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Effects of varied ionic calcium and phosphate on the proliferation, osteogenic differentiation and mineralization of human periodontal ligament cells *in vitro*

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Background and Objective: A number of bone-filling materials containing calcium (Ca^{2+}) and phosphate (P) ions have been used in the repair of periodontal bone defects; however, the effects that local release of Ca^{2+} and P ions has on biological reactions are not fully understood. In this study, we investigated the effects of various levels of Ca^{2+} and P ions on the proliferation, osteogenic differentiation and mineralization of human periodontal ligament cells (hPDLCs).

Material and Methods: The hPDLCs were obtained using an explant culture method. Defined concentrations and ratios of ionic Ca^{2+} to inorganic P were added to standard culture and osteogenic induction media. The ability of hPDLCs to proliferate in these growth media was assayed using the Cell Counting Kit-8. Cell apoptosis was evaluated by the fluorescein isothiocyanate–annexin V/propidium iodide double-staining method. Osteogenic differentiation and mineralization were investigated by morphological observations, alkaline phosphatase activity and Alizarin Red S/von Kossa staining. The mRNA expression of osteogenic related markers was analysed using RT-PCR.

Results: Within the ranges of Ca^{2+} and P ion concentrations tested, we observed that increased concentrations of Ca^{2+} and P ions enhanced cell proliferation and formation of mineralized matrix nodules, whereas alkaline phosphatase activity was reduced. The RT-PCR results showed that elevated concentrations of Ca^{2+} and P ions led to a general increase of *Runx2* mRNA expression and decreased alkaline phosphatase mRNA expression, but gave no clear trend on osteocalcin mRNA levels.

Conclusion: The concentrations and ratios of Ca^{2+} and P ions could significantly influence proliferation, differentiation and mineralization of hPDLCs. Within the range of concentrations tested, we found that the combination of 9.0 mM Ca^{2+} ions and 4.5 mM P ions were the optimal concentrations for proliferation, differentiation and mineralization in hPDLCs.

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Human periodontal ligament cells (hPDLCs) are highly specialized cells that reside within the periodontal ligament. These cells can differentiate into either cementoblasts to synthesize tooth root cementum or osteoblasts to synthesize alveolar bone, providing skeletal support for the tooth (1). Previous studies indicate that hPDLCs possess osteoblastic properties, such as high alkaline phosphatase (ALP) activity, capacity for production of bone-like matrix proteins and formation of mineralized nodules (1,2). Periodontitis is characterized by the loss of the supporting alveolar bone and the soft connective tissue attachment to the teeth, which can lead to loosening and subsequent tooth loss (3).

The regeneration of alveolar bone following its loss is one of the primary goals in the treatment of periodontitis. In many cases, filling and restoring alveolar bone defects using synthetic bone replacements is an accepted means of treatment (4), and bone substitutes, such as hydroxyapatite, tricalcium phosphate and bioceramic phosphates, have been proposed for use in the regeneration of alveolar bone defects. Calcium (Ca^{2+}) and phosphate (P) are the principal components of these ceramics-based filling materials or scaffolds (4–6), which exhibit various Ca^{2+}/P ratios and release profiles of Ca^{2+} and P ions into the local environment and interstitial fluids. It has previously been reported that Ca^{2+} and inorganic P ion concentrations exceed normal physiological concentrations at bone erosion sites (7). This free release of Ca^{2+} or P ions may have a significant impact on the local tissues and the nearby cell populations (8–10); the effects, however, of increased Ca^{2+} and P ion concentrations on hPDLCs with respect to periodontal regeneration have not previously been analysed.

In this study, we hypothesized that an elevation of Ca^{2+} and P ion concentrations in the local environment would significantly affect cell proliferation, differentiation and mineralization capacities of hPDLCs. The influence of different concentrations and ratios of Ca^{2+} and P ions on the proliferation, differentiation and mineralization of hPDLCs was therefore investigated *in vitro*.

Material and methods

Cell culture

The hPDLCs were obtained from healthy premolar teeth extracted for orthodontic reasons from subjects < 20 years of age. The experimental protocol used was approved by the Ethics Committee of Sun Yat-sen University, and informed consent was obtained from all subjects. Briefly, fresh periodontal ligament tissues were collected immediately after the teeth had been extracted in the clinic. The periodontal ligament tissue was removed from the middle third of the root using a sterile scalpel, and then dissected into small pieces and soaked in fresh Dulbecco's modified Eagle's medium (DMEM/High Glucose; Hyclone, Beijing, China) containing 20% fetal bovine serum (BioInd, Kibbutz Beit Haemek, Israel) and 2% (v/v) penicillin and streptomycin (Invitrogen, Grand Island, NY, USA) and plated out on tissue culture plastic. The explant cultures were incubated at 37°C in a humidified atmosphere of air containing 5% CO_2, and cells typically emerged 1–2 wk after plating. The cells were subcultured after reaching about 80% confluency through trypsinization (trypsin/EDTA; Invitrogen). Morphological analysis and immunocytochemical analysis were used to characterize the cell lineage. The primary antibodies of anti-vimentin (Boster, Wuhan, China) and anti-keratin (Cwbiotech, Beijing, China) were applied to the cultured cells for this characterization.

Calcium and phosphate supplements

The basal DMEM used in the experiments contained 1.8 mM Ca^{2+} ions and 0.9 mM P ions (Ca^{2+}/P ratio of 2.0) and served as the control. Defined amounts of ionic Ca^{2+} and P were added to the basal medium to test the cellular response. Calcium ions were prepared from $CaCl_2$ as a sterile 1.8 M solution in water, and P ions were obtained from Na_2HPO_4 and NaH_2PO_4 in a 4:1 molar ratio as a 0.9 M solution in water at neutral pH. All supplements were added fresh to the culture medium before being applied to the cell cultures. To analyse cell proliferation and osteogenic differentiation, Ca^{2+} and P ions were added to complete basal medium (DMEM containing 10% fetal bovine serum and 2% penicillin and streptomycin) and osteogenic differentiation medium [DMEM with 10% fetal bovine serum, 2% (v/v) penicillin/streptomycin, 10^{-8} M dexamethasone, 50 μg/mL ascorbic acid and 8 mM β-glycerophosphate]. The concentrations of Ca^{2+} ions and P ions added in the culture medium had to be optimized, because a Ca^{2+} concentration > 9 mM and a P concentration > 4.5 mM invariably led to significant cell death during the experimental period. Therefore, the concentration regime of Ca^{2+} and P ions tested in this study as follows.

As control: 1.8 mM Ca^{2+} + 0.9 mM P (Ca^{2+}/P ratio of 2.0).

Addition of Ca^{2+} only: 5.4 mM Ca^{2+} + 0.9 mM P (Ca^{2+}/P ratio of 6.0); and 9.0 mM Ca^{2+} + 0.9 mM P (Ca^{2+}/P ratio of 10.0).

Addition of P only: 1.8 mM Ca^{2+} + 2.7 mM P (Ca^{2+}/P ratio of 0.67); and 1.8 mM Ca^{2+} + 4.5 mM P (Ca^{2+}/P ratio of 0.4).

Addition of both Ca^{2+} and P: 5.4 mM Ca^{2+} + 2.7 mM P (Ca^{2+}/P ratio of 2.0); 5.4 mM Ca^{2+} + 4.5 mM P (Ca^{2+}/P ratio of 1.2); 9.0 mM Ca^{2+} + 2.7 mM P (Ca^{2+}/P ratio of 3.3); and 9.0 mM Ca^{2+} + 4.5 mM P (Ca^{2+}/P ratio of 2.0).

Cell proliferation assay

The hPDLCs were seeded at a density of $1 \times 10^4/cm^2$ in 96-well plates in complete growth medium, at five repeats for each group. After 24 h, the culture medium was replaced with the cell culture medium with the defined concentrations and ratios of Ca^{2+} and P ions described in the previous subsection. Changes in cell growth viability were analysed using the Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) on days 1, 3, 5 and 7 following subculturing into the defined media; these kits are considered to be more sensitive than those based on tetrazolium salts, such as

MTT [3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], XTT {2,3- bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide}, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] or WST-1[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. Briefly, the growth medium was removed and replaced with 15 μL of CCK-8 solution, then incubated at 37°C for 4 h in an incubator containing air supplemented with 5% CO_2. The absorbances of the supernatants were read on a plate reader at 490 nm.

Annexin V/propidium iodide double-staining assay

Cells were cultured in growth culture medium with defined concentrations and ratios of Ca^{2+} and P ions, as described above, for 14 d; then 1×10^5 cells were harvested and resuspended in PBS. Apoptotic cell death was detected by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA), using an Annexin-V-FITC Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA), which can permit double staining with recombinant FITC-conjugated annexin V and propidium iodide (PI), according to the manufacturer's instructions. Early apoptotic cells are annexin V^+/PI^-, whereas late apoptotic cells are annexin V^+/PI^+.

Alkaline phosphatase assay

Following 7, 14 and 21 d in culture, ALP activity was determined using an ALP activity assay kit (Jiancheng, Nanjing, China) according to the manufacturer's protocol. In brief, cells were washed three times with 10 mM PBS, and then 1 mL of cold 10 mM Tris–HCl buffer, pH 7.4, containing 0.1% Triton X-100 was added to each well, followed by lysis through two freeze–thaw cycles. Aliquots of these supernatants were placed into 24-well plates, to which 50 μL per well of an ALP substrate solution (2 mM $MgCl_2$ and 16 mM *p*-nitrophenyl phosphate) was added. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 50 μL of 200 mM NaOH, and the liberated *p*-nitrophenol was measured on a plate reader at 520 nm.

Cell-free controls and Alizarin Red S/von Kossa staining

A series of cell-free controls, corresponding to each treatment group, were conducted to determine whether spontaneous precipitation occurred as the result of supplementation with Ca^{2+} and P ions in conditions similar to that of the cell culture.

On days 5, 7 and 13 of culture, the process of mineralized matrix deposition formation was visualized using an Alizarin Red S staining kit (GENMED, Shanghai, China). A von Kossa staining kit (GENMED) was used to analyse mineralized matrix deposition on the cells after 21 d of culture. Images of the stained cells in each well were captured using an inverted phase-contrast microscope (OLYMPUS IX41, Olympus Corporation, Tokyo, Japan) at various magnifications.

Extraction of RNA and RT-PCR

Following 21 d of osteogenic induction culture, the expression levels of osteogenic markers were determined by RT-PCR. The total cellular RNA from each group was harvested using TRIzol Reagent (Invitrogen), and reverse transcribed using RevertAid™ M-MuLV Reverse Transcriptase (MBI/Fermentas, Burlington, ON, Canada). Target gene expression was normalized relative to the housekeeping gene *GAPDH*. The conditions for PCR were as follows: 94°C (3 min), then 28 cycles of 94°C (30 s), 60°C (30 s) and 72°C (20 s), with a final 3 min extension at 72°C. Forward and reverse primers are shown in Table 1.

Statistical analysis

Results are expressed as means ± SD. Means were analysed by one-way ANOVA using the SPSS 17.0 software package (IBM, Armonk, NY, USA). A value of $p < 0.05$ was accepted as statistically significant.

Results

Characterization of hPDLCs

Primary hPDLCs typically germinated within 5 d, and were confluent after approximately 2 wk. These monolayered cells exhibited a fibroblast-like morphology with a characteristic spindle shape. Immunofluorescence analysis showed these cells tested positive to vimentin (Fig. 1A) and negative to keratin (Fig. 1B), an indication that these were mesoderm-derived fibroblasts (Fig. 1).

Increased levels of Ca^{2+} and P ions promote late but not early proliferation of hPDLCs

The hPDLCs showed a maximal level of proliferation in the control media (1.8 mM Ca^{2+}, 0.9 mM P) after 24 h stimulation. Compared with this control group, the cell proliferation levels in all the other experimental groups were inhibited; the 1.8 mM Ca^{2+}, 4.5 mM P (Ca^{2+}/P ratio of 0.4) and 9.0 mM Ca^{2+}, 4.5 mM P (Ca^{2+}/P ratio of 2.0) groups showed significantly retarded growth ($p < 0.05$). Interest-

Table 1. Primer sequences used for RT-PCR

Gene	Primer	Size (bp)
GAPDH	Forward: CATGTTCCAATATGATTCCACC Reverse: GATGGGATTTCCATTGATGAC	88
Runx2	Forward: CCAACCCACGAATGCACTATC Reverse: TAGTGAGTGGTGGCGGACATAC	91
ALP	Forward: GCGCAGAGAAAGAGAAAGACCCCA Reverse: CCCATCCCATCTCCCAGGAACA	133
Osteocalcin (*OCN*)	Forward: CCTGAAAGCCGATGTGGT Reverse: GGCAGCGAGGTAGTGAAGA	148

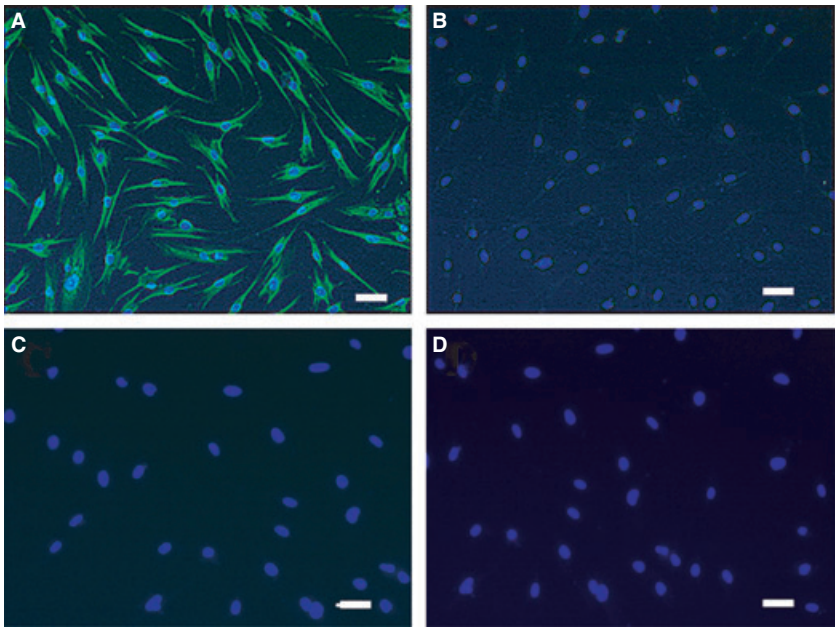

Fig. 1. Immunofluorescent staining of human periodontal ligament cells (hPDLCs). (A) Positive staining for vimentin (green color in cytoplasm). (B) Negative staining for keratin. (C and D) No staining was detected in the negative controls without primary antibodies (original magnification ×100). Bar = 50 μm

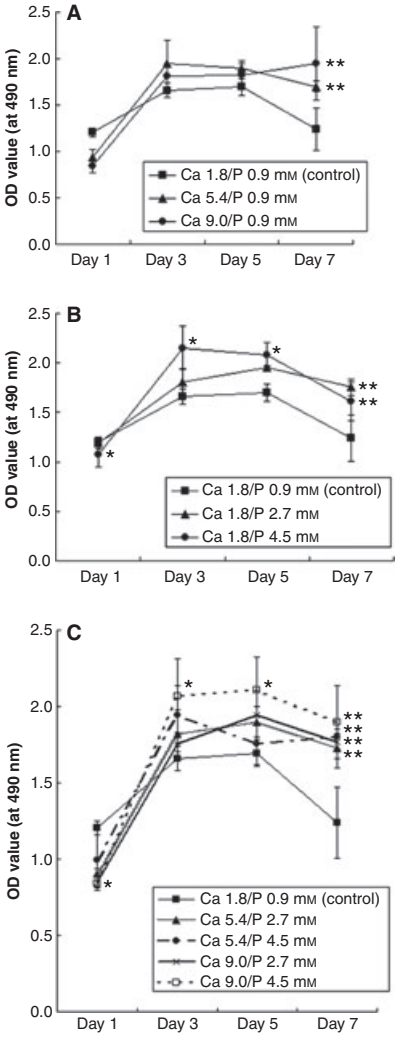

Fig. 2. Cell proliferation of hPDLCs cultured in growth medium. On day 1, cell proliferation in all experimental groups was inhibited. Supplementing the medium with Ca^{2+} and phosphate (P) ions enhanced cell proliferation on days 3, 5 and 7. Culturing the cells for longer periods of time resulted in more significant effects on cell proliferation. (A) Addition of Ca^{2+} ions alone. (B) Addition of P ions alone. (C) Addition of a combination of Ca^{2+} and P ions.*$p < 0.05$, **$p < 0.01$ vs. the corresponding control.

ingly, examination of cells on days 3, 5 and 7 revealed that Ca^{2+} and P ion supplements, to various degrees, appeared to enhance cell proliferation. We observed that when the cells were grown in the presence of increased levels of Ca^{2+} and P for longer periods of time, the cell proliferation levels tended to increase. At the early time points, there was no statistically significant difference in cell proliferation levels between experimental groups and the control ($p > 0.05$), except in the cases of treatment with 1.8 mM Ca^{2+}, 4.5 mM P and 9.0 mM Ca^{2+}, 4.5 mM P on days 3 and 5. However, after 7 d of culture in growth medium, all the experimental groups showed significantly increased cell proliferation in a concentration-dependent manner ($p < 0.001$; Fig. 2). It can be concluded that the ionic Ca^{2+} and inorganic P supplements were able to promote late but not early proliferation of hPDLCs.

Calcium and P ions affected apoptosis in hPDLCs

After treatment with increased Ca^{2+} or P ion supplements for 14 d, the hPDLCs exhibited a greater trend of cell apoptosis compared with controls (data not shown), and the rate of apoptosis significantly increased with higher combinations of Ca^{2+} and P ions. The early apoptosis rate increased from 8.24 to 82.31%, and the late apoptosis rate increased from 2.15 to 18.99% (Fig. 3).

Alkaline phosphatase activity is inhibited by elevated Ca^{2+} and P ion concentrations during both early and late cell proliferation

The hPDLCs cultured in control media (1.8 mM Ca^{2+}, 0.9 mM P) displayed the highest levels of *ALP* mRNA and ALP activity compared with the other experimental groups supplemented with Ca^{2+}/P ions, and at all the time points. As was expected, the specific ALP activity in the control group progressively increased with time in culture. The expression of ALP in all other groups was similar to the controls, and there were no obvious changes to ALP activity levels associated with either time in culture or media conditions (Fig. 4).

Increased Ca^{2+} and P ion levels in culture medium promote and accelerate the process of mineralization of hPDLCs

Mineral nodules first appeared after day 5 in the cell cultures grown in 9.0 mM Ca^{2+}, 2.7 mM P and 9.0 mM

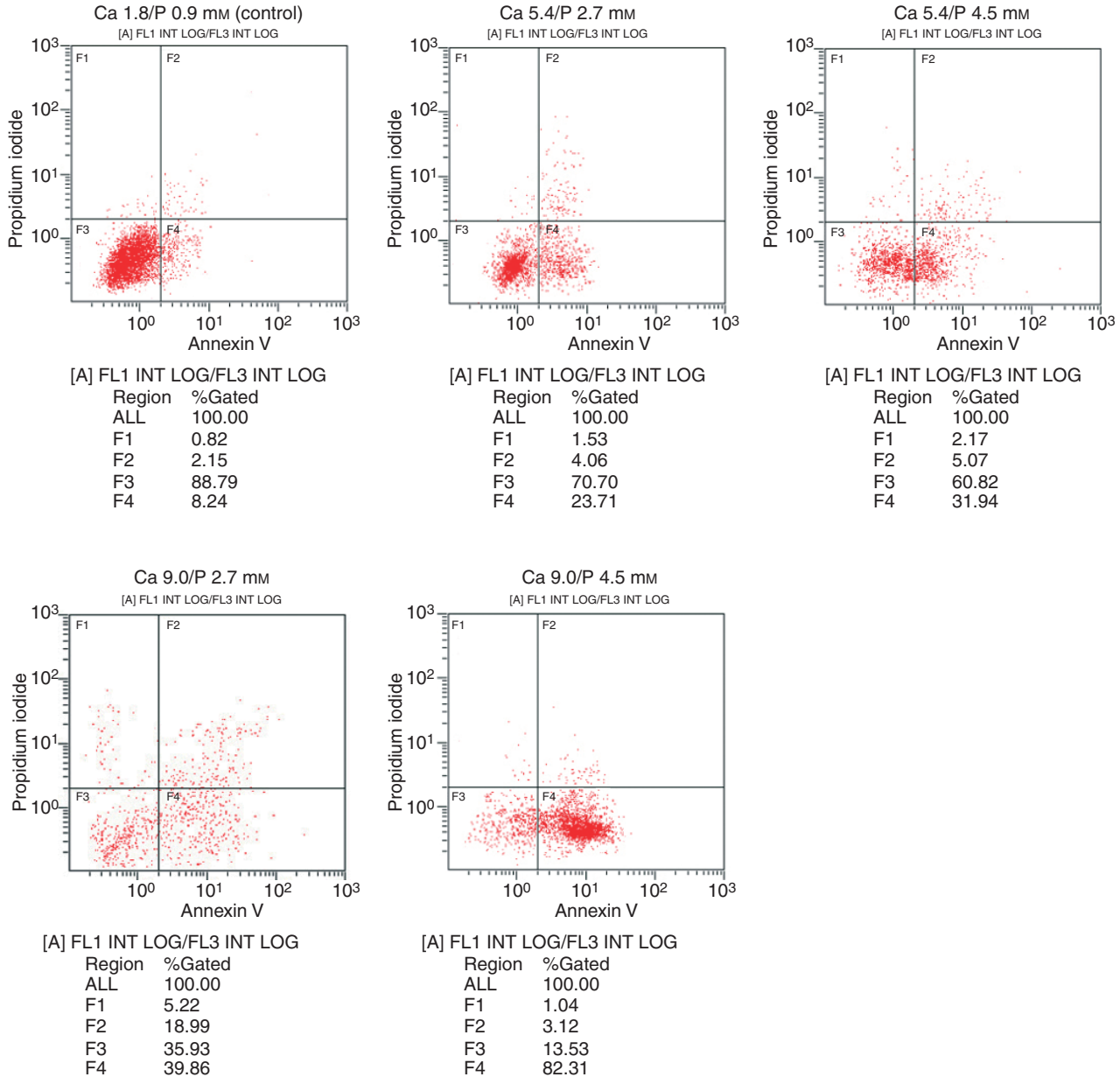

Fig. 3. Detection of cell apoptosis. Representative scatter graphs are shown. Apoptosis was quantified by FACS analysis after staining with annexin V and propidium iodide (PI) on day 14. Viable cells were annexin V^-/PI^- (F3), early apoptotic cells were annexin V^+/PI^- (F4), late apoptotic cells were annexin V^+/PI^+ (F2) and necrotic cells were annexin V^-/PI^+ (F1). The cells treated with higher combinations of Ca^{2+} and P ions showed higher apoptosis rates compared with the control group.

Ca^{2+}, 4.5 mM P medium. After 7 d in culture, nodules also appeared in the 5.4 mM Ca^{2+}, 2.7 mM P and 5.4 mM Ca^{2+}, 4.5 mM P groups; the most obvious mineralization nodes was found in the 9.0 mM Ca^{2+}, 4.5 mM P group. Control groups showed mineralization nodes on approximately day 13, by which time mineralized nodes stained by Alizarin Red S in all the other experimental groups had increased (Fig. 5). Mineralization images captured before and after von Kossa staining of day 21 mineralization are shown in Fig. 6.

Elevated concentrations of Ca^{2+} and P ions affect mRNA expression of osteogenic differentiation

The RT-PCR results for *Runx2*, *ALP* and *OCN* are shown in Fig. 7. Cells grown in the defined osteogenic media showed an upregulation of *Runx2* mRNA compared with the controls, whereas *ALP* mRNA levels were downregulated in all the experimental groups. The *OCN* mRNA levels were not as clear cut, showing increased expression in the 5.4 mM Ca^{2+}, 2.7 mM P and 9.0 mM Ca^{2+}, 4.5 mM P groups.

Discussion

Prior to commencing the experiment proper, the optimal concentrations of

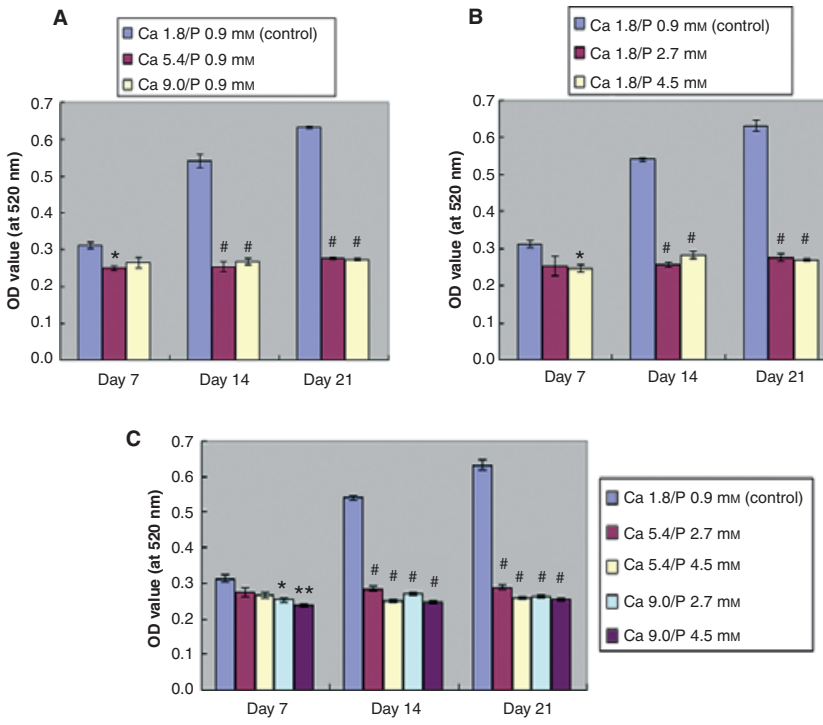

Fig. 4. Alkaline phosphatase (ALP) activity of hPDLCs cultured in osteogenic medium with varied Ca^{2+} and P ions concentrations. (A) Addition of ionic Ca^{2+} alone. (B) Addition of P ions alone. (C) Addition of a combination of Ca^{2+} and P ions. The control group (1.8 mM Ca^{2+}, 0.9 mM P) had the greatest activity relative to any other experimental group at any time point tested. The ALP activity increased progressively with culture time in the control group. The ALP activity in all experimental groups was similar, and there were no clear changes resulting from different culturing periods. $*p < 0.05$, $**p < 0.01$, $p < 0.001$ vs. the corresponding control.

Ca^{2+} and P ions were first determined based on cell survival rates over the duration of the experiment. Calcium ion concentration > 9 mM and P ion concentrations > 4.5 mM were found to induce significant cell death over this period. Based on these tests, the concentrations used in this study were in the range of 1.8–9.0 mM for Ca^{2+} and 0.9–4.5 mM for P. This study demonstrated that the cell proliferation of hPDLCs was enhanced by supplementation of the growth medium, within these concentration ranges, with Ca^{2+} ions and inorganic P ions, and various combinations of the two, in a time-dependent manner after a transient inhibition on day 1.

Calcium ions are ubiquitous intracellular messenger ions involved in the control of a number of cellular processes, including fertilization, mitosis, neuronal transmission, gene expression and cell death (11). Mammalian cells typically respond to extracellular Ca^{2+} through calcium sensing receptors (CaR); these are G-coupled receptors found abundantly in a wide range of cells (11,12). The effects of Ca^{2+} on cell proliferation in the present study are similar to those seen in similar experiments over the same concentration range using human adipose-derived stem cells and mouse osteoblasts (13–15). The most likely mechanism by which Ca^{2+} ions affect the behavior of these cells is via the activation of the CaR, which promotes cell proliferation and differentiation (11,12,16). The change in cell proliferation rates at different times seen in our study contrast with the study reported by

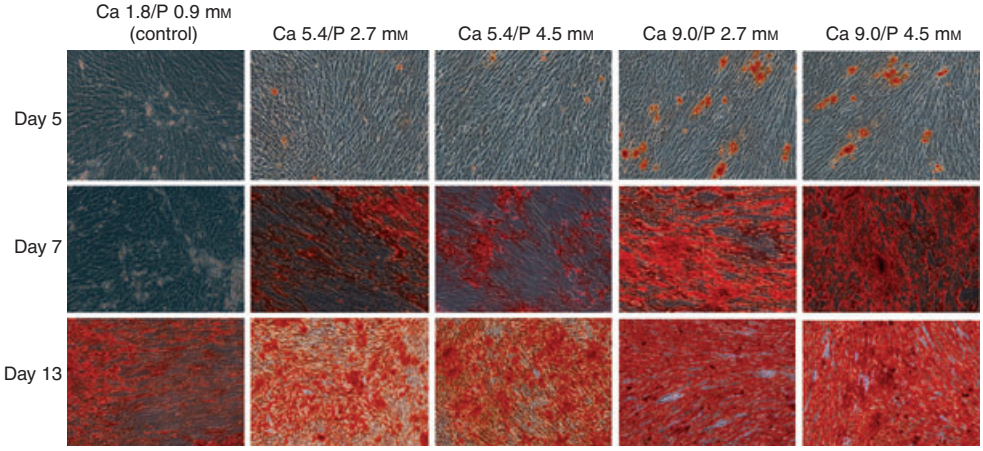

Fig. 5. The process of mineralization of hPDLCs in osteogenic medium with different Ca^{2+} and P ion supplements. Cell cultures in the 9.0 mM Ca^{2+}, 2.7 mM P and the 9.0 mM Ca^{2+}, 4.5 mM P groups first exhibited mineral nodules on day 5. After 7 d in culture, nodules also began to appear in the 5.4 mM Ca^{2+}, 2.7 mM P and the 5.4 mM Ca^{2+}, 4.5 mM P groups, with the most obvious mineralization nodes found in the 9.0 mM Ca^{2+}, 4.5 mM P group. Control groups showed mineralization nodes on approximately day 13, and at this time the mineralized nodes of all experimental groups were also increased (original magnification ×100). Bar = 50 μm

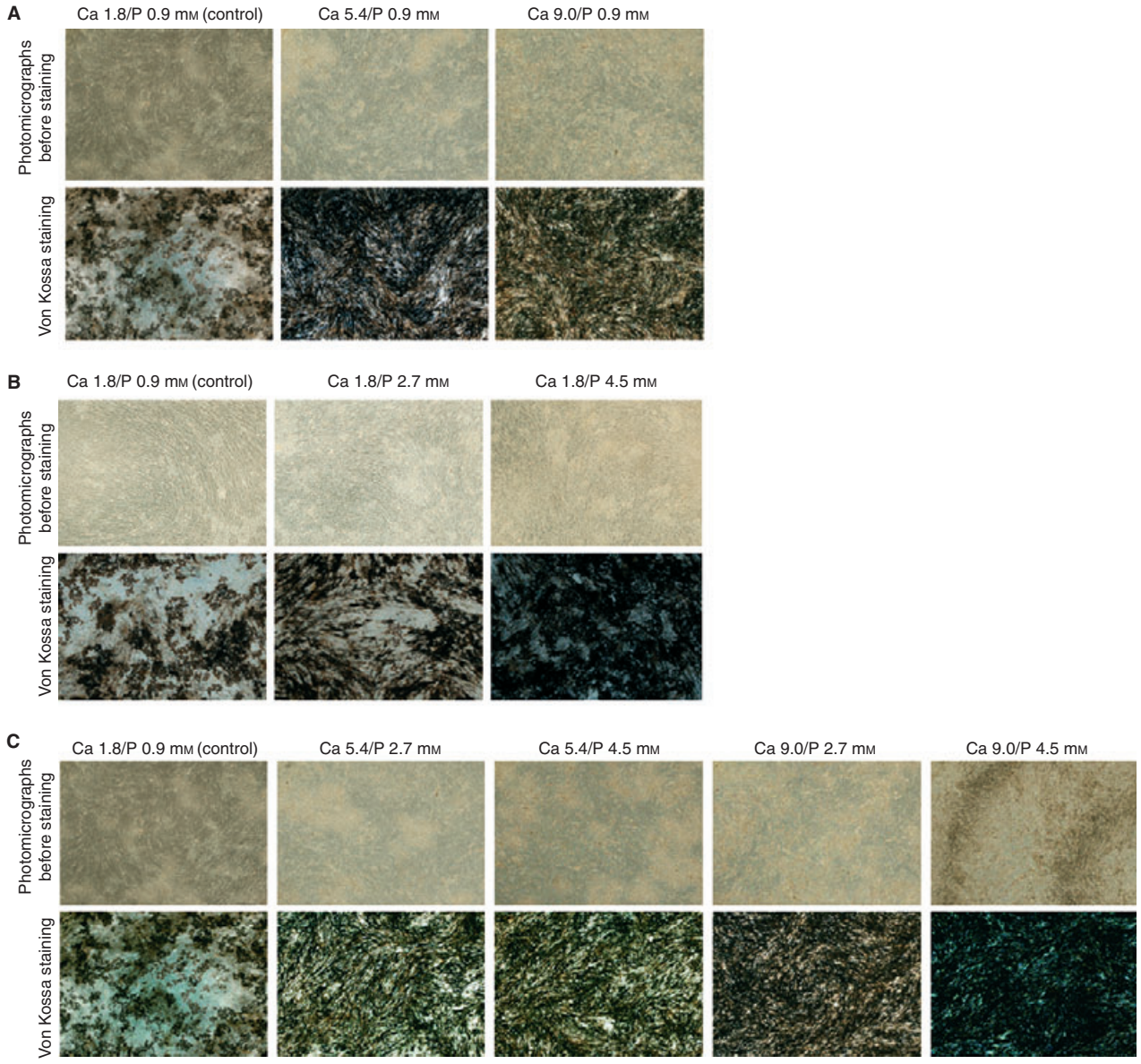

Fig. 6. Photos before staining and after von Kossa staining on day 21. (A) Addition of Ca^{2+} ions alone. The 9.0 mM Ca^{2+}, 0.9 mM P group exhibited the most mineralization nodes. (B) Addition of P ions alone. The 1.8 mM Ca^{2+}, 4.5 mM P group revealed the maximal degree of mineralization. (C) Addition of a combination of Ca^{2+} and P ions. The 9.0 mM Ca^{2+}, 4.5 mM P group showed the most obvious mineral nodules. Bar = 100 μm

Eklou-Kalonji *et al.* (17), who found that high extracellular Ca^{2+} ion concentrations (5, 7 or 10 mM) had a dose-dependent inhibitory effect on cell proliferation (17). Another group found that when inorganic P in the growth medium is at or above 3 mM, a small rise in Ca^{2+} concentration (0.1–1 mM) seemed to cause rapid apoptosis in both chondrocytes and osteoblasts (18); similar results were reported in further studies, in both osteoblasts (19) and chondrocytes (20), as a result of elevated phosphate levels. These findings, however, are by no means universal; Wu *et al.* (21) analysed chondrocyte cultures, at various stages of development, grown in different culture media and found no significant apoptosis in response to elevated phosphate concentration. It is quite possible that the different cell responses reported may be due to the various cell types used (hPDLCs, human adipose-derived stem cells, human osteoblasts or chondrocytes, porcine osteoblasts and mouse osteoblasts), as well as differences in experimental conditions. The temporary suppression of cell proliferation we observed on day 1, following exposure to Ca^{2+}/P ion supplementation of the medium, could be a cellular response to changes in the extracellular microenvironment.

Alkaline phosphase activity was very sensitive to elevated Ca^{2+} and P ion

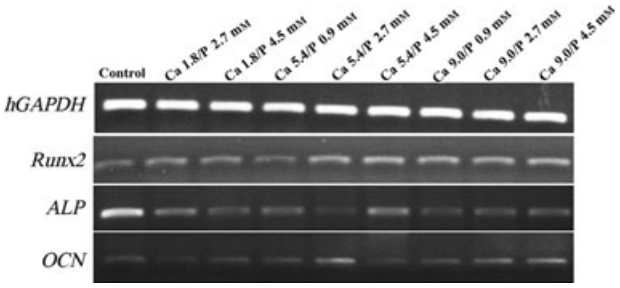

Fig. 7. RT-PCR analysis of osteogenic differentiation markers. Representative agarose gels are shown. In comparison to cells grown in control media, *Runx2* mRNA levels were upregulated in all experimental groups tested, whereas *ALP* mRNA levels were downregulated in all experimental groups. The osteocalcin (*OCN*) mRNA levels were increased only in the 5.4 mM Ca^{2+}, 2.7 mM P and 9.0 mM Ca^{2+}, 4.5 mM P groups.

concentrations and was inhibited in all the experimental groups. This inhibition, demonstrated by decreased mRNA expression and enzyme activity, was not an immediate response, but was sustained for the duration of the culture period and was consistent with that observe in similar studies with porcine osteoblasts (17), mouse MC3T3-E1 cells (22) and HBV155 osteoblast-like cells (23). These results all seem to indicate that suppression of ALP activity, by elevated Ca^{2+} and P ion concentrations, is independent of the ionic media concentrations of either factor, as well as the cell type or species origin. Alkaline phosphatase is a membrane-associated enzyme and an early stage osteoblastic differentiation marker; its precise role in differentiation is, however, unclear. In the groups with elevated levels of Ca^{2+} and P ions, we found mineralization to occur at an earlier stage but could not detect any changes in ALP activity at any of the time points analysed. There clearly is not a positive linear relationship between ALP expression levels *per se* and the timing of formation and quantity of extracellular mineralization in cultured hPDLCs. Tissue-nonspecific alkaline phosphatase is necessary and sufficient to trigger bone extracellular matrix mineralization, but beyond that is not required for subsequent calcification (24).

β-Glycerophosphate was supplemented at 8 mM for osteogenic induction. In our study, the ALP activity in the osteogenic cultures reflects only the influence of the P ions in the osteogenic media over and above the 8 mM β-glycerophosphate. It is therefore difficult to assess accurately what effects the different concentrations of P ions had on ALP activities. Other studies investigating the requirement for and timing of ALP and β-glycerophosphate in the process of mineralization have revealed that β-glycerophosphate, and therefore inorganic P ion levels, are both necessary to initiate mineralization; however, following its initiation, mineralization in osteoblasts will continue independent of the levels of both ALP and β-glycerophosphate (25–27). β-Glycerophosphate contains an organic phosphate group that is released by ALP to free inorganic P ions, which then provide the chemical potential for promoting mineral deposition. An excess of extracellular inorganic P ions is a competitive inhibitor of ALP activity (28).

Biological mineralization is accomplished primarily by osteoblasts, chondrocytes and odontoblasts (29). The biochemical mechanisms that initiate cell-mediated mineralization subsequent to the increase in Ca^{2+} and/or P ion levels are still subject to debate, but it seems that cell apoptosis and death induced by Ca^{2+} and/or P ions plays a role in the process of tissue mineralization (30). In the present study, the rate of cell apoptosis increased with higher Ca^{2+} and P ion concentrations, which also promoted and accelerated mineralization in hPDLCs. We are still uncertain of the underlying mechanism for this phenomenon, but we hypothesize that P ion-induced cell apoptosis somehow acts synergistically with extracellular Ca^{2+} ions (31).

Our investigation of the effects of Ca^{2+} and P ions on the mRNA expression of specific genes revealed that *ALP* was downregulated in all experimental groups. The expressions of *Runx2* and *OCN* were either upregulated or downregulated in an ion concentration-dependent manner. *Runx2*, for example, has a well-defined role as a transcription factor during the later stages of osteoblastic maturation and is required for normal osteogenesis, being active throughout the induction, proliferation and maturation of osteoblasts (32). The significantly higher expression of *Runx2* indicated that elevated Ca^{2+} and P ion concentrations may promote the osteogenic differentiation of hPDLCs. *OCN* is a late marker of mature osteoblasts, and its expression was seen in all experimental groups, although its expression was downregulated in most groups, with the exception of the 5.4 mM Ca^{2+}, 2.7 mM P and 9.0 mM Ca^{2+}, 4.5 mM P groups. It is conceivable that in our experimental system there is a cellular mechanism that maintains the fibroblastic phenotype of hPDLCs and stops them from differentiating into mature osteoblasts.

Conclusion

The data gathered from this study indicate that both Ca^{2+} and P ions can promote late but not early proliferation of hPDLCs, accelerate *in vitro* mineralization and inhibit ALP activity. There was no evidence of a linear relationship between *ALP* gene expression and the onset and quantity of *in vitro* extracellular mineralization in cultured hPDLCs. There are implications arising from these results with respect to the clinical use of biodegradable calcium phosphate bioceramics to repair periodontal bone defects, and also for hydroxyapatite cement paste-coated implants for achieving better osseointegration by the simultaneous local concentrations of Ca^{2+} and P ions. Within the range of Ca^{2+} and P ions concentrations tested in this study, the combined

concentrations of 9.0 mM of Ca^{2+} and 4.5 mM P (Ca^{2+}/P ratio of 2.0) most vigorously promoted *in vitro* hPDLC proliferation, differentiation and mineralization.

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