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Multiparameter analyses of three-dimensionally cultured tumor spheroids based on respiratory activity and comprehensive gene expression profiles

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ABSTRACT

Multicellular spheroids of human breast cancer cells (MCF-7) formed with two different three-dimensional (3D) culture methods were evaluated in detail on the basis of respiratory activity and high-throughput gene expression analysis. The spheroids formed with poly(dimethylsiloxane) (PDMS) microwell arrays indicated significant restriction of the spheroid size, whereas their respiratory activity was 2-fold greater than that formed with the hanging drop culture method. Fluidigm BioMark dynamic array was used for comprehensive and quantitative real-time polymerase chain reaction (qRT–PCR) analysis on the samples whose respiratory activity had been measured. Genes involved in cellular senescence and glucose metabolism indicated significantly higher values for the PDMS microwell culture method than for the hanging drop culture method ($P < 0.05$). Interestingly, samples formed with the PDMS microwell culture method showed stronger responses for glycolysis than those formed with the hanging drop method. These results illustrate the power of multiparameter analysis to characterize multicellular spheroids cultured in different microenvironments even if they have the same morphology.

It has become widely recognized that a three-dimensional (3D)[1] culture system reflects the clinical functions of cellular gene expression profiles and metabolic features more accurately than a two-dimensional (2D) culture system. The most commonly used 3D culture systems are multicellular spheroids because of their potential for high-throughput drug screening [1–3]. Spheroids have been traditionally formed by various culture methods with nonadherent surfaces, spinner flasks, or hanging drops [4]. These methods share the common feature of resisting cell–substrate interactions to activate cell adhesion molecules (CAMs) with homophilic cell–cell binding. Recently, microengineering systems have been used to form spheroids or embryoid bodies to control the microenvironmental signals [5–8]. In one of these approaches, microwell structures are fabricated with poly(dimethylsiloxane) (PDMS) using photolithography [9] to prevent cellular adhesion on the microwell surface and promote the subsequent formation of spheroids within microwells [10,11].

In general, the rate of cell proliferation in 3D culture systems is much lower than that in 2D systems. However, 3D culture systems have not been optimized to accurately measure the rate of cell proliferation in vivo. In addition, it is known that the rate of cell proliferation differs according to the methods and conditions of 3D culture systems, whereas the mechanisms underlying the functional differences in certain microenvironments have not been clarified [12]. Scanning electrochemical microscopy (SECM) has been applied as a reliable and highly sensitive method for measuring respiratory activity of individual spheroids [13], embryo bodies [14], or embryos [15]. Respiratory activity measured by SECM is noninvasive, and samples can be further evaluated by destructive assays such as molecular biological and biochemical techniques. Therefore, multifunctional characterization of spheroids has been expected to select high-quality cellular aggregate samples.

Multiparameter analysis is required for complex systems such as tissues, cell aggregates, and single cells [16]. Furthermore, hierarchical analysis may become a reliable standard to integrate genomic, transcriptomic, proteomic, and metabolomic information [17–19]. Recently, a dynamic array device integrating microfluidic circuits was used for comprehensive and quantitative analyses of gene expression profiles of tissue samples and single cells [20,21]. However, quantitative comparison of the gene expression profiles of multicellular tumor spheroids formed with different 3D culture methods has not been reported to date.

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[1] *Abbreviations used:* 3D, three-dimensional; 2D, two-dimensional; CAM, cell adhesion molecule; PDMS, poly(dimethylsiloxane); SECM, scanning electrochemical microscopy; cDNA, complementary DNA; PCR, polymerase chain reaction; TBP, TATA box binding protein; PCA, principal component analysis; TCA, tricarboxylic acid; PDH, pyruvate dehydrogenase; CoA, coenzyme A.

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In the current study, the function of the multicellular spheroids of the human breast cancer cell line (MCF-7) was evaluated on the basis of respiratory activity and comprehensive analyses of gene expression profiles. Two culture methods, hanging drop and PDMS microwell, were used to form spheroids. It was found that the respiratory activity of the spheroid formed with the PDMS microwell culture method was 2-fold greater than that formed with the hanging drop culture method when comparing spheroids of identical sizes, although the spheroids formed with the PDMS microwell culture method indicated a significant restriction of the spheroid size and a stronger response for glycolysis at the messenger RNA (mRNA) expression level.

Materials and methods

Cell culture

MCF-7 cells were supplied by the Institute of Development, Aging, and Cancer at Tohoku University (Sendai, Miyagi, Japan). They were cultured in 25-cm^2 cell culture flasks (Falcon) with RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 mg of streptomycin at 37 °C under a 5% CO_2 atmosphere. In experiments, cells were harvested with 0.25% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA, Gibco).

Fabrication of PDMS microwell array chips

SU-8 photoresist was spin-coated onto a silicon wafer. Ultraviolet (UV) light (365 nm) was applied through a photomask to create the SU-8 master mold. Toray Silpot-184 (Toray) was combined with the curing agent and degassed according to the manufacturer's instructions. It was then poured over the SU-8 mold and allowed to cure for 30 min at 80 °C. We fabricated PDMS microwell arrays to contain 2500 (50 × 50) microwells of five different geometries: width of 200 μm and depth of 100 μm; width of 200 μm and depth of 50 μm; width of 100 μm and depth of 100 μm; width of 100 μm and depth of 50 μm; and width of 50 μm and depth of 50 μm.

Two culture methods for spheroid formation

Single-cell suspensions were generated from trypsinized monolayers and diluted to the desired cell density. In the hanging drop culture method, 20 μl of cell suspension drop was spotted on a cap of a 60-mm cell culture dish (Falcon). On inversion of the cap, the cell suspension drops were held in place by surface tension. Cells in the drop accumulated by gravity and subsequently formed a single spheroid. Then, 1 ml of phosphate-buffered saline (PBS) was added to the 60-mm dish and incubated at 37 °C in a 5% CO_2 atmosphere. In the PDMS microwell culture method, 1 ml of cell suspension of either 1×10^5 or 5×10^5 cells/ml was seeded on a PDMS microwell array chip and placed in a 35-mm cell culture dish (Falcon) containing 2 ml of culture medium.

Respiratory activity measurement

Respiratory activity by single spheroid was measured with a commercial SECM system (HV405, Hokuto Denko). Spheroids were transferred individually into a plate composed of 6 cone-shaped microwells and filled with 3 ml of embryo respiration assay medium 2 (ERAM-2, Research Institute for the Functional Peptides, Yamagata, Japan). The temperature of the medium was maintained at 37 °C by a hot plate on the microscope stage. The XYZ stage (K701-20R, Suruga Seiki, Tokyo, Japan) and the potentiostat (HV405, Hokuto Denko) were controlled by a notebook computer (FMV-BIBLO-NE7/800). Oxygen reduction currents were monitored using a platinum disk microelectrode probe at −0.5 V versus Ag/AgCl. The electrode probe was scanned vertically from the side of the sample up to 160 μm and repeatedly for each sample up and down six times. The respiratory activity of the spheroid [F (mol s^{-1})] was obtained by multiplying the difference in oxygen concentration between the bulk and surface of the spheroid [ΔC (mol cm^{-3})] and sample radius [R (cm)] according to the spherical diffusion equation [22]:

$$F = \frac{D}{0.7} \times 2\pi\left(1 - \frac{1}{\sqrt{2}}\right)(1+\sqrt{2})R\Delta C \quad (1)$$

where D is the diffusion coefficient of oxygen (2.1×10^{-5} cm^2 s^{-1}). The concentration profile formed near the sample has been confirmed to allow for spherical diffusion with the center of the sphere at the peak of the cone-shaped microwell rather than the center of the sample. Therefore, the concentration profile was shown as a function of the distance $(1+\sqrt{2})$ from the peak of the cone-shaped microwell. The mean radius (R) of the spheroid was based on the microscope image and determined according to the equation $R = (1/2)(ab)^{1/2}$, where a and b are the two orthogonal diameters of the spheroid calculated by using ImageJ software [13,23].

High-throughput quantitative PCR

Total RNA from cultured spheroids was extracted using an RNeasy Micro Kit (Qiagen). Total RNA was reverse-transcribed into first-strand complementary DNA (cDNA) using a QuantiTect Reverse Transcription Kit (Qiagen). cDNA (2.5 μl) was mixed with 2× Taqman Preamp Master Mix (5 μl, Applied Biosystems) and 2.5 μl of STA Multiplex Primer Pool. Subsequently, cDNA was amplified using sequence-specific primers by denaturation at 95 °C for 15 s and annealing and amplification at 60 °C for 4 min for 14 cycles. Primer lists for gene expression analyses are listed in Table S1 of the Supplementary material, These preamplified products were diluted 5-fold prior to analysis with Fast Start Taqman Probe Master Mix and inventoried Taqman gene expression assays (Applied Biosystems) in 48.48 Dynamic Arrays on a BioMark System (Fluidigm). C_t values were calculated from the system software [BioMark Real-Time PCR (polymerase chain reaction) Analysis, Fluidigm], and genes with C_t values higher than 28 were removed from subsequent analyses (12 of 48 genes). The expression value of each gene relative to the endogenous control gene TATA box binding protein (TBP) was also calculated using the soft and $\Delta\Delta C_t$ methods.

Results and discussion

Respiratory activity of spheroids formed with hanging drop culture method

For the hanging drop culture method, spheroids were prepared with a concentration of either 100 or 500 cells per drop. Spheroids were formed on day 1 and serially propagated until 1 week (Fig. 1A). The initial concentration of cells in the starting suspension drop critically controlled the radius of the spheroid; for example, in a suspension drop with 500 cells, the radius of the spheroid was 216 ± 4 μm on day 7. We measured the respiratory activity (F) of spheroids on different days of cultivation (Fig. 1B). The respiratory activity of spheroids increased with increasing days in cultivation. The initial cellular concentration determines the respiratory activity for certain culture periods even after 7 days. We next plotted the respiratory activity as a function of the spheroid radius (R; Fig. 1C). The respiratory activity of spheroids was approximately