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Research Article

Growth and gene expression differ over time in alpha-linolenic acid treated breast cancer cells

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ABSTRACT

Scope: Heterogeneity of breast cancer (BC) subtypes makes BC treatment difficult. α-linolenic acid (ALA), rich in flaxseed oil, has been shown to reduce growth and increase apoptosis in several BC cell lines, but the mechanism of action needs further understanding.

Methods and results: Four BC cell lines (MCF-7, BT-474, MDA-MB-231 and MDA-MB-468) were incubated with 75 μM ALA+1 nM 17-β estradiol (E2) or 1 nM E2 only (control) for 24 h. MDA-MB-231 cells were additionally incubated at 6 and 12 h. Viable cell number was measured, and expression of genes related to BC (signaling pathways, cell cycle, apoptosis) was quantified by real-time PCR array. There was a reduction in growth of all ALA treated cell lines after 24 h, and in MDA-MB-231 cells this was time-dependent. Many genes were altered after 24 h, and these differed between cell lines. In MDA-MB-231 cells, several gene expression changes were time-dependent.

Conclusions: ALA reduces growth of BC cell lines, by modifying signaling pathways, which differ between BC molecular subtypes. The ALA effect on gene expression is dynamic and changes over time, indicating the significance of incubation period in detecting gene changes.

Introduction

Breast cancer (BC) remains a major health concern as it is the leading cancer killer of women worldwide [50]. Several issues, in particular heterogeneity between BC cases, lead to difficulty in determining prognosis and successful treatment of this disease. To assist physicians in treating patients, BC is now divided into several molecular subtypes based on the expression of cell estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). These subtypes consist of luminal A (ER+PR+HER2-), luminal B (ER+PR+HER2+), HER2 overexpressing (ER–PR–HER2+), and basal-like (ER–PR–HER2-) which many refer to as triple negative BC (TNBC) [52].

Aside from heterogeneity of BC, other difficulties with current BC therapies include ineffectiveness, development of drug resistance, negative side effects, and high cost [33,4,45,13]. Thus, an increasing number of patients are turning to complementary and alternative methods to combat BC such as incorporation of

Abbreviations: ALA, α-linolenic acid; BC, breast cancer; DHA, docosahexaenoic acid; E2, 17-β estradiol; EPA, eicosapentaenoic acid; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; n-3 PUFA, omega-3 polyunsaturated fatty acid; PR, progesterone receptor; SAT, saturated fatty acid; TNBC, triple negative breast cancer.

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specific food agents into their diets, with one of the most popular being flaxseed and its oil [1] containing approximately 57% alpha-linolenic acid (ALA), an n-3 polyunsaturated fatty acid (n-3 PUFA) [11].

Flaxseed oil (4% of diet) has been shown in several in vivo studies to significantly reduce the size of human ER+BC xenografts in mice [39,44]; however, there are inconsistencies in BC risk reduction and growth effects from flaxseed and flaxseed oil consumption from in vivo animal and epidemiological studies (reviewed in [48,29]). It was therefore suggested that flaxseed and flaxseed oil effects on BC may vary between the BC molecular subtypes. We have previously addressed this question, and found in vitro that there is some variation in growth, apoptosis induction, and incorporation of ALA into the cell phospholipids across BC cell lines, but overall, ALA does successfully reduce BC cell growth (>50%) regardless of cancer subtype, at doses as low as 50 μM treated for 96 h [49].

Attention is now focused on the potential mechanisms of action of ALA and other n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in reducing growth of different BC cell lines, including increasing lipid peroxidation, altering cell membrane receptor expression and activation, changing transcription factors, and regulating tumor suppressor genes such as phosphatase and tensin homolog (PTEN) [23,16,31]. Analysis of genes allows exploration into potential mechanisms of action, and measuring changes using real-time PCR array provides quick analysis of several potential mechanisms of action at the same time; however it does introduce researchers to several experimental variables, one of which is optimum treatment time to observe gene changes.

Cellular expression of genes is dynamic and constantly changing [3]. Due to this, researchers are turning to time-series gene expression studies which provide more accurate information of dynamic processes [3]. To fully capture the gene expression changes induced by ALA, it is important to first determine the optimal time after treatment to be extracting RNA and measuring expression, as this will likely vary between genes and cell types. Therefore, to further understand the effect of ALA on BC cells with different molecular subtypes, we initially treated four BC cell lines in vitro with ALA for 24 h and measured viability and gene expression changes by real-time PCR array (Study 1). To address the role of treatment time, MDA-MB-231 BC cells were treated with ALA for 6, 12, and 24 h and viability and gene expression were again measured (Study 2).

Materials and methods

Cell culture

Four commercial human BC cell lines from three different molecular subtypes (MCF-7, luminal A; BT-474, luminal B; MDA-MB-231, triple negative; MDA-MB-468, triple negative; American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and an antimycotic–antibiotic solution (Gibco) at 37 °C with 5% CO2. Cells were plated at a density of 9.5×10^4 to 1.28×10^5 cells/well in 6-well plates (Sarstedt, Nümbrecht, Germany) and allowed to adhere for 72 h.

Fatty acid treatment

ALA (free form, >99% pure, Sigma-Aldrich) was incubated with charcoal-stripped FBS (Sigma-Aldrich) for 1 h at 37 °C with 5% CO2. ALA–FBS was added to phenol-red free DMEM-F12 medium (Gibco) to a total of 75 μM ALA, 5% FBS, and 1 nM E2 (Sigma-Aldrich). Control medium contained no ALA but with the same concentration of FBS and E2. A concentration of 75 μM ALA was selected as it reduces growth by at least 50% in these four BC cell lines after 96 h treatment time [49], and is physiologically relevant as in vivo studies in mice showed serum concentrations over 100 μM after feeding a 10% FS or 4% flaxseed oil diet [15,28] and human serum reached 197.5 μM with intake of a 6 g/day ALA diet [2]. Treatment medium contained E2 to ensure usual growth in estrogen dependent cell lines.

Cell growth

Trypan blue exclusion was used to determine total cell number and total live cell number for control and ALA treated cells. In Study 1, after 24 h ALA treatment, cells were collected using trypsin–EDTA (Sigma-Aldrich), centrifuged and resuspended in 50 μL medium. A 10 μL aliquot was added to 10 μL of 0.4% trypan blue stain (Gibco) and total and viable cells were counted using a TC10 automated cell counter (Bio-Rad, Hercules, CA, USA). In Study 2, changes in cell growth of MDA-MB-231 cells were again tested after 6, 12, and 24 h of ALA treatment. For both studies, each treatment was run in triplicate, and the mean viable cell number of three wells for each treatment calculated as a % of the control viable cell number.

Gene expression

In Study 1, after 24 h of ALA treatment, cells were trypsinized, centrifuged, and immediately stored at −80 °C until needed. Extraction of total RNA was completed using the RNeasy mini kit with on-column DNase digest according to manufacturer's protocol (Qiagen, Frederick, MD, USA). The concentration and quality of the total RNA were estimated using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) at the University Health Network Microarray Centre (Toronto, Ontario, Canada). cDNA was synthesized using 0.5 μg RNA with the RT^2 First Strand kit (Qiagen) following manufacturer's protocol. For gene expression analysis, SYBR Green Mastermix (Qiagen) was added to the cDNA for each sample and 25 μL was loaded to each well of a customized RT^2 Profiler BC PCR array (Qiagen) measuring 88 genes of interest plus controls and reference genes, with 1 sample per plate. This array was selected as it screens for a wide range of BC pathways potentially altered by ALA including markers for apoptosis, angiogenesis, cell cycle regulation and metastasis. Gene expression was measured using the ABI 7900HT fast real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). In Study 2, gene expression after 6, 12 and 24 h ALA treatment of MDA-MB-231 cells was similarly analyzed.

Statistical analysis

For each cell line, unpaired Student's *t*-test was used to determine difference in growth and gene expression between control and ALA-treated cells at 24 h (Study 1). Two-way ANOVA was used to