ORIGINAL ARTICLE

Effects of Addition of Nano-hydroxyapatite to Highly-pressed Collagen on Osteogenic Differentiation in Osteoblastic SaOS-2 Cells

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Synopsis
We produced highly pressed nano-hydroxyapatite/collagen composites (P-nHAP/COL) by Newton press. The aim of this study was to investigate osteogenic differentiation profiles in osteoblastic SaOS-2 cells cultured on P-nHAP/COL and pressed collagen (P-COL) as control for 1, 2, 3 and 4 weeks. Total RNA were extracted from cultured cells, and expressions of osteogenic-differentiation related genes were evaluated with quantitative real-time (RT) PCR using primer sets of alkaline phosphatase (ALP), type 1 collagen (COL1), bone Sialo protein (BSP) and osteocalcin precursor (BGLAP) genes as well as β-actin gene. The cultured cells were also observed with scanning electron microscopy (SEM). RT-PCR analyses revealed that osteogenic differentiation was more accelerated on P-nHAP/COL than on P-COL, especially revealed by up-regulation with time of BGLAP gene. In SEM images, SaOS-2 cells on P-COL were either cubic or fibroblastic up to 4 weeks, but those on P-nHAP/COL appeared as granular or highly stretched. The obtained results suggested that P-nHAP/COL could be employed as a new osteo-conductive bone substitute material.

Key words: *Osteoblastic SaOS-2 cells, Nano-hydroxyapatite, Collagen composite, Newton press, Osteogenic differentiation*

Introduction

Hydroxyapatite/collagen (HAP/Col) composites have often been utilized as bone substitute materials in dentistry and orthopedic surgery for re-generation of damaged hard tissues [1]. HAP has the capability of excellent osteo-conduction and slow bio-degradation [2] while Col is the bio-absorbable scaffold to bind HAP [3].

We have already produced soft and porous nano hydroxyl-apatite/collagen composites (nHAP/COL), and reported that nHAP/COL sponges accelerated osteogenic differentiation of SaOS-2 cells [4] and facilitated active bone-formation in rat calvarial bone defects with respect to COL sponges up to 4 weeks [5]. Over 4 weeks up to 8 weeks, however, *in vivo* new bone formation diminished and disappeared, largely due to bio-absorption of nHAP particles from nHAP/COL sponges [5]. The breakthrough was, thus, ex0pected to render nHAP/COL

composites to have more prolonged osteo-conductive effect. It was considered that condensation by Newton press of the composite [6], which can highly embed nHAP particles to COL, might correspond to this request. There are, however, few systematic studies to evaluate osteo-conductive capability of P-nHAP/COL [6].

To achieve osteo-re-generation after implantation of biomaterials in bone defect area, it is required that osteoblasts adhere to and differentiate into a final matured stage in contact with materials [7] It has been known that osteoblasts differentiate following a characteristic step-wise sequence while secreting respective phenotype marker enzymes/proteins in multi-lineage differentiation (*i.e*. early stage ALP and COL1; and middle-to-late-stage BSP and BGLAP) [8]. Quantitative RT-PCR is a sophisticated technique to evaluate expressions of genes (mRNAs) which produce marker enzyme/proteins of osteoblasts cultured on implant materials [9].

The purpose of this investigation was, therefore, to evaluate expressions of four selected osteogenic-differentiation-related (*i.e*. ALP, COL1, BSP and BGLAP) genes in osteoblasts-like cells (SaOS-2) cultured on P-nHAP/COL and P-COL, using RT-PCR machine so that effects of the addition of nHAP to P-COL on osteogenic differentiation in osteoblastic cells could be better understood. Furthermore, SaOS-2 cells cultured on two test materials (P-nHAP/COL and P-COL) were observed with SEM so that cell morphological change during osteogenic differentiation of SaOS-2 cells could be clarified. This *in vitro* cell culture study was a first screening test prior to succeeding *in vivo* animal test.

Materials and methods

1. Preparation of P-nHAP/Col composites

Preparation of n-HAP/Col sponges is first briefly described, as follows [5]. Collagen pellets (NMP collagen PS, Nippon Meat Packers Inc., Tokyo, Japan) (1 g) were dissolved in distilled water (28 mL) in 50 mL polystyrene conical tube at 4°C. The produced acidic sol was neutralized by 0.1N NaOH solution (6.5 mL) in disposable rectangular plastic plate (100 × 70 × 12 mm) so that collagen gel of pH = 7.5 could be obtained. The n-HAP powder with average diameter of 40 nm (MHS-00405 type nano-SHAp, Sofsera, Tokyo, Japan) (1.5 g) was then manually mixed with collagen gel using plastic spatula. The n-HAP/collagen composite gel was then frozen at -80°C for 3 h, and freeze-dried with a freeze-drier (FD-5N, EYELA, Tokyo, Japan) for 12 h. The composite sponge (sheet) stored in a holed stainless steel case was subsequently cross-linked by de-hydrothermal treatment at 140°C for 24 h in a vacuum dry oven (VO-300, AS ONE, Tokyo, Japan).

Explanation of Newton Press (P) is secondary mentioned [6]. The Newton press machine (NT-100H, Sansho Industry, Osaka, Japan) (Fig. 1 a) enabled the material in the die, (whose inner dimensions were 9.95 mm diameter and 20 mm height), to be homogeneously pressed, while the manual and oil-generated pressure (29.4 kN (3,000 kgf)) were applied simultaneously from the top and bottom sides (*i.e*. co-axial bi-directional pressing) for 2 min. The resultant pressed specimens (Fig. 1 b) were then punched out to obtain disks (P-nHAP/COL) 6 mm in diameter and about 1 mm in height with a hole puncher.

As control, punched pressed collagen specimens without n-HAP (P-COL) were also produced.

Disks stored in exclusive pouche bags were sterilized by ethylene oxide gas, and kept in a vacuumed desiccator before cell culture tests.

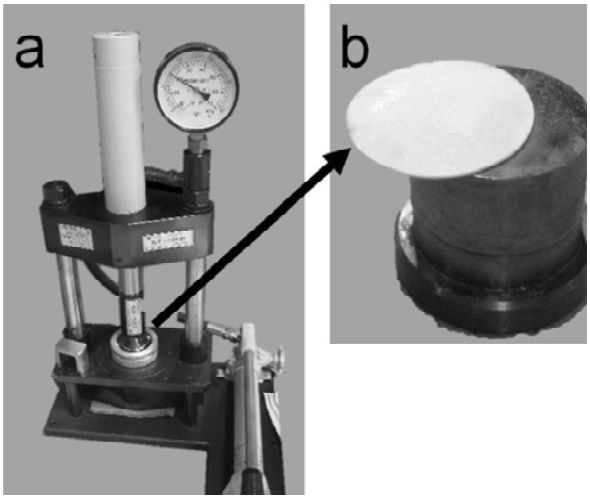

Fig. 1 (a) Newton press machine and (b) obtained pressed material.

SEM observations were preliminarily made on P-COL and P-nHAP/COL which were sputtered with Osmium (Fig. 2). On P-COL, many compressed clothes were layered on the surface at the low magnification (Fig. 2 upper-left side), and their micro-surface was grooved at the high magnification (Fig. 2 lower-left side), reflecting bundle of collagen fibers. On P-nHAP/COL, the surfaces were fully embedded by n-HAP particles at the low magnification (Fig 2 upper right side) whilst agglomerated or isolated nHAP particles were present on their detailed and pinpointed surface at the high magnification (Fig. 2 lower-right side).

2. Culture of SaOS-2 cells

Human osteoblast-like cells (SaOS-2) (RCB0428, Riken BioResource Center Cell Bank, Tsukuba, Japan) were regularly cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS, BI Biological Industries, Kibbutz Beit Haemek, Israel) and 2% antibiotic (penicillin), (Gibco, Carlsbad, CA, USA) in a 5% CO_2 incubator at 37℃. After sub-confluence, cells were collected by trypsinization with 0.05% Trypsin-EDTA (1X) (Gibco), and sub-cultured by 1:3 ratios.

Cell culture of SaOS-2 cells on P-nHAP/COL and P-COL specimens (3 disks each) was carried out in the following manner. SaOS-2 cells (1 x 10^6) in 2 mL media were inoculated on test materials held in wells of a 6-well polystyrene culture dish. Then, SaOS-2 cells on test materials were continuously

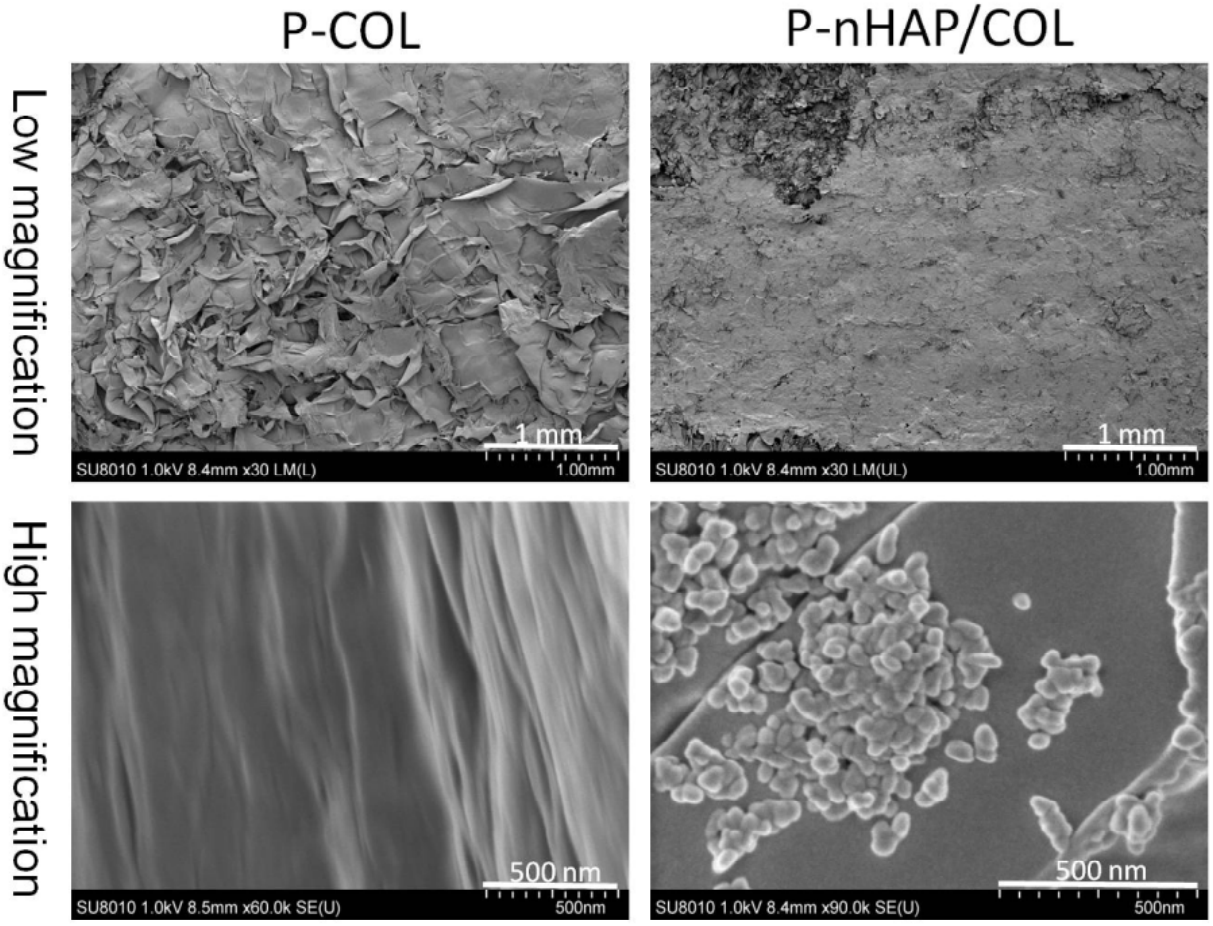

Fig. 2 SEM photo-micrographs of P-COL (left side) and P-nHAP/COL (right side) at low (upper side) and high magnifications (lower side).

cultured for 1, 2, 3 and 4weeks in a 5% CO_2 incubator at 37℃, while medium was exchanged twice per week. Culture tests for each condition were repeated three times.

3. Quantitative RT-PCR analyses

As osteogenic-differentiation-related phenotype markers, subsequent five genes were selected. While one gene, β-actin gene was used as a control, 1) ALP and COL1genes and 2) BSP and BGLAP genes were used as 1) early stage and 2) middle-to-late stage osteogenic differentiation phenotype markers, respectively [10].

Total RNAs were extracted from the cells using ISOGEN reagent (Nippon Gene, Tokyo, Japan). Reverse transcription was performed using the PrimeScript RT reagent Kit (TaKaRa, Ohtsu, Japan). The mRNA expression levels of five genes were determined by quantitative RT-PCR using SYBR Premix Ex Taq II (TaKaRa) and Thermal Cycler Dice Real Time System TP8000 (TaKaRa) and five primers (Table 1) designed by the Perfect Real Time support system (TaKaRa). The primer set for BGLAP was specially made upon our request while those for other four genes were ready-made in the support system (TaKaRa). For each test run, cDNA derived from 50 ng total RNA was used. After an initial denaturation at 95°C for 30 sec, a two-step cycle procedure was used (denaturation at 95°C for 5 sec, annealing and extension at 60°C for 30 sec) for 40 cycles. Gene expression levels were normalized according to the expression level of β-actin gene. Relative amounts (RQ values) of each mRNAs in each sample were calculated by ΔΔ Ct method. The gene expression analyses were duplicated. To ensure reproducibility, every sample was analyzed in triplicate. Data were presented as means ± standard deviation.

Statistics were carried out by Student *t* test at $\alpha = 0.05$ level of significance.

Table 1 Primers of five genes for quantitative real-time (RT)-PCR

Primers for		Sequences
β-Actin	F	TGGCACCCAGCACAATGAA
	R	CTAAGTCATAGTCCGCCTAGAAGCA
ALP	F	GGACCATTCCCACGTCTTCAC
	R	CCTTGTAGCCAGGCCCATTG
COL1	F	TCTAGACATGTTCAGCTTTGTGGAC
	R	TCTGTACGCAGGTGATTGGTG
BSP	F	GGCCACGATTTATCTTTACAAGCA
	R	TCACCCTCAGAGTCTTCATCTTCA
BGLAP	F	AGGTGCAGCCTTTGTGTCCA
	R	GGCTCCCAGCCATTGATACAG

4. SEM observations

SaOS-2 cells cultured on P-nHAP/COL and P-COL disks for 1 and 4 weeks were freeze-dried at room temperature for one hour, following fixation in 2.5% glutaraldehyde solution, fixation in 1% Osmium solution, dehydration in graded alcohols, infiltration by t-butyl alcohol and freezing at 0℃ for 12 hours. The cells on test materials were then sputtered with Osmium and observed with field-emission type SEM (SU8010, Hitachi, Ibaragi, Japan).

Results

1. Quantitative RT-PCR analyses

1) Early stage osteogenic differentiation marker (ALP and COL1) genes

Expressions of ALP gene in SaOS-2 cells on P-nHAP/COL increased from at 1 week to at 2 weeks, followed by gradual decline from at 2 weeks up to 4 weeks (Fig. 3), implying that the early osteogenic differentiation proceeded which

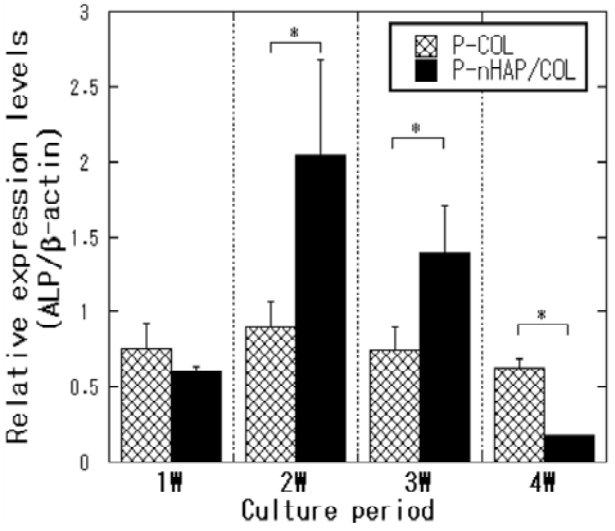

Fig. 3 Expressions of ALP gene in SaOS-2 cells cultured on P-COL and P-nHAP/Col for 1, 2, 3 and 4 weeks (* $p<0.05$).

peaked at 2 weeks. On the other hand, expres sions of ALP gene on P-COL remained almost similar from 1 week to 4 weeks (Fig. 3), implying that the early osteogenic differentiation was not completed.

Expressions of COL1 gene on P-nHAP/COL also increased from at 1 week to at 3 weeks, followed by gradual decline from at 3 weeks to at 4 weeks (Fig. 4), hinting that the early osteogenic differentiation proceeded which peaked at 3 weeks. In contrast, expressions of ALP gene on P-COL tended to slightly increase from 1 week to 4 weeks (Fig. 4), implying that the early osteogenic differentiation was still under process, yet.

2) Middle-to-late stage osteogenic differentiation marker (BSP and BGLAP) genes

Expressions of BSP gene on P-nHAP/COL significantly increased from at 1week to at 3 weeks, followed by sudden drop from at 3 weeks to at 4 weeks (Fig. 5), showing that the middle-to-late osteogenic differentiation advanced which peaked at 3 weeks. Expressions of BSP gene on P-COL, however, stagnated from at 1 week to at 3 weeks, followed by small increase from at 3 weeks to at 4 weeks (Fig. 5), meaning that the middle-to-late osteogenic differentiation was

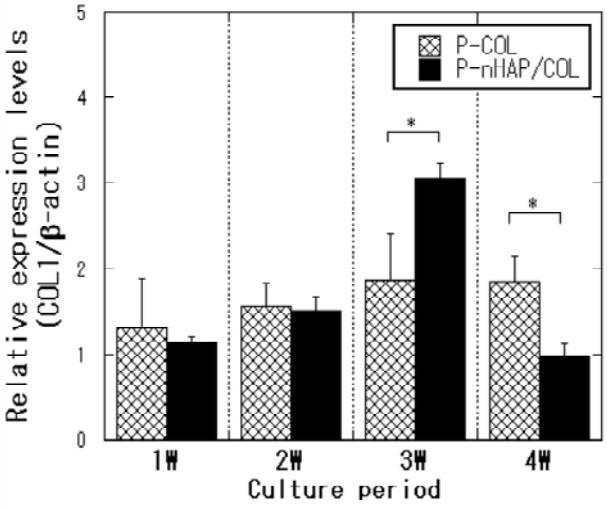

Fig. 4 Expressions of COL1 gene in SaOS-2 cells cultured on P-COL and P-nHAP/Col for 1, 2, 3 and 4 weeks (* $p<0.05$).

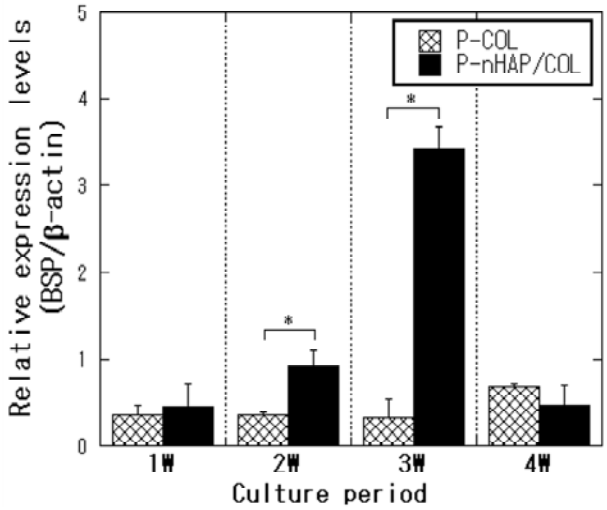

Fig. 5 Expressions of BSP gene in SaOS-2 cells cultured on P-COL and P-nHAP/Col for 1, 2, 3 and 4 weeks (* $p<0.05$).

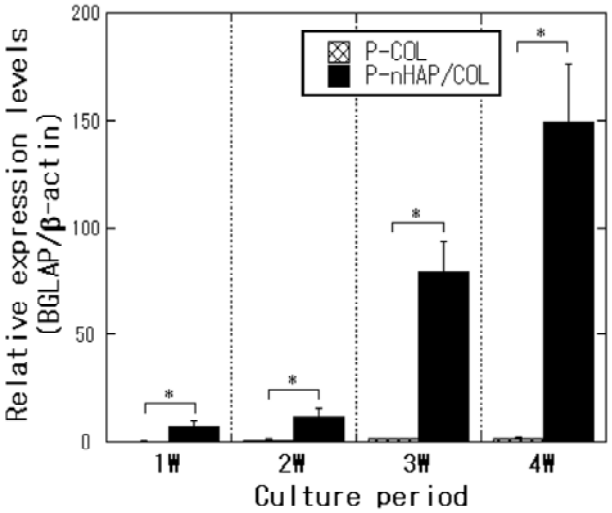

Fig. 6 Expressions of BGLAP gene in SaOS-2 cells cultured on P-COL and P-nHAP/Col for 1, 2, 3 and 4 weeks (* $p<0.05$).

under the process.

Expressions of BGLAP gene on P-nHAP/COL dramatically increased from at 1 week to at 4 weeks (Fig. 6), indicating that the late (last) osteogenic differentiation took place, which peaked at 4 weeks. Expression of BGLAP gene on P-COL slightly increased from at 1 week up to at 4 weeks with their magnitude very small (Fig. 6), meaning that the late osteogenic differentiation did not occur.

2. SEM observations

1) P-Col

SaOS-2 cells were either cubic or fibroblastic on P-Col at 1 week at the low magnification (Fig. 7 upper-left side), and the fibroblast had many projections, some of which were in contact with P-COL surfaces at the high magnification (Fig. 7 lower-left side). SaOS-2 cells on P-COL at 4 weeks were similar to those at 1 week, while the former cells were more fibroblastic at the low magnification (Fig. 7 upper-right side). The fibroblastic appearance at 4 weeks (Fig. 7 lower-right side) was basically analogous to those at 1 week. SaOS-2 fibroblasts held the basic cellular appearance from 1 week up to 4 weeks, suggesting that the osteogenic differentiation did not proceed well.

2) P-nHAP/Col

Osteoblastic SaOS-2 cells adhered and proliferated on P-nHAP/COL as round (major case) and spread (minor case) cells at 1 week at the low magnification (Fig. 8 upper-left side) while surfaces of round cells were lumpy at the high magnification (Fig. 8 lower-left side), associated intra-cellular absorption of nHAP particles [4]. At 4 weeks, numbers of adhered round cells diminished at the low magnification (Fig 8 upper-right side), but some cells (or secreted extracellular matrix) highly spread over P-nHAP/COL (especially over agglomerated nHAP particles) whilst round cells co-existed at the high magnification (Fig. 8 lower-right side).

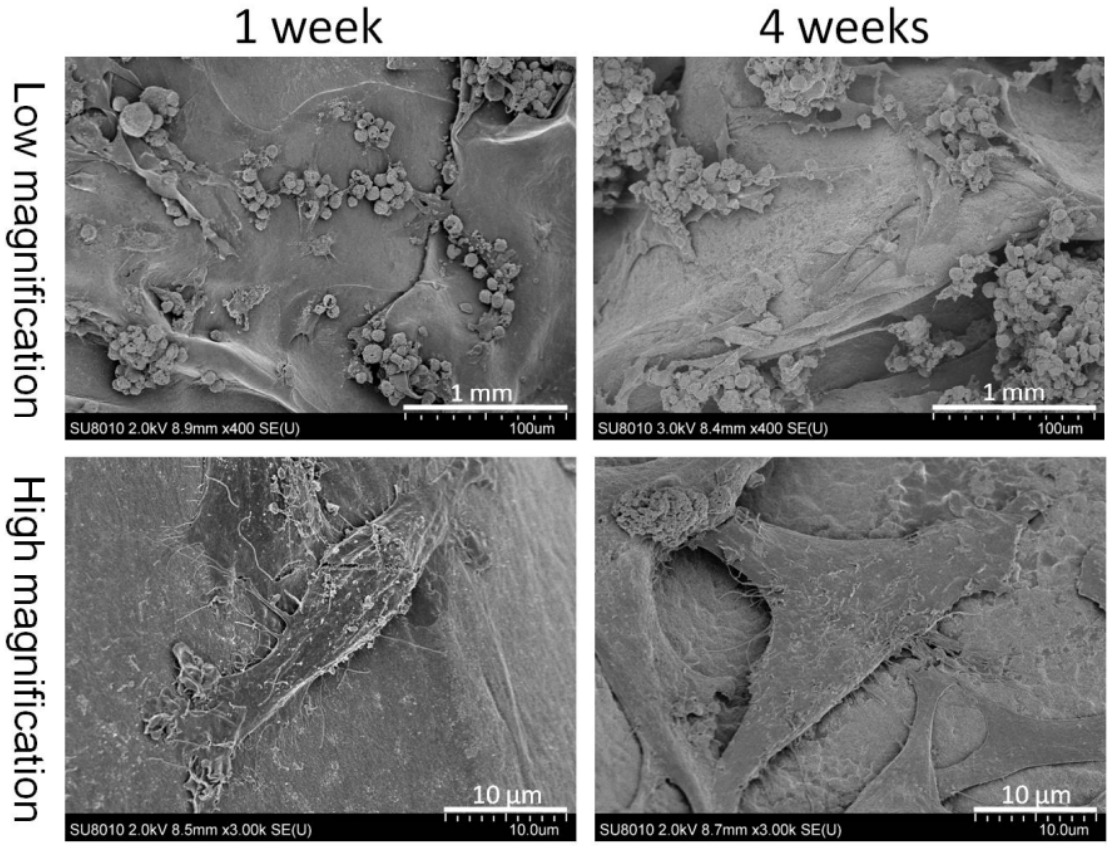

Fig. 7 SEM images of SaOS-2 cells on P-COL cultured for 1 and 4 weeks.

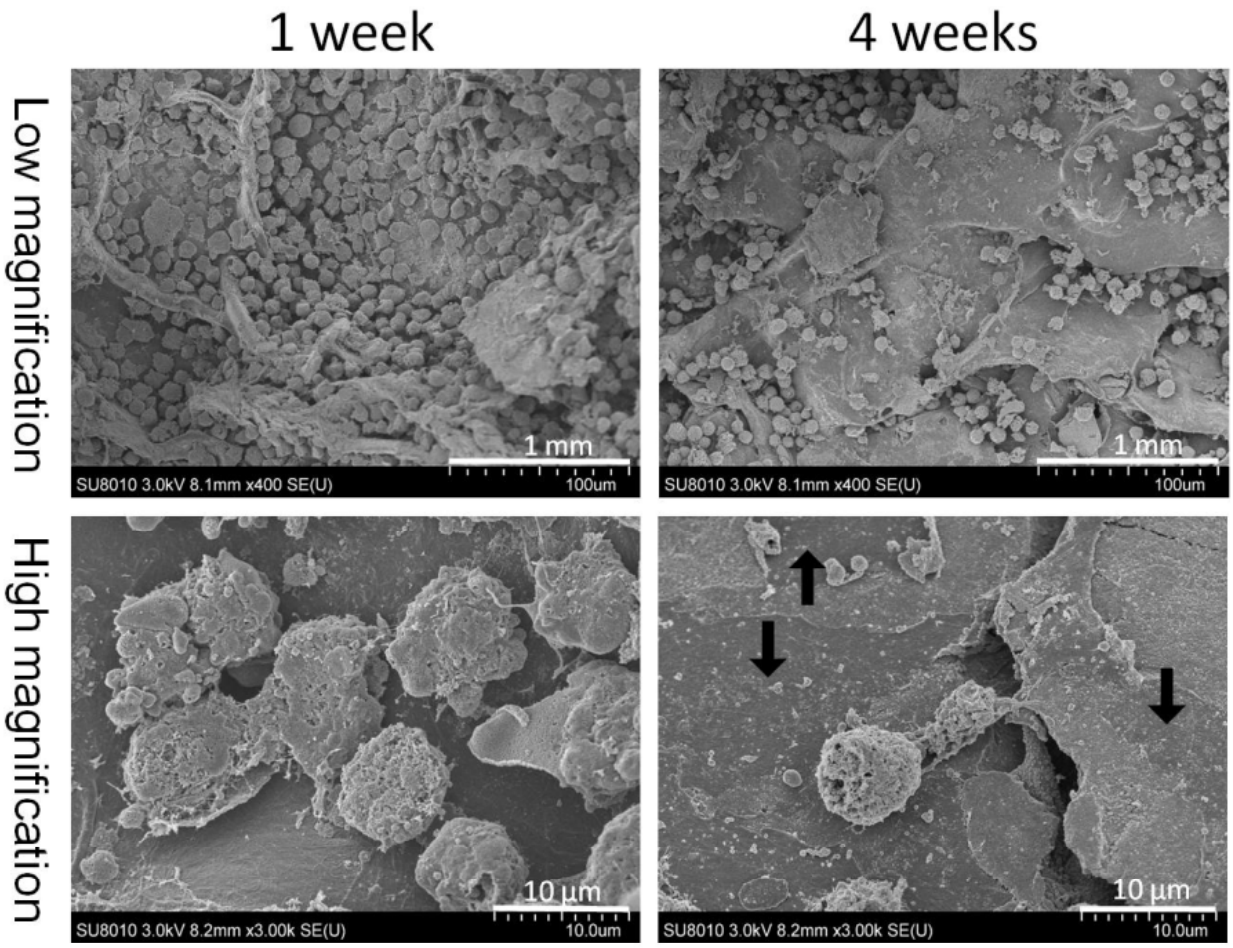

Fig. 8 SEM images of SaOS-2 cells on P-nHAP/COL cultured for 1 and 4 weeks.

Note: Black arrows indicate highly spread SaOS-2 cells and secreted extracellular matrix on P-nHAP/COL at 4 weeks (lower-right side).

Such cellular morphological change apparently reflects osteogenic differentiation of SaOS-2 cells [4].

Discussion

SaOS-2 cells are one of popular osteoblastic cells employed in the osteogenic differentiation studies [11]. We have already reported that osteogenic differentiation of SaOS-2 cells was more accelerated on apatite-coated titanium than on titanium by gene expression analyses using SaOS-2 cells [12]. Since SaOS-2 cells originally lied in the early stage osteogenic differentiation stage (*i.e*. ALP positive cells), it was considered easy to evaluate effects of materials on later-advancing osteogenic differentiation of SaOS-2 cells.

It became clear from the results obtained that osteogenic differentiation in SaOS-2 cells on P-nHAP/COL reached the final stage at 4 weeks, at which expressions of early stage osteogenic differentiation marker (ALP and COL1) genes and middle-to-late stage osteogenic differentiation marker (BSP) gene have passed their peaks (Figs. 3, 4 and 5), and those of late (last) stage osteogenic differentiation marker (BGLAP) gene reached the maximum values (Fig. 6). It can be, therefore, pointed out that P-nHAP/COL did considerably accelerate osteogenic differentiation of osteoblastic SaOS-2

cells, and was osteo-conductive. On the contrary, it also became evident that osteogenic differentiation of SaOS-2 cell on P-COL seemed to stay in the early-to-middle stages at 4 weeks, at which expressions of early stage osteogenic differentiation marker (ALP) gene remained almost constant from 1 week to 4 weeks (Fig. 3), and those of another early stage osteogenic differentiation marker (COL1) gene (Fig. 4) as well as middle-to-late stage osteogenic differentiation marker (BSP and BGLAP) genes (Figs. 5 and 6) increased with quite small quantities. These findings clarify that P-COL did not effectively accelerate the osteogenic differentiation of SaOS-2 cells. In other word, P-COL itself does not have strong osteo-conductive power [6].

SEM observations demonstrated the significant effect of the addition of nHAP to P-COL on the cellular morphology of SaOS-2 cells. SaOS-2 was more fibroblastic on P-COL, while their basic appearance was similar between at 1 week and at 4 weeks (Fig. 7). This phenomenon might arise from the stagnation of osteogenic differentiation of SaOS-2 cells on P-COL from at 1 week to at 4 weeks. On the other hand, nHAP in nHAP/COL apparently affected the cellular appearance of SaOS-2 cells. SaOS-2 cells were more angular granule-shape on nHAP/COL at 1 week (Fig. 8). This morphological change might be brought about by intra-cellular phagocytosis of nHAP particles. Such activity of osteoblasts was reported by other investigators [13, 14]. It could be speculated that intra-cellular digestion of nHAP particles might accelerate osteogenic differentiation of SaOS-2 cells [15], partly due to supply of both calcium [16] and phosphate ions, resulting in activation of cell signaling pathway (e.g. MAPK pathway) [17, 18]. At 4 weeks, cellular appearance of SaOS-2 cells significantly altered as a result of osteogenic differentiation. While round cells co-existed, cells highly spread over or secreted intercellular matrix covered the surface of SaOS-2 cells on P-nHAP/COL (Fig. 8). It also appeared that more proteins in culture medium adhered on agglomerated nHAP particles in P-nHAP/COL, leading to increased adhesion [19], spreading, proliferation and intensified osteogenic differentiation of SaOS-2 cells [20]. SaOS-2 cells well adhered to and spread over agglomerated nHAP particles in P-nHAP/COL, compared with surfaces of P-COL (Figs. 7 and 8).

Referring to porous nHAP/COL sponge, analogous experimental results were obtained. They also accelerated osteogenic differentiation of SaOS-2 cells [4]. Porous soft nHAP/COL sponge most accelerated the middle-to-late stage osteogenic differentiation marker gene (BSP) [4], while dense harder P-nHAP/COL most up-regulated the last stage osteogenic differentiation marker gene (BGLAP) (Fig. 6). The difference between nHAP/COL and P-nHAP/COL appears to arise from binding situation of nHAP to COL. Newton press (P) highly embedded nHAP particle to COL with higher volume fraction and physical agglomoration energy. The degradation rate of the composite (including bio-absorption rate of nHAP particles from the composite) might be, thus, decreased for dense P-nHAP/COL, compared with for porous nHAP/COL, which might cause P-nHAP/COL to be more osteo-conductive. It should be reminded that porous nHAP/COL had not enough osteo-conductive effect in rat critical-size calvarial bone defects [5].

Conclusion

We self-prepared highly pressed nano hydroxyapatite/collagen composites (P-nHAP/COL) and collagen control (P-COL) by Newton Press; conducted expression analyses of four osteogenic differentiation marker genes of SaOS-2 cells cultured on two materials for 1, 2, 3 and 4 with quantitative real-time PCR; and carried out SEM observations of the said cells cultured on two materials at 1 and at 4 weeks. The following experimental results were obtained:

(1) Osteogenic differentiation of osteoblastic SaOS-2 cells was significantly more accelerated on P-nHAP/COL than on P-COL.
(2) SaOS-2 cells phagocytized n-HAP particles in P-nHAP/Col, changing the cellular appearance to granulate or highly spread by osteogenic differentiation. Many SaOS-2 cells on P-COL remained, however, to be fibroblastic.
(3) It was supposed that P-nHAP/COL might be applied as a new osteo-conductive bone sub-

stitute material. *In vivo* animal tests and clinically related studies are highly anticipated for P-nHAP/COL in the near future.

There is no Conflict of Interest (COI) of all authors in this study.

Acknowledgements

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