Biochimica et Biophysica Acta 1853 (2015) 328–337

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

The inhibition of aromatase alters the mechanical and rheological properties of non-small-cell lung cancer cell lines affecting cell migration

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ARTICLE INFO

Article history:
Received 4 June 2014
Received in revised form 10 November 2014
Accepted 12 November 2014
Available online 18 November 2014

Keywords:
Non-small-cell lung cancer cell
Migration
Aromatase
Mechanical properties

ABSTRACT

Tumor invasion and metastasis are key aspects of non-small cell lung cancer (NSCLC). During migration, cells undergo mechanical alterations. The mechanical phenotype of breast cancer cells is correlated with aromatase gene expression. We have previously shown that targeting aromatase is a promising strategy for NSCLC. The aim of this study was to examine morphological and mechanical changes of NSCLC cells, upon treatment with aromatase inhibitor and correlate their ability to migrate and invade. In vitro experiments were performed using H23 and A549 NSCLC cell lines and exemestane was used for aromatase inhibition. We demonstrated that exemestane reduced H23 cell migration and invasion and caused changes in cell morphology including increased vacuolar structures and greater pleomorphism. In addition, exemestane changed the distribution of α-tubulin in H23 and A549 cells in a way that might destabilize microtubules polymerization. These effects were associated with increased cell viscosity and decreased elastic shear modulus. Although exemestane caused similar effects in A549 cells regarding viscosity and elastic shear modulus, it did not affect A549 cell migration and caused an increase in invasion. The increased invasion was in line with vimentin perinuclear localization. Our data show that the treatment of NSCLC cells with an aromatase inhibitor not only affects cell migration and invasion but also alters the mechanical properties of the cells. It suggests that the different origin of cancer cells is associated with different morphological characteristics and mechanical behavior.

1. Introduction

Non-small-cell lung cancer (NSCLC) is the most common malignancy worldwide in both sexes and it is responsible for more than one million deaths annually [1]. Despite the promising actions of new therapeutic regimens, the survival of NSCLC patients is still poor with more than 65% of patients with NSCLC presenting with advanced or metastatic disease at the time of diagnosis [2]. In fact, metastasis is the leading cause of death in these patients; thus unveiling that the mechanism of tumor progression and metastasis is of great importance [2]. Cancer cells have the ability to invade tissues and spread from their initial site to distant organs [3]. The initial steps of local invasion include the activation of signaling pathways that control cytoskeletal dynamics of tumor cells, the turnover of cell–matrix and cell–cell junctions. These steps are followed by active tumor cell migration followed by metastasis to distant organs [4].

It is now well documented that cell microenvironment regulates cell movement. The physical and chemical properties of the extracellular matrix facilitate the conversion of the cell surface mechanical cues to biochemical response and hence the ability of the cells to sense their microenvironment and adjust accordingly [5–7]. The mechanical response of cells during migration includes cell micro-structural changes, alteration in cell deformability and function as well as changes in cell ability to migrate [8]. Recent studies suggest that changes in the mechanical characteristics in cells reflect changes in cytoskeletal structure and composition that facilitate cell movement and/or migration [9–11]. In the case of cancer, cell stiffness has been postulated to play a critical role in transmigration through a basement membrane [8]. A series of biophysical techniques have been applied to probe the mechanical properties of cells. While cancer tissue has been found to be generally stiffer than the normal, recent studies have shown that malignant cells themselves are more compliant than normal cells [12,13]. However, correlation between cell mechanical properties and cancer

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http://dx.doi.org/10.1016/j.bbamcr.2014.11.016
0167-4889/

progression especially after treatment has not been examined thoroughly [14].

The aromatase is an enzyme complex that catalyzes the final step of estrogen synthesis in tissues, including breast and lung [15]. Exemestane is an irreversible steroidal aromatase inhibitor approved for postmenopausal women with breast cancer [16]. Previous studies, we and others have conducted, show that exemestane exerts an antitumor effect in NSCLC cell lines, inhibiting cell proliferation while reducing single cell migration [17,18]. Recent evidence has correlated the mechanical phenotype of breast cancer cells with aromatase gene expression. The authors included in cell phenotype the cell density, cell shape and response to various culture substrates and showed that these factors affect aromatase gene expression [19].

The aim of the current study is to address the morphological changes and mechanical properties of NSCLC cells upon treatment with exemestane and correlate them with the ability of cancer cells to migrate and invade. Cell migration and invasion was estimated using scratch-wound and invasion assay, respectively and the morphological observation of cells was performed using scanning electron microscopy (SEM). In order for the rheological and mechanical properties of the cell lines (cell viscosity, η, and elastic shear modulus, G, respectively) to be investigated, the micropipette technique was employed [20]. Micropipette aspiration (MA) is the most feasible and convenient one in this category, compared to AFM, the MA approach seems to be more reliable in representing the whole mechanical properties of a cell since it produces a deformation of the entire suspended cell, and it eliminates undesirable effects of cell–matrix interaction and more importantly the stiffness of substrate [20–23]. Single-cell stiffness has recently been recognized as a promising feature for assessment of the cell invasiveness and can provide new insights into the process of cancer metastasis [24].

2. Materials and methods

2.1. Cell culture and reagents

NSCLC cell lines H23 (adenocarcinoma) and A549 (squamous cell carcinoma) were purchased from the American Type Culture Collection (ATCC, LGC Standards, Wesel, Germany) and cultured as recommended by the manufacturer. Cells were cultured in RPMI 1640 medium with 2 mM of L-glutamine and supplemented with 1 mM of sodium pyruvate, 4.5 g/L of glucose, 1.5 g/L of sodium bicarbonate and 10% fetal bovine serum. The culture conditions used were 37 °C, 5% CO_2 and 100% humidity.

Aromatase inhibitor, exemestane (Aromasin) was obtained by Pfizer (Hellas). H23 and A549 cells were treated with exemestane as previously described [25]. Exemestane was applied to cell lines after cell attachment at the IC_{50} concentrations of 50 μM and 20 μM for H23 and A549 cells, respectively. Exemestane was then diluted in dimethyl sulfoxide (DMSO) with its final concentration in culture medium being 0.5%. After reaching 50% confluence, cells were washed with phosphate buffered saline (PBS) and incubated with phenol red-free medium (rf-medium) with 1% dextran-coated, charcoal-stripped serum (CSS) for 24 h to deplete estrogen. Then, exemestane was added to cells and the experiments were terminated at the indicated time point.

2.2. Scratch-wound assay

The effect of exemestane on collective cell migration was evaluated using 2D scratch-wound assay. Cells were seeded in 6-well plates at a density of 2×10^5 cells/well. After reaching 100% of confluence, cells were treated in the appropriate medium, rf-RPMI, with 1% CSS for 24 h. In the confluent cells' monolayer, an artificial gap was created with a yellow pipette tip. Cells were then rinsed several times with the appropriate medium to remove dislodged cells. Exemestane was finally added to cells and 24 h later images of living cells were captured using an inverted microscope at 4× magnification (Axiovert 40 CFL, AxioCam ERc, Zeiss, Germany).

2.3. Invasion assay

To evaluate the effect of exemestane on cell to invasion, a boyden chamber containing matrigel coated polycarbonate membranes with 8 μm (Invasion Chambers, BD Biosciences, Oxford, UK) was used. Cells were cultured with rf-RPMI supplemented with 1% CSS for 24 h. Exemestane was added to cells and 24 h later, cells were trypsinized and resuspended at 5×10^4/0.1 ml in rf-RPMI with 1% CSS. Cell solution was loaded in the upper chamber. The bottom chamber was filled with 0.6 ml of rf-RPMI with 10% CSS. 24 h later, the non-invading cells were removed from the upper compartment using a cotton swab. Transwell filters were fixed with saline-buffered formalin for 10 min and then in 100% methanol for 20 min. Cells were stained in toluidine blue solution for 10 min and washed twice in 1% PBS. Images of cells that have migrated through the matrigel coated filter were captured using an inverted microscope (Axiovert 40 CFL, AxioCam ERc, Zeiss, Germany) at a magnification of 20×.

2.4. Scanning electron microscopy (SEM)

The effect of exemestane in cell surface morphology was evaluated with scanning electron microscopy. Cells were seeded in an 8 well coverslip at a density of 3×10^4 cells/well and treated with exemestane for 24 h as described above. After the indicated incubation time, cells were washed once with PBS and were then fixed in 2.5% glutaraldehyde at room temperature for 20 min followed by an additional washing cycle. Subsequently, cells were dehydrated with 10%, 30%, 50%, 70%, 90% and 100% ethanol gradient for 20 min in each step. The samples were coated with gold and examined under scanning electron microscope (SEM, JEOL 6300) with an accelerating voltage of 20 kV.

2.5. Immunofluorescence assay

Cells were grown in 4-well coverslips (15×10^3 cells/well) as described above. After 24 h of incubation with exemestane, cells were fixed with saline-buffered formalin for 15 min and permeabilized with 0.1% Triton for 5 min. Blocking was performed with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 10% FBS for 1 h at 37 °C. After the incubation, cells were rinsed once with PBS for 5 min and then incubated with phalloidin–fluorescein isothiocyanate labeled (1:1000, Sigma-Aldrich, Inc., Germany), a mouse anti-a-tubulin (1: 500, Sigma-Aldrich, Inc., Germany) or a mouse anti-vimentin (1:100, Novocastra, UK) for 1 h at 37 °C. Cells were rinsed 3 × 5 min with PBS and then a chicken anti-mouse Alexa Fluor 594 (1:1000, Molecular Probe, Invitrogen Corporation, Camarillo, CA, USA) diluted in blocking solution and incubation for 30 min at 37 °C was followed. Cells were rinsed 2 × 5 min with PBS and then incubated for 5 min with Hoechst 33258 (Sigma-Aldrich, Inc., Germany); dilution in PBS was followed for nucleus staining and cells mounted on glass sides. Fluorescence was visualized using a Nikon microscope at 40× magnification.

2.6. Micropipette technique

2.6.1. Micropipette aspiration

The study of the rheological and mechanical properties of cancer cells included the measurement of cell viscosity, η, and the elastic shear modulus, G, of cells. A micropipette aspiration technique was used to partially aspirate the cell and obtain measurements of the resulted aspirated length for a given value of negative suction pressure being applied each time, following the method described by Evans et al. [26]. The instrumentation and experimental set up consisted of the borosilicate glass capillary micropipette, controlled by a mechanical