Original article

Calcium sulfate acts on stem cells derived from peripheral blood

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Calcium sulfate (CaS) is a highly biocompatible material and enhances bone formation in vivo. However, how CaS alters osteoblast activity to promote bone formation is incompletely understood.

To study how CaS can induce osteoblast differentiation in mesenchymal stem cells, the expression levels of bone related genes and mesenchymal stem cell markers were analyzed, using real time Reverse Transcription-Polymerase Chain Reaction.

CaS causes a significant induction of the bone related genes osteopontin (SPP1), osteocalcin (BGLAP) and collagen, type III, alpha 1 (COL3A1). The expression of ENG and FOSL1 were not significantly changed in stem cells treated with CaS respect to untreated cells, while RUNX1, COL1A1 and ALPL were significantly down expressed.

The results obtained lend to a better understand of the molecular mechanism of bone regeneration and as a model for comparing other materials with similar clinical effects.

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Key words: calcium sulfate, gene expression, stem cells, bone regeneration.

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INTRODUCTION

Several graft materials have been proposed in implants dentistry. Autogenous bone is the gold standard but usually donor oral sites have a limited amount of graft material. Consequently, surgeons harvest bone from extra-oral sites with increased morbidity and the need for general anesthesia[1,2]. An alloplastic material avoids the need for a second surgical field but it should be safe, resorbable, able to maintain space, and cheap[3,4]. Calcium sulfate (CaS) is highly biocompatible and it is one of the synthetic grafts with the longest clinical history (more than 100 years)[5-12]. It has been utilized in treating periodontal disease, endodontic lesions, alveolar bone loss, and maxillary sinus augmentation[3,4,13-18]. CaS has been used as a membrane to facilitate healing and to prevent the loss of other grafting materials[19]. When associated with other bone grafts it seems to have a favorable effect on osteogenesis[2,20]. CaS rapidly resorbs and leaves a calcium phosphate lattice which promotes osteogenic activity[21,22]. Ricci et al.[23] demonstrated that CaS induces new bone formation in dogs after 2 weeks and that it is almost completely resorbed after 1 month.

In previous studies we carried out a

genome wide screen of osteoblast-like cell line (MG-63) following treatment with CaS, using cDNA microarray. Several genes covering a broad range of functional activities, like signal transduction, differentiation, cell-cycle regulation, were significantly up-regulated[24]. Then the genetic effect of CaS was studied in the same cell system at post-transcriptional level, with microRNA microarray[25]. We identified miRNA that regulates the transduction of genes related to bone formation (TFIP11), skeletal development (MSX1, ADAMTS4, DLX5 FGFR1 COMP EN1 SHOX), cartilage remodeling (MATN1) and ossification (BMP1, BMP7, ALPL, PTH).

Because few reports analyze the genetic effects of CaS on stem cells[26], the expression of genes related to osteoblast differentiation were analyzed using cultures of mesenchymal stem cells derived from peripheral blood (PB-hMSCs) treated with CaS.

To investigate the osteogenic differentiation of PB-hMSCs, the quantitative expression of the mRNA of specific genes, like transcriptional factor (RUNX2), bone related genes (SPP1, COL1A1, COL3A1, BGLAP, ALPL, and FOSL1) and mesenchymal stem cells marker (ENG) were examined by means of real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR).

MATERIALS AND METHODS

a) Stem preparation

PB-hMSCs were obtained for gradient centrifugation from peripheral blood of healthy anonymous volunteers, using the Acuspin System-Histopaque 1077 (Sigma Aldrich, Inc., St Louis, Mo, USA). Firstly, 30 ml of heparinizated peripheral blood were added to the Acuspin System-Histopaque 1077 tube and centrifugated at 1000 x g for 10 minutes. After centrifugation the interface containing mononuclear cells was transferred in another tube, washed with PBS and centrifuged at 250 x g per 10 minutes. The enriched mononuclear pellets was resuspended in 10 ml of Alphamem medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with antibiotics (Penicillin 100 U/ml and Streptomycin 100 micrograms/ml - Sigma, Chemical Co., St Louis, Mo, USA) and amminoacids (L-Glutamine - Sigma, Chemical Co., St Louis, Mo, USA). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 Medium was changed after 24 hours. PB-hMSC were selected for adhesiveness and characterized as stem cells by immunoflorescence.

b) Immunofluorescence

Cells were washed with PBS for three times and fixed with cold methanol for 5 min at room temperature. After washing with PBS, cells were blocked with bovine albumin 3% (Sigma Aldrich, Inc., St Louis, Mo, USA) for 30 min at room temperature. The cells were incubated overnight sequentially at 4 °C with primary antibodies raised against CD105 1:200, mouse (BD Biosciences, San Jose, CA, USA), CD73 1:200, mouse (Santa Cruz Biotecnology, Inc., Santa Cruz, CA, USA), CD90 1:200, mouse (Santa Cruz Biotecnology, Inc., Santa Cruz, CA, USA), CD34 1:200, mouse (Santa Cruz Biotecnology, Inc., Santa Cruz, CA, USA). They were washed with PBS and incubated for 1 h at room temperature with secondary antibody conjugated-Rodamine goat anti-mouse 1:200 (Santa Cruz Biotecnology, Inc., Santa Cruz, CA, USA). Subsequently, cells were mounted with the Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and observed under a fluorescence microscope (Eclipse TE 2000-E, Nikon Instruments S.p.a., Florence, Italy).

c) Cell culture

PB-hMSCs at second passage were cultured in Alphamem medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 10% fetal calf serum, antibiotics (Penicillin 100 U/ml and Streptomycin 100 micrograms/ml - Sigma Aldrich, Inc., St Louis, Mo, USA) and amminoacids (L-Glutamine - Sigma Aldrich, Inc., St Louis, Mo, USA). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

For the assay, cells were collected and seeded at a density of $1x10^5$ cells/ml into 9 cm^2 (3ml) wells by using 0.1% trypsin, 0.02% EDTA in Ca++ - and Mg – free Eagle's buffer for cell release.

One set of wells were added with CaS (Surgiplaster, Classimplant, Rome, Italy) at the concentration of 0.001 mg/ml. Another set of wells containing untreated cells were used as control. The medium was changed every 3 days.

After seven days, when cultures were sub-confluent, cells were processed for RNA extraction.

d) RNA processing

Reverse transcription to cDNA was performed directly from cultured cell lysate using the TaqMAn Gene Expression Cells-to-Ct Kit (Ambion Inc., Austin, TX, USA), following manufacturer's instructions. Briefly, cultured cells were lysed with lysis buffer and RNA released in this solution. Cell lysate were reverse transcribed to cDNA using the RT Enzyme Mix and appropriate RT buffer (Ambion Inc., Austin, TX, USA). Finally the cDNA was amplified by real-time PCR using the included TaqMan Gene Expression Master Mix and the specific assay designed for the investigated genes.

e) Real time PCR

Expression was quantified using real time RT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene RPL13A and were expressed as fold changes relative to the expression of the untreated PB-hMSCs. Quantification was done with the delta/ delta calculation method[27]. Forward and reverse primers and probes for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA, USA) and are listed in **Table 1**.

All PCR reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer and 200 nM of the probe, and cDNA. The amplification profile was initiated by 10-minute incubation at 95°C, followed by two-step amplification of 15 seconds at 95°C and 60 seconds at 60°C for 40 cycles. All experiments were performed including non-template controls to exclude reagents contamination. PCRs were performed with two biological replicates.

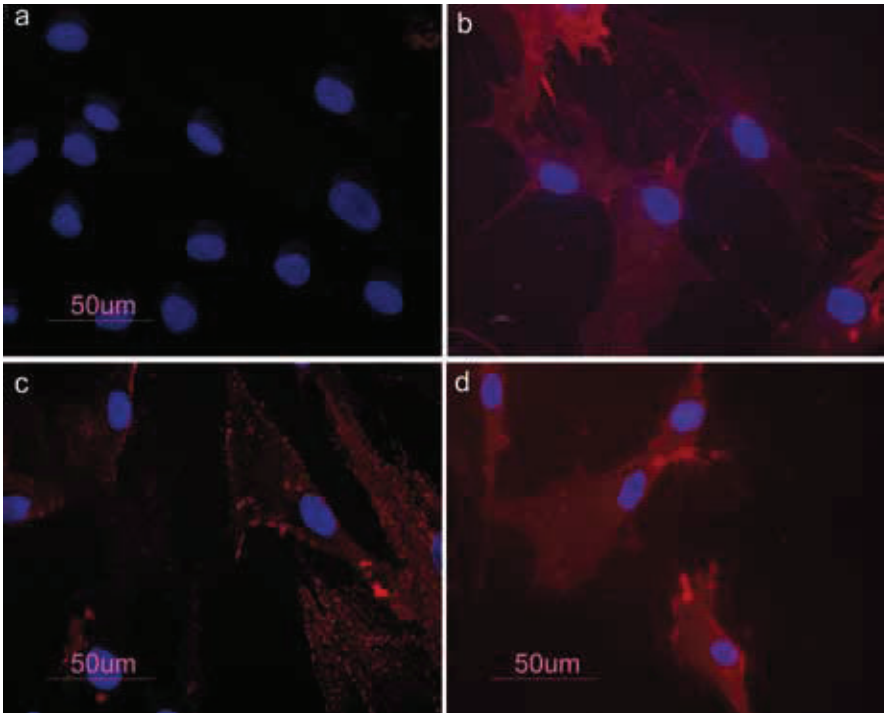
Gene symbol	Gene name	Primer sequence (5'>3')	Probe sequence (5'>3')
SPP1	osteopontin	F-GCCAGTTGCAGCCTTCTCA R-AAAAGCAAATCACTGCAATTCTCA	CCAAACGCCGACCAAGGAAAACTCAC
COL1A1	collagen type I alpha1	F-TAGGGTCTAGACATGTTCAGCTTTGT R-GTGATTGGTGGGATGTCTTCGT	CCTCTTAGCGGCCACCGCCCT
RUNX2	runt-related transcription factor 2	F-TCTACCACCCCGCTGTCTTC R-TGGCAGTGTCATCATCTGAAATG	ACTGGGCTTCCTGCCATCACCGA
ALPL	alkaline phospatasi	F-CCGTGGCAACTCTATCTTTGG R-CAGGCCCATTGCCATACAG	CCATGCTGAGTGACACAGACAAGAAGCC
COL3A1	collagen, type III, alpha 1	F-CCCACTATTATTTTGGCACAACAG R-AACGGATCCTGAGTCACAGACA	ATGTTCCCATCTTGGTCAGTCCTATGCG
BGLAP	osteocalcin	F-CCCTCCTGCTTGGACACAAA R-CACACTCCTCGCCCTATTGG	CCTTTGCTGGACTCTGCACCGCTG
CD105	endoglin	F-TCATCACCACAGCGGAAAAA R-GGTAGAGGCCCAGCTGGAA	TGCACTGCCTCAACATGGACAGCCT
FOSL1	FOS-like antigen 1	F-CGCGAGCGGAACAAGCT R-GCAGCCCAGATTTCTCATCTTC	ACTTCCTGCAGGCGGAGACTGACAAAC
RPL13A	ribosomal protein L13	F-AAAGCGGATGGTGGTTCCT R-GCCCCAGATAGGCAAACTTTC	CTGCCCTCAAGGTCGTGCGTCTG

Table 1. Primer and probes used in real time PCR

RESULTS

PB-hMSCs were characterized by immunofluorescence. The cell surfaces were positive for mesenchymal stem cell markers, CD105, CD90 and CD73 and negative for marker of hematopoietic origin, CD34 (Figure 1). Transcriptional expressions of several osteoblast-related genes (RUNX2, SPP1, COLIA1, COL3A1, BGLAP, ALPL and FOSL1) and mesenchymal stem cells marker (ENG) were examined after 7 days of supplement treatment with CaS (0.001 mg/ml). CaS enhanced the expression of bone related genes like COL3A1, BGLAP and SPP1. The treatment did not affect the mRNA expression of ENG and FOSL1 that were similarly in both treated and untreated PB-hMSCs. RUNX2, COL1A1 and ALPL were decreased in the presence of CaS at day 7.

Figure 1. PB-hMSCs by indirect immunofluorescence (Rodamine). Cultured cells were positive for the mesenchymal stem cell marker CD73 (b), CD90 (c), CD105 (d) and negative for the hematopoietic markers CD34 (a). Nucleuses were stained with DAPI. Original magnification x40.



DISCUSSION

CaS is a highly biocompatible material[5-12]. Solidified or crystallized CaS is very osteogenic *in vivo*. As the surface of CaS dissolves in body fluids, the calcium ions form calcium phosphate that re-precipitates on the surface forming an osteoblast "friendly" environment. How this "friendly" environment alters osteoblast activity to promote bone formation is incompletely understood. In order to get more inside how CaS acts on PB-hMSCs, changes in expression of bone related marker genes (RUNX2, SPP1, COLIA1, COL3A1, BGLAP, ALPL and FOSL1) and mesenchymal stem cells marker (ENG) were investigated by real-time RT–PCR.

Mesenchymal stem cells (MSCs) are defined as self-renewable, multipotent progenitors cells with the ability to differentiate, under adequate stimuli, into several mesenchymal lineages, including osteoblasts[28]. In our study, mesenchymal stem cells from human peripheral blood were isolated and characterized by morphology and immunophenotype. Isolated PB-hMSCs showed fibroblast-like morphology and were positive for MSCs surface molecules (CD90, CD105, CD73) and negative for markers of haematopoietic progenitors (CD34).

After 7 day of treatment with CaS the expression levels of osseodifferentiation genes were measured by relative quantification methods using real-time RT–PCR.

Two osteoblast-specific genes, SPP1 (an acid phosphoprotein involved in regulation of bone mineralization) and BGLAP (a bone specific protein involved in mineralization and bone resorption), that are generally express by osteoblasts in the early stages of their differentiation[29] were significantly upregulate in treated PB-hMSCs.

Expression of ENG and FOSL1 didn't have significant change in treated cells respect to control after 7 day of treatment with CaS. ENG (CD105) is a surface markers used to define a bone marrow stromal cell population capable of multilineage differentiation[30]. This gene is a receptor for TGF-β1 and -β3[31] and modulates TGF-β signaling by interacting with related molecules, such as TGF-β1, -β3, BMP-2, -7, and activin A. It is speculated that these members of the TFG-β superfamily are mediators of cell proliferation and differentiation and play regulatory roles in cartilage and bone formation[32]. The

disappearance of the CD105 antigen during osteogenesis suggests that this protein, like others in the TFG-β superfamily, is involved in the regulation of osteogenesis[33]. FOSL1 that encodes for Fra-1, a component of the dimeric transcription factor activator protein-1 (Ap-1), which is composed mainly of Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun proteins (c-Jun, JunB and JunD). AP-1 sites are present in the promoters of many developmentally regulated osteoblast genes, including alkaline phosphatase and collagen I. McCabe et al.[34] demonstrated that differential expression of Fos and Jun family members could play a role in the developmental regulation of bone-specific gene expression and, as a result, may be functionally significant for osteoblast differentiation. Kim et al.[35] studying the effect of a new anabolic agents that stimulate bone formation, find that this gene is activated in the late stage of differentiation, during the calcium deposition.

In our study FOSL1 and ENG were weakly down- and up-regulated respectively, probably because cells were at early stage of differentiation. RUNX2 and ALPL were down regulated in treated cells respect to control after 7 day of treatment with CaS. RUNX2 is the most specific osteoblast transcription factor and is a prerequisite for osteoblast differentiation and consequently mineralization. This result is comparable with data reported by Kim et al.[36] They showed that there was no BMP-2-mediated up-regulation of RUNX2 mRNA expression at days 3 or 7[36].

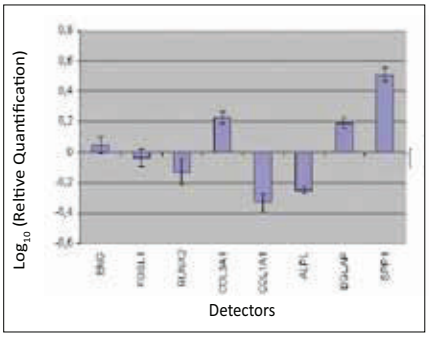


Figure 2. Gene expression analysis of PB-hMSCs after 7 days of treatment with CaS.

Alkaline phosphatase regulates mineralization of bone matrix. Several studies demonstrated that the potency of individual substances to induce alkaline phosphatase varies in a species-dependent manner. Glucocorticoids such as dexamethasone are potent inducers in human and rat stromal cells, but they have no effect on alkaline phosphatase activity in mouse stromal cells[37,38]. On the contrary, bone morphogenetic proteins (BMPs) are potent inducers of osteogenesis in both mouse and rat bone marrow stromal cells[39] but Diefenderfer et al showed that BMP-2 alone is a poor osteoblast inducer in human marrow derived stromal cells[40].

CaS also modulates the expression of genes encoding for collagenic extracellular matrix proteins like collagen type 1α1 (COL1A1). Collagen type1 is the most abundant in the human organism[41]. In our study COL1A1 is significantly down expressed as compared to the control when exposed to CaS probably because this gene is activated in the late stage of differentiation and are related to extracellular matrix synthesis.

On the contrary, COL3A1 was up-regulated in treated cells respect to control. COL3A1 encodes the pro-alpha1 chains of type III collagen, a fibrillar collagen that is found in extensible connective tissues[42].

CONCLUSIONS

The present study shows the effect of CaS on PB-hMSCs in the early differentiation stages: CaS is an inducer of osteogenesis on human stem cells as demonstrated by the activation of bone related genes: osteopontin (SPP1), osteocalcin (BGLAP) and COL3A1. Moreover, we chose to perform the experiment after 7 days in order to get information on the early stages of stimulation. It is our understanding, therefore, that more investigations with different time points are needed in order to get a global comprehension of the molecular events related to CaS action. The reported model is useful to investigate the effects of different substances on stem cells.

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