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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

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Abstract

Human mesenchymal stem cells (hMSCs) are pluripotent cells, and have been expanded and differentiated into several kinds of mesodermal tissue in vitro. In order to promote bone repair, the enhancement of proliferation and differentiation of hMSCs towards osteoblasts in vitro is recommended prior to therapeutic delivery. However, for clinical application, it is still unclear which method is more advance for tissue engineering to transplant undifferentiated or to some extent differentiated stem cell. Therefore, the present study aimed to investigate how osteogenic differentiation medium (ODM) affect the hMSCs cultured in 3D scaffold by 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 fabrication of scaffolds. 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. In 14 days ODM culture, significant increase in cell number and higher density of cell distribution in the scaffold were observed both static and dynamic cultivation compared to GM culture. Significant increase of ALP activity in 14 days ODM is

recognized in dynamic cultivation compared to static cultivation. Cells that BMP-2 was expressed were frequently observed for 14 days in dynamic culture compared with other conditions, and expression of osteopontin was confirmed in dynamic cultivation for both 7 days and 14 days. Results in this study revealed that both the proliferation and bone differentiation of hMSCs in 3D culture by RFB were accelerated by the culture of osteogenic differentiation medium, suggesting an advantageous future clinical application of RFB cell culture and cell transplantation for tissue engineering.

Introduction

Large bone defects caused by trauma, inflammation, tumors, or congenital abnormalities are often treated with autologous or allogenic bone grafts. The implantation of autologous bone grafts are the most popular due to their high performance in terms of osteogenesis, the only drawback being there limited availability due to donor site morbidity. Allogenic bone grafts are less attractive due to the risk of immunogenicity, donor-to-host transmission of disease (e.g. HIV), 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 creation of bioartificial tissues and even organs. Human mesenchymal stem cells (hMSCs) are pluripotent cells, can be readily isolated from adult donors with a 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 directly apply to clinical treatment. Static culturing of MSCs on porous scaffolds and maturation is the simplest method of developing 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, lack of influence from shear stress and mechanical loading, in static culture efficient osteoinduction

seems difficult to achieve. Accordingly, specialized dynamic culture systems, called bioreactors, have been used in bone tissue engineering. Such a dynamic three-dimensional (3D) culture system may represent a more physiological environment than a dish and 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, 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, allowing comparatively larger tissues to be constructed.[14-17] To allow even distribution of oxygen, culture medium is pumped to the center of the chamber from the periphery under low shear stress.

In order to promote bone repair as one approach, the enhancement of proliferation and differentiation of hMSCs towards osteoblasts in vitro is recommended prior to therapeutic delivery.[4] In the previous study, it is reported that the preosteoblast-like cells and hMSCs were expand uniformly over a 3D scaffold under dynamic cultivation using an RFB, and the hMSCs was not changed in cellular characteristics compare to static cultivation in DMEM without bone differentiation medium.[14,18] However, for clinical application, it is still unclear which method is more

advantageously for tissue engineering to transplant undifferentiated or to some extent differentiated stem cell.

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

Materials and Methods

Figure.1 shows a summary of the study protocol.

Culture of human MSCs

hMSCs derived from human bone marrow (PT-2501; Lonza Walkersville,) and donated by a 19-year-old man 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 unit/ mL penicillin–streptomycin (Gibco) was used as growth media (GM) for static and dynamic cultivation as well as preculture. On the other hand, GM supplemented with 50nM dexamethasone, 0.2mM ascorbic acid and 10mM b-glycerophosphate was used as an osteogenic differentiation medium (ODM) for static and dynamic cultivation.

Cells suspension containing 5.0×10^5 cells was seeded into 75cm^2 flasks, and 20 mL fresh culture medium 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. At 1-week 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).

Preculture

Method of cell seeding is based on Katayama's study.[18] Briefly, type 1 collagen sheets were placed in a 12-well plate and cell suspension (80μL) 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, they were turned over, and a further 80μL cell suspension containing 1.0×10^5 cells was added before a further 6 h incubation (Finally, total cell seeding density is 2.0×10^5 cells per sheet).

Dynamic cultivation

Figure.2 shows the RFB (Able) and RFB cell culture system used. To form a scaffold, 3 precultured sheets were placed in the RFB in layers after incubation for 12 h (6 h + 6 h). 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 14days in each of the GM and ODM as shown in Figure 1

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 2mL. 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.shows cultivation condition.

Histological analysis

Figure 3 shows cross section of layered 3 collagen sheets used for each analysis.

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

DNA-based cell count

At 7 days or 14 days of culture, scaffolds with 3 cultured sheets from the RFB were selected for a DNA-based cell count. They were divided into upper, middle, and lower areas from top to bottom (Fig.3). Single collagen sheets were also selected from the static cultivation, because precultured collagen sheet was not laminated in static cultivation. This method of cell counting was

selected based on an earlier study by this group.[18]

Total DNA was quantified with the ND-1000 (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 count of the 3 areas under dynamic cultivation was compared with that under static cultivation.

Alkaline phosphatase (ALP) activity

Scaffolds ware harvested from 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 30s after application of 200 μL Triton-X. The lysates obtained were centrifuged at 15,000rpm for 15min, and the supernatant was used as sample. ALP activity was assayed using LabAssay ALP (Wako). Sample absorbance was measured in a 96-well plate at 405nm. Amount of total protein in the sample was then examined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Finally, ALP activity was expressed as units/μg protein.

Immunocytochemical analysis

Immunocytochemical analysis was carried out at 7 days or 14 days 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 section 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 ab 63856), overnight below 4°C. The sections were washed in PBS and then incubated with the secondary antibody, peroxidase-labeled anti-mouse IgG polyclonal antibody (Histofine Simple Stain MAX-PO, MULTI, Nichirei), for 30 min and 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 and the sections were observed using a universal photomicroscope (Axiophot 2).

Statistical analysis

The DNA-based cell count and ALP activity was statistically analyzed using a one-way analysis of variance, followed by the multiple-comparison with fisher’s LSD.

Results

Histological analysis

Figure 4 and 5 shows optical micrographs of hematoxylin-eosin staining. The cells were distributed densely in the scaffold both static and dynamic culture by ODM at 7 days of culture. (Fig.4). A higher cell density was observed in ODM under dynamic culture and no difference was

observed between static and dynamic in GM. For 14 days culture, cells are distributed more densely under both static and dynamic culture by ODM. (Fig.5) Compare to static culture, a higher cell density was observed in both GM and ODM under the dynamic culture.

DNA-based cell count

The DNA-based cell count of each area is shown in Figure 6 and 7. Figure 6 shows the comparison of cell numbers in each area (upper, middle, lower) under dynamic cultivation. No significant difference in cell numbers was observed among the 3 areas.

The number of cells increased for 14 days compared to 7 days on both static and dynamic culture in the ODM. Comparison of cell numbers between GM and ODM under static and dynamic cultivation at 7 days and 14 days of cultivation is shown in Figure 7. On dynamic cultivation, mean of 3 areas was chosen for comparison with that under static cultivation because of no significant difference in cell numbers among the 3 areas. At 7 days of cultivation, no significant difference in cell numbers between GM and ODM was found under either static or dynamic culture. On the other hand, a significant difference in cell numbers between GM and ODM was observed under both static and dynamic culture (approximately 1.7-1.9 times) in ODM for 14days (** $p < 0.01$). Significant increases in cell numbers under dynamic cultivation were noted in both GM and ODM for 7 and 14

days compare to static culture (approximately 1.4-1.8 times) (*p< 0.05, **p < 0.01).

ALP activity

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

Immunocytochemical analysis

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

Discussion

This study aimed to investigate the effects of osteogenic differentiation medium on hMSCs seeded in 3D scaffold 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.

As reported by Yoshinari et al., there is a possibility that the culture medium cannot penetrate the scaffold equally due to calcification under long term 3D culture of hMSCs in osteogenic differentiation medium using RFB.[19] To avoid the influence, we set the culture period as 7 days and 14 days.

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

Compared to static culture, dynamic culture demonstrated significantly increased cell numbers and higher cell distribution density in scaffold regardless culture time and culture medium. It is possibly due to a more efficient delivery of nutrients and exchange of gas, along with the

elimination of metabolic waste under dynamic cultivation.[20, 21] Accordingly, the cell death associated with usual 3D culture is partially prevented by the dynamic cultivation.[22]

In this study, ALP activities as well as expression of BMP-2 and osteopontin were analyzed to investigate how ODM affects osteogenic differentiation of hMSCs in 3D culture by RFB. ALP activity and BMP-2 are known as early markers of osteoblastic differentiation.[22, 23] By culturing osteogenic differentiation medium, increase of ALP activity was observed in all experiment conditions and there is a significant difference between static and dynamic culture for 14 days. ALP activity is upregulated and the bone differentiation speed is promoted in dynamic cultivation compared to static cultivation. Dynamic culture 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 and the shear stress are also important for cell differentiation.

BMP-2 was confirmed by color development in all condition, and especially in 14 days dynamic culture, higher cell density and stronger color development were observed. This phenomena are also in agreement with the prior observation that dynamic culture improves BMP-2 expression of hMSCs in 3D scaffolds.[28] Osteopontin is reported as secreted by osteoblasts at an early stage of bone development and promotes cell attachment necessary for mineralization of the matrix.[29] In this study,

expression of osteopontin was only 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 the fluid flow in the dynamic culture[30], and the osteoblasts are sensitive even to limited mechanical influences.[10]

Flow shear stress increases with an increase in the perfusion speed, which stimulates proliferation of cells and the formation of the extracellular matrix, including collagen, under dynamic cultivation.[31] However, the benefit of flow shear stress to 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.

I n conclusion, the results in this study revealed that by culturing 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 the preosteoblast-like cells may reduce the recognition of a foreign substance as well as the hindrance of medium penetration in the scaffold caused by the calcification, indicating that the cultivation used in this study is believed to be better for bone tissue engineering compared to using the conventional cultured bone that is calcified scaffold *in vitro*. The possibility and effectivity to use ODM for 3D culture of hMSCs by RFB is confirmed, which suggested an advantageous future clinical application of RFB cell culture and cell

transplantation for tissue engineering.

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Figure legends

TABLE 1. Cultivation condition

FIG. 1. A flowchart of the present study.

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

FIG.3. Cross section of layered 3 collagen sheets used for each analysis. Scaffolds in RFB were divided horizontally and perpendicularly into 9 areas consisting of 3 sheets (from top to bottom: upper, middle, and lower) × 3 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 3 sheets (upper, middle, and lower).

FIG.4. Typical optical micrographs of specimens stained with hematoxylin–eosin. (7days)

(A) GM static cultivation.(B) GM dynamic cultivation. (C) ODM static cultivation. (D) ODM dynamic cultivation. (×200; scale bar:100μm)

FIG.5. Typical optical micrographs of specimens stained with hematoxylin–eosin.(14days)

(A) GM static cultivation.(B) GM dynamic cultivation. (C) ODM static cultivation. (D) ODM dynamic cultivation. (×200; scale bar:100μm)

FIG.6. Comparison of cell numbers (DNA-based cell count) in each area under dynamic cultivation. No significant difference was observed among the 3 areas. Data are expressed as mean ± SD over 5 cultures.

FIG.7. Comparison of cell numbers between GM and ODM under static and dynamic cultivation. Under dynamic cultivation, mean of 3 areas was chosen for comparison with that under static cultivation. Data are expressed as mean ± SD over 5 cultures.

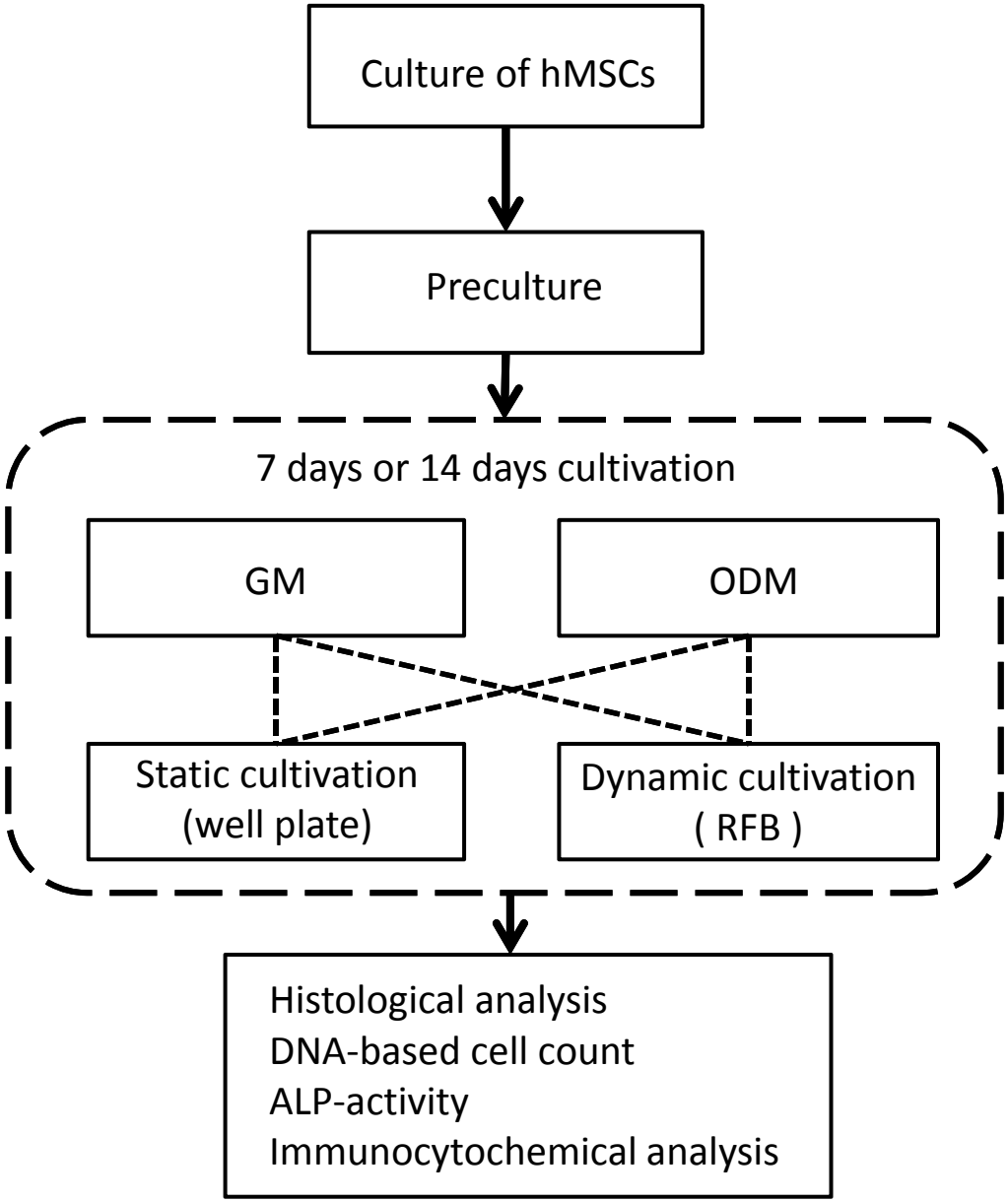
FIG.8. Comparison of ALP activity (units/μg protein) between GM and ODM under static and dynamic cultivation. Under dynamic cultivation, mean of 3 areas was chosen for comparison with that under static cultivation. Data are expressed as mean ± SD over 5 cultures.

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:50μm)

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

TABLE 1. Cultivation condition

	static	dynamic
Cell number	2.0×10^5 cells / scaffold	
Temperature	37℃	
CO_2	5%	
pH		7.4
DO		6.86
Medium flow late		3ml / min
Medium volume	2ml / well	100ml / day
Medium change	Every 3 days	Changed daily after 3days
scaffold	1 sheet / well	3 sheets / reactor



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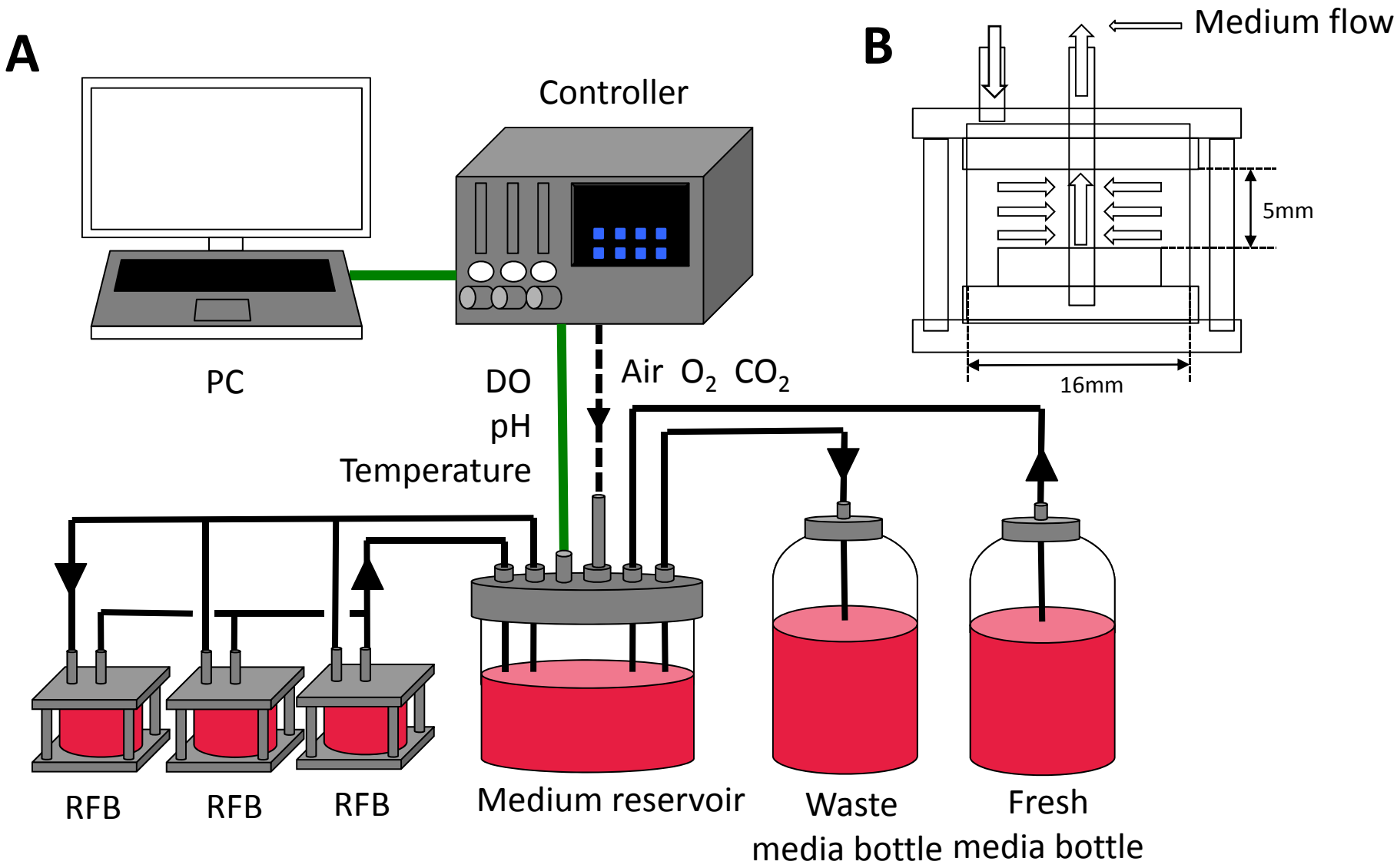

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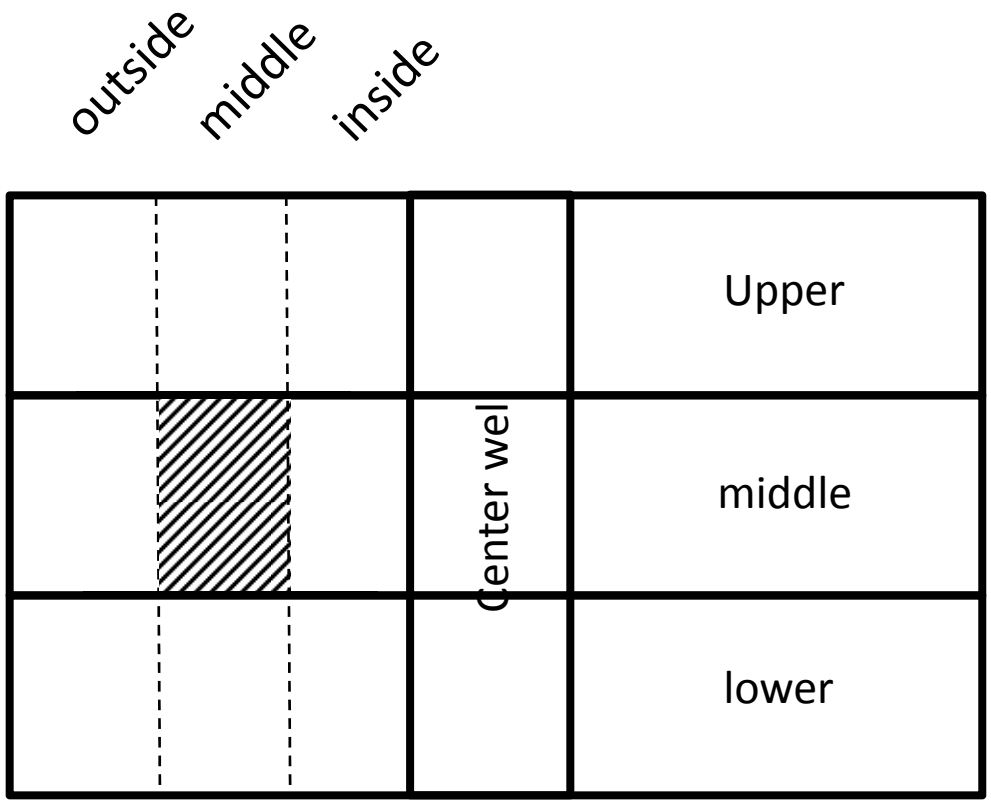

FIG.3. Cross section of layered 3 collagen sheets used for each analysis. Scaffolds in RFB were divided horizontally and perpendicularly into 9 areas consisting of 3 sheets (from top to bottom: upper, middle, and lower) × 3 areas (inside, middle, and outside). Histological analysis and immunocytochemical analysis were performed using the middle area of the middle sheet (shaded area). DNA based cell count and ALP activity were evaluated using 3 sheets (upper, middle, and lower).

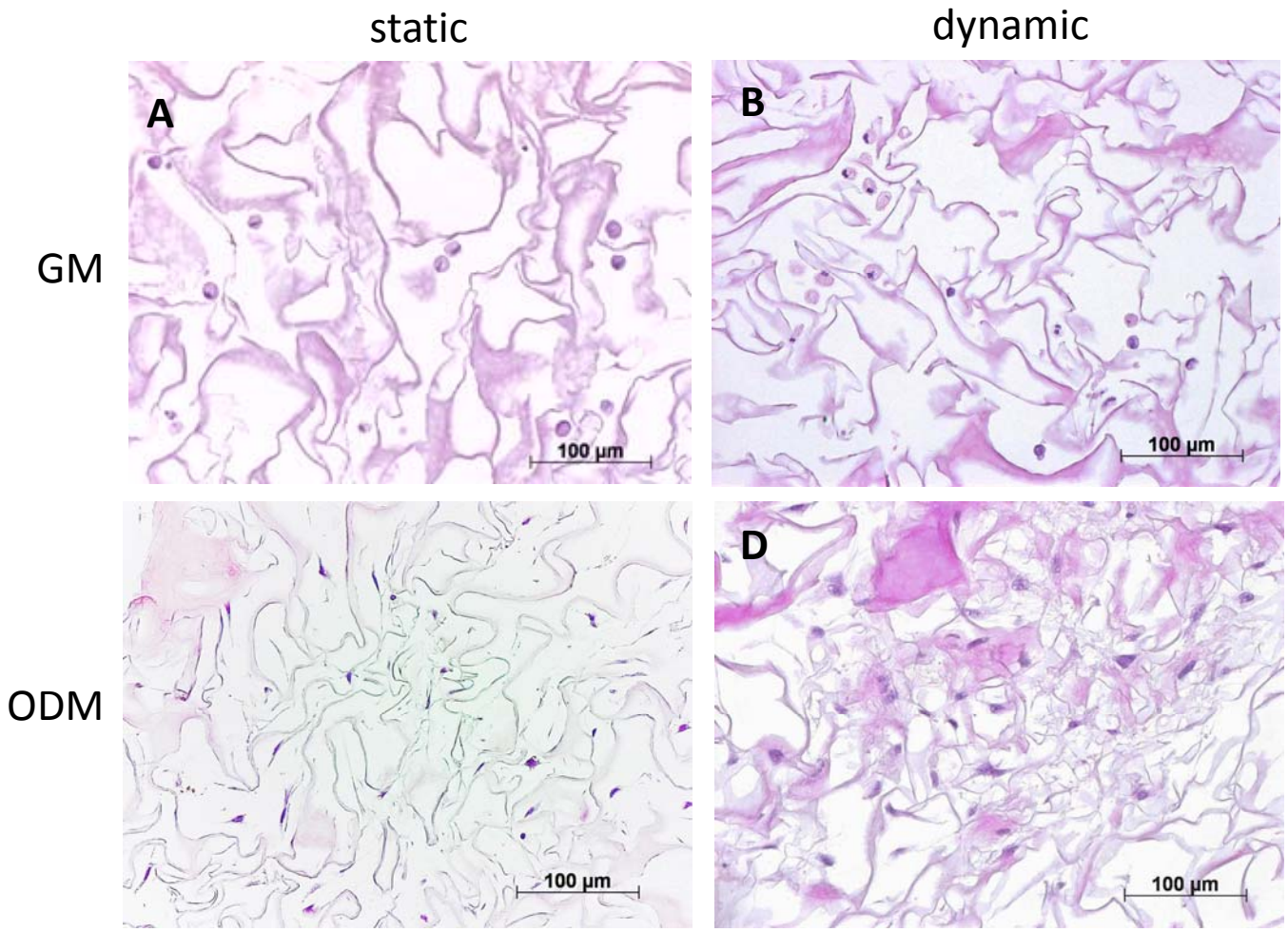

FIG. 4. Typical optical micrographs of specimens stained with hematoxylin–eosin.(7days)

(A) GM static cultivation.(B) GM dynamic cultivation. (C) ODM static cultivation. (D) ODM dynamic cultivation. (× 200; scale bar:100μm)

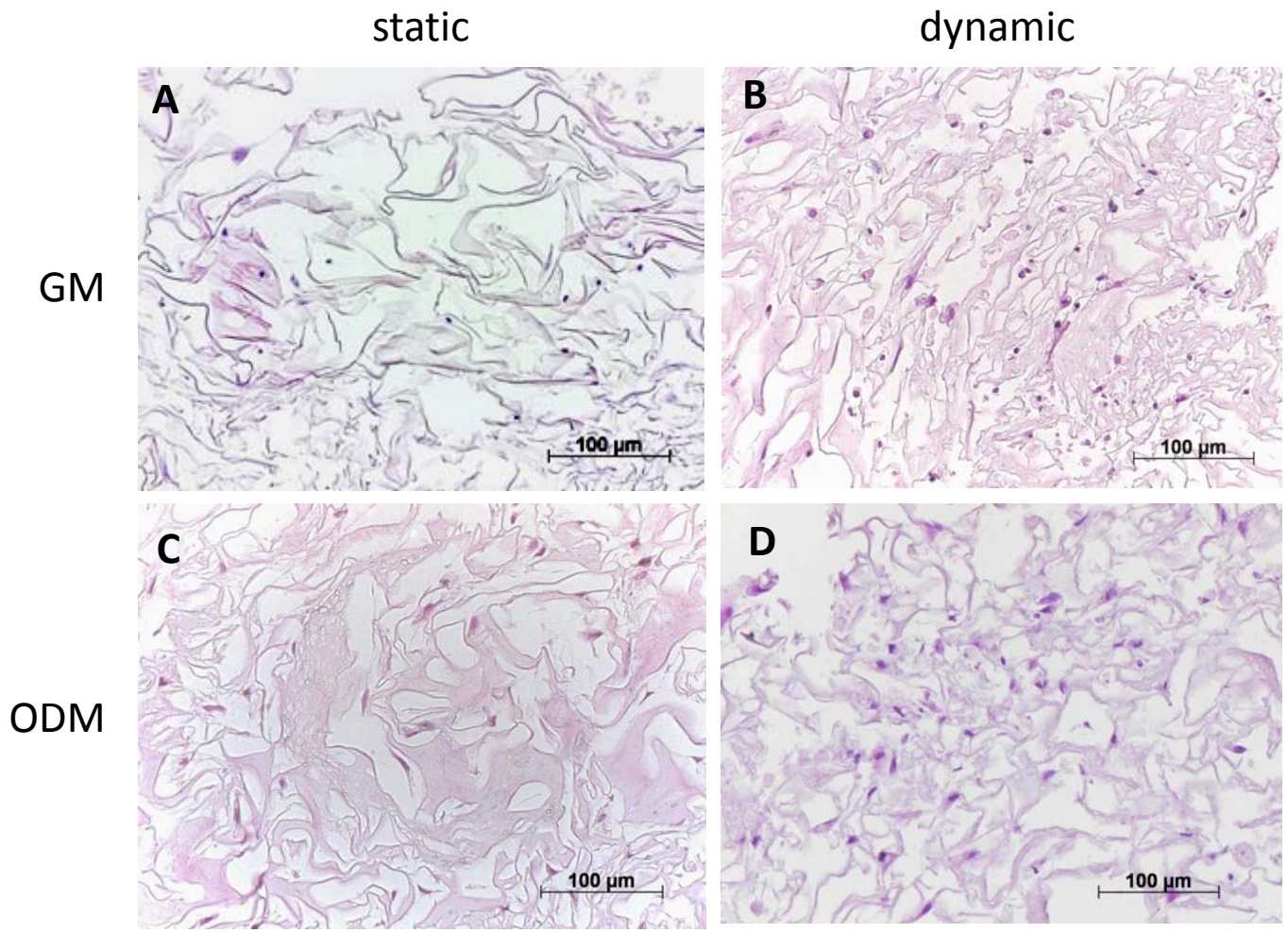

FIG.5. Typical optical micrographs of specimens stained with hematoxylin–eosin.(14days) (A) GM static cultivation.(B) GM dynamic cultivation. (C) ODM static cultivation. (D) ODM dynamic cultivation. (× 200; scale bar:100μm)

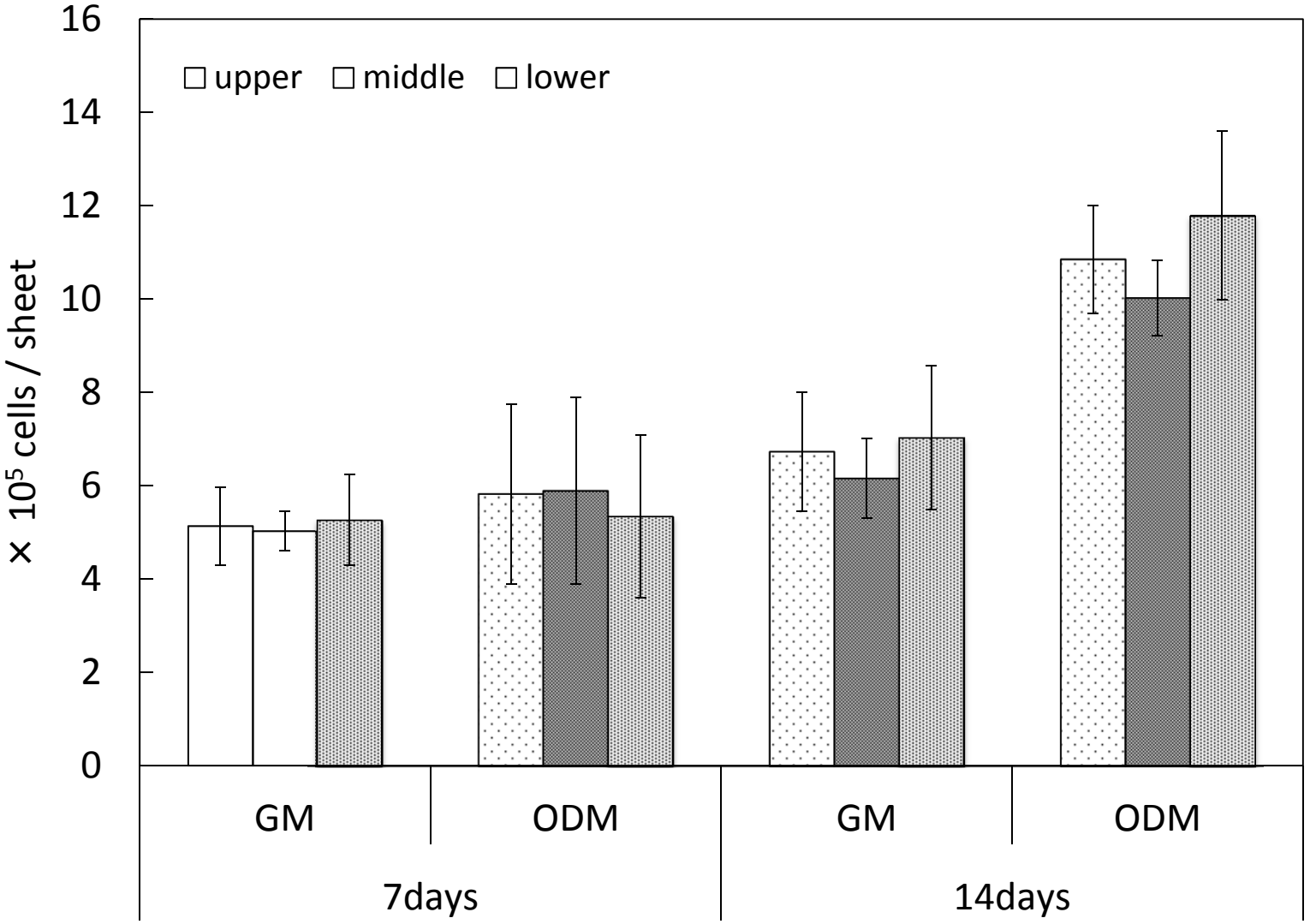

FIG.6. Comparison of cell numbers (DNA-based cell count) in each area under dynamic cultivation. No significant difference was observed among the 3 areas. Data are expressed as mean ± SD over 5 cultures.

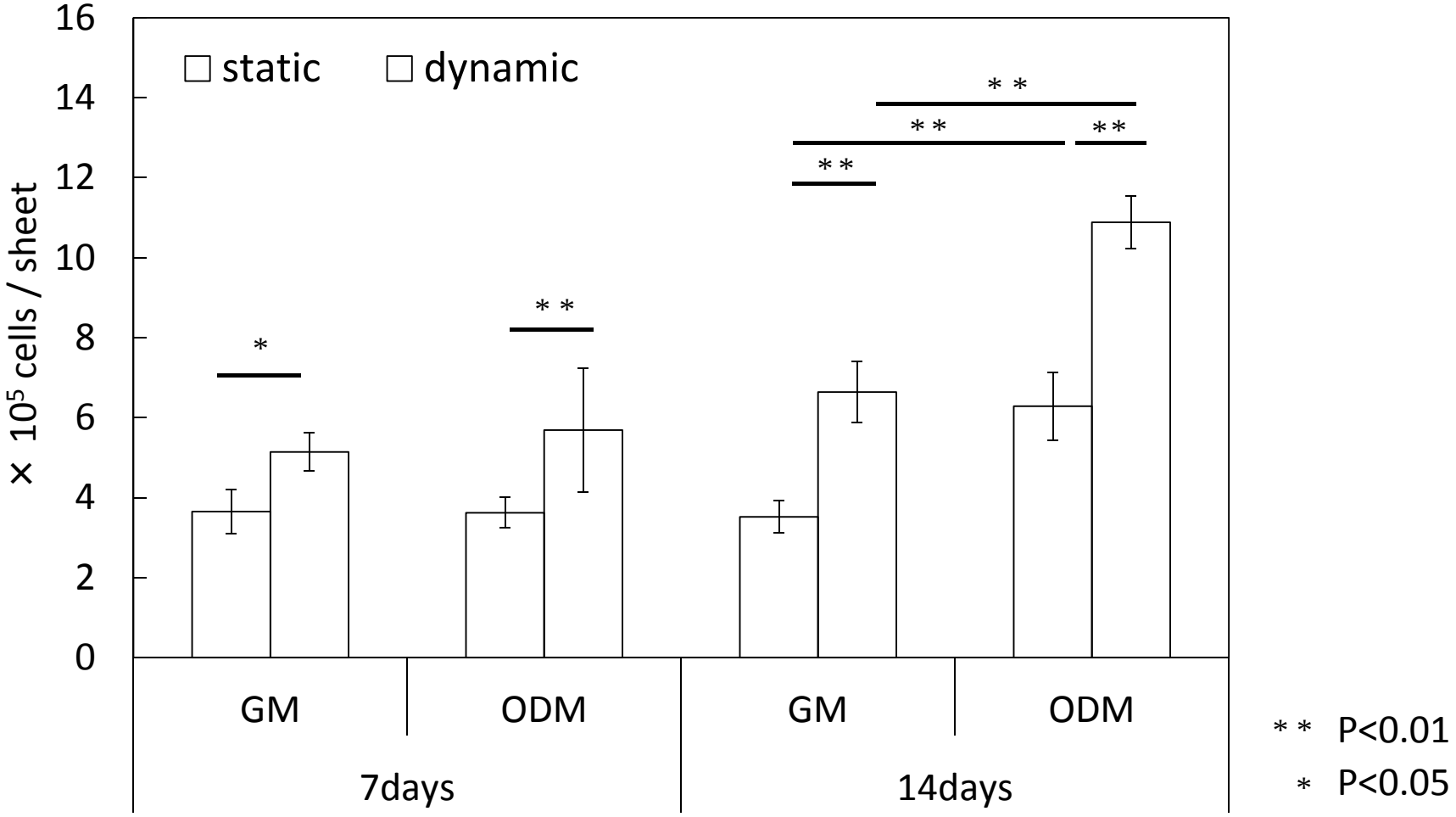

FIG.7. Comparison of cell numbers between GM and ODM under static and dynamic cultivation. Under dynamic cultivation, mean of 3 areas was chosen for comparison with that under static cultivation. Data are expressed as mean ± SD over 5 cultures.

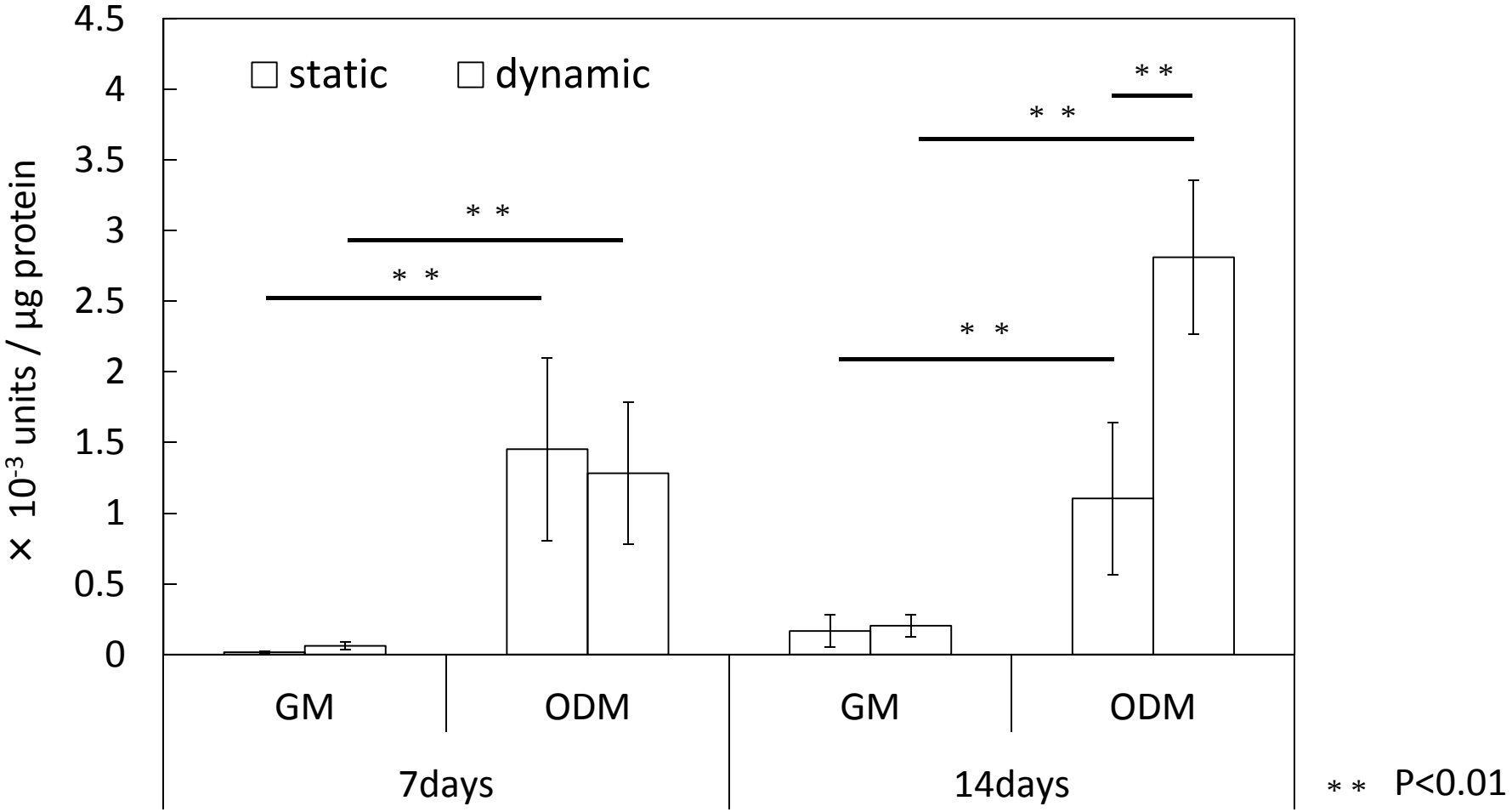

FIG. 8. Comparison of ALP activity (units/μg protein) between GM and ODM under static and dynamic cultivation. Under dynamic cultivation, mean of 3 areas was chosen for comparison with that under static cultivation. Data are expressed as mean ± SD over 5 cultures.

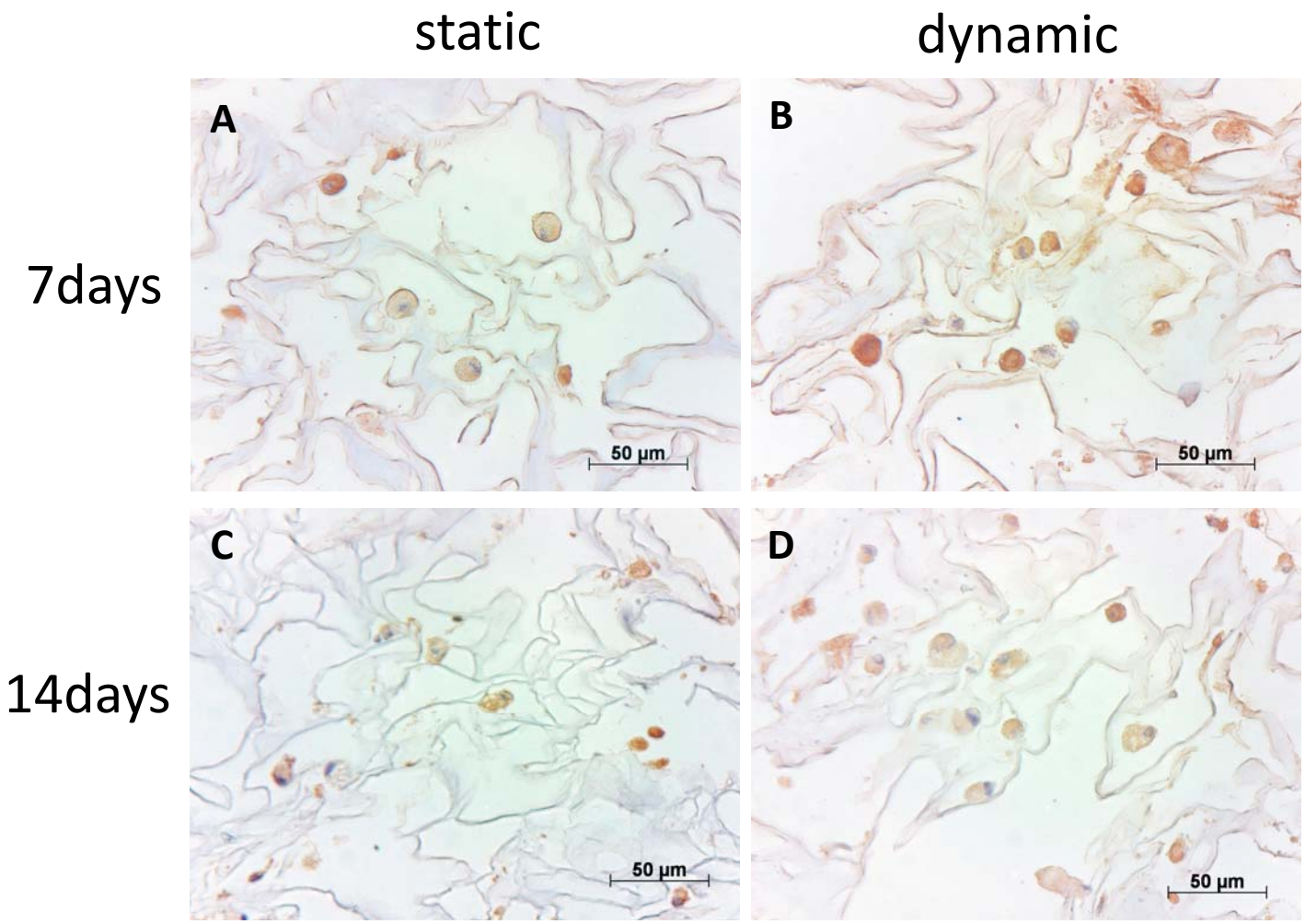

FIG.9. Typical optical micrographs of specimens stained with BMP-2 antibodies in ODM. (A) 7 days static cultivation.(B) 7 days dynamic cultivation. (C) 14 days static cultivation. (D) 14 days dynamic cultivation. (× 320; scale bar:50μm)

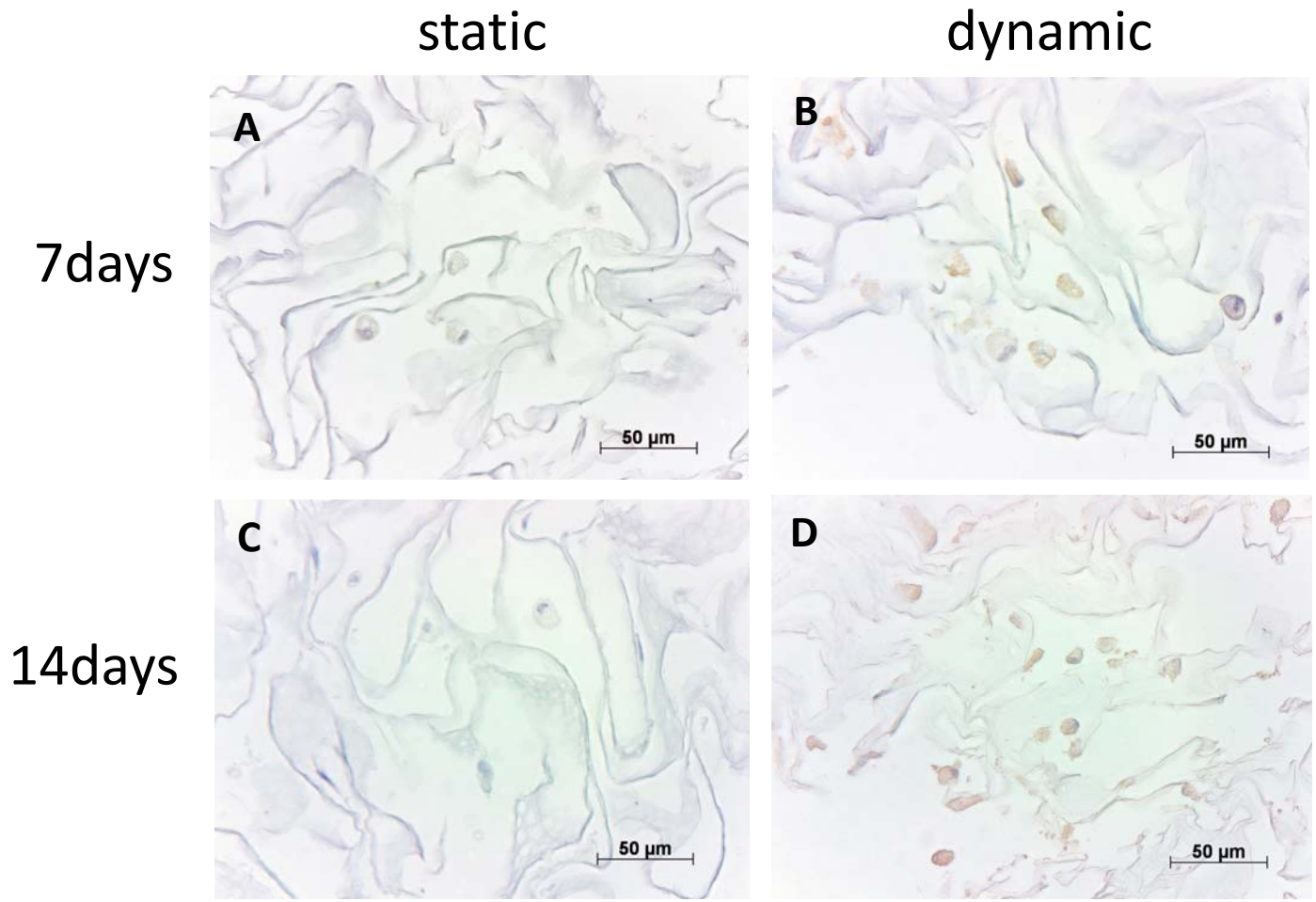

FIG.10. Typical optical micrographs of specimens stained with osteopontin antibodies in ODM.
(A) 7 days static cultivation.(B) 7 days dynamic cultivation. (C) 14 days static cultivation. (D) 14days dynamic cultivation. (× 320; scale bar:50μm)