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Anti-mitochondrial therapy in human breast cancer multi-cellular spheroids

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ABSTRACT

During multi-cellular tumor spheroid growth, oxygen and nutrient gradients develop inducing specific genetic and metabolic changes in the proliferative and quiescent cellular layers. An integral analysis of proteomics, metabolomics, kinetomics and fluxomics revealed that both proliferative- (PRL) and quiescent-enriched (QS) cellular layers of mature breast tumor MCF-7 multi-cellular spheroids maintained similar glycolytic rates (3–5 nmol/min/10^6 cells), correlating with similar GLUT1, GLUT3, PFK-1, and HKII contents, and HK and LDH activities. Enhanced glycolytic fluxes in both cell layer fractions also correlated with higher HIF-1α content, compared to MCF-7 monolayer cultures. On the contrary, the contents of the mitochondrial proteins GA-K, ND1, COXIV, PDH-E1α, 2-OGDH, SDH and F1-ATP synthase (20 times) and the oxidative phosphorylation (OxPhos) flux (2-times) were higher in PRL *vs.* QS. Enhanced mitochondrial metabolism in the PRL layers correlated with an increase in the oncogenes h-Ras and c-Myc, and transcription factors p32 and PGC-1α, which are involved in the OxPhos activation. On the other hand, the lower mitochondrial function in QS was associated with an increase in Beclin, LC3B, Bnip3 and LAMP protein levels, indicating active mitophagy and lysosome biosynthesis processes. Although a substantial increase in glycolysis was developed, OxPhos was the predominant ATP supplier in both QS and PRL layers. Therefore, targeted anti-mitochondrial therapy by using oligomycin (IC_{50} = 11 nM), Casiopeina II-gly (IC_{50} = 40 nM) or Mitoves (IC_{50} = 7 nM) was effective to arrest MCF-7 spheroid growth without apparent effect on normal epithelial breast tissue at similar doses; canonical anti-neoplastic drugs such as cisplatin and tamoxifen were significantly less potent.

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1. Introduction

Accelerated glycolysis, even in the presence of saturated O_2 concentration is a common characteristic of all studied neoplasias [reviewed in 1,2]. However, the relevance of oxidative phosphorylation (OxPhos) supporting cancer growth has been documented for a variety of cancer cell lines [1, reviewed in 3] and experimental models, including bi-dimensional [3,4] and tri-dimensional systems (also called human tumor multi-cellular spheroids – MCTS – which resemble early stages of solid tumor) [5–7], and xenografts in mice [8,9]. In this regard, it has been described that OxPhos supports the high ATP demand required during the early stages of HeLa and Hek293 MCTS growth [7]. However, the addition of mitochondrial inhibitors such as Casiopeina-IIgly (CasII-gly) only induces a partial diminution of tumor spheroid growth, indicating that HeLa and Hek293 MCTS proliferation may also be supported by glycolysis [7].

The metabolic reprogramming of mature MCTS (close to 1 mm diameter) involves a marked increase in HIF-1α-activated glycolysis and severe OxPhos depression [7]. The molecular mechanism associated with the glycolytic activation triggered by the development of a hypoxic microenvironment in the tumor core, involves the HIF-1α stabilization and enhancement in the glycolytic gene transcription, protein contents, enzyme activities and flux [reviewed in 10].

Solid tumors develop a tri-dimensional structure that favors the formation of nutrients and oxygen gradients, which in turn promotes the formation of three regions with clearly different phenotypes [11,12]. These three well-defined regions (the proliferative, quiescent and the necrotic center) have been analyzed as a whole, *i.e.*, in mature, entire MCTS (HeLa, Hek293, U343MG, EMT6/Ro, BT474, DU145, T47-D) in which growth rate, morphology and physiology have been determined [7,13–21]. However, the phenotypic characteristics determined in

Abbreviations: Atg7, autophagy-related gene 7; Bnip 3, Bcl2/adenovirus EIB 19 kD-interacting protein 3; COXIV, cytochrome oxidase subunit IV; GA-K, glutaminase-K; GLUT, glucose transporters; HK, hexokinase; HIF-1α, hypoxia inducible factor-1 alpha; LAMP, lysosome-associated membrane proteins; LDH, lactate dehydrogenase; LC3B, autophagy marker light chain 3 isoform B; MCT-1, monocarboxylate transporter-1; MCTS, multi-cellular tumor spheroids; ND1, NADH dehydrogenase (complex I) subunit 1; PDH, pyruvate dehydrogenase; PGC-1α, peroxisome proliferator activated receptor gamma coactivator-1 alpha; PRL, proliferative cell layers of tumor spheroids; QS, quiescent cell layers of tumor spheroids; SDH, succinate dehydrogenase; 2OG, 2-oxoglutarate; 2OGDH, 2-oxoglutarate dehydrogenase

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whole spheroids may not reflect the intrinsic signature of each region inside the tumor derived from the large metabolite and oxygen fluctuations throughout the spheroid [11,21].

Regarding energy metabolism, scarce information is available on its cellular zonation across MCTSs. Freyer [16,22] described a severe diminution in the rhodamine123 retention, and presumably oxygen consumption, in both outer and inner cellular layers of EMT6 mouse mammary carcinoma and 9L rat glioma MCTSs in comparison to cells grown as monolayers [16,22]. Unfortunately, the rhodamine123 concentration used in these studies promotes severe perturbation to the OxPhos by acting both as an uncoupler and an inhibitor of the ATP/ADP translocator and ATP synthase [23,24], making difficult the interpretation of results. In addition, the cellular oxygen consumption measurements in MCTSs have not usually been corrected by using a respiratory chain inhibitor or oligomycin to discard the O_2-uptake by non-mitochondrial enzymes, whose activity is 2.5–5 times increased in tri-dimensional models [25,26].

In solid tumors and probably in MCTS models, prolonged hypoxia promotes a metabolic symbiosis in which the lactate overproduced by the highly glycolytic and hypoxic cells (*i.e.*, inner, and presumably, quiescent cell layers) is actively consumed by the blood-closer and well-oxygenated cells (*i.e.*, external layers) for oxidative metabolism. This metabolic switch involves the over-expression of, at least, two proteins in the oxidative tumor cells, which are required for the massive lactate uptake (MCT-1) and for the rapid cytosolic lactate oxidation (LDH-B) to generate pyruvate, which in turn, enters into the mitochondria for generation of reducing equivalents and ATP [27]. These observations strongly suggest that the micro-area, in which the tumor cell lies inside MCTS, may modify the energy cellular metabolism. Therefore, the identification of the principal energy supply pathway for each cellular population in the MCTSs, and solid tumors, and hence the use of specific and potent metabolic inhibitors against this particular pathway may be considered as a potential anti-tumor strategy.

To analyze the bioenergetics of the MCTS external and inner cellular layers, proteomic analysis, kinetic determinations and metabolic fluxes of OxPhos and glycolysis were performed in disaggregated mature spheroids. In parallel, the expression patterns of several transcription factors involved in the modulation of glycolysis and mitochondrial metabolism were also analyzed. Once the principal ATP producer was identified, specific anti-tumor therapy was designed for the entire mature MCTS using permeable and selective inhibitors to diminish tumor growth. In parallel, canonical chemotherapy drugs were evaluated on MCTS growth for comparative purposes. Results of the present study may contribute to the better understanding of the energy metabolism changes in the basic unit of tumor growth and provide guidance in the design of more appropriated targeted clinical treatment strategies.

2. Materials and methods

2.1. Monolayer and spheroid cultures

Breast human tumor stage-3 MCF-7 (1×10^7 cells/dish) and the normal epithelial breast MCF-12A cells (American Type Culture Collection; Rockville, MD, USA) were grown in Dulbecco-MEM medium supplemented with 10% fetal bovine serum (GIBCO; Rockville, MD, USA) plus 10,000 U penicillin/streptomycin (SIGMA; Steinheim, Germany) and placed under a humidified atmosphere of 5% CO_2/95% air at 37 °C during 3–4 days until confluence of 80–90% was reached. The genotyping of the MCF-7 cells (National Institute of Genomic Medicine, Mexico) used in the present study revealed that they are already a subclone because they only share 5 out of 14 canonic allelic markers with the original MCF-7 clone (American Type Culture Collection; Rockville, MD, USA). Afterwards, cells were gently detached from the culture dish by a 2–3-min exposure to 3 mL of 0.25% trypsin/EDTA (GIBCO), followed by washing with fresh Krebs–Ringer medium (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.4 mM $CaCl_2$, 1 mM H_2PO_4, 25 mM HEPES, pH 7.4) and centrifugation at $300 \times g$ for 2–4 min at room temperature [28].

MCF-7 and MCF-12A spheroids were formed by using the liquid overlay modified technique [29,30]. Briefly, 1×10^5 cells were seeded in 2% (w/v) agarose-coated Petri dishes. Once spheroids reached a diameter of 100 ± 57 (MCF-7; n = 140) or 197 ± 30 (MCF-12A; n = 80) μm, medium was replaced with fresh medium and placed under slow (20–50 rpm) orbital shaking for additional 14–18 (MCF-7) or 9–10 (MCF-12A) days at 37 °C under 95% air/5% CO_2. Fresh medium was added every 2–3 days to remove cellular debris and non-well formed spheroids. The size of breast tumor and non-tumor spheroids was measured at different culture times with a graduated reticule (1/10 mm; Zeiss, Thornwood, NY, USA) in an inverted phase contrast microscope (Zeiss).

2.2. Selective disaggregation of MCF-7 spheroids

Mature spheroids (863 ± 64 μm diameter after 20 days of culture; n = 140; Fig. 1A) were sequentially trypsinized using the standard dissociation method [13] to separate both external (proliferative) and internal (quiescent + apoptotic) cell populations. Briefly, 20–40 spheroids were exposed to 5 mL 0.25% trypsin/EDTA under gentle orbital agitation (20–50 rpm) at 37 °C for 3 min. Then, two fractions were collected: a supernatant containing proliferative cells and a bottom constituted by the quiescent cells. Both cellular fractions were gently washed with fresh medium and centrifuged at $34{,}000 \times g$ for 5 min, at 37 °C. The cellular bottoms were re-suspended in fresh D-MEM and stored at room temperature until use. Cellular viability was determined by using the blue trypan method [4], which yielded values of 87 ± 8 and $> 95 \pm 5$% for quiescent (QS) and proliferative (PRL) cell-enriched layers, respectively. Cellular protein was determined by using the Biuret assay as described elsewhere [4].

To determine the growth capacity of each isolated MCTS (QS or PRL) fraction, the cells (5×10^4 cells) were seeded in 1 mL D-MEM in 24 multi-well plates and incubated at 37 °C, under 5%CO_2/95% air. Cellular numbers were quantified every 48 h until 144 h of culture by using the trypan blue assay [4].

2.3. Immunohistochemical analyses of the MCTS

Mature spheroids were fixed by incubating with 10% paraformaldehyde at 4 °C overnight and washed with fresh phosphate buffer. Afterwards, spheroids were embedded in paraffin and sectioned into 2 μm thick layers. Cut layers were stained with the primary Ki-67 antibody (Santa Cruz, CA, USA) at a final dilution of 1:70 for 20 min or with hematoxylin & eosin (H&E). The Ki67 and H&E detection was performed in the automated BenchMark ULTRA system (Roche, Tucson, AZ, USA) using the ultraView Universal DAB detection system (Ventana Medical System Inc, Tucson, AZ, USA) following established protocols [31].

2.4. Determination of metabolic fluxes

For OxPhos flux, oligomycin-sensitive respiration was determined at 37 °C in both quiescent (3.5–4×10^6 cells) and proliferative (1–1.5×10^6 cells) cell fractions by using a Clark-type electrode in an air-saturated Krebs–Ringer medium plus 5 mM glucose. To reveal the activity of the respiratory chain, KCN-sensitive respiration was also determined. For glycolysis, both outer (proliferating) and inner (quiescent) cell-enriched layers (1–30×10^6 cells) were pre-incubated for 10 min in 3 mL Krebs–Ringer medium under orbital shaking (150 rpm, 37 °C) and afterwards, 5 mM glucose was added. After 3 min, samples were precipitated with 3% (v/v) ice-cold perchloric acid and neutralized with 3 M KOH/0.1 M Tris. Neutralized samples were used for lactate, ATP and ADP determinations [32]. L-Lactate generated by glycolysis was determined by using lactate dehydrogenase following the NADH formation at 340 nm [33].

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