

MELANOMA SPHEROIDS AS A MODEL FOR CANCER IMAGING STUDY*

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In contrast to standard 2D cell cultures, spheroids are three-dimensional (3D) models which can mimic natural conditions of cancer growth and metabolism. Their complex structure can be investigated and analyzed using fluorescence microscopy and micro-tomographic imaging (micro-CT) as a new technique. In this study, we show application of two different melanoma cell lines (WM115 and WM266) with different biological characteristics to form spheroids by a hanging drop method.

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1. Introduction

Since the 1930s, when George Otto and Margaret Gey developed and implemented their “roller tube” technique to grow cells *in vitro*, a two-dimensional (2D) system for cell culturing has been established to replace the formerly used Maximow hanging drop assemblies [1, 2]. Since then, monolayer cell cultures have been used as a common model in biological studies for many years due to many advantages of this type of cell culture. They use easy to handle culture dishes or flasks, are accessible for microscopic observations and accurate for quantitative studies. Nevertheless, a 2D cell culture system is not representative when a solid tumor model description is needed. Therefore, three-dimensional (3D) cell culture systems were introduced to better mimic tissue conditions. Spheroids, formerly known as spherical aggregates, have been used since the 1940s. The intensive use of them as a model for cancer research has been applied since the 1980s by

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Mueller–Klieser and Sutherland [3, 4] to study a cellular environment in the context of the interrelationship between tumor-specific micromilieu, cellular metabolism, proliferation and viability. As the first, the multicellular tumor spheroids of melanoma cells were developed by Folkman and Hochberg in 1973 [5], and since then almost 5000 papers have been published in this field with the phrase “tumor spheroids” in the title or abstract, and during last 3 years, the number of such papers oscillated between 500 and 600 papers per year (see Fig. 1 (A)).

The aim of the present investigation was to establish a melanoma spheroid model for imaging *in vitro* study of cancer for the further investigation in radiopharmaceutical testing.

2. Methods

2.1. Spheroid culture

Primary melanoma cell line (WM115) and malignant melanoma cell line (WM266) samples were obtained from the Department of Glycoconjugate Biochemistry, Institute of Zoology and Biomedical Research of the Jagiellonian University in Kraków [6]. Cells in different densities were cultured in 15 μ l volume medium RPMI 1640 (Gibco, 21875091) supplemented with fetal calf serum (10%) using a hanging drop method to form spheroids (see Fig. 1 (B)). Finally, shaped spheroids were observed between 24 to 72 hours after seeding, depending on the cell line and density.

2.2. Spheroid imaging techniques

The structure of spheroids was determined under the inverted optical microscope (Olympus, IX-81, Japan) (see Fig. 1 (C)). For viability testing, spheroids were stained with different fluorochromes, including fluorescein diacetate (FDA) and propidium iodide (PI) (see Fig. 1 (E)).

Spheroid shape, structure and characteristics were determined by microtomography (micro-CT). Additionally, we evaluated the diameter, rate of growth and viability of spheroids with contrast microscopy and different dyes for fluorescence microscopy. Micro-CT investigation was carried out with a Bruker SkyScan 1172 scanner with X-ray energy set to 40 keV without physical filtration. Images were captured with spatial resolution between 1–2 μ m per pixel. As a contrasting agent, the most common micro-CT staining solution Lugol (I₃K) was used.

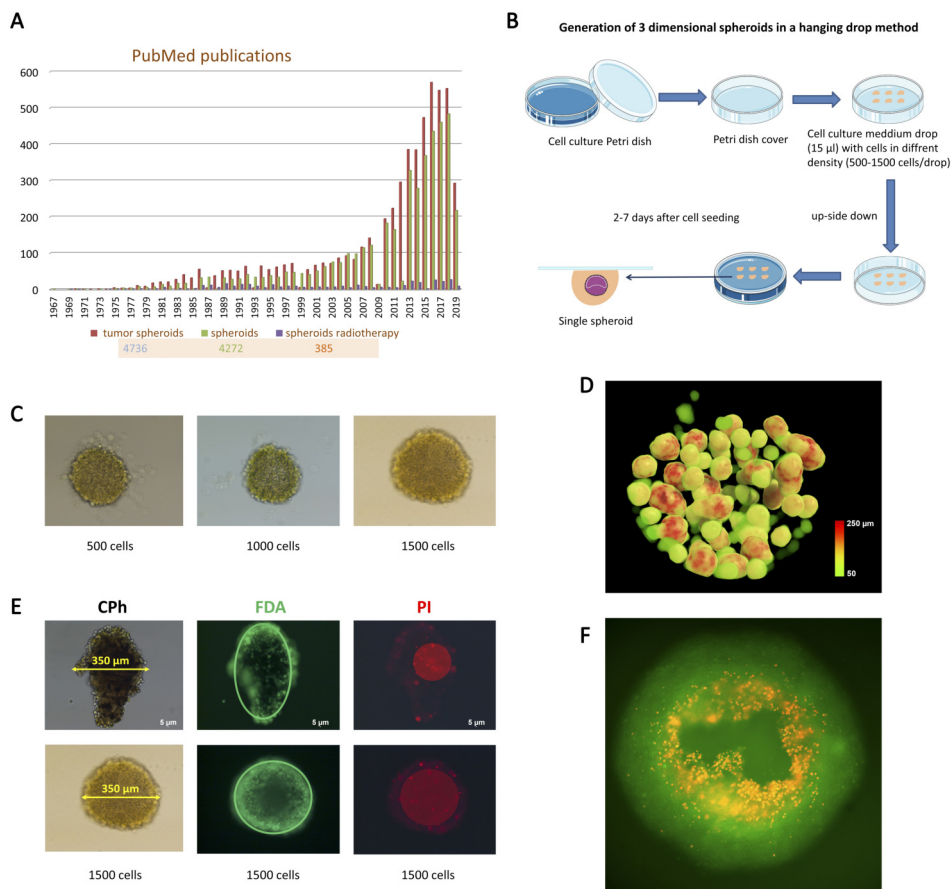


Fig. 1. (Color online) Cancer spheroids methodology and results. (A) Number of scientific papers available in the NHI data base (PubMed) classified by a year of publishing with key words “tumor spheroids”, “spheroids” or ‘spheroids and radiotherapy” in an abstract or title records; (B) A workflow for a hanging drop methodology to culture cancer spheroids; (C) Contrast phase images of melanoma WM266 cell line spheroids seeded with different starting cell density; (D) Micro-CT reconstruction of a cancer spheroid cluster formed by the WM266 cell line; (E) Contrast phase (CPh) images of WM115 (up) and WM266 (down) cell lined and viability test showing life cell (middle/green, FDA — fluorescein diacetate) and death cell (right/red, PI — propidium iodide) biological; (F) necrotic zone of a spheroid stained with red — PI, and life cells in green — FDA).

3. Results and discussion

3.1. Spheroid size and shape

The size and shape of growing spheroids were diverse depending on the cell number to start seeding and cell line malignant properties. A spheroid diameter usually varies from around 250 μm (WM115 cell line) to around 350 μm (WM266) in the 72th hour after seeding (see Fig. 1 (E)). Both these cell lines have the same BRAF mutation (p.V600E; ENST00000288602), confirmed by the COSMIC data base, which is responsible for uncontrolled growth, and they differ from each other with unique 102 mutations for WM115 and 97 mutations for WM266 [7]. This specificity has important physiological and biochemical implications: WM266 cells migrate faster, are enriched with $\alpha 5\beta 1$ glycoprotein (integrin), and have a significantly higher level of $\alpha 2,3$ -linked sialic acid residues. In contrast, the adhesion efficiencies of WM115 cells were significantly lower than those of WM266 cells [8]. In our study, the WM266 cell line forms bigger and better shaped spheroids in comparing to WM115 cell line. In clinic, WM115 and WM266 were originated from the same patient. They represent a different cancer growth phase, respectively: a primary WM115 cell line represented radial/vertical growth and lymph node metastasis was represented by metastatic WM266 cell line. We also confirmed a spheroid shape and integrity by micro-CT. WM266 gave us a good resolution spheroid images in a scale between 50 and 250 μm (see Fig. 1 (D)).

3.2. Spheroid viability

It is well-known phenomenon that a spheroid diameter limits spheroid cell viability due to the limitation in O_2 and CO_2 diffusion or nutrition factors and metabolites exchange [9]. The other limitation is a space. Spheroids form the multi-layered structures with the outer layer which is close to the nutrient supplies and O_2 ; the inner layer is hypoxic and mildly acidic. These layers differ with respect to their density. The metabolite and nutrient concentrations create a gradient which depends on the distance from the nutrient supply and cell density (packing). Nutrients and oxygenation are external (environmental factors), but cell density is an intrinsic feature of each cell line that influences different spheroid cell lines viability [10, 11]. The density $f(s)$ of living cells defined as the fraction of live cells per unit volume decreases exponentially as the distance (s) from the surface of the cell cluster. In our study, we visualized this phenomenon using fluorescence dye FDA/PI method (see Fig. 1 (F)). In the theoretical study of Milotti *et al.* (2012) [10], it was approximated by an exponential function with decay length λ determined by experiment

$$f(s) = \exp(-s/\lambda). \quad (1)$$

The parameter λ is not a constant, but a weakly decreasing function of tumor size, with the higher rate of slope for diameters between 150 and 250 μm . The λ parameter is also different for different cell lines forming spheroids showing its complexity depending on the cancer biology [10, 12].

4. Conclusions

Cellular heterogeneity, layered structure, growth kinetics, cell–cell signaling and gene expression made the spheroids suitable model for cancer response to radiotherapy. By means of standard and new techniques (micro-CT and PALS), which has been recently proposed in our papers, spheroids can be intensively explored to study cancer sensitivity to radiation as a more realistic 3D cell culture model [13, 14].

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