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Effects of low crystalline carbonate apatite on proliferation and osteoblastic differentiation of human bone marrow cells

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Abstract Carbonated apatite (CO_3Ap) is the inorganic component of bone. We have proposed a new method for the fabrication of CO_3Ap blocks based on a dissolution-precipitation method using a synthetic precursor. The aim of this study is to examine the effects of low crystalline CO_3Ap on initial cell attachment, proliferation and osteoblastic differentiation of human bone marrow cells (hBMCs) using sintered hydroxyapatite and tissue culture plates as controls. Initial cell attachment and proliferation were assessed with a MTT assay. Expression of osteoblastic markers was examined by reverse transcription-polymerase chain reaction. XRD and FT-IR results showed formation of B-type carbonate apatite with lower crystallinity. No difference was observed for initial cell attachment between HAp and CO_3Ap discs. hBMSC attached more significantly on tissue culture plate than on HAp and CO_3Ap discs. The number of cells on HAp was higher than that on CO_3Ap until day 7, after which the number of cells was similar. hBMSC proliferated more significantly on tissue culture plate than on HAp and CO_3Ap discs. In contrast, hBMCs incubated on CO_3Ap demonstrated much higher expression of osteoblastic markers of differentiation, such as type I collagen, alkaline phosphatase, osteopontin and osteocalcin, than hBMCs on HAp. On the tissue culture plate, they were not any change throughout the culture period. These results demonstrated that low crystalline CO_3Ap exhibit higher osteoinductivity than HAp.

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1 Introduction

In general, autogenous bone grafting is regarded as an initial choice for bone defects because the graft is already supplied with osteogenic cells, osteoinductive growth factors, and an osteoconductive scaffold [1]. However, damage to and morbidity of the donor site are inevitable, and the available volume and shape of bone is limited. Additionally, reconstruction of large bony defects with autogenous bone grafting is impossible [2]. Allogenous bone grafting is currently the second alternative strategy. However, infections, immunogenic rejection and risk of disease transmission remain unsolved problems with allogenous bone grafting [3].

Currently, many types of synthetic bone substitutes such as calcium phosphate ceramics, collagen, bioactive glasses, and biodegradable polymers have been used in clinics or studied for the treatment of bone defects [4–11]. Among these materials, calcium phosphate ceramics, especially hydroxyapatite (HAp), have been widely used for the reconstruction of lost bone tissue in orthopaedic surgery and maxillofacial surgery [12, 13]. Although HAp shows excellent biocompatibility and good osteoconductivity, the key drawback of HAp is its stability in the bone as foreign substance. In other words, HAp is difficult to be resorbed in the bone defect, and as a result HAp introduces a risk for

secondary infection. Therefore, biodegradable osteoconductive materials have been the focus of recent research [14].

It should be noted that the apatite found in human bone is not stoichiometric HAp, $Ca_{10}(PO_4)_6(OH)_2$ but carbonate apatite (CO_3Ap; $Ca_{10-a}(PO_4)_{6-b}(CO_3)_c(OH)_{2-d}$) that contains 6–8 weight % carbonate in its apatitic structure [15]. Because CO_3Ap is known to be resorbed by osteoclasts [15, 16], it has received considerable attention as a bone replacement for the treatment of bony defects. For clinical applications, CO_3Ap blocks must be made because apatitic powder is known to cause inflammatory response even though bulk apatite shows excellent tissue response [17].

Generally, apatite blocks are prepared by sintering at high temperature. However, thermal decomposition occurs and carbon dioxide (CO_2) is liberated when CO_3Ap powder is sintered [18, 19]. Additionally, the crystallinity of the apatite becomes high during the sintering process. We have previously proposed a new method to fabricate low crystalline, porous CO_3Ap blocks based on a dissolution-precipitation reaction using a synthetic precursor [20–23]. This method consisted of two processes. First, a precursor block is fabricated. For example, calcium hydroxide compact was exposed to CO_2 to fabricate a calcite block, which is used as a precursor [24]. The calcite block thus prepared cannot be washed out even when the block is immersed in an aqueous solution. Next, the precursor block was immersed in a phosphate salt solution. As CO_3Ap is thermodynamically more stable than calcite in aqueous phosphate salt solutions at neutral and alkaline pH, the calcite block is transformed into a low crystalline CO_3Ap block while retaining its macroscopic structure based on a dissolution-precipitation reaction. CO_3Ap thus prepared is thought to potentially have better osteoinductivity than sintered Hap because CO_3Ap is closer in chemical composition to bone mineral.

In this study, we isolated human bone marrow cells (hBMCs) from a patient and examined the effects of low crystalline CO_3Ap on initial cell attachment, proliferation, and osteoblastic differentiation of hBMCs.

2 Materials and methods

2.1 Preparation of CO_3Ap disc

CO_3Ap discs were prepared by the dissolution-precipitation reaction using calcite blocks as precursors as reported previously [20–24]. In short, commercially obtained calcium hydroxide ($Ca(OH)_2$, Nacalai tesque, Kyoto, Japan) compact (10 mm in diameter, 2 mm in thickness) prepared using a uniaxial press at 5 MPa was exposed to CO_2 at room temperature for 3 days. The calcite disc thus prepared cannot be washed out even when immersed in a liquid phase and so was immersed in 1 mol/L disodium hydrogenphosphate (Na_2HPO_4, Nacalai tesque) solution at 60 °C for 14 days, allowing for a compositional transformation based on the dissolution-precipitation reaction from calcite to CO_3Ap.

The composition and crystallinity of CO_3Ap was evaluated by means of powder X-ray diffraction (XRD) and Fourier transform infrared spectrometry (FT-IR). XRD patterns of the specimens were recorded with a vertically mounted diffractometer system (RINT 2500V, Rigaku Co., Tokyo, Japan) using counter-monochromatised CuK_α radiation generated at 40 kV and 100 mA. Specimens were scanned from 10° to 60° 2θ in continuous mode at a scanning rate of 2°/min. FT-IR spectra were measured with a KBr disc method using the FT-IR spectrometer (SPECTRUM 2000LX, Perkin Elmer Co., Ltd., Kanagawa, Japan).

2.2 Isolation of human bone marrow cells

hBMCs were isolated from a patient (45-year-old woman) who had given her informed consent undergoing mandibular reconstructive surgery using a modification of previously reported methods [25]. A bone marrow fragment obtained from an iliac bone was washed with phosphate-buffered saline (PBS) to remove blood and plated onto a 60-mm tissue culture dish coated with type I collagen (COL) (Iwaki glass, Tokyo, Japan). The dish was incubated in a humidified atmosphere of 5 % CO_2, 95 % air at 37 °C in culture medium consisting of alpha-modified minimum essential medium (alpha-MEM, Sigma, St. Louis, MO, USA) supplemented with 10 % foetal bovine serum (FBS, Cambrex Bio Science, Walkersville, MD, USA), penicillin (100 units/mL, Invitrogen, Grand Island, NY, USA), streptomycin (100 μg/mL, Invitrogen) and amphotericin B (0.25 μg/mL, Invitrogen) with medium changes every 2 days. After 2 weeks in culture, cells grown from the explants had reached semi-confluence, and the cells were trypsinised and subcultured in culture medium. Cells from the fourth to sixth passages were used for the studies, because a decrease in their osteoblastic differentiation capability of bone marrow stromal cells has been shown to occur with the passage increase.

2.3 Initial cell attachment and cell proliferation

Initial cell attachment and cell proliferation were assessed. In short, each disc was placed on a tissue culture plate (48-well cell-culture cluster, Corning, New York, NY, USA), and 400 μL of culture medium was poured into each well. Finally, hBMCs were seeded on each disc at a density of 2×10^4 cells/cm^2 and incubated in a humidified

atmosphere of 5 % CO_2, 95 % air at 37 °C. Cell viability was measured by the MTT assay at each culturing period as described previously [8]. The values of absorbance from MTT assays were indirectly related to cell number. Initial cell attachment was assessed after 8 h of incubation, and cell proliferation was assessed at 1, 3, 5, 7 and 10 days. To minimise the number of the cells that attached to the side of bottom of the well as well as the under-surface of the disc, cell suspensions of hBMCs were gently added to each well, and the disc was replaced in a new culture well at MTT assays [8]. At each culturing period, 200 μL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Dojin Chemical, Kumamoto, Japan) reagent was added to each sample and incubated at 37 °C for 4 h. The formazan product was solubilised in 200 μL of dimethylsulfoxide (DMSO, Nacalai tesque), and absorbances were measured on a microplate reader (MULTISKAN JX, Thermo Electron, Waltham, MA, USA, reference wavelength: 650 nm; test wavelength: 570 nm). Background absorbance produced by wells containing no cells was subtracted from all samples. Sintered HAp discs and tissue culture plates were used as controls. The experiment was repeated three times (n = 9), and data from one representative experiment are presented. The error bars represent the standard deviation of the measurements.

2.4 Osteoblastic differentiation

To evaluate osteoblastic differentiation of hBMCs, we measured mRNA expression levels of type I COL, alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OCN). hBMCs were plated at a density of 1×10^5 cells/cm^2 on the CO_3Ap and HAp discs. At each culturing period (7, 14, 21 days), each disc was moved into a new tissue culture plate to extract RNA from cells attached to the disc, and total RNA was prepared from cells using ISOGEN (Nippon Gene, Osaka, Japan) according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using the Superscript™ One-Step RT-PCR System (Invitrogen). The primers used in this study are summarised in Table 1. PCR products were subjected to 1.2 % agarose gel electrophoresis and visualised by staining with ethidium bromide. mRNA expression of osteoblastic markers was analysed and normalised to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results are representative of three identical RT-PCR experiments.

2.5 Statistical analysis

Data are expressed as the mean values ± standard deviation (mean ± SD). Statistical analysis was carried out by Students' t test, and significance was considered at $p < 0.05$.

Table 1 Primer sequence used for RT-PCR analysis

Gene	Primer sequence	Product size (bp)
COL		
F	atccgcagtggcctcctaat	414
R	tcccctcaccctcccagtat	
ALP		
F	acgtggctaagaatgtcatc	476
R	ctggtaggtgatgtcctta	
OPN		
F	catctcagaagcagaatctcc	313
R	ccataaaccacactatcacctc	
OCN		
F	catgagagccctcaca	315
R	agagcgacaccctagac	
GAPDH		
F	ccacccatggcaaattccatggca	598
R	tctagagggcaggtcaggtccacc	

3 Results

3.1 CO_3Ap disc

Figure 1 summarises the XRD patterns of the sintered HAp discs and CO_3Ap discs used in this study. XRD patterns of $Ca(OH)_2$ and $CaCO_3$ are shown for comparison. The XRD peaks of the sintered HAp disc showed a typical apatitic pattern with no identifiable peaks that is detected in the 32°–35° (2θ) region. For comparison, CO_3Ap also showed the same XRD patterns as those of HAp. The spectra

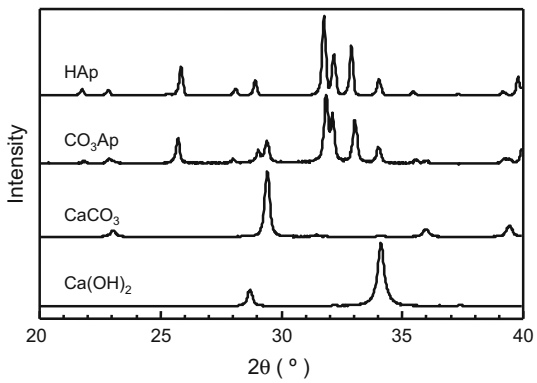

Fig. 1 XRD patterns of CO_3Ap. The XRD patterns of the powder phase of final product showed slightly broader peaks than HAP approximately 32° 2θ. The final product consists of an apatite structure with low crystallinity

obtained for the CO_3Ap had a shape that is a typical apatitic pattern with lower crystallinity when compared to sintered HAp. Although no peaks corresponding to $Ca(OH)_2$ were observed, a small peak corresponding to $CaCO_3$ was observed.

Figure 2 summarises the FT-IR spectra of sintered HAp discs and CO_3Ap discs used in this study. Sintered HAp showed a typical FT-IR spectra. CO_3Ap also showed an apatitic FT-IR spectra. It should be noted that CO_3 peaks were observed in addition to apatitic peaks in the case of CO_3Ap. Peaks observed at 1,410 and 1,450 cm^{-1} indicated B-type carbonate apatite, in which CO_3 is replaced with PO_4 and the CO_3Ap found in bone apatite. The hydroxyl groups found in sintered HAp were not detected in CO_3Ap.

3.2 Initial cell attachment

Figure 3 shows the cell viability of hBMCs initially attached on the CO_3Ap disc, HAp disc and tissue culture plate after incubation for 8 h. No significant difference was observed between HAp and CO_3Ap discs.

3.3 Cell proliferation

Figure 3 summarises the cell viability of hBMCs on the CO_3Ap disc, HAp disc and tissue culture plate at 1, 3, 5, 7 and 10 days. The viability of cells on the CO_3Ap and HAp discs gradually increased throughout the culture period except for day 1. The decrease in cell viability was observed at day 1, because all cells seeded to each well cannot be attached on the discs and some cells were attached to the side of bottom of the well. The number of cells on the HAp disc was greater when compared to the CO_3Ap disc at days 3, 5 and 7. No significant difference was observed at days 1 and 10.

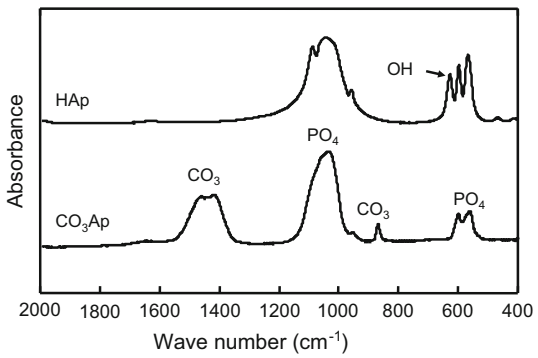

Fig. 2 FTIR spectra of CO_3Ap. The spectra revealed broad doublet absorbance peaks approximately 1,450 and 1,410 cm^{-1}, and a peak at 875 cm^{-1} was clearly observed. The spectra of the HAp disc is shown for comparison. The concentration of carbonate in CO_3Ap, calculated from spectra recorded in the linear absorption mode, was approximately 15.5 wt%

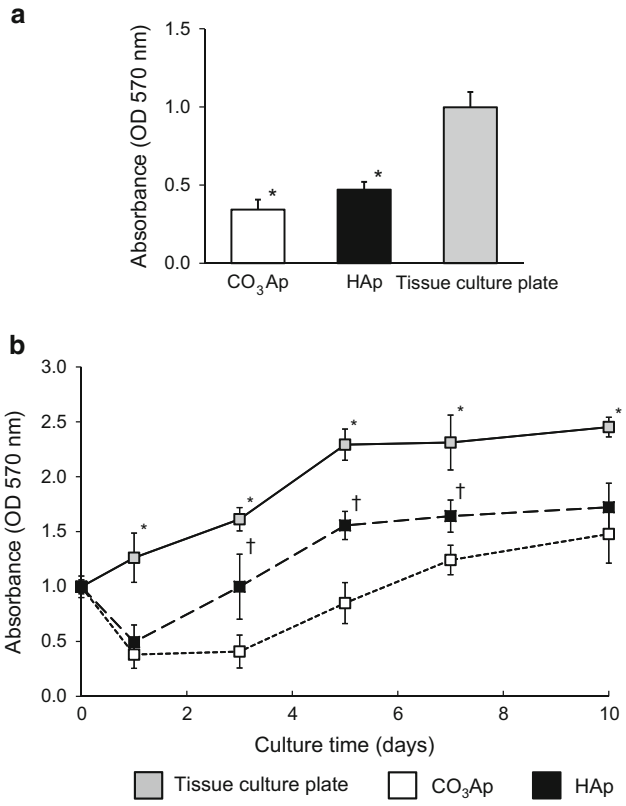

Fig. 3 Initial cell attachment and proliferation of hBMCs. **a** hBMCs were directly seeded at a density of 2×10^4 cells/cm^2 on the CO_3Ap disc (*open square*), HAp disc (*filled quare*) and tissue culture plate (*grey quare*) and incubated for 8 h. Cell attachment was assessed with a MTT assay. *Asterisk* indicates a significant difference compared to the tissue culture plate. The experiment was repeated three times (n = 9), and data from one representative experiment are presented. **b** The number of cells proliferated on the CO_3Ap disc (*open circle*), HAp disc (*filled circle*) and tissue culture plate (*grey circle*) for 1, 3, 5, 7 and 10 days were examined with a MTT assay. hBMCs were seeded at a density of 2×10^4 cells/cm^2. The number of cells on the CO_3Ap and HAp discs gradually increased throughout the culture period. *Asterisk* indicates a significant difference compared CO_3Ap disc and HAp disc. *Dagger* indicates a significant difference compared CO_3Ap disc. The experiment was repeated three times (n = 9), and data from one representative experiment are presented

3.4 Osteoblastic differentiation

Expression of osteoblastic marker genes such as COL, ALP, OPN and OCN in hBMCs cultured on the CO_3Ap disc, HAp disc and tissue culture plate for 7, 14 and 21 days was analysed semi-quantitatively using RT-PCR as shown in Fig. 4a. Data obtained from densitometric analysis of ethidium bromide-stained agarose gels were normalised to the corresponding GAPDH level and are shown in Fig. 4b as the relative expression level.

hBMCs on the CO_3Ap disc exhibited significantly higher mRNA levels for all osteoblastic markers, including COL, ALP, OPN and OCN, compared to hBMCs on the HAp disc,

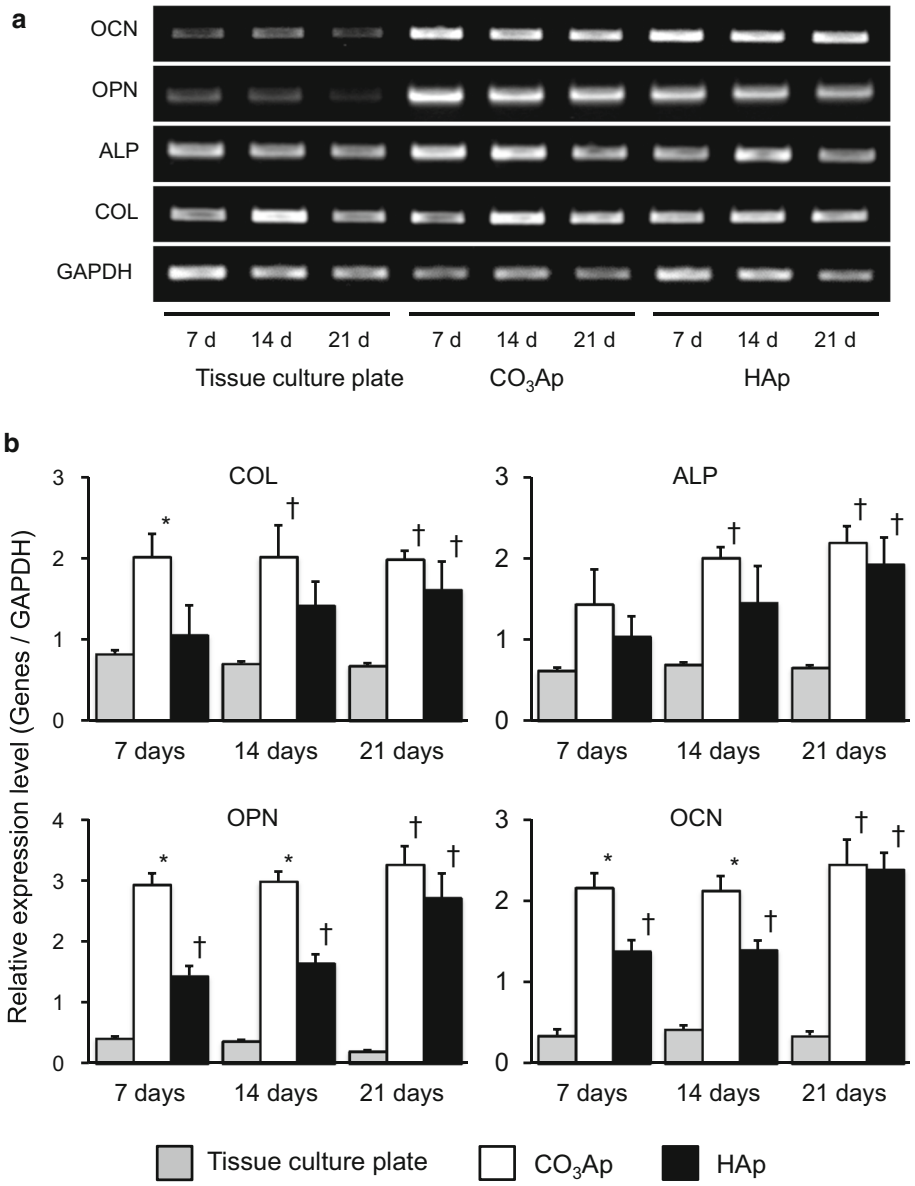

Fig. 4 Expression of osteoblast marker genes. **a** mRNA levels of type I collagen (COL), alkaline phosphatase ALP, osteopontin (OPN) and osteocalcin (OCN) were assessed in hBMCs cultured on the CO_3Ap disc, HAp disc and tissue culture plate for 7, 14 and 21 days. **b** Relative mRNA expression levels normalised to GAPDH level. The mRNA expressions of osteoblastic markers, such as ALP, COL, OPN and OCN, in the cells on the the CO_3Ap disc (*open square*), HAp disc (*filled quare*), and tissue culture plate (*grey quare*) were assessed using RT-PCR. *Asterisk* indicates a significant difference compared HAp and tissue culture plate. *Dagger* indicates a significant difference as compared tissue culture plate. The results are representative of three identical RT-PCR experiments

except for the day 7 ALP value, which showed no significant difference. The expression of mature osteoblastic markers, such as OPN and OCN, were high. However, differences in expression between the CO_3Ap and HAp discs disappeared gradually, and there were no differences at day 21. On the tissue culture plate, the expression of osteoblastic markers in culture plastic is much lower than in the ceramic samples, and their expression levels were unchanged throughout the culture period.

4 Discussion

Bone marrow contains osteoprogenitor cells originating from mesenchymal stem or progenitor cells that have the potential to differentiate into several mesenchymal lineages including bone, cartilage, muscle, tendon and fat [26]. Cells isolated from bone marrow provide an excellent source of proliferative osteoprogenitor cells that can be induced to differentiate into mature osteoblasts [27, 28]. Therefore, bone marrow cells have been widely employed as a suitable in vitro model for the evaluation of interactions between osteoblastic cells and biomaterials used in orthopaedic and maxillofacial surgeries [11, 23, 25, 27]. It should be noted that, when bone substitution materials were used to fill in bone defects, a fibrin blood clot has formed around materials and releases growth factors and biological signals to recruit the bone marrow cells onto the surface of the biomaterial. Therefore, the behaviour of bone marrow cells on the surface of bone substitution

materials is important for the reconstruction of bone defects. Thus, in this study, we isolated human bone marrow cells from a patient and examined the effects of low crystalline CO_3Ap on their initial cell attachment, cell proliferation and osteoblastic differentiation.

Generally, new bone formation, induced by osteoblastic cells, is characterised by a sequence of events involving recruitment of osteoprogenitor cells, attachment, proliferation, and differentiation into mature osteoblasts [28, 29]. With respect to the initial cell attachment of hBMCs, no significant difference was observed between CO_3Ap and HAp (Fig. 3). However, both CO_3Ap and HAp show a lower initial cell attachment compared to tissue culture plates (Fig. 3). Obviously, tissue culture plates do not show osteoinductivity. Therefore, the relationship between initial cell attachment and osteoinductivity may be limited.

Similarly, proliferation of hBMCs on the surface of CO_3Ap and HAp was lower than that on tissue culture plates (Fig. 3). In this case, CO_3Ap showed a lower value even when compared to HAp for day 3 to day 7. However, at day 10, the number of cells was similar (Fig. 3). The relationship between proliferation and differentiation of osteoprogenitor cells is controversial. Regarding this relationship, Stein GS et al. have demonstrated that, when cell proliferation is inhibited in mouse osteoblast-like cells, expression of a specific subset of osteoblast phenotype markers was increased [30]. Owen et al. have also demonstrated that decreased cell proliferation is related to the progression of osteoblast differentiation in rabbit bone marrow cells [31]. Additionally, Alliot-Licht et al. have shown reduced cell proliferation and differentiation in mouse osteoblast-like cells following apatite release from HAp [32]. Ozawa and Kasugai have also demonstrated that rat bone marrow cells, when cultured on HAp, show osteoblastic differentiation when compared to tissue culture plates regardless of cell proliferation [33]. Although it is well known that cell proliferation is required for new bone formation [30–32], decreased cell proliferation may be related to an osteoinductivity issue in this case.

The results obtained in the present study demonstrate clearly that CO_3Ap and HAp upregulate osteoblastic differentiation of hBMCs when compared to tissue culture plates. It should be noted that the relative expression levels of hBMCs genes incubated on CO_3Ap and HAp were higher than that on tissue culture plates for all differentiation makers, such as type I COL, ALP, OPN and OCN regardless of incubation time as shown in Fig. 4. The results for sintered HAp are consistent with previously reported results reported [4–8]. In addition, the results obtained in this study and previous studies seem reasonable because sintered HAp demonstrates osteoinductivity whereas tissue culture plates show no osteoinductivity [6, 11].

A noteworthy point in the present study was that the relative expression level of osteoblastic differentiation marker genes in hBMCs incubated on CO_3Ap was higher than that on HAp (Fig. 4). In this study, we examined the expression of type I COL, ALP, OPN and OCN to evaluate the osteoblastic differentiation of hBMCs. Type I COL is the most abundant bone matrix protein and is expressed in immature and mature osteoblasts. ALP is involved in bone mineralisation by providing free calcium and phosphate for HAp crystal growth. Generally, type I COL and ALP are thought to be early markers of osteogenesis [28, 29]. On the other hand, OPN and osteocalcin, which are major noncollagenous protein components of bone extracellular matrix, are synthesised and secreted by differentiated osteoblastic cells. These two proteins are thought to be late-stage markers of bone formation and bone mineralisation [28, 29]. In this case, expression levels of all these genes in hBMCs incubated on CO_3Ap were higher than that on HAp, suggesting that CO_3Ap has a better ability to promote osteoblastic differentiation of hBMCs compared to HAp.

We found very interesting behaviour with respect to expression of OPN and osteocalcin, which are late stage markers of osteogenesis, in hBMCs. It should be noted that the expression levels of OPN and OCN in hBMCs cultured on CO_3Ap were higher compared to HAp after 7 days of culture. Definite sequential expression of osteoblast differentiation marker genes was identified at three distinct periods of osteoblast phenotype development: proliferation, maturation and extra-cellular matrix synthesis, and matrix mineralisation [28, 29]. OPN and OCN are bone-synthesised proteins known to be associated with extracellular matrix mineralisation. OPN is expressed during both the active proliferation period and the mineralisation period, and OPN expression reaches a maximum during the mineralisation period [34]. On the other hand, OCN is expressed by mature osteoblasts only in the post-proliferative period [35] and is maximally expressed during the mineralisation period [36]. Several studies suggest that OPN and OCN are involved in the regulation of mineral deposition and are a marker of mature osteoblasts [37, 38]. The results in the present study clearly demonstrated that the expression levels of both OPN and OCN in hBMCs cultured on CO_3Ap were higher than that on HAp at 7 days culture, suggesting that hBMCs cultured on CO_3Ap differentiated into a mature osteoblast mineralised bone extracellular matrix. In addition, considering that CO_3Ap showed a lower cell proliferation rate when compared to HAp up to day 7, CO_3Ap promotes osteoblastic differentiation of hBMCs earlier than HAp. It appears that there is a good possibility that our low crystalline CO_3Ap shows a higher osteoinductivity than sintered HAp. The results have been obtained with the sample of a single patient. Further studies using samples from different patients are needed.

5 Conclusions

In conclusion, we found that our low crystalline CO_3Ap had an ability to promote osteoblastic differentiation of human bone marrow cells earlier than sintered HAp. Taking into account the fact that CO_3Ap has osteoinductivity and can be resorbed in vivo, CO_3Ap may be a superior candidate as a bone substitution material and scaffold for bone regeneration compared to sintered HAp.

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