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ORIGINAL ARTICLE

Masato Tamai, PhD · Kazuo Isama
Ryusuke Nakaoka, PhD · Toshie Tsuchiya, PhD

Synthesis of a novel β-tricalcium phosphate/hydroxyapatite biphasic calcium phosphate containing niobium ions and evaluation of its osteogenic properties

Abstract To promote the osteogenic properties of osteoblasts, we synthesized a hydroxyapatite (HAp) with β-tricalcium phosphate (β-TCP) biphasic calcium phosphate containing Nb ions (NbTCP/HAp). NbTCP/HAp was prepared by annealing precipitates obtained by coprecipitation of an aqueous solution of $Ca(NO_3)_2$ and a mixture of $(NH_4)_2HPO_4$ and aqueous Nb solution. The precipitates can be regarded as a calcium-deficient HAp, the PO_4 sites of which are partly occupied by Nb ions. NbTCP/HAp was successfully synthesized by thermal decomposition of the precipitates. NbTCP/HAp enhanced the calcification of normal human osteoblasts (NHOst), and the amount of calcified tissue increased in proportion to the Nb ion concentration in the NbTCP/HAp. The alkaline phosphatase (ALP) activity of NHOst was also enhanced by NbTCP/HAp. Because Nb ions significantly enhance the ALP activity of NHOst, calcification by NbTCP/HAp is considered to be due to enhancement of ALP activity induced by Nb ions dissolved from NbTCP/HAp. These results indicate that NbTCP/HAp can be an effective bone repair material.

Key words Tissue engineering · Bone · Osteoblasts · Calcium phosphate · Nb ions

Introduction

Bone tissue engineering offers a promising alternative strategy for healing severe bone injuries by utilizing the body's natural biological response to tissue damage in conjunction with engineering principles. Osteogenic cells, growth factors, and biomaterial scaffolds form the foundation of the many bone tissue engineering strategies employed to achieve regeneration of damaged bone tissue. An ideal biomaterial scaffold will provide mechanical support to an injured site and also enhance osteogenic differentiation to encourage bone growth.[1] To develop biomaterial scaffolds with optimal performance, understanding the interactions between osteoblasts and scaffolds is extremely important.

Hydroxyapatite [HAp, $Ca_{10}(PO_4)_6(OH)_2$] and related calcium phosphate ceramics, e.g., β-tricalcium phosphate [β-TCP, β-$Ca_3(PO_4)_2$], have good biocompatibility with bone tissue because their chemical compositions are very similar to the mineral phase of human bone. It is well known that these calcium phosphate ceramics can be biologically bonded to natural bone. In fact, it has been reported that porous materials composed of HAp, β-TCP, or β-TCP/HAp biphasic calcium phosphate are useful for bone tissue regeneration because of their osteoconductivity.[2–6] It has also been reported that β-TCP/HAp biphasic calcium phosphate shows better osteoconductivity than HAp or β-TCP alone.[7,8] Therefore, this material has been actively studied for use as a scaffold for bone tissue regeneration.

In a previous study, Nb ions were reported to lower cytotoxicity[9] (IC_{50} of Nb ions for L929 fibroblasts is 3.63×10^3), and we reported that Nb ions significantly promoted the calcification of normal human osteoblasts (NHOst).[10] Furthermore, we succeeded in synthesizing a hydroxyapatite containing Nb ions (NbHAp) and showed that NbHAp has the potential to promote alkaline phosphatase (ALP) activity, an important factor in the generation of new bone, in NHOst.[11] In this study, to further promote the cell activity of osteoblasts, we synthesized β-TCP/HAp biphasic calcium phosphate containing Nb ions and investigated interactions between β-TCP/HAp biphasic calcium phosphate and NHOst in vitro.

Materials and methods

Synthesis and characterization of β-TCP/HAp biphasic calcium phosphate containing Nb ions

Reagent grade $Ca(NO_3)_2$, $(NH_4)_2HPO_4$, and $NbCl_5$ (Wako, Osaka, Japan) were used without purification. NbTCP/HAp

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M. Tamai · K. Isama · R. Nakaoka · T. Tsuchiya (✉)
Division of Medical Devices, National Institute of Health Sciences,
1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
Tel. +81-3-3700-4842; Fax +81-3-3707-6950
e-mail: tsuchiya@nihs.go.jp

samples were prepared by annealing precipitates obtained from coprecipitation of an aqueous solution of $Ca(NO_3)_2$ with a mixture of $(NH_4)_2HPO_4$ and an aqueous solution of Nb as described below. $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$ were completely dissolved in distilled water. The aqueous Nb solution was prepared by mixing distilled water and $NbCl_5$ dissolved in 5% hydroxyacetone and 5% 2-aminoethanol.[12] A 0.2 M $(NH_4)_2HPO_4$ aqueous solution was combined with 0.01 M $NbCl_5$ and stirred with a magnetic bar at Nb/(Nb + P) molar ratios of 0.0000, 0.0167, or 0.1667. The pH of the mixture was adjusted to 10 using 1 N NaOH throughout the reaction, and 0.2 M $Ca(NO_3)_2$ was slowly dropped into the mixture (20 ml/min). The amount of 0.2 M $Ca(NO_3)_2$ solution was adjusted to a Ca/(Nb + P) molar ratio of 1.6 in order to synthesize β-TCP/HAp biphasic calcium phosphate, followed by stirring the suspension for 24 h at room temperature. The precipitates were centrifuged at 3600 rpm for 5 min and washed with distilled water. The resulting precipitates of Nb/(Nb + P) with molar ratios of 0.0000, 0.0167, and 0.1667 were named NbHAp-0, NbHAp-I, and NbHAp-II, respectively. These precipitates were then annealed at 800°C for 2 h (temperature increase: 5°C/min) and named NbTCP/HAp-0, NbTCP/HAp-I, and NbTCP/HAp-II, respectively. The NbTCP/HAp samples obtained were characterized by X-ray diffraction analysis (XRD, Rint2000, Rigaku, Tokyo, Japan) with Cu K_α radiation (40 kV, 50 mA). The XRD profiles of 2θ angles between 20° and 60° with a step interval of 0.01° were collected at a scanning rate of 4°/min. Also, measurement of the lattice parameter was carried out using the 211, 112, and 300 planes of HAp, and data for the lattice parameter were collected with a scan rate of 0.025°/min. The observed interplanar spacing was corrected using elemental Si as a standard material.

Concentrations of Ca, P, and Nb ions in the precipitate were estimated by inductively coupled plasma analysis (ICP, HP4500, Hewlett-Packard, CA, USA) after the precipitate was dissolved in HNO_3 solution. Microstructural evaluation of the precipitates was performed by scanning electron microscopy (SEM, JSM-5800LV, JEOL, Tokyo, Japan; acceleration voltage: 25 kV) and energy-dispersive X-ray spectroscopy (EDX) (LV5800, JEOL).

Osteogenic effects of NbTCP/HAp

NbTCP/HAp pellets were fabricated to investigate their effects on the osteogenic function of osteoblasts. In total, 100 mg of powdered NbTCP/HAp was put into a stainless steel mold and uniaxially pressed at 30 MPa for 1 min to form a pellet 0.5 mm in thickness and 12 mm in diameter. The pellets were sintered at 800°C for 2 h (temperature increase: 5°C/min).

NHOst were purchased from BioWhittaker (Walkersville, MD, USA) and maintained in d-minimumessential medium (αMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS, Kokusai Sinyakyu, Tokyo, Japan) in incubators at 37°C in a humid atmosphere with 5% CO_2. All assays were performed using αMEM containing 10% FCS supplemented with 10 mM β-glycerophosphate.

Cells were seeded on the pellets as described below. Each NbTCP/HAp pellet was immersed in 1 ml culture medium in a well of a 24-well cell culture plate (Corning, Corning, NY, USA) and incubated at 37°C for 24 h. After discarding the medium, 300 µl of new culture medium was put into each well, followed by 1 ml of NHOst suspension (4×10^4 cell/ml), and incubation was carried out for 4 h. Finally, the cell-seeded NbTCP/HAp pellet was transferred to a new well of a 24-well plate with 1 ml of the test medium and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 7–14 days.

Extracts from various NbTCP/HAp samples were prepared to investigate their effects on dissolved ions. NbTCP/HAp powder (100 mg/ml) was added to the culture medium (αMEM) containing 10% FCS and immersed at 37°C for 24 h. After changing the medium, the suspensions were stirred by a shaker at 200 rpm for 72 h at 37°C. The suspension was centrifuged at 3600 rpm for 5 min, and the supernatant was collected to use as an extract for an osteogenesis test in vitro. The atomic concentrations of Nb in the extract were measured by ICP.

An NHOst suspension (4×10^4 cells/ml) was added to culture wells and incubated for 4 h. After the NHOst had adhered to the well, the suspension medium was discarded and 1 ml of the extract supplemented with 10 mM β-glycerophosphate was added. The NHOst were incubated at 37°C in a saturated humid atmosphere with 5% CO_2 for 7–14 days.

We also examined the effect of Nb ions on the osteogenesis of NHOst. A solutionn of 0.2 µM $NbCl_5$/αMEM and serial dilutions were prepared. In addition to the experiment using the extracts indicated above, NHOst were cultured in $NbCl_5$/αMEM supplemented with β-glycerophosphate for 7–14 days.

Proliferation of NHOst cells in each experiment was estimated by a TetraColor One assay (Seikagaku, Tokyo, Japan), which incorporates an oxidation–reduction indicator based on detection of metabolic activity. After a 7-day incubation, the culture medium was discarded and 2% TetraColor One/αMEM solution was added to each well and was incubated for 2 h. The absorbance of the supernatant at 450 nm was measured using a µQuant spectrophotometer (Bio-tek, Winooski, VT, USA) to estimate the proliferation of the test cells. After estimating the proliferation, the cells were washed with phosphate-buffered saline [PBS(–)], followed by the addition of 1 ml of 0.1 M glycine buffer (pH 10.5) containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 4 mM *p*-nitrophenylphosphate sodium salt. The absorbance of the added buffer at 405 nm after 5 min incubation at room temperature was detected to evaluate the ALP activity of the test cells. After measurement of ALP, the NHOst cultured in the extract were washed with PBS(–) three times and the calcium phosphate deposited by NHOst was estimated. The amount of deposited calcium phosphate dissolved in 0.1 N HCl solution was determined by a Wako Calcium C test kit (Wako), which is based on the *o*-cresolphthalein complex color development method. The NHOst in all assays were stained in 5% Giemsa solution and observed by light microscopy (Nikon, Eclipse TE300, Tokyo, Japan) to confirm

Table 1. Chemical composition and characteristics of the precipitates prepared in this study

Sample	Phase	Annealing temperature	Theoretical composition[a]		Measured composition[a]		Color of precipitate	Lattice parameter[b]	
			Ca/(P + Nb)	Nb/(P + Nb)	Ca/(P + Nb)	Nb/(P + Nb)		*a*-axis (nm)	*c*-axis (nm)
NbHAp-0	HAp		1.60	0.000	1.60	–	White	–	–
NbHAp-I	HAp		1.60	0.017	1.56	0.013	Pale yellow	–	–
NbHAp-II	HAp		1.60	0.167	1.56	0.077	Buff yellow	–	–
NbTCPHAp-0	β-TCP + HAp	800°C	1.60	0.000	1.60	–	White	0.939	0.687
NbTCP/HAp-I	β-TCP + HAp	800°C	1.60	0.017	1.56	0.013	White	0.942	0.689
NbTCP/HAp-II	β-TCP + HAp	800°C	1.60	0.167	1.56	0.074	White	0.943	0.690

HAp, hydroxyapatite; NbHAp, hydroxyapatite containing Nb ions; TCP, tricalcium phosphate
[a] Molar ratio
[b] Lattice parameter for HAp

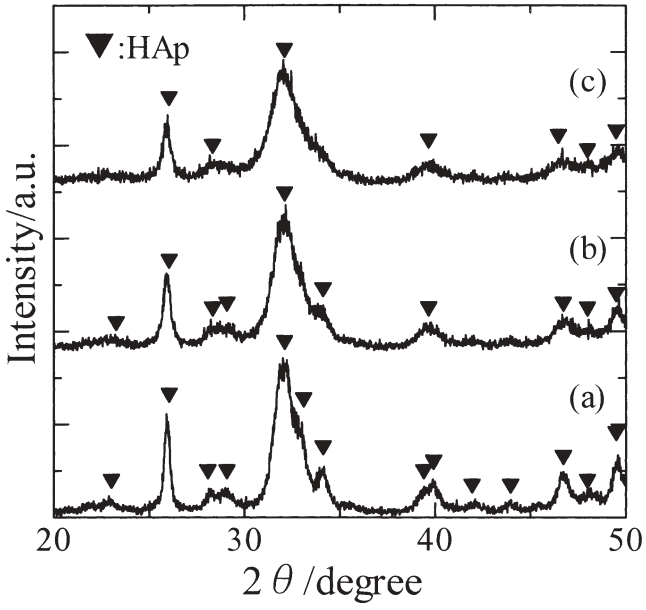

Fig. 1. X-ray diffraction (XRD) patterns of the precipitates with a Ca/(P + Nb) molar ratio of 1.50: *a*, Nb/(Nb + P) = 0; *b*, Nb/(Nb + P) = 0.0167; and *c*, Nb/(Nb + P) = 0.1667. *Triangles* represent XRD peaks due to the crystal structure of hydroxyapatite (HAp)

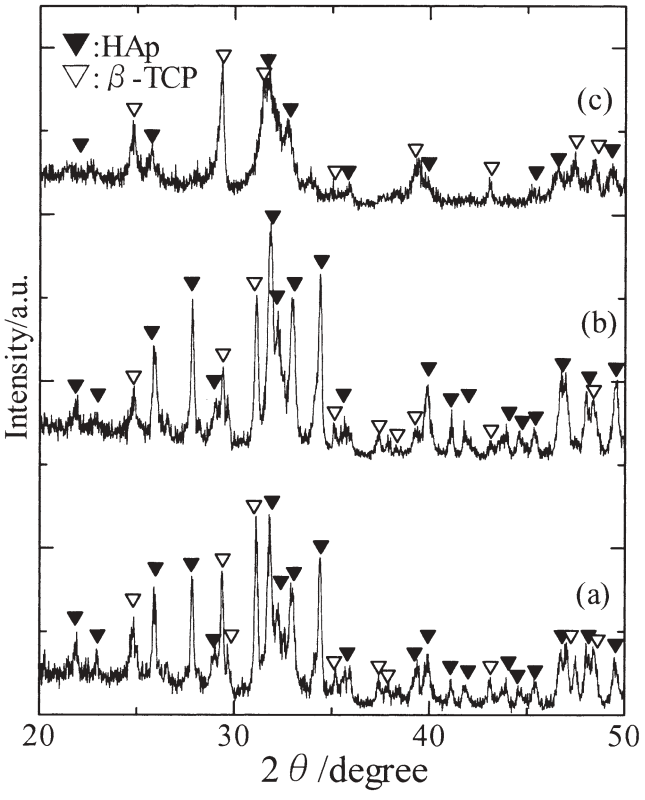

Fig. 2. XRD patterns of the annealed precipitates with a Ca/(P + Nb) molar ratio of 1.50: *a*, Nb/(Nb + P) = 0; *b*, Nb/(Nb + P) = 0.0167; and *c*, Nb/(Nb + P) = 0.1667. These precipitates were annealed at 800°C. β-*TCP*, β-tricalcium phosphate

their proliferation. All results were expressed as mean values ± SD and were analyzed statistically with Student's *t* test.

Results

XRD patterns of the precipitates prepared in this study are shown in Fig. 1. The XRD indicated that precipitates with Nb/(Nb + P) molar ratios from 0 to 0.167 had a monolithic apatite structure, irrespective of the Nb/(Nb + P) molar ratio of the starting solution, although the level of crystallite decreased as the Nb content increased. XRD patterns of the precipitates with various Nb/(Nb + P) molar ratios annealed at 800°C are shown in Fig. 2. The level of crystallites of the precipitates was high due to the annealing, and their diffraction peaks were composed of those of both HAp and β-TCP. Interestingly, the crystallite level decreased when the Nb level increased.

The chemical compositions and characteristics of the precipitates prepared in this study are summarized in Table 1. Both the Ca/(Nb + P) and the Nb/(P + Nb) molar ratios in precipitates measured by ICP approximately agreed with their theoretical values, except for the Nb/(P + Nb) molar ratio of NbTCP/HAp-II: the measured Nb/(P + Nb) molar ratio of NbTCP/HAp-II was 0.074, which is lower than the theoretical value of 0.167. The lattice parameter of the HAp phase in NbTCPHAp increased with increasing Nb content.

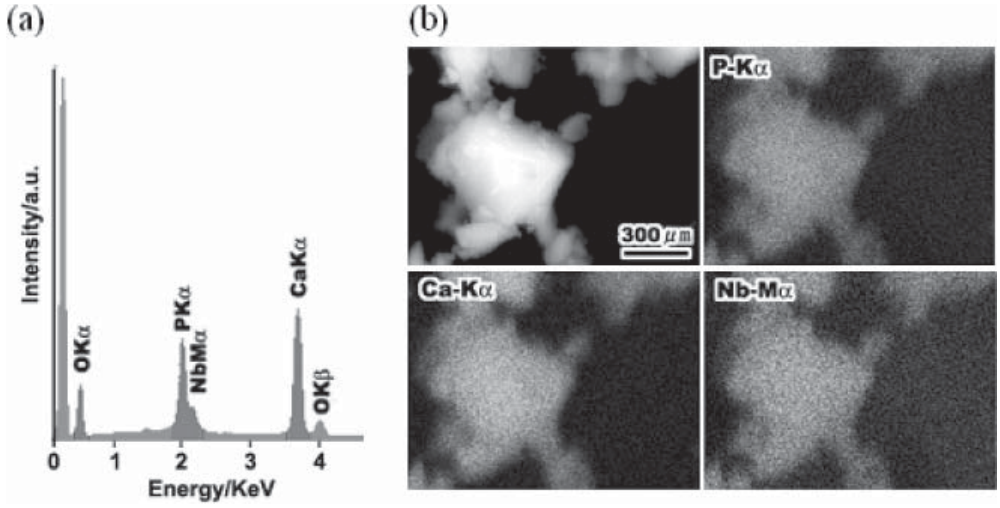

Fig. 3. Scanning electron microscopy–energy-dispersive X-ray spectroscopy spectra of NbTCP/HAp-II annealed at 800°C (**a**) and their mapping images from P-Kα, Ca-Kα, and Nb-Mα lines (**b**)

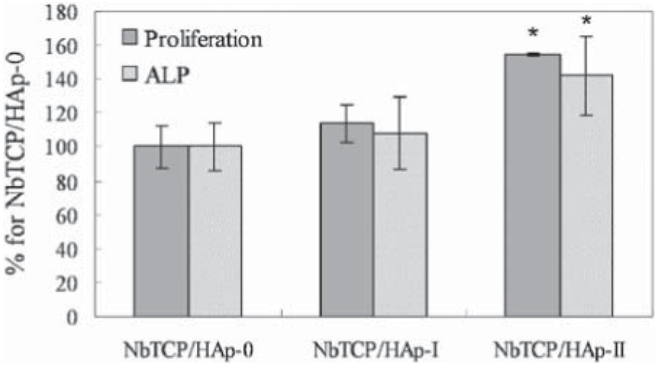

Fig. 4. Proliferation and alkaline phosphatase (*ALP*) activity of normal human osteoblasts (NHOst) cultured on various kinds of NbTCP/HAp pellets. *$P < 0.01$ against NbTCP/HAp-0 (without Nb ions)

The lattice parameters of NbTCP/HAp-0 without Nb ions were 0.939 nm for the *a*-axis and 0.687 nm for the *c*-axis, while those of NbTCP/HAp-II were 0.943 nm for the *a*-axis and 0.690 nm for the *c*-axis. In addition, the color of the precipitates became dark yellow as the Nb/(P + Nb) molar ratio increased, while the annealed precipitates of NbTCP/HAp were white.

SEM observation of the precipitates before annealing revealed that all precipitates were present as aggregates composed of primary particles of less than 1 μm in diameter, irrespective of the Nb/(P + Nb) molar ratio. Figure 3a shows SEM-EDX spectra of NbTCP/HAp-II. The EDX spectrum of Nb Mα was separated from the P K_α line and could be observed at 2.17 KeV, although its intensity was weak. The mapping images of the P-K_α, Ca-K_α, and Nb-M_α lines are shown in Fig. 3b. As shown in Fig. 3b, Nb ions were present at the same site as the Ca and P ions, suggesting that the Nb ions were homogenously distributed in the aggregates.

The proliferation and ALP activity of NHOst cultured on various kinds of NbTCP/HAp pellets is shown in Fig. 4. The proliferation of NHOst cultured on NbTCP/HAp-II pellets was approximately 60% higher than that on NbTCP/HAp-0 without Nb ions ($P < 0.01$). As shown in Fig. 5, many

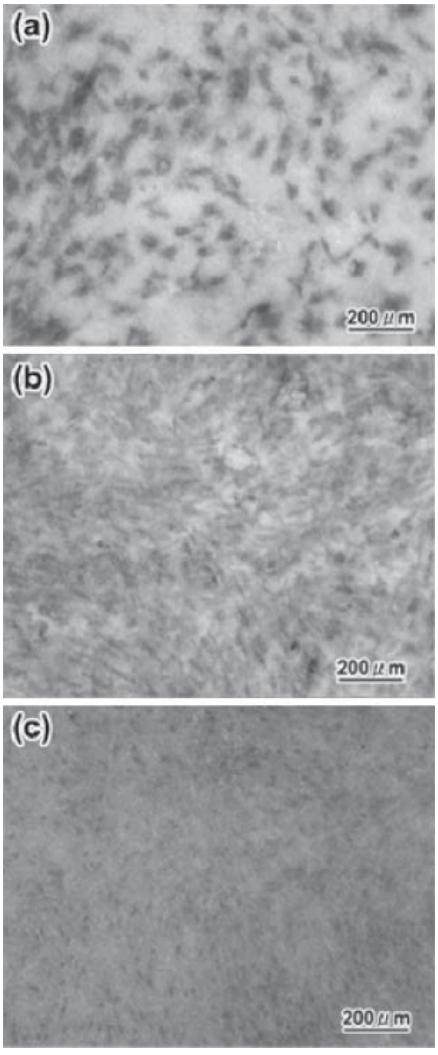

Fig. 5. Light microscopic images of NHOst cultured on various NbTCP/HAp samples for 7 days: *a*, NbTCP/HAp-0; *b*, NbTCP/HAp-I; and *c*, NbTCP/HAp-II. NHOst were stained by Giemsa solution

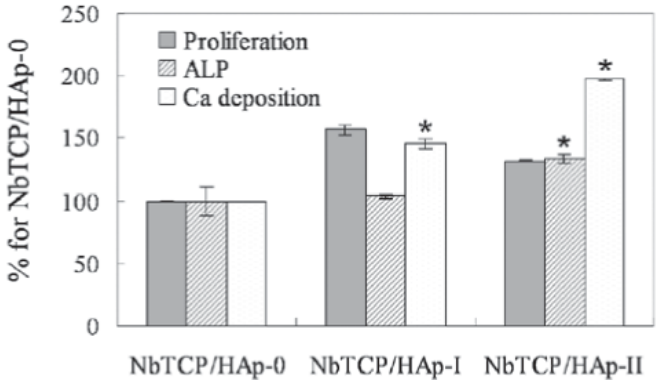

Fig. 6. Osteogenic properties (proliferation, ALP activity, and Ca deposition) of NHOst cultured in extracts from various NbTCP/HAp samples for 14 days. *P < 0.01 against NbTCP/HAp-0 (without Nb ions)

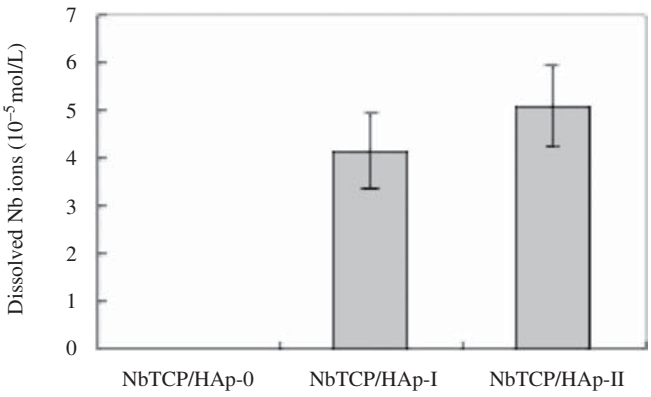

Fig. 7. Concentrations of Nb ions in extracts from various NbTCP/HAp samples. The concentration of Nb ions in cell culture medium was measured by inductively coupled plasma analysis

NHOst adhered to and spread on NbTCP/HAp-I and -II, while little spreading of NHOst was observed on HAp. In addition, as shown in Fig. 4, NHOst cultured on the NbTCP/HAp-II pellets expressed high ALP activity, compared with those cultured on NbTCP/HAp-0. Figure 6 shows the proliferation, ALP activity, and Ca deposition of NHOst cultured in extracts from various NbTCP/HAp samples for 14 days. Like the NHOst cultured on pellets, NHOst cultured in the extract from NbTCP/HAp-II expressed higher ALP activity than those in the extract from NbTCP/HAp-0. Furthermore, the amount of deposited calcium from NHOst increased with increasing Nb ion concentration in NbTCP/HAp, and the calcium deposition in the extract from NbTCP/HAp-II was twice that in the extract from NbTCP/HAp-0.

Figure 7 shows the concentration of Nb ions in extracts from NbTCP/HAp samples. It was found that Nb ions were released into the cell culture medium at concentrations of the order of 1×10^{-5} mol/l. To investigate the effect of Nb ions on NHOst function, NHOst were cultured in a medium containing Nb ions. The dependence of osteogenesis by NHOst on Nb ion concentration is shown in Fig. 8. Nb ions did not affect the proliferation of NHOst, but the ALP activity and Ca deposition of NHOst proceeded proportionally when the concentration of Nb ions was more than 1×10^{-5} mol/L.

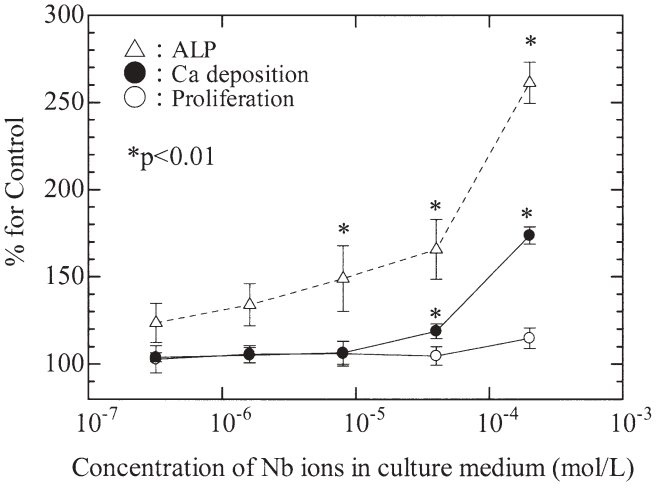

Fig. 8. Relationship between concentration of Nb ions in culture medium and osteogenic properties of NHOst. *P < 0.01 against cell culture medium without Nb ions

Discussion

Characterization of NbTCP/HAp biphasic calcium phosphate ceramics

As summarized in Table 1, before annealing the precipitates, the NbHAp samples were hydroxyapatite with low levels of crystallite. The hydroxyapatite structure is known to be very tolerant of ionic substitution.[12] Ca^{2+} ions, PO_4^{3-} ions, and OH^- ions can be replaced, partly or completely, by various cationic or anionic ions. Notably, as shown in Table 1, the lattice parameter of HAp increased when the Nb content in NbTCP/HAp was high. This fact suggests that Nb ions are taken into the apatite lattice. If a substitution of an Nb^{5+} ion for a Ca^{2+} ion in HAp occurred, the lattice parameter should decrease, since the ionic radius of Ca^{2+} and Nb^{5+} are 0.1 nm and 0.064 nm, respectively. Therefore, the possibility of substitution of Nb ions for Ca ions is low. On the other hand, although the structure of Nb ions in aqueous solution is not fully understood at present, it has been reported that Nb ions in solution are not present as Nb^{5+} but as niobiumate acid, $H_xNb_6O_{19}^{(8-x)-}$ ions (x = 0,1,2) for basic conditions,[14,15] and the niobiumate acid cluster ($H_xNb_6O_{19}^{(8-x)-}$) was polymerized or dissociated depending on the pH and ion concentration.[15] According to these reports, $H_4NbO_6^{3-}$ anionic monomer can exist in basal and low Nb concentrations (<0.08 M). Since the Nb concentration in this study was 0.01 M, Nb ions would exist as $H_4NbO_6^{3-}$ anionic monomers. $H_4NbO_6^{3-}$ may be substituted at the PO_4 site since the PO_4 site in HAp can be replaced by anionic

atomic groups. In addition, the ionic radius of the $H_4NbO_6^{3-}$ monomer and PO_4 are approximately 0.30 nm and 0.23 nm, respectively, suggesting that an increase in lattice parameter of NbTCP/HAp is ascribed to the substitution of PO_4 sites by this monomer in HAp. Furthermore, the fact that both the Ca/(Nb + P) and Nb/(P + Nb) molar ratios of the precipitates, as measured by ICP, approximately agreed with their theoretical values may support this hypothesis. Despite the theoretical Nb/(Nb + P) ratio being 0.1667, the Nb/(Nb + P) molar ratio in NbTCP/HAp-II was about 0.07, which suggests that the maximum amount of substituted Nb ions at the PO_4 site is around 0.07.

The Ca/(P + Nb) molar ratio in the NbHAp obtained in this study was lower than that of the stoichiometric value of 1.67 for HAp. Hydroxyapatite having a lower Ca/P molar ratio is known as calcium-deficient hydroxyapatite [Ca-def HAp, $Ca_{10-Z}(HPO_4)_Z(PO_4)_{6-Z}(OH)_{2-Z}$, Z = 0–1]. Therefore, NbHAp can be regarded as a Ca-def HAp in which the PO_4 sites are partly occupied by Nb ions.

Ca-def HAp decomposes to stoichiometric HAp and β-TCP at temperatures above 600°C according to the following reaction:[16,17]

$$Ca_{10-Z}(HPO_4)_Z(PO_4)_{6-Z}(OH)_{2-Z}\cdot nH_2O \rightarrow (1-Z)Ca_{10}(PO_4)_6(OH)_2 + 3Z\cdot\beta\text{-}Ca_3(PO_4)_2 + Z\cdot nH_2O$$

The above thermal decomposition reaction occurred during the annealing of NbHAp, resulting in a lower Ca/P molar ratio than the stoichiometric value of HAp because of partial β-TCP formation. In addition, the homogenously distributed Nb ions in NbTCP/HAp may result from thermal diffusion of Nb ions during the thermal decomposition process.

Osteogenesis of NHOst cultured on NbTCP/HAp

In this study, NbTCP/HAp showed potential to promote calcification of NHOst. This study indicated that osteogenic behavior of NHOst cultured on NbTCP/HAp pellets was consistent with that of NHOst cultured in extracts from the pellets, suggesting that dissolved ions from the NbTCP/HAp pellets affect calcification of NHOst. As shown in Fig. 7, Nb ions were apparently released from NbTCP/HAps and dissolved in the medium at concentrations of the order of 1×10^{-5} mol/l. When 4×10^{-5} mol/l of $NbCl_5$ was added to the culture medium, Ca deposition clearly increased (Fig. 8). Therefore, the enhancement of Ca deposition is considered to be due to the dissolved Nb ions. One possible mechanism for enhancement of calcification is discussed below.

ALP is known to play an important role in the calcification of bone.[18–20] Generally, the calcification of bone mineral occurs in the matrix vesicles budding from the surface of osteoblasts.[21] The nucleation of biological apatite, which is the initial stage of calcification, occurs due to the reaction between inorganic PO_4^{3-} ions produced by the ALP and calcium ions in matrix vesicles.

NHOst cultured on the NbTCP/HAp pellets containing Nb ions expressed high ALP activity compared with those cultured on HAp without Nb ion. Similarly, it was found that NHOst cultured in an extract from NbTCP/HAp containing Nb ions expressed higher ALP activity than those in the extract from HAp without Nb ions. These results suggest that Nb ions affect the enhancement of ALP activity. Based on the above calcification mechanism in matrix vesicles, the enhancement of calcification might result from the enhancement of ALP activity due to dissolved Nb ions from NbTCP/HAp. The enhancement of ALP activity increases the production of inorganic PO_4^{3-} ions, and then the inorganic PO_4^{3-} ions produced may be taken into the matrix vesicles. The subsequent nucleation of biological hydroxyapatite occurs due to a reaction of Ca ions and inorganic PO_4^{3-} ions, followed by calcification. Although we cannot deny that Nb ions directly promote calcification by NHOst unrelated with ALP expression, the essence of the calcification enhancement by NbTCP/HAp may be the enhancement of ALP activity by Nb ions dissolved from NbTCP/HAp. The biological effect of Nb ions on NHOst is under investigation. Although further studies are necessary to clarify the mechanism of enhanced calcification by Nb ions, this study strongly suggests that NbTCP/HAp is a more promising material for use as a bone tissue engineering scaffold than HAp.

Conclusion

In order to promote the osteogenicity of osteoblasts, we synthesized a combination of HAp and β-TCP biphasic calcium phosphate containing Nb ions (NbTCP/HAp). The NbTCP/HAp samples were prepared by annealing precipitates obtained by coprecipitation of an aqueous solution of $Ca(NO_3)_2$ with a mixture of $(NH_4)_2HPO_4$ and aqueous Nb solution. The precipitates obtained by the coprecipitation process can be identified as Ca-def HAp, the PO_4 sites of which are partly occupied by Nb ions. NbTCP/HAp samples were successfully obtained by thermal decomposition of the precipitates.

NbTCP/HAp enhanced calcification of NHOst. The enhancement of calcification of NbTCP/HAp was ascribed to the enhancement of ALP activity due to the dissolved Nb ions from NbTCP/HAp.

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