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Bone Nodule Formation of MG63 Cells is Increased by the Interplay of Signaling Pathways Cultured on Vitamin D_3-Entrapped Calcium Phosphate Films

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Abstract: Since vitamin D_3 is an important regulator of osteoblastic differentiation, a presently-established vitamin D_3-entrapped calcium phosphate film (VCPF) was evaluated for hard tissue engineering. The entrapped vitamin D_3 more rapidly induced bone nodule formation. To characterize the cellular events leading to regulations including faster differentiation, signal transduction pathways were investigated in osteoblastic MG63 cells at a molecular level. Major signaling pathways for MG63 cell proliferation including phosphatidylinositol-3-kinase, extracellular signal-regulated kinase, c-Jun N-terminal kinase and focal adhesion kinase pathways were markedly down-regulated when cells were cultured on calcium phosphate film (CPF) and VCPF. This agreed with our earlier observations of the immediate delay in proliferation of MG63 cells upon culture on CPF and VCPF. On the other hand, the p38 mitogen-activated protein kinase (p38 MAPK) and protein kinase A (PKA) pathways were significantly up-regulated on both CPF and VCPF. CPF alone could simulate differential behaviors of MG63 cells even in the absence of osteogenic stimulation and entrapment of vitamin D_3 within CPF further amplified the signal pathways, resulting in continued promotion of MG63 cell differentiation. Interplay of p38 MAPK and PKA signaling pathways likely is a significant event for the promotion of differentiation and mineralization of MG63 cells.

Key words: calcium phosphate coating, $1\alpha,25(OH)_2$ vitamin D_3, osteoblast, p38 MAPK, PKA

Abbreviations: CPF (calcium phosphate film), VCPF ($1\alpha,25(OH)_2$ vitamin D_3-entrapped calcium phosphate film), PI3-kinase (phosphatidylinositol 3-kinase), PDK1 (phosphoinositide-dependent protein kinase1), ERK (extracellular signal-regulated protein kinase), JNK (c-Jun N-terminal kinase), PKA (protein kinase A)

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INTRODUCTION

In the development of biomaterials used for bone substitutes, it is desirable to promote differentiation of osteoblasts and bone regeneration. These biological events relevant to bone formation are strictly regulated by various biological factors. In tissue engineering, surface properties of bone substitutes including chemical composition and surface roughness are crucial in implant success. Appropriately, attempts have been made to enhance the biocompatibility of biomaterials via surface modifications. One of these attempts involved a hydroxyapatite (HA) coating, since HA has a chemical structure similar to natural bone and shows excellent biocompatibility inducing acceleration of bone regeneration (Bagambisa et al., 1990; Gregoire et al., 1990; Kilpadi et al., 2001). *In vitro* culture of osteoblasts on CP films can recapitulate *in vivo* cellular behaviors such as adhesion, proliferation and differentiation. In general, CP delays proliferation but accelerates differentiation (Okumura et al., 2001; Chuo et al., 2005; Mo et al., 2005a, 2005b). The interaction between cells and a biomaterial generates signal transduction pathway-mediated stimulations that significantly affect cellular behavior. Nevertheless, osteoblastic responses to CP containing compounds, including HA, and related signal transduction pathways have been rarely explored so far.

Vitamin D is an important regulator of bone metabolism. The most biologically active form of vitamin D is $1\alpha,25$-dihydroxy vitamin D_3 (vitamin D_3), which is synthesized after two consecutive hydroxylations of skin-derived

vitamin D_3 in the liver (C-25 position) and in the kidney (C-1a position) (Driel et al., 2004). It is well-known that vitamin D_3 plays an important role in the regulation of the early stages of human osteoblast differentiation by promoting the expression of differentiation regulatory genes, such as core binding factor alpha 1 (cbfα1), inhibitor of the MyoD family (I-mfa) and Notch (Lian and Stein, 1999; Tsuji et al., 2001; Schnabel et al., 2002; Drissi et al., 2002; Viereck et al., 2002). In addition, *in vitro* studies have established that vitamin D_3 promotes osteoblastic differentiation by inhibiting cell proliferation and increasing alkaline phosphatase activity (Manolagas et al., 1981; Narisawa et al., 1997; Hui and Tenenbaum, 1998). Since the formation of extracellular matrix and mineralization are pre-requisites for the bone formation, vitamin D_3 is critical for the bone nodule formation due to its role in type I collagen expression and alkaline phosphatase activation.

Recently, we developed a method to synthesize CP film (CPF) on the surface of solid substrates and demonstrated that this inorganic film can accelerate differentiation and bone-like nodule formation in osteoblast-like MG63 cells (Mo et al., 2005a). In addition, we prepared vitamin D_3-entrapped CPF (VCPF) to endow multiple functionalities on CPF. The prepared VCPF did not seriously perturb the physical characteristics of CPF (Jung et al., 2009). In our earlier investigation, we reported that osteoblastic proliferation, differentiation and bone-like nodule formation are significantly promoted in the presence of vitamin D_3 (Jung et al., 2009). This is highly desirable for a biomaterial used as bone substitutes. In the present study, we aimed to identify the signal transduction pathways responsible for the above-mentioned cellular events. At the same time, we attempted to investigate the effect of VCPF-entrapped vitamin D_3 on the signal transduction pathways. Here, we report that modulations of signal transduction pathways by the stimulations originate from CPF and VCPF.

To understand the biological basis of such a profound effect of VCPF on bone nodule formation, we studied the signal transduction pathways of the ostoeblastic MG63 cells cultured on VCPF. We characterized the signaling pathways responsible for cell proliferation, adhesion and differentiation, and found them to be significantly regulated by culturing either on CPF or VCPF. Most interestingly, we found that the protein kinase A (PKA) signaling pathway was activated by VCPF. We suggest that the elucidation of VCPF-induced amplification of signaling pathways can be further applied to bone tissue engineering for the development of a successful bone implant.

MATERIALS AND METHODS

Preparation of CPF and VCPF films

VCPFs were prepared on the surface of polystyrene culture plates as previously reported (Mo et al., 2005a, 2005b). In brief, a supersaturated calcium and phosphate ionic solution was prepared. To form CPF, the ionic solution was applied directly to the polystyrene surface at 4 and 37°C for 60 and 90 min, respectively. To prepare VCPF, the above ionic solution was mixed with vitamin D_3 to produce a final D_3 concentration of 1 μM. After treatment, sample surfaces were rinsed three times with double-distilled water (ddH_2O), followed by drying in the clean bench.

Cell culture

MG63 human osteoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antimycotic solution in a humidified 5% CO_2 atmosphere at 37°C. Prior to performing experiments, cells were harvested with 0.5% trypsin-EDTA, washed, and seeded on the sample surfaces.

Antibodies

Antibodies specific for the phosphorylated form of extracellular signal-regulated protein kinase (ERK; #9106), c-Jun N-terminal kinase (JNK; #9251), p38 (#9211), focal adhesion kinase (FAK; #3281), phosphoinositide-dependent protein kinase1 (PDK1; #3061), Akt1 (#9271), and PKA (#4781), and antibodies recognizing ERK (#9102), JNK (#9258), p38 (#9219), FAK (#3285), PDK1 (#3062), Akt1 (#9272), and PKA (#4782) were purchased from Cell Signaling Technology (Beverley, MA). Anti-β-actin antibody (ab6276) was purchased from Abcam (Cambridge, MA).

Field-emission scanning electron microscopy and energy-dispersive X-ray spectroscopy

The surface morphologies of CPF and VCPF were observed by scanning electron microscopy (SEM) using a JSM-5800 apparatus (JEOL, Tokyo, Japan). The micrographs were taken at a magnification of 10,000×. Energy-dispersive X-ray spectroscopy (EDS) was used to determine CPF and VCPF chemical compositions.

Immunoblotting

MG63 cells were disrupted in a lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 10 μg/mL leupeptin, 5 μg/mL aprotinin and 1% NP-40). The protein concentrations of cell lysates were determined by a commercial Bradford assay (Bio-Rad, Hercules, CA). Twenty micrograms protein was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the resolved proteins were transferred to Immobilion-P (Millipore, Bedford, MA). The membrane was blocked in blocking buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) with 5% skim milk, and was incubated with

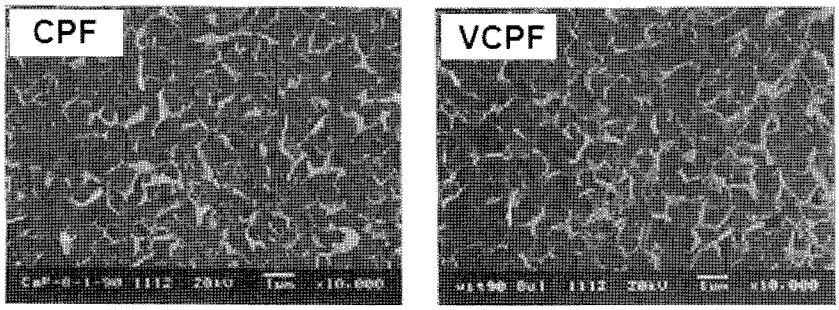

Fig. 1. Scanning electron microscopy of CPF and VCPF. Micrographs of CPF and VCPF illustrating the highly porous surface structure of the surfaces. The concentration of vitamin D_3 used for the preparation of VCPF was 1 μM. The micrographs provided evidence that the morphological characteristics were not changed significantly by the incorporation of vitamin D_3. (Bar represents 1 μm)

primary antibodies, followed by enhanced chemiluminescence detection using horseradish peroxidase-conjugated secondary antibodies (MILLIPORE) using the manufacturer's protocol.

Osteoblast bone-like nodule formation

VCPF incorporating 1 μM vitamin D_3 was utilized for bone-like nodule formation. Vitamin D_3 induced differentiation was analyzed using von Kossa staining. MG63 cells were seeded on CPF and VCPF at a cell density of 2×10^4 per well. As a comparison, MG63 cells were seeded on polystyrene culture plate (Contr) and CPF in vitamin D_3-containing culture media. After continuous culturing for 12 days, cells were fixed using 10% formaldehyde for 10 min and rinsed with ddH_2O. After addition of 1 mL of 5.5% silver nitrate, each sample was exposed to sunlight for 30 min, rinsed with ddH_2O, and 7.2% sodium thiosulfate was added. After 5 min, the samples were rinsed with ddH_2O and dehydrated with ethanol.

RESULTS

Incorporation of vitamin D_3 into CPF does not significantly change the physical properties of the film

Since surface morphology is important for cellular responses, SEM was used to examine tthe surface morphology of VCPF and CPF (Fig. 1). Both CPF and VCPF prepared in this study demonstrated a highly porous surface structure and the morphology of VCPF was not appreciably different from CPF (Fig. 1). Calcium to phosphorus ratios of obtained from EDS measurements were similar (2.202 for CPF, 2.097 for VCPF). The results suggest that the incorporation of vitamin D_3 did not significantly change the chemical composition of VCPF in comparison to CPF.

Bone nodule formation is enhanced by vitamin D_3 entrapped in VCPF

Vitamin D_3 induces osteoblast differentiation, which drives the formation of bone tissues including bone nodules. We developed the VCPF to enhance the bone forming potential of osteoblastic MG63 cells in culture. It was of interest to examine whether the entrapped vitamin D_3 could actually induce differentiation of osteoblast-like MG63 cells and bone nodule formation, as occurs with soluble vitamin D_3. Our results demonstrated that bone-like nodules were rapidly formed both on CPF and VCPF, whereas no nodules were observed in the plain culture dish (Contr). No nodules were found from the plain culture plate (Contr) and nodules were found on CPF (Fig. 2A). The latter result is unusual because the cells were cultured in a growth medium lacking differentiation agents. On VCPF, nodule formation was further accelerated compared to that of CPF (Fig. 2A). To examine the mineralization of the bone-like nodules, von Kossa staining was used. As shown in Fig. 2B, the nodules stained dark brown, which indicated the presence of calcium ions. For Contr samples, the intensity of staining was very weak. On the other hand, the nodules formed on CPF and VCPF were intensely brown stained indicating mineralized nodule formation. To test whether MG63 cells cultured in the presence of soluble vitamin D_3 in the medium could also form bone-like nodules, von Kossa staining was performed on CPF samples. Less bone-like nodule formation was evident (Fig. 2B). At the same time, MG63 cell proliferation was significantly reduced, strongly indicating that nodule formation by MG 63 cells can be induced by CPF and accelerated by the vitamin D_3 entrapped within VCPF. The results are consistent with the suggestion that both CPF and vitamin D_3 can synergistically affect bone-like nodule formation.

CP containing film leads to inactivation of FAK, ERK and JNK pathways

Because osteoblast adhesion is a critical event for the bone formation (Kim et al., 2007), we first tested whether the signaling pathways leading to cell adhesion were activated. Since integrins interact with extracellular matrices and ultimately lead to the formation of focal complexes and focal adhesions (Clark and Brugge, 1995; Xiao et al.,

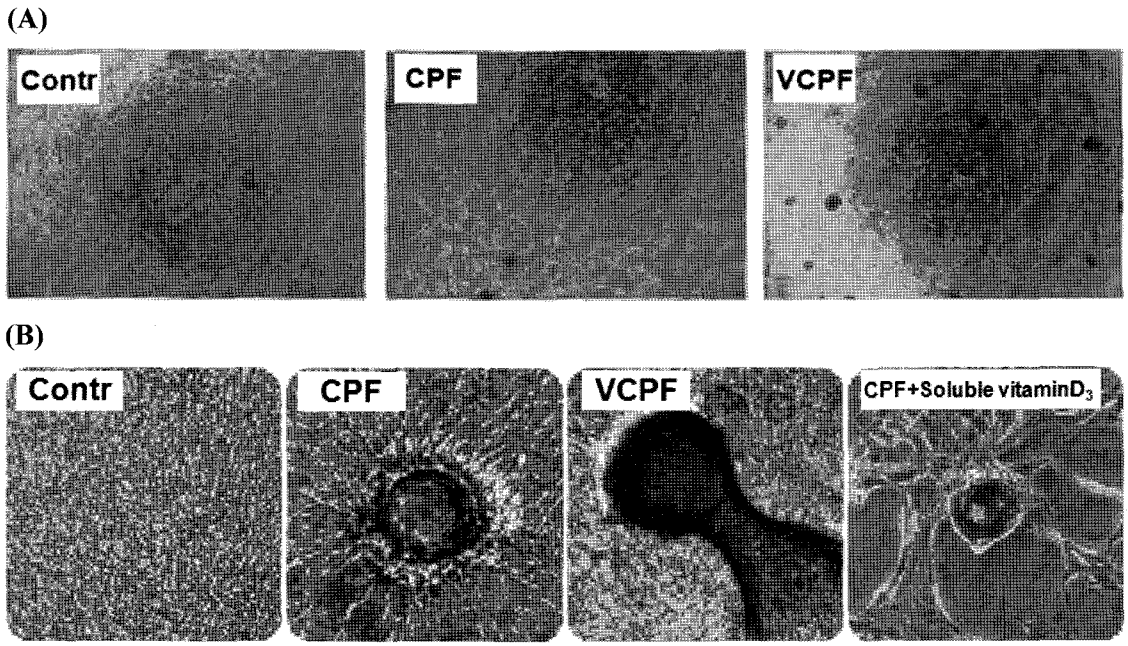

Fig. 2. Bone-like nodule formation of MG63 cells in VCPF. (A) MG63 cells were cultured on plain culture dish (Contr), CPF and VCPF for 7 days. No nodules were found on plain culture plate. Formation of bone-like nodules was further accelerated in the presence of vitamin D_3. (B) Representative result from von Kossa staining of bone-like nodules formed by MG63 cells. MG63 cells were cultured for 12 days on Contr, CPF, or VCPF, or CPF with soluble vitamin D_3-dissolved in the culture medium.

1998), we tested the activation status of FAK in MG63 cells. Immunoblotting with anti-phospho-FAK showed that FAK was basally phosphorylated in MG63 cells when they were cultured on plain culture dishes. However, complete inactivation of FAK was evident when the cells were cultured either on CPF or VCPF (Figs. 3A and 3B). Since the phosphorylation of FAK ultimately activates the MAPK signaling pathways (Zhu and Assoian, 1995; Reiske et al., 2000; Howe et al., 2002), we also tested whether FAK inactivation affected the typical MAPK signaling pathways, such as ERK and JNK. As shown in Figs. 3C and 3D, ERK and JNK signaling pathways were basally activated in MG63 cells on the plain culture plate, whereas they were mostly inactivated upon culturing either on CPF or VCPF. This result was consistent with the suggestion that the culture environment causes inactivation of FAK and downstream ERK and JNK proliferation signaling pathways in MG63 cells, which must be responsible for the low rate of proliferation of MG63 cells cultured on either CPF or VCPF.

CP film leads to inactivation of PI3-kinase pathway

Little is known about the change of PI3-kinase activation in osteoblasts cultured on CPs. Since the PI3-kinase pathway is the main pathway mediating cell survival signal, we compared the activation of PI3-kinase signaling of MG63 cells cultured on the plain culture dish (Contr), CPF and VCPF (Fig. 4A). Immunoblotting was performed with phosphorylation specific anti-Akt1 and PDK1 antibodies. PI3-kinase pathway was basally activated in the cells cultured on the plain culture dish. When the cells were cultured on CPF or VCPF, however, the PI3-kinase pathway was immediately down-regulated (Fig. 4B). More interestingly, endogenous protein levels of PDK1 and Akt1 were significantly reduced upon culture on CPF and VCPF. This result suggests that CPF or VCPF may somehow provide inadequate survival conditions, thus, inducing the degradation or reduced expression of Akt1 and PDK1 proteins.

CP film leads to activation of p38 MAPK pathway

Since we observed the almost inactivation of some of MAP kinase signal pathways (ERK and JNK), we next tested whether other MAPK signal pathways or bone lineage differentiation signaling pathways were activated in the MG63 cells cultured on CPF and VCPF. It was previously reported that bone forming hormones induce p38 activation during the differentiation of osteoblasts (Hu et al., 2003; Rey et al., 2007). Hence, we tested whether CPF or VCPF could activate the p38 signaling pathways (Fig. 5A). We performed Western blot analysis of MG63 cell extracts with anti-phospho-p38 and anti-p38 antibodies. The basally low level of p38 signaling was significantly enhanced when the cells were cultured with on CPF or VCPF (Fig. 5B), clearly indicating that CPF induced p38 activation, which is involved in differentiation of MG63 cells.

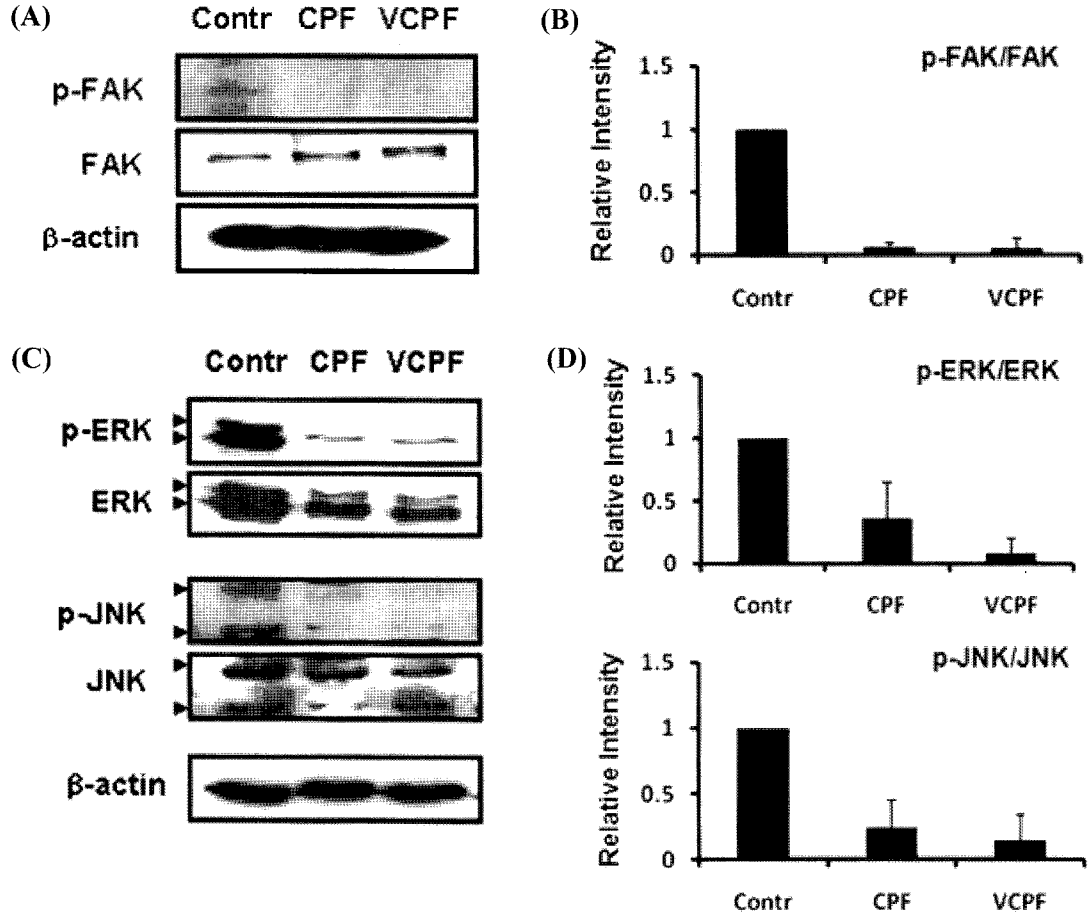

Fig. 3. Western blot analysis of the FAK, ERK and JNK kinase pathways. (A) Phosphorylation of FAK was analyzed with anti-phospho-FAK antibody. Anti-FAK antibody was used as a control. Cells were cultured on a plain culture dish (Contr), CPF, or VCPF for 2 days, and whole cell extracts were prepared. For each lane, 20 μg of protein was loaded. (B) Graphical representation of the data shown in (A). Degree of phosphorylation among total FAK protein is shown as bars. Results are the average of three independent experiments. (C) Phosphorylation of ERK and JNK was analyzed with anti-phospho-ERK and anti-phospho-JNK antibody, respectively. Anti-ERK and JNK antibodies were also used as controls. Anti-β-actin antibody was used as a loading control. (D) Graphical representation of the data shown in (C). Degrees of phosphorylation among total ERK or JNK proteins are shown as bars. Results are the average of three independent experiments.

CP film and vitamin D_3 synergistically activates the PKA pathway

Since we observed extremely fast bone-like nodule formation on VCPF (Fig. 2B), it was appropriate to examine the signaling pathways relevant to osteoblastic differentiation. Since PKA can be activated by vitamin D_3 (Schwartz e al., 1992; Lohmann et al., 1999), we prepared whole cell extract from MG63 cells cultured on plain culture dish (Contr), CPF and VCPF for 2, 5, and 7 days, and performed immunoblotting with anti-phospho PKA antibody. Fig. 5C depicts representative results from a Western blot. Even though transient inactivation of PKA was found on day 2, we consistently observed activation of PKA by CPF and VCPF, with more pronounced effect by VCPF after 5 and 7 days of culture (Fig. 5D). These results suggested that CPF and vitamin D_3 entrapped within the VCPF might synergistically activate the PKA signaling pathway, leading to the greatly accelerated nodule formation by MG-63 cells in this condition. This finding could provide the molecular basis for the significant differentiation of MG63 cells and fast nodule formation cultured on VCPF.

DISCUSSION

Presently, we established a vitamin D_3-entrapped calcium phosphate film (VCPF), which could modulate cellular responses on the surface of implant materials. Since the VCPF-induced signal transduction pathway is ill-understood, we explored the signal transduction pathways of MG63 cells cultured on CPF and VCPF. We found that rates of adhesion and proliferation were significantly different when MG63 cells were cultured on CPF or VCPF for 1-2 days. So we explored the adhesion and proliferation related signal transduction pathways when MG63 cells were cultured for 2 days. Bone-like nodules were first formed after 4 days culturing on CPF; however, the rate of nodule formation was significantly faster on VCPF. So we explored the PKA signal transduction pathway when

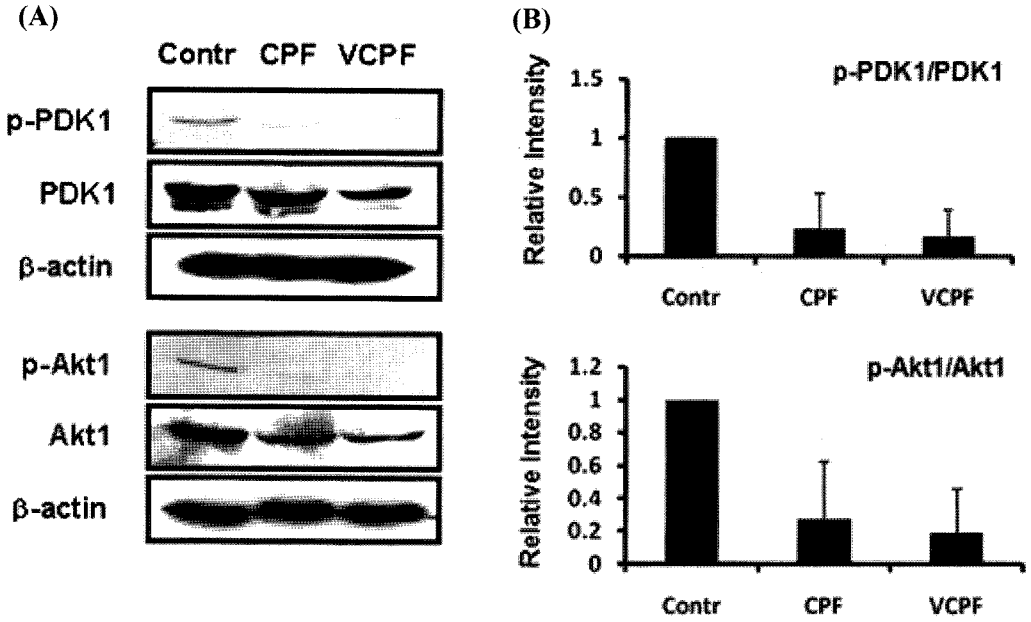

Fig. 4. Western blot analysis of the PI3-kinase pathway. (A) Phosphorylation of PDK1 and Akt1 was analyzed with anti-phospho-PDK1 and anti-phospho-Akt1 antibody, respectively. Anti-PDK1 and Akt1 antibodies were used as controls. Anti-β-actin antibody was used as a loading control. Cells were cultured on a plain culture dish (Contr), CPF, or VCPF for 2 days, and whole cell extracts were prepared. For each lane, 20 μg of protein was loaded. (B) Graphical representation of the data shown in (A). Degrees of phosphorylation among PDK1or Akt1 total proteins are shown as bars. Results are the average of three independent experiments.

MG63 cells were cultured for 2, 5 and 7 days. As well, the PKA signal pathway was promoted more in VCPF cultured cells. Since it has been reported that the surface roughness and vitamin D_3 are somehow related to the activation of PKA (Lohmann et al., 1999), we anticipated that activation of PKA signaling must be synergistically activated both by CPF and VCPF. As expected, CPF activated PKA and vitamin D_3-entrapped in CPF synergistically activated the PKA signaling pathway, which greatly accelerated nodule formation.

The p38 MAPK pathway is reported to be a critical signaling pathway for osteoblastic differentiation when the cells are induced by several osteotropic factors, including serum growth factors and bone morphogenetic protein-2 (Hu et al., 2003; Rey et al., 2007). Moreover, p38 activation is required for the expression of alkaline phosphatase and matrix calcification (Hu et al., 2003; Rey et al., 2007). However, it is unclear whether the p38 MAPK pathway is regulated by CPF or VCPF. Presently, both CPF and VCPF stimulated the p38 signal, but vitamin D_3 in VCPF did not affect the p38 signal in osteoblastic MG63 cells. The interplay of signal transduction pathways activated by the surface-induced p38 signal and vitamin D_3-induced PKA signal could account for the effects shown on the fast bone nodule formation of osteoblastic MG63 cells.

Since the extracellular matrix exerts stringent control on the proliferation of normal cells through interaction with the adhesion complex, mitogenic signaling pathways are expected to be activated by the integrin-mediated focal adhesion complex (Clark and Brugge, 1995; Zhu and Assoian, 1995). We found a low rate of proliferation (Jung et al., 2009) and inactivation of ERK, JNK, and FAK signaling pathways of MG63 cells upon culture on CPF or VCPF. This result indicates that the surface properties of CPF are unfavorable for cell adherence, which results in a low level of proliferation through the inactivation of ERK and JNK. MG63 cells are likely to be involved in a post-proliferative stage of osteoblast development, even after short incubation in CPF or VCPF.

Since cell death was not evident, we expected that a main cell survival signal, such as PI3-kinase pathway, must remain activated in MG63 cells cultured on CPF or VCPF. Contrary to this expectation, complete shut-down of the PI3-kinase pathway was apparent. A reasonable interpretation of this result might be that integrin-mediated focal adhesion signal could be the upstream signal for Akt1 and PDK1 signal. Interestingly, we found that endogenous protein levels of PDK1 and Akt1 were also decreased upon culturing on CPF and VCPF. It is likely that the surface properties of CPF somehow activated an unknown degradation signal for the Akt1 and PDK1 proteins. We expect that the identification of VCPF-induced cell signaling will provide valuable information for the future development of osteoconductive biomaterials for tissue engineering or bone implant.

In this study, von Kassa staining revealed astonishingly fast bone-like nodule formation from MG63 cells cultured on VCPF. Nodule formation was more pronounced in the

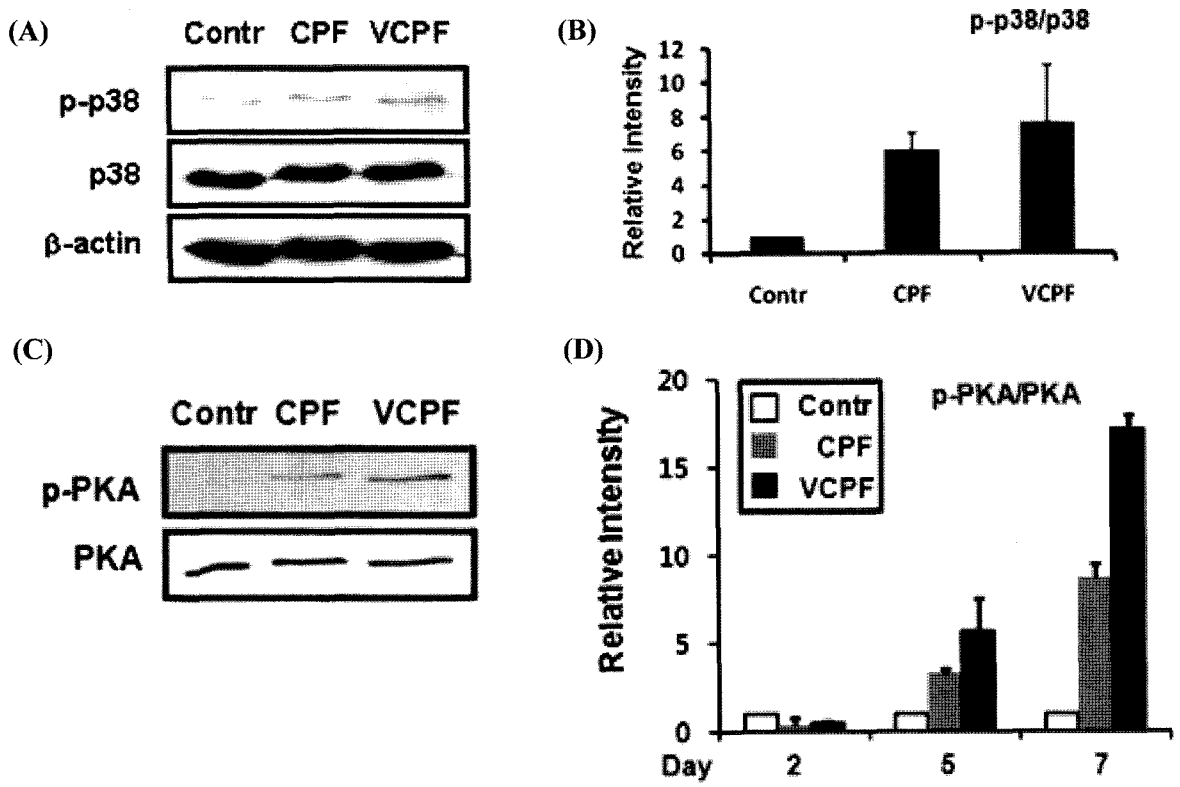

Fig. 5. Western blot analysis of p38 and PKA activation. (A) Phosphorylation of p38 was analyzed with anti-phospho-p38 antibody. Anti-p38 antibody was used as a control and anti-β-actin antibody was used as a loading control. (B) Degree of phosphorylation of p38 protein is presented by the graphical bars. Results are the average of three independent experiments. (C) MG63 cells were cultured on a plain culture dish (Contr), CPF, and VCPF for 7 days, and whole cell extracts were prepared at the indicated time. Phosphorylation of PKA was analyzed with anti-phospho-PKA antibody and anti-PKA antibody was also used as controls. (D) MG63 cells were cultured on a plain culture dish (Contr), CPF, and VCPF for 2, 5, or 7 days. The degree of phosphorylation of PKA protein is represented by graphical bars. Results are the average of three independent experiments

presence of vitamin D_3 within the CP matrix. However, when vitamin D_3 was dissolved in the culture medium instead of being entrapped in CPs, the degree of nodule formation was much less compared to cells cultured on CPF and VCPF. Furthermore, MG63 cell proliferation in the presence of vitamin D_3 in the culture medium was significantly suppressed, in accordance with other reports (Bonewald et al., 1992; Mulkins et al., 1983).

In conclusion, the present study demonstrates that entrapment of vitamin D_3 within a CP matrix can provide an environment that is more conducive to the differentiation of osteoblast-like MG63 cells.

ACKNOWLEDGMENTS

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REFERENCES

Bagambisa FB, Joos U, and Schilli W (1990) Interaction of osteogenic cells with hydroxylapatite implant materials in vitro and in vivo. *Int J Oral Maxillofac Implants* 5: 217-226.

Bonewald LF, Kester MB, Schwartz Z, Khare A, Johnson TL, Leach RJ, and Boyan BD (1992) Effects of combining transforming growth factor beta and 1,25- dihydroxyvitamin D3 on differentiation of a human osteosarcoma (MG-63). *J Biol Chem* 267: 8943-8949.

Chou YF, Huang W, Dunn JC, Miller TA, and Wu BM (2005) The effect of biomimetic apatite structure on osteoblast viability, proliferation, and gene expression. *Biomaterials* 26: 285-295.

Clark EA and Brugge JS (1995) Integrins and signal transduction pathways: the road taken. *Science* 268: 233-239.

van Driel M, Pols HA, and van Leeuwen JP (2004) Osteoblast differentiation and control by vitamin D and vitamin D metabolites. *Curr Pharm Des* 10: 2535-2555.

Drissi H, Pouliot A, Koolloos C, Stein JL, Lian JB, Stein GS, and van Wijnen AJ (2002) 1,25-$(OH)_2$-vitamin D_3 suppresses the bone-related Runx2/Cbfa1 gene promoter. *Exp Cell Res* 274: 323-333.

Gregoire M, Orly I, and Menanteau J (1990) The influence of

calcium phosphate biomaterials on human bone cell activities. An in vitro approach. *J Biomed Mater Res* 24: 165-277.

Howe AK, Aplin AE, and Juliano RL (2002) Anchorage-dependent ERK signaling-mechanisms and consequences. *Curr Opin Genet Dev* 12: 30-35.

Hu Y, Chan E, Wang SX, and Li B (2003) Activation of p38 mitogen-activated protein kinase is required for osteoblast differentiation. *Endocrinology* 144: 2068-2074.

Hui M and Tenenbaum HC (1998) New face of an old enzyme: alkaline phosphatase may contribute to human tissue aging by inducing tissue hardening and calcification. *Anat Rec* 253: 91-94.

Jung JY, Hong YJ, Choi YS, Jeong S, Lee WK (2009) A new method for the preparation of bioactive calcium phosphate films hybridized with 1alpha, 25-dihydroxyvitamin D_3. *J Mater Sci Mater Med* DOI 10.1007/s10856-009-3817-y

Kilpadi KL, Chang PL, and Bellis SL (2001) Hydroxylapatite binds more serum proteins, purified integrins, and osteoblast precursor cells than titanium or steel. *J Biomed Mater Res* 57: 258-267.

Kim JB, Leucht P, Luppen CA, Park YJ, Beggs HE, Damsky CH, and Helms JA (2007) Reconciling the roles of FAK in osteoblast differentiation, osteoclast remodeling, and bone regeneration. *Bone* 41: 39-51.

Lian JB and Stein GS (1999) The developmental stages of osteoblast growth and differentiation exhibit selective responses of genes to growth factors (TGF beta1) and hormones (vitamin D and glucocorticoids). *J Oral Implantol* 19: 95-105.

Lohmann CH, Sagun Jr R, Sylvia VL, Cochran DL, Dean DD, Boyan BD, and Schwartz Z (1999) Surface roughness modulates the response of MG63 osteoblast-like cells to 1,25-$(OH)_2D_3$ through regulation of phospholipase A2 activity and activation of protein kinase A. *J Biomed Mater Res* 47: 139-151.

Mo JM, Kim SU, Shin HY, Im DS, Jung IH, Ko JS, and Lee WK (2005a) Biological responses of osteoblastic HOS cells to titanium- and calcium phosphate-coated films. *J Ind Eng Chem.* 11: 507-514.

Mo JM, Shin HY, Jung IH, Ko JS, McGuire J, and Lee WK (2005b) Characterization of low-crystalline calcium phosphate films formed on silica glass surface. *J Ind Eng Chem* 11: 261-266.

Manolagas SC, Burton DW, and Deftos LJ (1981) 1,25-Dihydroxyvitamin D_3 stimulates the alkaline phosphatase activity of osteoblast-like cells. *J Biol Chem* 256: 7115-7117.

Mulkins MA, Manoagas SC, Deftos LJ, and Sussman HH (1983) 1, 25-Dihydroxyvitamin D3 increases bone alkaline phosphatase isoenzyme levels in human osteogenic sarcoma cells. *J Biol Chem* 258: 6219-6225.

Narisawa S, Frohlander N, and Millan JL (1997) Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. *Dev Dyn* 208: 432-446.

Okumura A, Goto M, Goto T, Yoshinari M, Masuko S, Katsuki T, and Tanaka T (2001) Substrate affects the initial attachment and subsequent behavior of human osteoblastic cells (Saos-2). *Biomaterials* 22: 2263-2271.

Reiske HR, Zhao J, Han DC, Cooper LA, and Guan JL (2000) Analysis of FAK-associated signaling pathways in the regulation of cell cycle progression. *FEBS Lett* 486: 275-280.

Rey A, Manen D, Rizzoli R, Ferrari SL, and Caverzasio J (2007) Evidences for a role of p38 MAP kinase in the stimulation of alkaline phosphatase and matrix mineralization induced by parathyroid hormone in osteoblastic cells. *Bone* 41: 59-67.

Schnabel M, Fichtel I, Gotzen L, and Schlegel J (2002) Differential expression of Notch genes in human osteoblastic cells. *Int J Mol Med* 9: 229-232.

Schwartz Z, Dennis R, Bonewald LF, Swain LD, Gomez R, and Boyan BD (1992) Differential regulation of prostaglandin E2 synthesis and phospholipase A2 activity by 1,25-$(OH)_2D_3$ in three osteoblast- like cell lines (MC-3T3-E1, ROS ROS 17/2.8, and MG-63). *Bone* 13: 51-58.

Tsuji K, Kraut N, Groudine M, and Noda M (2001) Vitamin D(3) enhances the expression of I-mfa, an inhibitor of the MyoD family, in osteoblasts. *Biochim Biophys Acta* 1539: 122-130.

Viereck V, Siggelkow H, Tauber S, Raddatz D, Schutze N, and Hufner M (2002) Differential regulation of Cbfa1/Runx2 and osteocalcin gene expression by vitamin-D_3, dexamethasone and local growth factors in primary human osteoblast. *J Cell Biochem* 86: 348-356.

Xiao G, Wang D, Benson MD, Karsenty G, and Franceschi RT (1998) Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. *J Biol Chem* 273: 32988-3294.

Zhu X and Assoian RK (1995) Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. *Mol Biol Cell* 6: 273-282.

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