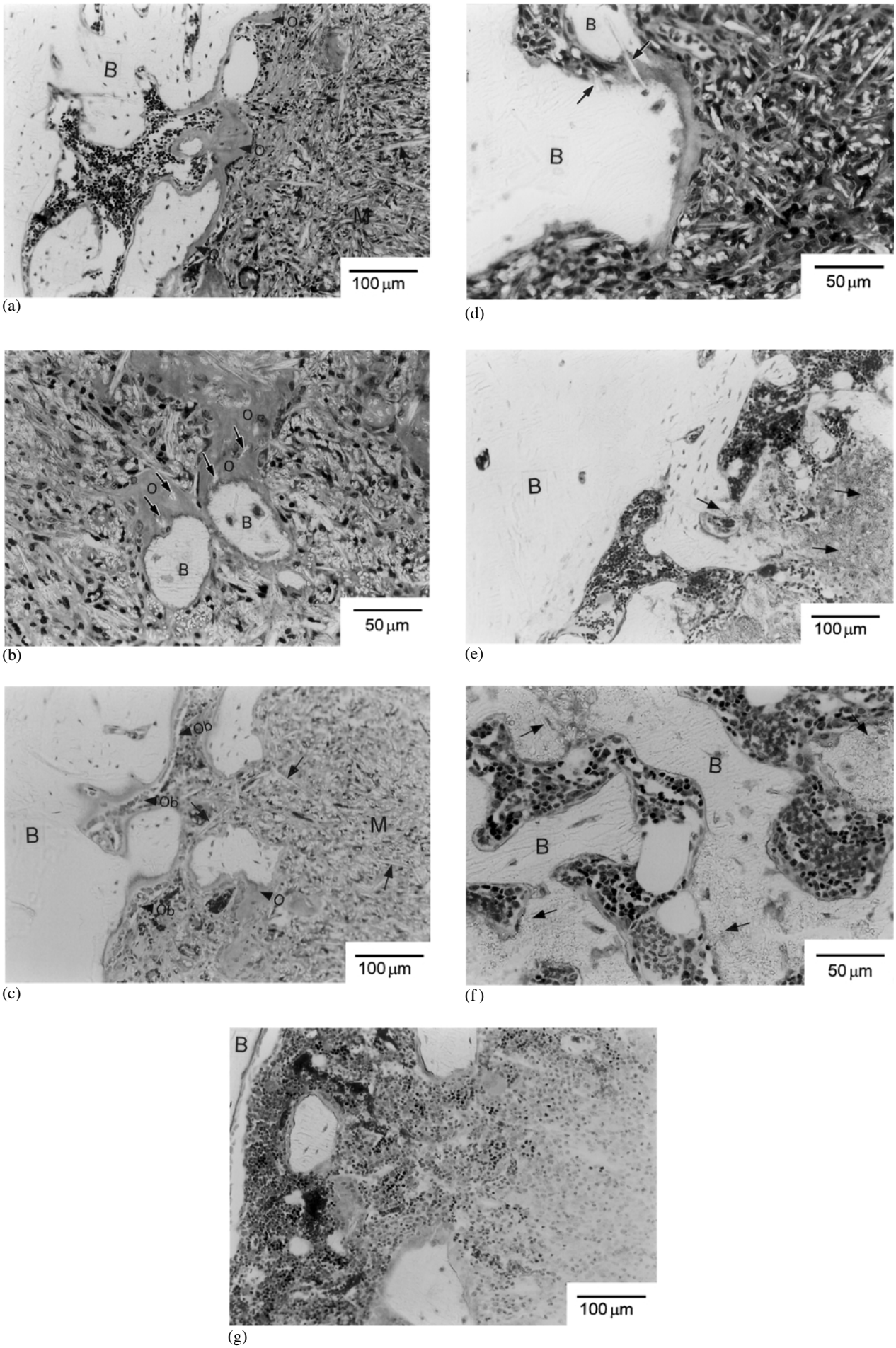
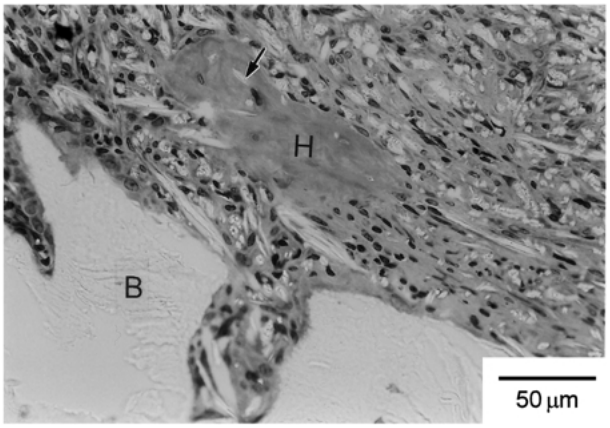
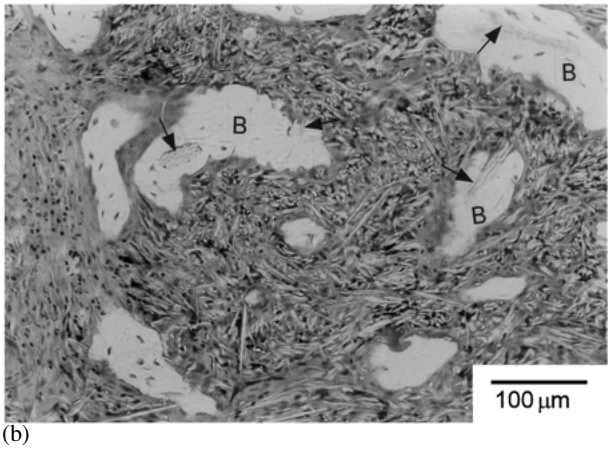


Figure 2 Histological photographs of the interface between the cancellous bone and the defect site after 4 weeks implantation. (a,b) CPF, (c,d) CPF-t, (e,f) CCW and (g) saline vehicle. The arrows show the implanted materials. B: bone, O: osteoid, Ob: osteoblast, M: mussa consisting of CPF or CPF-t and fibroblasts.



(a)



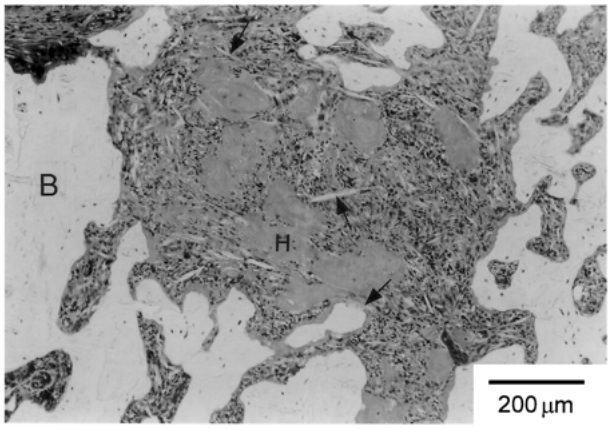
(b)

Figure 3 Histological photographs of the hole in which (a) CPF and (b) CPF-t were implanted after 8 weeks. The arrows in (a) and (b) show the implanted materials. The hyaline materials and the cancellous bone including CPF and CPF-t are seen in (a) and (b), respectively. B: bone, H: hyaline material.

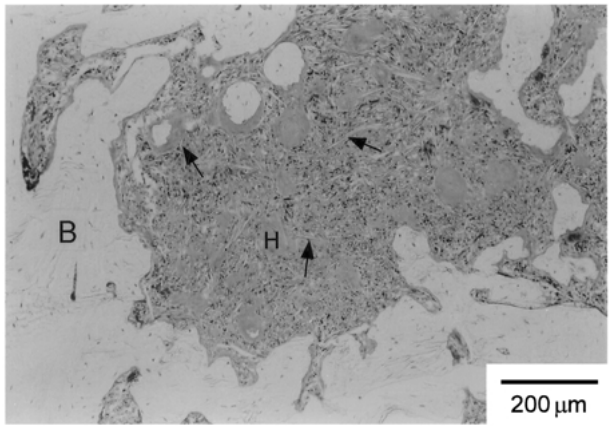
bone including CCW decreased in comparison with the specimens in which CCW was implanted for four weeks.

Fig. 4(a)–(c) shows the histological micrographs of the hole in which CPF, CPF-t and CCW were implanted after 12 weeks. As shown in Fig. 4(a) and (b), the cancellous bones were observed to be developed towards the center of the hole. In the center of the hole shrunk by the new bone formation, hyaline materials were observed. They are suggested to be calcified, resulting in formation of the cancellous bones. It is suggested that the mussa consisting of CPF or CPF-t and fibroblasts formed at early stages start to be converted gradually into new cancellous bone at the marginal zone of mussa and that the bone formation is developed towards the center of mussa. The hole was filled with the cancellous bone tissue (Fig. 4(c)). No CCW can be seen in most of the specimens except a few specimens. It is suggested that CCW is resorbed already at an early stage after implantation and that subsequently new bone tissue is formed. However, some implanted sites, which were not completely filled with new bone tissue, were also seen. This finding may be originated from insufficiency of the implantation dose or excretion of CCW from the implantation site.

Many researchers have tried to improve mechanical properties of calcium phosphate ceramics and polymers by introducing various fibrous products such as metallic



(a)



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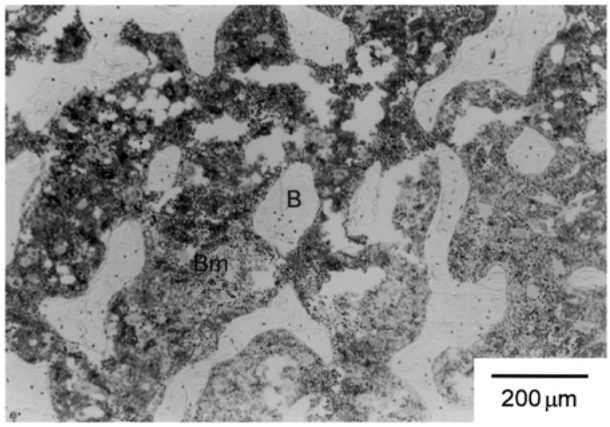

(c)

Figure 4 Histological photographs of the whole defect area after implantation of (a) CPF, (b) CPF-t and (c) CCW for 12 weeks. The arrows in (a) and (b) show the implanted materials. B: bone, Bm: bone marrow cells, H: hyaline material.

fibers, carbon fibers, potassium titanate whiskers and silicon nitride whiskers [7–9, 18]. Although such fibrous materials have mechanically high strength, almost all of them show poor biocompatibility except for HA whiskers [19] and bioresorbable glass fibers [20], to our knowledge. The results in the present work suggest that CPF, CPF-t and CCW have great potential as new types of biocompatible fibers.

From clinical observations, no abnormalities were found in all rats during 12 weeks. All animals showed normal weight gain and unusual observations such as inflammation reactions and myeloid anemia were not found in the rats at each implantation period. Although

an infiltration of neutrophiles was observed in bone marrow of a few rats, hematological values were normal in the peripheral blood.

Concluding remarks

Implantations of three types of fibrous calcium compounds into bone marrow of rat tibiae were carried out. The results of the clinical observations, the normal body weight increase, the hematological findings and the histological observations of implanted sites imply that each fiber has not produced negative effects for the living body and did not show any injuries to the bone tissue. These fibers are expected to show no toxicity.

Cancellous bone can be actively formed around CPF and CPF-t, and CCW is resorbed at an early stage after implantation. CPF and CPF-t are expected to be stable in a living body for a long term, while CCW is biodegradable. The above results may be originated from the chemical composition of the fibers and its surfaces.

We expect that these fibers are potential candidates for development of new biomaterials such as composites associating calcium phosphates or organic compounds. Since CPF and CPF-t are high-strength, fibers with biocompatibility, they are promising as reinforcing materials for preparing the various biomaterials. Using these fibers, we have already successfully prepared novel, unique materials such as high-strength calcium phosphate ceramics having a low modulus of elasticity [10], porous ceramics with a CPF skeleton [21] and polylactic acid composites [11]. CCW may be useful as a filler to reinforce biodegradable polymers.

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