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A new method for the preparation of bioactive calcium phosphate films hybridized with 1α,25-dihydroxyvitamin D_3

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Abstract The primary goal of this investigation was to develop a calcium phosphate film hybridized with 1α,25-dihydroxyvitamin D_3 for the improvement of osteoconductivity of bone substitutes. The hybrid films (hCaP) were prepared at the different concentrations of 1×10^{-10}, 1×10^{-8}, and 1×10^{-6} M designated as hCaPL, hCaPM, and hCaPH, respectively. The change of the hormone concentration during the preparation of the hybrid films did not cause significant variations on the physical properties of hCaPs, i.e. surface morphology and roughness. On the other hand, X-ray photon spectroscope (XPS) measurements revealed that the concentration change affected the chemical composition of the hybrid films. Recruitment of osteoblast-like MG-63 cells was considerably improved on hCaPs compared to tissue culture plate (TCP). However, cell proliferation on hCaPs was substantially suppressed and inversely proportional to the hormone concentration used. It was observed that bone-like nodules which consisted of bead-like components and well-developed matrix were rapidly formed on hCaPs. Masson's trichrome and safranin-O stainings elucidated that the bead-like components were MG-63 cells. Safranin-O staining showed that proteoglycan was produced actively. These results indicate that the cells cultured on hCaPs were strongly stimulated by the hormone to produce proteoglycan which can be considered as an induction of premature bone formation. The number of the nodules was increased with hormone concentration and most pronounced at the hCaPH. Gene expression patterns of alkaline phosphatase (ALP), transforming growth factor-β (TGF-β), and osteopontin (OPN) were strongly modulated by hybridized the hormone. For ALP and OPN, gene expressions were activated earlier on hCaPs than untreated calcium phosphate (CaP) confirming the effect of the hybridization was substantial. The TGF-β gene expression was immediately activated after seeding but difference between samples was not significant suggesting that the gene expression was modulated not by the hormone hybridization but by CaP itself. As a result, hybridization of $1,25(OH)_2D_3$ with CaP can be a potentially strong candidate to promote osteoconductivity of implant materials.

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1 Introduction

The surface property of biomaterials is one of the crucial factors that determine the success of an implant material. The cellular responses such as cell attachment, spreading, motility, proliferation, and differentiation can be modulated by the surface properties of implants. Several materials, i.e. titanium, bioglass, polymeric compounds, calcium phosphates, and composites, have been extensively investigated as bone substitutes [1–3]. Calcium phosphates including hydroxyapatite have been widely used for orthopedic and dental implants due to their excellent biocompatibility and biointegrative properties. The mechanical toughness and fracture strength of calcium phosphates, however, limit their use from load-bearing

purposes. One way to overcome this weakness is to form calcium phosphate coating on the solid substrates such as titanium or its alloys in order to utilize the excellent bone forming ability of calcium phosphate. A number of coating methods have been introduced including plasma spraying [4, 5], electrodeposition [6], sputtering [7] and sol–gel dip coating [8]. Meanwhile, most of the coatings were prepared under the conditions different from physiological condition, for example, high temperature [9, 10], which resulted in the reduction of biocompatibility and increase in crystallinity [11].

1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is a group of steroids naturally occurring substance that can be synthesized in the skin under the influence of ultraviolet light of sun. This hormone can be obtained from food sources such as fatty fish. Active form of $1,25(OH)_2D_3$ can move into cells and bind to a specific gene such as a hormone response element to stimulate the osteogenic gene expression [12]. $1,25(OH)_2D_3$ has been reported to play a key role in regulating osteoblast responses. $1,25(OH)_2D_3$ inhibits cell proliferation but increases specific activity of ALP [13–18], synthesis of osteopontin [19] and osteocalcin [20]. ALP activity was increased on titanium but decreased on tissue culture plate (TCP) when MG-63 cells were stimulated by $1,25(OH)_2D_3$ [18]. $1,25(OH)_2D_3$ is known to inhibit type I collagen and bone sialoprotein synthesis [21–23]. However, it is also reported that cellular responses were dependent on the treatment procedure with $1,25(OH)_2D_3$. Owen et al. reported that treatments with $1,25(OH)_2D_3$ during proliferation phase of rat calvarial cell cultures can significantly inhibit the mRNA expression for type I collagen and alkaline phosphatase [24]. In addition, osteocalcin and osteopontin genes expression were suppressed and cells did not form mineralized nodules [24–26]. Therefore, cellular responses to $1,25(OH)_2D_3$ are complex and case-dependent.

In this study, we developed a new method to prepare CaP films hybridized with $1,25(OH)_2D_3$ at different concentrations under the condition mimicking physiological environments such as temperature and pH. In order to evaluate the feasibility of these films as a potential candidate for the improvement of bone regeneration, we examined hormone-responsiveness of osteoblastic MG-63 cells to the hybrid films.

2 Materials and methods

2.1 Preparation of hybrid films of $1,25(OH)_2D_3$ and calcium phosphate

Hybrid films composed of $1,25(OH)_2D_3$ and CaP were prepared on the surface of TCP according to the following procedure. $1,25(OH)_2D_3$ (D1530, Sigma) pre-dissolved in absolute ethanol was diluted in ddw. Calcium and phosphate ionic solution was prepared in ddw in which the concentrations of calcium and phosphate ions were 4.52 mM and 3.73 mM, respectively. These solutions were vigorously mixed followed by filtration for the removal of homogeneous precipitations. In order to form hybrid films of $1,25(OH)_2D_3$ and calcium phosphate, the filtered solution was applied to the TCP. All the treatment steps were performed at cold condition. The TCP containing ionic solution was stored in 4°C for 60 min followed by incubating under 5% CO_2 at 37°C for 90 min. After incubation, the solution was discarded and the treated TCP was rinsed with excess amount of ddw and then dried in clean bench overnight. The concentrations of $1,25(OH)_2D_3$ used in this study were 1×10^{-10}, 1×10^{-8}, and 1×10^{-6} M which were designated as hCaPL, hCaPM, and hCaPH, respectively.

2.2 Field-emission scanning electron microscopic (FE-SEM) analysis

The surface morphology of the CaP and hCaPs was observed with field emission scanning electron microscope (JSM-6700F, Jeol) at the acceleration voltage of 15 kV. Prior to morphological observation, the CaP and hCaPs were sputter-coated with gold and the images were obtained at the magnification of 30,000.

2.3 Atomic force microscopy (AFM) analysis

A scanning probe microscope (AutoProbe CP Research System, Thermomicroscope, USA) was used to measure the surface roughness of hCaPs. The values for average surface roughness of CaP and hCaPs were determined.

2.4 X-ray photoelectron spectroscopic (XPS) analysis

For the determination of chemical elements constituting of hCaPs, samples were examined using X-ray photoelectron spectrometer (AXIS-NOVA, Kratos Inc.). The XPS spectrophotometer employed a standard monochromatic Al Kα excitation source ($h\nu = 1486.6$ eV). For the comparison purpose, XPS spectrum for $1,25(OH)_2D_3$ adsorbed on the surface of TCP was obtained. Prior to XPS measurements, samples were dried under ambient environments. Adventitious contaminants were removed by sputtering with argon ions.

2.5 Cell culture

Osteoblast-like MG-63 cell was purchased from ATCC. MG-63 cell is a human osteoblast sarcoma and displays

various characteristics of osteoblast. MG-63 cells were seeded on a TCP containing Dulbecco's modified eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and 0.5% antibiotics diluted from a stock solution containing 5000 U/ml penicillin, 5000 U/ml streptomycin and then incubated at 37°C in an atmosphere of 5% CO_2. Culture media was changed at a regular interval. Prior to experiments, cells were harvested using trypsin–EDTA and pre-determined number of cells was seeded on the samples.

2.6 Cell attachment assay

Attachment assay was performed in order to examine the interaction between MG-63 cell and samples. MG-63 cells were seeded both on TCP and hCaPs at 1×10^4 cells per well and then allowed to attach for 4, 8 and 12 h. The cell attachment was quantified using crystal violet assay kit (C6158, Sigma-Aldrich) according to manufacturer's instruction. After desired contact time, the media was aspirated from the well followed by washing with PBS twice. The culture plate was incubated at 4°C for 10 min after addition of 0.25 ml 70% cold ethanol followed by washing with PBS twice. Pre-made 0.05% crystal violet solution (12.5 ml 0.2% Crystal violet stock solution + 37.5 ml ddw) was added to the well and incubated at a room temperature. After 10 min, the well was washed with ddw for three times and 1% SDS solution was added for cell lysis. Aliquot obtained from the well was transferred to 96-well plate and the optical density was measured at 570 nm using a microplate reader. All measurements were triplicated.

2.7 Cell proliferation assay

In order to examine the effect of $1,25(OH)_2D_3$ entrapped within CaP film on the proliferation of MG-63 cells, cells were seeded at the cell density of 5×10^3 cells per well and then cultured for 3, 5, and 7 days. At desired culture period, 300 μl of mixture of 10% of CCK-8 solution and 90% of growth media was added into the culture after removal of growth media. Then, the plates were incubated for 3 h. The amount of MG-63 cells grown on each sample was determined by measuring the optical density at 450 nm on the microplate reader with a reference wavelength at 600 nm. All measurements were triplicated.

2.8 Microscopic and histochemical analysis on bone-like nodules

During the culture of MG-63 cells on hCaPs, the formation of bone-like nodules was observed using phase contrast microscope. The number of bone-like nodules was counted and the experimental data point was presented as the mean ± SEM of three cultures. Micrographs of the nodules were taken during the culture at a regular interval. Structural details of the nodule were examined using SEM. For SEM observation, culture was rinsed with PBS and then fixed with formaldehyde. Then the culture was stored deep freezer at −82°C overnight and then freeze–dried for 1 day. The dried culture was coated with gold using plasma spray coater. SEM micrographs were taken at the magnification of 300× and 3000×. In order to analyze the components constituting the nodules, we performed histochemical staining including Masson's trichrome staining and safranin-O (Grogg Chemie AG, Deisswil, Switzerland) on the culture according to manufacturer's instruction. Histochemical staining using alizarin red S was performed in order to examine the effect of the incorporation of $1,25(OH)_2D_3$ within CaP matrix. MG-63 cells were plated on TCP, hCaPL, hCaPH, and pure CaP formed on TCP at the cell density of 20,000 cells per well and continuously cultured for 12 days. For the pure CaP formed on TCP, 1×10^{-6} M $1,25(OH)_2D_3$ was dissolved in culture media instead of incorporating $1,25(OH)_2D_3$ within CaP film to examine the difference between incorporation of $1,25(OH)_2D_3$ within CaP film and presence in culture media. The culture media was changed in a regular interval. After 12 days of culture, media was removed and washed with PBS for two times followed by fixing with 10% formaldehyde for 10 min. Each sample was washed with ddw twice and then incubated in 2% alizarin red S. After 10 min incubation, each sample was washed PBS twice and dehydrated in 70, 95, and 100% ethanol.

2.9 Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from the MG-63 cells cultured for 5, 10, and 15 days on CaP and hCaPs using Tissue RNA PrepMate (K-3080, Bioneer, Korea) according to the manufacturer's instruction. The untreated CaP was used as a control. The amount of total RNA was determined by absorbance at 260 nm and the purity of the RNA was confirmed by the ratio between the absorbance values at 260 and 280 nm representing the amount of RNA and total protein, respectively. 2 μg total RNA and 20 pmole oligo dT_{20} (20 mer, N-7053, Bioneer, Korea) were mixed with RT premix (AccuPower RT Premix, K-2042, Bioneer, Korea) followed by filling reaction volume up with DEPC-DW. cDNA was synthesized at 60 min at 42°C and 5 min at 94°C using thermal cycler (FTGENE2D, Techne, UK). The cDNA was used as a template for the amplification of osteoblast phonotypic genes using gene-specific primers for ALP, TGF-β, and OPN. Base sequences of the primers were listed in the Table 1. PCR was performed by mixing

Table 1 Primer sequences used in PCR

Gene	Primer	Primer sequence (5′–3′)	Size (bp)	No. of cycles
ALP	Forward	ACGTGGCTAAGAATGTCATC	475	40
	Reverse	CTGGTAGGCGATGTCCTTA		
TGF-β	Forward	TACCTGAACCCGTGTTGCTC	294	30
	Reverse	CGGTAGTGAACCCGTTGATG		
OPN	Forward	ACCTCACACATGGAAAGCGA	257	40
	Reverse	CTGTGGAATTCACGGCTGAC		
GAPDH	Forward	AAGTGGATATTGTTGCCATC	454	30
	Reverse	ACTGTGGTCATGAGTCCTTC		

5 μl of cDNA and 20 pmol of gene-specific forward and reverse primers in PCR premix (AccuPower PCR Premix, K-2014, Bioner, Korea) containing 2.5 units Taq DNA polymerase, 250 μM dNTP mix, 10 mM Tris–Cl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye. Aliquots of PCR products were loaded on 2% agarose gel for the confirmation of the expressed genes using nucleic acid staining kit (C-833, Bioneer, Korea). PCR reaction condition was 5 min at 94°C for initial denaturation followed by cycles of 30 s at 94°C for denaturation, 30 s at 55°C for annealing, and 1 min at 72°C for extension. Number of reaction cycles for each primer was indicated in Table 1.

2.10 Statistical analysis

Each data point presented in figures represents the mean ± SEM of three individual cultures. Statistical difference of between groups was analyzed using *t*-test. Statistical differences were considered to be significant when the *P* values ≤ 0.05.

3 Results

3.1 Surface characterization by FE-SEM and AFM

The surface morphology of the samples was observed using FE-SEM (Fig. 1). hCaP films demonstrated highly porous three-dimensional structures but did not show significant variations in physical appearance although the films were prepared at different $1,25(OH)_2D_3$ concentrations. We further examined the hCaPs using AFM whether the hormonal concentration affected the surface roughness of the hCaPs since the surface roughness is one of factors that significantly affect the proliferation and differentiation of osteoblast

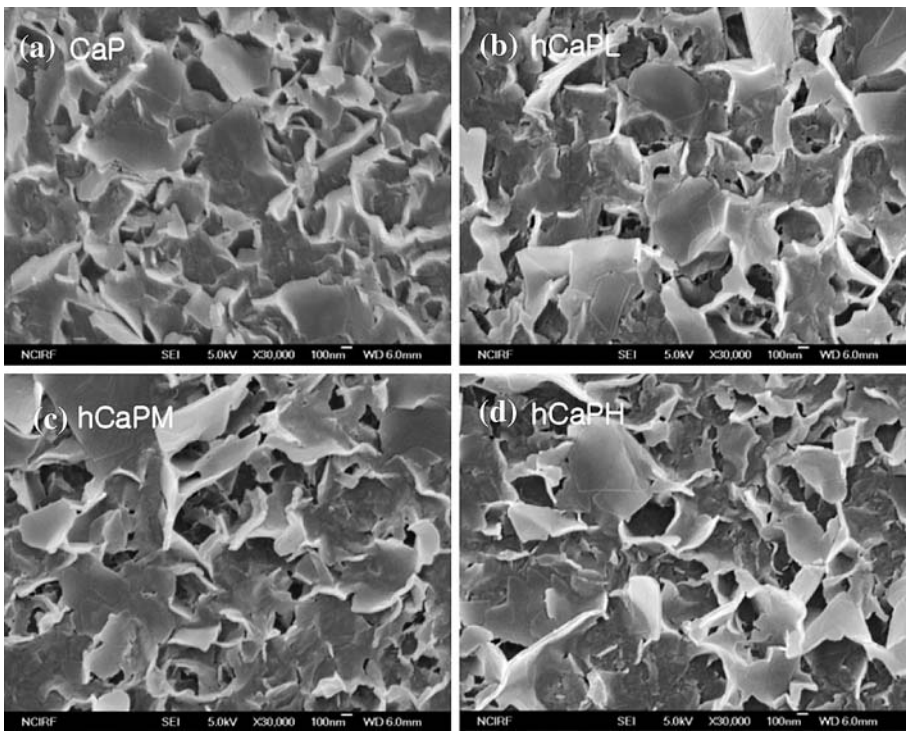

Fig. 1 FE-SEM micrographs of the hCaPs: **a** CaP film prepared in the absence of $1,25(OH)_2D_3$. Hybrid films of hCaPL (**b**), hCaPM (**c**), and hCaPH (**d**) were prepared at 1×10^{-10}, 1×10^{-8}, and 1×10^{-6} M $1,25(OH)_2D_3$ (Bar: 100 nm)

[27, 28]. The values of surface roughness were measured as 0.62, 0.68 and 0.66 μm for hCaPL, hCaPM, and hCaPH, respectively indicating that the hybridized films prepared at different $1,25(OH)_2D_3$ concentrations did not introduce significant variations on surface roughness. Accordingly, the physical properties of hCaPs were not significantly altered due to the hybridization of $1,25(OH)_2D_3$ and CaP.

3.2 XPS analysis on the chemical states of the hybrid films

XPS measurements were performed in order to examine the chemical composition of hCaPs. The atomic percentages of the constituent elements were listed in Table 2 showing that hCaPs consisted of calcium, phosphorus, oxygen and carbon elements. The ratio of atomic percentage of calcium and phosphorus (Ca/P ratio) widely used for the characterization of calcium phosphates was measured as 1.20, 1.16 and 1.24 for hCaPL, hCaPM, and hCaPH, respectively. These values are considerably lower than those of octacalcium phosphate (1.33), tricalcium phosphate (1.50), and hydroxyapatite (1.67). As $1,25(OH)_2D_3$ concentration was increased, the atomic percentage of carbon was increased but those of calcium and phosphorus were decreased. XPS spectra for chemical elements were subject to peak analysis after correction using aliphatic carbon peak at 284.6 eV as an internal standard. Peaks for chemical elements of hCaPs were deconvoluted using peak analysis program. The binding energies of subpeaks for O1s, C1s, Ca2p, and P2p were listed in Table 3. In all cases, the position of binding energy was not significantly changed. XPS spectrum obtained from $1,25(OH)_2D_3$ adsorbed on TCP and compared with spectra from hCaPH. Figure 2a displayed the comparison of spectra for C1s. The spectral peak became broader after hybridization. For oxygen, the center of spectral peak was considerably shifted due to the hybridication (Fig. 2b). These results are believed to be due to the hybridization which caused changes in chemical composition of carbon and oxygen elements. Peaks for C1s, O1s, Ca2p and P2p of hCaPH were deconvoluted using peak analysis program and presented in Fig. 2c, d, e, and f, respectively. Figure 2c shows the peak of C1s XPS spectra contained two different chemical moities. Peak attributable to carbonate was found at 289 [29]. For oxygen, subpeak at 531.3 should be associated with the oxygen atom in PO_4^{2-} (Fig. 2d) [30]. Regarding calcium, the Ca2p peak contained two subpeaks located at 347.9 and 351.5 which were typical for calcium and observed in hydroxyapatite [31]. For phosphorus, the subpeak of P2p spectrum at 133.3 can be assigned to phosphorus [32].

Table 2 Atomic percentage measured from hCaPs by XPS measurements

Elements	Atomic percentage		
	hCaPL	hCaPM	hCaPH
C	21.5	24.3	26.2
O	46.8	44.9	45.4
Ca	17.2	16.6	15.7
P	14.4	14.2	12.7

3.3 Cell attachment

For an implant material, it is important to have a capability to recruit cells to the surface of the material from surrounding tissues upon implantation. Attachment assay can be used to estimate the ability of the material. We examined MG-63 cells attachment on TCP (control), hCaPL, hCaPM and hCaPH (Fig. 3). Attachments of MG-63 cells on all the hCaPs were at least 1.5-fold greater than that on TCP throughout culture periods. These results suggest that hybridized films strongly promote MG-63 cell attachment.

3.4 Cell proliferation

MG-63 cells were cultured on TCP and hCaPs for 3, 5 and 7 days. On the contrary to the stimulatory effect of hCaPs on attachment, proliferation on hCaPs was significantly delayed compared to TCP (Fig. 4). Until day 5, the number of cells grown on TCP was more than 2-fold greater than that on the hCaPs. Culture on TCP appeared to reach stationary phase on 7 days of culture. However, cells on hCaP films grew much slower and appeared to be on exponential growth phase until day 7. Although the proliferation of the cells was delayed on hCaPs, the cell number was gradually increased in the course of culture time. The delay was in a

Table 3 Binding energies determined from deconvolution of XPS spectra for C1s, O1s, Ca2p, and P2p

Sample	Elements								
	C1s			O1s		Ca2p		P2p	
hCaPL	284.6 (44.0)	286.6 (44.3)	289.8 (11.7)	531.1 (53.5)	532.9 (46.5)	348.2 (70.6)	351.8 (29.4)	133.2 (35.1)	134.8 (64.9)
hCaPM	284.7 (51.2)	286.7 (37.6)	290.1 (11.2)	530.1 (51.2)	532.9 (48.8)	348.3 (70.9)	351.9 (29.1)	132.9 (34.7)	134.6 (65.3)
hCaPH	284.6 (59.7)	286.5 (28.8)	289.5 (11.5)	531.4 (68.1)	533.0 (31.9)	347.9 (68.1)	351.5 (31.9)	133.5 (50.3)	134.7 (49.7)

Numbers in parenthesis represent the percentage of the subpeak area

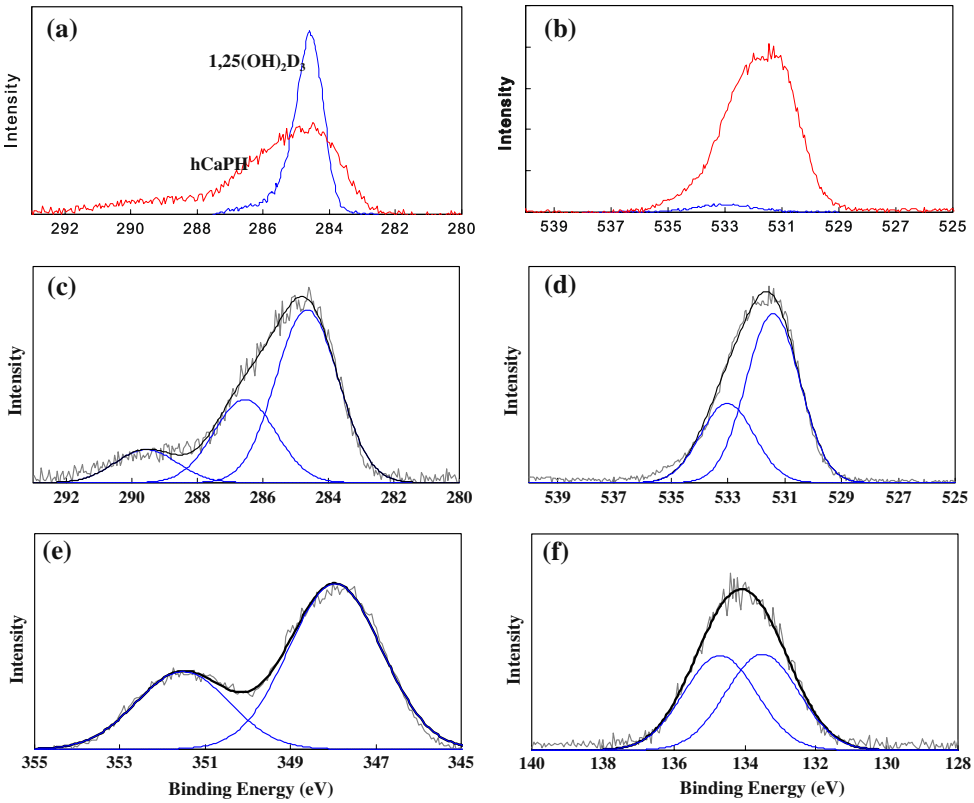

Fig. 2 XPS spectra analysis on chemical elements of the hybrid film. XPS spectra for C1s (**a**) and O1s (**b**) were obtained from hCaPH and $1,25(OH)_2D_3$ adsorbed on TCP. Spectral analysis on the peaks of constituent elements of hCaPH: C1s (**c**), O1s (**d**), Ca2p (**e**), and P2p (**f**)

hormone concentration-dependent manner. This result is in agreement with other reports that the cell proliferation was inhibited by $1,25(OH)_2D_3$. However, different from current study, the hormone was dissolved in culture media [33, 34]. Therefore, this result demonstrated that the $1,25(OH)_2D_3$ hybridized with CaP maintained the biological functions even after hybridization.

3.5 Formation of bone-like nodules resembling bone-like nodule

During the culture of MG-63 cells on hCaPs, we observed that nodules were rapidly formed. The SEM observation on the morphology of the nodule formed on culture days 7 demonstrated that the nodule consisted of extensively

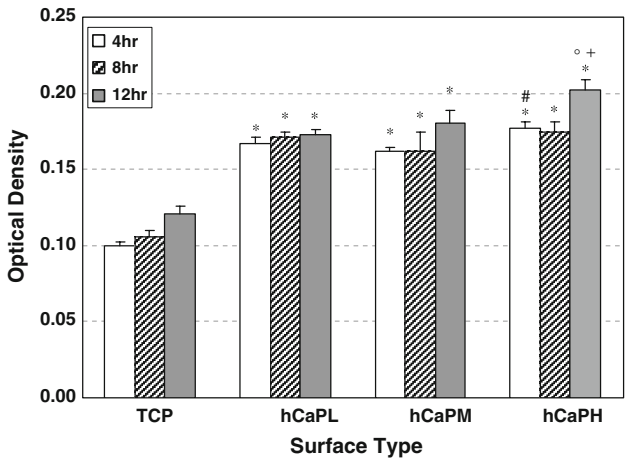

Fig. 3 Adhesion of MG-63 cells on TCP and hCaPs for 4, 8, and 12 h. Duration of incubation was indicated within the figure legend. All measurements were triplicated. * $P < 0.01$, TCP versus hCaPs; # $P < 0.01$, hCaPM versus hCaPH; ◦ $P < 0.05$, hCaPL versus hCaPH; + $P < 0.01$, hCaPM versus hCaPH

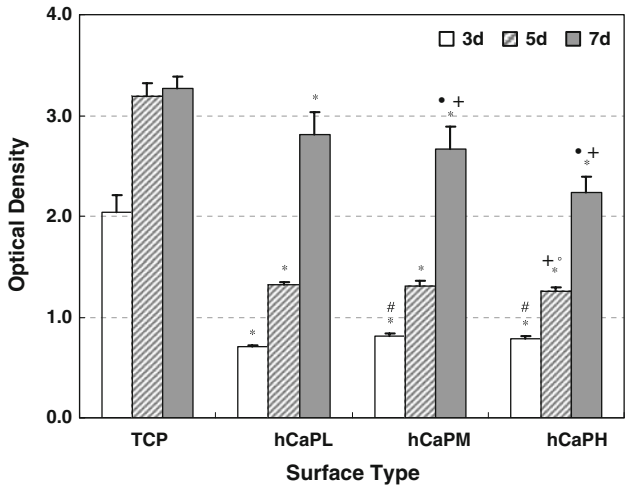

Fig. 4 Proliferation of MG-63 cells on TCP and hCaPs were determined after 3, 5, and 7 days of culture. All measurements were triplicated. * $P < 0.01$, TCP versus hCaPs; # $P < 0.01$, hCaPL versus hCaPM and hCaPH; ◦ $P < 0.05$, hCaPL versus hCaPH; + $P < 0.01$, hCaPL versus hCaPM and hCaPH; • $P < 0.01$, hCaPM versus hCaPH

developed matrix within which numerous bead-like components were embedded (Fig. 5a). We could also observe that numerous cells gathered on the rim of the nodule. Detailed morphology of the nodule was displayed in the inset of the Fig. 5a. Upon Masson's trichrome staining, the bead-like components were stained in violet color revealing that the bead-like components were MG-63 cells (Fig. 5b). Safranin-O staining elucidated that proteoglycan was extensively expressed (Fig. 5c). The development of

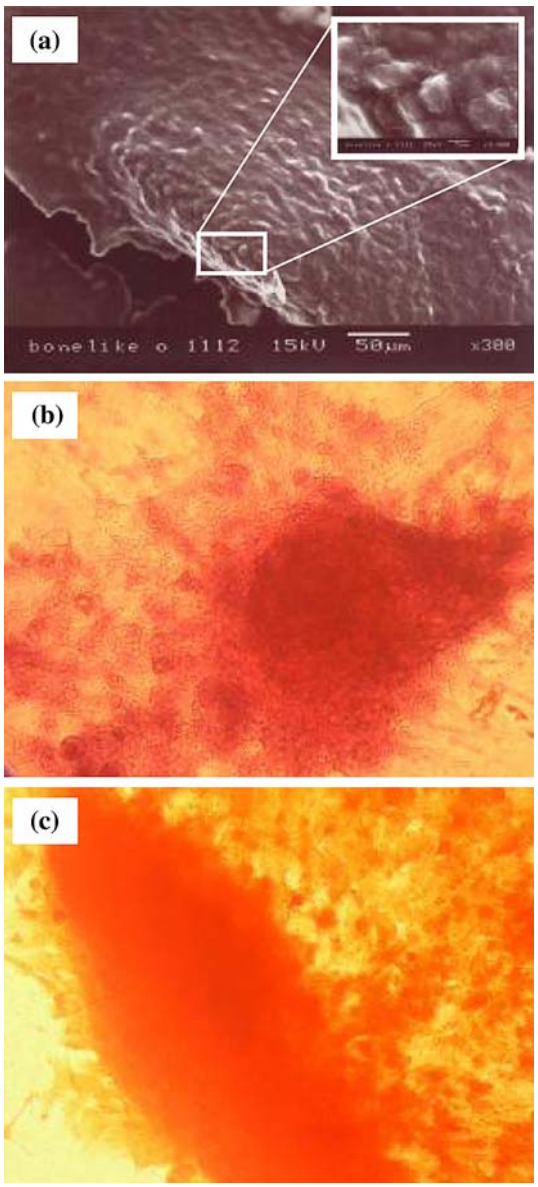

Fig. 5 SEM observation and histological staining on the cluster observed in hCaPs. **a** Micrograph of a cluster obtained from 7 days of culture on hCaP. Bar = 50 µm; **b** Masson's trichrome staining displays the cluster was stained in violet indicating that the cluster was consisted of MG-63 cells. **c** Safranin-O staining produced intensive red color illustrating that the cells constituting the cluster was actively expressing proteoglycan

the nodules on sample surfaces was monitored in the course of culture time using phase contrast microscope at 7, 14, and 21 days of culture (Fig. 6). No nodules were observed from the TCP throughout culture period (Fig. 6a–c). On the other hand, a number of bone-like nodules began to form within 3 days of culture. The development of the nodules on hCaPL, hCaPM and hCaPH are illustrated in Fig. 6d–f, g–i and j–l, respectively. Nodule size was also increased with culture time. The number of nodules formed after 7 days of culture is plotted in Fig. 7. The number of nodules was increased with increase of $1,25(OH)_2D_3$ concentration used at the preparation. The nodule number was strikingly higher on hCaPH compared to hCaPL and hCaPM implying that 1×10^{-6} M was the most influential for the nodule formation. In order to examine the mineralization of bone-like nodule, we performed alizarin red S staining. As shown in Fig. 8a, the culture on TCP was not stained at all indicating the absence of calcium ions. On the other hand, the cultures on hCaPL and hCaPH were stained with increase of $1,25(OH)_2D_3$ concentration (Fig. 8b, c). At the same time, bone-like nodules were heavily stained indicating that the nodules contain significant amount of calcium ions. When MG-63 cells were cultured on the pure CaP film in the culture media where 1×10^{-6} M of $1,25(OH)_2D_3$ was dissolved, the bone-like nodules were formed but less compared to those of hCaPL and hCaPH (Fig. 8d). At the same time, the proliferation of MG-63 cells was greatly suppressed compared to other cases.

3.6 Modulation of marker gene expressions by the hybridization of $1,25(OH)_2D_3$ and CaP

We examined the effect of the hybridization of $1,25(OH)_2D_3$ with CaP on the expression patterns of osteogenic marker genes of ALP, TGF-β, and OPN. The untreated CaP film was used as a control for the comparison of the effect of hybridization.

ALP is one of the marker proteins produced during the early stage of osteoblast differentiation [35]. Hence ALP activity is widely used as a typical indication of osteoblastic differentiation. At day 5, the ALP gene expression was activated on hCaPs earlier than on CaP and the expression level was in the order of hCaPH, hCaPL, and CaP (Fig. 9a). The gene expression was increased in all sample surfaces until day 10. The expression levels were slightly decreased on day 15. This result suggests that ALP gene expression was quickly activated both on CaP and hCaPs but expression level was stronger on hCaPs than CaP due to the presence of $1,25(OH)_2D_3$ within CaP.

TGF-β is one of the major growth factors present in bone matrix and plays a significant role in the promotion of osteoblastic differentiation and bone matrix [36, 37]. In our

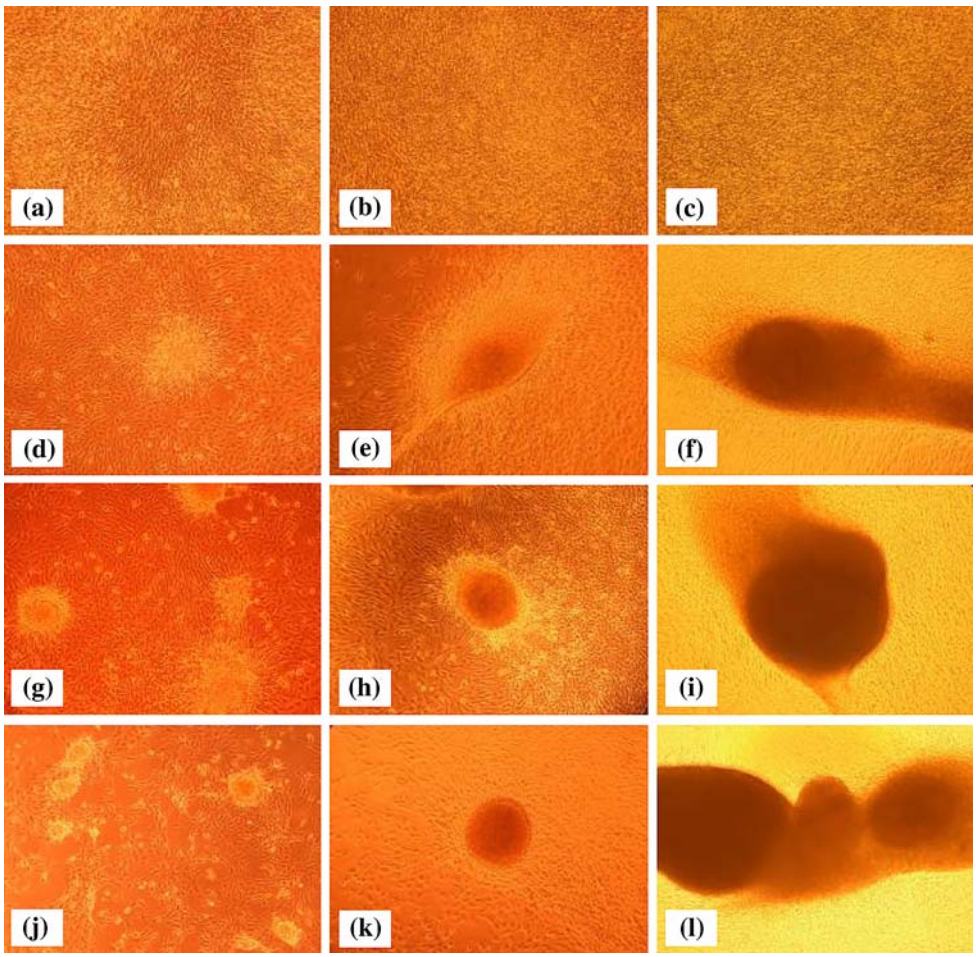

Fig. 6 Phase contrast micrographs of MG-63 cell were taken at 7, 14 and 21 days of culture on TCP (**a**, **b**, **c**), hCaPL (**d**, **e**, **f**), hCaPM (**g**, **h**, **i**), and hCaPH (**j**, **k**, **l**), respectively. The number of cellular clusters was increased with $1,25(OH)_2D_3$ concentration. Cluster size was increased with culture time displaying greatly developed clusters on 21 days of culture (Magnification = 100×)

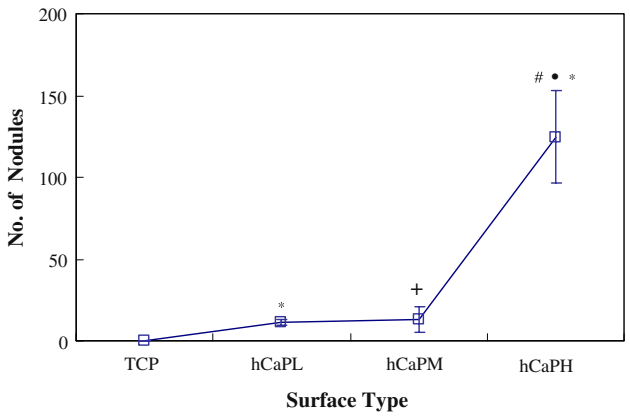

Fig. 7 The number of cellular clusters determined after 7 days of culture was presented in terms of mean value with standard deviation. All measurements were triplicated. * $P < 0.01$, TCP versus hCaPs; $^{+}$ $P < 0.05$, TCP versus hCaPM; $^{\#}$ $P < 0.01$, hCaPL versus hCaPH; $^{\bullet}$ $P < 0.01$, hCaPM versus hCaPH

study, the TGF-β gene expressions on CaP and hCaPs were activated from day 5 and maintained at elevated level until 15 (Fig. 9b).

OPN is synthesized during active proliferation and on the early stage of mineralization [38]. Therefore, the OPN gene expression pattern can provide a useful information regarding the responsiveness in osteoblast to the hybridized $1,25(OH)_2D_3$. The gene expression pattern of OPN was considerably different from ALP and TGF-β. At day 5, OPN gene expression was dramatically increased on hCaPH compared to CaP or hCaPL (Fig. 9c). At day 10, it was interesting to observe that the expression level on hCaPH was dramatically suppressed whereas the level on hCaPL reached peak level. The expression level on CaP was lower than hCaPs and slightly decreased. At day 15, the gene expression was no longer detectable indicative of complete suppression of the gene expression on hCaPH. The expression level on hCaPL was greatly reduced and the expression on CaP was similar to that of day 10. Therefore, it can be seen that the presence of $1,25(OH)_2D_3$ played a crucial role to the modulation of TGF-β gene expression pattern. At the same time, the degree of modulation on gene expression is expected to be amplified if we can increase the $1,25(OH)_2D_3$ concentration within hCaP.

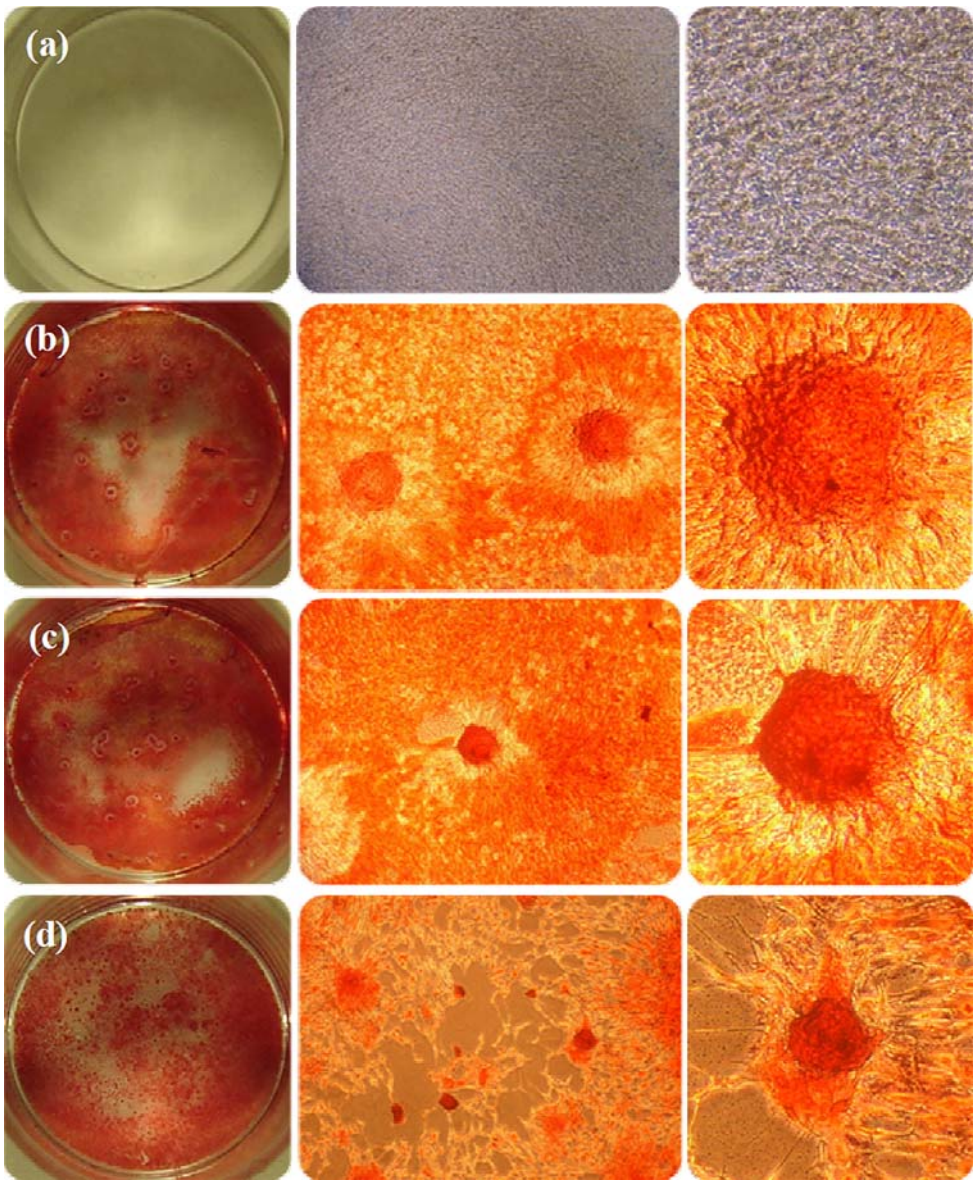

Fig. 8 Alizarin red staining of bone-like nodules formed by MG-63 cells after culturing for 12 days on TCP (**a**), hCaPL (**b**), hCaPH (**c**) and pure CaP (**d**). Figures in first column represent the photograph of culture plate. The figures in second and third columns represent stained bone-like nodules

4 Discussion

The primary objectives of this study were to develop a hybrid film composed of organic–inorganic compounds and to examine the feasibility of the film for the improvement of biocompatibility and osteoconductivity of implant materials. In order to achieve this goal, we developed a new hybrid film of $1,25(OH)_2D_3$ and CaP and examined the responsiveness in osteoblast-like MG-63 cells to $1,25(OH)_2D_3$ present within the hCaP. To examine the effect of $1,25(OH)_2D_3$ concentrations used to prepare the hybrid films on MG-63 cell responses, the hybrid films were prepared at different hormone concentrations. To our knowledge, hybridization of $1,25(OH)_2D_3$ and CaP has not been explored yet and it will be very meaningful to examine the feasibility of the hybridized film for the modification of surface properties of implant materials. In addition, this method can be further utilized to develop new systems of organic–inorganic hybrid films.

According to SEM observations and AFM measurements, the surface morphology of hCaPs displayed a highly porous three-dimensional structure which can provide increased surface area to which attachment molecules and cells can readily interact. It will be a favorable condition for bone regeneration mechanisms. The difference of surface roughness between hCaPs was negligibly small so that the surface roughness of the hCaPs was not a significant factor that can differentiate the cellular responses. Therefore, the physical properties of hCaPs were not be significantly altered within the $1,25(OH)_2D_3$ concentration range used in this study. The chemical states examined by XPS measurements confirmed that the prepared films were

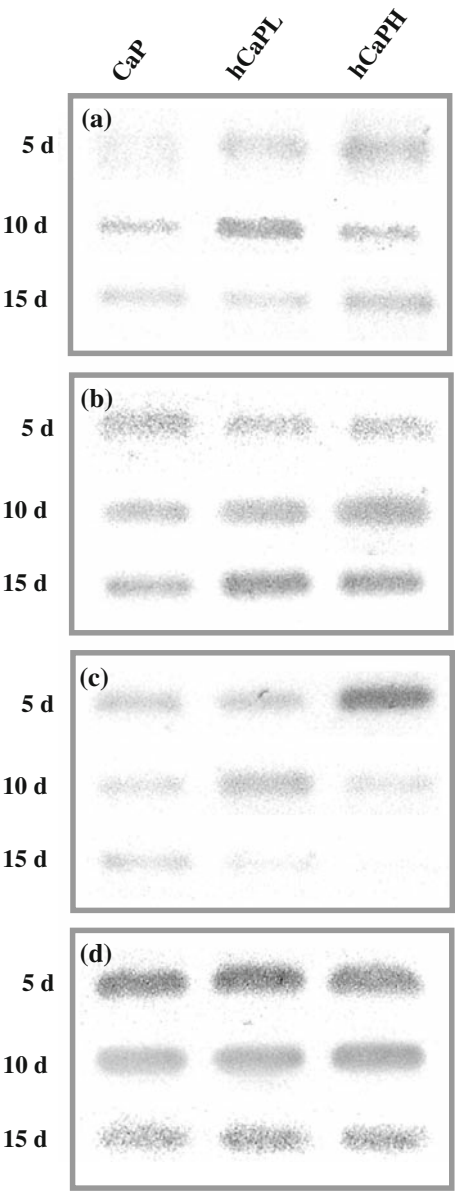

Fig. 9 Gene expression patterns of ALP (**a**), TGF-β (**b**) and OPN (**c**) obtained from CaP, hCaPL, and hCaPH after culturing 5, 10 and 15 days. House-keeping gene, GAPDH (**d**), was used as an internal standard obtained from corresponding days of culture, respectively

hybridized form of $1,25(OH)_2D_3$ and CaP. The atomic percentage of carbon element was increased in response to the increase of $1,25(OH)_2D_3$ concentration due to the hybridization of $1,25(OH)_2D_3$ with CaP. The atomic percentages of calcium and phosphorus were decreased with increase of $1,25(OH)_2D_3$ concentration. Therefore, the presence of $1,25(OH)_2D_3$ clearly affected the chemical properties of the hybrid films. We further examined the effect of the changes of chemical properties of hCaPs with regard to attachment, proliferation and marker gene expression pattern.

Recruitment of cells to the implant surface is one of crucial factors for successful bone formation. The attachment of MG-63 cell was increased at least two-fold on hCaPs compared to TCP whereas there were no significant differences between hCaPs. Therefore, the increase of $1,25(OH)_2D_3$ present in CaP did not affect the recruitment of MG-63 cells to the hybrid films, which is probably due to the constant physical properties of hCaPs. On the contrary to our observation, it was reported previously that osteoblasts attached faster and to a greater extent on TCP than on hydroxyapatite [39]. Therefore, the surface property of hCaPs prepared in this study can be excellent for cell recruitment. For bone regeneration, it will be desirable that the attached cells can readily proliferate and differentiate on the implant [40].

The proliferations of MG-63 cells were considerably delayed on hCaPs compared to TCP. The number of cells was 2-fold greater than those of hCaPs by day 5. The inhibitory effect of the $1,25(OH)_2D_3$ on the proliferation of MG-63 cells was stronger as $1,25(OH)_2D_3$ concentration was increased. This result is in an agreement with the other reports that the proliferations of MG-63 cells and fetal rat calvarial cells were inhibited in the presence of $1,25(OH)_2D_3$ in a dose-dependent manner of the hormone dissolved in culture media [33, 34]. Coupled with our result, this study demonstrates that the biological function of $1,25(OH)_2D_3$ has been maintained intact even if the hormone was hybridized with CaP.

We observed that bone-like nodules were rapidly forming on hCaPs. Microscopic observations of these nodules displayed structurally similar aspects of bone-like nodules reported by others [41, 42]. The SEM observations elucidated that the nodule was composed of extensively developed matrices. On the other hand, no such nodules were found in TCP. Histochemical stainings such as Masson's trichrome method and safranin-O elucidated that the nodules were collection of MG-63 cells expressing extensive amount of proteoglycan which is considered to play a significant role in bone formation mechanism. This result strongly suggests that hCaPs can provide strong stimulation to MG-63 cells to actively synthesize proteoglycan. Alizarin red S staining demonstrated that the nodules contain a significant amount of calcium ion when MG-63 cells were cultured on $1,25(OH)_2D_3$-incorporated CaPs while no sign of staining was observed from the cells cultured on TCP. As a comparison, MG-63 cells were cultured on the pure CaP film with the culture media in which $1,25(OH)_2D_3$ was dissolved. The proliferation of MG-63 cells was significantly suppressed compared to other cases, which is in a good agreement with other reports [13–18]. These cells still produced nodules but the extent of nodule formation was much less than those of hCaPs.

We should emphasize the fact that these results were obtained from hybridization of $1,25(OH)_2D_3$ and CaP in the absence of differentiation factors including $1,25(OH)_2D_3$ in the culture media. For now, we can not fully understand the formation mechanism of the bone-like nodules. However, it is clear that the cells were stimulated by hCaP to form bone-like nodules. At the same time, cells were stimulated to actively synthesize proteoglycan necessary for bone regeneration. We expect that osteoblastic differentiation can be further promoted so that bone regeneration can be accelerated if we culture osteoblasts on hCaPs in the presence of differentiation factors.

In order to examine the effectiveness of the hybrid films, we investigated the hormone responsiveness in osteoblast-like MG-63 cells cultured on hCaPs. The expression patterns of osteogenic marker genes, such as ALP, TGF-β, and OPN, were examined in response to the hybridized films and compared to those on CaP. It was reported that ALP activity was increased upon treatment with $1,25(OH)_2D_3$ [13, 34]. Boyan et al. also reported that ALP activity was increased in response to the increase of $1,25(OH)_2D_3$ coupled to surface roughness [18]. According to FE-SEM and AFM examination, the surface roughness of hCaPs was not so different from each other so that the effect of surface roughness can be excluded in our study. This condition allowed us to evaluate the effect of the hybridized $1,25(OH)_2D_3$ within hCaPs on marker gene expression patterns. The current result revealed that initiation of ALP gene expression was faster on hCaPs than CaP. This result is in agreement with the previous reports [13, 34].

Lohmann et al. reported that the TGF-β gene expression was not affected by $1,25(OH)_2D_3$ but it was enhanced on rougher surface [33]. On the contrary to other reports, the expression level was activated from the early stage of culture both on CaP and hCaPs in our study. The expression pattern was slightly higher on hCaPs compared to CaP. Therefore, the effect of the presence of $1,25(OH)_2D_3$ within hCaPs did not greatly stimulate the gene expression, which is in agreement to other reports. However, the expression level appeared to be promoted by CaP itself.

OPN is a bone sialoprotein expressed during the proliferation and early stage of osteoblast differentiation [43, 44]. $1,25(OH)_2D_3$ stimulates the gene expression [45] and OPN protein production in osteoblastic cells [19]. Our result displayed that OPN mRNA expression was up-regulated both on CaP and hCaPs, which is in agreement with other reports. Interestingly, expression level reached at different culture time. For hCaPH, the peak level of expression was achieved on day 5 while that was on day 10 on hCaPL. After achievement of peak level, the expression was completely suppressed with culture time. For CaP, the expression level was maintained from day 5 throughout culture period. However, the expression level was much lower than that of hCaPs. This result suggests that cells cultured on hCaPs enter differentiation phase earlier due to the effect of $1,25(OH)_2D_3$ within hCaPs. This phase change was dependent on the concentration of $1,25(OH)_2D_3$ used in the preparation of hCaPs. Therefore, we can expect that the cellular responses can be modulated by the hybridization of $1,25(OH)_2D_3$ and CaP. At the same time, we can also expect that the expression level can be amplified if cells were cultured on hCaPs with culture media supplemented with differentiation factors.

5 Conclusion

Hybridization of $1,25(OH)_2D_3$ and CaP did not cause significant variations in the physical properties of hCaPs, probably due to the extremely low concentration of $1,25(OH)_2D_3$ used in the preparation of the hybrid films. However, the hybridization demonstrated strong effects on the cellular responses including the rapid formation of cellular nodules and hormone-responsiveness of osteoblast-like MG-63 cells. Cell recruitment capability was greatly enhanced but proliferation was declined. Cells cultured on CaP and hCaPs were forming nodules of which formation was $1,25(OH)_2D_3$ concentration-dependent. The nodules examined with histochemical methods elucidated that the they consisted of cells and that proteoglycan was actively synthesized in the culture on hCaPs. Proteoglycan synthesis can be indicative of the induction of the osteoblastic differentiation which is greatly promoted by the presence of $1,25(OH)_2D_3$. Hybridization of $1,25(OH)_2D_3$ and CaP also affected the osteogenic marker gene expression patterns, such as ALP, TGF-β, and OPN. In the absence of differentiation factors within the culture media, cells were strongly stimulated by the $1,25(OH)_2D_3$ present within hCaPs. The effect of the hormone was more pronounced if the hormone concentration was increased. We expect that the biological functions of these hybridized films can be further significantly enhanced if we use differentiation media. At the same time, the hybridized films can be utilized for the surface modification of biomaterials in order to improve osteoinduction of bone substitutes.

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References

1. Finke B, Luethen F, Schroeder K, Mueller P, Bergemann C, Frant M, et al. The effect of positively charged plasma polymerization on initial osteoblastic focal adhesion on titanium surfaces. Biomaterials. 2007;28:4521–34.

2. Jiang G, Evans ME, Jones IA, Rudd CD, Scotchford CA, Walker GS. Preparation of poly(epsilon-caprolactone)/continuous bioglass fibre composite using monomer transfer moulding for bone implant. Biomaterials. 2005;26:2281–8.
3. Link D, van den Dolder J, van den Beucken J, Wolke J, Mikos A, Jansen J. Bone response and mechanical strength of rabbit femoral defects filled with injectable CaP cements containing TGF-β1 loaded gelatin microparticles. Biomaterials. 2008;29:675–82.
4. Borsari V, Fini M, Giavaresi G, Rimondini L, Consolo U, Chiusoli L, et al. Osteointegration of titanium and hydroxyapatite rough surfaces in healthy and compromised cortical and trabecular bone: in vivo comparative study on young, aged, and estrogen-deficient sheep. J Orthop Res. 2007;25:1250–60.
5. Dong Z, Khor K, Quek C, White T, Cheang P. TEM and STEM analysis on heat-treated and in vitro plasma-sprayed hydroxyapatite/Ti-6Al-4V composite coatings. Biomaterials. 2003;24:97–105.
6. Shirkhanzadeh M, Azadegan M, Stack V. Fabrication of pure hydroxyapatite and fluoridated-hydroxyapatite coatings by electrocrystallization. Mater Lett. 1994;18:211–4.
7. Wolke J, van der Waerden J. In vivo dissolution behavior of various RF magnetron-sputtered Ca-P coatings on roughened titanium implants. Biomaterials. 2003;24:2623–9.
8. Gan L, Wang J, Pilliar R. Evaluating interface strength of calcium phosphate sol-gel-derived thin films to Ti6Al4V substrate. Biomaterials. 2005;26:189–96.
9. Gross K, Berndt C. Thermal processing of hydroxyapatite for coating production. J Biomed Mater Res A. 1998;39:580–7.
10. Nelea V, Pelletier H, Iliescu M, Werckmann J, Craciun V, Mihailescu I, et al. Calcium phosphate thin film processing by pulsed laser deposition and in situ assisted ultraviolet pulsed laser deposition. J Mater Sci: Mater Med. 2002;13:1167–73.
11. Okumura A, Goto M, Goto T, Yoshinari M, Masuko S, Katsuki T, et al. Substrate affects the initial attachment and subsequent behavior of human osteoblastic cells(Saos-2). Biomaterials. 2001;22:2263–71.
12. Delmas PD. Biochemical markers of bone turnover for the clinical assessment of metabolic bone disease. Endocrinol Metab Clin North Am. 1990;19:1–18.
13. Mulkins MA, Manolagas SC, Deftos LJ, Sussman HH. 1α, 25-dihydroxyvitamin D_3 increases bone alkaline phosphatase isoenzyme levels in human osteogenic sarcoma cells. J Biol Chem. 1983;258:6219–25.
14. Franceschi RT, James WM, Zerlauth G. 1α, 25-dihydroxyvitamin D_3 specific regulation of growth, morphology, and fibronectin in a human osteosarcoma cell line. J Cell Physiol. 1985;123:401–9.
15. Franceschi RT, Linson CJ, Peter TC, Romano PR. Regulation of cellular adhesion and fibronectin synthesis by 1 alpha, 25-dihydroxyvitamin D_3. J Biol Chem. 1987;262:4165–71.
16. Lian J, Stewart C, Puchacz E, Mackowiak S, Shalhoub V, Collart D, et al. Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. Proc Natl Acad Sci. 1989;86:1143–7.
17. Bonewald LF, Kester MB, Schwartz Z, Swain LD, Khare A, Johnson TL, et al. Effects of combining transforming growth factor beta and 1α, 25-dihydroxyvitamin D_3 on differentiation of a human osteosarcoma (MG-63). J Biol Chem. 1992;267:8943–9.
18. Boyan BD, Batzer R, Kieswetter K, Liu Y, Cochran DL, Szmuckler-Moncler S, et al. Titanium surface roughness alters responsiveness of MG63 osteoblast-like cells to 1α, 25-(OH)2D_3. J Biomed Mater Res. 1998;39:77–85.
19. Prince CW, Butler WT. 1, 25-dihydroxyvitamin D_3 regulates the biosynthesis of osteopontin, a bone-derived cell attachment protein, in clonal osteoblast-like osteosarcoma cells. Coll Relat Res. 1987;7:305–13.
20. Price PA, Baukol SA. 1, 25-dihydroxyvitamin D_3 increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. J Biol Chem. 1980;255:11660–3.
21. Wong GL, Luben RA, Cohn DV. 1, 25-dihydroxycholecalciferol and parathormone: effects on isolated osteoclast-like and osteoblast-like cells. Science. 1977;197:663–5.
22. Raisz LG, Maina DM, Gworek SC, Dietrich JW, Canalis EM. Hormonal control of bone collagen synthesis in vitro: inhibitory effect of 1-hydroxylated vitamin D metabolites. Endocrinology. 1978;102:731–5.
23. Kream BK, Rowe D, Smith MD, Maher V, Majeska R. Hormonal regulation of collagen synthesis in a clonal rat osteosarcoma cell line. Endocrinology. 1986;119:1922–8.
24. Owen TA, Aronow MS, Barone LM, Bettencourt B, Stein GS, Lian JB. Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology. 1991;128:1496–504.
25. Ishida H, Bellows CG, Aubin JE, Heersche JN. Characterization of the 1α, 25-(OH)2D_3-induced inhibition of bone nodule formation in long-term cultures of fetal rat calvaria cells. Endocrinology. 1993;132:61–6.
26. Shi Y, Worton L, Esteban L, Baldock P, Fong C, Eisman JE, et al. Effects of continuous activation of vitamin D and Wnt response pathways on osteoblastic proliferation and differentiation. Bone. 2007;41:87–96.
27. Kim HJ, Kim SH, Kim MS, Lee EJ, Oh HG, Oh WM, et al. Varying Ti-6Al-4V surface roughness induces different early morphologic and molecular responses in MG63 osteoblast-like cells. J Biomed Mater Res A. 2005;74:366–73.
28. Boyan BD, Lohmann CH, Sisk M, Liu Y, Sylvia VL, Cochran DL, et al. Both cyclooxygenase-1 and cyclooxygenase-2 mediate osteoblast response to titanium surface roughness. J Biomed Mater Res. 2001;55:350–9.
29. Moulder JF, Stickle WF, Sobol PE, Bomben KD (1995) In: Chastain J, King RC, editors. Handbook of x-ray photoelectron spectroscopy. Minnesota: Physical Electronics, Inc.
30. Kaciulis S, Mattogno G, Pandolfi L, Cavalli M, Gnappi G, Montenero A. XPS study of apatite-based coatings prepared by sol-gel technique. Appl Surf Sci. 1999;151:1–5.
31. Ide-Ektessabi A, Yamaguchi T, Tanaka Y. RBS and XPS analyses of the composite calcium phosphate coatings for biomedical applications. Nucl Instrum Methods Phys Res B. 2005;241:685–8.
32. Ferraz MP, Monteiro FJ, Santos JD. CaO-P2O5 glass hydroxyapatite double-layer plasma-sprayed coating: in vitro bioactivity evaluation. J Biomed Mater Res. 1999;45:376–83.
33. Lohmann CH, Bonewald LF, Sisk MA, Sylvia VL, Cochran DL, Dean DD, et al. Maturation state determines the response of osteogenic cells to surface roughness and 1, 25-dihydroxyvitamin D_3. J Bone Miner Res. 2000;15:1169–80.
34. Matsumoto T, Sowa Y, Ohtani-Fujita N, Tamaki T, Takenaka T, Kuribayashi K, et al. p53-independent induction of WAF1/Cip1 is correlated with osteoblastic differentiation by vitamin D_3. Cancer Lett. 1998;129:61–8.
35. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, et al. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol. 1990;143:420–30.
36. Bonewald LF, Mundy GR. Role of transforming growth factor beta in bone remodeling: a review. Connect Tissue Res. 1989;23:201–8.

37. Sporn MB, Roberts AB. Peptide growth factors are multifunctional. Nature. 1988;332:217–9.
38. Sodek J, Ganss B, McKee MD. Osteopontin. Crit Rev Oral Biol Med. 2000;11:279–303.
39. Irie K, Zalzal S, Ozawa H, Nanci A. Morphological and immunocytochemical characterization of primary osteogenic cell cultures derived from fetal rat cranial tissue. Anat Rec. 1998;252: 554–67.
40. De Bruijn JD, Van Den Brink I, Mendes S, Dekker R, Bovell Y, Van Blitterswijk C. Bone induction by implants coated with cultured osteogenic bone marrow cells. Adv Dent Res. 1999;13: 74–81.
41. Butler WT. The nature and significance of osteopontin. Connect Tissue Res. 1989;23:123–36.
42. Nagata T, Bellows CG, Kasugai S, Butler WT, Sodek J. Biosynthesis of bone proteins [SPP-1 (secreted phosphoprotein-1, osteopontin), BSP (bone sialoprotein) and SPARC (osteonectin)] in association with mineralized-tissue formation by fetal-rat calvarial cells in culture. Biochem J. 1991;274:513–20.
43. Oldberg A, Jirskog-Hed B, Axelsson S, Heinegard D. Regulation of bone sialoprotein mRNA by steroid hormones. J Cell Biol. 1989;109:3183–6.
44. Lynch MP, Stein JL, Stein GS, Lian JB. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. Exp Cell Res. 1995;216:35–45.
45. Beck GR Jr. Inorganic phosphate as a signaling molecule in osteoblast. J Cell Biochem. 2003;90:234–43.