The Influence of Bioactive Inorganic Materials on Osteopontin Expression in Rat Calvarial Osteoblast Culture

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Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HA_p] and titanium(Ti) metal are known to be excellent materials with high affinities for natural bone through the apatite. Tricalcium phosphate [$Ca_3(PO_4)_2$, TCP] is a promising alternative material because it is similar to HA_p in its physical properties and biocompatibility. To examine the influence of hydroxyapatite, tricalcium phosphate, pure titanium and pre-treated titanium on osteopontin expression in osteoblasts, RNA was extracted from proliferated and cultured osteoblast cells and OPN mRNA expression was observed by RT-PCR.

Key Words : Hydroxyapatite, Tricalcium phosphate, Titanium, Osteopontin expression

Introduction

Calcium phosphate is an essential element of bone, cartilage, and dental enamel and is commonly found in tissues as a form of apatite. Most of the calcium phosphate in the bone is present as hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HA_p] or its modified form. Therefore, synthesized HA_p can be used as a substitute for aged or destroyed bone since it is very similar to natural HA_p in both structural and chemical aspects.[1] The Ca, P and O sites of natural HA_p are partially substituted with other ions. Although the substitution ratio is small, these replacements have a significant influence on surface charge, structure, strength, and solubility. Hence, in manufacturing an HA_p alternative for use in bone treatments, the structure and form of natural bone should be duplicated as closely as possible. Moreover, although synthesized HA_p has good biocompatibility, it also has high brittleness and low tensile strength, which causes many problems in its use immediately after sintering.

Recently, some other materials have been developed for use in bone replacements. Tricalcium phosphate [$Ca_3(PO_4)_2$, TCP] is a promising alternative material because it is similar to HA_p in its physical properties and biocompatibility and becomes integrated with natural tissues after absorption. TCP can be present in two different states: a high-temperature form, α-TCP and low-temperature form, β-TCP; the transition temperature between α and β is 1,120-1,180 °C. The α form is used for the synthesis of bone cement due to its high reactivity with water, and the β form is used as a bone implant material.

Metals, such as the Co-Cr alloy, stainless steel and high molecular substances, such as polymethyl metacrylate, silicone and high-density ethylene implants, have been widely used for the substitution of bone and dental enamel. The disadvantage of these materials is that they demonstrate inadequate affinity for live tissues and can release metal ions or monomers, which can cause harmful effects to the human body; because of these problems, their application range is limited.

According to several established papers, calcium phosphate ceramics are useful artificial alternative materials for hard tissues, having been applied in various medical fields.[2-6] Titanium and titanium alloy have been reported through various studies that it spontaneously builds up a bone-like apatite layer on the surface of the inner body and can connect to natural bone through the apatite layer if heated, allowing an amorphous sodium titanate layer to form by reacting with NaOH.[7-9] However, in the studies of bioactive inorganic materials on osteoblastic proliferation and osteopontin expression in osteoblast culture, in particular, the entirety of published reports for various inorganic materials, rarely if ever have these been found. Therefore, in this study, gene expression pattern analysis by RT-PCR were used to examine the effects of bioactive inorganic materials (HA_p, TCP, pure titanium and pre-treated titanium) which have high affinities to the human body on osteopontin expression in osteoblasts.

Experimental Section

Preparation of Hydroxyapatite. 0.075 mol of $Ca(NO_3)_2 \cdot 4H_2O$ (Ducksan, > 97%) was used as the calcium source by dissolving it in ethanol. 0.045 mol of $P(OC_2H_5)_3$ (Fluka, > 97%) was used for phosphorous source by dissolving it in a mixture of ethanol and distilled water and adjusting the Ca/P ratio to 1.67 (this is the theoretical mol ratio of HA_p). For the hydrolysis of $P(OC_2H_5)_3$, H_2O was added to create a ratio ($[H_2O]/[P(OC_2H_5)_3]$) of 3.3. Next, the solutions were mixed and stirred at 500 rpm for about 2 h, and the solution, prepared by the addition of ammonium hydroxide, was stirred for 8 h. The hydrolysis and condensation reaction of $P(OC_2H_5)_3$ is slow enough that the solution was maintained in a stable liquid state even after 7 days. The solution was dried at 100 °C for 72 h and the powder was prepared by drying at 1,000 °C for 2 h, upon which HA_p was obtained. Synthesized HA_p powder was palletized by a pressure of

0.70 kg/cm^2 with a diameter of 13 mm and a height of 9.3 mm.

Preparation of Tricalcium Phosphate. $CaHPO_4 \cdot 2H_2O$ and $CaCO_3$ (Nacalai tesque. INC) was used for the synthesis of TCP powder. First, calcium hydrogen phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$. DCPD) was prepared by adding 50 g of calcium carbonate to 1 L of distilled water and then adding 1 L of 0.5 M phosphate solution drop-wise with stirring. The pH of the solution was maintained below 4, and the resulting precipitate was separated through a vacuum filter. The precipitate was dried at 120 °C for 24 h. When the synthesized $CaHPO_4 \cdot 2H_2O$ powder was heated at about 200 °C for 1 h, $CaHPO_4$ was formed as a result of dehydration. A slurry with a 1.5:1 mol ratio was produced by the addition of 2 mol $CaHPO_4$ and 1 mol $CaCO_3$ to 272 mL distilled water. This slurry was separated through a vacuum filter, and palletized by a pressure of 0.70 kg/cm^2 with a diameter of 13 mm and a height of 9.3 mm. α-TCP was obtained by heating the prepared pallet at 1,400 °C for 4 h and then quickly cooling it. Also, β-TCP was obtained by heating the prepared pallet at 1,100 °C for 24 h and then cooling it at ambient temperature.

Pretreatment of Titanium Metal (Modified Ti). A block (25 mm × 25 mm × 0.5 mm) of unoxidized titanium (Ti > 99.99%, Aldrich Chemicals) was prepared and soaked in 60 °C 5.0 M NaOH solution for 24 h. The pure titanium was washed with distilled water and dried at 40 °C for 24 h under atmospheric pressure, then heated for 1 h at 600 °C.

Characterizations. To identify the synthesized powder and examine its structure and crystal phase, FT-IR (Avata 360E.S.P) and X-ray diffractometer (Shimatsu, XRD-6000) analysis was performed. A Scanning Electron Microscope (Hitachi, S-4700) was used to observe the fine structure and particle size of calcium phosphate, and surface area was analyzed using a BET Machine (Gemini 2375). Inductively coupled plasma atomic emission spectrometry (Perkin Elmer, ICP/OES OPTIMA 3300DV) was used for quantitative analysis to examine the composition of the final product.

Simulated Body Fluid Deposition.[10-14] α- and β-TCP pallets, with a diameter of 13 mm and height of 1.8 mm, respectively, were soaked in 30 mL simulated body fluid to determine whether apatite is produced in this fluid. The simulated body fluid was prepared by dissolving NaCl, $NaHCO_3$, KCl, K_2HPO_4, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and Na_2SO_4 in distilled water and then passing the solution through an ion-exchange resin. The pH was adjusted to 7.25 by the addition of 50 mM trishydroxymethyl aminomethane [$(CH_2OH)_3CNH_2$] buffer and 45 mM hydrochloric acid (HCl). The temperature of the simulated body fluid was maintained at 36.5 °C. Table1 displays the ion concentrations for both the simulated body fluid and human plasma solution.

Osteoblast Cell Proliferation. A mouse pre-osteoblast cell line (MC 3T3) was cultured in a-MEM (10% FBS, 1X penicillin-streptomycin) medium at 37 °C with 95% humidity, 95% air, and 5% CO_2. The pure titanium and pretreated metal samples were added to 10 mm plates, and the MC 3T3 cells were serially diluted by 1×10^6. After 6, 24, and 36 h, samples were removed from the plates, put into 50 mL tubes, and incubated with T-EDTA for 5 min at 37 °C. The cells were then counted.

Total RNA Isolation and RT-PCR. Total RNA was extracted from the MC 3T3 cells of each group using TRIZOl (Invitrogen), and cDNA was synthesized using invitrogen. The primer sequences for the OPN gene were 5'-ATT TGC TTT TGC CTG TTT GG-3' and 5'-CAT CGT CAT CAT CAT CG-3', and primers for the internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-GTC GTG GAG TCT ACT GGT GT-3' and 5'-CAA AGT TGT CAT TGA GAG CA-3'. RNA was examined through the following serial experiments: PCR reaction, agarose gel electrophoresis, ethium bromide staining, visualization under a UV illuminator, and analysis with an image analyzer.

Results and Discussion

Table 2 displays the average size, specific surface area, and composition of the synthesized calcium phosphate particles. The average size of the particle and the specific surface area increase in the following order: α-TCP < β-TCP < HA_p. Moreover it has been found that the composition of the products nearly agree with the stoichiometric compositions. These theoretical compositions are as follows: HA_p (Ca: 39.89%, P: 18.50%, O: 41.40%, H: 0.21%), α- and β-TCP (Ca: 38.76%, P: 19.97%, O: 41.27%).

Generally, the main problem in the preparation of HA_p was the heat-instability. HA_p is converted into other compounds, such as CaO, above 1,000 °C. Also, when the equivalence ratio is not exact, HA_p cannot be taken. However, in this study, HA_p powder was synthesized using a sol-gel method at room temperature, and this powder has excellent

Table 1. Ion concentrations of simulated body fluid (SBF)

Ion Concentration (mM)		
Ion	Simulated fluid	Blood plasma
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	147.8	103.0
HCO_3^-	4.2	27.0
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5

Table 2. Physical properties of calcium phosphate

Division	Hydroxyapatite	α-TCP	β-TCP
An average particle size (μm)	28-32	3.0-4.0	23-25
BET surface area (m^2/g)	2.42	1.05	1.48
Composition analysis (%)			
Ca	40.00	38.90	38.80
P	18.30	19.10	19.20
O	41.50	42.00	42.00
H	0.20		

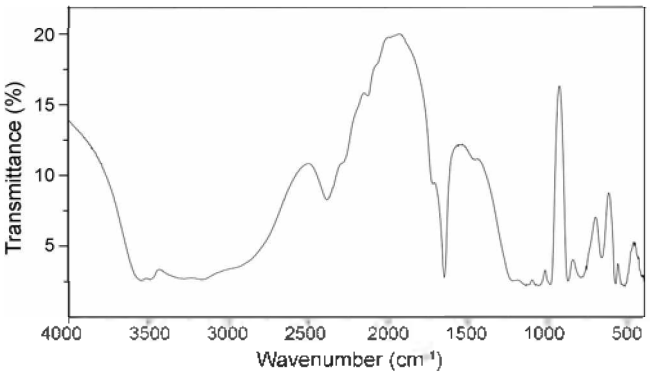

Figure 1. FT-IR spectrum of calcium hydrogenphosphate dehydrate (DCPD).

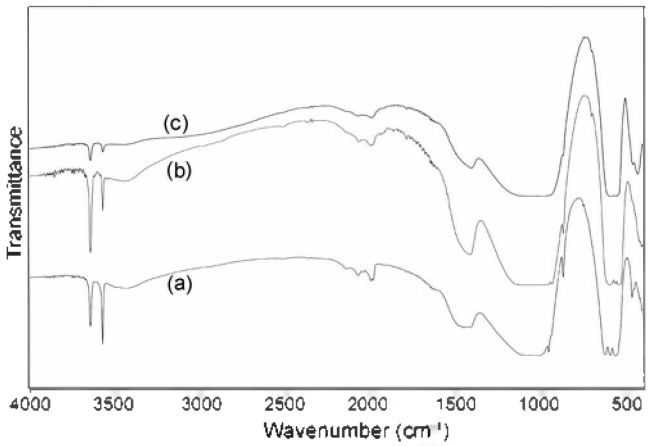

Figure 2. FT-IR spectra of heat-treated calcium phosphates. (a) HA_p (b) β-TCP (c) α-TCP.

properties. At each temperature, TCP shows a relatively good crystal phase, and it was confirmed that predominantly α- and β-TCP were present without HA_p or CaO.

Figures 1 and 2 show the FT-IR spectra of dibasic (DCPD) and calcium phosphates. Strong absorption bands at 602 and 571 cm^{-1} are asymmetric O-P-O bending modes (ν4), and the weak absorption bands at 473 and 436 cm^{-1} are symmetric O-P-O bending modes (ν2). The strong and broad absorption band at 1,045 cm^{-1} is observed as an asymmetric P-O stretching mode (ν3), and the small absorption band at 960 cm^{-1} is observed as a symmetric P-O stretching mode (ν1).

The XRD pattern for the synthesized calcium phosphate is shown in Figure 3. Figure 3(a) shows very pure HA_p without TCP, CaO, or SiO_2 after sintering for 2 h at 1,000 °C. Figure 3(b) and 3(c) show the XRD pattern for the α- and β-TCP powders. α- and β-TCP samples were sintered at 1,400 °C and 1,100 °C respectively for 24 h and show relatively good crystal phases for each temperatures.

Figure 4 shows the SEM analysis for calcium phosphates powders. Figure 4(a) shows the plate-typed particle. Figure 4(b) shows HA_p, in which unique acicular-typed particles are found, and 4(c) and 4(d) show α- and β-TCP, respectively, displaying a large increase in the glassy phase. These results reveal the excellent properties for the materials used

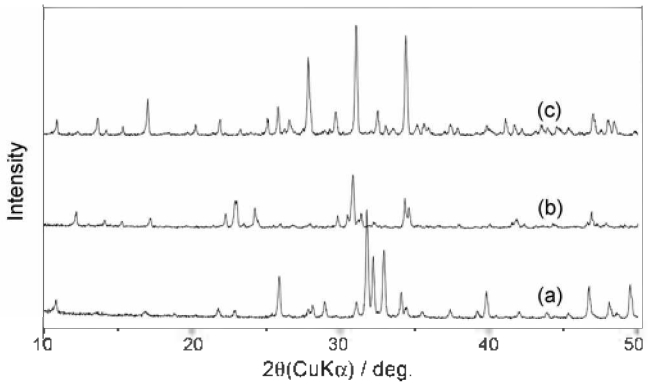

Figure 3. X-ray diffraction patterns of the heat-treated calcium phosphates. (a) HA_p (b) α-TCP (c) β-TCP.

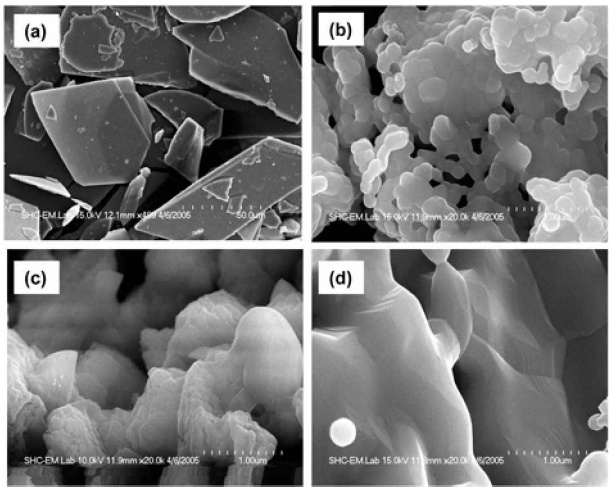

Figure 4. FE-SEM photographs of the heat-treated calcium phosphates powders. (a) DCPD (b) HA_p (c) α-TCP (d) β-TCP

in this study. Figure 4(c) shows that the α-TCP was obtained by decomposition, condensation, and sintering of the dried powder at the respective heated temperatures. The increase in the glassy phase and widely positioned pores can be seen. It is thought that these pores could indicate CO_2 gas escaping at about 1,000 °C and dehydration of HA_p at 800 °C. Figure 4(d) shows β-TCP, where 5-10 μm of the smallest particles are composed of secondary particles through condensation, and it has been observed that the secondary particles' size and shape are irregular. On the surface, there are pores due to the decomposition of $CaCO_3$.

Figures 5 and 6 display the results of the simulated body fluid soak. Post-soaking samples were washed with distilled water. X-ray diffraction patterns for α-TCP post-soaking in simulated body fluid are shown in Figure 5. The apatite peak, which was not observed in the pre-soaking sample, appeared after 7 days of soaking time. Figure 6 shows the X-ray diffraction patterns for β-TCP post-soaking in simulated body fluid. The apatite peak for this sample appeared at 3 days of soaking time. SBF is already oversaturated with apatite, once the apatite nucleates, it arbitrarily grows using the surrounding calcium and phosphate. Comparing Figures 5 and 6, it showed that β-TCP showed an apatite peak about

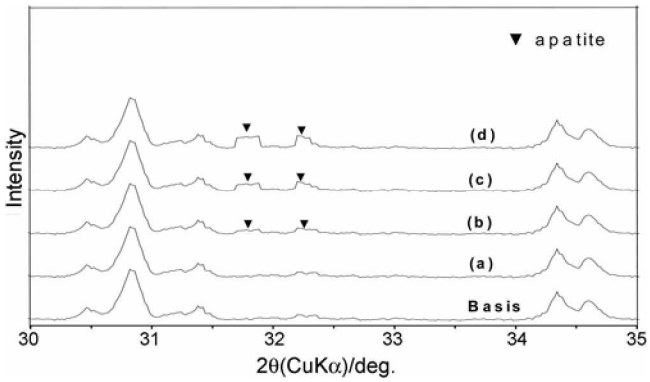

Figure 5. XRD patterns of α-TCP pallets that were soaked in SBF for (a) 3 days (b) 7 days (c) 10 days, and (d) 15 days.

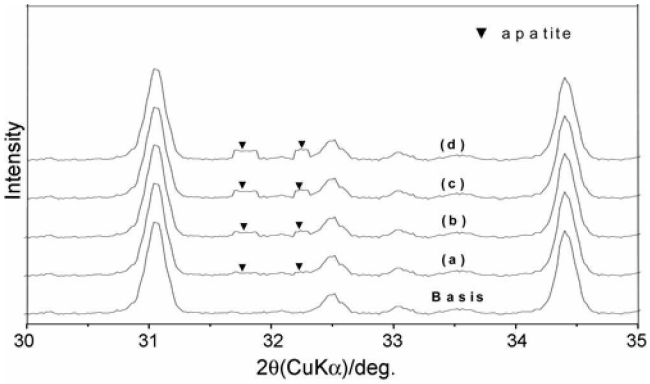

Figure 6. XRD patterns of β-TCP pallets that were soaked in SBF for (a) 3 days (b) 7 days (c) 10 days, and (d) 15 days.

4 days faster than α-TCP. This may be based on the difference of surface area. The apatite formation varies greatly with heat treatment. This apatite formation generally decreases with increase of heat treatment temperature, since the heat treatment temperature of α-TCP is higher than that of β-TCP. Therefore, α-TCP has a smaller surface area than β-TCP.

Figure 7 displays the cell count. The cells were prepared by dispensing 1×10^6 osteoblasts onto both pure titanium and pre-treated titanium, then incubating for 6, 24, and 36 h, and then they were removed. With OPN expression, the number of osteoblasts increased on both pure titanium and pre-treated titanium over time. Although pure Ti was pre-treated, there was no result showing that cell proliferation might be prominent compared to pure Ti. Merely a tendency for increase in cell proliferation as time increased.

Figures 8 and 9 show the OPN and GAPDH mRNA expression levels for the control, HA_p, TCP, pure Ti, and pre-treated Ti conditions after 6, 24, and 48 h of incubation. Control was cultivated on a cell culture plate and other samples were cultured on the condition of HA_p, TCP, Ti and pre-treated Ti. The bar graph presents the ratio of OPN/GAPDH. The control in both Figures 8 and 9 was the same sample, therefore, there was a correlation between them. Control had expression after 6 and 24 h incubation, however, there was a decrease after 48 h. HAp had a similar result to the control after 6 h incubation, however, there was a decrease

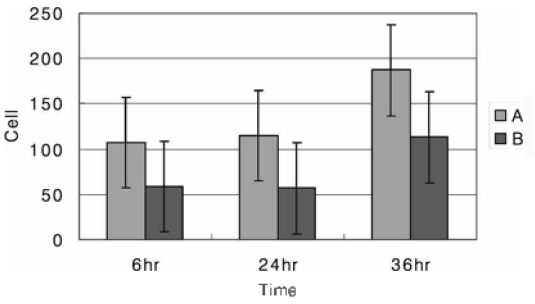

Figure 7. Cell count of osteoblasts attached to Ti and pre-treated Ti cultured for 6, 24, and 36 h. (A) Ti (B) pre-treated Ti.

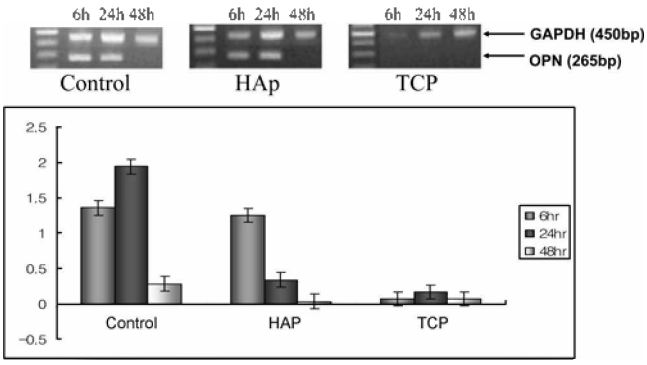

Figure 8. Gel electrophoresis of RT-PCR products amplified from extracts of cellular RNA, OPN mRNA (199bp), and GAPDH mRNA for HAp and TCP.

over time. β-TCP showed no sign of expression. After 6 h incubation, the expression amount of pure Ti was similar to that of control, however, that of pre-treated Ti was more than control; and after 24 h, the amount was less than control. After 48 h, only Ti and pre-treated Ti were expressed. With those results, it could be expressed in HAp, Ti and pre-treated Ti, but not in TCP. As the incubation time was increased, a tendency of increase in expression amount of OPN mRNA did not correspond to control. It is thought that the expression amount of biomaterial during cell culture is related to the feature of biomaterial in promotion of cell proliferation and durability of bioactivity. In TCP, there was bioactivity, however it did not meet those conditions during cell culture, and therefore it was not expressed. In HAp, it was expressed in the early stage of cell culture and was decreased with time. In Ti and pretreated Ti, those were expressed in the early stage and were maintained with time. Therefore, it is considered that Ti and pretreated Ti do not only promote cell proliferation, but also have durability in cell activity. Also the expression results for calcium phosphate and titanium may be explained by osteoconduction, which stimulates bone formation, and this process is thought to be the result of cytotoxicity of biocompound. The reasons for this are not clear, but Hyakuna *et al.*[15] reported that cytotoxicity occurred from the reduction of Ca, P, and albumin and the differences in chemical composition and pore ratio of ceramics. In addition, Matsuda *et al.*[16] reported that alkali ions secreted from ceramics slow the growth rate of cultured cells. However, as pointed out by Wilson *et al.*[17] it may be

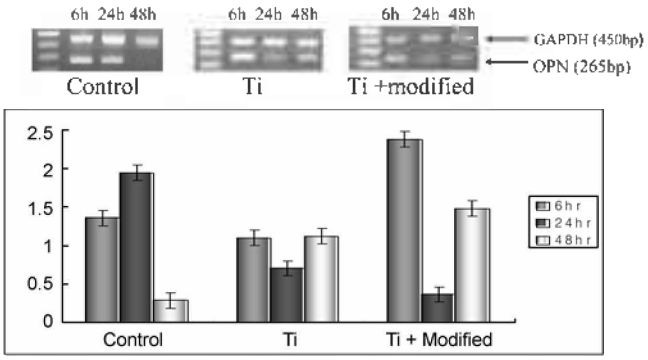

Figure 9. Gel electrophoresis of RT-PCR products amplified from extracts of celluar RNA, OPN mRNA (199bp), and GAPDH mRNA for Ti and Ti + modified.

expected that synergic events occur *in vivo* due to the buffering effects of body fluid, and this results in a difference from cell culture experiments.

The cell maintains homeostasis by interacting with other cells or components of the extracellular matrix. These cell-to-extracellular interactions are achieved through protein receptors attached to the cell surface; the integrin family is an example of this class of receptors. Integrin is involved in cell differentiation, proliferation, and migration and interacts with other cells through the adhesion-promoting protein RGD tripeptide (Arg-Gly-Asp) base. Examples of proteins that have the RGD base on the surface are bone sialoprotein, fibronectin (FN), laminin, osteopontin (OPN) and osteonectin (ON), among others. There are differences in the distribution of the RGD base and the mechanism of its action depending on the cell type. The osteoblast is an adhesion cell type, and it has been determined that the extracellular OPN, FN, and ON proteins are involved in substrate adhesion to the cell surface.

Bone tissue is composed of bone cells of the osteoblast and extracellular matrix; this matrix consists mainly of phosphoproteins, such as type 1 collagen, osteopontin (OPN), fibronectin (FN), and vitronectin. Osteoblasts express FN, collagen, and TGF-β at the proliferation stage, alkaline phosphatase at the initial stage of differentiation, and osteopontin and osteocalcin at the bone formation stage late in differentiation. Moreover, it has been known that the osteoblast forms bone tissues through the proliferation and differentiation stages by adhering to the extracellular matrix, and intracellular protein is concerned to adhering osteoblasts to the matrix. These proteins have common base sequences for RGD, and cellular receptors, such as integrin on the cell membrane, recognize these RGD patterns and transmit signals into the cell. OPN, which is also called 44-kDa phosphoprotin or bone sialoprotin, is an adhering molecule for osteoblasts and osteoclasts and is present in the kidney, heart and lung, as well as bone tissues. It has been reported that this molecule plays an important role in tissue formation and wound healing through the processes of cell adhesion, proliferation, differentiation, metastasis, and embryogenesis. Moreover, OPN is expressed throughout the osteoblast proliferation, differentiation and bone-formation stages, and its expression ratio is known to increase as differentiation progresses toward bone formation. If osteoblasts are stimulated mechanically, OPN expression increases.

Conclusion

Calcium phosphates were synthesized by a sol-gel reaction and bioactivity of TCP was confirmed by a simulated body fluid soaking test. Also, compositions of the synthesized calcium phosphates were nearly concord with stoichiometric compositions. According to the examination of the influence of the hydroxyapatite, tricalcium phosphate, pure titanium and pre-treated titanium on OPN expression in osteoblasts by osteoblast cell proliferation, RNA extract and RT-PCR, it was found that hydroxyapatite, pure titanium and pre-treated titanium are materials that promote osteoblasts differentiation and play an important role in up-regulating OPN expression. In pure titanium and pre-treated titanium, those were expressed in the early stage and were maintained with time. Therefore, it is considered that Ti and pretreated Ti do not only promote cell proliferation, but also have durability in cell activity.

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