Effect of $\beta$-TCP Size on Bone-Like Layer Growth and Adhesion of Osteoblast-Like Cells in Hydroxyapatite/$\beta$-TCP Composites

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Hydroxyapatite (HAp)-20 vol% $\beta$-tricalcium phosphate ($\beta$-TCP) composites were prepared. The effect of $\beta$-TCP size on bone-like layer growth and adhesion of osteoblast-like cells on the composites was systematically studied in vitro. When the composites were soaked in a simulated body fluid, the formation and growth rates of the bone-like layer increased with increasing $\beta$-TCP size, even if the volume fraction of $\beta$-TCP was constant. Moreover, selective dissolution of $\beta$-TCP phase and formation of the bone-like layer around the phase were frequently observed. Higher Ca$^{2+}$ concentration due to the fast dissolution of $\beta$-TCP beneath the sample surface resulted in faster formation and growth of the bone-like layer, especially in the samples containing $\beta$-TCP powders larger than 100$\mu$m. MC3T3-E1 osteoblast-like cells preferentially adhered to $\beta$-TCP phase in HAp/$\beta$-TCP composites because of the enrichment of Ca$^{2+}$ ions around $\beta$-TCP.

Keywords: hydroxyapatite, $\beta$-tricalcium phosphate, biomaterials, osteoblast, simulated body fluid

1. Introduction

Biphasic calcium phosphate (BCP) composed of hydroxyapatite (HAp) and $\beta$-tricalcium phosphate ($\beta$-TCP) is known to exhibit faster bone growth and better biocompatibility than HAp alone.$^{1,2}$ Faster dissolution of $\beta$-TCP led to enrichment of Ca$^{2+}$ and PO$_4^{3-}$ ions around the composites and activated cells resulting in the faster bone growth. The optimum volume fraction of $\beta$-TCP for obtaining good biocompatibility was examined in detail and was determined to be 20–30%.$^{1,2}$ However, the effect of $\beta$-TCP size$^3$ on biocompatibility has rarely been taken into consideration. Osteoblast cells with about 10$\mu$m in diameter played an important role in the construction of human bone.$^{4,5}$ Biocompatibility of biomaterials correlated with the cell behaviour, especially the cell adhesion to them.$^{4,5}$ The adhesion and spreading of osteoblast cells depended strongly on the biomaterials. Thus, there is a possibility that osteoblast cells preferentially adhere to $\beta$-TCP or HAp in the composites, which may influence the biocompatibility of the composites. In the present study, the size of $\beta$-TCP in HAp-20 vol% $\beta$-TCP composites was systematically changed in order to investigate the effect of $\beta$-TCP size on bone-like layer growth and adhesion of osteoblast-like cells in vitro.

2. Experimental Procedure

$\beta$-TCP powder ($\beta$-TCP-100, Taihei Chemical Industrial Co.) was graded by stainless sieves with different mesh sizes and classified into three groups: fine powder $< 281$ mesh, middle powder between 100 and 140 mesh and coarse powder between 30 and 100 mesh. The mean diameters of fine, middle and coarse $\beta$-TCP powders were determined to be 4.3, 110.1 and 274.2$\mu$m, respectively. HAp powder (HAp-100) was mixed in a ball mill with the graded $\beta$-TCP powders which have a volume fraction of 20%. The homogeneity of mixed powders was examined by X-ray diffractometry (XRD). The pellets were prepared from the mixed powders by uniaxial pressing and were sintered at 1373 K for 2 hours. HAp/$\beta$-TCP composites containing fine, middle and coarse $\beta$-TCP powders were designated as F, M and C samples. After the sintering, HAp/$\beta$-TCP composites were mechanically polished by #2000 emery paper to avoid the effect of surface roughness$^6$ and then soaked in a simulated body fluid (SBF) at 309.5 K. Table 1 represents the chemical composition of SBF used in the present study.$^7$ Change in calcium concentration in SBF solution with soaking time was measured by an atomic absorption spectrometer (AAS). A bone-like layer formed on HAp, which was reported to be poorly crystallized carbonated apatite,$^8$ was observed by a scanning electron microscope (SEM). MC3T3-E1 osteoblast-like cells were cultured in Dulbecco’s modified eagle medium (DMEM) in an incubator containing 10% CO$_2$ at 310 K. The suspension of MC3T3-E1 cells was placed in a 96-well culture microplate with HAp/$\beta$-TCP composites. After the cell cultivation for 2.5 hours, the composites were rinsed by phosphate buffered saline (PBS) solution. Then, the cells adhering to the HAp/$\beta$-TCP samples were stained with 0.04% crystal violet. The absorbance of the

Table 1 Ion concentration of SBF solution (Kokubo solution$^7$) and human blood plasma (HBP).

<table>
<thead>
<tr>
<th>Ions</th>
<th>SBF, mmol/L</th>
<th>HBP, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>142.0</td>
<td>142.0</td>
</tr>
<tr>
<td>K$^+$</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>148.8</td>
<td>103.0</td>
</tr>
<tr>
<td>HCO$_3^-$</td>
<td>4.2</td>
<td>27.0</td>
</tr>
<tr>
<td>HPO$_4^{2-}$</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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solution which corresponds to the number of adhering cells on the specimens was measured by a MTP-32 microplate reader (CORONA Electric). For comparison, pure HAp and β-TCP samples sintered at 1373 K for 2 hours were also subjected to the cell attachment test. Statistical analysis of the data was done by Student’s t-test, and the differences in the data were considered to be significant when \( p < 0.05 \). The preferential site of the cell adhesion was examined by SEM.

3. Results and Discussion

3.1 HAp/β-TCP composites with different β-TCP sizes

Figure 1 shows XRD profiles of HAp/β-TCP mixed powders with different β-TCP sizes. All the X-ray peaks in the profiles can be indexed with respect to β-TCP and HAp. Moreover, the peak height ratio of β-TCP and HAp is independent of β-TCP size. This means that the powders are homogeneously mixed and there is little contamination during ball milling. The SEM micrographs of the sintered samples are shown in Fig. 2. β-TCP particles are composed of larger grains (Fig. 2(a)) and show darker contrast (Figs. 2(b) and (c)) compared with HAp.

3.2 Bone-like layer growth

Figure 3 shows SEM micrographs of a bone-like layer formed on HAp/β-TCP sintered composites soaked in SBF for 12, 24 and 336 hours. At a soaking time of 12 hours, preferential dissolution of β-TCP is clearly observed in the F and M samples as shown in Figs. 3(a) and (b), respectively. In contrast, there exists the thin layer of bone-like crystals in the C sample (Fig. 3(c)). The bone-like layer is scarcely seen in the F sample even after 24 hours (Fig. 3(d)), while bone-like crystals are preferentially formed around β-TCP phase in the M sample soaked for 24 hours (Fig. 3(e)). In contrast, homogeneous formation of the bone-like layer is observed in the C sample as shown in Fig. 3(f). After 336 hours, a thin bone-like layer is created in the F sample, while thickening of this layer is remarkably observed in the M and C samples. Thus, formation rate of the bone-like layer increased in increasing order of F, M and C samples.

Ca\(^{2+}\) concentration of SBF solution with F, M and C samples was measured by AAS and is plotted against soaking time in Fig. 4. This concentration in SBF solution varied in four stages: (1) an increase by selective dissolution of β-TCP immediately beneath the sample surface, (2) a decrease by preferential formation of the bone-like layer around β-TCP, (3) an increase by dissolution of HAp and inner β-TCP, and (4) a decrease by homogeneous formation of a bone-like layer. Ca\(^{2+}\) increase in the first stage in M and C samples is obviously larger than that in the F samples. Moreover, Ca\(^{2+}\) concentration in F samples decreases more rapidly after 1 hour than that in the M and C samples. In the third and fourth stage, the SBF solution with M and C samples contains many more Ca\(^{2+}\) ions than that with the F samples. In general, higher Ca\(^{2+}\) concentration leads to higher formation rate of the bone-like layer. A large amount of Ca\(^{2+}\) ions in the M and C samples is believed to have caused faster formation and growth of the bone-like layer in these samples.

The dependence of bone-like layer formation on β-TCP size was discussed as follows. β-TCP is more soluble in SBF solution than HAp. Fast dissolution of β-TCP contacting the sample surface occurred, while HAp and inner β-TCP only slightly dissolved in the early stage of soaking. From a stereological viewpoint, the area fraction of β-TCP is the same as the volume fraction, i.e. 20%. However, total volume of β-TCP attaching to the surface increases with an increase in size as schematically illustrated in Fig. 5. The mean diameter of randomly sectioned sphere \( \bar{d} \) is given by

\[
\bar{d} = \frac{\pi D}{4}
\]

where \( D \) is the diameter of sphere. If the surface area of the
sample is $S$, the total surface area of $\beta$-TCP is written as $1/5 S$. Number of $\beta$-TCP immediately beneath the surface ($n$) is given by,

\[ n = \frac{4S}{5\pi d^2} \]  

(2)

Volume of a $\beta$-TCP powder ($v$) of which diameter is $D$, is written as

\[ v = \frac{\pi D^3}{6} \]  

(3)

Since $\beta$-TCP powder is randomly sectioned, total volume of this powder beneath the sample surface is given by

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**Fig. 3** SEM micrographs of HAp-20 vol% $\beta$-TCP composites soaked in SBF solution for 12 (a-c), 24 (d-f) and 336 (g-i) hours; (a, d, g) F, (b, e, h) M and (c, f, i) C samples.

**Fig. 4** Variation in $\text{Ca}^{2+}$ concentration of SBF solutions with soaking time.

**Fig. 5** Schematic illustration of preferential dissolution of $\beta$-TCP beneath the sample surface.
3.3 Osteoblast-like cell adhesion

Absorbance of the solution containing MC3T3-E1 cells adhering to HAp/β-TCP composites was measured by a microplate reader and is shown in Fig. 6. The absorbance of the solution with pure β-TCP is higher than that with pure HAp. This means that the number of adhering cells is higher on β-TCP. In general, higher Ca\(^{2+}\) concentration in the vicinity of the soaked samples results in strong adhesion of osteoblast-like cells, though too much calcium is toxic both \textit{in vitro}\(^9\) and \textit{in vivo}.\(^{10}\) β-TCP is more soluble than HAp and releases Ca\(^{2+}\) ions, which is consistent with the present experimental results. In the present study, the osteoblast-like cells were cultured only for 2.5 hours; thus the cell death due to the toxicity of calcium was insignificant, resulting in the good cell adhesion on β-TCP. However, the cell cultivation with pure β-TCP for long time led to the cell death and consequently the cells adhering on HAp-20 vol% β-TCP was more stable than those on pure β-TCP.\(^9\) Accordingly, the optimum volume fraction of β-TCP was 20–30% from the viewpoint of the cell adhesion. However, there is no significant difference among HAp/β-TCP composites with different β-TCP sizes. Therefore, β-TCP size did not play an important role in the number of adhering cells. However, β-TCP particles in HAp/β-TCP composites acted as a preferential adhering site for osteoblast-like cells as shown in Fig. 7. Random adhesion of osteoblast-like cells is observed in the F sample (Fig. 7(a)) while the cells tend to adhere to β-TCP in the M and C samples (Figs. 7(b) and (c)). Moreover, the cells adhering on β-TCP spread (Fig. 7(d)) better than on HAp (Fig. 7(d)), suggesting that binding force between the cell and β-TCP is strong. It should be noted that the preferential cell adhesion on β-TCP was not significant in fetal bovine serum (FBS)-doped suspension, though the results are not shown here. In general, osteoblast cells attach to HAp through interaction between the cell surface receptors and adhesion ligands. Integrin, one of the receptors, is activated by Ca\(^{2+}\) ions.\(^{11}\) Fast release of Ca\(^{2+}\) ion from β-TCP led to the increase in number of adhering cells and strong bonding between the cells and β-TCP, especially in the M and C samples. In contrast, Ca\(^{2+}\) enrichment around β-TCP particles in the F samples was not enough for the preferential cell adhesion to β-TCP. Moreover, the size of β-TCP particles (4.3 μm) was smaller than that of MC3T3-E1 cells, which resulted in the random adhesion of the cells. It is well known that the cell network through cadherins plays an important role in the fulfillment of the cell function. Thus, there is a possibility that patterning of the β-TCP phase is effective in an acceleration of the function of osteoblast cells. Since β-TCP can be easily obtained from HAp by heating, laser lithography, for example, may be applicable for the patterning of the β-TCP.

4. Conclusions

Bone-like layer growth and osteoblast-like cell adhesion on HAp-20 vol% β-TCP with different β-TCP sizes were examined \textit{in vitro}. The following conclusions were reached.

(1) The formation and growth speed of the bone-like layer increased with increasing β-TCP size, even if the volume fraction of β-TCP was constant. Selective dissolution of β-TCP and formation of the bone-like layer around the particles were also observed.

(2) Higher Ca\(^{2+}\) concentration due to fast dissolution of β-TCP beneath the sample surface resulted in faster formation and growth of the bone-like layer, especially in the M and C samples.

(3) MC3T3-E1 cells preferentially adhered to β-TCP because of enrichment of Ca\(^{2+}\) ions around β-TCP. This implies that cell arrangement can be controlled by the substrate.

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REFERENCES

3) “β-TCP size” means the size of β-TCP grain assembly which corresponds to the starting powders. For instance, β-TCP composed of a few hundred grains shows a black contrast in Fig. 2(b). On the other hand, each β-TCP grain can be seen due to grain boundary grooving in a high magnification image of Fig. 2(a).

Fig. 7 SEM micrographs of MC3T3-E1 cells adhering to F (a), M (b) and C (c) composites. (d) and (e) show the cells adhering to HAp and β-TCP phase.