

Effect of magnesium and calcium phosphate coatings on osteoblastic responses to the titanium surface

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PURPOSE. The aim of this study was to evaluate the surface properties and *in vitro* bioactivity to osteoblasts of magnesium and magnesium-hydroxyapatite coated titanium. **MATERIALS AND METHODS.** The magnesium (Mg) and magnesium-hydroxyapatite (Mg-HA) coatings on titanium (Ti) substrates were prepared by radio frequency (RF) and direct current (DC) magnetron sputtering. The samples were divided into non-coated smooth Ti (Ti-S group), Mg coating group (Ti-Mg group), and Mg-HA coating group (Ti-MgHA group). The surface properties were evaluated using scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS). The surface roughness was evaluated by atomic force microscopy (AFM). Cell adhesion, cell proliferation and alkaline phosphatase (ALP) activity were evaluated using MC3T3-E1 cells. Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed. **RESULTS.** Cross-sectional SEM images showed that Mg and Mg-HA deposition on titanium substrates were performed successfully. The surface roughness appeared to be similar among the three groups. Ti-MgHA and Ti-Mg group had improved cellular responses with regard to the proliferation, alkaline phosphatase (ALP) activity, and bone-associated markers, such as bone sialoprotein (BSP) and osteocalcin (OCN) mRNA compared to those of Ti-S group. However, the differences between Ti-Mg group and Ti-MgHA group were not significant, in spite of the tendency of higher proliferation, ALP activity and BSP expression in Ti-MgHA group. **CONCLUSION.** Mg and Mg-HA coatings could stimulate the differentiation into osteoblastic MC3T3-E1 cells, potentially contributing to rapid osseointegration. [*J Adv Prosthodont* 2013;5:402-8]

KEY WORDS: Biocompatible materials; Surface-Coated materials; Surface properties; Alkaline phosphatase; Bone sialoprotein; Osteocalcin

INTRODUCTION

Titanium (Ti) alloys are extensively used in orthopaedic and dental implants due to corrosion resistance, biocompatibility, machinability and load bearing capability. In order to improve the biocompatibility, several surface treatment and coating methods have been developed. Hydroxyapatite (HA) coating on titanium substrate is desirable for biomedical applications owing to its bone forming ability.¹ Recently, magnesium (Mg) alloys or Mg-coated surfaces on metallic substrates were introduced in medical applications.^{2,3} A few studies have reported an increase in the attachment and functions of osteoblast on the Mg surface.^{4,5} On the other hand, there are some concerns regarding the fast degradation of Mg as a response to the body fluid. Mg dissolution leads to an increase in pH, reducing the biological activities of

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osteogenic cells. Therefore, several studies have been performed to strengthen the Mg surface with coatings and surface treatments.^{2,6} They showed that the calcium-phosphate coating provided magnesium with a good surface bioactivity and increased new bone formation at the implant/bone interface. Therefore, a surface coating is an effective method for reducing the drawbacks of magnesium and improving the surface bioactivity.

The aim of this study was to evaluate the surface properties and *in vitro* bioactivity to osteoblasts of magnesium (Mg) and magnesium-hydroxyapatite (MgHA) coated titanium compared to non-coated titanium. In the present study, Mg and MgHA coating layers were developed on the Ti substrates by radio frequency (RF) and direct current (DC) magnetron sputtering, while in other studies electrochemical deposition was used to develop Mg coating layers.

MATERIALS AND METHODS

Target preparation

Synthetic HA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) disc (CERAC Co., USA, 127 mm diameter, 10 mm thickness) and pure Mg metal disc (127 mm diameter, 5 mm thickness, 99.9% purity) were used as the target. The Ca/P atomic ratio of HA disc was approximately 1.68.

RF magnetron sputtering

Commercially pure machined titanium (grade II) discs were prepared (12 mm and 25 mm diameter, 1 mm thickness). The discs were wet ground with 240, 400, and 600 grit silicon carbide paper, and then cleaned ultrasonically in acetone and ethanol for 10 minutes each, with deionized water rinsing between applications of each solvent.

RF magnetron sputtering was carried out using L-210HS-F (Anelva Corp., USA). Mg deposition was performed by DC sputtering for 10 minutes. A 127 mm-diameter high purity Mg (99.99%) target was mounted parallel to the substrate, at a distance of 47 mm in the deposition chamber. The sputtering chamber was evacuated to a pressure $<1 \times 10^{-5}$ Pa using an oil diffusion pump with a liquid nitrogen trap. Argon gas (99.999%) was then introduced into the chamber using a mass flow controller (66.7 Pa). The HA films were grown in a vacuum at room temperature on single side of the synthetic HA disc. The HA deposition was performed by RF magnetron sputtering for 5 hours (100W). The inlet flow rate of the sputtering gas, argon (Ar), was kept at 100 sccm. The base pressure of the system was 3×10^{-4} Pa. During deposition, the Ar flow rate was fixed at 120 sccm and the O_2 flow rate was varied from 0 to 30 sccm, which caused a change in the total pressure in the chamber in the range from 1.5 to 1.7 Pa. The total pressure was kept constant during deposition. After coating, the samples were divided into 3 groups. Group Ti-S was a non-coated smooth Ti surface. Group Mg was a Ti surface coated with Mg by DC sputtering. Group Ti-MgHA was a Ti

surface coated with Mg by DC sputtering and HA by RF magnetron sputtering.

Characterization of the coatings

Scanning electron microscopy (SEM, S-4700, Hitachi, Japan) was used to evaluate the surface morphology. The surface composition was examined by X-ray photoelectron spectroscopy (XPS.MULTILAB2000SYSTEM, SSK Co., USA). Surface roughness (Rrms: root-mean-square roughness) was evaluated by atomic force microscopy (AFM, Nano Scope IIIa, Digital Instrument, USA).

Cell culture

Mouse MC3T3-E1 cells (ATCC, Rockville, MD) were cultured in T-75 flasks in alpha minimum essential medium (α -MEM, Invitrogen Co., USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/mL penicillin, and 100 mg/mL streptomycin (Invitrogen Co., USA) at 37°C in humidified atmosphere of 5% CO_2 -95% air.

Morphological evaluation of cell spreading by SEM

MC3T3-E1 cells were evaluated by SEM for cell attachment and growth. The cells were seeded in a 12 well plate at a density of 1×10^4 cells/mL in α -MEM. After incubation for two days, the dishes were washed three times with phosphate buffered saline and fixed with 2.5% glutaraldehyde in 100 mM cacodylate buffer. The samples were dehydrated in increasing concentrations of ethanol, air-dried, and mounted on aluminum stubs and coated with platinum.

Cell proliferation assay

The MC3E3-T1 cells were cultured on Ti-S, Ti-Mg and Ti-MgHA surfaces (12 mm diameter) in 12 well plates at a density of 1×10^4 cells/mL in α -MEM. After incubation, cell proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 AQueous, Promega, USA). Formazan accumulation was quantified by the absorbance at 490 nm using an enzyme-linked immunosorbent assay plate reader (Microplate manager, BIO-RAD, USA).

Alkaline phosphatase (ALP) activity

To measure the ALP activity, MC3T3-E1 cells were seeded on Ti-S, Ti-Mg and Ti-MgHA discs (12 mm diameter) in a 12 well plate at a density of 1×10^4 cells/mL in α -MEM. The ALP activity was determined on day 7. Briefly, the cells were lysed in Triton 0.1% (Triton X-100) in PBS, frozen at -70°C and then thawed. 100 μL of the cell lysates was mixed with 200 μL of 10 mM p-nitrophenol phosphate and 100 μL of 1.5 M 2-amino-2-methyl-1-propanol buffer, and then incubated for 60 minutes in an oven at 60°C. The

ALP activity was measured from the absorbance reading at 405 nm with a spectrophotometer (SmartSpec, BIO-RAD, USA).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

All sample discs (25 mm diameter) were placed in the 6 well tissue culture dishes under aseptic conditions. Subsequently, 1.0×10^5 cells/mL in α -MEM were seeded into each well and incubated for 7 days. The total RNA was isolated on day 7 using the methodology described by the manufacturer. PCR was then performed using amplication primer sets (Sigma-Genosys, USA) for bone sialoprotein (BSP, 1068 base pairs (bp): forward, 5'-AACAAATCCGTGC CACTCA-3'; reverse, 5'-AACAAATCCGTGCCACTCA-3'), collagen type-I (COL-I, 250 bp: forward, 5'-TCTCCA CTCTTCTAGGTTTCCT-3'; reverse, 5'-TTGGGTCATTTCCAC ATGC-3'), osteocalcin (OCN, 198bp: forward, 5'-TCTGAC AAACCTTCATGTCC-3'; reverse, 5'-AAATAGT GATAC CGTAGATGCG-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 418 bp: forward, 5'-CACC ATGGAG AAGGCCGGGG-3'; reverse, 5'-GACGGACA CATTGG GGGTAG-3') genes. A semi-quantitative comparison with

GAPDH was performed to assess the changes in BSP, COL-I and OCN gene expression in the titanium specimens using a Gel-Doc imaging system (BIO-RAD, USA). These experiments were performed in duplicate and 2 samples per group were used for each experiment.

Statistical analysis

One-way analysis of the variance followed by a Scheffe's test was used to assess the data for surface roughness, cell proliferation and ALP activity. SPSS (v. 12.0) software package was used to determinethe statistical significance. $P < .05$ was considered significant.

RESULTS

Surface characteristics

SEM images showed that uniform Mg and Mg-HA layers with relatively well-developed columnar structures were developed on the Ti surface (Fig. 1). The thickness of Mg and Mg-HA layer were 11 and 20 μ m, respectively, as shown in the cross-sectional images (Fig. 1B and Fig. 1D). The surface chemical composition of Ti-Mg and Ti-MgHA

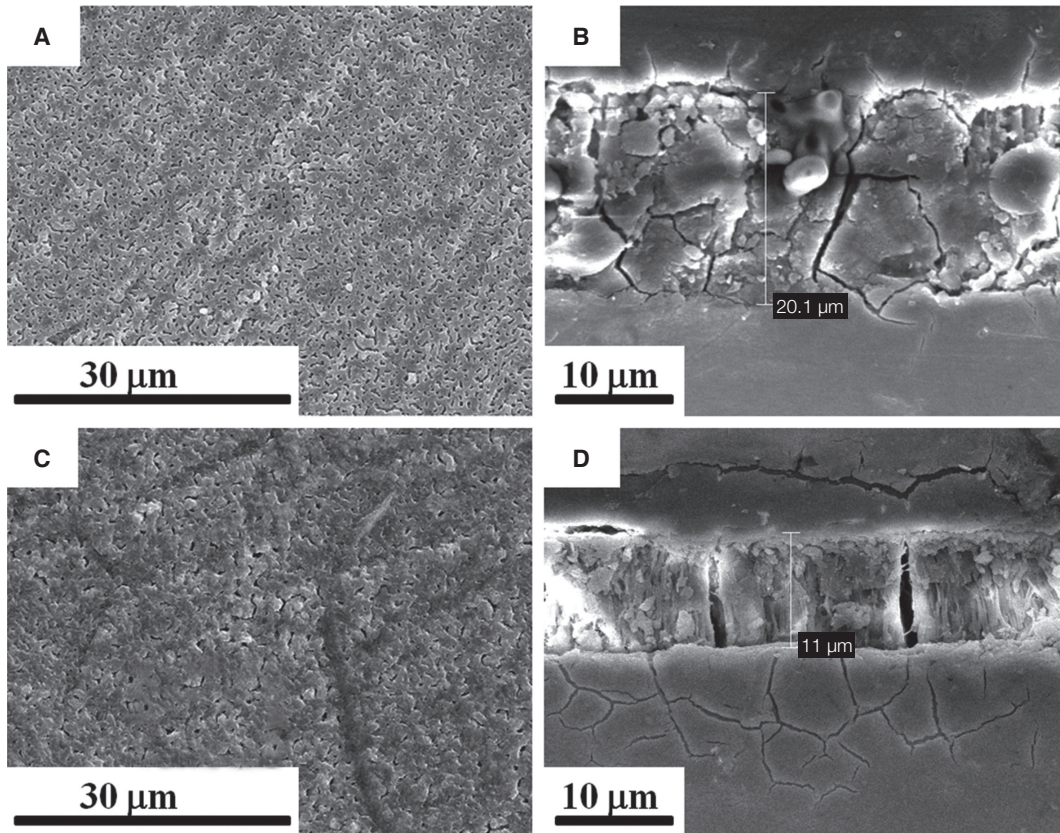


Fig. 1. Scanning electron microscopy (SEM) images of (A and B) Ti-Mg and (C and D) Ti-MgHA. ((A and C) Overview, (B and D) Cross-sectional view).

was analyzed by XPS (Fig. 2). The main element peaks of Ti-S were Ti and O. Ti-Mg showed Mg peak, while Ti-MgHA showed Ca and P peaks in addition to Mg peak.

Profile roughness measurements revealed 0.24, 0.25, and 0.25 μm of root mean square (RMS) roughness in Ti-S, Ti-Mg and Ti-MgHA, respectively (Table 1). The surface roughness appeared to be similar in all groups.

Table 1. Surface roughness of the coating surfaces

Type of Ti surface	RMS Roughness (μm)
Ti-S	0.24 \pm 0.08
Ti-Mg	0.26 \pm 0.03
Ti-MgHA	0.25 \pm 0.05

Ti-S: non-coated smooth titanium surface, Ti-Mg: magnesium coated surface, Ti-MgHA: magnesium and hydroxyapatite coated surface, RMS: root mean square.

Cell response

For each specimen, the cell morphology was examined by SEM after 48 h of cell seeding. In this study, MC3T3-E1 cells adhered tightly to the surfaces of the Ti-S, Ti-Mg and Ti-MgHA surfaces. The cells were spread extensively, flattened with elongated shapes and connected to adjacent cells by filopodia (Fig. 3). No morphological difference was observed between Ti-S, Ti-Mg, and Ti-MgHA.

Cell proliferation was measured by aMTS assay (Fig. 4). On day 3, the Ti-Mg and Ti-MgHA surfaces showed a proliferation rate of 104% to 107%, respectively, compared to Ti-S ($P > .05$). On day 5, the optical densities on the three investigated surfaces were significantly higher than those observed on day 3 ($P < .05$). On day 5, Ti-Mg and Ti-MgHA showed a proliferation rate of 112% to 124%, respectively, compared to Ti-S ($P > .05$). The cells on Ti-Mg and Ti-MgHA showed 50-60% higher ALP levels than those on Ti-S ($P < .05$, Fig. 5)

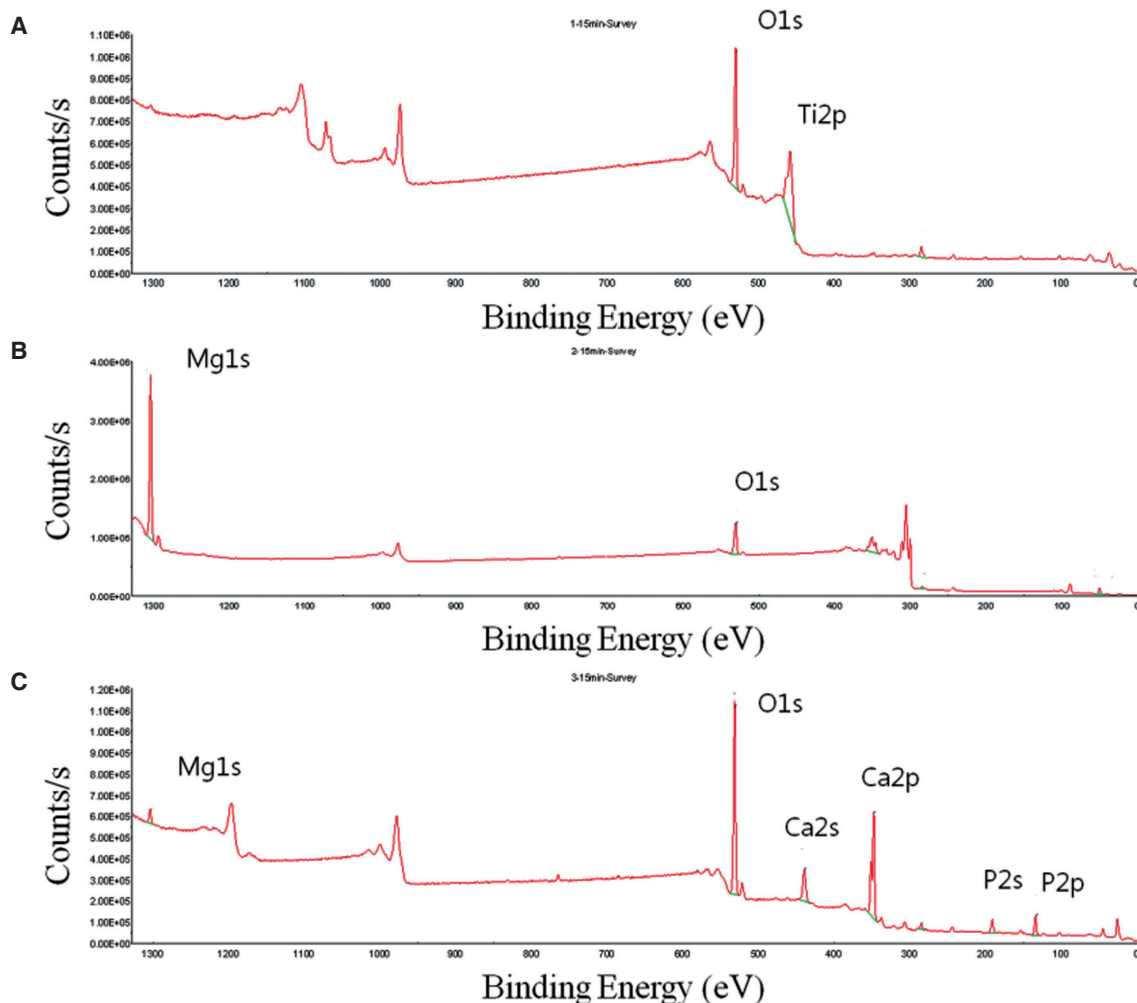


Fig. 2. XPS profile of (A) Ti-S, (B) Ti-Mg, and (C) Ti-MgHA.

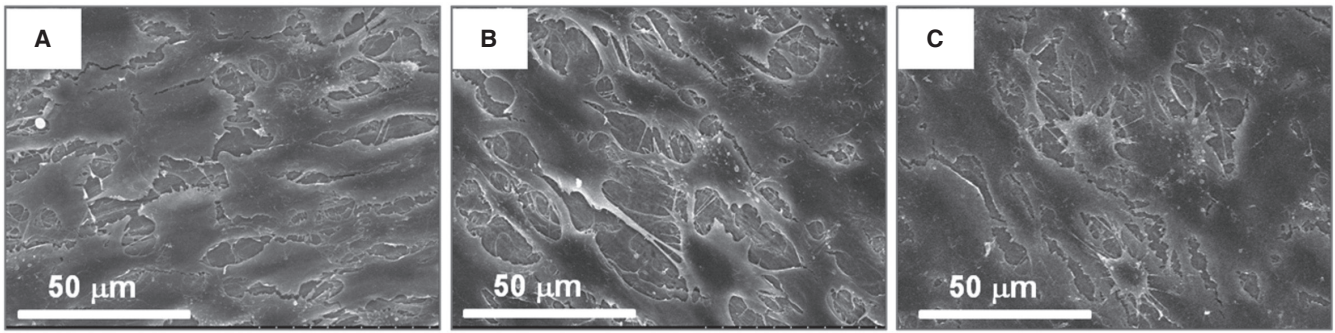


Fig. 3. SEM images of cell adhesion on the titanium surfaces at 2 days. MC3T3-E1 cells on (A) Ti-S, (B) Ti-Mg surface, and (C) Ti-Mg-HA surface.

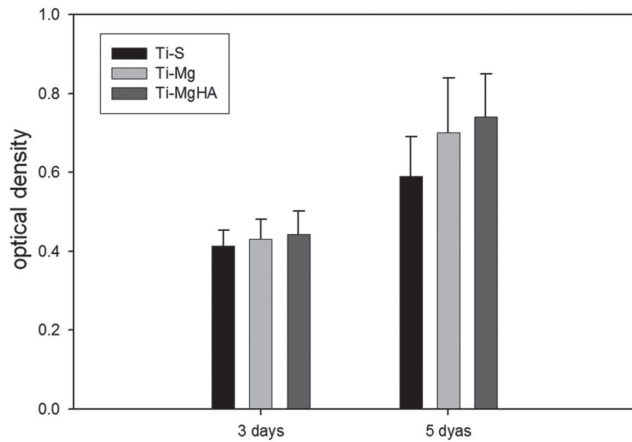


Fig. 4. Cell proliferation assay after 3 and 5 days of Ti-S, Ti-Mg, and Ti-MgHA.

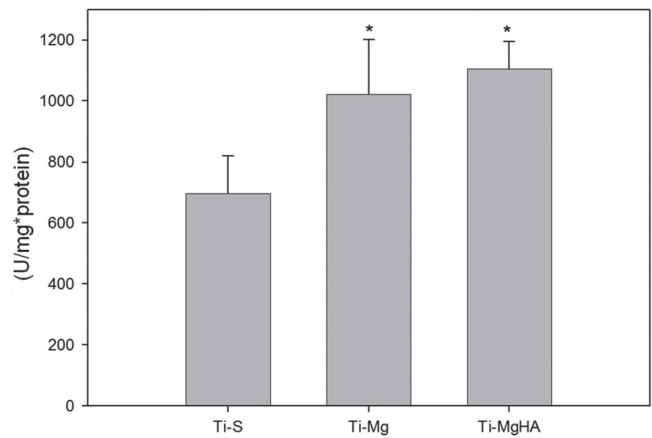


Fig. 5. Alkaline phosphatase activity (U/mg*protein) after 7 days of Ti-S, Ti-Mg, and Ti-MgHA.

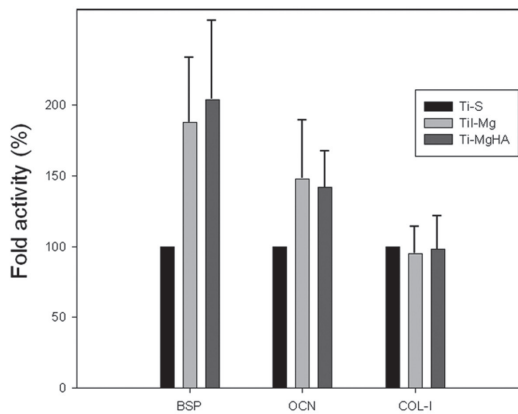
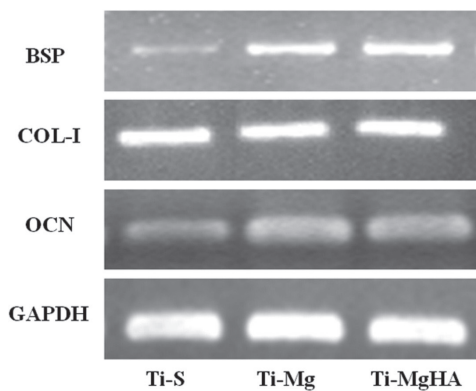


Fig. 6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of bone sialoprotein (BSP), collagen type I (COL-1), osteocalcin (OCN), and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mRNA expression in MC3T3-E1 cells.

RT-PCR analysis

Fig. 6 compares the patterns BSP, COL-I, and OCN mRNA expression of the cells cultured on Ti-Mg, Ti-MgHA, and Ti-S at 7 days. The cells on Ti-S, Ti-Mg, and Ti-MgHA expressed the COL-I gene well. The cells on

Ti-Mg and Ti-MgHA increased mRNA expressions of BSP and OCN more than the cells on Ti-S. BSP mRNA expression on Ti-Mg and Ti-MgHA increased approximately 1.8-fold and 2.1-fold respectively. OCN mRNA expression on Ti-Mg and Ti-MgHA increased approximately 1.5-fold and 1.4-fold, respectively (Fig. 6).

DISCUSSION

The main element peaks of Ti-S were Ti and O. Ti-Mg showed Mg peak while Ti-MgHA showed Ca and P peaks in addition to Mg peak. Because Ca and P are elements of HA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), these results imply Mg and HA layers were well developed on the Ti substrate.

No obvious morphological difference was observed among cells attached on Ti-S, Ti-Mg, and Ti-MgHA surfaces. Cell proliferation measurement presented a tendency that Ti-Mg and Ti-MgHA surfaces showed higher proliferation rate than Ti-S, although the difference was not significant. In addition, the difference between Ti-Mg and Ti-S or between Ti-MgHA and Ti-S increased with time.

Surface roughness is one of the factors influencing on cell responses.⁷ However, both coating groups and Ti-S showed little difference in surface roughness in the present study, while surface composition showed a clear difference. Therefore, it can be suggested that the result of cell proliferation measurement in Ti-Mg and Ti-MgHA surfaces might be attributed to the different surface chemistry.

Although there was no significant difference among the groups, the Ti-Mg and Ti-MgHA surface tended to show a higher proliferation level than Ti-S and Mg-HA coating tended to enhance cell proliferation more than Mg coating. Surface modification with Mg was reported to increase the level of osteoblastic cell adhesion and proliferation, which were associated with integrin receptors.⁸ Ibasco *et al.*³ also reported that calcium-phosphate coating formed on a Mg coated Ti surface enhanced the osteoblast viability up to 8 days. This can explain why the Ti-Mg and Ti-MgHA surface in the present study showed a higher proliferation level with respect to the Ti-S surface. Sampaio *et al.*⁹ and Hashimoto *et al.*¹⁰ showed osteoblasts grow faster on high-crystallinity HA coatings than uncoated Ti surface. The calcium-phosphate layers on the Ti substrate also enhance cell attachment and proliferation.

Regarding coating methods, we mainly focused on the releasing ion from the coatings, so post-deposition heat treatment was not performed. Hulshoff *et al.*¹¹ have suggested that magnetron-sputtered Ca-P coatings show the similar result of bone healing as the plasma-sprayed Ca-P coatings and there was little difference in amount of bone between heat-treated coating and amorphous coating at 9 weeks. Due to the difference of the thermal expansion coefficients of titanium and Ca-P coating, post deposition heat treatment causes delamination of Ca-P coating, leading to the failure of implants clinically. Dissolution of amorphous coating may promote precipitation of Ca-P apatite layer from the locally supersaturated fluids. Barrère *et al.*¹² described that the Ca-P deposition on titanium surface is needed chemical bonding of nanosized clusters and stabilized by magnesium ions. Mg ion also promotes the adherence of the precipitated Ca-P apatite layers.

The osteoblastic cells initially increase their number and produce an extracellular matrix. The differentiation phase then follows, which is characterized by the high levels of

alkaline phosphatase (ALP) production and modifications of the matrix.¹³ In this study, the cells on Ti-Mg and Ti-MgHA after 7 days showed 50 and 60% higher ALP levels than those on Ti-S, respectively ($P < .05$). This implies that the surface chemical compositions appear to affect the ALP activity, indicating the facilitation of osteoblastic differentiation. Increased ALP activity shows the increase in cell differentiation onto the chemical modification of Ti substrate.

Bone-associated markers, such as bone sialoprotein (BSP) and osteocalcin (OCN) were evaluated by semi-quantitative RT-PCR to evaluate the effect of Mg and MgHA coating on bone-associated markers. COL-I is an essential component of the extracellular matrix that is required before mineralized matrix formation. In this study, the cells on Ti-S, Ti-Mg, and Ti-MgHA expressed the COL-I gene well. BSP and OCN are secreted by osteoblasts and regulate mineralization and maturation.¹⁴ The higher levels of BSP and OCN mRNA expression with increased ALP activity indicated a more differentiated phenotype for the MC3T3-E1 on Ti-Mg and Ti-MgHA, compared to the cells on Ti-S.

Zreiqat *et al.*^{5,8} examined the osteogenic response to an Mg-ion-modified metal surface and reported enhanced osteoblastic differentiation. The improved osteogenic response contributes to the increased expression of integrin receptors of Mg-ion-modified metal surface. The integrin receptors were reported to increase the level of cell proliferation and promote the osteoblastic differentiation of bone cells. A HA film coated on biomaterials also increased the level of osteoblastic cell proliferation and differentiation, which was associated with a high level of fibronectin receptors.¹⁴ In this study, MC3T3-E1 cells were closely attached to the coated Ti surface and Mg and HA might facilitate integrin-mediated proliferation and differentiation.

Mg corroded too rapidly *in vivo*. To improve the corrosion resistance of Mg, many studies regarding different types of coating and surface treatment were performed.^{2,6} Magnetron sputtering was used to produce homogeneous and dense crystalline HA coating.¹⁵ In this study, a magnesium-hydroxyapatite coating surface had a dense and high crystalline structure, which might inhibit the rapid dissolution of Mg. Consequently, the slowed dissolution of Mg on the Ti substrate prevented an abrupt change in pH and Mg concentration in the culture media. Our pilot study showed that the Ti-MgHA surface in distilled water for 30 days still contained Mg, Ca, and P elements on the surface, but Ti-Mg did not contain Mg on the surface. Therefore, a Mg HA coating might reduce the dissolution rate of Mg and effectively enhance the osteogenic response. On the other hand, the experimental time period in this study was up to 7 days, which might be short for evaluating the response of osteoblasts to Mg and Mg HA coated Ti surface. Further *in vivo* and *in vitro* studies will be needed to determine the stability and other biological features of Ti with Mg HA coatings.

In addition, the present study did not compare the stability of coating layers developed by magnetron sputtering and other coating methods. However, the thicknesses of

coating layers were higher than reported in other studies.

In summary, Mg and Mg HA coating by RF and DC magnetron sputtering was found to stimulate differentiation into osteoblasts of MC3T3-E1 cells, potentially contributing to rapid osseointegration. HA coating on the Ti-Mg surface might be helpful for protection of Mg coating by forming a dense and high crystalline layer to be hardly degraded.

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