A Highthroughput Production of Composite Breast Tumoroids:
A Tool for Investigation of Cellular Heterogeneity and Drug Delivery

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Abstract

Breast tumors are typically heterogeneous and contain diverse subpopulations of tumor cells with differing phenotypic properties. This study has developed an *in vitro* coculture-based three-dimensional breast tumor model that studies the effects of mixing heterogeneous tumor cell populations. Breast cancer cell lines of different phenotypes (MDAMB231, MCF7 and ZR751) were cocultured in a rotating wall vessel (RWV) bioreactor to form a large number of heterogeneous tumoroids. Prior to each experiment, cells were labeled with cell tracker dyes to allow for time-course fluorescence microscopy to monitor cell aggregation. Histological sections of the tumor spheroids were stained with hematoxylin and eosin (HE), progesterone receptor (PR), E-cadherin (E-cad) and proliferation marker, ki67. Results showed that heterogeneous tumoroids reflected the composition the growth rate, invasion potential, and spatial distributions of heterogeneous tumor spheroids were highly dependent on cell composition. A suitable *in vitro* model for studying tumor-cell heterogeneity and reciprocal interactions will accelerate understanding of tumor cell phenotype population dynamics.

Introduction

Tumor heterogeneity
- Resistance to chemotherapeutic (Desoize et al., 1998)
- Variety of subpopulations of cells (Fidler et al., 1978)

Multicellular spheroids (MCS) (Sutherland et al., 1970)
- Micrometastases
- Avascular tumor regions
- Resistance to cytotoxic drugs

Microenvironmental effects (Sutherland., 1988)
- Proliferation
- Metabolism
- Differentiation

Other applications (Mueller-Klieser W., 1997)
- Embryogenesis
- Artificial tissue modeling and engineering

Methodology

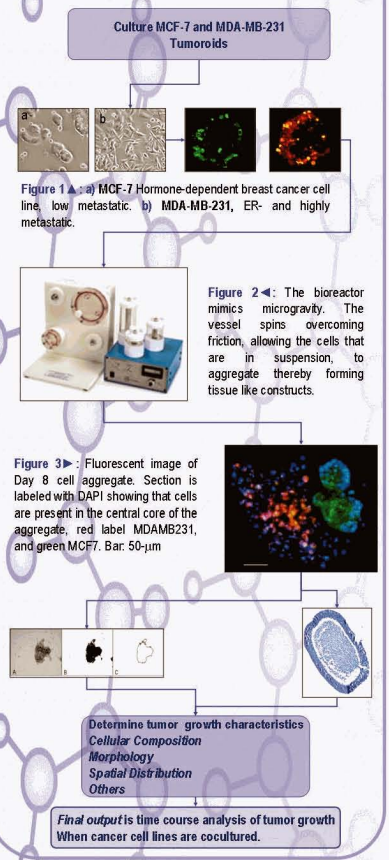

Figure 1▲: a) **MCF-7** Hormone-dependent breast cancer cell line, low metastatic. b) **MDA-MB-231,** ER- and highly metastatic.

Figure 2◄: The bioreactor mimics microgravity. The vessel spins overcoming friction, allowing the cells that are in suspension, to aggregate thereby forming tissue like constructs.

Figure 3►: Fluorescent image of Day 8 cell aggregate. Section is labeled with DAPI showing that cells are present in the central core of the aggregate, red label MDAMB231, and green MCF7. Bar: 50-μm

Results

Coculture Model

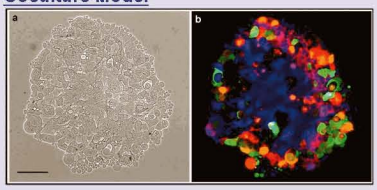

Figure 4◄: Day 1 cell aggregate of 1:1:1 coculture of MDAMB231, ZR751 and MCF7 breast cancer cells formed in the RWV. a) Image taken using phase contrast microscopy. b) Fluorescent labeling of three cell lines reveals spatial distribution, where the least aggressive cell line (blue: MCF7) is located in the center, and the more aggressive (red: MDAMB231) at the periphery. *Bar: 30 μm*

Time-Course of Tumoroid Evolution

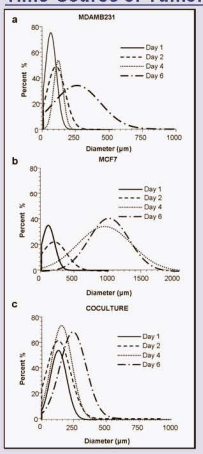

Figure 5◄: Size distribution for cell aggregates cultured in RWV. a) MDAMB231 cell aggregates seeded initially at 0.2 × 10^6 cells per ml in 10 ml RPMI-1640 medium for days 1, 2, 4 and 6. b) MCF7 cell aggregates seeded initially at 0.2 × 10^6 cells per ml in 10 ml RPMI-1640 medium for days 1, 2, 4 and 6. c) 1:1 coculture cell aggregates seeded initially at 0.2 × 10^6 cells per ml in 10 ml (0.1 × 10^6 each cell line) RPMI-1640 medium for days 1, 2, 4 and 6.

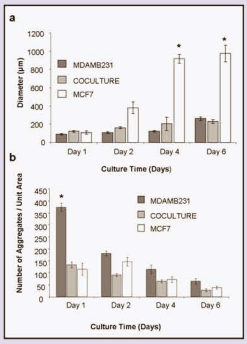

Figure 6►: a) Average diameter of the cell aggregates cultured in RWV follow an exponential growth pattern. cell aggregates displayed the typical solid tumor growth pattern of an exponential growth period followed by a plateau with little or no growth. b) Number of aggregates per unit area cultured in RWV and obtained from phase contrast images.The figure shows the mean of three different experiments (*P < 0.05).

Spatial Distribution of Tumor Heterogeneity

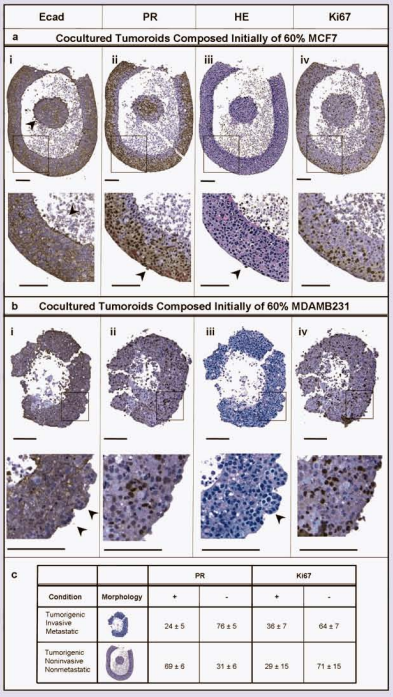

Figure 8▲: Hematoxylin-eosin (HE) and immunohistochemical staining of progesterone receptor (PR), E-cadherin (E-cad) and proliferation marker ki67 are shown of day 8 stained 10μm thick paraffin sections through spheroid cocultures of three breast cancer cell lines formed in the RWV, and composed of different cell concentrations at initial density of 2×10^6 cells per ml show a viable cell layer and necrotic core. Cell concentrations are a)0.5 × 10^6 cells per ml MCF7, 1 × 10^6 cells per ml MDAMB231 and 0.5 × 10^6 cells per ml ZR751 and b)1 × 10^6 cells per ml MCF7, 0.5 × 10^6 cells per ml MDAMB231 and 0.5 × 10^6 cells per ml ZR751. c) Differential expression of PR and ki67 between noninvasive, nonmetastatic condition versus invasive and metastatic. Bar: *100μm*

Cellular Composition of Heterogeneous Tumoroids

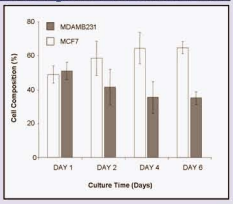

Figure 7►: Cellular composition of the heterogeneous tumoroids. Initially, cells were seeded at 1:1 ratio at 0.2 × 106 cells per ml. MDAMB231 and MCF7 cell lines were relabeled red and green respectively prior to mixing. At each time point, aggregates were collected, and cells were counted using time-course fluorescent microscopy. The figure shows the mean of three different experiments (*P < 0.05).

Migration Potential of Heterogeneous Tumoroids

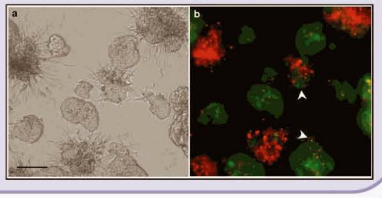

Figure 9►: Migration potential of tumors is highly dependent on cellular composition. a) Phase contrast image of tumor spheroids composed of MDAMB231 and MCF7 cells. b) Fluorescent micrograph of the same field demonstrating the identity of the cells comprising the morphologically different tumor spheroids. Tumors composed primarily of MDAMB231 (red) show invasion of the surrounding collagen matrix, in contrast to tumors which are primarily composed of MCF7 (green). *Bar: 100μm*

Conclusions

In this study, we developed a novel high-throughput heterogeneous spheroid model *in vitro*. Initial cell density of cultures as well as cell ratio composition are important parameters that have an effect on cellular adhesion and aggregation ability, invasion, spatial distribution, size and viability. Cell-cell adhesions vary according to cell type and also coculture conditions and for this reason studies of tumor classification, composed of multiple cell types, should take into consideration these parameters. MDAMB231 cells had much slower adhesion rates than that of MCF7 due to different adhesion surface molecules. The observations come to verify the preexisting differential adhesion hypothesis by Ramsey et al., which proposed that a motile population will spontaneously tend to replace weaker intercellular adhesions with stronger ones until it approaches that configuration in which adhesive bonding is maximized taking also into account that in the case when different cell populations are mixed, cells expressing similar adhesion molecules will aggregate each other with homophilic bonds affecting in this way the cellular organization of heterogeneous spheroids. The coculture model presented here corresponds to early stages of tumor growth in the absence of vasculature. As such it provides an excellent model for investigating early stages of tumor development. The ability to monitor the growth and spatial distribution of the cancer cells in a high throughput *in vitro* system will aid our understanding of the early stages of early cancer development. The model system developed here can be used in investigating the impact of cell phenotype heterogeneity on global gene expressions profiles (microarray data) as well as developing *in vitro* drug testing and drug delivery systems. Effective therapeutic agents and trials for the treatment of malignant tumors should target the metastatic subpopulations of cells. Heterogeneous *in vitro* models could account for the mixed responses to detection, treatment and evaluation protocols as a result of tumor heterogeneity. Moreover, metastatis potential into different tissue types can be considered with this system by infusing composite tumoroids into microfluidic channels coated with cultured cells of other tissue types.