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Regulation of bone-related genes expression by bone-like apatite in MC3T3-E1 cells

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Abstract Bone-like apatite on HA/TCP ceramics sintered at 1,100 °C (HT1) and 1,200 °C (HT2) could be obtained via immersing substrates into simulated body fluid (SBF) for 3 days. When MC3T3-E1 preosteoblastic cells cultured on the surface of the bone-like apatite for 3 days, SEM observations revealed cell membrane features with secreted crystals very similar to in vivo bone formation during intramembranous ossification with a direct bone apposition on the ceramics. According to semi-quantitative RT-PCR method, mRNA expressions of osteocalcin (marker of late-stage differentiation) and type 1 collagen were increased in cultures with HT1S and HT2S when compared to HT1 and HT2 after cultured for 6 days. The results indicated that bone-like apatite had the ability to support the growth of osteoblast-like cells in vitro and to promote osteoblast differentiation by stimulating the expression of major phenotypic markers. Taken together, our findings will be helpful in understanding the mechanism of osteoinductivity of calcium phosphate ceramics and in constructing more appropriate biomimetic substrate.

Introduction

Calcium phosphate ceramics have been found osteoinductivity via ectopic bone formation for decades [1–3]. It is well known that bioactive materials form a bone-like apatite layer on their surfaces in the living body, and bond to bone through a apatite layer [4, 5] and the apatite coating can reduce fibrous tissue encapsulation [6], promote bone ingrowth [7], enhance direct bone contact [7–9], and has also been shown to promote differentiaiton of bone marrow stromal cells along osteogenic lineage [10]. In order to enhance the bone-bonding ability of non-bioactive materials, in vitro biomimetic apatite coating methods involving the immersion of material substrates in simulated body fluids (SBF) at physiological temperatures have been developed by Kokubo et al [11–13]. The formation of bone-like apatite has been regarded as a vital factor affecting bone formation in vivo [14], Therefore, investigation of bone-like apatite on osteoblasts gene expression in vitro will be helpful in revealing the mechanism of osteoindcutivity of Calcium phosphate ceramics.

In this paper, the influence of bone-like apatite on mRNA expression of bone-related gene in MC3T3-E1 preosteoblastic cells, such as osteocalcin, osteopontin and type 1 collagen, was investigated.

Materials and methods

Materials

Biphasic porous calcium phosphate ceramics (HA/α-TCP = 60/40) were prepared by foaming with H_2O_2, and sintered at 1,100 and 1,200 °C (named HT1 and HT2 respectively). Then the plate-shaped ceramics ($\phi 2 \times 12$ mm) were fabricated and cleaned by ultrasonication. Formation of apatite on the ceramics were performed by immersed the samples in SBF solution at a temperature of 37 °C for 3 days (HT1S and HT2S respectively). Shape of the apatite and

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microstructure on the samples were observed by SEM (Fig. 1). The four samples were ultrasonic washed and autoclaved before seeded with cells.

Cell culture

MC3T3-E1 preosteoblastic cells were cultured in regular culture media consisting of Dulbecco's Modified Eagle Medium (DMEM, High Glucose; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; TBS Corp., China) and antibiotics in a humidified atmosphere of 95% air and 5% CO^2 at 37 °C. In the experiments, MC3T3-E1 cells were trypsinized and plated onto the four kinds of Ca/P ceramics at a density of 2×10^4 cells/cm^2 (24-well plate, Corning, PA), before they were harvested at different time point, Culture media were changed every three days (Fig. 2).

Cell morphology

MC3T3-E1 cells were seeded on different flake-shape ceramics in 24-well plate at a concentration of 2×10^4 cells/well. For SEM, after cultured for 48 h, samples were rinsed with PBS and fixed for 1 h with 2.5% glutaraldehyde in phosphate buffer saline (PBS, pH = 7.2). The cultures were subsequently rinsed and progressively dehydrated with alcohol. They were processed for critical point drying in CO2 and then coated with gold. Samples were examined under scanning electron microscopy.

RNA extraction and RT-PCR

MC3T3-E1 cells were seeded on various ceramics in 24-well plate at a concentration of 1×10^5 cells/well. Osteocalcin, type 1 collagen and osteopontin, which were looked as matured osteoblast cell markers and indicated cell differentiation, were determined by semi-quantitative RT-PCR method after cultured for 6 days (Fig. 3). Total RNA was isolated using Trizol regent (Invitrogen). RNase-free DNaseI was used to eliminate genomic DNA contamination in RNA samples. Purified RNA was stored at –20 °C in 0.5 ml ethanol before use.

Complementary DNA (cDNA) was synthesized from 1 μg of total RNA with a 60 min incubation at 37 °C using M-MLV reverse transcriptase (Gibco BRL) and oligo-(dT_{18}) primer in 20 μL of reaction system.

Amplification was performed in a gradient thermocycler (Eppendorf, Germany) using Taq-plus DNA polymerase (Takara corp., China) with the primers shown in Table 1. To perform a semi-quantitative analysis of samples, a series of PCR cycles were subjected to evaluate the linear amplification range for each primer set (Fig. 4). PCR program was carried out at 94 °C for 3 min followed with 35 cycles at 94 °C for 60 s, 56 °C for 60 s and 72 °C for 60 s, then extended at 72 °C for 10 min. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) gene was participated in RT-PCR reaction as an internal reference. All RT-PCR experiments repeated 3 times. RT-PCR products were resolved on a 1.0% agarose gel stained with ethidium bromide. Primer and PCR product size of every gene was given in Table 1.

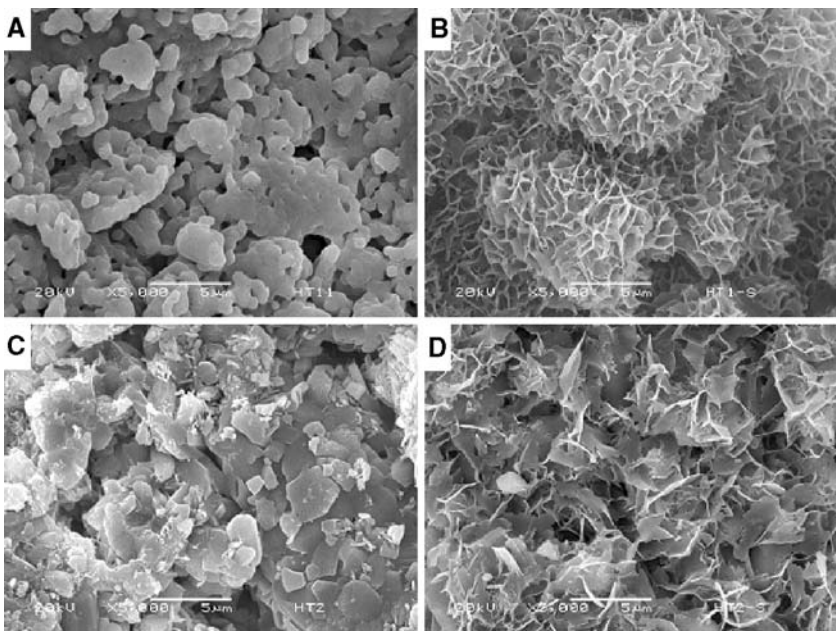

Fig. 1 Surface SEM morphology of HA/TCP ceramics before and after immersion in SBF for 3 days (**A**. HT1; **B**. HT1S; **C**. HT2; **D**. HT2S)

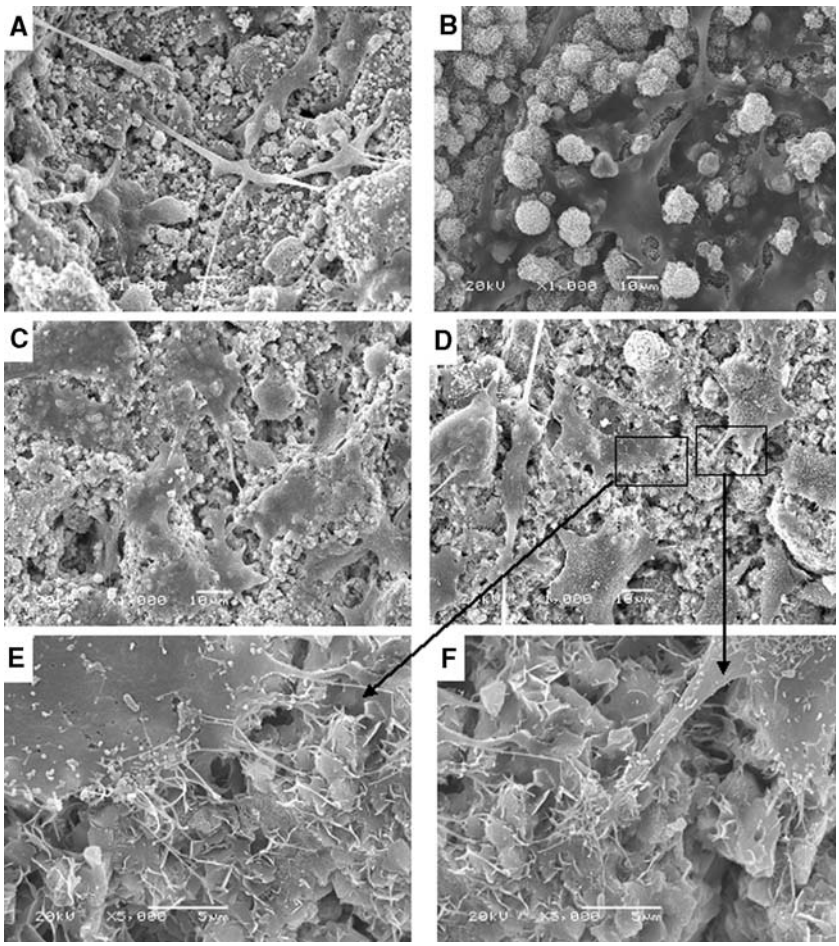

Fig. 2 Typical cell morphology was observed after cultured on the surface of HT1 (**A**), HT1S (**B**), HT2 (**C**) and HT2S (**D**) for 3 days; cell had been spreading well on the 4 samples, and crystals were found to secret by those cells with round-like shape growth on bone-like apatite of HT2S (**E**, **F**)

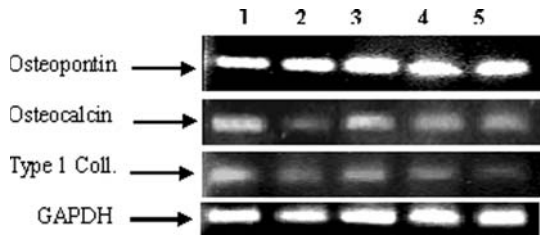

Fig. 3 RT-PCR analysis for gene expression of Osteopontin, Osteocalcin and type I collagen in 3T3-E1 preosteoblast cells cultured for 6 days on different samples (1. HT1S; 2. HT1; 3. HT2S; 4. HT2; 5. negative control). PCR products were visualized in ethidium bromide stained gels and normalized by GAPDH gene, quantitative expression was analyzed with Quantity One software (version: 4.4.0, Bio-Rad)

Results and discussion

Under SEM, the bone-like apatite had been deposited on the surface of the two kinds of ceramics, and the shape of the apatite was plate-like. The MC3T3-E1 preosteoblast cells were adhered and grown on the surface of the HA/TCP ceramics by culture 3 and 6 days. The apatite layer on the surface of the ceramics helped spreading and promoted the extension of cellular projections along the tough surfaces. Some crystals were found on the cell membrane secreted by the cells with round-like shape growth on bone-like apatite of HT2S. These crystals were speculated by its shape and position on the cell membrane, were hydroxyapatite crystals. In the complex mature process of osteoblast in the

Table 1 Primers utilized for RT-PCR amplification

Target	forward primer	Reverse primer	product size	GenBank accession no.
OCN	TCTGACAAAGCCTTCATGTC	AAATAGTGATACCGTAGATG	198	L24431
COL1	TCTCCACTCTTCTAGTTCCT	TTGGGTCATTTCCACATGC	268	NM_007743
OPN	ACACTTTCACTCCAATCGTC	TGCCCTTTCCGTTGTTGTCC	240	NM_009263
GAPDH	ACTTTGTCAAGCTCATTTCC	TGCAGCGAACTTTATTGATG	267	NM_001001303

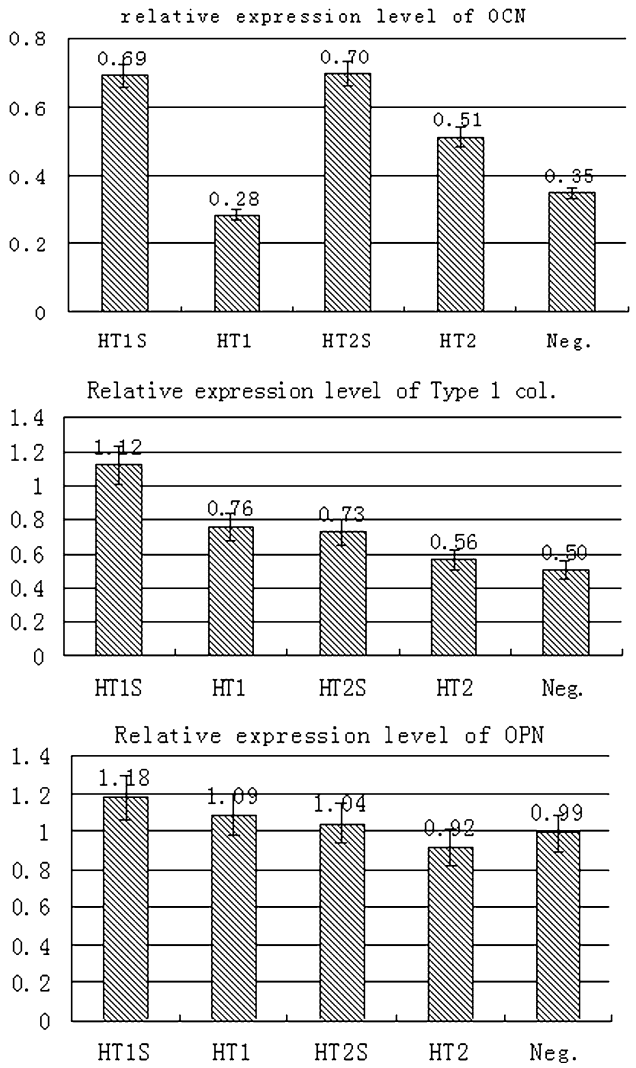

Fig. 4 Semi-quantitative RT-PCR results of osteocalcin, Type 1 collagen and osteopontin gene expression. 3T3-E1 preosteoblast cells were cultured on different samples for 6 days. The *y*-axis of the figures was the gene expression results normalized by GAPDH

extracellular matrix, the appearance of the hydroxyapatite was a major mature feature [15]; the observations proved that these cells were differentiated towards osteocytes and were very similar to in vivo bone formation during intramembranous ossification with a direct bone apposition on the ceramics.

After 6 days culture, the osteocalcin mRNA expression of MC3T3-E1cells grown on HT1S surface was 0.69 fold normalized by GAPDH gene and was similar to those cell cultured on HT2S (0.70 fold), distinct higher than that of cells grown on HT1 (0.28 fold) and HT2 (0.51 fold). Type 1 collagen mRNA expression was highest in HT1S, next in HT2S, followed by HT1 and HT2. These results suggested that bone-like apatite on its surface of biophasic ceramics were more effective to promote osteoblast differentiation with high expression of bone special markers. Osteopontin mRNA expressions were similar in all the four samples; it indicated that the difference in apatite layer appeared not to be an important factor in the regulation of osteopontin within MC3T3-E1cells.

In osteogenesis, bone-related genes are strictly regulated to ensure correct temporo-spatial expression. The temporo-spatial expression of each gene has a unique expression profile which lead to either cell differentiation and osteogenesis or cell growth and proliferation. Surface topography and chemistry can affect these gene expression and lead to different cell response. Yu-Fen Chou et al found that an apatite coating on poly (lactide-co-glycolide) (PLGA) scaffold could upregulate expression of osteocalcin and bone sialoprotein at 4 weeks [16]. They also had compared five types of biomimetic apatite on osteoblast gene expression, and found that the large plate-like apatite induced highest expression of mature osteogenic markers osteocalcin according to quantitative real-time RT-PCR [17]. Calcium phosphate ceramics without addition of any bone growth factor or living cell could induce bone formation [3, 18, 19], the necessary material factors for the osteoindcution maybe: 3-D porous structure and the formation of bone-like apatite surface/interface specific characteristics [20] Taken together, our results confirmed the affect of bone like apatite on gene expression in MC3T3-E1cells and would be helpful in understanding the mechanism of osteoinductivity of calcium phosphate ceramics and in constructing more appropriate biomimetic substrate.

Conclusions

In this work, bone-like apatite had formed on the surface of HA/TCP ceramics sintered at 1,100 and 1,200 °C, after immersed into SBF for 3 days. Bone-like apatite enhanced mRNA expression of osteocalcin and type 1 collagen gene in MC3T3-E1 preosteoblast cells after cultured for 6 days. The results suggested these cells had been induced to differentiation toward mature osteoblast. Expression of osteopontin was not affected by bone-like apatite. Deposition of a biomimetic apatite layer throughout the porous structure of ceramics represents a practical method of controlling surface topography and chemistry property. Biomimetic apatite layer can accelerate osteoblast to mature and differentiate. In vitro data demonstrate that calcium phosphate ceramics modified with precipitated apatite were more suitable for osteoblast adhesion and differentiation and could provide more appropriate substrates for repairing bone fractures such as non-unions and cavitational defects.

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