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Original article

Effect of osteogenic differentiation medium on proliferation and differentiation of human mesenchymal stem cells in three-dimensional culture with radial flow bioreactor

Itsurou Nishimura [a], Ryuichi Hisanaga [c], Toru Sato [c], Taichi Arano [a], Syuntaro Nomoto [c], Yoshito Ikada [d], Masao Yoshinari [b, *]

[a] Department of Crown and Bridge Prosthodontics, Division of Oral Implant Research, Oral Health Science Center, Tokyo Dental College, Chiba, Japan
[b] Division of Oral Implant Research, Oral Health Science Center, Tokyo Dental College, Chiba, Japan
[c] Department of Crown and Bridge Prosthodontics, Tokyo Dental College, Chiba, Japan
[d] Division of Life Science, Nara Medical University, Kashihara, Japan

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ABSTRACT

Human mesenchymal stem cells (hMSCs) are multipotent cells, and have been expanded and differentiated into several kinds of mesodermal tissue in vitro. In order to promote bone repair, enhancement of the proliferation and differentiation of hMSCs into osteoblasts in vitro is recommended prior to therapeutic delivery. However, for clinical applications, it is still unclear which method is more advanced for tissue engineering: to transplant undifferentiated cells or partially differentiated stem cells. Therefore, the present study aimed to investigate how osteogenic differentiation medium (ODM) affects hMSCs cultured in a 3D scaffold using a radial-flow bioreactor (RFB) besides cell growth medium (GM). To produce precultured sheets, the hMSCs were first seeded onto type 1 collagen sheets and incubated for 12 h, after which they were placed in the RFB for scaffold fabrication. The culture medium was circulated at 3 mL/min and the cells dynamically cultured for 1 week at 37 °C. Static cultivation in a culture dish was also carried out. Cell proliferations were evaluated by histological analysis and DNA-based cell count. Alkaline phosphatase (ALP) activity, immunocytochemical analysis with BMP-2, and osteopontin on the hMSCs in the collagen scaffold were performed. After 14 days of ODM culture, a significant increase in cell number and a higher density of cell distribution in the scaffold were observed after both static and dynamic cultivation compared to GM culture. A significant increase in ALP activity after 14 days of ODM was recognized in dynamic cultivation compared with that of static cultivation. Cells that BMP-2 expressed were frequently observed after 14 days in dynamic culture compared with other conditions, and the expression of osteopontin was confirmed in dynamic cultivation after both 7 days and 14 days. The results of this study revealed that both the proliferation and bone differentiation of hMSCs in 3D culture by RFB were accelerated by culture in osteogenic differentiation medium, suggesting an advantageous future clinical applications for RFB cell culture and cell transplantation for tissue engineering.

1. Introduction

Large bone defects caused by trauma, inflammation, tumors, or congenital abnormalities are often treated with autologous or allogeneic bone grafts. Implantation of autologous bone grafts is the most popular treatment method due to their high performance in terms of osteogenesis, the only drawback being their limited availability due to donor site morbidity. Allogeneic bone grafts are less attractive because of the risk of immunogenicity, donor-to-host transmission of disease (e.g., HIV), and graft failure as a consequence of the reduced osteoinductivity of allograft bone [1].

Recently, cell-based tissue engineering has drawn much interest as an alternative to these approaches, offering the potential for the

* Corresponding author.
E-mail address: yosinari@tdc.ac.jp (M. Yoshinari).
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creation of bioartificial tissues and even whole organs. Human mesenchymal stem cells (hMSCs) are multipotent cells, can be readily isolated from adult donors with little damage, and are inducible osteoprogenitor cells, making them the cell of choice in bone tissue engineering and regeneration [2,3]. However, the amount of hMSCs harvested from donor tissue is limited to that which is to be applied to clinical treatment. Static cultivation of MSCs on porous scaffolds and maturation is the simplest method of building a cell-scaffold complex for in vivo implantation [4]. Several studies have reported low seeding efficiencies and non-uniform cell distributions within scaffolds owing, in part, to the manual- and operator-dependent nature of the process [5–7]. Moreover, because of a lack of influence from shear stress and mechanical loading, efficient osteoinduction in static culture seems difficult to achieve. Accordingly, specialized dynamic culture systems, called bioreactors, have been utilized in bone tissue engineering. Such a dynamic three-dimensional (3D) culture system may represent more of a physiological environment than a dish and demonstrate that fluid flow is an important component for seeding and culturing BMSCs in 3D environments [8–11]. This increased interest in tissue engineering has led to the development of various types of equipment for the construction of bioreactors, including spinner flasks, rotating wall vessels, and direct perfusion bioreactors, all of which have been extensively investigated in bone tissue engineering [12,13].

The radial-flow bioreactor (RFB) has shown the ability to maintain an even cell culture environment by radial provision of the medium, enabling the construction of comparatively larger tissues [14–17]. To enable even distribution of oxygen, culture medium is pumped from the periphery to the center of the chamber under low shear stress.

As one approach of promoting bone repair, the enhancement of proliferation and differentiation of hMSCs towards osteoblasts in vitro is recommended prior to therapeutic delivery [4]. In a previous study, it was reported that preosteoblast-like cells and hMSCs were expand uniformly over a 3D scaffold under dynamic cultivation using an RFB, and the cellular characteristics of the hMSCs were not changed in comparison to static cultivation in DMEM without bone differentiation medium [14,18]. However, for clinical applications, it is still unclear which method is more advantageous for tissue engineering: to transplant undifferentiated or, to some extent, differentiated stem cells.

Therefore, the present study aimed to investigate how osteogenic differentiation medium (ODM) affects hMSCs cultured in 3D scaffolds by a radial-flow bioreactor (RFB).

2. Materials and methods

Fig. 1 is a summary of the study protocol.

2.1. Culture of human MSCs

hMSCs derived from human bone marrow (PT-2501; Lonza Walkersville, MD, USA) and donated by a 19-year-old male were passaged 5 times for use in this study. Dulbecco's Modified Essential Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Sigma–Aldrich) and 100 units/mL penicillin-streptomycin (Gibco) was used as growth media (GM) for static and dynamic cultivation as well as for preculture. On the other hand, GM supplemented with 50 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM b-glycerophosphate was used as an osteogenic differentiation medium (ODM) for static and dynamic cultivation.

A cell suspension containing 5.0×10^5 cells was seeded into 75 cm^2 flasks, and 20 mL fresh culture medium was added to each flask. Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was changed every 3 days. After 1 week of cultivation, before reaching confluence, the cells were harvested by trypsin treatment and seeded onto type 1 collagen sheets (Gunze) (pore size, 70–110 μm; porosity, 80%–95%; diameter, 12 mm; thickness, 3 mm).

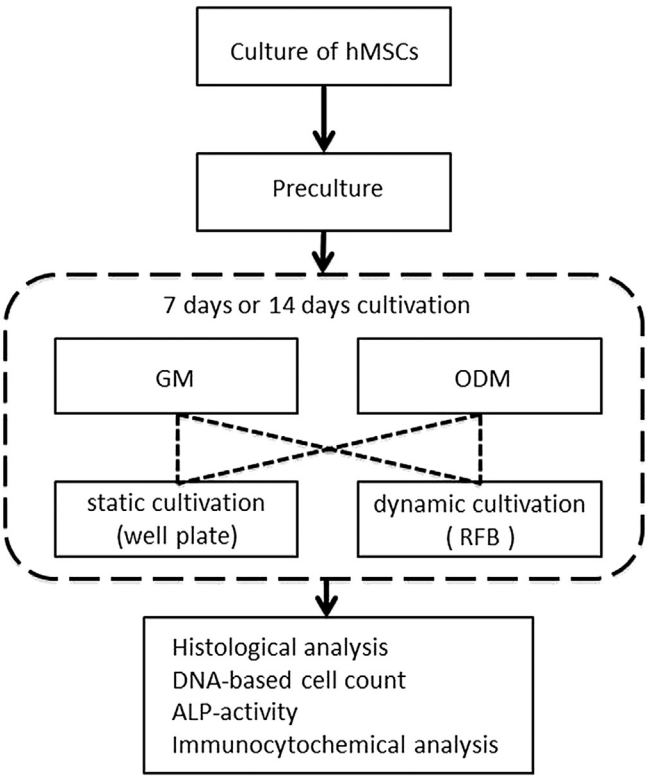

Fig. 1. Flowchart of the present study.

2.2. Preculture

To optimize appropriate pre-culture method for initial cell attachment with high rate of cell density to the collagen sheets, a pre-culture assay was performed that involve turning over the sheets according to the previous study [14,18]. Briefly, type 1 collagen sheets were placed in a 12-well plate and a cell suspension (80 μL) containing 1.0×10^5 cells was seeded onto them. The sheets were then incubated in a humidified atmosphere at 37 °C with 5% CO_2 for 6 h. Next, the sheets were turned over, and a further 80 μL cell suspension containing 1.0×10^5 cells was added before a further 6 h incubation. (The final cell seeding density was 2.0×10^5 cells per sheet.)

2.3. Dynamic cultivation

Fig. 2 shows the RFB (Able) and RFB cell culture system used. To form a scaffold, three precultured sheets were placed in the RFB in layers after incubation for 12 h (6 h + 6 h). The temperature (37 °C), pH (7.4), and dissolved oxygen (DO, 6.86 ppm) in the medium reservoir were controlled and monitored. The medium volume was maintained at 100 mL. After commencement of culture, the medium was changed every day from the third day onward. The medium flow rate was set at 3 mL/min. Culture was carried out for 7 days and 14 days in each of the GM and ODM, as shown in Fig. 1.

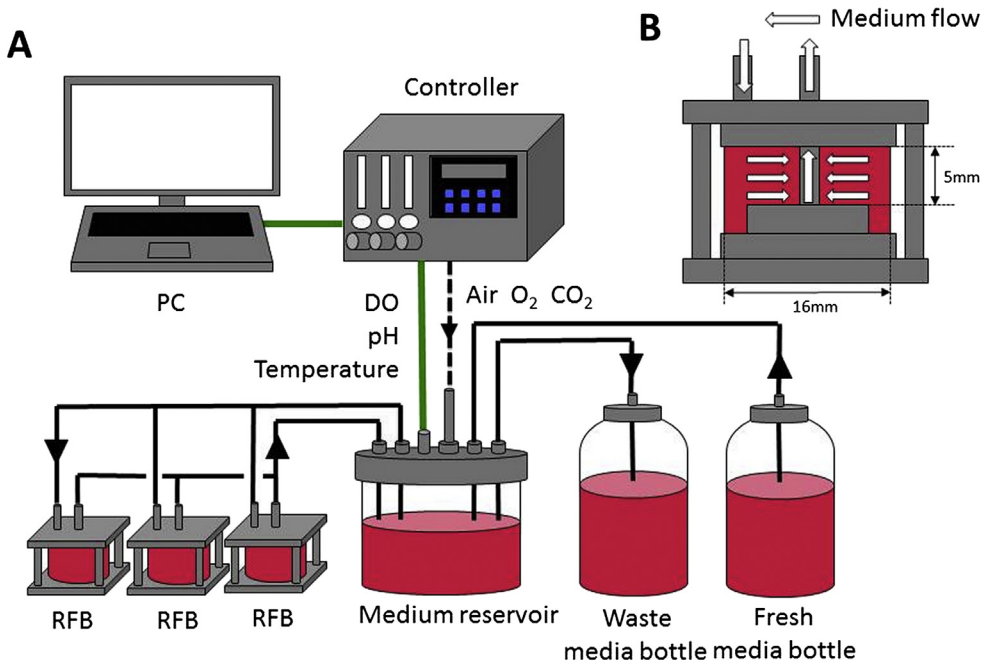

Fig. 2. Radial-flow bioreactor (RFB) system used in this study. (A) Schematic diagram of total system. The medium was circulated between the RFB and medium reservoir using a circulation pump. During the experiment, dissolved oxygen (DO), pH, and temperature of the medium were monitored and controlled. Volume of the chamber medium was maintained at 100 mL, and fresh medium was added continuously. (B) Schematic diagram of RFB. The medium in the RFB flows from the periphery to the center of the reactor chamber.

2.4. Static cultivation

The preculture protocol for static cultivation was the same as that for dynamic cultivation. An individual precultured sheet was placed in each well of a 12-well plate. The culture medium was maintained at 2 mL. Culture was carried out in a humidified atmosphere at 37 °C and 5% CO_2 for 7 days and 14 days in each of the GM and ODM with no control of DO or pH values. The culture medium was changed every 3 days. Table 1 lists the cultivation conditions.

2.5. Histological analysis

Fig. 3 shows a cross section of the three layered collagen sheets used for each analysis.

Histological analysis was carried out at 7 days or 14 days after commencement of culture. Scaffolds that were harvested after culture were fixed with 10% neutral-buffered formalin and dehydrated through a series of graded ethanols. After being embedded in paraffin, 4-μm thick sections were prepared from both types of specimens and stained with hematoxylin-eosin (H–E staining). Finally, they were observed with a universal photomicroscope (Axiophot 2, Carl Zeiss).

Table 1
Cultivation conditions.

	Static	Dynamic
Number of cells	2.0×10^5 cells/scaffold	2.0×10^5 cells/scaffold
Temperature	37 °C	37 °C
CO_2	5%	
pH		7.4
DO		6.86
Medium flow rate		3 ml/min
Medium volume	2 ml/well	100 ml/day
Medium change	Every 3 days	Daily after 3days
Scaffold	1 sheet/well	3 sheets/reactor

2.6. DNA-based cell count

At 7 days or 14 days after commencement of culture, scaffolds with three cultured sheets from the RFB were selected for DNA-based cell count. They were divided into upper, middle, and

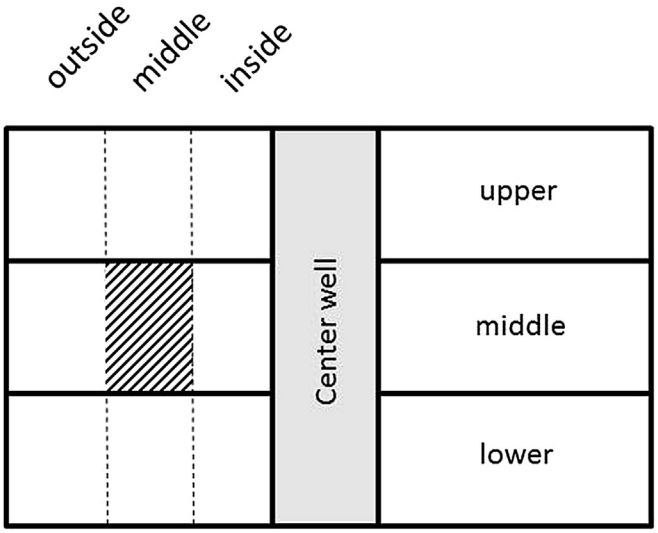

Fig. 3. Cross section of three layered collagen sheets used for each analysis. Scaffolds in the RFB were divided horizontally and perpendicularly into nine areas consisting of three sheets (from top to bottom: upper, middle, and lower) × three areas (inside, middle, and outside). Histological analysis and immunocytochemical analysis were performed using the middle area of the middle sheet (shaded area). DNA-based cell count and ALP activity were evaluated using three sheets (upper, middle, and lower).

lower areas from top to bottom (Fig. 3). Single collagen sheets were also selected from the static cultivation because the precultured collagen sheets were not laminated in the static cultivation. This method of cell counting was selected based on an earlier study by this group [18].

Total DNA was quantified using the ND-1000 spectrophotometer (NanoDrop Technologies). The cell number was then calculated using a working curve based on the cell number and total DNA. The mean DNA-based cell counts of the three areas under dynamic cultivation were compared with those under static cultivation.

2.7. Alkaline phosphatase (ALP) activity

Scaffolds ware harvested from the RFB and placed in a 12-well plate. The scaffolds were rinsed with cold phosphate-buffered saline (PBS), cut into small fragments, and sonicated for 30 s after application of 200 μL Triton-X. The lysates obtained were centrifuged at 15,000 rpm for 15 min, and the supernatant was used as the sample. ALP activity was assayed using the LabAssay ALP kit (Wako). Sample absorbance was measured in a 96-well plate at 405 nm. The amount of total protein in the sample was then determined with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Finally, ALP activity was expressed as units/μg protein.

2.8. Immunocytochemical analysis

Immunocytochemical analysis was carried out at 7 days or 14 days after commencement of culture in ODM. Proteins in the collagen sheet were visualized with antibodies of BMP-2 and osteopontin. The sections were washed in 10 nmol/L with pH 7.4 phosphate-buffered saline (PBS) and endogenous peroxidase activity was blocked by incubating the sections with 0.3% H_2O_2 in methanol for 30 min. The sections were then reacted with the primary antibodies, BMP-2 (diluted 1:250) (Abcam ab14933), and osteopontin (diluted 1:140) (Abcam ab63856) overnight below 4 °C. The sections were washed in PBS, incubated with the secondary antibody, peroxidase-labeled anti-mouse IgG polyclonal antibody (Histofine Simple Stain MAX-PO, MULTI, Nichirei), for 30 min, and then washed with PBS. Subsequently, the sections were stained with 3,3′-diaminobenzidine (DAB Substrate Kit, Nichirei), washed in sterilized water, and counterstained with hematoxylin. The sections were then dehydrated according to established procedure after which they were observed using a universal photomicroscope (Axiophot 2).

2.9. Statistical analysis

The DNA-based cell count and ALP activity was statistically analyzed using a one-way analysis of variance, followed by multiple-comparison tests with Fisher's LSD method using a statistical analysis for Excel (Excel statistics 2006, SSRI, Japan).

3. Results

3.1. Histological analysis

Figs. 4 and 5 show optical micrographs of hematoxylin-eosin staining. The cells were densely distributed in the scaffold of both the static and dynamic culture by ODM after 7 days of commencement of culture (Fig. 4). A higher cell density was observed in ODM under dynamic cultivation; no difference was observed between static and dynamic cultivation in GM. After 14 days of culture, cells were distributed more densely under both static and dynamic cultivation by ODM. (Fig. 5) Compared to that under static cultivation, a higher cell density was observed in both the GM and ODM under dynamic cultivation.

3.2. DNA-based cell count

DNA-based cell counts of each area are shown in Figs. 6 and 7. Fig. 6 shows a comparison of cell numbers in each area (upper, middle, lower) under dynamic cultivation. No significant difference in cell numbers was observed among the three areas.

The number of cells increased for 14 days compared to that for 7 days under both static and dynamic cultivation in the ODM. A comparison of cell numbers between GM and ODM under static and dynamic cultivation after 7 days and 14 days of culture is shown in Fig. 7. Under dynamic cultivation, a mean of three areas was chosen for comparison with that under static cultivation because there was no significant difference in cell numbers among the three areas. After 7 days of culture, no significant difference in cell numbers between GM and ODM was found under either static or dynamic cultivation. On the other hand, a significant difference in cell numbers between GM and ODM was observed under both static and dynamic cultivation (approximately 1.7–1.9 times) in ODM for 14 days ($^{**}p < 0.01$). Significant increases in cell numbers under dynamic cultivation were noted in both GM and ODM for 7 and 14 days compared to static cultivation (approximately 1.4–1.8 times) ($^{*}p < 0.05$, $^{**}p < 0.01$).

3.3. ALP activity

A comparison of ALP activity is shown in Fig. 8 ALP activity was hardly observed in GM under both static and dynamic cultivation. In ODM, ALP activity increased under both dynamic and static cultivation after 7 days as well as after 14 days. Only a significant difference in ALP activity was observed between static and dynamic cultivation after 14 days (approximately 2.5 times) ($^{**}p < 0.01$) even though no significant difference was observed after 7 days.

3.4. Immunocytochemical analysis

The results of immunocytochemical analysis in ODM are shown in Figs. 9 and 10. BMP-2 was confirmed by cell color development in both static and dynamic cultivation at 7 days and 14 days (Fig. 9). In particular, cells that expressed BMP-2 were frequently observed for 14 days in dynamic cultivation compared with other conditions. Expression of osteopontin was observed in dynamic cultivation for both 7 and 14 days, but not in static cultivation. (Fig. 10).

4. Discussion

This study aimed to investigate the effects of osteogenic differentiation medium on hMSCs seeded in 3D scaffolds under a perfusion culture by RFB. The results showed that the three-dimensional culture of hMSCs in RFB with osteogenic differentiation medium accelerated both cell proliferation and osteogenic differentiation.

In general, MSCs have heterogeneous population and cannot easily be induced to differentiate into some lineage. Therefore, several lots of MSCs should be used for the cell-therapy experiment. In the present study, MSCs derived from human bone marrow according to the previous study that MSCs were confirmed as positive for CD29, CD44, CD71, CD105 and CD166; and negative for CD34, CD45 in agreement with the manufacturer's (Lonza) information [18].

Stiehler et al. have been already reported that 3-D flow culture induced proliferation and differentiation of hMSC in scaffold [28].

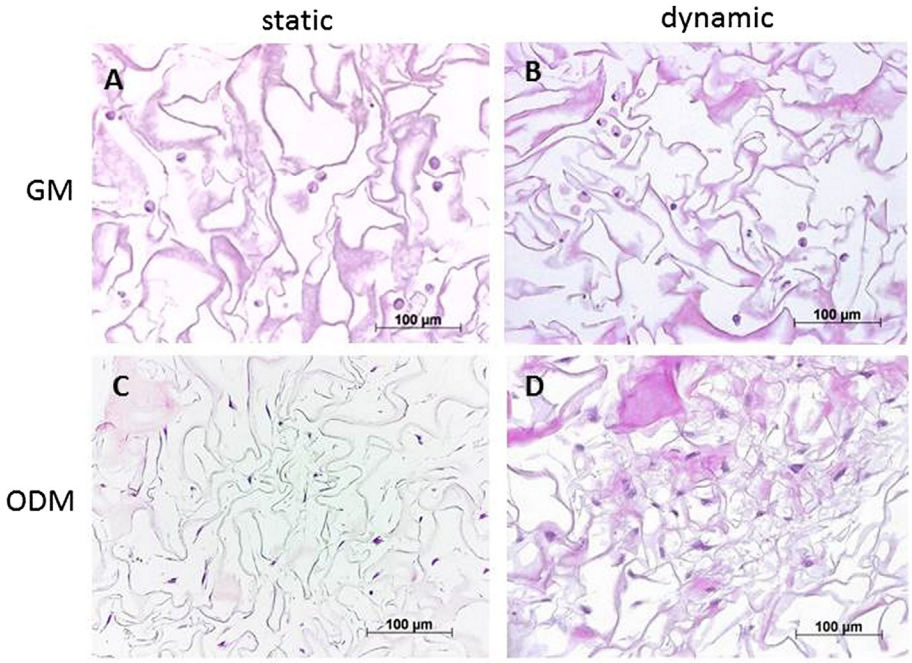

Fig. 4. Typical optical micrographs of specimens stained with hematoxylin–eosin. (7 days). (A) GM static cultivation. (B) GM dynamic cultivation. (C) ODM static cultivation. (D) ODM dynamic cultivation. (×200; scale bar: 100 μm).

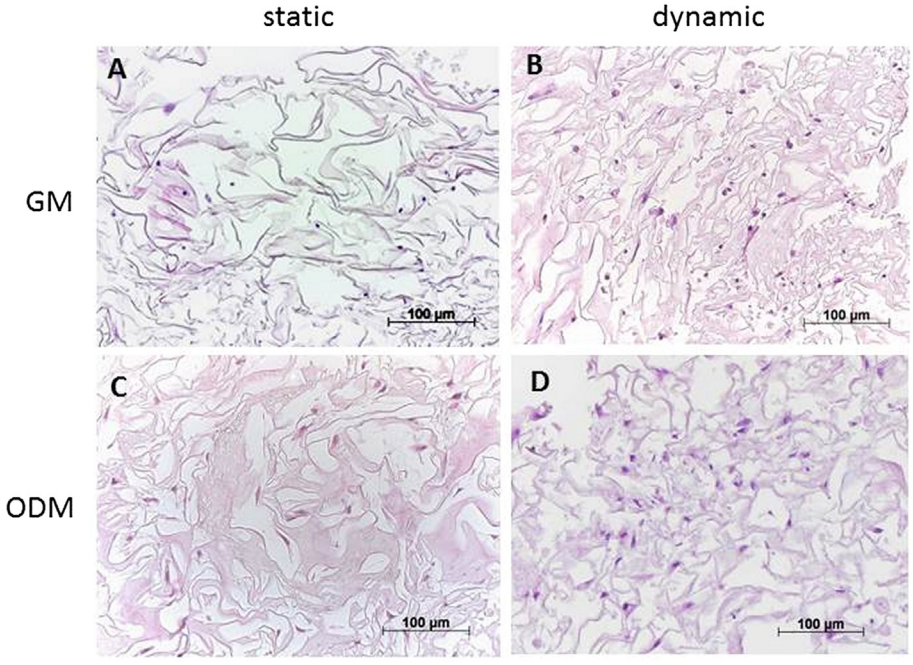

Fig. 5. Typical optical micrographs of specimens stained with hematoxylin–eosin. (14 days). (A) GM static cultivation. (B) GM dynamic cultivation. (C) ODM static cultivation. (D) ODM dynamic cultivation. (×200; scale bar: 100 μm).

Major differences in dynamic 3-D culture methods between Stiehlers' study and this study were; spinner flasks (the former) or radial flow bioreactor (the latter) in bioreactor, and PLGA (the former) or collagen (the latter) as scaffold. The radial flow bioreactor has distinguishing characteristics compared to spinner flasks that the medium is made to flow from the periphery to the center of the bed under low shear stress, allowing even greater efficiency of delivery of nutrients and gas exchange, as well as of elimination of metabolic waste products, indicating the usefulness for generating tissues for the treatment of large defects. The collagen scaffold is believe to be biocompatible and bioabsorbable compared with PLGA, and thus, easy to form the extracellular matrix for tissue generation.

As reported by Yoshinari et al., there is a possibility that the culture medium cannot penetrate the scaffold equally because of calcification under long-term 3D culture of hMSCs in osteogenic

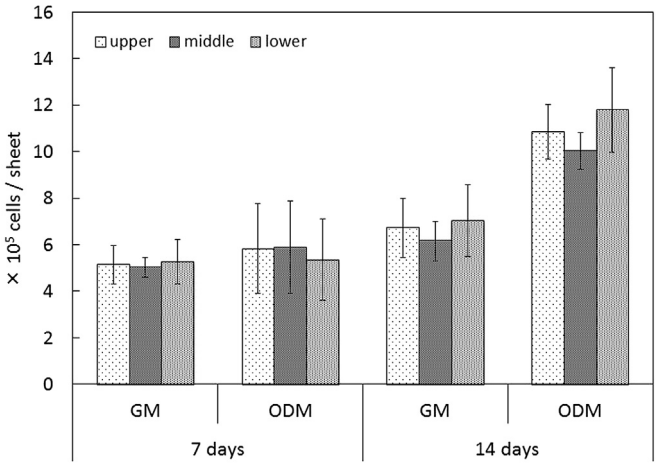

Fig. 6. Comparison of cell numbers (DNA-based cell count) in each area under dynamic cultivation. No significant differences were observed among the three areas. Data are expressed as mean ± SD over five cultures.

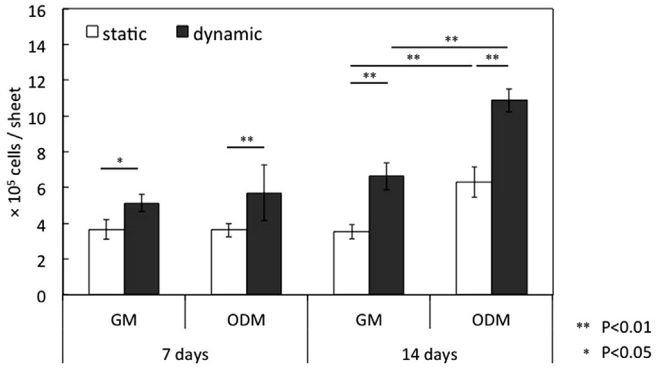

Fig. 7. Comparison of cell numbers between GM and ODM under static and dynamic cultivation. Under dynamic cultivation, a mean of three areas was chosen for comparison with that under static cultivation. Data are expressed as mean ± SD over five cultures.

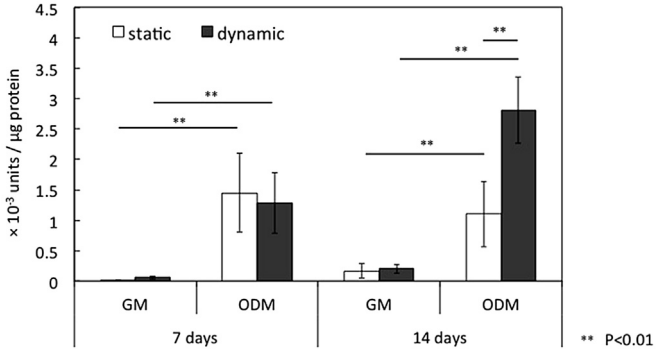

Fig. 8. Comparison of ALP activity (units/μg protein) between GM and ODM under static and dynamic cultivation. Under dynamic cultivation, a mean of three areas was chosen for comparison with that under static cultivation. Data are expressed as mean ± SD over five cultures.

differentiation medium using RFB [19]. To avoid such influence, we set the culture period as 7 days and 14 days.

In this study, by culturing in osteogenic differentiation medium, cell proliferation was significantly promoted in both static and dynamic cultivation for 14 days while not for 7 days. A possible explanation for these result is that ODM induces hMSCs to differentiate to preosteoblast-like cells, which can expand more quickly than can totally undifferentiated hMSCs. However, it is still unclear in which stage of differentiation the proliferation speed increases and when the ability to proliferate is lost [14,18].

Compared to static cultivation, dynamic cultivation demonstrated significantly increased cell numbers and higher cell distribution density in the scaffolds regardless of culture time and culture medium. The possible cause for such differences under dynamic cultivation is a more efficient delivery of nutrients and exchange of gas along with the elimination of metabolic waste [20,21]. Accordingly, the cell death associated with usual 3D culture is partially prevented by dynamic cultivation [22].

In this study, ALP activities as well as the expression of BMP-2 and osteopontin were analyzed to investigate how ODM affects the osteogenic differentiation of hMSCs in 3D culture by RFB. ALP activity and BMP-2 are known as early markers of osteoblastic differentiation [23,24]. By culturing in osteogenic differentiation medium, an increase of ALP activity was observed in all experimental conditions and there was a significant difference between 14 days of static and dynamic cultivation. ALP activity is upregulated and the speed of bone differentiation is promoted in dynamic cultivation compared to that in static cultivation. Dynamic cultivation of hMSCs by RFB is believed to facilitate osteogenic differentiation due to shear stress caused by medium perfusion and enhanced delivery of ODM [25–27]. Effective circulation of ODM provides necessary differentiation medium to hSMCs in scaffolds uniformly; the shear stress is also important for cell differentiation.

BMP-2 was confirmed by color development under all conditions. In particular, after 14 days of dynamic cultivation, higher cell density and stronger color development were observed. These phenomena are also in agreement with the prior observation that dynamic cultivation improves BMP-2 expression of hMSCs in 3D scaffolds [28]. Osteopontin, which is reported as being secreted by osteoblasts at an early stage of bone development, promotes cell attachment necessary for mineralization of the matrix [29]. In this study, the expression of osteopontin was confirmed in dynamic cultivation for both 7 days and 14 days, but not in static cultivation. This finding is consistent with previous reports that osteopontin can be readily induced by fluid flow in dynamic cultivation [30] and that osteoblasts are sensitive even to limited mechanical influences [10]. Since BMP-2 was confirmed in both static and dynamic cultivation and the expression of osteopontin was recognized at 7 days and 14 days, hMSCs were considered to be differentiated to the osteoblast-like cells or preosteoblasts.

Flow shear stress increases with an increase in perfusion speed, which stimulates cell proliferation and the formation of the extracellular matrix, including collagen, under dynamic cultivation [31]. However, the benefits of flow shear stress on the proliferation of hMSCs may depend on the flow rate and the type of bioreactor, cell, scaffold, or medium [32,33]. Accordingly, further study is necessary to clarify the appropriate perfusion speed for the combination of materials.

In conclusion, the results in this study revealed that by culturing with osteogenic differentiation medium, both the proliferation and bone differentiation of hMSCs were accelerated in 3D culture with dynamic cultivation using RFB. Thus, the method using preosteoblast-like cells may reduce the recognition of foreign substances as well as the hindrance of medium penetration in the scaffold caused by calcification. This indicates that the cultivation used in this study is believed superior for bone tissue engineering compared to the use of conventional cultured bone that is calcified scaffold in vitro. The possibility and efficacy

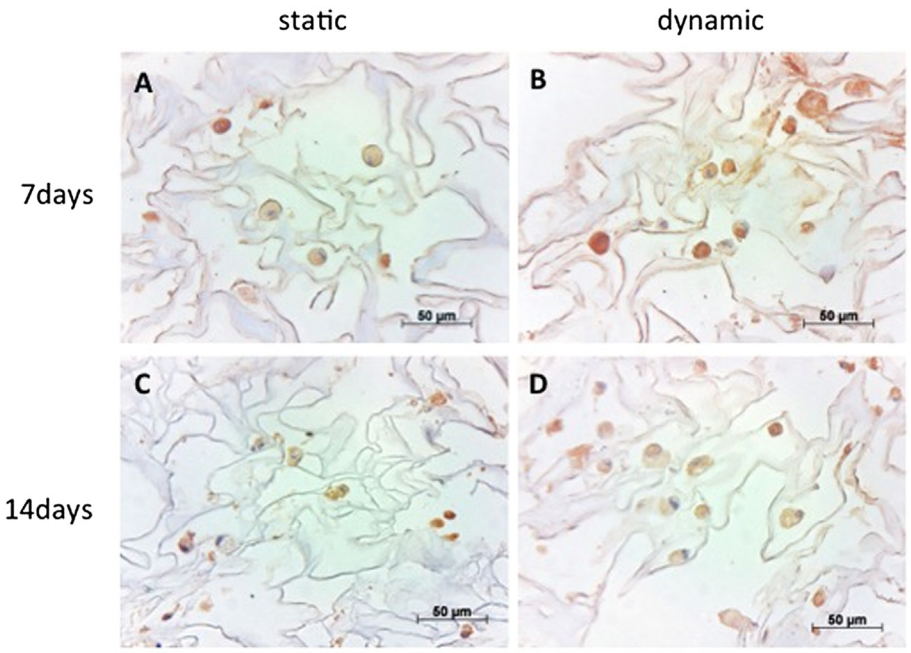

Fig. 9. Typical optical micrographs of specimens stained with BMP-2 antibodies in ODM. (A) 7 days static cultivation. (B) 7 days dynamic cultivation. (C) 14 days static cultivation. (D) 14 days dynamic cultivation. (×320; scale bar: 50 μm).

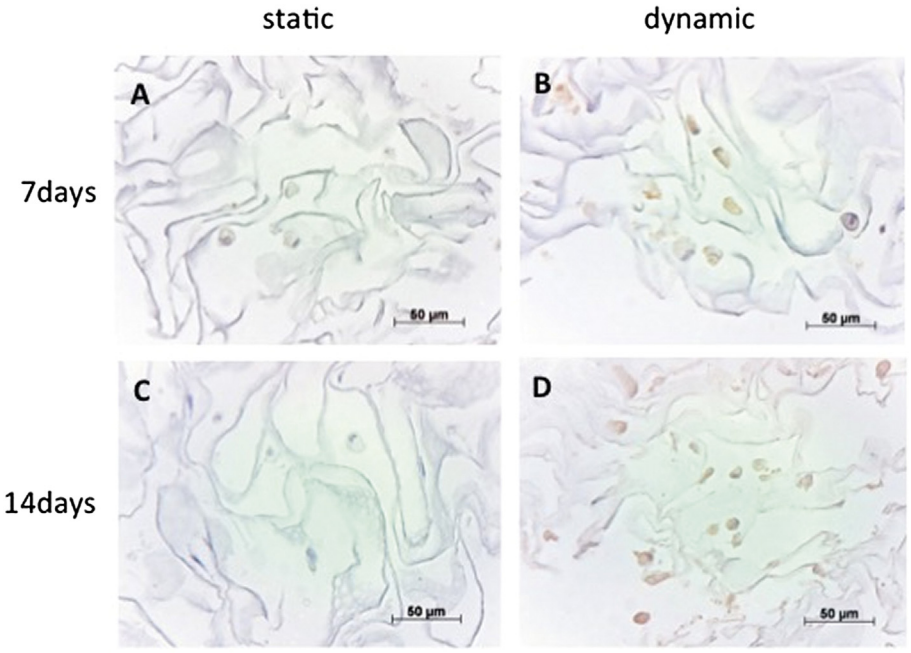

Fig. 10. Typical optical micrographs of specimens stained with osteopontin antibodies in ODM. (A) 7 days static cultivation. (B) 7 days dynamic cultivation. (C) 14 days static cultivation. (D) 14 days dynamic cultivation. (×320; scale bar: 50 μm).

of using ODM for the 3D culture of hMSCs by RFB has been confirmed, suggesting advantageous future clinical applications of RFB cell culture and cell transplantation for tissue engineering.

Conflict of interest

The authors have no conflict of interest to declare.

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