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Development of Spheroids in Co-culture of Prostate Tumor with Human Fibroblasts,using the Hanging-Drop Technique with Plate Inversion for Analysis of Gamma Irradiation by ^{60}Co

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1. Introduction

Three-dimensional (3D) culture has proven to be a useful and powerful tool for biomedical research in recent decades. Unlike two-dimensional (2D) cultures, 3D cultures have minor limitations on cell growth. This is due to their similarity to living systems and cellular interactions. The hanging drop technique in cell culture is a commonly used approach to create conditions that favor the formation of three-dimensional (3D) structures of cells in culture. This technique investigates cellular interactions, cell migration, cell differentiation and tissue formation in conditions closer to the in vivo environment. Hanging drop is often used in 3D cultures of fibroblasts, cells found in connective tissue that play a fundamental role in the production of extracellular matrix and maintenance of tissue structure. This technique performs a comprehensive analysis of cellular interactions, migration, differentiation, and tissue morphogenesis in an environment that more accurately mimics in vivo conditions. Murine fibroblasts are commonly employed in this context due to their ability to secrete key components of the extracellular matrix, such as collagen, fibronectin, and elastin. This secretion contributes to the formation and three-dimensional organization of a more complex extracellular matrix in response to pathological stimuli.

Prostate carcinomas are the most prevalent malignant tumor type among men, regarding up to 70.000 cases / year in Brazil [2]. Although fibroblasts are normal stromal cells present in the prostate, they play an important role in the tumor microenvironment and progression of prostate cancer. Fibroblasts in the prostate normally provide structural support and secrete extracellular matrix molecules that maintain tissue integrity. However, during carcinogenesis, fibroblasts adjacent to the tumor may undergo abnormal activation, resulting in a population of cancerous fibroblasts (also known as cancer-activated fibroblasts or CAFs). These CAFs play a crucial role in remodeling the tumor microenvironment, promoting prostate cancer progression through the production of growth factors, pro-inflammatory cytokines and enzymes that facilitate tumor cell invasion and metastasis. The present study will use the LNCap (Lymph Node Cancer Prostate) lineage, FGC clone (ATCC CRL-1740) as a tumor line, which is a lineage of Invasive adenocarcinoma isolated from the lymph node of a 50-year-old Caucasian patient and which is positive for the expression of androgens and estrogens receptors.

The interaction between prostate cancer and human fibroblasts with gamma radiation, specifically using ^{60}Co, is an area of active research in radiation oncology, with the aim of damaging the DNA of these cells and preventing their ability to reproduce, causing damage to fibroblast DNA, leading to cell death or activation of signaling pathways that can have effects on surrounding tumor cells. A detailed understanding of the effects of radiation on fibroblasts and their interaction with prostate cancer cells is crucial to optimizing the effectiveness of radiotherapy.

2. Methodology

Cell cultures

HF002-J (murine embryonic fibroblasts) cells were a kind gift from Dr. Monica Beatriz Mathor (CETER / IPEN) and LNCap cells were cultured at 37ºC in a humid atmosphere containing 5% CO_2, maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics. When reaching 60-70% confluence, the cells were detached using a 0.05% trypsin solution. Spheroids were prepared using the hanging-drop technique adapted from 440 µL of medium containing cell variations ranging from 2×10^3 to 6×10^4 cells per well of a 96-well plate were deposited, generating a positive meniscus. The plate was inverted and incubated as described [1].

Irradiation

Cells in suspension or adhered were irradiated in PBS (Phosphate Buffered Saline) for gamma-ray irradiation at doses ranging from 0.5 to 16 Gy of gamma radiation form a ^{60}Co source, under sterile conditions and at room temperature. The irradiations were carried out using a GammaCell 220 model equipment (Irradiation Unit of Canadian Atomic Energy Commission, Ltd.) located at the Radiation Technology Center of IPEN/CNEN-SP, using lead shielding corresponding to 90% radiation attenuation.

Fluorescence microscopy assays

Cells in 96-well plates were stained with Hoechst 33342 solution (10 mg/mL) and SYTOX® Green (0.4 µM) and left in the incubator for 60 minutes. The material was visualized and photographed at 4X magnification (Nikon 4X/0.20, Plan Apo, CFI/6) using an automated fluorescence microscope (INCell 2500 HS, Cytiva) at 24, 48, and 72 hours. 20 to 50 acquisitions of each spheroid were performed, in focal planes 4 to 15 µm apart, generating stacks of images. Cell nuclei were highlighted by blue fluorescence, labeled by Hoechst 33342, and nuclei of non-viable cells were found to be highlighted in green, labeled by SYTOX® Green. The proportions between viable and non-viable cells were a quantitative parameter of spheroid cell viability. Morphological parameters such as cross-sectional area of nuclei, volume, fluorescence intensity, sphericity, and roundness were also evaluated.

3. Results and Discussion

The present study aimed to evaluate the development of cellular spheroids after 4 days of culture using different cell preparations. Our results demonstrated that the preparations used produced compact spheroids, characterized by homogeneous sizes in the range of 500 to 1000 µm. When analyzing the images obtained by wide-field fluorescence microscopy, we observed that the proportions of unviable cells labeled with fluorophores varied significantly according to the initial amount of cells used in the preparations. Notably, increasing the initial number of cells resulted in a proportional increase in the number of non-viable cells present in the formed spheroids.

These results suggest that the initial cell density can affect the development and viability of the formed spheroids. It is possible that higher cell densities could led to greater competition for nutrients and space, resulting in greater cell mortality and less viable spheroids.

4. Conclusions

An initial prototype of spheroids from human fibroblast cells that can resemble tissues in vivo due to

their cellular interactions was shown, thus providing a useful tool for the study of antitumoral drugs and treatments.

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