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Cytocompatibility of calcium phosphate coatings deposited by an ArF pulsed laser

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Abstract In the current studies, we deposited ultra-thin hydroxyapatite films on a pure titanium substrate by pulsed laser deposition, and we examined the effects of these surfaces on rat bone marrow (RBM) cells. This method allowed deposition of 500-, 2000-, and 5000-Å-thick hydroxyapatite films. X-ray diffraction showed that the amorphous films recrystallized to a hydroxyapatite crystal structure after annealing. The proliferation of RBM cells was unaffected by the hydroxyapatite films, but osteocalsin and alkaline phosphatase mRNA and protein levels were elevated in cells grown on 2000- and 5000-Å-thick films. These results indicate that ultra-thin hydroxyapatite films generated by pulsed laser deposition are better at promoting osteogenesis than pure titanium surfaces.

Introduction

Hydroxyapatite (HA) is a bioactive implant material that enhances early bone formation [1, 2]. Although various techniques for coating titanium-based implant devices with HA have been reported [3, 4], plasma-spray is currently the only one that is commercially available [5]. However, plasma-sprayed HA films have several drawbacks, including poor surface adherence and crystallinity [5–7].

Many other methods, such as magnetron sputtering [3], ion beam-assisted deposition [4], chemical vapor phase deposition [8], sol-gel [9], biomimetic [10], and pulsed-laser deposition (PLD) exist for coating of surfaces. PLD [11, 12] allows deposition of very thin films, control of surface roughness, and ablation of any material. As a result, PLD provides strong bonding between the film and substrate. A previous study [11] showed that PLD produces an ultra-thin HA film with improved mechanical properties, including increased tensile strength and decreased film thickness. However, the biocompatibility of titanium implants coated with an ultra-thin HA film by PLD is not currently known.

This study was therefore carried out to examine the cellular response to titanium substrates coated with an ultra-thin HA film by PLD. For these studies, we examined the proliferation and differentiation of rat bone marrow (RBM) cells cultured on HA films of various thicknesses.

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Materials and methods

Starting material

HA disks were used as targets for PLD. The raw HA powder (Japan Chemical Co., Sapporo, Japan) was pressed at 150 MPa in a cylindrical steel die to form compacts 20 mm in diameter and 2 mm in thickness.

Preparation of the titanium surfaces

Titanium disks are 15 mm in diameter and 1 mm thick were certified according to the JIS Grade II specifications. Discs were prepared by manually parallel polishing using silicium carbide papers (grade 80–4000) and automatic mirror polishing using 3- to 0.05-μm Al_2O_3 particles. Deposition of HA targets was carried out in water vapor generated by bubbling O_2 gas through a water bath at 0.8 m Torr (1.1×10^{-1} Pa). An ArF excimer laser (wavelength [λ] = 193 nm and pulse width [τ] = 20 ns) operating at a repetition rate of 10 Hz was used for deposition. The film was deposited at substrate temperature of 300°C and at a rate of 10 nm/min. The film thickness was estimated on the basis of the deposition rate. Film thicknesses of 500, 2000, and 5000 Å were prepared and annealed by heating at 380°C for 1 h at heating and cooling rates of 1.5°C/min in water vapor generated by bubbling O_2 gas through a water bath. After annealing, the crystallinity of the coatings was characterized by X-ray diffraction (XRD). Prior to analysis and in vitro experiments, test samples were cleaned by a series of 10-min ultrasound treatments in pure acetone, pure alcohol, and distilled water. For cell culture experiments, test samples are sterilized in an autoclave. Four samples of each group are used for test.

Cell culture

Because most bone implant materials are implanted into adult bone that is in direct contact with bone marrow tissue, bone marrow cell cultures from adult rats are the useful for investigating the effect and safety of new implant materials. Rat bone marrow (RBM) cells proliferate and differentiate into a phenotype that expresses bone cell markers forms mineralized nodules in vitro

RBM cells were harvested from the tibias and femurs of 6- to 8-week-old male Wistar rats (weight 150–175 g). Briefly, rats were euthanized using 4% isofluorane, and the bones were aseptically excised from the hind limbs. External soft tissue was discarded, and the bones were placed in 50 ml of α-Eagles's minimal essential medium (α-MEM) supplemented with penicillin (850 U/mL) for approximately 15 min. This concentration of antibiotics was 4-fold higher than the normal concentration used for cell culture and to avoid contamination during the harvesting process. The proximal end of the femur and the distal end of the tibia were clipped. An 18-gauge needle was inserted into the hole in the knee joint in each bone, and the marrow was flushed out of the shaft with α-MEM. The resulting marrow pellet was broken up by trituration, and the cell suspensions from all bones were combined in a centrifuge tube.

Cell proliferation

RMB cells were cultured in 75 cm^2 flasks in α-MEM. When monolayers reached confluence, the cells were enzymatically lifted from the flask with trypsin. Cells (1.5×10^4/ml) were seeded on the samples in the wells of 24-well tissue culture plates containing α-MEM supplemented with 10% fetal bovine serum. The cells were incubated for 3 days in a CO_2 incubator at 37°C. A Cell Titer 96 nonradioactive assay (Promega Corporation, Madison, WI, USA) was used to assess cell proliferation. This assay determines the number of viable cells by measuring the mitochondrial conversion of 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium) (MTS) to a colored compound. MTS/PMS reagent (a component of the assay kit) was added to each well of the 24-well plate and incubated for 60 min at 37°C in a CO_2 incubator. The absorbance was determined at a wavelength of 490 nm using a Maxline ELISA Plate Reader (Molecular Devices, CA, USA).

Cell differentiation

RBM cells (4×10^4 cells/ml) were seeded on coated and non-coated titanium disks placed in the wells of 24-well tissue culture plates containing α-MEM supplemented with 10% fetal bovine serum. The cells were incubated for 3 days in a CO_2 incubator at 37°C. The medium was removed from the wells, and the cells were cultured in α-MEM containing 10% fetal bovine serum and osteogenic supplements (10 mM β-glycerophosphate, 50 mg/l ascorbic acid, and 10^{-8} M dexamethasone) for 14 or 21 days. At each time point, the conditioned medium was removed from the wells for analysis of osteocalsin (OCN) production.

Quantitative analysis of gene expression

Total RNA (ribonucleic acid) was isolated from the cell cultures using a Total RNA extraction kit (Trizol, Invitrogen, CA, USA). Single-strand cDNA was synthesized from 1 μg of total RNA using an oligo$(dT)_{20}$ primer (Toyobo, Osaka, Japan) and a ReverTra Ace-α first strand cDNA synthesis kit (Toyobo). For quantitative real-time PCR (polymerase chain reaction), sequences for the ALP and OCN probes and primers were designed using Primer Express Software 1.0 (Applied Biosystems, Ca, USA). The internal fluorogenic

probes were labeled at the 5′-end with FAM™ as a reporter dye (Applied Biosystems) and at the 3′-end with TAMRA™ as a quencher dye (Applied Biosystems). S-18, a probe for a housekeeping gene (Applied Biosystems), was labeled at its 5′-end with VIC® as a reporter dye and with TAMRA at its 3′-end as a quencher dye. This allowed simultaneous determination of the concentration of both the target message and S-18 within a single reaction. Thermal cycling and fluorescence detection were performed in a real-time Light Cycler (ABI Prism 7700 sequence detection system, Applied Biosystems). The thermal cycling conditions were as follows: initial denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The amounts of the targets were determined by comparing the signals with a standard curve. The values were normalized to the expression of the S-18 housekeeping gene, and the fold increase was expressed compared to cells cultured on 24-well plastic plates.

Assay of alkaline phosphatase (ALP) activity

The RBM cells were washed with phosphate buffered saline (PBS) and then frozen and thawed three times. The lysates obtained from the cells were homogenized with Tris (10 mM, pH 7.4) buffer and sonicated for 15 min on ice. A fluorimetric assay was performed to ascertain the total amount of DNA. Immediately prior to analysis, Hoechst 33528 solution (DOJINDO Laboratories, Kumamoto, Japan) was added to the sample tubes, and the contents were mixed. The fluorescence was then quantified on CytoFluor 4000 fluorescence plate reader (Applied Biosystems) with an excitation wavelength of 350 nm and an emission wavelength of 455 nm.

The remaining half of the lysates was used for measurement of ALP activity. ALP activity was assessed colorimetrically using *p*-nitrophenyl phosphate as the substrate and at pH 10.3 (Sigma, St. Louis, MO, USA). Conversion of the substrate by ALP was quantified at 405 nm using a Maxline ELISA Plate Reader (Molecular Devices). The amount of ALP activity was normalized by the amount of total DNA in the cell lysate.

Assay for OCN production

The sandwich enzyme immuno assay used in this study is specific for rat OCN and can measure its levels directly in cell culture supernatants. The OCN levels in the conditioned media were measured using a commercially available immunoassay kit (Rat OCN RIA Kit; Biomedical Technologies, MA, USA) as described previously. The amount of OCN was normalized by the amount of DNA in the cell lysate.

Statistical analyses

One-way analysis of variance (ANOVA) was used to determine the statistical significance of the differences between different film thicknesses. When ANOVA detected a statistically significant difference, a Scheffé test was used to examine the significance of the differences between individual groups.

Results

Structure of HA films

X-ray diffractograms of the coatings are shown in Fig. 1. Prior to annealing, the 500-, 2000-, and 5,000-Å coatings did not show a crystalline phase signal. After annealing, a few peaks in the X-ray diffractograms of the three coatings corresponded with HA. The results also show that the intensity of the peaks increased with the thickness of the films.

Effect of the films on cell proliferation

Figure 2 shows the proliferation of RBM cells grown on the various samples. Cell proliferation was measured using a MTS assay. The various HA coatings did not have a significant effect on the proliferation of the cells.

Effect of the films on the expression of ALP and OCN genes

We next assessed the expression of ALP (Fig. 3) and OCN (Fig. 4) genes by quantitative real-time PCR. After 14 days, the various coatings did not have a significant effect on the expression of ALP mRNA. After 21 days, the mean value was significantly higher in cells grown on a 2000-Å film than in the other cells.

The highest level of OCN mRNA was obtained in cells cultured on a 2000-Å film for 14 days. After 21 days, the highest level of OCN mRNA was also observed in cells on the 2000-Å film. At this point, the level of OCN mRNA was significantly higher than in cells on a 500-Å film or bare titanium, but there was not significant difference with cells grown on a 5000-Å film.

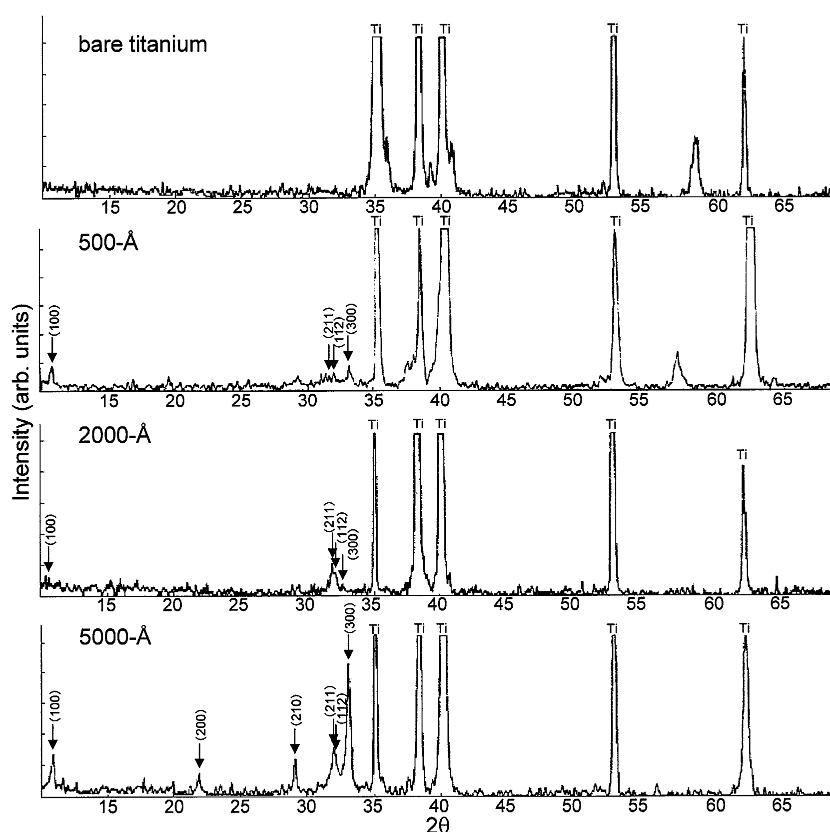

Fig. 1 XRD patterns recorded from bare titanium or thin films of HA deposited by PLD on titanium surfaces at a thickness of 500, 2000, and 5000 Å

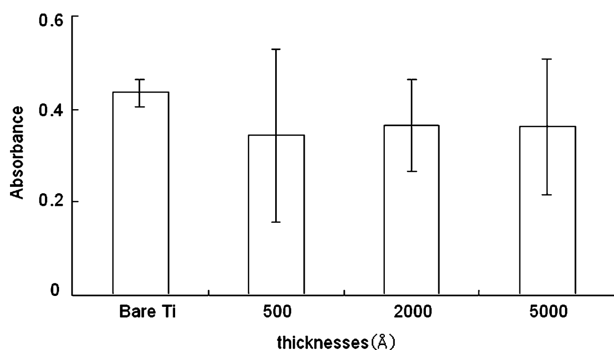

Fig. 2 RBM cell proliferation after 3 days of culture on bare titanium or on thin films of HA deposited by PLD on titanium surfaces at a thickness of 500, 2000, and 5000 Å

Effect of the films on ALP activity

Figure 5 shows the results from an assay of ALP activity. At 14 days, quantitative differences in ALP activity were not apparent between any of the conditions. However, when cultured for 21 days, cells on 2000 or 5000-Å films had higher ALP activity than cells on 500-Å films or bare Ti, but there was no difference between cells grown on 2000- and 5000-Å films.

Effect of the films on OCN production

Figure 6 shows the effect of the films on OCN production as assessed by EIA. The production of OCN by cells cultured on 2000- and 5,000-Å films for both 14 and 21 days was significantly higher than cells cultured on a 500-Å film or bare Ti, but the differences between cells on 2000- and 5000-Å films and between the 500 Å-film and bare titanium were not significant.

Discussion

In the current studies, we used ArF PLD to generate various thicknesses of HA on a highly polished titanium substrate. Growth of the RBM cells on HA films did not enhance their proliferation, but it did increase the levels of mRNA and protein for bone-related genes.

The coatings examined in this study were produced at a lower temperature (300°C) to avoid the oxidation of titanium. Arias et al. [13] demonstrated that the laser wavelength (193 nm) is short enough that the ablated material is only constituted by atoms, ions and small molecules. The XRD data showed that the amorphous coatings produced by PLD recrystallized to

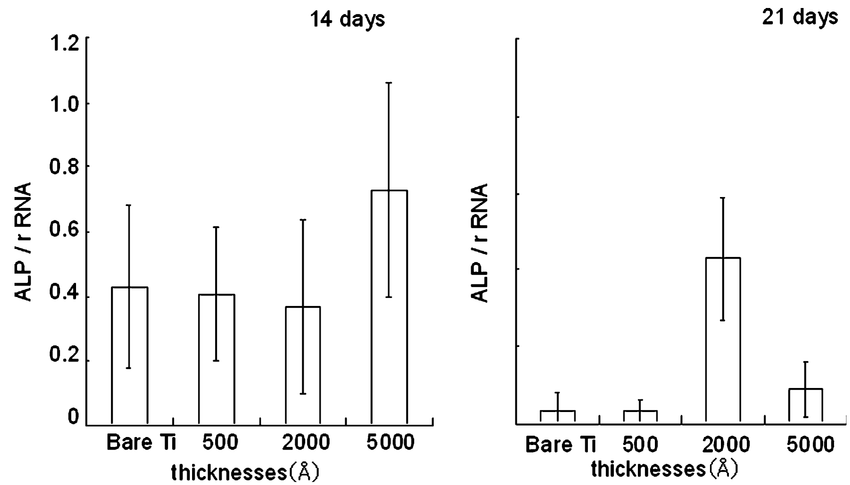

Fig. 3 Expression of ALP mRNA by RBM cells after 14 and 21 days of culture on bare titanium or on thin films of HA deposited by PLD on titanium surfaces at a thickness of 500, 2000, or 5000 Å. Bars represent the fold increase in ALP mRNA expression compared to that in cells cultured on plastic plates

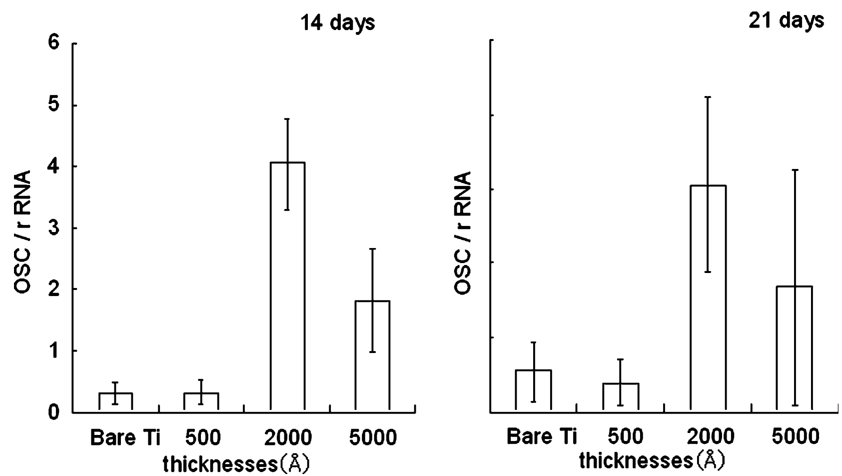

Fig. 4 Expression of OCN mRNA by RBM cells after 14 and 21 days of culture on bare titanium or on thin films of HA deposited by PLD on titanium surfaces at a thickness of 500, 2000, or 5000 Å. Bars represent the fold increase in OCN mRNA expression compared to that in cells cultured on plastic plates

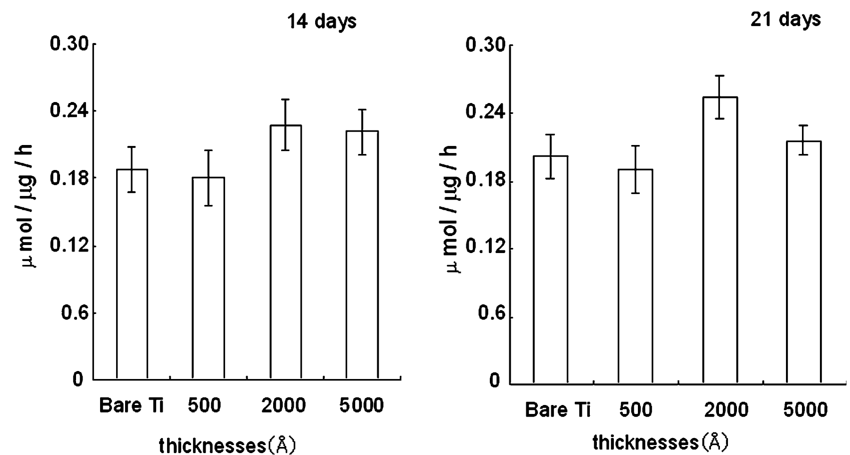

Fig. 5 ALP activity expressed by RBM cells after 14 and 21 days of culture on bare titanium or on thin films of HA deposited by PLD on titanium surfaces at a thickness of 500, 2000, or 5000 Å. Bars represent the µmol of ALP per µg of DNA

a HA crystal structure after heating for 1 h at 380°C. The degree and rate of recrystallization were time- and temperature-dependent, with effective recovery of the crystalline structure occurring between 500 and 700°C [7, 14]. However, a previous study [7] reported that treatment at high temperature decreases the integrity and interfacial bond strength of the HA coatings, which could increase the likelihood of cracking. On the other hand, Lynn et al. [14] showed that temperatures as low as 400°C allows partial recovery of the crystal-

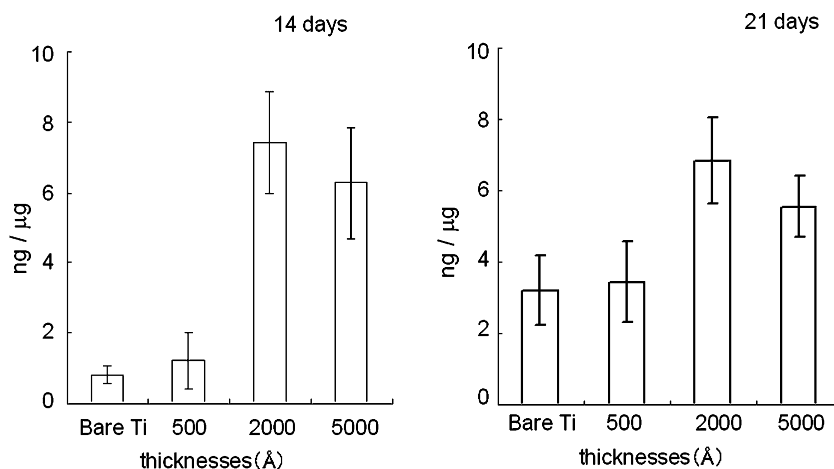

Fig. 6 OCN production by RBM cells after 14 and 21 days of culture on bare titanium or on thin films of HA deposited by PLD on titanium surfaces at a thickness of 500, 2000, or 5000 Å. Bars represent the ng of OCN per μg of DNA

line HA structure but that it is inferior to recovery at higher temperatures. The combination of low heat and the ArF PLD method used here may be useful for apatite formation on titanium because it provides a thin film with good crystallinity.

The HA films obtained in this study were between 500 and 5000 Å thick, which is thinner than not only coatings obtained by plasma-spray and the sol-gel technique but also coatings previously reported with PLD [15]. The different crystalline and mechanical properties of the coating were due to control of the PLD deposition parameters, including the wavelength and fluence of the leaser beam, the water vapor pressure in the chamber, and the substrate temperature [13, 16, 17].

To protect the surface, the coating must guarantee good impermeability [15]. According to the XRD patterns from the 500-Å coating, typical peaks for apatite were observed. As previously described [11], the adhesion properties of the coatings improve as their thicknesses decrease. Thus, because very thin coatings were obtained in the current studies, the deposition parameters used here may provide good bond strength between the HA coating and the titanium surface.

The RBM cell culture system is a powerful alternative to in vivo studies of bone biology for understanding osteogenesis [18]. RBM cells have long been recognized as the source of osteoprogenitor cells, and they can be induced to differentiate with various factors, including ascorbic acid, sodium β-glycerophosphate, and dexamethasone [19]. Therefore, the RBM culture system may be a useful model for studies of bone replacement biomaterials, for example for investigating the effect of direct contact of bone marrow tissue in adult bone with dental implants [18]. Vehof et al. [20] used the RBM culture system to show that cells on calcium phosphate-coated substrates produce a more mineralized matrix than cells cultured on titanium.

In the present study, according to the MTS assay, the presence of crystallized HA films did not affect the proliferation of RBM cells. In general, apatite formed on titanium results in increased adsorption of serum proteins compared to a titanium surface. Specifically, HA films induce cell attachment and proliferation [21] due to the adsorption of a high quantity of fibronectin [22, 23]. Chou et al. [24] reported that high-crystallinity HA coatings enhanced the proliferation of human gingival fibroblasts compared to uncoated titanium. On the other hand, similar to our current results, Ozawa et al. [25] found that DNA levels were not significantly different in RBM cells cultured on HA and pure titanium. Finally, Hulshoff et al. [26] reported that bone marrow cells form a more mineralized extracellular matrix on magnetron-sputtered HA-coated titanium than uncoated titanium but that the proliferation of the cells was inhibited by the HA coating.

Differentiating osteoblasts are known to synthesize ALP and noncollagenous extracellular bone matrix proteins such as an OCN. Thus, ALP and OCN are useful markers of osteogenesis [25, 27]. In vitro studies have demonstrated that ALP is expressed during the proliferative period of extracellular matrix maturation and that the expression of OCN occurs later during the period of extracellular matrix mineralization [28]. Up to 7 days, there was little induction of osteogenic markers (data not shown), but their levels increased between 7 and 14 days. At 21 days, the levels were still elevated. Induction of ALP is usually associated with preproliferative osteoblasts, but at 21 days, they may not longer be in the same state of differentiation [29]. On the other hand, the primary RBM cells used here retain their osteogenic features even after they have differentiated into osteoblast cells [30].

Previous studies have reported that HA coatings made using PLD promote the activation of osteoblast differentiation [29, 31]. Similarly, in the present study, the different thickness of HA films significantly affected the mRNA and protein levels for bone-related genes. In particular, cells cultured on 2000-Å films had a higher level of ALP and OLP mRNA and protein. Why the different thicknesses have different effects on the differentiation of RBM cells is unclear, but it is well-known that the composition and crystallinity of HA films influences osteoblast differentiation [29]. If a high crystallinity of HA films induces cell differentiation, the highest level of mRNA and protein for bone-related genes would be expected to be found in cells cultured on 5000-Å films. Fernandez-Pradas et al. [15] reported that HA coatings obtained by KrF PLD have (β-tricalcium phosphate) XRD peaks in addition to the HA peaks and that these additional peaks increase with the thickness of the films.

We found a good correlation between ALP mRNA and protein levels. For example, on day 14, the changes in the ALP mRNA and proteins levels were not significant for any of the films. However, the levels of OCN mRNA and protein did not correlate well. The production for OCN protein by cells cultured on the 5000-Å film was enhanced at 21 days compared to bare titanium or the 500-Å film, but mRNA expression for OCN was not enhanced. At 14 days, there was no difference in the OCN production between cells on the 2000- and 5000-Å films, but a difference was observed in the mRNA levels. This may be due to the persistence of the OCN protein in the medium after the mRNA has been degraded; in other words, the half-life for the protein may be much longer than for the mRNA. Furthermore, it is possible that the mRNA expressed at a given time might not have yet been fully translated into protein.

Many arguments have been made both in opposition to and in favor of the use of HA coatings [26]. This debate has been fed by the failures of clinical implants, supposedly as a result of poor coating-substrate adherence and problems with the crystallinity of the coating [5–7]. Our previous studies [11, 12] have reported that ArF PLD generates thin ceramic coatings on implants with good adhesion to the substrate and good crystallinity. In addition, the lower annealing temperature used in the present study allows the effective recovery of the crystalline structure. Very recently, Ohashi [32] showed in animal studies that implants with a 500-Å HA film deposited with ArF PLD show adequate bone conduction at the early stage. Using another calcium phosphate coating method, Mohammadi et al. [33] found that a 1000-Å film on Ti implants elicited an improved early bone response compared with that obtained with a 20,000-Å coating.

In conclusion, the present study showed that the expression of osteoblastic markers is higher in RBM cells grown on a 2000-Å HA coating than on bare titanium. This suggests that integration of titanium implants in bone tissue should be improved if they are coated with thin films of HA. PLD is particularly useful in this regard because it can generate very thin films with good substrate adhesion and crystallinity.

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