The influence on gene-expression profiling of osteoblasts behavior following treatment with the ionic products of sintered β -dicalcium pyrophosphate dissolution

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Abstract

Sintered dicalcium pyrophosphate (SDCP) is biocompatible to bone tissue both in the in vivo and in vitro model. However, the molecular mechanisms that mediated these processes have yet to be identified. In this study, we investigated the influence of SDCP ions on in vitro osteoblasts behavior. The powder of sintered β -dicalcium pyrophosphate (SDCP) was dissolved by HCl and then diluted into different concentration of solutions by culture medium used in the osteoblast cell culture. The effects of various concentration of SDCP on bone cell activities were evaluated by using MTT assay. For the differentiation of osteoblasts, alkaline phosphatase (AP) staining, von Kossa stain for mineralized nodules and bone markers messenger ribonucleic acid (mRNA) isolation and identification were performed at 3 h, days 1, 3, 7 and 14.

In the presence of 10^{-8} M SDCP for 14 days, the osteoblasts population was still significantly higher than that of control. In the qualitative analysis for the formation of AP staining colonies and mineralization nodules formation were not affected by SDCP ions. When osteoblasts cultured in the presence of 10^{-8} M SDCP ions, the osteocalcin mRNA expression was up-regulated; while the collagen, osteonectin and osteopontin mRNA expression were down-regulated.

In this study, we demonstrated that the elevated concentration of calcium and pyrophosphate ions can activate genes of the bone cells. This study will contribute to a better understanding of cell/biomaterial interactions and mechanisms that SDCP affect the bone cells.

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1. Introduction

Recent advancement in orthopedic surgery can be attributed to the revolution in biomaterials. Initially, the choice of biomedical materials for use in the body was dependent on those already available off the shelf. Later, many of the materials selected proved to be either pathogenic or toxic. Whereas second-generation biomaterials were designed to be either resorbable or bioactive, the next generation of biomaterials is combining these

two properties. Third-generation bioactive glasses and macroporous foams are being designed to activate genes that stimulate regeneration of living tissues [1].

Sintered dicalcium pyrophosphate (SDCP) appear to be biocompatible to bone tissue in the in vivo animal model [2]. Host tissue responses to this material are generally assessed by morphological and histological examinations of the implant site in order to evaluate their biocompatibility with respect to cytotoxicity. It is difficult to exam in vivo reaction of a specific cell to the substrate because numerous cell populations and chemical factors are involved in implantation. In order to determine the sequences of events and the parameters influencing the interactive process, in vitro studies on bone cell culture models are used to determine

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biocompatibility and to investigate bone and biomaterial interactions. Osteoblastic cells (responsible for bone formation) are used most frequently [3–7] and osteoclasts (cell responsible for bone resorption) are also used but less frequently [8,9].

In the *in vitro* model, SDCP has been proved to be more biocompatible than hydroxyapatite [10]. Recent work in our institute has also demonstrated that the ingestion of either bisphosphonate [11] or SDCP decreased the bony porosity and increased bone mineral contents in the long bones of ovariectomized rats [12]. In the later study, we demonstrated that SDCP can induce apoptosis of osteoclasts in an ultrastructural model characterized by changes of the nucleus accompanied by degradation of cellular organelles [13]. However, the molecular mechanisms that mediated these processes have yet to be identified. Furthermore, it has not been possible to define whether stimulation of bone synthesis by SDCP occurs through direct contact between substrate and cells or through soluble ions released by SDCP during its resorption.

Collagen Type I is synthesized from procollagens that are secreted in the extracellular space. Type I collagen is the most abundant collagen in vertebrates, forms 90% of the organic mass of bone, and is produced in large quantities by osteoblasts [14]. Osteocalcin is one of the most abundant non-collagenous proteins in bone, but the biologic function of osteocalcin has not been precisely defined. Because of the specific interaction with hydroxyapatite, osteocalcin has been postulated to affect the growth or maturation of Ca^{2+} -phosphate mineral phases. More recently, it was reported that osteocalcin may act in combination with other hydroxyapatite-binding proteins as a signal for bone remodeling [15]. Osteopontin is thought to promote or regulate the adhesion, attachment, and spreading of osteoclasts to the bone surface during bone resorption [16,17]. It is known to be produced by osteoblasts [18–20], as well as osteoclasts [19,21,22]. Another protein abundant in bone with potential roles in regulating cellular activities, albeit not in binding the cells, is osteonectin [23]. Studies *in vitro* have shown roles of osteonectin in modulating cell division and cell migration [23] and perhaps initiating active mineralization in normal skeletal tissue [24]. Bone sialoprotein, similar to osteopontin as a sulfate-protein secreted by osteoblasts, is an early marker of osteoblasts differentiation that plays significant roles in regulating biomineralization [23]. Type I collagen expression is considered an early differentiation marker of the osteoblast phenotype [25–27]; while the expression of osteocalcin, osteopontin, and osteonectin were late differentiation marker as they associated with mineralization of the bone extracellular matrix [28].

In this study, we investigated the influence of SDCP ions on *in vitro* osteoblasts behavior. This paper reports the evaluation of the bioactivity and biocompatibility of

SDCP prepared at different concentrations (10^{-2} – 10^{-14} M). The biocompatibility has been evaluated by means of cytotoxicity and cytocompatibility tests. Cell proliferation as well as the expression of some biochemical parameters of osteoblastic phenotype (alkaline phosphatase (AP) activity, type I collagen, osteocalcin, osteonectin, and osteopontin production) have been monitored.

2. Materials and methods

2.1. Preparation of sintered β -dicalcium pyrophosphate solutions

The powder of sintered β -dicalcium pyrophosphate (SDCP) was prepared as previously described [2]. In short, the β -DCP powder (β -DCP: $\text{Ca}_2\text{P}_2\text{O}_7$, Sigma, St. Louis, MO, USA) mixed with 5 wt% $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ in water and dried at 70°C for 3 days. The well-mixed and dried cake was grounded and sieved to obtain 40–60 mesh particles. The sieved particles were placed in a platinum crucible and heated up to 930°C at a heating rate of $3^\circ\text{C}/\text{min}$ in a conventional Ni–Cr coiled furnace and then maintained in air for 1 h after the sintering temperature of 930°C was reached. The obtained SDCP was dissolved by 12 N hydrogen chloride and then diluted into different concentration of solutions by culture medium used in the bone cell culture. In the first part of this study, the effects of various concentration of SDCP ions on bone cell activities were evaluated by using MTT assay as described below. The highest concentration of SDCP used in this study was 1.0×10^{-2} M, because when SDCP ions were higher than 1.0×10^{-2} M, the buffering capacity of medium was exceeded and the calcium phosphate precipitations appeared. Thirteen different concentrations (1.0×10^{-2} – 1.0×10^{-14} M) were tested for 1, 3, 7 and 14 days period. While in the control cultures, an equivalent and molarity of HCl was added.

2.2. Osteoblast cell culture

Sequential digestion of newborn Wistar-rat calvaria was performed by using a modification of the methods described by Wong and Cohn [29]. Briefly, after pretreatment of the dissected calvaria with 4 mM $\text{Na}_2\text{-EDTA}$ in a pre-warmed (37°C) solution containing 137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4 , pH 7.2 (solution A), for 10 min three times, the fragments were sequential digested with collagenase (180 U/ml, Sigma, St. Louis, MO, USA) in solution A with EDTA. The sequential digestion consisted of four times' treatments for 5 min followed by two times' treatments for 10 min. The cells released after each treatment were immediately harvested by centrifugation and resuspended in culture

medium. Unambiguous identification of cell populations as osteoblasts is complex since none of the parameters used for defining osteoblasts-like cells are unique to this cell types [30]. The presence of AP, an early marker of osteoblasts [31], is used to assess the osteoblastic character of the isolated cells [29,32–34].

2.3. Colorimetric MTT (Tetrazolium) assay for cell viability [35]

The mitochondria activity of the bone cells after exposure to various concentrations of SDCP was determined by colorimetric assay which detects the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma catalog no. M2128, Sigma Co., St. Louis, MO, USA) to formazan. For the assay, 2.5×10^4 cells/well were incubated (5% CO₂, 37°C) in the presence of various concentration of SDCP ions. After various time intervals the supernatant was removed, 100 µl/well of MTT solution (1 mg/ml in test medium) was added and the wells were incubated at 37°C for 4 h to allow the formation of formazan crystal. Again the supernatant was removed and acid-isopropanol (100 µl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on Micro Elisa reader (Emax Science Corp., Sunnyvale, CA, USA), using a test wavelength of 570 nm against a reference wavelength of 690 nm. Plates were normally read within 1 h after adding the isopropanol.

2.4. Osteoblast differentiation

Osteoblasts cultured in the media in the presence of dexamethasone have been shown to be capable of synthesizing and mineralizing an extracellular matrix and to form AP in vitro [36]. To subculture, the cells were washed twice with sterile PBS followed by treatment with 1:1 mixture of 0.03% collagenase (25 U/ml in DMEM only; type VII, Sigma, St. Louis, MO, USA) and 0.05% trypsin (Sigma, St. Louis, MO, USA) for 20 min at 37°C in 5% CO₂. The resulting cell suspension was then passed and centrifuged at 1500 rpm for 5 min to pellet the cells. The supernatant was removed and the pellet resuspended in α -minimal essential media (α -MEM; Sigma, St. Louis, MO, USA). A concentration of 1×10^6 cells/100 µl was added to 35 mm wells of a 6-well plate. The osteoblasts were incubated at 37°C in 5% CO₂ for 24 h. After 24 h, the media were changed and the cells were incubated in α -MEM supplemented with 10% fetal calf serum (FCS; Gibco BRL, Rockville, MD, USA), antibiotics (gentamicin 50 µg/ml, penicillin G 100 µg/ml [Gibco BRL, Rockville, MD, USA]), L-ascorbic acid (50 µg/ml Gibco BRL, Rockville, MD, USA), supplemented with 5 mM

β -glycerophosphate (Sigma, St. Louis, MO, USA) and 10^{-8} M dexamethasone (Sigma, St. Louis, MO, USA). The day of subculture was day zero. From day one of culture, 10^{-8} M SDCP solution was added. The medium was changed every 3–4 days; AP staining, von Kossa stain for mineralized nodules and bone markers messenger ribonucleic acid (mRNA) isolation and identification were performed at 3 h, day 1, 3, 7 and 14.

2.5. Alkaline phosphatase activity determination

After fixing the cells, the dishes were incubated for 30 min in TRIS Buffer (0.2 M, pH 8.3) with AS-MX phosphate (Sigma, St. Louis, MO, USA) as a substrate and Fast Blue (Sigma, St. Louis, MO, USA) as a stain [37]. The AP positive cells stained blue/purple. For each experiment, a minimum of three dishes was counted and the experiments were repeated three times.

2.6. The von-Kossa staining on mineralized nodules formation

Since the calcium usually co-precipitate with the phosphate ion in the in vitro culture condition, mineralization of the nodules in the cultures was assessed using von-Kossa stain. The matrix was washed with PBS, and cultures were treated with 5% silver nitrate solution 100 µl/well in the dark at 37°C for 30 min. The excess silver nitrate solution was then completely washed away using double-distilled H₂O and the culture plate was exposed to sodium carbonate/formaldehyde solution for few minutes to develop color. Mineralized and unmineralized nodules could be distinguished separately: mineralized nodules by their von-Kossa-positive staining (dark brown center and light brown peripheral area), and unmineralized nodules by their surface layer of cuboidal cells, light brown staining, and three-dimensional structure. To evaluate bone nodule area precisely, the von-Kossa-stained areas were viewed by light microscopy. For each experiment, a minimum of three dishes was counted and the experiments were repeated three times.

2.7. Messenger ribonucleic acid (mRNA) isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

After a predetermined time, the RNA was isolated from osteoblast cells by an acid guanidine method using RTIzol Reagent (Gibco BRL, Rockville, MD, USA) and reverse transcribed to cDNA. For the quantification of mRNA, the method developed by Nakajima et al. was used [38]. Briefly, 10-fold titrated cDNA (1 µg RNA–1 pg RNA) was amplified by polymerase chain reaction (PCR). The amplification procedure consisted of 35 cycles (95°C for 1 min, 58°C for 1 min,

72°C for 10 min) with the oligonucleotide primer sets shown in Table 1. The cDNA amplified by PCR was fractionated by electrophoresis in agarose gel. Relative expression were evaluated by detection of specific band (39, Collagen type I: 1208 bp; 40, Osteocalcin: 462 bp;

41, Osteonectin: 890 bp; 42, Osteopontin: 941 bp) visualized by ethidium bromide staining.

2.8. Statistical analysis

All data are expressed as mean±standard deviation and were analyzed by analysis of variance (one-way ANOVA). Statistical significance was determined by Bonferroni's *t*-test . Probability values less than 0.05 were considered significant.

3. Results

3.1. Quantitative analysis of osteoblast cell counts

MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] is a pale yellow substrate that produces a dark blue formazan product when incubated with living cells. MTT ring is cleaved in active mitochondria, and the reaction occurs only in living cells [35]. Fig. 1 shows the effect of various concentrations of SDCP ions on osteoblast cells viability measured by MTT assay. When osteoblast cells cultured with 10⁻² M or 10⁻³ M

Table 1 Oligonucleotide of 5' and 3' primers of targets	
Collagen type I	1208 bp [39]
5' Primer	5'-ATGGT GCTCC TGGTG CCA-3'
3' Primer	5'-TGTAT TCGAT GACTG TCTTG-3'
Osteocalcin	462 bp [40]
5' Primer	5'-ATGAG GACCC TCTCT CTGCT CACT-3'
3' Primer	5'-ACCGT TCCTC ATCTG GACTT TA-3'
Osteonectin	890 bp [41]
5' Primer	5'-CTT AGA TCA CCA GAT CCT TGT TGA TG-3'
3' Primer	5'-CCC AGC ATC ATG AGG GCC TGG ATC TT-3'
Osteopontin	941 bp [42]
5' Primer	5'-CAA CCA TGA GAC TGG CAG TGG TTT GC-3'
3' Primer	5'-GCC TCT TCT TTA ATT GAC CTC AGA AG-3'

Fig. 1. The effect of various concentrations of SDCP ions on osteoblast cells viability (N = 12). When osteoblast cells cultured with 10⁻² M or 10⁻³ M SDCP for one day, there was significant decrease in the formation of formazan; while in the 10⁻⁴ M or 10⁻¹⁰ M concentration of SDCP, the formation of formazan was significantly increased. At the 10⁻⁸ M concentration of SDCP, the beneficial effect on the osteoblasts attained peak at the end of 3 days' culture, although it did not attain the significant level. (Shaded bar mean the difference attained a statistically significant difference, i.e., P<0.05.)

Fig. 2. Photomicrograph of AP staining for the differentiation of osteoblasts in the medium containing 5 mM β -glycerophosphate and 10^{-8} M dexamethasone. At 3 h after differentiation medium, there was little AP positive staining colony was found in the culture. The AP positive staining colonies first appeared at the 1st day's culture, and then progressively increased as the culture period passed, and attained a significant degree at the 14th day's culture. Similar result was observed in the group cultured with 10^{-8} M concentration of SDCP ions. (Control: control group; Exp. Study group with 10^{-8} M concentration of SDCP ions; bar = 200 μ m.)

SDCP for one day, there was significant decrease in the formation of formazan; while in the 10^{-4} M or 10^{-10} M concentration of SDCP, the formation of formazan was significantly increased in the first day's culture (Fig. 1). At the 10^{-8} M concentration of SDCP, the beneficial effect on the osteoblasts attained to the end of 14 days' culture, although it did not attain the significant level at the 3rd and 7th days' culture (Fig. 1). We selected the 10^{-8} M concentration of SDCP ions for the further biochemical study.

3.2. Alkaline phosphatase staining and mineralized nodules formation

When cultured in the medium containing 5 mM β -glycerophosphate and 10^{-8} M dexamethasone, the os-

teoblasts differentiated as the cultured period increased. At 3 h after differentiation medium, little AP positive staining colony was found in the culture. The AP positive staining colonies first appeared at the 1st day's culture of control groups, and then progressively increased as the culture period passed, and attained a significant degree at the 14th day's culture (Fig. 2). Similar result was observed in the group cultured with 10^{-8} M concentration of SDCP ions (Fig. 2). The addition of SDCP ions on the osteoblast cultures also did not affect the formation of mineralization nodules, and similar results were observed on the von-Kossa staining (Fig. 3). The qualitative analysis of ALP and mineralization were repeated in triplicate which showed similar result. The formation of AP positive staining colonies and mineralization nodules formation in the

Fig. 3. Photomicrograph of von-Kossa staining for the mineralized nodules formation of the osteoblasts in the medium containing 5 mM β -glycerophosphate and 10^{-8} M dexamethasone. At 3 h after differentiation medium, there little von-Kossa positive staining colony was found in the culture. The von-Kossa positive staining colonies first appeared at the 1st day's culture, and then progressively increased as the culture period passed, and attained a significant degree at the 14th day's culture. Similar result was observed in the group cultured with 10^{-8} M concentration of SDCP ions. (Control: control group; Exp. Study group with 10^{-8} M concentration of SDCP ions; bar = 200 μ m.)

osteoblast cultures were not significantly affected by SDCP ions.

3.3. Reverse transcriptase-polymerase chain reaction

In this model, osteoblasts have been known to express type I collagen, osteonectin and osteopontin mRNA at the 3 h culture, then attained their maximal expression at the first day's culture. The osteocalcin mRNA expression was quite low in the first 3 days' culture and then appeared at the 7th day's culture. In the presence of on 10^{-8} M SDCP ions, type I collagen, osteonectin and osteopontin mRNA expression were down-regulated at the 3 h culture and then returned to

that of control level at the day 1. The osteocalcin mRNA expression was up-regulated in the first 3 days' culture and returned to that of control level at the day 7 (Fig. 4). When osteoblasts cultured in the presence of 10^{-8} M SDCP ions, the osteocalcin mRNA expression was up-regulated; while the collagen, osteonectin and osteopontin mRNA expression were down-regulated than that of the control.

4. Discussion

Continuous advances in the field of implantable calcium phosphate bioceramics have produced impres-

Fig. 4. The effect of various concentrations of SDCP ions on bone markers expression by RT-PCR. In this model, osteoblasts have been known to express collagen type I, osteonectin and osteopontin mRNA at the 3 h culture, then attained their maximal expression at the first day's culture. The osteocalcin mRNA expression was quite low in the first 3 days' culture and then appeared at the 7th day's culture. In the presence of on 10^{-8} M SDCP ions, collagen type I, osteonectin and osteopontin mRNA expression were down-regulated at the 3 h culture and then returned to that of control at the day 1. The osteocalcin mRNA expression was up-regulated in the first 3 days' culture and returned to that of control at the day 7. When osteoblasts cultured in the presence of 10^{-8} M SDCP ions, the osteocalcin mRNA expression was up-regulated; while the collagen, osteonectin and osteopontin mRNA expression were down-regulated than that of the control. (Con.: control group; Exp. Study group with 10^{-8} M concentration of SDCP ions; Col: Type I collagen; OC: osteocalcin; ON: osteonectin; OPN: osteopontin.)

sive progress with respect to their biocompatibility and have shown an ability to promote tissue formation. Biodegradable biomaterials have found a wide variety of applications in clinical medicine. Certain biodegradable calcium phosphates have been used as bone substitutes to avoid the complications associated with bone grafts, such as the shortage of supply, the immunogenicity, and the transfer of disease with the graft [43–46]. One of the main advantages of biodegradable substances is that it obviates the need for eventual surgical removal. SDCP has been proved to have great potential as an *in vivo* biodegradable bone substitute [2,47]. Recent work in our institute has also demonstrated that the ingestion of SDCP decreased the bony porosity and increased bone mineral contents in the ovariectomized rats [12]. In the *in vitro* model, SDCP has been proved to be more biocompatible than hydroxyapatite [10]. Later, we demonstrated that SDCP can induce apoptosis of osteoclasts in an ultrastructural model characterized by changes of the nucleus accompanied by degradation of cellular organelles [13]. However, it has not been possible to define the molecular mechanisms that mediated these processes; whether stimulation of bone synthesis by SDCP occurs through direct contact

between substrate and cells or through soluble ions released by SDCP during is still unknown. In the previous study, it has been shown that osteoclast differentiation and activation are thought to be mediated through modulation of paracrine factors produced by osteoblastic lineage cells [48]. In this study, we investigated the influence of SDCP ions on *in vitro* osteoblasts behavior.

From MTT assay of this study, we observed cells proliferation was significantly ($p < 0.05$) increased in the first day's culture when osteoblasts were cultured with 10^{-4} – 10^{-10} M concentration of SDCP ions. At the 10^{-8} M concentration of SDCP, the beneficial effect on the osteoblasts attained to the end of 14 days' culture, although it did not attain the significant level at the 3rd and 7th days' culture (Fig. 1). The concentration of 10^{-8} M SDCP ions was selected for the further biochemical study.

The differentiation of osteoblastic cells in culture involves a programmed developmental sequence [25,26]. The proliferation of cells in culture usually follows a standard pattern, with an initial lag-phase (period of adaptation during which the cell replaces elements of the cell surface and extracellular matrix lost during trypsinization), followed by the log-phase (phase of exponential increase in cell number, during which osteoblastic cells are relatively undifferentiated), and the plateau phase (in which cell division is balanced by cell loss and bone cell phenotypic markers are expressed). In general, cell cultures are most consistent and uniform in the log-phase, and sampling at day 6 gives a high yield and reproducibility of proliferative activity in osteoblast cells [49,50]. The increased cell number with 10^{-8} M SDCP ions at day 1 possibly was caused by an increased growth rate during the lag-phase or an extended lag-period, implying a stimulation effect of SDCP ions on the cells during this phase. The cell number was quite similar between the groups with and without 10^{-8} M SDCP ions at days 3 and 7, implying the stimulation effect of SDCP ions on the cells possibly was not evident during the log-phase. The increased cell number with 10^{-8} M SDCP ions appeared again at day 14, implying a stimulation effect of SDCP ions on the cells during the plateau phase.

To assess the influence of surface composition on bone cell specific markers, AP staining, von-Kossa staining on mineralized nodules formation, collagen Type I, osteocalcin, and osteonectin production were measured. Osteoblast differentiation is a multistep series of events modulated by an integrated cascade of gene expression that initially supports proliferation and the sequential expression of genes associated with the matrix maturation, and mineralization of the bone extracellular matrix [28]. AP expression is considered an early differentiation marker of the osteoblast phenotype, while the von-Kossa stain of mineralized nodules

formation represented the end differentiation marker of the osteoblasts. In this study, the differentiation of osteoblasts was induced when β -glycerophosphate and dexamethasone were added into the culture medium [36,51], the degree of differentiation increased as the cultured period increased (Figs. 2 and 3) and cultures of osteoblasts also had detectable calcium deposition, as seen on von-Kossa staining by days 7–14 after the cells reached confluency [52]. However, the formation of AP positive staining colonies and mineralization nodules formation in the osteoblast cultures were not significantly affected by adding of 10^{-8} M SDCP ions. Although the von-Kossa stain is a measure of phosphate ion precipitation, the possibility of ectopic mineralization due to the cultures been grown in the presence of the exogenous calcium phosphate and β -glycerophosphate can be excluded since the similar results were observed in the control samples.

Recent research shows that there is genetic control of the cellular response of osteoblasts to bioactive glasses. Several families of genes are up-regulated within 48 h of the exposure of primary human osteoblasts to the ionic dissolution products of bioactive glasses [53]. The activated genes express numerous proteins that influence all aspects of differentiation and proliferation of osteoblasts. RT-PCR analysis was used in this study to evaluate the cell–biomaterials interactions at the genetic level. For the evaluation of RNA expression, northern blotting analysis is used as usual, but this requires more than 1 μ g mRNA for one sample; i.e., over 10^7 cells are needed to obtain 1 μ g mRNA, so it is difficult to use northern blotting for studying cell–biomaterials interactions [54]. In fact, RT-PCR analysis seemed to fit our purpose, as it needs only a small amount (pg) of mRNA for detection.

When osteoblasts cultured in the presence of 10^{-8} M SDCP ions, the osteocalcin mRNA expression was up-regulated during the first 3 days' culture; while the collagen, osteonectin and osteopontin mRNA expression were down-regulated in the first 3 h culture (Fig. 4). In the current study, the amount of osteocalcin mRNA expression attained its highest amount at the day 7. The osteocalcin, osteonectin and osteopontin mRNA expression reached their maximal earlier than the mineralization process, indicating an increased proportion of non-collagenous proteins, including osteocalcin, osteonectin and osteopontin being bound to the collagen matrix or hydroxyapatite mineral [15,55].

Despite encouraging preliminary in vivo reports about improvement with the use of SDCP in treatment of osteoporosis after ovariectomy, basic-science and controlled study reported to date still have not established yet. In this study, we demonstrated that elevated concentration of calcium and pyrophosphate ions can activate genes of the bone cells. This study will contribute to a better understanding of cell/biomaterial

interactions and mechanisms that SDCP ions affect the bone cells. Although our study had limitations and our findings are preliminary, continued and advanced study on the alterations in gene expression of bone cells by SDCP will provide a basis for understanding the observed the bone cells responses to various biomedical and pharmacological interventions.

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References

- [1] Hench LL, Polak JM. Third-generation biomedical materials. *Science* 2002;295:1014–7.
- [2] Lin FH, Lin CC, Lu CM, Liu HC, Sun JS, Wang CY. Mechanical properties and histological evaluation of sintered beta- $\text{Ca}_2\text{P}_2\text{O}_7$ with $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ addition. *Biomaterials* 1995;16:793–802.
- [3] Gregoire M, Orly I, Menanteau J. The influence of calcium phosphate biomaterials on human bone cell activities. An in vitro approach. *J Biomed Mater Res* 1990;24:165–77.
- [4] Davies JE. In vitro modeling of the bone/implant interface. *Anat Rec* 1996;245:426–45.
- [5] Kawai N, Niwa S, Sato M, Sato Y, Suwa Y, Ichihara I. Bone formation by cells from femurs cultured among three-dimensionally arranged hydroxyapatite granules. *J Biomed Mater Res* 1997;37:1–8.
- [6] Acil Y, Terheyden H, Dunsche A, Fleiner B, Jepsen S. Three-dimensional cultivation of human osteoblast-like cells on highly porous natural bone mineral. *J Biomed Mater Res* 2000;51:703–10.
- [7] Zamboni G, Losito I, Triffitt JT, Zamboni CG. Detection of collagen synthesis by human osteoblasts on a tricalcium phosphate hydroxyapatite: an X-ray photoelectron spectroscopy investigation. *J Biomed Mater Res* 2000;49:120–6.
- [8] Beckham CA, Greenlee Jr TK, Crebo AR. Bone formation at a ceramic implant interface. *Calcif Tissue Res* 1971;8:165–71.
- [9] Yamada S, Heymann D, Bouler JM, Daculsi G. Osteoclastic resorption of biphasic calcium phosphate ceramic in vitro. *J Biomed Mater Res* 1997;37:346–52.
- [10] Sun JS, Tsuang YH, Liao CJ, Liu HC, Hang YS, Lin FH. The effects of calcium phosphates particles on the growth of osteoblast. *J Biomed Mater Res* 1997;37:324–34.
- [11] Harris ST, Gertz BJ, Genant HK, Eyre DR, Survill TT, Ventura JM, DeBrock J, Ricerca E, Chesnut III CH. The effect of short term treatment with alendronate on vertebral density and biochemical markers of bone remodeling in early postmenopausal women. *J Clin Endocrinol Metab* 1993;76:1399–406.
- [12] Sun JS, Huang YC, Tsuang YH, Chen LT, Lin FH. Sintered dicalcium pyrophosphate increases bone mass in the ovariectomized rats. *J Biomed Mater Res* 2002;59:246–53.
- [13] Sun JS, Huang YC, Lin FH, Chen LT. The effect of sintered dicalcium pyrophosphate on osteoclast metabolism: an ultrastructural study. *J Biomed Mater Res* 2003;64A:616–21.
- [14] Von der Mark K. Structure, biosynthesis, and gene regulation of collagens in cartilage and bone. In: Seibel MJ, Robins SP, Bilezikian JP, editors. *Dynamics of bone and cartilage metabolism*. San Diego: Academic Press; 1999. p. 3–29.

- [15] Gunberg CM, Nishimoto SK. Vitamin K-Dependent proteins of bone and cartilage. In: Seibel MJ, Robins SP, Bilezikian JP, editors. *Dynamics of bone and cartilage metabolism*. San Diego: Academic Press; 1999. p. 45–53.
- [16] Reinholt FP, Hulthén K, Oldberg A, Heinegård D. Osteopontin—a possible anchor of osteoclasts to bone. *Proc Natl Acad Sci USA* 1990;87:4473–5.
- [17] Ikeda T, Nomura S, Yamaguchi A, Suda T, Yoshiki S. In situ hybridization of bone matrix proteins in undecalcified adult rat bone sections. *J Histochem Cytochem* 1992;40: 1079–88.
- [18] Weinreb M, Shinar D, Rodan GA. Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. *J Bone Mineral Res* 1990;5:831–42.
- [19] Merry K, Dodds R, Littlewood A, Gowen M. Expression of osteopontin mRNA by osteoclasts and osteoblasts in modelling adult human bone. *J Cell Sci* 1993;104:1013–20.
- [20] Hirakawa K, Hirota S, Ikeda T, Yamaguchi A, Takemura T, Nagoshi J, Yoshiki S, Suda T, Kitamura Y, Nomura S. Localization of the mRNA for bone matrix proteins during fracture healing as determined by in situ hybridization. *J Bone Mineral Res* 1994;9:1551–7.
- [21] Tezuka K, Sato T, Kamioka H, Nijweide PJ, Tanaka K, Matsuo T, Ohta M, Kurihara N, Hakeda Y, Kumegawa M. Identification of osteopontin in isolated rabbit osteoclasts. *Biochem Biophys Res Commun* 1992;186:911–7.
- [22] Dodds RA, Ali N, Pead MJ, Lanyon LE. Early loading-related changes in the activity of glucose 6-phosphate dehydrogenase and alkaline phosphatase in osteocytes and periosteal osteoblasts in rat fibulae in vivo. *J Bone Mineral Res* 1993;8: 261–7.
- [23] Lane TF, Sage EH. The biology of SPARC, a protein that modulates cell–matrix interactions. *FASEB J* 1994;8:163–73.
- [24] Termine JD, Kleinman HK, Whitson SW, Conn KM, McGarvey ML, Martin GR. Osteonectin, a bone-specific protein linking mineral to collagen. *Cell* 1981;26:99–105.
- [25] Stein GS, Lian JB. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. *Endocr Rev* 1993;1: 424–42.
- [26] Stein GS, Lian JB, Owen TA. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. *FASEB J* 1990;4:3111–23.
- [27] Lynch MP, Stein JL, Stein GS, et al. 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.
- [28] Stein GS, Lian JB, Stein JL, Van Wijnen AJ, Montecino M. Transcriptional control of osteoblast growth and differentiation. *Physiol Rev* 1996;76:593–629.
- [29] Wong GL, Cohn DV. Target cells in bone for parathormone and calcitonin are different: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. *Proc Natl Acad Sci USA* 1975;72:3167–71.
- [30] Bellows CG, Aubin JE, Heersche JNM, Antosz ME. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *Calcif Tissue Int* 1986;38:143–54.
- [31] Caplan AI, Pechak DG. The cellular and molecular embryology of bone formation. In: Peck C, editor. *Bone and mineral research*, Vol. 5. Amsterdam: Elsevier; 1987. p. 117–83.
- [32] Hall AK, Dickson IR. The effects of parathyroid hormone on osteoblast-like cell from embryonic chick calvaria. *Acta Endocrinol* 1985;108:217–23.
- [33] Begley CT, Doherty MJ, Kankey DP, Wilson DJ. The culture of human osteoblasts growth upon bone graft substitutes. *Bone* 1993;14:661–7.
- [34] Doherty MJ, Schlag G, Schwarz N, Mollan RAB, Nolan PC, Wilson DJ. Biocompatibility of xenogeneic bone, commercially available coral, a bioceramic and tissue sealant for human osteoblasts. *Biomaterials* 1994;15:601–8.
- [35] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J Immunol Methods* 1983;65:55–63.
- [36] Gundle R, Beresford JN. The isolation and culture of cells from explants of human trabecular bone. *Calcif Tissue Int* 1995;56: S8–10.
- [37] Burnstone M. Histochemical observation on enzymatic processes in bone and teeth. *Ann NY Acad Sci* 1960;85:431–44.
- [38] Nakajima T, Aono H, Hasunuma T, Yamamoto K, Maruyama I, Nosaka T, Hatanaka M, Nishioka K. Overgrowth of human synovial cells driven by the human T cell leukemia virus type I tax gene. *J Clin Invest* 1993;92:186–93.
- [39] Genovese C, Rowe D, Kream B. Construction of DNA sequences complementary to rat alpha-1 and alpha-2 collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D. *Biochemistry* 1984;23: 6210–6.
- [40] Lian J, Stewart C, Puchacz E, Mackowiak S, Shalhoub V, Collart D, Zambetti G, Stein G. Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. *Proc Natl Acad Sci USA* 1989;86:1143–7.
- [41] Lee MJ, Saijoh K, Nestler EJ, Duman RS, Sumino K. Regional differences in expression of osteonectin mRNA after administration of calcium to rats. Full staff reviewed, 1995, unpublished.
- [42] Oldberg A, Franzen A, Heinegård D. Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly Asp cell binding sequence. *Proc Natl Acad Sci USA* 1986;83:8819–23.
- [43] Bos GD, Goldberg VM, Zika JM, Heiple KG, Powell AE. Immune response of rats to frozen bone allografts. *J Bone Jt Surg* 1983;65A:239–46.
- [44] Hollinger JO, Battisone GC. Biodegradable bone repair materials: synthetic polymers and ceramics. *Clin Orthop* 1986;207: 290–305.
- [45] Mankin HJ, Gebhardt MC, Tomford WW. The use of frozen cadaveric allografts in the management of patients with bone tumors of the extremities. *Orthop Clin N Am* 1987;18:275–89.
- [46] Urist MR, Dawson E. Intertransverse process fusion with aid of chemosterilized autolyzed antigen-extracted allogeneic (AAA) bone. *Clin Orthop* 1981;154:97.
- [47] Lin CC, Liao CJ, Sun JS, Liu HC, Lin FH. Prevascularized bone graft cultured in sintered porous beta-Ca₂P₂O₇ with 5wt% Na₄P₂O₇·10H₂O addition ceramic chamber. *Biomaterials* 1996; 17:1133–40.
- [48] O'Brien EA, Williams JH, Marshall MJ. Osteoprotegerin is produced when prostaglandin synthesis is inhibited causing osteoclasts to detach from the surface of mouse parietal bone and attach to the endocranial membrane. *Bone* 2001;28: 208–14.
- [49] Freshney RI. *Culture of Animal Cells*. Edition 4. New York: Wiley-Liss; 2000. p. 309–28.
- [50] Goldring SR, Wang JT. Molecular biological approaches to investigate cell/biomaterial interactions. In: Davies JE, editor. *The bone–biomaterial interface*. Toronto: University of Toronto Press; 1991. p. 241–9.
- [51] Coelho MJ, Fernandes MH. Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteoblastic differentiation. *Biomaterials* 2000;21:1095–102.

- [52] Harris SA, Enger RJ, Riggs BL, Spelsberg TC. Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. *J Bone Miner Res* 1995;10:178–86.
- [53] Ioannis D, Xynos ID, Edgar AJ, Buttery LDK, Hench LL, Polak JM. Gene-expression profiling of human osteoblasts following treatment with the ionic products of Bioglass[®] 45S5 dissolution. *J Biomed Mater Res* 2001;55:151–7.
- [54] Kishida A, Kato S, Ohmura K, Sugimura K, Akashi M. Evaluation of biological response to polymeric biomaterials by RT-PCR analysis. *Biomaterials* 1996;17:1301–5.
- [55] Raif EM, Harmand MF. Molecular interface characterization in human bone matrix: I. biochemical and IR spectroscopic studies. *Biomaterials* 1993;14:978–84.