A scaffold-free surface culture of B16F10 murine melanoma cells based on magnetic levitation

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Abstract Multicellular spheroids are obtained in a variety of three-dimensional (3D) culture systems without the use of supporting scaffold. We present here a 3D culture method that resulted in a multicellular sheet under scaffold-free conditions. A floating disk-shaped 3D culture was prepared by magnetic levitation of B16F10 cells that has ingested Fe_3O_4 -containing fibroin microspheres. The melanoma disk grew up to 19 mm in diameter and the thickness was ranged between 80 and 100 μm . The 3D culture was filled with closely packed cells that were proliferating exponentially at a specific growth rate of $\mu = 0.015 \text{ h}^{-1}$. Approximately half of the cells were Ki-67 positive with no detectable levels of apoptotic or autophagic cells. However, the percentage of propidium iodide-permeable cells was $8.5 \pm 1.2 \%$, which was probably due to physical damage in the cell membrane caused by Fe_3O_4 -containing microspheres under a strong magnetic field. Melanin production increased by a factor of 3.0–3.7 in the 3D culture, due to an increased population of pigmented cells. This study presented a surface 3D culture of B16F10 cells without the use of a scaffold based on magnetic levitation.

Keywords Fe_3O_4 nanoparticle · Magnetic levitation · Melanoma sheet · Three-dimensional culture

Introduction

Three-dimensional (3D) cell culture is receiving more attention since it provides a cellular environment more consistent with that in vivo (Page et al. 2013). Approaches to 3D cell culture are divided into scaffold-free and scaffold-based systems. Cells in scaffold-free conditions are expected to form their own extracellular matrix and to maximize cell to cell contact. Scaffold-free 3D spheroids were obtained from melanocytes using the hanging drop method (Kelm et al. 2003), non-adherent surfaces (Hirschhaeuser et al. 2010), and under microgravity environments (Marrero et al. 2009). These melanoma spheroids have been used to investigate tumourigenic potential (Fang et al. 2005) and to construct an organotypic skin melanoma model for in vitro drug testing (Vörsmann et al. 2013). Tumour spheroids have been shown to have a layered structure consisting of a necrotic center, quiescent cells on the rim area, and proliferating cells in the outer layer (Jiang et al. 2005).

This study demonstrated a 3D culture of B16F10 cells in a form of a multicellular sheet at the air medium interface without the use of any support. The

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morphology of the 3D cultures differed significantly from those obtained previously. There has been no report on scaffold-free 3D culture forming multicellular sheet except bioprinting (Tan et al. 2014) and centrifugal cell seeding method (Way et al. 2011). Multicellular aggregates with irregular shapes were obtained in a liquid/liquid culture system supported with oxygenated perfluorodecalin (Pilarek et al. 2013). Air–liquid interface cell culture has decades of history, but requires a physical support on which the cells can adhere (Aufderheide et al. 2016). Therefore, the floating multicellular sheet of B16F10 cells presented in this study cannot be prepared by the previous 3D culture methods. The growth and structure of B16F10 multicellular sheet may differ from those of 3D spheroids, since 3D culture microenvironment can be determined by the diffusive supply of nutrient and oxygen.

These prompted us to investigate the size and shape of the multicellular structure prepared from B16F10 cells that were treated with Fe_3O_4 -containing microspheres. The morphology was also examined by using paraffin wax-embedded sections of the 3D culture. We also investigated the growth kinetics and melanin synthesis of B16F10 cells in 3D environments.

Materials and methods

Materials

A water-dispersible Fe_3O_4 nanopowder (637,105, <50 nm in diameter; Aldrich, Milwaukee, WI, USA) was used to prepare Fe_3O_4 -containing fibroin microspheres, as reported previously (Lee and Hur 2014). The Fe_3O_4 content in the fibroin microspheres was determined by thermal gravimetric analysis (SDT Q600 TGA/DSC; TA Instruments, Austin, TX, USA) at a heating rate of 10 °C/min in air.

Magnetic levitation of B16F10 cells

The mouse melanoma B16F10 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco's modified Eagle's medium (Lonza, Walkersville, MD, USA) with 4.5 g/l glucose and 4 mM L-glutamine, supplemented with 10 % fetal calf serum (Lonza). The Fe_3O_4 -containing microsphere suspension (1 mg/ml) was sterilized by

autoclaving and added to a subconfluent culture of B16F10 cells in a T25 flask at a final concentration of 0.09 mg/ml, followed by incubation for 24 h. The cells were then dissociated using trypsin/EDTA solution and resuspended in fresh medium. The suspension was then transferred to a Petri dish (60 × 15 mm; SPL, Pocheon, Korea) or a six-well culture plate (SPL) and incubated with one or two disk-shaped neodymium magnets (30 × 5 mm; Nsmagnet, Seoul, Korea) on top of the plate, of which magnetic field strength is 217.3 ± 3.0 mTesla at a point 5.5 mm from the surface. The magnetically levitated cell assembly at the air-medium interface was carefully transferred to a new culture plate or replenished with fresh medium every 2 days. The 3D culture was dissociated with trypsin/EDTA solution and analyzed for cell counting with a cell counter (Luna automated cell counter; Logos Biosystems, Gyeonggi, Korea). Trypsinized cells were also stained with propidium iodide or fluorescein diacetate for cell viability analysis and for microsphere uptake analysis using flow cytometry (FACSCalibur, BD Biosciences, San Diego, CA, USA). Matlab software (Mathworks, Natick, MA, USA) was used to analyze raw data and to generate scatter dot plots of forward scatter, side scatter, and fluorescence signals.

Analysis of multicellular structure

The microspheres were observed by field emission scanning electron microscopy (SEM; S-4800; Hitachi, Tokyo, Japan) and by transmission electron microscopy (TEM; KEO 912AB, Carl Zeiss, Jena, Germany). B16F10 cell aggregates were washed with cold phosphate-buffered saline, fixed in 4 % formaldehyde for 30 min, dehydrated using an ethanol series, and then embedded in paraffin wax or epoxy resin. The samples in epoxy resin were sectioned and then stained with uranyl acetate and lead citrate for examination by TEM. The paraffin wax-embedded sections were deparaffinized and stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, sections were stained using the anti-Ki-67 antibody (550,609; BD Biosciences), anti-caspase-3 antibody (AVARP00021_T100; Aviva Systems Biology, San Diego, CA, USA), anti-LC3 antibody (ab58610; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or isotype control antibody for 1 h at room temperature. The sections were incubated with

Polymer-HRP secondary antibodies and visualized using the Polink-2 HRP Plus Broad DAB Detection System (GBI Labs, Mukilteo, WA, USA).

Melanin deposition was detected by a Fontana–Masson stain Kit (ScyTek Lab., Logan, UT, USA) according to the manufacturer's instructions. Melanin contents of B16F10 cells were measured according to a previously published method (Lee et al. 2010). Briefly, cellular melanin was solubilized using 1 N NaOH at 4 °C for 24 h and determined spectrophotometrically at 405 nm, with synthetic melanin (Sigma-Aldrich M8631) as a standard.

Results

Magnetic levitation of B16F10 cells

Fibroin microspheres entrapping Fe₃O₄ nanoparticles were prepared by the oil-in-water emulsion dehydration method, which ranged from 0.6 to 1.2 µm in diameter. The Fe₃O₄ content was determined to be 10.8 ± 0.8 % (w/w) by thermal gravitational analysis (Supplemental Fig. S1). A growing culture of B16F10 cells was incubated with the microspheres for 24 h, dissociated and harvested. The cell suspension (~10⁶ cells) was incubated under a magnetic field for 7 days (Fig. 1a). This resulted in the development of a multicellular aggregate at the air–liquid interface of the culture medium (Fig. 1b). The aggregate had the shape of a thin disk with a rough edge and had a dark color showing melanin production. The color of the culture medium also changed to dark brown within 2 days after medium replenishment (Fig. 1c). A disk-shaped multicellular structure was routinely obtained in 2 days under magnetic levitation, which grew to a diameter of ~19 mm within 7 days with daily replenishment of the culture medium (Fig. 1d). A bar-shaped thin multicellular culture was obtained when bar magnets were used (Supplemental Fig. S2). Accordingly, magnetic attraction force appeared to determine the shape of the 3D culture at the air–medium interface.

Figure 2a shows a representative disk-shaped melanoma sheet, 15.7 mm in diameter, with uneven pigmentation. The TEM micrograph of the 3D culture shows circular dark spots with a size comparable to microspheres in the cytoplasm, indicating cellular uptake of Fe₃O₄-containing microspheres (Fig. 2b).

H&E staining revealed that the 3D culture was composed of closely packed cells with a thickness of 90.5 ± 0.5 µm (Fig. 2c). The compactness of the cells was influenced by the strength of the magnetic field and by the Fe₃O₄ content in the fibroin microspheres used for magnetic levitation. A higher magnification of the H&E image (Fig. 2d) showed that cells were densely packed together and that some contained dark spots. TEM analysis of the 3D culture confirmed the intracellular localization of Fe₃O₄-containing microspheres. These results confirm that 3D cultures can be obtained by magnetic levitation of B16F10 cells that have ingested Fe₃O₄-containing microspheres. The morphology of the 3D culture was significantly different from that of other scaffold-free 3D cultures, which formed a spherical body of multicellular aggregates (Kelm et al. 2003; Hirschhaeuser et al. 2010; Souza et al. 2010). In our previous study, the same method of magnetic levitation was used for preparing a 3D culture of murine fibroblast 3T3 cells, which also resulted in a multicellular spheroid (Lee and Hur 2014).

Factors affecting the 3D culture morphology

The present method was used to prepare multicellular aggregates from varying numbers of cells (2.7×10^4 – 3.8×10^6 cells) in a six-well culture plate. A series of multicellular aggregates with different sizes and shapes was harvested after 8 days (Fig. 3a). The aggregate size increased in a stepwise manner in response to increasing initial cell numbers (Fig. 3b). The apparent areas of large aggregates were 63.6, 70.7, and 78.5 mm², of which the cell numbers were 9.6×10^5 , 1.9×10^6 , and 3.8×10^6 cells, respectively. Thus, the cell density per area of the thin disks was in the range of 4.0 – 6.1×10^5 cells/cm², which was several times higher than that of a normal monolayer culture determined to be 1×10^5 cells/cm² under the same culture conditions. The analysis is consistent with the observation of the H&E-stained section in Fig. 2a, in which the thickness is the size of several cells.

Small aggregates were darker than larger ones, as seen in Fig. 3a, suggesting that small aggregates were thicker. The apparent cell density of small aggregates ranged from 1.2 to 5.2×10^6 cells/cm², which was 3–10× higher than those of thin aggregates, indicating that small aggregates were not thin, flat multicellular

Fig. 1 Graphical overview of the procedure for magnetic levitation of B16F10 cells fed with Fe_3O_4 -containing microspheres (a), photographic images of magnetically levitated cells in a 60-mm culture plate with two neodymium magnets on the top (b), disk-shaped multicellular aggregates (c, d)

structures. Thus, the 3D morphology appeared to be determined by the number of cells present in the 3D culture.

Varying amounts of Fe_3O_4 -containing microspheres were added to 5.3×10^5 cells and incubated under a magnetic field to allow aggregate formation. The resulting 3D cultures had a disk-shaped morphology, except when cell cultures were prepared with cells treated with less than 0.01 mg/ml of microspheres (Fig. 4). The size and number of cells in the 3D cultures increased with the number of microspheres added to cells. The multicellular disk appeared more perforated as the number of microspheres added to the cells was reduced. Therefore, more than 2.0×10^5 cells were fed with microspheres to a final concentration of 0.1 mg/ml to prepare 3D cultures for the following experiments.

Cell proliferation in the 3D culture

The size of B16F10 multicellular aggregate increased during magnetic levitation as shown in Fig. 5a, which prompted us to count the number of cells dissociated

from the 3D cultures (Fig. 5b). The total cell number increased exponentially during the first 5 days and reached a plateau of $\sim 2.0 \times 10^6$ cells. After the exponential phase, the color of fresh medium turned to brown within 24 h, indicating that lactic acid accumulation had inhibited further growth. The specific growth rate (μ) of the 3D culture was determined to be 0.015 h^{-1} (doubling time of 46.2 h), whereas the specific growth rates of cells in monolayer culture was 0.052 h^{-1} (Supplemental Fig. S3). The rates are comparable to the reported specific growth rate of B16F10 cells, i.e., 0.035 h^{-1} (Ohira et al. 1994).

Cell proliferation was also confirmed by immunohistochemistry for Ki-67 expression in the cells of the 3D culture. The percentage of Ki-67-positive cells was determined to be 46.8 % in a section of a B16F10 melanoma sheet (Fig. 6a). The proliferating cells had no particular distribution pattern and were scattered across the section with varying intensities of staining. Sections of the 3D culture were also evaluated to confirm cell survival by immunohistochemistry for caspase-3 and LC3 expression, as indications of apoptosis and autophagy that might occur in response

Fig. 2 A photographic image of a disk-shaped 3D culture of B16F10 cells based on magnetic levitation (a), TEM micrograph showing Fe_3O_4 -containing microspheres (arrows) in the

cytoplasm of cells in the 3D culture (b), and micrographic images of a cross-section of the 3D culture sheet stained with hematoxylin and eosin (c, d)

to nutrient starvation in the 3D culture of densely packed cells. Figure 6b, c shows that no cells were positively stained with anti-caspase-3 and anti-LC3 antibodies when compared with isotype control. Microspheres were also stained dark brown (Fig. 6d), since Fe_3O_4 nanoparticles have peroxidase-like activity (Wu et al. 2011).

B16F10 cells exhibited an exponential growth in the 3D environment, but the rate decreased to less than half of the proliferation rate of cells in monolayer culture. Immunohistochemistry indicated that the diffusional limitation of nutrients or oxygen was not substantial enough to cause any severe starvation response in the cells in the 3D environment. The decreased rate of cell proliferation was observed only in 3D cultures; the proliferation rate was not affected by cellular uptake of microspheres alone, but by subsequent magnetic levitation. Therefore, investigating the viability of cells in 3D environments was necessary.

Cell viability in 3D cultures

We investigated the effect of microsphere uptake and subsequent magnetic levitation on cell viability in 3D cultures. Flow cytometric analysis of cells stained with propidium iodide (PI), shown in Fig. 7, revealed that the percentage of PI-positive cells in the 3D culture was $8.5 \pm 1.2 \%$, higher than those of cells treated with microspheres ($2.2 \pm 1.6 \%$), cells incubated under a magnet ($2.1 \pm 0.4 \%$), and control cells ($3.0 \pm 1.0 \%$). This shows that the highly fluorescent PI-positive fraction increased only in the cells dissociated from the 3D culture. Thus, pipetting damage during 3D culture dissociation was tested but the percentages of PI-positive cells rarely increased after repeated pipetting of the B16F10 cell suspension using 1-ml pipette tips (supplemental Fig. S4).

The analysis also showed that cells that ingested microspheres exhibited increased side scatter, and also that cell viability based on membrane integrity was

Fig. 3 Photographic images **(a)** and the area and number of cells in 3D cultures **(b)** prepared by magnetic levitation for 8 days with varying initial numbers of B16F10 cells

unaffected by microsphere uptake. To verify B16F10 cell resilience to microsphere uptake, Fe_3O_4 -containing microspheres were labeled with Alexa 647, and the labeled microspheres were used to prepare a 3D culture. The 3D culture was dissociated and stained with fluorescein diacetate (FDA) and PI, and used to investigate the correlation between cell viability versus microsphere uptake in two-dimensional scatter plots of FDA or PI versus Alexa 647 fluorescence using flow cytometry. Figure 8 shows the vertical distribution of scattered events of nonfluorescent FDA-negative cells and highly fluorescent PI-positive

cells irrespective of microsphere fluorescence, indicating that microsphere uptake had no effect on the viability of cells in monolayer culture and in 3D culture. The percentages of PI-positive and FDA-negative cells were 13.1 and 21.9 %, respectively, in the 3D culture obtained by magnetic levitation for 2 days, which was higher than those of control cells treated with microspheres. These results show that cellular uptake of Fe_3O_4 -containing fibroin microspheres had no effect on cell viability, but that cell death occurred in the 3D environments after subsequent magnetic levitation.

Fig. 4 Photographic images (a) and the area and number of cells in 3D culture (b) prepared by magnetic levitation for 8 days using B16F10 cells treated with varying concentrations of Fe_3O_4 -containing microspheres

Enhanced melanogenesis under 3D conditions

The intracellular melanin content of B16F10 cells in a 3D culture was determined to be 12.8 ± 5.1 pg/cell, a factor of 3.7 and 3.0 greater fold higher than that of cells in monolayer culture and that of microsphere-treated cells in monolayer culture, respectively (Fig. 9a), which explains the dark color of the 3D culture. Since microscopic examination revealed that the 3D culture contained varying degrees of pigmented cells (Fig. 9b), we enumerated the percentage of pigmented cells from dissociated cell suspensions. The percentage of pigmented cells in a 3D culture under magnetic levitation for 2 days was

39.2 ± 17.0 %, which was significantly higher than that of cells in a monolayer culture and that of microsphere-treated cells in a monolayer culture, which were 2.6 ± 1.4 and 13.4 ± 3.7 %, respectively (Fig. 9c). A section of the 3D culture was stained using the Fontana–Masson technique (a melanin-specific stain) to visualize cells that produce melanin (Fig. 9d). The technique shows that the 3D culture was composed of nonpigmented cells, cells containing small melanin particles in the cytoplasm, and some cells full of dark stains.

The results indicate that melanin synthesis significantly increased in B16F10 cells under 3D conditions due to an increase in the number of melanogenic cells.

Fig. 5 Photographs of 3D cultures showing a radial growth of B16F10 cells at the air-medium interface (a) and the growth kinetics of the 3D culture with an exponential phase at a specific growth rate (μ) of 0.015 h^{-1} (b)

Thus, the recovery of the melanogenic phenotype of B16F10 cells was allowed in the 3D culture conditions of the present study. In monolayer culture, the color of B16 melanoma cells fades with cell passage unless induced by α -melanocyte-stimulating hormone (Lee et al. 2010). Melanogenesis is modulated by various intracellular signaling mechanisms in response to a stimulus such as ultraviolet irradiation (Park et al. 2009). Glucose depletion also enhances intracellular melanin as well as tyrosinase activity and expression (Cedrola et al. 2004). Therefore, the complex 3D

environments, including microsphere uptake, might have been responsible for the recovery of melanogenesis.

Discussion

Here, we present a 3D culture of B16F10 cells, shaped like disks, which appeared similar to the radial growth of fungi at an air-medium interface, a phenomenon rarely observed in animal cell cultures. The planar

Fig. 6 Immunohistochemical staining of 3D culture sections using the anti-Ki-67 antibody (a), anti-caspase3 antibody (b), anti-LC3 antibody (c), and isotype control (d)

Fig. 7 Two-dimensional scatter plots of propidium iodide-stained B16F10 cells cultured for 4 days with or without a neodymium magnet on the *top* of the culture plate and with or without addition of microspheres into the culture plate

morphology was very different from the multicellular spheroids usually obtained using other 3D culture methods. Although morphological transformation

from multicellular disks to spheres has been observed during magnetic levitation of 3T3 cells (Souza et al. 2010; Lee and Hur 2014), the 3D culture of B16F10

Fig. 8 Scatter plots of FL4-height (FL4-H; Alexa 647 fluorescence) versus FL1-height (FL1-H; fluorescein diacetate (FDA) fluorescence; upper row) and versus FL3-height (FL3-H; propidium iodide (PI) fluorescence; bottom row) of cells, cells treated with Alexa 647-labeled microspheres (0.1 mg/ml) and 3D culture prepared by magnetic levitation for 2 days

Fig. 9 Melanin contents of B16F10 cells, treated with Fe_3O_4 -containing microspheres and 3D culture prepared by magnetic levitation for 4 days (a), a light microscope image of planar 3D culture showing uneven pigmentation (b), the percentages of pigmented cells (c), and 3D culture section stained with Fontana–Masson (d)

cells in this study retained the initial planar shape. In tumor spheroids, cells are migrating from the outer well-nourished region toward the necrotic central core (McElwain and Pettet 1993), and collective cell migration has been considered as a hallmark of tissue remodeling events (Friedl and Gilmour 2009). Thus, cell migration seems critical for the morphological change from planar to spheroid. Lung cancer cell migration can be inhibited by arginine-conjugated albumin microspheres, although the mechanism of migration inhibition has not been identified (Lee et al. 2013). Accordingly, the planar morphology and radial growth of the 3D culture could be associated with the loss of motility of B16F10 cells that had taken up Fe_3O_4 -containing microspheres.

In tumor spheroids, proliferating cells are usually observed at the periphery, chemical gradients (e.g. of oxygen, nutrients, and catabolites) are at diameters between 200 and 500 μm , and a central secondary necrosis appears typically at sizes greater than 500 μm (Hirschhaeuser et al. 2010). However, Ki-67 positive proliferating cells were randomly distributed across the section of a 3D culture that had a planar shape with a thickness of less than 100 μm , in which B16F10 cells were growing exponentially but with a decreased specific growth rate. Thus, the results imply that mass transfer limitation was not critical to cause apoptotic or autophagic responses, but that a diffusion-limited nutrient supply resulted in slow proliferation in the 3D culture. Nevertheless, we cannot exclude the possibility that nutrient limitation also triggered cell death under the 3D conditions, since 3D cell viability decreased after subsequent magnetic levitation. Another possibility is that magnetically attracted microspheres were forced towards the magnet and may have damaged membrane integrity. The cause of cell death in the 3D culture remains to be investigated.

A planar-shaped, multilayer 3D culture floating at an air-medium interface has not been previously demonstrated. Although the present method provides only a simple form of multicellular organization, it can be the basis for constructing a more complex multicellular architecture. Thus, the present method offers a way to manufacture more complex multilayer tissue constructs.

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