Influence of shaking culture on the biological functions of cell aggregates incorporating gelatin hydrogel microspheres

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The objective of this study is to investigate the influence of shaking culture on the biological functions of cell aggregates incorporating gelatin hydrogel microspheres in terms of the microspheres/cells ratio. The mixture of MC3T3-E1 cells and the microspheres was cultured in the U-bottomed wells of 96-well plate pre-coated with poly (vinyl alcohol) (PVA) to form cell aggregates incorporating microspheres. When incubated in the static or shaking culture, the size of cell aggregates increased with amounts of gelatin hydrogel microspheres but was similar between the two cultures. At the smaller ratio of microspheres to cells, the viability of cell aggregates under the shaking culture was significantly higher than that of static culture. On the other hand, there was no significant difference in the viability between them at the higher ratio. Gelatin hydrogel microspheres enabled to enhance ATP and mitochondrial activities of cell aggregates under the shaking culture. The effect was high at the smaller microspheres/cells ratio. It is concluded that the shaking culture was promising to allow cells to enhance their activities.

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[Key words: Tissue engineering; Gelatin hydrogel microspheres; Cell aggregates; Shaking culture methods; Biological functions]

Cells are often cultured in two-dimensional (2D) systems such as a cell culture dish or plate, and the 2D culture systems are known as a conventional cell culture. It is easy to culture and to observe cells under the 2D systems. However, the 2D cell culture systems are not suitable for researches of cell proliferation, cell differentiation, regenerative medicine, and drug discovery because the 2D systems are quite different from the inner body and the local environment. In the body tissues, most cells tend to form cell aggregates, resulting in enhanced cell differentiation (1), the metabolic activity (2), cell—cell interaction (3), and production of extracellular matrix proteins (4). Therefore, recently, development of three-dimensional (3D) cell culture technologies have been reported in order to mimic the local environment of cells in living tissues (5—8). However, there are some disadvantages of the 3D cell culture systems. For example, little of oxygen and nutrients are supplied to cells present in the center of cell aggregates during growth of cell aggregates, resulting in cell death (9,10). Therefore, it is impossible to culture cell aggregates over a long time period because of cell death. However, in order to investigate cell proliferation or differentiation, it is necessary to culture cell aggregates over a long time period. To tackle this serious problem, we have developed cell aggregates incorporating hydrogel materials, resulting in protected cells aggregated from the lack of oxygen and nutrients in the long-term culture (11). Gelatin, which is recognized as a biodegradable biomaterial, is used for many fields such as medical, pharmaceutical, and cosmetic application (12—32). In this study, gelatin was selected because it is easy to form a hydrogel and its biosafety has been proven (12).

Shaking culture methods are often used in cell culture to enhance biological functions such as improvement of cell availability (33), promotion of bone proliferation from mesenchymal stem cells (34), and release of more types of proteins from mesenchymal stem cells (35). Because the shaking culture methods supply more oxygen and nutrients to cells compared with the static culture methods. However, the influence of shaking culture on the biological functions of cell aggregates incorporating gelatin hydrogel microspheres (GMs) has been never investigated. The objective of this study is to investigate the influence of shaking culture on the biological functions of cell aggregates. Following MC3T3-E1 cells were cultured with GMs at different mixing ratios of microspheres/cells under the static or shaking culture, the sizes, morphologies, ATP, mitochondrial activities, and oxygen concentration of cell aggregates were evaluated.

MATERIALS AND METHODS

Preparation of GMs GMs were prepared by the chemical crosslinking of gelatin in a water-in-oil emulsion state according to the method previously reported (24). Briefly, an aqueous solution (20 ml) of 10 wt % gelatin (isoelectric point 5.0, weight-averaged molecular weight = 100,000, Nitta Gelatin Inc., Osaka, Japan) was preheated at 40°C, followed by stirring at 300 rpm for 10 min to prepare the water-in-oil emulsion. The emulsion temperature was decreased at 4°C for the natural gelation of gelatin solution to obtain non-crosslinked

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hydrogel microspheres. The resulting GMs were washed three times with cold acetone in combination with centrifugation (5000 rpm, 4°C, 5 min) to completely exclude the residual oil. Then, GMs were fractionated by size using sieves with apertures of 32 and 53 μm (Iida Seisakusho Ltd., Osaka, Japan) and air dried at 4°C. Then, non-crosslinked and dried GMs (200 mg) were treated in a vacuum oven at 140°C to allow to dehydrothermally crosslink for 72 h. The picture of GMs in the swollen state was taken with a microscope (BZ-X710, Keyence Ltd., Osaka, Japan). The size of 100 microspheres for each sample was measured using the computer program Image J (NIH Inc., Bethesda, MD, USA) to calculate the average diameters.

Cell culture experiments　MC3T3-E1 cells of a pre-osteoblast line derived from mouse (KAC Co., Ltd., Kyoto, Japan) were cultured in alpha minimum essential medium (αMEM) (Invitrogen Inc., Carlsbad, CA, USA) supplemented with 10 vol % fetal calf serum (Thermo Inc., Waltham, MA, USA), penicillin (50 U/mL), and streptomycin (50 U/mL) (standard medium) and cultured at 37°C in a 95 % air−5 % carbon dioxide atmosphere. The culture medium was changed every 2 days and the confluent cells were subcultured through trypsinization.

Preparation of cell aggregates incorporating various amounts of GMs and their culture under shaking or static culture　A poly (vinyl alcohol) (PVA) sample (the degree of polymerization = 1800 and the saponification = 88 mol %) kindly supplied from Unichika (Tokyo, Japan) was dissolved in phosphate-buffered solution (PBS) (pH 7.4, 1wt %). The PVA solution was added to each well of round-bottomed (U-bottomed) 96-well culture plate (100 μl/well) and incubated at 37°C for 15 min. Then, the solution was removed by aspiration and the wells washed twice with PBS (100 μl/well). Gelatin microspheres dehydrothermally crosslinked for 72 h and MC3T3-E1 cells were separately suspended in the standard medium. After the suspensions of GMs (0 or 1×10^3, 2×10^3, 3×10^3, and 4×10^3 microspheres/ml, 50 μl) and cell suspensions (2×10^4 cells/ml, 100 μl) were mixed, the mixture was added to the wells coated. After 2 days (cell aggregates were formed), some samples were cultured by an orbital shaker (Bellco Glass, Inc., Vineland, NJ, USA) at 30 rpm until over the time periods of 21 days, and then the medium was changed on the first day and every 3 days until the end of experiments. Experiments for each sample were performed three wells independently unless otherwise mentioned.

Morphologies and size of cell aggregates incorporating various amounts of GMs under shaking or static culture　In order to investigate the influence of the amounts of GM incorporated under the shaking culture on the morphologies and size of cell aggregates in the long-term culture, the morphologies and the size of cell aggregates were evaluated. The pictures of cell aggregates without GM or with various amounts of GM incorporated under the shaking or static culture were taken with a microscope (CKX41, Olympus Ltd., Tokyo, Japan). The size of cell aggregates was measured using the computer program Image J (NIH Inc.) to calculate the average diameter.

Evaluation of ATP activity of cell aggregates under shaking or static culture　The ATP activity of cell aggregates was determined by using Katamari ATP assay kit (Fujifilm Wako Pure Chemical Co. Ltd., Osaka, Japan) 7, 14, and 21 days after incubation. The number of cells in cell aggregates was determined by the fluorometric quantification of cellular DNA according to the method reported by Rao et al. (36). Briefly, the samples were lysed in 30 mM sodium citrate-buffered saline solution (SSC) (pH 7.4) containing 0.2 mg/ml sodium dodecyl sulfate (SDS) at 37°C for 12 h with occasional mixing. The cell lysate (30 μl) was mixed with a dye solution (70 μl; 30 mM SSC, 10 μg/ml Hoechst 33,258 dye), and then the fluorescent intensity of mixed solution was measured in a fluorescence spectrometer (F-2000, Hitachi Ltd., Tokyo, Japan) at excitation and emission wavelengths of 355 and 460 nm, respectively. The calibration curve between the DNA and cell number was performed by use of cells of known numbers.

Evaluation of mitochondrial activity of cell aggregates under shaking or static culture　At different time intervals, cell aggregates were taken into 2 ml of the tube, and 400 μl of the medium was added. Then, 40 μl of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Nacalai Tesque Inc., Kyoto, Japan) was added to each tube, and the tubes were incubated for further 4 h to allow the mitochondria of cells to reduce the yellow MTT into dark-blue formazan crystals. The absorbance of individual wells (100 μl) was measured at 450 nm using microplate reader (F-2000, Hitachi Ltd.). The mitochondrial activity per cell of cell aggregates was calculated as the mitochondrial activity per cell of cell aggregates without GM 7 days after incubation under the static culture of 1.

Measurement of oxygen concentration of cell aggregates under shaking or static culture　The oxygen concentration of cell aggregates incorporating GMs was measured by using an O_2 sensor probe (Fibox4, Taitec Co., Saitama, Japan). Cell aggregates 14 days after incubation were sensed by the sensor probe (the diameter of 200 μm) as carefully as possible not to break or miss cell aggregates (on the dish), and then oxygen concentration was measured. One unit of cell aggregates was used per the measurement.

Statistical analysis　All the statistical data were expressed as the mean ± standard error of the mean. The data were analyzed by t-test or Tukey's test to determine the statistical significance of difference between experimental results which was accepted at the p value of < 0.05.

RESULTS

Characterization of GMs　Fig. 1 shows the microscopic pictures of GMs. The GMs were of spherical shape and had a smooth surface. The size of microspheres in the swollen condition was 50.5 ± 6.7 μm.

Culture of cell aggregates incorporating various amounts of GMs under shaking or static culture　Fig. 2A−E shows that the light microscopic pictures of MC3T3-E1 cell aggregates 7, 14, and 21 days after incubation with various amounts of GMs incorporated under the shaking or static culture. Fig. 2F shows the size of cell aggregates 7, 14, and 21 days after incubation without GMs or with various amounts of GMs incorporated. The size of cell aggregates increased upon increasing the amount of GMs in the cell aggregates. At the same amount of GMs, the size of cell aggregates did not change, irrespective of the culture condition. However, the size of cell aggregates incorporating GMs 21 days after incubation was small compared with that 7 and 14 days. GMs looked to be present in the cell aggregates 60 days after incubation (Fig. S1). Table 1 shows that cell number of cell aggregates 7, 14, and 21 days after incubation with various amounts of GMs incorporation under the shaking or static culture. The cell number of cell aggregates did not change, irrespective of the culture condition and the numbers of GMs incorporation.

ATP activity of cell aggregates incorporating GMs under shaking or static culture　Fig. 3 shows the ATP activity of cell aggregates 7, 14, and 21 days after incubation with various amounts of GMs under the shaking or static culture. When cell aggregates incorporating 0 or 1×10^3 and 2×10^3 microspheres/ml of GMs were prepared, the ATP activity of cell aggregates 7, 14, and 21 days after incubation under the shaking culture was higher than that under the static culture (Fig. 3A−C). However, when cell aggregates incorporating 3×10^3 and 4×10^3 microspheres/ml of GMs were prepared, the ATP activity was not significantly different from that under the static culture, irrespective of the culture period (Fig. 3D, E). In addition, the ATP activity of cell aggregates incorporating 3×10^3 and 4×10^3 microspheres/ml of GMs 21 days after incubation was low compared with that 7 and 14 days later (Fig. 3D, E). Moreover, the ATP activity of cell aggregates incorporating GMs was much higher than that of GMs-free culture (Fig. 3F).

Mitochondrial activity of cell aggregates incorporating GMs under shaking or static culture　Fig. 4 shows the mitochondrial activity of cell aggregates 7, 14, and 21 days after incubation with various amounts of GMs under the shaking or
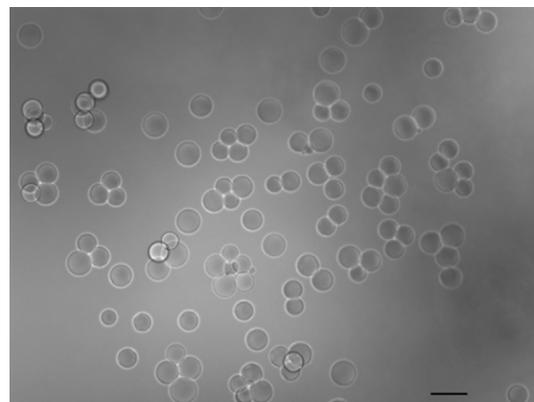

FIG. 1. Microscopic picture of GMs dispersed in water. The GMs were dehydrothermally crosslinked for 72 h at 140°C. Scale bar: 100 μm.
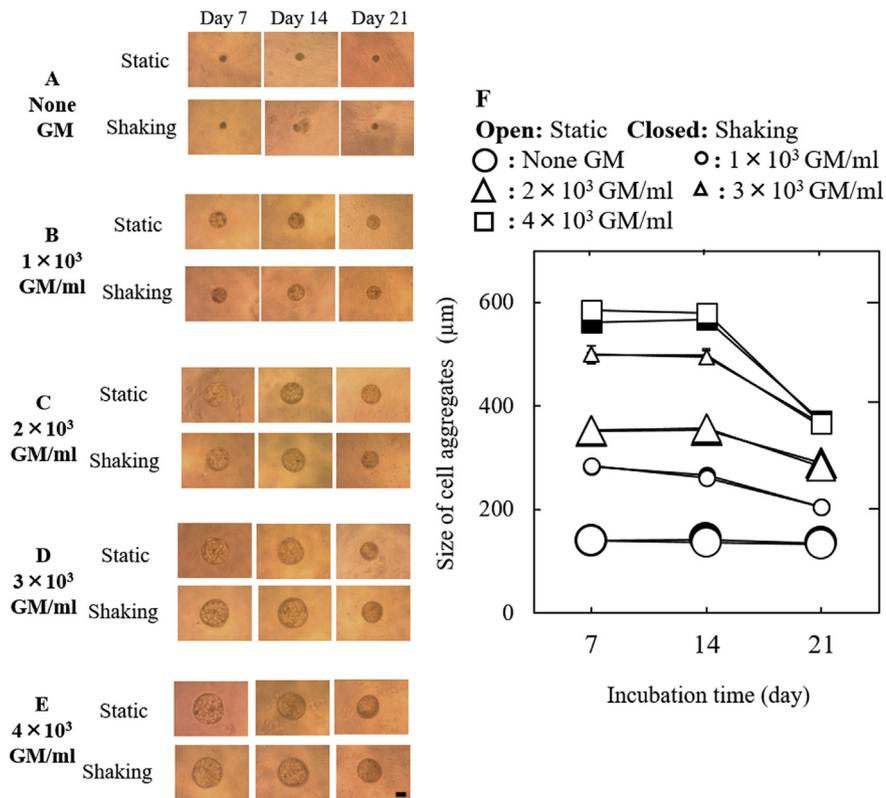
FIG. 2. Light microscope pictures of MC3T3-E1 cell aggregates 7, 14, and 21 days after incubation without GM (A) or with 1×10^3 (B), 2×10^3 (C), 3×10^3 (D), and 4×10^3 GM/ml (E) of GM under the static culture or shaking culture. Scale bar: 200 μm. (F) The sizes of MC3T3-E1 cell aggregates 7, 14, and 21 days after incubation without GM (large open circles) and with 1×10^3 (small open circles), 2×10^3 (large open triangles), 3×10^3 (small open triangles), and 4×10^3 GM/ml (open squares) of GM under the static culture, or without GM (large closed circles) and with 1×10^3 (small closed circles), 2×10^3 (large closed triangles), 3×10^3 (small closed triangles), and 4×10^3 GM/ml (closed squares) of GM under the shaking culture.

static culture. The tendency was similar to that of ATP activity (Fig. 3A–E). When cell aggregates incorporating 0 or 1×10^3 and 2×10^3 microspheres/ml of GMs were prepared, the mitochondrial activity of cell aggregates 7, 14, and 21 days after incubation under the shaking culture was higher than that under the static culture (Fig. 4A–C). However, when cell aggregates incorporating 3×10^3 and 4×10^3 microspheres/ml of GMs were prepared, the mitochondrial activity of cell aggregates was not significantly different from that under the static culture, irrespective of the culture period (Fig. 4D, E). Moreover, the mitochondrial activity of cell aggregates incorporating 4×10^3 microspheres/ml of GMs 21 days after incubation was low compared with that 7 and 14 days later (Fig. 4E). However, the mitochondrial activity of cell aggregates incorporating GMs was not always higher than that of GMs-free culture (Fig. 4F).

The oxygen concentration of cell aggregates incorporating GMs under shaking or static culture Fig. 5 shows the relative oxygen concentration of the cell aggregates incorporating 1×10^3 (A) and 4×10^3 (B) microspheres/ml of GMs 14 days after incubation under the shaking or static culture. The oxygen concentration of cell aggregates incorporating 1×10^3 microspheres/ml of GMs 14 days after incubation under the shaking culture was higher than that under the static culture (Fig. 5A). However, the oxygen concentration of cell aggregates incorporating 4×10^3 microspheres/ml of GMs under the shaking culture was not significantly different from that under the static culture (Fig. 5B).

DISCUSSION

GMs enable the cells to improve their viability and functions in cell aggregates. Preparation of cell aggregates incorporating microspheres such as gelatin, poly (lactic-co-glycolic acid) (PLGA), and alginate has been reported (13,15,37–39). Among these materials, in this study, gelatin was selected because of a cell adhesion ability. In addition, the oxygen and nutrients permeation through the water phase of GMs is expected (39). Among the microspheres properties, the mixing ratio of microspheres to cells was focused because the number of microspheres would be one of the most important factors contributing to the oxygen and nutrients permeation for cell aggregates. Shaking culture methods are also often introduced in cell culture to improve the cells viability and functions because the oxygen and nutrients permeation is expected by the dynamic motion of medium (33–35). Here, the influence of

TABLE 1. Cell number of MC3T3-E1 cell aggregates 7, 14, and 21 days after incubation without GM or with 1×10^3, 2×10^3, 3×10^3, and 4×10^3 GM/ml of GM under the static or shaking culture.

Number of GM	Culture condition	Day 7 (cells)	Day 14 (cells)	Day 21 (cells)
None GM	Static	14477 ± 1759	14465 ± 2913	15341 ± 4498
	Shaking	14197 ± 1786	14684 ± 2286	15165 ± 5078
1×10^3 GM/ml	Static	14282 ± 2499	15572 ± 2330	16272 ± 4720
	Shaking	14157 ± 3183	15747 ± 3138	16131 ± 3431
2×10^3 GM/ml	Static	14741 ± 2333	16400 ± 4200	15963 ± 2088
	Shaking	16571 ± 2109	16764 ± 1172	17134 ± 3989
3×10^3 GM/ml	Static	15157 ± 2445	17471 ± 3237	16896 ± 2459
	Shaking	15159 ± 2405	17048 ± 1044	17866 ± 4031
4×10^3 GM/ml	Static	15387 ± 2454	17078 ± 5891	18286 ± 1408
	Shaking	14950 ± 1583	17792 ± 1030	17888 ± 3424

The data represent averages from triplicate experiments.
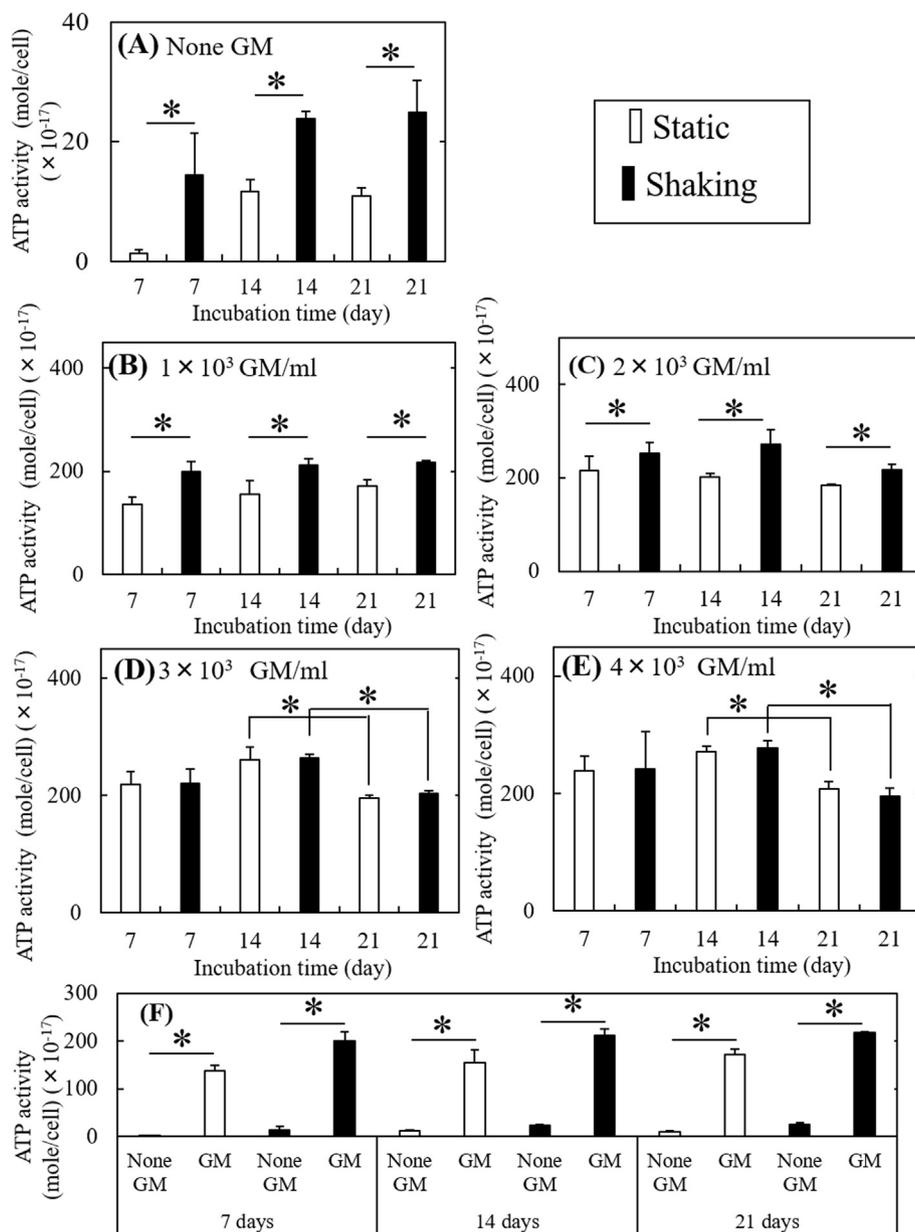
FIG. 3. ATP activity of MC3T3-E1 cells in cell aggregates without (A) or with 1×10^3 (B), 2×10^3 (C), 3×10^3 (D), and 4×10^3 GM/ml (E) of GM 7, 14, and 21 days after incubation under the static (open bars) or shaking culture (closed bars). $p < 0.05$; significant against ATP activity between the two groups. (F) ATP activity of MC3T3-E1 cells in cell aggregates without or with 1×10^3 GM/ml of GM 7, 14, and 21 days after incubation under the static (open bars) or shaking culture (closed bars). $p < 0.05$; significant against ATP activity between the two groups.

the two factors, the shaking culture and GM, on the biological functions of cell aggregates were evaluated.

In our previous study, the GMs incorporation for MCT3T3-E1 cell aggregates was characterized to optimize (39). In this study, the effect of culture procedures on the functions was evaluated. Therefore, the same MC3T3-E1 cell line was used. In this study, the GMs with diameters of 32–53 μm were used (Fig. 1). This is because the size range of GMs is demonstrated to be appropriate to form cell aggregates (39). In addition, the GMs dehydrothermally crosslinked for 72 h were used. The previous study demonstrates that the crosslinking condition was suitable for this purpose although the crosslinking extent of GMs increased with an increase in the crosslinking time (39).

We prepared various types of cell aggregates incorporating GMs by changing the mixing ratio of GMs to cells under the shaking or static culture. The cell aggregates were formed only for the U-bottomed well, in contrast to the flat-bottomed one. It is conceivable that a U-shaped bottom well allows cells to accumulate in the center of well. As a result, the frequency of cell–cell contact would increase, resulting in the better formation of cell aggregates (11). In addition, cell aggregates with or without the GMs incorporation were heavy enough to spontaneously sink down the bottom of each well. Even if the shaking was added, only the culture medium would be moved. The number of GMs did not significantly affect the formation of cell aggregates. However, cell aggregates were not formed when cells were prepared with more than 4×10^3 microspheres/ml of GMs (i.e., cells:GMs = 10:1) (Fig. S2). This proportion would be an upper limit to form cell aggregates incorporating GMs because cell–cell interaction would be weaker in the presence of too much GMs. In addition, the culture condition
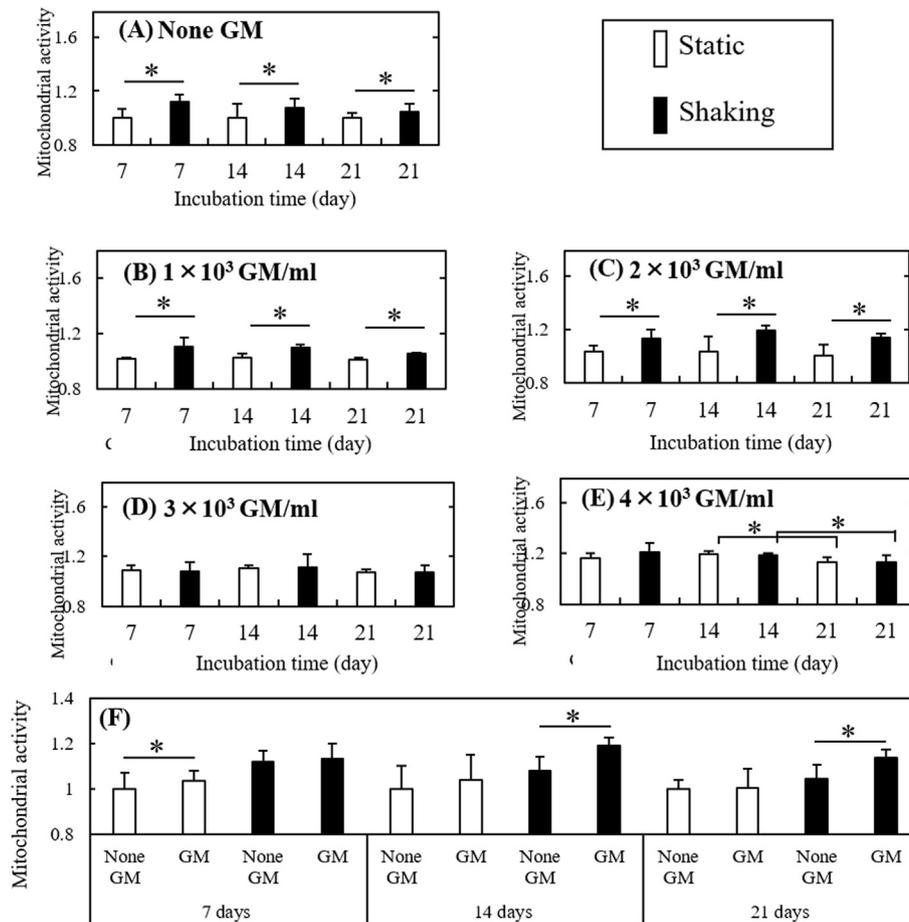
FIG. 4. Mitochondrial activity of MC3T3-E1 cells in cell aggregates without (A) or with 1×10^3 (B), 2×10^3 (C), 3×10^3 (D), and 4×10^3 GM/ml (E) of GM 7, 14, and 21 days after incubation under the static (open bars) or shaking culture (closed bars). $p < 0.05$; significant against mitochondrial activity between the two groups. (F) Mitochondrial activity of MC3T3-E1 cells in cell aggregates without or with 2×10^3 GM/ml of GM 7, 14, and 21 days after incubation under the static (open bars) or shaking culture (closed bars). $p < 0.05$; significant against mitochondrial activity between the two groups.
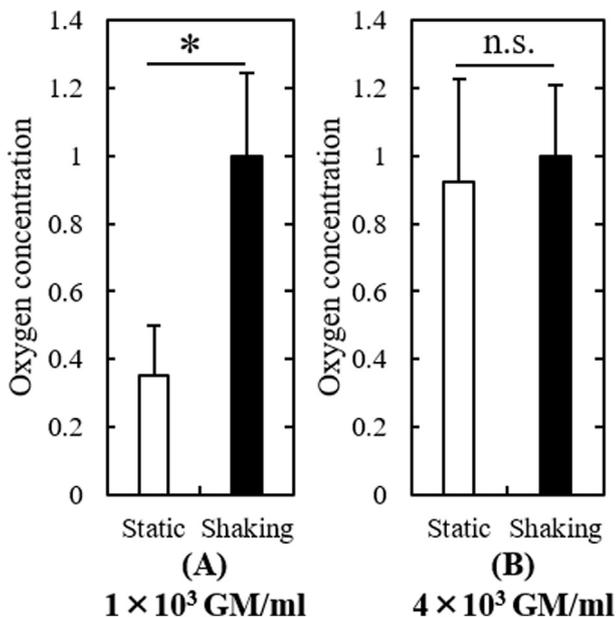

FIG. 5. Relative oxygen concentration of MC3T3-E1 cell aggregates incorporating 1×10^3 (A) and 4×10^3 GM/ml (B) of GM 14 days after incubation under the static (open bars) or shaking culture (closed bars). $p < 0.05$; significant against oxygen concentration between the two groups.

(shaking or static culture) did not significantly affect the size and morphologies of cell aggregates incorporating the same number of GMs (Fig. 2). However, the mixing ratio of GMs to cells affected the size and morphologies. The increased ratio of GMs to cells increased both of them (Fig. 2). It is possible that considering the amount of GMs, the size of cell aggregates is modified. The size of cell aggregates incorporating GMs 21 days after incubation was small compared with that 7 and 14 days, irrespective of the number of GMs or the culture conditions. GMs were seen within cell aggregates clearly 7 and 14 days after incubation, but the frame of GMs within cell aggregates 21 days after incubation was unclear because of their degradation (Fig. 2B–E). We can say with certainty that the degradation of GMs made the size of cell aggregates smaller. Moreover, it seems that GMs existed in cell aggregates 60 days after incubation because the size of cell aggregates was about 250 μm (> 150 μm: the size of cell aggregates without GMs) (Fig. S1). The results suggest that the GMs degradation is one of the important factors to form cell aggregates. For drug discovery or regenerative medicine using cell aggregates for a longer time period, GMs with a slower degradation should be used. GMs of slower degradation could be prepared for longer time periods of crosslinking (39). For the drug discovery, cell aggregates for about 2 weeks or longer would need to survive. On the other hand, for the application to tissue regeneration, longer time periods would be needed although it depends on the therapeutic purpose. The time period needed to maintain the functions should be optimized in terms of the application. The cell number of cell aggregates 7, 14, and 21 days after

incubation did not change, irrespective of the culture conditions and the number of GMs incorporation (Table 1). This reason is not clear at present.

ATP and mitochondrial activities of cell aggregates were investigated as a measure of biological functions (Figs. 3 and 4). The amounts of ATP per cells were calculated by dividing the number of cells into cell aggregates. ATP activity is often evaluated as a metabolic level of cells (40). However, we think that the level of ATP activity does not always indicate the metabolism of mitochondria. The active glycolysis may make ATP activity higher. To evaluate as the metabolic functions of cell aggregates, the ATP and mitochondrial activities of cell aggregates were measured. First, when cell aggregates were prepared without GMs, the ATP activity was much lower than that of cell aggregates incorporating GMs, irrespective of the culture conditions (Fig. 3F). It is demonstrated that when the amounts of ATP and mitochondrial activities are higher, biological functions are higher (40). The results strongly support our previous studies. GMs enable to facilitate to supply oxygen and nutrients to cells, which lead to making cells alive (11). However, the mitochondrial activity of cell aggregates incorporating GMs was not always high compared with that of aggregates without GMs (Fig. 4F). The reason is not clear at present. Furthermore, when cell aggregates without or with lower amounts of GMs (1×10^3 and 2×10^3 microspheres/ml) were prepared under the shaking culture, the ATP and mitochondrial activities of cell aggregates were significantly different from that under the static culture (Figs. 3A–C and 4A–C). It is suggested that shaking culture is the most important factor when the influence of GMs on cell aggregates is lower. However, in case of cell aggregates incorporating larger amounts of GMs (3×10^3 and 4×10^3 microspheres/ml), the ATP and mitochondrial activities of cell aggregates were not significantly different between the static and shaking culture (Figs. 3D, E and 4D, E), and the difference between static and shaking culture decreased upon increasing the amounts of GMs (Figs. 3A–C and 4A–C). Taken together, it is highly conceivable that the influence of GMs on the biological functions of cell aggregates was stronger than under the shaking culture. The tendency of mitochondrial activity was similar to that of ATP activity. Furthermore, ATP activities of cell aggregates incorporating larger amounts of GMs (3×10^3 or 4×10^3 microspheres/ml) and mitochondrial activities of cell aggregates incorporating larger amounts of GMs (4×10^3 microspheres/ml) 21 days after incubation were low compared with those 7 and 14 days later because of degradation of GMs (Figs. 3D, E, and 4E). Furthermore, the ATP and mitochondrial activities of cell aggregates incorporating various amounts of GMs under the shaking culture at 5–20 rpm were not significantly different (Fig. S3). This indicates that when the shaking speed was lower, the shaking culture is not always an important factor to affect the functions of cell aggregates. However, the effect of the shaking culture on the biological functions of cell aggregates incorporating GMs at 20–30 rpm was observed (Figs. 3 and 4). At the shaking rate more than 30 rpm, a leakage of culture medium from each well was observed in the experimental system of this study. In addition, in order to evaluate the effect of the number of GMs or the shaking culture on the oxygen supply to cell aggregates, oxygen concentration of the cell aggregates incorporating a lower (1×10^3 microspheres/ml) or larger (4×10^3 microspheres/ml) amount of GMs 14 days after incubation under the shaking or static culture was measured (Fig. 5). The relative oxygen concentration of cell aggregates incorporating 1×10^3 microspheres/ml of GMs under the shaking culture was higher than that under the static culture (Fig. 5A). However, the concentration of cell aggregates incorporating 4×10^3 microspheres/ml of GMs under the shaking culture was not significantly different from that under the static culture (Fig. 5B). The findings support the results of ATP or mitochondrial activities (Figs. 3 and 4). It is likely that more effective allowance of oxygen supply to

cells was observed for the lower amount of GMs incorporated. On the other hand, when a larger amount of GMs was incorporated into cell aggregates, enough oxygen would be supplied, irrespective of the culture conditions. However, there is one point to be considered in this study. It is unclear the distance which the probe can reach in cell aggregates. Therefore, the relative oxygen concentration was introduced (oxygen concentration of cell aggregates under the shaking culture = 1).

Regarding the depth effect of the medium, when cell aggregates incorporating various amounts of GMs were cultured in 200 μl of the medium, the morphologies, size, and the biological functions of cell aggregates were not significantly different from those in 150 μl (Fig. S3).

It was demonstrated that the shaking culture enables to facilitate to supply oxygen or nutrients of culture medium into cells, which leads to enhancing the biological function (33). On the other hand, it is reported that there is a relationship between the biological functions of cells and the supply of oxygen or nutrients (11). Considering the findings, it is likely that the shaking culture enables to supply the oxygen and nutrients into cell aggregates, leading to an enhanced cell function. In addition, oxygen and nutrients would be permeated through the water phase of GMs matrices (11,39). However, the shaking culture did not improve the biological functions of cell aggregates incorporating a large amount of GMs. This may be explained in terms of the amount of GMs incorporated. Since the GMs are present in the cell aggregates at a large enough volume, the water phase necessary for oxygen and nutrient supply would be large enough for cell functions in aggregates (11,39,41). As the result, the shaking of medium may not affect the cell functions very much.

Recently, the cell transplantation of cell aggregates has been reported and increasingly noted to enhance their viability and the therapeutic efficacy (42,43). It is needed to improve the efficiency of cell therapy such as the incorporation of growth factors into GMs (39). Furthermore, the shaking culture should be introduced to enhance the biological functions of cell aggregates and mimic the body environment of the blood circulation. Thus, a combination of biomaterials and shaking culture is important to improve the efficacy of tissue regeneration or drugs.

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