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Hydrolysis of Biopex-R-allografts cement and the osteoblastic response to the cement

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A composite of clinically used Biopex-R and allografts was hydrolyzed in calf serum to obtain hydroxyapatite (HAP), while Biopex-R was hydrolyzed in calf serum to obtain octacalcium phosphate (OCP) and HAP. The products from Biopex-R-allografts composite as well as the Biopex-R exhibited rough surfaces. After the hydrolysis, MC3T3-E1 cells were cultured on the Biopex-R-allografts composite. Adherence of cells on Biopex-R-allografts composite is greater in number than on the Biopex-R. The composition of the resulting products affected the cell growth.

Key words: calcium phosphate, bone cement, allograft, octacalcium phosphate, cell adhesion.

Introduction

There has been considerable interest in the development of paste-like calcium phosphate cements because they have excellent biocompatibility and are easily handled [1-4]. In particular, Biopex that consists of α-tricalcium phosphate, teracalcium phosphate monoxide, dicalcium phosphate dihydrate, and hydroxyapaite has been used clinically as a bone-cement due to its fast hardening and excellent mechanical strength [5]. Although Biopex showed biocompatibility, its resorption hardly occurred in vivo [6]. By the addition of allografts to Biopex, most of the Biopex-allografts composite was resorbed by 24 weeks after implanting in a rabbit [7]. In the hydrolysis of the Biopex-allografts composite, the allografts controlled the local pH to produce HAP and amorphous calcium phosphate that differed from the products of Biopex [8]. The addition of allografts to the bone cement is effective for the resorption of the cement.

Recently, Biopex-R which can be stored at room temperature has been developed. For improvement of the stability of Biopex, magnesium phosphate is added to the powder component of Biopex and $NaHSO_4$ is added to the liquid component. As the result, Biopex-R shows not only thermal stability but also faster hardening than Biopex.

In the present study, we have examined the hydrolysis of Biopex-R-allogarfts composite in calf serum and the cell response using MC3T3-E1 osteoblast cells. Remarkable differences between the Biopex-R-allogarfts composite and Biopex-R were found.

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Experimental procedures

Materials

Biopex-R was purchased from Mitsubishi Materials Co., Ltd. Biopex-R consists of powder and liquid components. The powder component is a mixture of α-tricalcium phosphate (α-TCP), teracalcium phosphate monoxide (TetCP), dicalcium phosphate dihydrate (DCPD), hydroxyapaite (HAP), and magnesium phosphate. The liquid component is an aqueous solution containing sodium succinate, sodium chondroitin sulfate, and sodium hydrogensulfite. Allografts were excised from femurs and tibiae of rabbits. The allografts were sterilized in a saline solution at 80°C for 10 minutes and then were stored at -80°C. When the allografts were to be used, they were thawed and finely ground in a mortar and pestle.

Method

4.3 g of calcium phosphates powder of Biopex-R was mixed with 1.7 g of allografts by mortar and pestle. The mixture was added to 1.9 ml of a liquid component to give a Biopex-allografts composite paste. The resulting paste was loaded in Teflon cells of 6.0 mm in diameter and 4.0 mm in length. After keeping for 30 minutes at room temperature, the pellets of Biopex-allografts composite were taken out from the Teflon cells. Pellets of Biopex-R were prepared by a similar method.

Cell adhesion

MC3T3-E1 osteoblast cells obtained from the Riken Cell Bank (Tokyo, Japan) were cultured in α-minimum essential medium (α-MEP) supplemented with 10% fetal bovine serum (FBS) at 37°C in a fully-humidified atmosphere with 5% CO_2.

Four pellets of Biopex-allografts composite and four pellets of Biopex were stored in separate 96-wells plate. To the wells, 200 μl of calf serum was added and then the 96-wells plate was kept at 37°C in a fully humidified atmosphere with 5% CO_2 for 12 days. The calf serum was changed every 3 days. After 12 days, the calf serum was removed and the pellets were washed twice with 100 μl of 0.25% phosphate-buffered saline solution (PBS). Subsequently, 6×10^3 cells were diluted into 200 μl of α-MEP supplemented with 10% FBS and then the suspension was added to the wells containing the pellets and incubated at 37°C in a fully-humidified atmosphere with 5% CO_2 . After 3 days and 15 days, the media was removed and two of the pellets were washed two times with 100 μl of 0.25% PBS and lyophilized each time.

The lyophilized pellets were stained with 5 ml of 0.05% toluidine blue for 30 min and then washed twice in 5 ml of 95% ethanol and washed with 5 ml of anhydrous ethanol.

Microscopical examination

The cells attached on the pellets were observed by a digital microscope (VH-6300, KEYENCE). The morphologies of the cells-attached surfaces of the pellets were observed by scanning electron microscopy (SEM, JSM-5510S, JEOL).

The cells-attached surfaces of the pellets were also examined by X-ray powder diffractometry (XRD, RU-200B, Rigaku Co., Ltd.) with CuKα radiation generated at 50 kV and 150 mA.

Results

Figure 1 shows XRD patterns of the samples after soaking in calf serum and the successive cultivation.

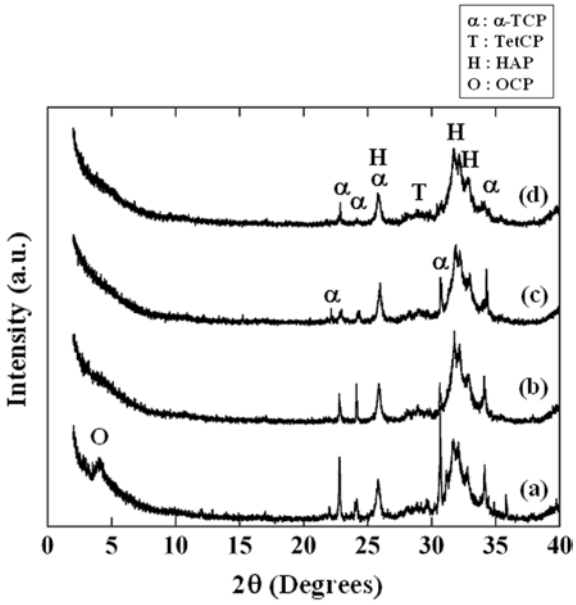

Fig. 1. XRD patterns of (a) Biopex-R after soaking in calf serum for 7 days and (b) after cultivating for 15 days, Biopex-R-allografts composite after soaking in calf serum for (c) 7 days and (d) after cultivating for 15 days.

From these observations, it is understood that the parent materials; α-TCP and TetCP existed in the composite. In the XRD pattern of the Biopex-R-allografts composite after soaking in calf serum for 7 days, the peaks observed for α-TCP are (1 5 0) plane at 22.2°, (2 4 1) plane at 22.9°, (1 3 2) plane at 24.2°, (1 7 0) plane at 30.8°, and (0 4 3) plane at 34.3°. The peaks observed for TetCP are (0 4 0) plane at 29.8° The peak intensities of HAP; (0 0 2) plane at 25.8°, (1 0 2) plane at 28.2°, (2 1 0) plane at 28.9°, (2 1 1) plane at 31.8°, (1 1 2)

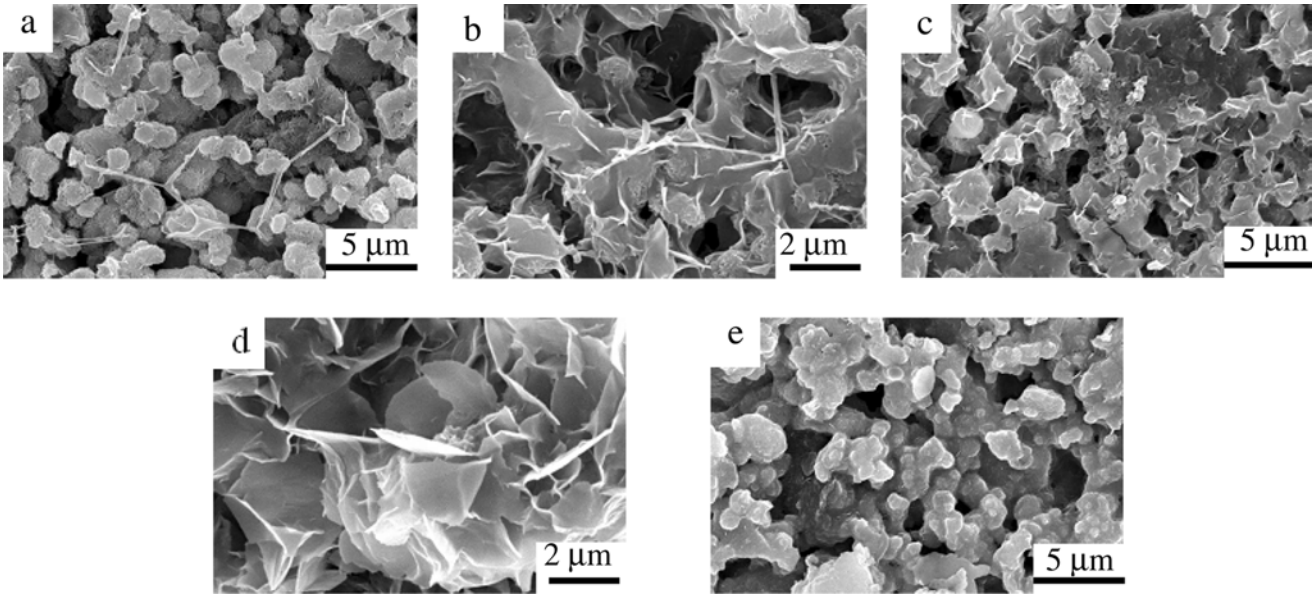

Fig. 2. SEM photographs of (a), (b) Biopex-R-allografts composite after soaking in calf serum for 7 days, (c), (d) Biopex-R-allografts composite after cultivating for 15 days, and (e) Biopex-R after cultivating for 15 days.

plane at 32.2°, and (3 0 0) plane at 32.9° increased, while the peaks from DCPD disappeared. These broad peaks from HAP suggest the formation HAP with relatively low crystallinity. In the hydrolysis of Biopex-R after soaking for 7 days, the DCPD phase disappeared, whereas α-TCP and TetCP remained, Besides the HAP with low crystallinity, OCP; (0 1 0) plane at 4.0° was observed. After cultivation for 15 days, α-TCP, and TetCP decreased in both cements and the resulting OCP was transformed.

The intensity ratios of $\alpha\text{-TCP}_{241}/\text{HAP}_{300}$ are as follows. Biopex-R-allografts composite after soaking for 7 days; 1.5, after culturing for 15 days; 0.5, Biopex-R after soaking for 7 days; 0.24, after culturing for 15 days; 0.24. These results suggest that the hydrolysis of the Biopex-R-allografts composite gradually proceeded.

Figure 2 shows SEM photographs of the samples after soaking in calf serum and the successive cultivation. Allografts were not recognized because of the deposition of HAP on the surface. After soaking for 7 days, particles were connected with each other (a) and leaf-like crystallites were partly deposited (b) in Biopex-R-allografts composite. After additional culturing for 15 days, the particles became larger and the leaf-like crystallites grew. Similar accumulation on the particles resulting from Biopex was recognized, however, the leaf-like crystallites were not found.

Figure 3 shows digital microscope photographs of the Biopex-R-allografts composite and Biopex-R after cultivation of MC3T3-E1 cells. After culturing for 3 days, the number of adherent cells on the Biopex-R-allografts composite were greater than that on Biopex-R. After culturing for 15 days, the aggregation of adherent cells on the Biopex-R-allografts composite increased, whereas on Biopex-R there was no change.

Discussion

We recently reported the mechanism of hydrolysis of the Biopex-allografts composite and of Biopex in physiological solutions and concluded that the allografts controlled the local pH [8]. In the hydrolysis of Biopex, OCP and HAP were formed partly through amorphous calcium phosphate and the resulting crystals were plate-like crystals concomitant with a small amount of needle-like crystals. In the hydrolysis of the Biopex-allogarfts composite, needle-like HAP was formed partly through amorphous calcium phosphate. In the present cements, similar reaction products were formed; OCP and HAP from Biopex-R and HAP from Biopex-R-allografts. Therefore, the allografts controlled the local pH in a similar way as in the Biopex system.

During the soaking and the successive cultivation, the starting calcium phosphates were transformed and the enlargement of particles was observed, however, HAP did not increase so much, as is evident from the XRD pattern. These results suggest that amorphous calcium phosphate (ACP) was formed rather than HAP in both Biopex-R and Biopex-R-allografts cements.

Compared with Biopex and the Biopex-allgrafts composite, the hydrolyses of Biopex-R and of the Biopex-R-allografts composite gave remarkable differences;

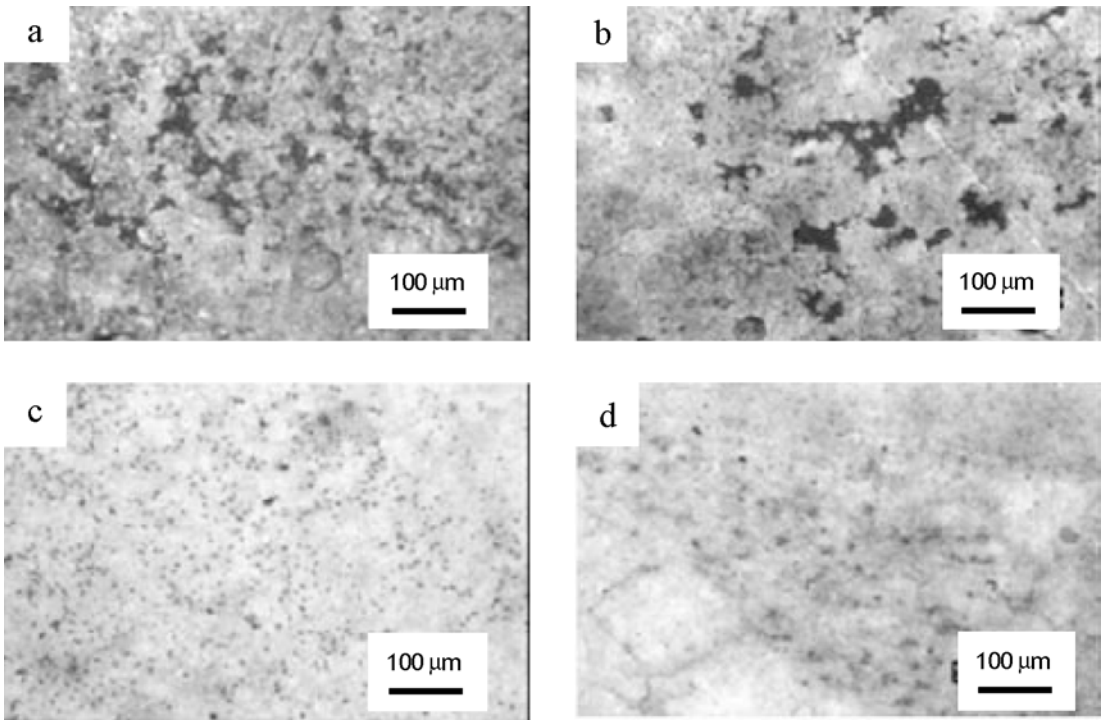

Fig. 3. Digital microscope photographs of Biopex-R-allografts composite after cultivating for (a) 3 days and (b) 15 days, Biopex-R after cultivating for (c) 3 days and (d) 15 days.

the formation of non-crystalline particles and enhanced formation of amorphous calcium phosphate. These differences are attributed to the presence of magnesium ions [9].

In the present cell adhesion experiments, the cells did not adhere on allografts but on the hydrolysis products, because the allografts were completely covered with deposited calcium phosphates. The cell adhesion and growth on the Biopex-R-allografts composite differed from those on Biopex-R. The roughness of the cement surface is known to be responsible for the initial cell adhesion [10]. As the Biopex-R-allografts composite cement showed a rough surface as well as Biopex-R, the composition of the resulting products is crucial for the cell adhesion. It has been known that the composition affects the cell adhesion on the cement [11]; HAP and β-TCP show excellent cell adhesion and growth, however, OCP, DCPD, TetCP and α-TCP show poor cell adhesion and growth. Calcium phosphates, which dissolve to yield a higher concentration of HPO_4^{2-}, tend to show poor cell adhesion. In the hydrolysis of Biopex-R OCP and ACP, which were formed at slightly acidic conditions, it may contain a higher concentration of HPO_4^{2-} and degrade to yield a higher concentration of HPO_4^{2-}. Since the hydrolysis still proceeded during the cultivation, a higher concentration of HPO_4^{2-} would result. This HPO_4^{2-} production may inhibit the cell adhesion.

Conclusions

The Biopex-R-allogarfts composite was hydrolyzed in calf serum to give ACP and HAP. Mg^{2+} ions promoted the formation of ACP and allografts affected the composition of the resulting products. The cell adhesion to the resulting products of the Biopex-R-allogarfts composite with MC3T3-E1 osteoblast cells gave better results than the products of Biopex-R. The allografts behave as a buffer and may control the concentration of HPO_4^{2-}, which is required for the cell adhesion. The simple addition of allogrfts to the bone cement is an effective method in terms of the cell affinity, availability, and handling.

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