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GPI/AMF inhibition blocks the development of the metastatic phenotype of mature multi-cellular tumor spheroids

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ABSTRACT

Epithelial–mesenchymal transition (EMT) and cellular invasiveness are two pivotal processes for the development of metastatic tumor phenotypes. The metastatic profile of non-metastatic MCF-7 cells growing as multicellular tumor microspheroids (MCTSs) was analyzed by determining the contents of the EMT, invasive and migratory proteins, as well as their migration and invasiveness potential and capacity to secrete active cytokines such as the glucose phosphate isomerase/AMF (GPI/AMF). As for the control, the same analysis was also performed in MCF-7 and MDA-MB-231 (highly metastatic, MDA) monolayer cells, and in stage IIIB and IV human metastatic breast biopsies. The proliferative cell layers (PRL) of mature MCF-7 MCTSs, MDA monolayer cells and metastatic biopsies exhibited increased cellular contents (2–15 times) of EMT (β-catenin, SNAIL), migratory (vimentin, cytokeratin, and fibronectin) and invasive (MMP-1, VEGF) proteins *versus* MCF-7 monolayer cells, quiescent cell layers of mature MCF-7 MCTS and non-metastatic breast biopsies. The increase in metastatic proteins correlated with substantially elevated cellular abilities for migration (18-times) and invasiveness (13-times) and with the higher level (6-times) of the cytokine **G**PI/AMF in the extracellular medium of PRL, as compared to MCF-7 monolayer cells. Interestingly, the addition of the GPI/AMF inhibitors erythrose-4-phosphate or 6-phosphogluconate at micromolar doses significantly decreased its *extracellular* activity (>80%), with a concomitant diminution in the metastatic protein content and migratory tumor cell capacity, and with no inhibitory effect on tumor lactate production or toxicity on 3T3 mouse fibroblasts. The present findings provide new insights into the discovery of metabolic inhibitors to be used as complementary therapy against metastatic and aggressive tumors.

1. Introduction

Once tumor metastasis progresses, the response to conventional anti-cancer therapy significantly declines contributing to decrease the cancer patient's life quality and leading to an increase in fatal outcomes [1,2]. Therefore, the search for specific metastasis biomarkers [3] and in consequence, strategies directed against metastatic progression has emerged as an urgent need in clinical research [2,4–6]. It has been demonstrated that the epithelial–mesenchymal transition (EMT), a mechanism developed by epithelial cancer cells to acquire a metastatic phenotype, plays an essential role during solid tumor progression towards metastasis [7,8]. The EMT onset involves the secretion of some growth factors such as EGF, HDGF, b-FGF, insulin-like growth factor, and some cytokines (BMP, TGF-β, and VEGF) [9,10], all of which drive the metastatic processes through diverse signaling pathways (Snail-1, Slug, Twist, β-catenin, matrix metalloproteinase, vimentin, N-cadherin, fibronectin, urokinase PAR receptor, and cytokeratin) and downstream activation [11,12]. However, these same growth factors and cytokines are also expelled by some normal cells (keratinocytes and epithelial cells) during wound healing and tissue regeneration, activating specific repair mechanism. Thus, the anti-cancer strategy to block the secretion of these molecules may result disadvantageously for the host cells [4,13].

Recently, it has been documented that some metabolic pathway enzymes such as the glucose phosphate isomerase (GPI), when secreted to the extracellular milieu, act as a potent cytokine (autocrine motility factor, AMF) [14,15]. Although its role as a metastasis inducer has not been clearly demonstrated, a significant increase (4–6 times *vs.* no EMT-

Abbreviations: EGF, epidermal growth factor; EMT, epithelial–mesenchymal transition; ERI4P, erythrose-4-phosphate; b-FGF, basic fibroblast growth factor; HDGF, hepatocyte-derived growth factor; GPI/AMF, glucose phosphate isomerase/autocrine motility factor; LDH, lactate dehydrogenase; MMP-1, matrix metalloproteinases-1; MCTS, multi-cellular tumor spheroids; PRL, proliferative cell layers; QS, quiescent cell layers; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; 6PG, 6-phosphogluconate

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induced cells) of the extracellular GPI/AMF content in EMT-induced fibrosarcoma and pancreatic carcinoma has been documented, which in turn promotes the acquisition of metastatic phenotypes [16,17]. In fact, it has been shown that the use of GPI inhibitors such as erythrose-4-phosphate or D-mannose-6-phosphate diminishes the tumor migratory and invasive capacities in bi-dimensional cultures of several breast cancer lines (SK-BR3, BT-474 and Zr-75R) without apparent effect on normal cells [15,18–21], suggesting that the GPI inhibition could be a selective strategy to block tumor metastasis. However, monolayer cultures cannot accurately reproduce the behavior of cancer cells within solid tumors regarding the cyclic exposure to O_2, glucose, other nutrients, and growth factors, and the distinct transcriptional and biochemical regulation imposed by the formation of metabolite gradients between the cell layers near the blood vessels and those localized more than 100 μm away from them [22,23].

Therefore, the present study focused on elucidating the effect of secreted GPI/AMF on EMT, migratory and invasive tumor cell processes in an experimental model that resembles the initial stages of solid tumor formation: the multi-cellular tumor spheroids (MCTSs) [24]. In this regard, no studies have been performed in MCTSs where migration, invasiveness and EMT are simultaneously analyzed. In addition, the role of cytokines such as GPI/AMF on tumor migration, invasiveness and EMT has not been evaluated, which takes relevance considering that its enzymatic inhibition by erytrose-4-phosphate or 6-phosphogluconate may diminish the EMT process and in consequence may block metastatic progression. In addition, to assess whether the findings on the acquisition of metastatic phenotype using MCTSs have physiological relevance, the GPI/AMF content and EMT and protein migration profiles were also determined in metastatic human breast tumor biopsies (stages IIIB and IV).

2. Material and methods

2.1. Human breast tumor tissue

Ten female patients (from 40 to 80 years old) diagnosed with infiltrating ductal breast carcinoma (IDC) were selected from a cross-sectional study based in a medical protocol approved by the Ethics and Research Committees of the Instituto Nacional de Cancerología (INCAN), México. The patients were punctured with a tru-cut biopsy needle in the absence of local anesthesia. Histopathology was performed on hematoxylin and eosin stained biopsy slides. Each specimen was analyzed by visual inspection using standard light microscope by experimented pathologists [25]. According to routine pathological procedure to determine Scarf Bloom Richardson differentiation state and the presence of lymph nodes [26], the 10 samples were classified as no-metastatic stage 1 (n = 1); stage IIB (n = 1); potentially metastatic stage IIIB (n = 7) and metastatic stage IV (n = 1).

Non-tumor tissue was surgically withdrawn from selected areas of normal breast tissue of the 3 patients and stored in liquid nitrogen in the INCAN Tumor Bank for 12–24 months [27]. Non-tumor samples were further validated as non-tumorigenic by assessing immunohistochemistry (IHC) negativity towards Her2 and hormone receptor.

Samples from biopsies (0.4–1.8 mg total cellular protein) were placed in liquid nitrogen and kept at −70 °C until their use. For western blotting, frozen tumor and normal samples were powdered, resuspended and homogenized in 0.6 mL 25 mM Tris–HCl buffer, pH 7.4, *plus* 1 mM PMSF (phenyl methanesulfonyl fluoride), 1 mM EDTA and 5 mM DTT, and centrifuged at 2000 *g* for 30 min at 4 °C. Afterwards, supernatants were recollected and protein content was determined by using the Lowry assay. The protein contents of GPI, VEGF, MMP1 and vimentin were determined (see Western blot assay section below) with specific monoclonal antibodies (1:500–1:1000 dilutions; Santa Cruz Biotechnology, Santa Cruz California, USA).

2.2. MCF-7 spheroids formation

Human breast tumor MCF-7 cells (1×10^5/mL) were grown in Dulbecco-MEM medium supplemented with 10% fetal bovine serum (GIBCO, Rockville, USA) and 10,000 U penicillin/streptomycin (Sigma; Steinheim, Germany) and placed in a humidified atmosphere of 5% CO_2/95% air at 37 °C for 3–4 days until confluence of 80–90% was reached. Afterwards, cells were treated with 0.25% trypsin/EDTA (GIBCO, Rockville, USA) for 2–3 min and washed by centrifugation (300 ×*g*) with fresh PBS [28]. MCF-7 MCTSs were formed from monolayer cultures by using the liquid overlay modified technique [29]. Briefly, 1×10^5 cells were seeded in 2% (w/v) agarose-coated Petri dishes. Once spheroids (n = 140) reached a diameter of 100 ± 57 μm (after 5 days of culture), the medium was replaced with fresh DMEM medium and placed under slow (20–50 rpm) orbital shaking for 14 days at 37 °C in 95% air/5% CO_2. The old medium was replaced with fresh medium each 2 to 3 days to remove cellular debris and non-formed spheroids. MCF-7 spheroid size was measured daily with a graduated reticule (1/10 mm; Zeiss, NY, USA) in an inverted phase contrast microscope (Zeiss, NY, USA).

2.3. Selective disaggregation of MCF-7 spheroids

Mature MCF-7 MCTSs (730 ± 40 μm diameter; n = 120) were recollected at days 16–18 of culture and sequentially trypsinizated by using a modified dissociation method [30,31] to separate both external (proliferative, PRL) and internal (quiescent, QS) cellular subpopulations. Briefly, 20–50 spheroids were exposed to 0.25% trypsin/EDTA solution under smooth orbital shaking at 37 °C. Two fractions were collected after 3 min of incubation, a rich-proliferative (supernatant) and rich-quiescent (bottom) cell subpopulations [31]. Afterwards, both cellular fractions were washed by differential centrifugation at 3400 ×*g* by 5 min, 37 °C. The cellular bottoms were resuspended in fresh DMEM. Using the blue trypan method [32], the cellular viability was higher than 85% and 95% for QS and PRL, respectively. Cellular protein for western blot assays was determined by using the Biuret method as previously described [32].

2.4. Cell proliferation and viability assays

Proliferation assays for QS and PRL layers were performed as described elsewhere [31]. Both QS and PRL-rich fractions (5×10 cells/mL) were cultured in DMEM in 24-multiwell plates and incubated at 37 °C. For comparative purposes the proliferation rates of MCF-7 and MDA-MB-231 monolayer cells (both at 5×10^4 cells/mL) were also determined at 24, 48, 72 and 96 h of culture. Cellular viability was 87 ± 8 and >95 ± 5% for QS and PRL cell fractions, respectively, whereas for MCF-7 and MDA-MB-231 monolayer cells viability was higher than 98%.

2.5. Invasiveness assays

PRL and QS cells from MCF-7 spheroids were placed in the upper compartment of 96-multiwell Boyden chamber at a final concentration of 5×10^4 cells/well in non-serum DMEM medium (Trevigen Inc., Helgerman, USA). After 24 h, the number of cells that have migrated to the lower chamber compartment was determined with 60 nM calcein AM (acetomethylester). After 60 min incubation, fluorescence was detected at 485 nm excitation and 520 nm emission in a microplate reader (Nunclon ™) [33]. For control assays, MCF-7 and MDA-MB-231 monolayer cells (both at 5×10^4 cells/mL) were used.

2.6. Migration assays

Cellular migration was evaluated by using (A) visualization by light microscopy of living spindle-like cells well-attached and spread around

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