Development and application of three-dimensional in vitro multicellular spheroids to study the efficiency of anticancer compounds

Alföldi Róbert

Theses of Ph.D. dissertation

Doctoral School of Biology
Faculty of Science and Informatics
University of Szeged

Supervisors:

László G. Puskás, Ph.D., D.Sc.
Scientific adviser, BRC, Szeged
CEO, Avidin Ltd. Szeged

Gábor János Szebeni, Ph.D.
Senior scientist, BRC, Szeged

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1. Introduction

As cancer is one of the leading causes of death, in parallel with the development of anticancer compounds, we need to improve the *in vitro* and *in vivo* screening methods to reliably test the new cytostatic and cytotoxic molecules that inhibit tumor growth. These new models shall allow us to test the effects of anticancer agents in a complex way, not only on tumor cells, but also in their complex environment that model the real in vivo tumor's microenvironment as well.

The major drawback of the routinely used two-dimensional (2D) cell culture methods today is that cell-cell and cell-ECM interactions of the artificially engineered monolayer cells are under-represented. According to the latest results, data obtained from two-dimensional cultures are questioning the biological relevance of these drug assays because difficult to predict the clinical efficacy of the tested drugs and adapt these data for both clinical therapy and research.

A key advantage of three-dimensional (3D) cell culture methods is that cells can grow in multiple layers and, as a result, their polarity does not become equatorial. These changes will not only result in morphological differences, but their metabolic processes will also be closer to living organisms, giving a more realistic picture of *in vivo* conditions. Cells in these multicellular spheroids receive different gradient of nutrients, growth factors and oxygen depending on their distance from capillaries, which mimics the microenvironment of cells in real tumors.

The aim of my Ph.D. thesis was to explore which *in vitro* cell culture method could most accurately mimic the normal *in vivo* tissue conditions and compare them to routinely used conventional two-dimensional cell cultures. First, we set up a novel matrix-based culturing method, where the three-dimensional pancreatic islets were embedded in a collagen matrix *ex vivo* and were compared with mouse pancreatic islets grown as a monolayer cell culture. Cell viability, glucagon and insulin production were measured. Then, a comprehensive study was performed with tumor cells grown in different matrices and culture conditions. We investigated the growth kinetics, viability, gene, and protein expression of A549 human lung adenocarcinoma cells grown in different ways *in vitro* and compared them with subcutaneously injected A549 xenograft tumors. Finally, we investigated the two- and three-dimensional cultures of human MCF-7 and mouse 4T1 breast cancer cells upon imidazo[1,2-b]pyrazole-7-carboxamide treatment to clarify how different culture conditions affect the efficacy of different drugs.

2. Aims

The main goal of my work was to determine which *in vitro* cell culturing method most accurately models the normal physiological state of cells or specific tissues, which could be used even in high-throughput studies. I compared the different 3D models with the conventionally used two-dimensional cell cultures.

To elucidate these assumptions, our goals were the followings:

- investigate the cell viability, glucagon and insulin production of mouse pancreatic islets grown in a conventional monolayer cell culture versus islet cells embedded in three-dimensional RAFT collagen matrix,

- monitor the growth kinetics, viability, gene and protein expression of A549 human lung carcinoma cells grown *in vitro* under different 2D and 3D culture conditions or *in vivo* subcutaneously grafted into mice,

- study the viability of differently cultured (2D and 3D conditions) human MCF-7 and mouse 4T1 breast tumor cells for testing the efficacy of newly synthesized drug candidate molecules

- determine the IC50 value of drug candidate molecules on 2D and 3D cultured MCF-7 and 4T1 cells

3. Methods

- Two-dimensional cell culturing
- Mouse pancreatic islet isolation and maintenance
- Production of multicellular spheroids grown in an ECM-like matrix using RAFT™ 3D Multicellular Cell Culture System (RAFT ™ MCS)
- Three-dimensional microcarrier coating
- Three-dimensional cell culturing using suspension culture method with and without microcarriers
- Production of multicellular tumor spheroids with ultra-low attachment method
- Determination of living cell number with Trypan blue dye
- Cell viability measurement by resazurin assay
- Apoptosis analysis by flow cytometry
- Cell cycle analysis by flow cytometry
- Confocal laser scanning microscopy
- A549 Xenograft Tumor Model
- Gene expression analysis
- Single cell mass cytometry (CyTOF)
- Cluster analysis
- Statistical analysis

4. Results

4.1. The viability of RAFT embedded cells was better than that of mouse pancreatic islet cells cultured in monolayer or suspension

The viability and morphology analysis of pancreatic islets maintained under different culture conditions were examined at five time points on the first, fourth, seventh, tenth and eighteenth days of culturing.
Cells were visualized by live-cell dye Calcein violet AM, the Annexin V was applied to detect phosphatidyl-serine exposure of apoptotic cells and Propidium Iodide detected the necrotic cell death.
Between the fourth and seventh day, continuous morphological changes were observed in the monolayer culture. A slow migration of fibroblast-like cells was observed in RAFT^{TM} MCS-s, the islets maintained their morphology throughout the experiment.
As for the viability of different cultures, we found that the mouse pancreatic islets maintained in standard suspension culture, similar to RAFT^{TM} islets, retained their spheroid morphology but showed an accumulation of propidium iodide positive necrotic regions after the seventh day of culture. The 2D pancreatic islets underwent necrotic damage most often within the first four days, as evidenced by the massive propidium iodide staining. In contrast, the RAFT^{TM}-embedded islets showed scattered and low intensity Annexin V staining localized mainly at the periphery of the islets throughout the eighteen-day culture period.

4.2. The insulin and glucagon production of the collagen embedded RAFT^{TM} islets was preserved for 18 days

The insulin production was measured by the expression of Ins-1 and Ins-2 genes. The expression of Ins-1 was significantly increased in RAFT^{TM} islets compared to suspension (SC) (* $p < 0.05$) or 2D (** $p < 0.01$) cultures. The Ins-2 also showed an elevated expression in RAFT^{TM} compared to SC (* $p < 0,05$) or 2D (* $p < 0.05$) cultures, respectively. Although immunofluorescent staining of glucagon showed signal intensity in RAFT^{TM} until the 10th day, Gcg gene expression was almost undetectable in all cultures.

Indirect immunofluorescent staining was used to validate qRT-PCR data and to assess insulin and glucagon production during culturing. Islets were disintegrated with diminished insulin and glucagon expression in the monolayer cultures by day 7th. The insulin and glucagon production showed intense signal and it was sustained continuously not only in the standard SC but also in RAFT^{TM} for 18 days. The RAFT^{TM} cultures showed the brightest hormone staining over the 18 day culture period. This result is superior to previously published results with a peptide amphiphile nanostructured gel-like scaffold which maintained islet viability and hormone production for 14 days only.

4.3. The gene expression of 3D in vitro cultured A549 human lung adenocarcinoma cells is closer to *in vivo* than that of the conventionally cultured 2D monolayer cells

The expression pattern of 624 tumor-specific genes was analyzed in cells isolated from xenograft tumors versus conventional 2D cell culture, The most differentially expressed 60 genes and two additional genes, SLC2A1 (GLUT1, glucose transporter) and SLC16A3 (MCT4, lactic acid transporter) were selected for further studies. To determine the closest culture method that mimicked the gene expression profile of non-small cell lung cancer cells in vivo, hierarchical clustering was performed on both 4^{th} and 9^{th} days of all the studied 2D and 3D culture methods. We found that the long-term (9 day) maintenance of three-dimensional cultures (3D Spheroid, 3D Nutrisphere, 3D Cytodex3) clustered closer to xenograft, which suggests that these cell cultures more closely mimic the real tumor tissue.

4.4. The protein marker profile of 3D in vitro cultured A549 human lung adenocarcinoma cells is closer to in vivo xenograft tumors than that of the 2D culture

Twelve cancer markers (TMEM45A, MCT4, CD66 (CEACAM5), GLUT1, CA9, CD24, TRA-1-60, CD326, EpCAM, Galectin-3, CD274, PD-L1 and EGFR) were studied by single cell mass cytometry from long-term (9 days) 2D monolayer and 3D Cytodex3, 3D Nutrisphere cultures and compared to early and late stage solid A549 tumors. Representative multi-dimensional data analysis (visualization of stochastic neighbor embedding, viSNE) revealed cell-relatedness based on common marker expression by simultaneous analysis of all 12 markers at single cell resolution in 2D, 3D (3D Cytodex3 or 3D Nutrisphere) cultures and in vivo (early or late stage) tumors.

Merging the viSNE graphs of 2D, 3D and in vivo samples by multiparametric (12 proteins) single cell mass cytometry results delineated a map with three different “islands” representing 2D, 3D and in vivo conditions with minimal overlap. Both the segmentation and the area of the maps were proportional with heterogeneity of single cells in terms of the expression of the twelve studied tumor markers within a cohort. Standard 2D as the smallest tSNE island represented the poorest heterogeneity far from the 3D or in vivo condition.

4.5. The three-dimensional culture of human MCF-7 and mouse 4T1 breast cancer cells are more resistant to antitumor agents tested in a multicomponent drug library screening

The antitumor activity of imidazo[1,2-b]pyrazole-7-carboxamide compounds was investigated in 2D and 3D cultures of human MCF-7 mammary adenocarcinoma and mouse 4T1 mammary carcinoma cells. Cell viability was detected fluorometrically using resazurin assay, compared the activity of novel molecules to known antitumor compound doxorubicin, which was used as a positive control in our studies. Small molecules that showed IC_{50} activity below 10 μM in at least one culture were considered active, those above 10 μM were considered inactive, as their clinical relevance would most likely be questioned in further drug development steps. Based

on these criteria, 16 of the 45 newly synthesized small molecules were found to be active on the two breast tumors used in our experiments. Six of them showed a minimum two-and-a-half-fold difference in activity between 2D and 3D cell cultures.

The difference in the effect of the active molecules was in agreement with the data reported in the literature, i.e., multicellular 3D cell cultures were less sensitive both to the newly generated active substances and to doxorubicin used as a positive control in our experiments.

5. Summary

The main goal of my work was to investigate different multicellular spheroids generated with different three-dimensional *in vitro* cell culture techniques in order to demonstrate their advantages over the two-dimensional culture method widely used today.

5.1.
The use of RAFT™ provided excellent results in preserving islet spheroid viability, structure integrity and insulin, glucagon production for at least 18 days *ex vivo*.
Based on our data, RAFT can be a promising tool both in research and therapy as well, as there is a great demand in the clinic for therapies where diabetic patients can replace their insulin supply with insulin-producing xenograft islets implanted in their bodies. To the best of our knowledge, our group's findings was first to report the successful maintenance of pancreatic islets using RAFT™ technique *in vitro*.

5.2.
Gene expression analysis by nanocapillary qRT-PCR (624 genes) and 1536 well high-throughput qRT-PCR (62 genes) resulted in the selection of lung cancer markers associated with higher (TMEM45A, SLC16A3, CD66, SLC2A1, CA9, CD24) or lower (EGFR) expression in vivo or in 3D in vitro models compared to 2D monolayer cultures

5.3.
The implemented multidimensional single cell proteomic profiling revealed that 3D (Cytodex3 and Nutrisphere) cultures represented a transition from 2D to in vivo situation by intermediate marker expression of TRA-1-60, TMEM45A, pan-keratin, CD326, MCT4, Gal-3, CD66, GLUT1, CD274. In 3D systems CA9, CD24, EGFR showed higher expression than *in vivo*.

5.4.
Using human MCF-7 and mouse 4T1 mammary tumor cells, we demonstrated the different chemoresistance of 2D and 3D cell cultures. The performed screening investigated that 6 out of the 16 active small imidazo[1,2-b]pyrazole-7-carboxamides molecules had different antitumor activity with minimum two and a half fold difference in potency using 2D and 3D in vitro cultures of same cell lines. In these cases, 3D cultures were less sensitive to the agent of interest, including the widely used and well-known doxorubicin. Our findings showed the reduced chemosensitivity of multicellular spheroids.

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Publications

Related to the thesis:

1. **Single Cell Mass Cytometry of Non-Small Cell Lung Cancer Cells Reveals Complexity of In vivo And Three-Dimensional Models over the Petri-dish. Alföldi R**, Balog JÁ, Faragó N, Halmai M, Kotogány E, Neuperger P, Nagy LI, Fehér LZ, Szebeni GJ, Puskás LG. Cells. 2019 Sep 16;8(9). pii: E1093. doi: 10.3390/cells8091093. **IF(2019): 5.656**

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Conference abstract:

1. Róbert Alföldi: **Single cell mass cytometry of non-small cell lung cancer cells reveals the complexity of in vivo and three-dimensional models over the Petri-dish.**
   3rd German Mass Cytometry User Forum, Berlin, Germany, January 23-24, 2019
2. Róbert Alföldi: **Increased expression of MCT4 and GLUT-1 in early events of spheroid formation of adenocarcinomic epithelial cells A549.** Annual Meeting of the Hungarian Biochemical Society, Szeged, Hungary, August 28-31, 2016

Oral presentations:

1. Róbert Alföldi: **Single cell mass cytometry of non-small cell lung cancer cells reveals the complexity of in vivo and three-dimensional models over the Petri-dish.**
   48th Annual Meeting of the Hungarian Society for Immunology, Bükfürdő, Hungary, October 16-18, 2019