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In vitro study on the osteogenesis of normal human osteoblasts cultured on the discs of various kinds of calcium phosphate ceramics

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Keywords: Calcium phosphate ceramics, osteogenesis, cytotoxicity,

Abstract. We estimated effects of various CP ceramics on the properties of normal human osteoblasts (NHOst) as well as a viability of V79 fibroblasts. In the present study, five kinds of CP ceramics, namely, hydroxyapatite (HAp) flouroapatite (FAp), α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP), were tested. Cytotoxicity test was carried out using V79 fibroblasts by colony assay system. The amounts differentiation level of NHOst was estimated from alkaline phosphatase (ALP) activity and osteocalcin.
From the results of colony assay, FAp and α-TCP showed strong cytotoxicities on V79 cells. The results from the proliferation studies of NHOst with CP ceramics were consistent with the results of colony assay. In addition, the ALP activities of NHOst with CP ceramics after 1week culture were significantly suppressed in comparison with that of NHOst alone. The osteocalcin amounts produced from NHOst cultured on β-TCP was the highest among five kinds of CP ceramics.

Introduction

Calcium phosphate (CP) ceramics have been studied to utilize as the scaffolds for repairing bone defects. For instance, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) or β-tricalcium phosphate (β-$Ca_3(PO_4)_2$, β-TCP), can be biologically bonded to natural bones and their porous materials are effective for restoration of bone defects [1]. Fluoroapatite ($Ca_{10}(PO_4)_6F_2$, FAp) has been reported to have a potential of novel bone repairing materials with high stability *in vivo*, since solubility of FAp is lower than that of HAp [2]. In addition, CP cement is also promising for bone repair and it is well known that α-tricalcium phosphate (α-$Ca_3(PO_4)_2$, α-TCP) or tetracalcium phosphate ($Ca_4(PO_4)_2O$, TTCP) are starting materials for the harden reaction of the bone cement [3].

To develop biomaterials for utilizing for bone tissue, various properties, e.g. biological, physical or chemical property, should be satisfied. Among them, biological safety and osteogenesis properties, e.g. proliferation and differentiation of the osteoblasts, should be important factors to provided to the biomaterials. However, understandings of the biological interaction between osteoblasts and various CP ceramics are few, since the interaction has not been studied under the same experimental condition in detail. Therefore, we estimated effects of various CP ceramics on the properties of normal human osteoblasts (NHOst) as well as a viability of V79 fibroblasts in this study.

Materials and Methods

Materials

Cytotoxicity and osteogenesis of NHOst on five kinds of CP ceramics, namely, HAp, FAp, α-TCP, β-TCP and TTCP (Wako chem. Co. Ltd., Tokyo, Japan), were evaluated. 0.25g of CP powders was put into stainless mold and uniaxially pressed at 30MPa for 1 min to form pellets. The dimensions of the obtained CP pellet were 1mm in thickness and 12mm in diameter. CP pellets were sterilized by the autoclave 121°C for 20 min.

Cytotoxicty test on CP ceramics

Cytotoxicity test was carried out using Chinese hamster V79 lung fibroblasts by the colony assay system. V79 cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd.,) with 10% fetal calf serum (FCS, Intergen company) and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

The method of cell seeding was shown below; At first, each CP pellets were placed in each culture wells of 24 well culture plates (Corning Co. Ltd.) and 300μl of culture medium was added into each well. Next, 50 cells/300μl of culture medium was added into each well and incubated for 4 h at 37°C. Finally, 400μl of culture medium was added and incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 7days.

In order to investigate the cell adhesive property on the CP ceramics, the culture medium was changed after cultivations for 4 h and incubated for 7days. The removed culture medium was transferred to another well of the plate and incubated for 7days as well.

Cytotoxicity of extracts from CP ceramics was also investigated in this study. Suspensions of CP ceramics in the culture medium (100mg/mL) were stirred at 37°C for 3days in 150rpm. The suspensions were centrifuged and the supernatants were collected to use as test extracts. The cytotoxicity test was carried out culturing 50 V79 cells in 1ml of the extracts and incubated at 37 °C for 7days.

After 7days, the cells were fixed in methanol and the number of the V79 colonies was counted after staining cells with 5%-Giemsa solution. In addition, the pH of the medium after 7-days culture was measured to estimate effect of the pH of the medium on the cytotoxicity test.

Osteogenesis evaluation of NHOst cultured on CP ceramics

NHOst were purchased from BioWhittaker Inc.(Walkersville,MD). The NHOst were maintained in alpha minimum essential medium (αMEM, Gibco, Grand Island, NY) containing 10%-FCS. incubators at 37 °C in a humidified atmosphere with 5% CO_2. All assays were performed using αMEM containing 10%-FCS supplemented with 10mM beta-glycerophosphate. Similar to the method of the cytotoxicity test, each CP pellets were placed in 24-well culture plates (Corning Co. Ltd.) and 300μl of culture medium was added into each well, followed by addition of 1ml of cell suspension (4×10^4 cells/ml) into each well.

Proliferation of NHOst cells cultured on various kinds of CP ceramics was estimated by Tetracolor One assay (Seikagaku Co., Ltd. Tokyo, Japan), which incorporates an oxidation reduction indicator based on detection of metabolic activity. After 7-days incubation, 2%-TetraColor One/αMEM solution was added to each well, followed by 2h incubation. The absorbance of the supernatant at 450nm was estimated using μQuant spectrophotometer (Bio-tek Instrument, Inc., Winooski, VT). After estimating the proliferation, the cells were washed by phosphate–buffered saline (PBS(-)), followed by addition of 1ml of 0.1M glycine buffer (pH=10.5) containing 10mM $MgCl_2$, 0.1mM $ZnCl_2$ and 4mM p-nitrophenylphosphate sodium salt. After incubating at room temperature for 5min, the absorbance of 405 nm of glycine buffer was detected using μQuant spectrophotometer to evaluated alkaline phosphatase (ALP) activity of the test cells. The amount of Osteocalcin produced by NHOst was evaluated using Gla-type Osteocalcin EIT kit (Takara. Co., Ltd.). The structural change of CP before and after autoclave sterilization or culture were investigated by powder X-ray diffraction (XRD) analysis and scanning electron microscopy(SEM). XRD analysis was carried out (Rigaku Co., Ltd. / RINT 2000) with the CuK_α radiation at 40kV, 50mA. SEM observations were performed (JEOL / JSM-5800LV) with an accelerating voltage of 25kV.

Results

Cytotoxicity of various CP ceramics

The results of the cytotoxicity test of CPs are summarized in table 1. Notably, the colonies were hardly formed on FAp and α-TCP pellets and the ratios of the colony formation against V79-alone culture were 22.6% and 0.0%, respectively. In addition, the ratios of the colony formation on the HAp, β-TCP and TTCP pellets were 58.1%, 57.3% and 78.4%, respectively. From these results, it is suggested that V79 cells were viable and adhered on the pellet after for 4h after seeding despite of the type of CP ceramics, irrespective of the type of CP ceramics. On the other hand, the cytotoxicity test of extracts from CPs revealed that the tendency of their cytotoxicity was almost the same as that of the respective CP pellets themselves (table1).

Proliferation and differentiation of NHOst cultured on CP ceramics

The effects of various kinds of CP ceramics on the osteogenesis of NHOst are represented in table2. The effects of the CPs on proliferation were consistent with those on the colony formation. Similar to the cytotoxicity test, the proliferation of NHOst was inhibited on FAp and α-TCP pellets. ALP activities of NHOst on CP ceramics after 7-days culture were significantly suppressed in comparison with that of NHOst alone. On the other hand, the osteocalcin amounts produced from NHOst were influenced by the type of CP ceramics. NHOst on β-TCP showed the highest Osteocalcin production among five kinds of CP ceramics.

4. Discussion

The fact that less formation of colonies was observed on FAp and α-TCP pellets suggests that they are strongly cytotoxic. It is suggested that the differences in the colony formation on various CP pellets are ascribed to difference in extract properties from the CP related with the composition or crystal structure (table1). In addition, proliferation of NHOst also was inhibited on FAp and α-TCP. The pH values of culture medium after incubation for 7 days are shown in table1. As shown in the table, the pH of culture medium after incubation with FAp pellets is almost the same as that of HAp, while the pH of the α-TCP medium is much lower than other CP ceramics. In order to considering the reason of the low pH of the culture medium with α-TCP pellet, a surface structural change of α-TCP before and after incubation was analyzed by SEM. SEM images of α-TCP after extraction treatment are shown in Fig.1.

Table1. Cytotoxicity test of various CP ceramics.

Samples	Composition	Formation of colony		pH of medium after culturing	Ca concentration[1] /ppm
		On pellets /%	Extraction / %		
V79 alone	-	100.0±4.5	100.0±13.9	7.12	-
HAp	$Ca_{10}(PO_4)_6(OH)_2$	58.1±12.8	84.6±15.1	7.24	0.19
FAp	$Ca_{10}(PO_4)_6F_2$	22.6±20.9**	26.9±8.6*	7.20	0.17
α-TCP	α-$Ca_3(PO_4)_2$	0.0*	7.6±5.1*	6.76	72.62
β-TCP	β-$Ca_3(PO_4)_2$	57.3±6.9	81.1±19.3	7.40	1.27
TTCP	$Ca_4(PO_4)_2O$	78.2±5.0	93.7±6.8	7.65	0.58

*p<0.01 against V79 alone, **p<0.05 against V79 alone, 1)The Ca ions concentration was extracted Ca ions from CP ceramics in PBS(-), which were measured by inductivity coupled plasma-atomic emission spectroscopy.

Table2. Osteogenesis of NHOst cultured on various kinds of CP ceramics.

Samples	Proliferation / %	Differentiation level	
		ALP activity / %	Osteocalcin / %
NHOst alone	100±7.9	100±4.4	100±46.2
HAp	63.2±3.5	20.4±1.8*	81.1±31.0
FAp	42.9±19.5	1.24±0.3*	47.2±20.7
α-TCP	18.3*±2.5	17.9±3.8*	110.7±18.8
β-TCP	56.0±4.7	6.3±3.2*	177.1±78.4**
TTCP	82.3±27.3	17.5±4.6*	114.8±4.0

*p<0.01 against NHOst alone, **p<0.01 against HAp

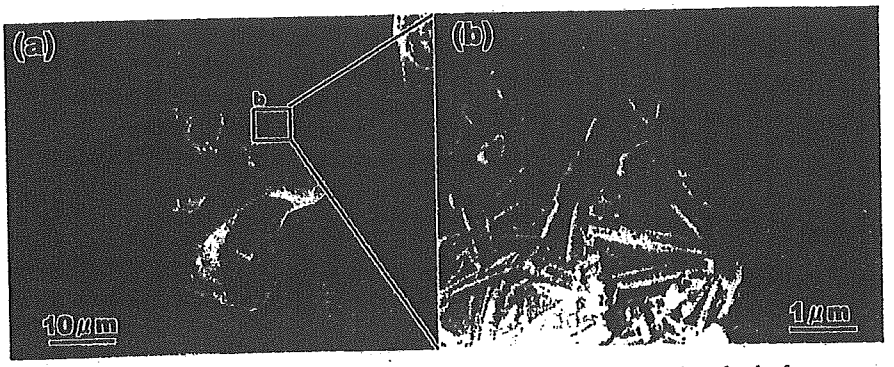

Fig.1. SEM images of α-TCP after extract treatment. (a) whole image and (b) enlarged image of the area enclosed by a rectangle in (a)

Before the extraction, a particle size of α-TCP was about 10μm and its surface was smooth. However, as shown in Fig.1, whisker-like precipitates of 1-2μm in length and 2-300nm in width are observed at the surface of α-TCP after the extraction, although there is no change in its particle size. It is well known that slightly water-soluble calcium phosphates convert to HAp in aqueous solution with high pH value. Since the solubility of α-TCP is higher than that of other calcium phosphates, the α-TCP converts to HAp rapidly as following the reaction.

$$10Ca_3(PO_4)_2+6H_2O \rightarrow 3Ca_{10}(PO_4)_6(OH)_2+2H_3PO_4$$

According to the report of this conversion [4], HAp produced by the above reaction has whisker-like morphology. Therefore, the whisker-like precipitates can be regarded as HAp, so that it is considered that the above conversion occurs at the surface of the α-TCP during incubation. In this case, phosphoric acid is produced as a byproduct in the conversion reaction and the phosphoric acid caused the decrease in the pH of solution. Morita and co-workers [5] have reported that low pH itself could be clastogenic to mammalian cells and the pH of 50% V79 cell survival was 6.5 for 24h incubation. Therefore, it is suggested that the cytotoxicity of α-TCP was mainly due to the pH decreasing resulting from an increase of the phosphoric acid ion produced by the hydrolysis conversion from α-TCP to HAp.

On the other hand, FAp has the same crystal structure of HAp but the hydroxyl ions in HAp substituted by fluorine ions. Since difference of the colony formation on various CP ceramics would be due to eluted substances from CP as described above, the cytotoxicity of FAp would be due to eluted fluoride ions from FAp.

Effects of CP ceramics on osteogenesis function of NHOst are shown in table2. As shown in the table, ALP activities of NHOst were significantly suppressed on CP ceramics irrespective of their type and the amount of osteocalcin on β-TCP was the highest among five kinds of CP ceramics. Since it is well known that osteocalcin express in maturated stage of differentiation level of NHOst, these results suggest that maturation of NHOst proceeds on β-TCP. . The differences of maturation of NHOst on various kinds of CP ceramics may be related with the amount of extracted Ca^{2+} and/or PO_4^{3-} ions.

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Cytotoxicity of Various Calcium Phosphate Ceramics

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Keywords: Calcium phosphate ceramics, Cytotoxicity,

Abstract. The cytotoxicity of five calcium phosphate ceramics, hydroxyapatite (HAp), flouroapatite (FAp), α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP), was investigated. Based on the guidelines of biological test for medical devices in Japan, a cytotoxicity test of these calcium phosphates was carried out using Chinese hamster V79 lung fibroblasts. The cytotoxic study revealed that FAp and α-TCP showed high cytotoxicities. From various analyses, it was considered that the cytotoxicity of the FAp was due to fluorine ions extracted in a culture medium and the cytotoxicity of α-TCP resulted from a decrease in pH of the medium by the phosphoric acid, which produced by hydrolysis of the α-TCP.

Introduction

From the view point of biological affinity to bone, calcium phosphate (CP) ceramics have been studied to utilize for many purposes in a medical field. For instance, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) and β-tricalcium phosphate (β-$Ca_3(PO_4)_2$, ß-TCP), are known to be biologically bonded to natural bones and their porous materials have been studied for effective restoration of bone defects.[1,2] Fluoroapatite ($Ca_{10}(PO_4)_6F_2$, FAp) has been reported to have a potential of novel bone repairing materials with high stability *in vivo*, since solubility of FAp is lower than that of HAp.[3,4] In addition, CP cement is also promising for bone repair and it is well known that α-tricalcium phosphate (α-$Ca_3(PO_4)_2$, α-TCP) and tetracalcium phosphate ($Ca_4(PO_4)_2O$, TTCP) are starting materials for the harden reaction of the bone cement.[5,6]

To develop biomaterials for utilizing for bone tissue, various properties, e.g. biological, physical and chemical property, should be satisfied. Among them, biological safety is important for the biomaterials. Since only a few studies which discuss the cytotoxicity of calcium phosphate ceramics have been reported, the cytotoxicity of CP ceramics is worthy to be investigated in order to design bioceramics with good biological safety for medical application. Therefore, the cytotoxicities of five calcium phosphate ceramics, hydroxyapatite (HAp), flouroapatite (FAp), α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP) were investigated.

Materials and Methods

Materials

Five kinds of CP ceramics, HAp, FAp, α-TCP, β-TCP and TTCP were purchased from Wako chem. Co. Ltd. CP powders (0.25 g) was put into stainless mold and uniaxially pressed at 30MPa for 1 min to form a pellet. The dimensions of the obtained CP pellet were 1mm in thickness and 12mm in diameter. CP pellets were sterilized by an autoclave at 121°C for 20 min.

Cytotoxicty test on CP ceramics

Cytotoxicity test was carried out using Chinese hamster V79 lung fibroblasts by a colony assay system. V79 cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd.) with 10% fetal calf serum (FCS, Intergen Co. Ltd.) and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

The method of cell seeding in the cytotoxicity test of CP ceramics was shown below; each CP pellets were placed in each culture wells of 24 well culture plates (Corning Co. Ltd.) and 300μl of culture medium was added into each well. Then, 50 cells/300μl of the cell suspension in the

culture medium were added into each well and incubated at 37°C for 4 h. Finally, 400μl of the culture medium was added into each well and the plates were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 7days. In order to investigate a cell adhesive property on the CP ceramics, the culture medium was changed after 4 h and further incubated for 7days. The removed culture medium was transferred to another well of a new plate and incubated for 7days as well.

Cytotoxicity of extracts from CP ceramics was also investigated in this study. Suspensions of CP ceramics in the culture medium (100mg/mL) were stirred at 37°C for 3days under the rotation condition at 150rpm. The suspensions were centrifuged and the supernatants were collected as test extracts. In addition, media with various pH values were prepared using HCl solution to investigate an effect of pH on cell survival. Fifty V79 cells in 1ml of the extracts or the medium with different pH value were incubated at 37 °C for 7days.

After 7-day incubation, the cells were fixed in methanol and the number of the V79 colonies was counted after staining cells with 5%-Giemsa solution to estimate the cytotoxicity of the test sample. In addition, the pH of the medium after 7-days culture was measured to estimate the effect of the pH of the medium on the cytotoxicity test.

Characterization of CP ceramics

The structural changes of CP before and after an autoclave-sterilization or an incubation at 37°C culture were investigated by powder X-ray diffraction (XRD) analysis and scanning electron microscopy (SEM). XRD analysis was carried out (Rigaku Co., Ltd. / RINT 2000) with the CuK_α radiation at 40kV, 50mA. SEM observations were performed (JEOL / JSM-5800LV) with an accelerating voltage of 25kV.

Results and Discussion

Cytotoxicity of various CP ceramics

From XRD analysis, no structural changes of CPs were observed after an autoclave sterilization. After staining CP pellets, it was observed that cell colonies were formed on various CP ceramics pellets (Fig.1(a)). The results of the cytotoxicity test of CPs are shown in Fig.1(b). The cell colonies were hardly formed on FAp and α-TCP pellets and the ratios of the colonies formed on these pellets against V79-alone culture were 22.6% and 0.0%, respectively. In addition, the ratios of the colonies on the HAp, β-TCP and TTCP pellets were 58.1%, 57.3% and 78.4%, respectively. As no colonies were observed after 7-day culture of the removed medium in cell adhesion studies of CP ceramics, these results suggested that V79 cells can adhere and be viable on these pellets, irrespective of the type of CP ceramics. Figure 2 shows the formation of colonies cultured in extract from CP ceramics. The cytotoxicity test of extracts from CPs revealed that the tendency of their cytotoxicities was similar to that of the cytotoxicities on the respective CP pellets themselves (Fig.1(b)).

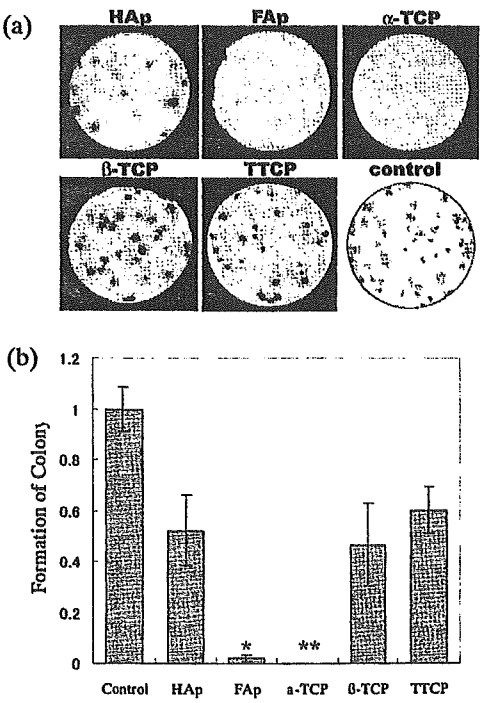

Fig.1. The appearance of colonies on various CP pellets (a) and their colony formation ratios (b). (*p<0.05 against for V79 alone, **p<0.01 against for V79 alone)

The fact that less formation of colonies was observed on FAp and α-TCP pellets suggests that they are highly cytotoxic. In addition to results shown in Fig.2, it is suggested that the differences in the colony formation ratio on various CP pellets are ascribed to difference in extract properties from the CP, which may be related with the composition or crystal structure. As shown in Table 1, the pH of culture medium after incubation with FAp pellets is almost the same as that of HAp, while the pH of the α-TCP-incubated medium is much lower than that of the other CP ceramics-incubated media.
In order to consider the reason of the low pH of the a-TCP-incubated medium, a surface structural change of α-TCP before and after incubation was analyzed by SEM. SEM images of α-TCP before and after extraction treatment are shown in Fig.3. Before extraction, a particle size of α-TCP was about 10μm and its surface was smooth (Fig.3(a) and (b)). However, whisker-like precipitates of 1-2μm in length and 2-300nm in width were observed at the surface of α-TCP after the extraction, although there was no change in its particle size (Fig.3(c) and (d)). It is well known

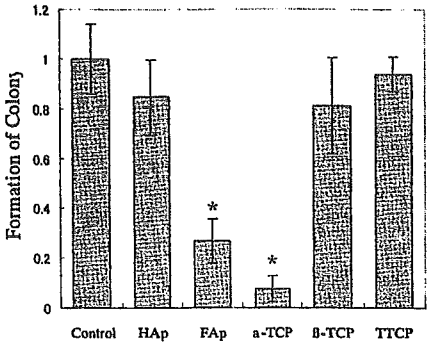

Fig.2. Formation of colony cultured in extract from various CP ceramics. (*p<0.01 against for V79 alone)

Table 1. The pH and Ca concentration of culture medium after incubation.

Samples	pH of medium after culturing	Ca concentration /ppm
V79 alone	7.12	-
HAp	7.24	0.19
FAp	7.20	0.17
α-TCP	6.76	72.62
β-TCP	7.40	1.27
TTCP	7.65	0.58

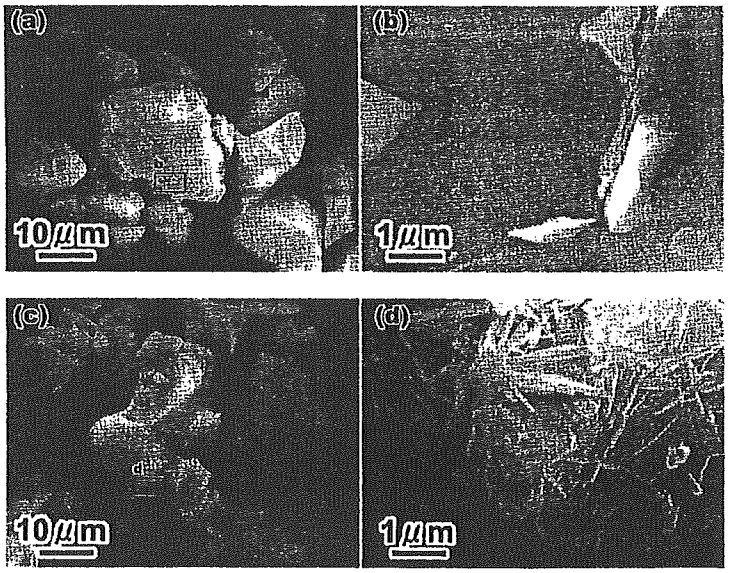

Fig.3. SEM images of α-TCP before (a), (b) and after extract treatment (c), (d).
(a) and (c) are whole image of before and after extract treatment, respectively. (b) and (d) are enlarged image of the area enclosed by a rectangle in (a) and (c), respectively.

that calcium phosphates convert to HAp in aqueous solution with high pH value. Since the solubility of α-TCP is higher than that of other calcium phosphates, α-TCP rapidly converts to HAp as follows;

$$10Ca_3(PO_4)_2+6H_2O \rightarrow 3Ca_{10}(PO_4)_6(OH)_2+2H_3PO_4$$

According to the report of this conversion [7], HAp produced by the above reaction has whisker-like morphology. Therefore, the whisker-like precipitates in Fig.3 (d) can be regarded as HAp, so that it is considered that the above conversion occurs at the surface of the α-TCP during incubation.

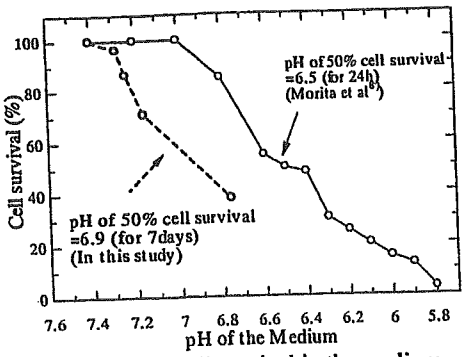

Fig.4. V79 cell survival in the medium with various pH values.

In this case, phosphoric acid is produced as a byproduct in the conversion reaction and the phosphoric acid causes the decrease in pH of the solution. As shown in Fig.4, Morita and co-workers[8] have reported that low pH itself could be clastogenic to mammalian cells and the pH of 50% V79 cell survival was 6.5 for 24h incubation. In the present colony assay system, the pH of 50% V79 cell survival was 6.9 for 7-days incubation. In addition, we confirmed that phosphoric acid showed no or weak cytotoxicities under our present experimental conditions. Therefore, it is suggested that the cytotoxicity of α-TCP is mainly due to the pH decrease resulting from an increase of the phosphoric acid ion by the hydrolysis conversion from α-TCP to HAp.

On the other hand, FAp has the same crystal structure of HAp but the hydroxyl ions in HAp substituted by fluorine ions. Since it is probable that difference of the colony formation on various CP ceramics are due to eluted substances from CP as described above, the cytotoxicity of FAp would be due to eluted fluoride ions from FAp. In conclusion, this study has revealed that FAp and α-TCP have a cytotoxicity, while TTCP has lower cytotoxicity than other calcium phosphates. To develop biomaterials made from calcium phosphate, further studies are necessary to clarify their cytotoxic mechanisms.

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Novel Calcium Phosphate Ceramics : The Remarkable Promoting Action on the Differentiation of the Normal Human Osteoblasts

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Keywords: Hydroxyapatite, Niobium ion, Osteoblast, Alkaline phosphatase activity

Abstract.
To promote the activity of normal human osteoblasts (NHOst), the novel HAp ceramics containing Nb ions (NbHAp) were synthesized by wet chemical process, which reacting aqueous solution containing a mixture of $Ca(NO_3)_2$, $(NH_4)_2HPO_4$, and the Nb aqueous solution. X-ray diffraction patterns indicated that NbHAp had a monolithic apatitic structure, although crystallite decreased as Nb content increased. From inductively coupled plasma analysis, maximum amount of Nb ions in the sample was almost 8.2atom% of P ions. The NbHAps were presented as aggregates and composed of fine crystal of <1μm in diameter. Nb ions in NbHAp were uniformly distributed in the aggregates. Furthermore, high-resolution XPS spectra of Nb $3d_{5/2}$ indicated that Nb ions in the HAp were presented as Nb^{5+}. These results suggested that Nb ions were at PO_4 site in crystal structure of HAp. When NHOst were cultured with the NbHAp, their ALP activity were twice as much as that of NHOst cultured with HAp without Nb ions.

Introduction

Tissue engineering takes advantages of the combined use of cultured living cells and scaffolds to deliver vital cells to the damaged site of the patient. Some tissue engineering approaches have been devised to repair large bone defect. In developing of the scaffold for bone tissue, the interaction between osteoblasts cells and scaffolds are much important. To achieve the restoration the bone tissue at early stage, the scaffold is required to have the ability of promoting proliferation and mineralization.

It is well known that hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) ceramics can be biologically bonded to natural bones and have been studied to utilize as the scaffolds. In addition, the structure is very tolerant of ionic substitutions and Ca^{2+} ions, PO_4^{3-} ions and OH^- ions can be replaced by various cationic or anionic ions, partly or completely[1]. For example, K^+, Mg^{2+} and Sb^{3+}, can substituted for Ca ions and CO_3^{2-} and VO_4^{3-} can substituted for PO_4^{3-} ions, completely or partially. Thus various kinds of ion substitutions can be made to synthesize novel modified-HAps.

Recently, our co-workers reported that niobium (Nb) ions have the significant effect which promotes the proliferation and differentiation of normal human osteoblastic cells (NHOst)[2]. In the present study, therefore, we attempted to synthesize the novel HAp ceramics containing Nb ions (NbHAp) to promote the activity of NHOst and investigated the interaction between NbHAp and NHOst.

Materials and Methods

Synthesis of Nb containing HAp

The NbHAp was synthesized by wet chemical process, which reacting aqueous solution containing a mixture of $Ca(NO_3)_2$, $(NH_4)_2HPO_4$, and the Nb aqueous solution. The reagent grade $Ca(NO_3)_2$, $(NH_4)_2HPO_4$ and $NbCl_5$ (Wako Pure Chemical Industries, Ltd) were used without purification. The metal ion chemical reagent was completely dissolved in an exact amount of distilled water. The Nb aqueous solution was prepared by the mixing of distilled water and $NbCl_5$ solution, which dissolved in 5vol%-hydroxyaceton and 5vol%-2-aminoethanol[3].

0.2M-$(NH_4)_2HPO_4$ and 0.01M $NbCl_5$ solutions were mixed and stirred with a magnetic bar. The Nb/(Nb+P) molar ratio of the mixing solution was set to 0.0000, 0.0167 and 0.1667. The pH of the mixing solution was adjusted to 10 using 1N-NaOH. 0.2M-$Ca(NO_3)_2$ was slowly dropped in the mixing solution (20ml/min) The ionic content of those starting solutions are shown in table 1. The pH was monitored and the reaction was terminated at pH 10.0. After the reaction, the suspension was stirred for 24h at room temperature. The precipitates were centrifuged at 3000rpm for 5min and washed with distilled water. The obtained apatites were annealed at 800°C for 2h (heating rate: 5°C/min). In this study, those precipitates obtained by reaction of $Ca(NO_3)_2$ solution and the mixing solution with different Nb/(Nb+P) molar ratio of 0.000, 0.0167 and 0.1667 are named HAp , NbHAp-I and NbHAp-II, respectively.

Characterization of NbHAp

The NbHAps were characterized by X-ray diffraction analysis (XRD, Rigaku, Rint2000). Ca, P and Nb ions concentrations in apatites are measured by inductively coupled plasma (ICP, Hewlett-Packard, HP4500). Microstructural evaluation was performed by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) (JEOL, LV5800). The chemical state of Nb ions in HAp was investigated by X-ray photon spectroscopy (XPS, Shimadzu, ESCA-3200).

Osteogenesis evaluation of NHOst cultured with NbHAp

NHOst were purchased from BioWhittaker Inc.(Walkersville,MD). The NHOst were maintained in alpha minimum essential medium (αMEM, Gibco, Grand Island, NY) containing 10%-FCS in incubators at 37°C in a humidified atmosphere with 5% CO_2. All assays were performed using αMEM containing 10%-FCS supplemented with 10mM beta-glycerophosphate. NHOst cells (4×10^4 cells/well/ml) were co-cultured with 5mg of the apatites for 7days to evaluated the effects of the apatites on NHOst.

Proliferation of NHOst cells cultured with the apatites was estimated by Tetracolor One assay (Seikagaku Co., Ltd. Tokyo, Japan), which incorporates an oxidation reduction indicator based on detection of metabolic activity. After 7-days incubation, 2%-TetraColor One/αMEM solution was added to each well, followed by 2h incubation. The absorbance of the supernatant at 450nm was estimated using μQuant spectrophotometer (Bio-tek Instrument, Inc., Winooski, VT). After estimating the proliferation, the cells were washed by phosphate–buffered saline (PBS(-)), followed by addition of 1ml of 0.1M glycine buffer (pH=10.5) containing 10mM $MgCl_2$, 0.1mM $ZnCl_2$ and 4mM p-nitrophenylphosphate sodium salt. After incubating at room temperature for 5min, the absorbance at 405 nm was detected using the μQuant spectrophotometer to evaluated alkaline phosphatase (ALP) activity of the test cells.

Results and Discussion

XRD patterns of NbHAps prepared by wet chemical process are shown in Fig.1(a). Irrespective of Nb/(Nb+P) molar ratio in starting solution, the precipitates were identified as monolithic HAp.

Table1. The ionic content of starting solution and the composition of the obtained precipitates.

Samples	Ionic content of Starting Solution*,			Theoretical Ca/(Nb+P)**	Nb/(Nb+P)**		Color of Precipitates
	Ca	PO_4	Nb		Theoretical	Measured***	
HAp	60.0	36.0	0.0	1.67	0.0000	-	White
NbHAp-I	60.0	35.4	0.6	1.67	0.0167	0.015	Pale yellow
NbHAp-II	60.0	30.0	6.0	1.67	0.1667	0.082	Buff yellow

*mmol, **Molar ratio, ***The precipitates were dissolved with HCl and the ionic concentration of HCl solutions were measured by ICP.

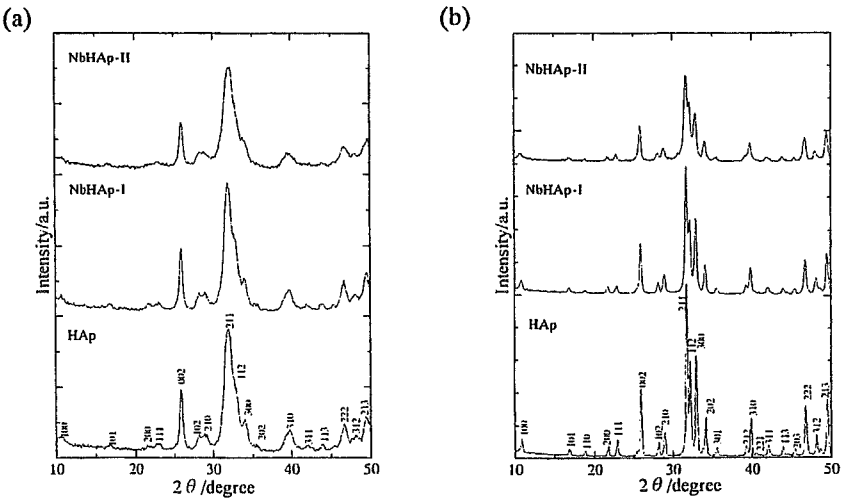

Fig.1. XRD patterns of HAp and NbHAp-I and NbHAp-II before (a) and after (b) annealing(800°C, 2h).

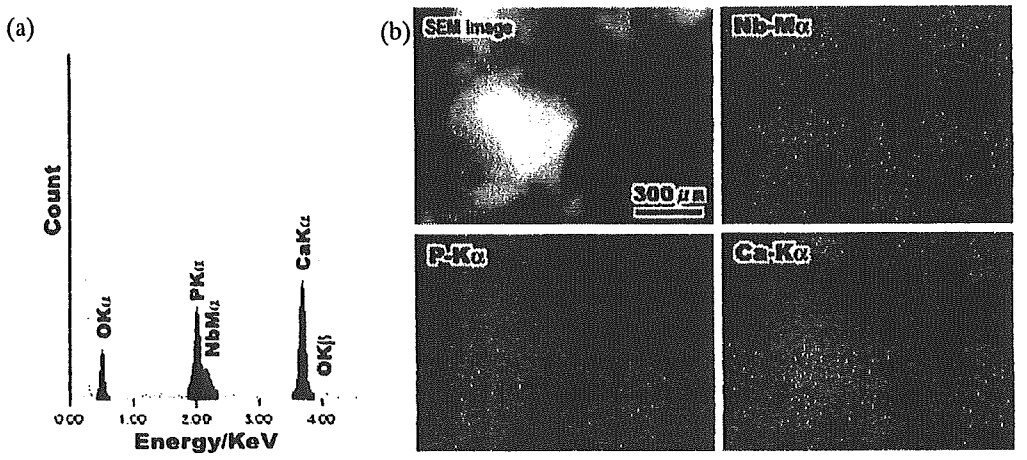

Fig.2. SEM-EDX analysis of NbHAp-II. ((a) An EDX spectrum and (b) SEM image and element mapping images of Nb, Ca and P).

As shown in Table1, the Nb/(Nb+P) molar ratio of NbHAp-I and NbHAp-II were 0.015 and 0.082, respectively. SEM observation revealed that the precipitates were present as aggregates composed of primary particles of less than 1μm in diameter.

XRD patterns of NbHAps annealed at 800°C are shown in Fig.1(b). The crystalline of the precipitates become high by the annealing and XRD patterns of all annealed NbHAps could be identified as monolithic apatitic structure. It is noted that the crystallite of the NbHAp decreased as Nb content increased. Figure 2(a) shows an EDX spectra of the whole region of SEM image in Fig.2(b). The EDX spectrum from Nb M_α was separated from P K_α line and could be observed at 2.17KeV, although the intensity of the spectra was weak. The mapping image of Nb, Ca and P ions are shown in Fig.2(b). As shown in Fig.2(b), Nb ions were present at the same site of Ca and P ions. Based on these observations, Nb ions are suggested to be uniformly distributed in the

aggregates. High-resolution XPS spectrum of Nb $3d_{5/2}$ of NbHAp-II annealed at 800°C is shown in Fig.3. The peak of XPS spectra due to $3d_{5/2}$ of Nb ions from annealed NbHAp-II is at 208.3eV. Since XPS peak of $3d_{5/2}$ due to Nb^{2+} from NbO and Nb^{5+} from Nb_2O_5 appears at 203.5eV and 207.2eV, respectively, the Nb ions in NbHAp can be identified as Nb^{5+}.

These results suggest that the NbHAp has apatitic structure containing Nb ions and the Nb ions are homogenously distributed in the grain. Generally, Nb^{5+} ions in the solution is not present as Nb^{5+} but as niobiumate acid, $H_xNb_6O_{19}^{(8-x)-}$ ions (X=0,1,2)[4]. The PO_4 in HAp can be replaced by anionic atomic group, e.g. CO_3^{2-}, VO_4^{3-} and AsO_4^{3-}. Therefore, it is probable that Nb ions are substituted in PO_4 site in HAp. However, measured Nb/(Nb+P) molar ratio in NbHAp-II was 0.082, despite their theoretical Nb/(Nb+P) ratio of 0.1667, suggesting that the value of the measured ratio might be the maximum amount of Nb ions in PO_4, practically.

Since Nb ions are expected to have an effect to promote the proliferation and ALP activity of osteoblastic cells, the NbHAps have a potential to promote the ALP activity of osteoblastic cells.

Figure 4 shows ALP activity of NHOst cultured with annealed NbHAps. As shown in Fig.4, NHOst cultured with the NbHAp expressed the ALP activities twice as much as that of NHOst cultured with HAp without Nb ions. It is well known that ALP is often expressed when fracture of bone is repaired *in vivo*. Furthermore, from the recent study, it has revealed that the ALP contributed to mineralization in bone formation[5]. Therefore, this enhancement in ALP activity of NHOst by NbHAp suggests that the NbHAp can promote the mineralization of bone formation.

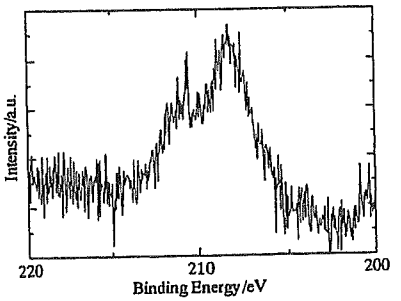

Fig.3. High-resolution XPS spectrum of Nb $3d_{5/2}$ of NbHAp-II annealed at 800°C.

Conclusion

We have succeeded to synthesize novel HAp containing Nb ions. The NbHAp would be a solid solution, which Nb ions were in PO_4 site in HAp and could enhance the ALP activity in NHOst.

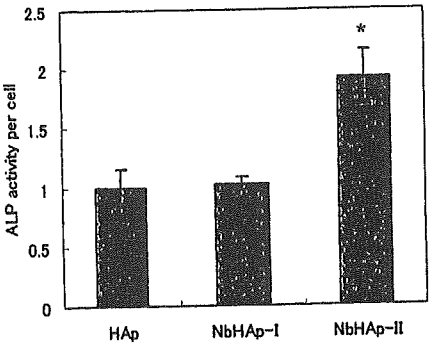

Fig.4. ALP activity of NHOst cultured with annealed NbHAp
*p<0.05 against HAp (without Nb ions)

Acknowledgment

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Enhancement of Differentiation and Homeostasis of Human Osteoblasts by Interaction with Hydroxyapatite in Microsphere Form

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Key words: osteoblasts, differentiation, homeostasis, hydroxyapatite, biocompatibility

Abstract. The aseptic loosening of artificial joints with associated periprosthetic bone resorption may be partly due to the suppression of osteoblast function to form new bone by wear debris derived from the joint. To assess the effect of wear debris on osteoblasts, we cultured normal human osteoblasts (NHOst) in contact with several kinds of microspheres as models of wear debris. The NHOst in contact with polystyrene, polyethylene, and alumina microspheres showed a lower differentiation level than NHOst alone as estimated from the amounts of deposited calcium. On the other hand, hydroxyapatite particles enhanced the differentiation of NHOst. In addition, sintered hydroxyapatite enhanced expression of osteocalcin mRNA and gap junctional communication of NHOst. This study suggests that polystyrene, polyethylene, and alumina microspheres have the potential to disorder not only the differentiation but also the homeostasis of NHOst in contact with them. However, hydroxyapatite enhanced the differentiation as well as the homeostasis of NHOst, even in microsphere form, suggesting its good biocompatibility as biomaterials for bone tissues.

Introduction

Biomaterials implanted into the harsh environment of the body cannot maintain their original shape, or even their desired function, resulting in undesirable side effects. One good example is the aseptic loosening of artificial joints observed in many patients who underwent a total joint replacement 5 to 25 years ago. Many researchers have reported that aseptic loosening with associated periprosthetic bone resorption is partly due to the activation of macrophages and osteoclasts by wear debris from the artificial joint [1-3], but few researches have focused on the interaction between wear debris and osteoblasts, especially normal human osteoblasts [4]. In this study, normal human osteoblasts were cultured in contact with various kinds of microspheres made from polymers or ceramics used as model wear debris, and the effects of the microspheres' characteristics and interaction conditions were discussed in regard to the proliferation, differentiation and homeostasis maintenance of the osteoblasts.

Materials and Methods

Microspheres. Monodispersed polystyrene (PS) microspheres with different diameters (0.1, 0.5, 1, 5, and 10 μm) were kindly supplied by Japan Synthetic Rubber Co., Ltd. (Tokyo, JAPAN). Low-density polyethylene (PE) microspheres were kindly provided by Sumitomo Seika chemicals Co., Ltd. (Tokyo, JAPAN). Alumina (Al_2O_3) microspheres were obtained from the Association of Powder Process Industry and Engineering. Sintered and un-sintered hydroxyapatite (HAp) microspheres (7.2 μm in diameter) were prepared and supplied by Ube Material Industries, Ltd. (Chiba, Japan). Determined by Multisizer II (Coulter Electronics Inc., Hialeah, FL), the average diameters of PE and alumina microspheres were found to be 6.4 and 5.1 μm, respectively. Sterile microspheres and microsphere-coated plates were prepared by the method previously reported [5]. The obtained microspheres and microsphere-coated plates (20 μg/well) were subjected to the assays.

Cellular Assays. Normal human osteoblasts (NHOst) were purchased from BioWhitteker Inc. (Walkersville, MD). The cells were maintained using alpha minimum essential medium (Gibco) containing 20% fetal calf serum (FCS) in incubators (37°C, 5%-CO_2-95%-air, saturated humidity).

All assays were carried out using the medium supplemented with 10mM β-glycerophosphate. NHOst ($2x10^4$ cells/well/500 μl medium) were cultured on the microsphere-coated plates for estimating the effect of the microspheres from the bottom of the cells. To estimate the effect of microspheres on cells adhered to the culture plates, the NHOst were cultured with microsphere-containing medium (20 μg/500 μl medium) after they had adhered to the collagen-coated plates. The cell number ratio of NHOst cultured with microspheres was evaluated using the alamar Blue™ assay (BioSource International, Inc., Camarillo, CA), which incorporates an oxidation-reduction indicator based on the detection of metabolic activity, according to manufacturer's instruction.

The level of alkaline phosphatase (ALP) activity of the NHOst and the amounts of calcium deposited during a 7-day incubation were evaluated to estimate differentiation level of NHOst as previously reported [6]. In addition, RT-PCR was performed to detect the expression of osteocalcin mRNA in the NHOst (primers for human osteocalcin [7]; forward 5'CATGAGAGCCCTCACA3' and reverse 5'AGAGCGACACCCTAGAC3'; product size 307-bp).

Gap junctional intercellular communication (GJIC), which is a function that plays an important role in maintaining cell and tissue homeostasis by exchanging low molecular weight molecules [8], among NHOst co-cultured with microspheres were evaluated using FRAP assay as previously reported [9].

All data were expressed as the mean value ± the standard deviation (SD) or the standard error of means (SEM) of the obtained data as indicated in all figures and tables. The Fisher-Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

Results and Discussion

Figure 1 shows the effect of the diameter of pre-coated polystyrene microspheres on proliferation, the ALP activity of co-cultured NHOst cells, and the amounts of deposited calcium on the NHOst. To compare the effect of the microspheres on the ALP activities and the calcium amounts for each NHOst, the obtained data were standardized based on the cell number ratio co-cultured with the microspheres. As shown in figure, suppression on ALP activity of NHOst and the amounts of deposited calcium were observed when 0.1 μm and 5 μm microspheres were co-cultured. When the microspheres were added after cell adhesion, they did not show a significant inhibitory effect on the functions of NHOst (data not shown). By pre-coating of the microspheres on the bottom of the test plates, the area they occupied became larger as their diameter became smaller. This increase in the microsphere occupied area would affect many functions of the test cells, resulting in the inhibitory effect of the 0.1μm microspheres on the function of NHOst when the same quantity of microspheres was coated. On the other hand, the suppression of ALP activity of NHOst and calcium deposition by pre-coated 5 μm PS microspheres suggests that not only the area they occupied but also their size may cause the unique inhibitory activity of the 5 μm PS microspheres. It is well known that the size of a microsphere plays an important role in phagocytosis [10], although it is unclear that there is the same size dependence on phagocytosis by the NHOst as by macrophages. In addition, our previous study suggested that even fibroblasts were likely to phagocyte microspheres of a specific

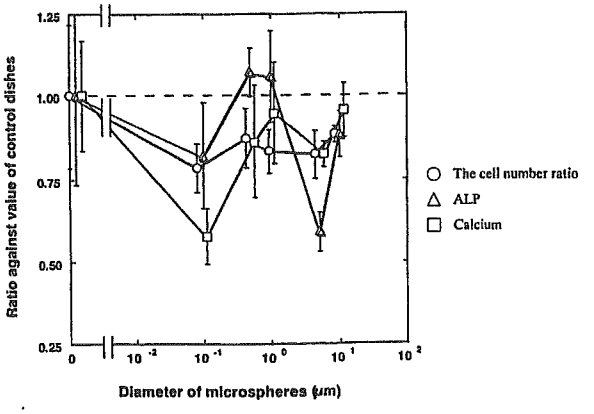

Figure 1. Effects of diameter of pre-coated PS on various functions of NHOst. Data are shown as the means±SD

diameter [5,9]. Taking into account our findings about the inhibitory effects of various microspheres on the functions of NHOst, it is probable that NHOst can phagocyte PS microspheres as well as macrophages, and in particular, may phagocyte microspheres 5 μm in diameter. Moreover, the effect of the added PS microspheres suggests that NHOst better recognize the microspheres from their lower than upper side. This may explain the reduced functions of NHOst co-cultured with the pre-coated 5 μm PS microspheres.

To estimate the effect of the material composing the microspheres, NHOst were cultured for 1 week on pre-coated PS, PE, alumina and HAp microspheres, all of which have a diameter of around 5 μm. Table 1 shows their number ratio and ALP activities, and the calcium amounts. Pre-coated PS, PE and alumina microspheres showed the potential to suppress functions of NHOst although some of these data did not show statistical differences against NHOst without microspheres. However, when NHOst were cultured with pre-coated HAp, the amount of calcium deposited was almost twice that detected in the cells without microspheres. It was observed that HAp microspheres have no potential to deposit calcium after a 1-week incubation without NHOst (data not shown). Therefore, the increase in calcium deposition by pre-coated HAp may be due to the enhancement in the differentiation of NHOst in contact with HAp. As expected, added various microspheres affected NHOst in a similar manner but less than the pre-coated microspheres (data not shown). We have hypothesized that GJIC of cells in contact with various biomaterials can be used as an index for estimating the biocompatibility of many kinds of biomaterials [5,6,9,11]. In addition, osteoblasts have been reported to communicate with one another *via* GJIC function, and the function is believed to be critical to the coordinated cell behavior necessary in bone tissue development [8,12]. Therefore, effects of these microspheres on the communication of co-cultured NHOst were estimated to consider the relation between this function and the differentiation of NHOst. The FRAP assay revealed that HAp microspheres enhanced the GJIC level of NHOst to 1.8 times as much as that of NHOst alone but others slightly inhibited it, indicating HAp has a potential to enhance homeostasis maintenance function of the NHOst as well as their differentiation. Details of the microspheres effects on GJIC of NHOst will be reported elsewhere [13]. These results indicated that the materials of microspheres affected the differentiation of co-cultured NHOst as well as the diameter of microspheres and their contact with the cells. In addition, microspheres made from HAp, which is a major component of bone tissue and has been shown to have good biocompatibility as bone substitute implants [14], may have the potential to enhance the differentiation of osteoblasts. These results suggest that the estimation of the effects of biomaterials in microsphere form on in vitro cell function may be useful for their in vivo biocompatibility evaluation.

We estimated the effect of sintering, normally used to harden HAp, on the function of NHOst. The estimation revealed that both HAp microspheres enhanced the amount of calcium deposited although the ALP activity of the cells decreased. In addition, when the un-sintered HAp microspheres were incubated with NHOst, the calcium deposition was observed more than sintered HAp. As another index of the differentiation of the NHOst, mRNA expression levels of osteocalcin, which is a well-known protein detected in

Table 1. Effects of a 1-week incubation with pre-coated microspheres on various functions of NHOst.
(Amounts of microspheres = 20 μg/well)

	Control	Polystyrene	Polyethylene	Alumina	Hydroxy Apatite (Sintered)
Diameter (μm)		5.0	6.4	5.1	7.2
The cell number ratio (%)	100.0±5.5	88.2±2.2	92.2±1.3	82.4±2.8	83.0±2.3
Percent ALP activity (activity/proliferation)	100.0±4.7	79.2±5.6	72.7±3.6*	58.2±5.7*	73.8±6.0*
Percent deposited calcium (Calcium percent/proliferation)	100.0±3.7	97.3±4.2	82.3±3.7	90.3±7.8	163.3±18.5*(a

Data are shown as the mean value ± SEM (n = 4 to 22)
* $p < 0.01$, against control group
(a) $p < 0.05$, against NHOst co-cultured with polyethylene and alumina microspheres

differentiated osteoblasts [15], were determined using the RT-PCR technique. Figure 2 shows time profiles of osteocalcin mRNA expression in NHOst cultured with pre-coated PS, PE, alumina, and two kinds of HAp microspheres. As shown in the figure, only the cells co-cultured with sintered HAp microspheres expressed osteocalcin mRNA after a 1-day incubation, while those co-cultured with other microspheres did not express the mRNA. This finding suggests that sintered HAp microspheres have the potential to induce osteocalcin production from NHOst. Neither spontaneous calcium deposition was observed by the incubation of sintered nor un-sintered HAp microspheres without NHOst, so that it is possible that the un-sintered HAp degrade in culture medium with NHOst, resulting in an increase of calcium concentration in the culture medium that enhances the calcium deposition by the NHOst. Therefore, it is suggested that sintered HAp can induce the differentiation of NHOst, and may be a suitable material for inducing osteogenesis rather than un-sintered one.

In conclusion, microspheres made from various materials had an effect on the differentiation of NHOst. The level of the effect varied with the size, amount, and composition of the microspheres. Microspheres made from PS, PE and alumina showed a potential to suppress the proliferation and the differentiation of co-cultured NHOst. On the other hand, microspheres made from HAp, especially sintered HAp, enhanced the differentiation of co-cultured NHOst, and showed their potential to maintain their homeostasis. Estimating the effect of various microspheres on the differentiation of osteoblasts will provide valuable information on the effects of wear debris from artificial hip joints as well as estimating their effects on osteoclast function.

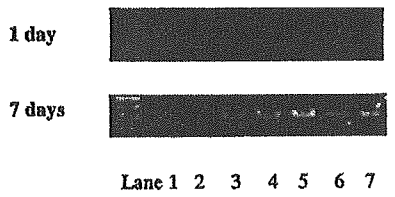

Figure 2. Expression of osteocalcin mRNA extracted from NHOst cultured on various microsphere pre-coated dish. Lane 1: Collagen-coated culture dish, 2: methanol-treated dish, 3: PS, 4: PE, 5: alumina, 6: un-sintered HAp, 7: sintered HAp.

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The effect of hyaluronic acid on insulin secretion in HIT-T15 cells through the enhancement of gap-junctional intercellular communications

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Abstract

The transplantation of bioartificial pancreas has the potential to restore endogenous insulin secretion in type I diabetes. The bioartificial pancreas is constructed in vitro from cells and a support matrix. Hyaluronic acid (HA) is an extremely ubiquitous polysaccharide of extracellular matrix in the body and plays various biological roles. It has been suggested that high molecular weight (HMW) HA increases in the function of gap-junctional intercellular communications (GJIC) and the expression of connexin-43 (Cx43). To determine whether the function of pancreatic β-cells is affected by gap junctions after HMW HA-treatment, we exposed HIT-T15, a clonal pancreatic β-cell line, in various concentrations of HA for 24 h, and then detected the insulin secretion and content, using an insulin assay kit by ELISA technique. The cellular functions of GJIC were assayed by dye-transfer method using the dye solution of Lucifer yellow. HA-treatment resulted in the enhancement of GJIC function, the increase of insulin release and insulin content. The results obtained in this study suggest that HA-coating increases the insulin secretion of HIT-T15 cells by the enhancement of Cx43-mediated GJIC. The results give useful information on design biocompatibility of HA when is used as a biomaterial for bioartificial pancreas.
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Keywords: Hyaluronic acid; Gap-junctional intercellular communications; HIT-T15 cells; Insulin; Bioartificial pancreas

1. Introduction

Type I diabetes is caused by the autoimmune destruction of the β-cells. All patients with type I diabetes require daily insulin shots for the control of glucose levels. However, the insulin therapy cannot inhibit the development of serious chronic complications. The pancreas transplantation has been expected to be the most promising approach toward treating diabetes. The bioartificial pancreas is constructed in vitro from insulin-secreting cells or islets and a support matrix by a tissue engineering method. The frequently used matrix materials are alginate and agar [1,2]. Although bioartificial pancreatic constructs contain insulin-secreting cells entrapped in agar or alginate matrix implanted into the peritoneal cavity of the diabetic patient, mice, and dog, can restore normoglycemia and markedly abate diabetic symptoms, there are important questions in the structural integrity of support matrix, metabolic activity and viability of cells or islets, and late vascular thrombosis [1,2]. Therefore, the new matrix biomaterials, which mimic the functions of extracellular matrix (ECM), need to be researched.

Hyaluronic acid (HA) is an extremely ubiquitous member of the nonsulfated glycosaminoglycan ECM molecule family and is thought to play various biological roles particularly in growth, adhesion, proliferation, differentiation, and cell migration [3,4]. More importantly, the receptor for HA-mediated motility regulates gap-junction

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channel and connexin-43 (Cx43) expression by its actions on focal adhesions and the associated cytoskeleton [5]. In addition, Park and Tsuchiya [6] have reported that high molecular weight (HMW) HA-coating can enhance the function of gap-junctional intercellular communications (GJIC). The insulin secretion from pancreatic β-cells is a multicellular event depending on their interaction with neurotransmitters and numerous signal molecules carried by blood and also direct interactions between cell–cell and cell–matrix contacts by gap-junctional channels, which mediate exchanges of molecules smaller than 1000 Da, such as ions, small metabolites, and second messengers between adjacent cells. The latter interactions are thought to be crucial regulatory mechanisms of insulin secretion [7–9], and the pharmacological blockade of GJIC markedly decreases insulin release [8]. However, the effects of HMW HA as biomaterials of support matrix on functions of pancreatic β-cells and gap-junctional channel remain unclear.

In the present study, we investigated the effects of HMW HA on the function of GJIC, the expression of Cx43, insulin content, and insulin secretion using HIT-T15 cells in vitro. These results suggest that HMW HA can be used as the biomaterial for the development of a bioartificial pancreas: design biocompatibility of HA depends on the molecular-weight size of HA, and its application method and concentration.

2. Materials and methods

2.1. Materials

Lucifer yellow was purchased from Molecular Probes (Eugene, OR). HA (1680 kDa) and TetraColor ONE (WST-8) were supplied by Seikagaku Industries, Ltd. (Tokyo, Japan). ELISA insulin assay kit was obtained from Morinaga Seikagaku Co. (Yokohama, Japan). Bovine serum albumin (BSA) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Krebs–Ringer bicarbonate (KRB) buffer (pH 7.4), fetal bovine serum (FBS), and anti-Cx43 were purchased from Sigma Chemical Co. (St. Louis, MO). β-actin antibody was obtained from Cell Signaling Technology Inc. (Tokyo, Japan). Roswell Park Memorial Institute (RPMI) 1640 medium was from Nissui pharmaceutical Co. (Tokyo, Japan). All other chemicals used were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of media and culture dishes

The HA polysaccharide was dissolved in distilled water at a concentration of 4 mg/ml. Each of the 35-mm culture dish (Falcon 1008, Becton Dickinson) was coated at a final concentration of 0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml. The HA-coated dishes were dried further under sterile air flow at room temperature for 12 h before use. In order to investigate the effect of HA-addition on the functions of HIT-T15 cells, different media were prepared at a final concentration of 0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml. HA-treatment is performed to cells for 24 h.

2.3. Cells and cell culture

A hamster pancreatic β-cell line, HIT-T15 (HIT-T15 cells, Dainippon Pharmaceutical Co., Japan), was cultured in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 100 IU penicillin-G and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The subculture cells were seeded at a density of $1.0–5.0 \times 10^5$ cells/ml in multiwell plates or culture dishes. When they reached more than 80% confluence, the cells were used for various studies. Throughout the cell growth period the culture media were replaced every 2 days.

2.4. Measurement of cell viability

To evaluate the affect of HMW HA on cell viability of HIT-T15 cells, HIT-T15 cells (1×10^5) were incubated into the various concentrations of HA-coated 24-well plates, or after the cells were seeded onto 24-well plates and pre-incubated in a 10% FBS/RPMI 1640 medium overnight, the medium was exchanged for 10% FBS/HA/RPMI 1640 medium prepared. After 24 h of HA-treatment, the cell viability was determined by the WST-8 reduction assay, according to the manufacturer's instructions. Control cells received fresh medium without HA.

2.5. Measurement of insulin release and insulin content

HIT-T15 cells were treated as described above. After pre-incubating for 30 min at 37 °C in KRB buffer, no glucose cells were stimulated for 60 min with 11.1 mM glucose in KRB buffer. The medium was collected, centrifuged for 5 min at 3000*g*, and the supernatant was frozen at −80 °C for insulin release assay. Cultures were then extracted for 24 h at 4 °C in acid-ethanol and the extracts also frozen for determination of insulin and protein content. Insulin was determined by ELISA insulin kit with rat insulin as standard, according to the manufacturer's instructions. Protein content was measured by the BCA protein assay reagent kit with albumin as standard (PIERCE). Values of secreted insulin were normalized to protein content.

2.6. Measurement of dye transfer

Gap junction-mediated communication between β-cells regulates the insulin secretion and insulin biosynthesis. Because HMW HA-coating increased the insulin release and insulin content but not HA-added, we tested whether the HA-coating increases the insulin secretion and insulin content have a relationship with gap junctions between HIT-T15 cells. HIT-T15 (5×10^5) cells were exposed to the HA-coated (0.1, 0.25, and 0.5 mg/dish) 35-mm glass coverslip (Ashland, MA) and incubated for 24 h to evaluate dye coupling using Lucifer yellow. The cells were rinsed with phosphate-buffered saline [PBS(+)] containing Ca^{2+}/Mg^{2+}, and 3 ml of PBS(+) containing 1% BSA and 10 mM HEPES (pH 7.4) were added to keep a sufficient pH stability under the microscope. The junctional coupling of HIT-T15 cells was determined by injecting Lucifer yellow into individual cells within monolayer clusters. Injections were performed on a phase-contrast microscope with InjectMan NI2 and microinjector FemtoJet (Eppendorf AG, Germany) using glass micropipette that were filled with a 4% solution of Lucifer yellow CH (MW 457.2) dissolved in 0.33 M lithium chloride, as previously described [11]. An injection pressure of 6.5 psi for 200 ms was used for each injection. The coupling extent was evaluated by counting dye-transferred cells at 2 min after microinjection. There was no leakage of injected dye into the medium.

2.7. Western blot analysis

HIT-T15 cells were grown into the various concentration of HA-coated 100-mm plastic dishes (0.1, 0.25, and 0.5 mg/dish) (FALCON 3003; Falcon) for 24 h, rinsed with Ca^{2+}/Mg^{2+}-free PBS(−) and then lysed in CelLytic™-M lysis/extraction reagent (Sigma). Protein content was measured by the BCA protein assay reagent kit (PIERCE). Samples of total extracts (20 μg protein/lane) were fractionated by eletrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The contents of the gels were transferred to PVDF membranes (Clear Blot Membrane-P). Membranes were saturated for 2 h at room temperature in Block Ace (Dainippon Pharmaceutical Co.,

Japan) and then were incubated with antibodies directed against Cx43 (1:1000) and β-actin (1:1000) as the primary antibody overnight at 4 °C. After repeated rinsing in PBS–Tween, the immunoblots were incubated with a peroxidase-conjugated antibody against rabbit (1:5000) at room temperature for 1 h. Membranes were developed by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech).

3. Results

3.1. Cell viability

In order to evaluate the affect of HMW HA on cell viability, HIT-T15 cells were incubated with HA-coated (0.01, 0.05, 0.1, 0.5, and 1.0 mg/dish) or -added (0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml) for 24 h. After 24 h exposure to HA-added, there was no significant change in the viable HIT-T15 cell number at the low concentration of HA-added ($\leqslant$1.0 mg/dish) compared to control. In contrast, after 24 h of incubation, the cell viability of HIT-T15 cells grown on high concentration HA-coated dishes ($\geqslant$1.0 mg/dish) was significantly less than on low concentration HA-coated and control (Fig. 1). Therefore, all further studies were conducted using low concentration of HA ($\leqslant$0.5 mg/dish).

3.2. Insulin secretion and insulin content

HIT-T15 cells, retain glucose-stimulated insulin secretion, showed an increase in insulin secretion as a function of stimulation. Thus, their insulin output was 2.73±0.36 and 3.90±0.41 pg/μg protein in the base and glucose-stimulation (11.1 mM), respectively (n = 9 dishes from three independent experiments). When these cells were exposed to a low concentration of HA-coating (0.1, 0.25, and 0.5 mg/dish) for 24 h, their insulin secretion was significantly increased in the presence of glucose-stimulation (Fig. 2). However, in contrast, when HIT-T15 cells were incubated with HA-addition for 24 h, the increasing effect was not exhibited. The insulin secretion was without a difference between control and HA-addition (Fig. 2). On the other hand, after acid-ethanol extraction, we found that the insulin content of the HIT-T15 cells grown onto the HA-coated dishes was significantly increased but not HA-added (Fig. 3).

GJIC and Cx43 are thought to be crucial regulatory mechanisms of insulin secretion and insulin content. As described above, HA-coating increased insulin secretion and insulin content of the HIT-T15 cells. In addition, Park and Tsuchiya [6] reported that HMW HA-coating can enhance the function of GJIC in normal human dermal fibroblasts but not HA-addition. Hence, all further studies on the mechanism of insulin secretion and insulin content were conducted using HA-coating.

3.3. Dye transfer

We assessed the function of GJIC using Lucifer yellow by counting the number of dye-transferred cells at 2 min

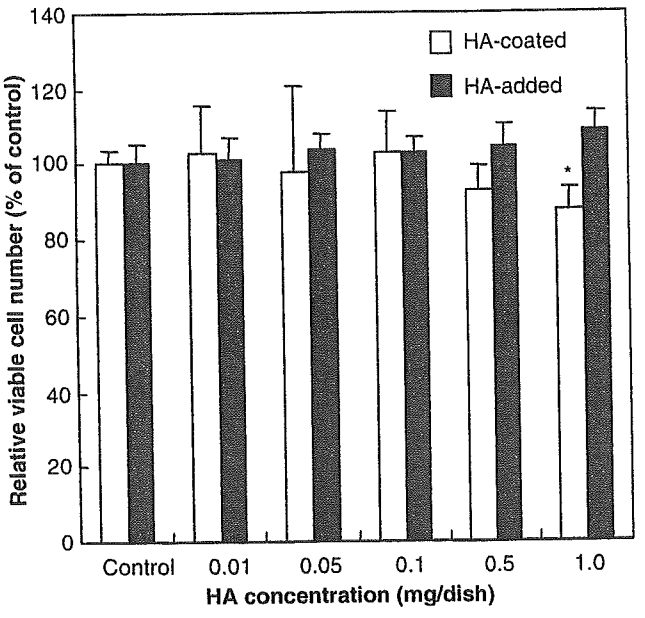

Fig. 1. Concentration-dependent effects of HA-treatment on viability of HIT-T15 cell. After HIT-T15 cells were incubated with HA-coated or HA-added for 24 h, the viable cell numbers of HIT-T15 cell were determined by WST-8 assay as described in methods. Each value denotes the mean±S.D. of three separate experiments. *$P\leqslant0.05$ compared to control under the HA-coated condition.

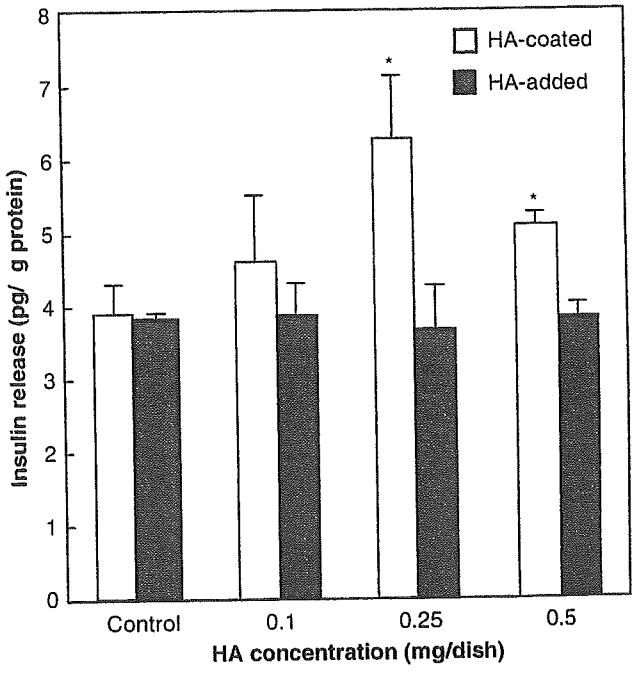

Fig. 2. Insulin secretion from HIT-T15 cells by HA-treatment. HIT-T15 cells were incubated with HA-coating (□) or HA-added (■) for 24 h and then stimulated for 60 min with 11.1 mM glucose in KRB buffer. The released insulin in the spent medium was determined by ELISA insulin kit. Each value denotes the mean±S.D. of three separate experiments. *$P\leqslant0.05$, compared to control in the presence of glucose.