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Aromatase induction in tamoxifen-resistant breast cancer: Role of phosphoinositide 3-kinase-dependent CREB activation

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ABSTRACT

Estrogens are important for the development and growth of estrogen receptor (ER)-positive breast cancer, for which anti-estrogen therapy is one of the most effective treatments. However, its efficacy can be limited by either *de novo* or acquired resistance. Aromatase is a key enzyme for the biosynthesis of estrogens, and inhibition of this enzyme leads to profound hypoestrogenism. Here, we found that the basal expression and activity of aromatase were significantly increased in tamoxifen (TAM)-resistant human breast cancer (TAMR-MCF-7) cells compared to control MCF-7 cells. We further revealed that aromatase immunoreactivity in tumor tissues was increased in recurrence group after TAM therapy compared to non-recurrence group after TAM therapy. Phosphorylation of Akt, extracellular signal-regulated kinase (ERK), and p38 kinase were all increased in TAMR-MCF-7 cells. Inhibition of phosphoinositide 3-kinase (PI3K) suppressed the transactivation of the aromatase gene and its enzyme activity. Furthermore, we have also shown that PI3K/Akt-dependent cAMP-response element binding protein (CREB) activation was required for the enhanced expression of aromatase in TAMR-MCF-7 cells. Our findings suggest that aromatase expression is up-regulated in TAM-resistant breast cancer via PI3K/Akt-dependent CREB activation.

Introduction

Breast cancer is one of the most common malignancies worldwide and estrogen is the primary hormone stimulant in the uncontrolled growth of estrogen receptor (ER)-positive breast cancer [1,2]. A clinical study revealed that the concentration of estrogen in breast tumor tissues is several-fold higher than that in the plasma of postmenopausal breast cancer patients [3] and most of these patients show a resistance to tamoxifen (TAM), a representative ER antagonist [4,5]. Aromatase is a member of cytochrome P450 superfamily important for sexual development, whose function is to aromatize androgens to produce estrogens. In humans, the gene CYP19, located on chromosome 15q21.1, encodes the aromatase enzyme [6], and circulating C19 steroid precursors are essential substrates for extragonadal estrogen synthesis [7]. Aromatase inhibitors have become useful for the management of ER-positive breast cancer patients [8–10].

Numerous studies have described the regulation of aromatase expression and its relevance in breast cancer development. Generally, aromatase is expressed at a higher level in human breast cancer tissues than in normal breast tissues [11,12], and aromatase expression is specifically regulated through the alternative use of exon 1 and multiple promoters [13,14]. In addition, growth factors secreted by breast cancer cells stimulate aromatase expression in both breast cancer and adjacent adipose tissues [15].

Despite an initial response to TAM, the majority of ER-positive patients will frequently relapse. Hence, TAM resistance is a major challenge in the management of breast cancer patients [4]. Aromatase inhibitors such as letrozole and exemestane are well tolerated, more effective than TAM in postmenopausal breast cancer patients [16], and considered as a therapeutic option for TAM-resistant breast cancer. Several clinical trials have revealed a significant

Abbreviations: ER, estrogen receptor; PI3K, phosphoinositide 3-kinase; CREB, cAMP response element binding protein; ERK, extracellular signal-regulated kinase; GR, glucocorticoid receptor; PTEN, phosphatase and tensin homolog; PGE2, phostaglandin E2; TAM, tamoxifen; COX-2, cyclooxygenase-2; EP1, PGE2 receptor 1; EP2, PGE2 receptor 2; 5-Aza, 5-aza-2′-deoxycytidine; MAPK, mitogen-activated protein kinase.

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benefit in sequential treatment with an aromatase inhibitor after TAM administration over TAM-only therapy [17,18]. However, the regulation of aromatase expression regulation in TAM-resistant breast cancer is poorly understood. In this study, we demonstrate for the first time that expression and activity of aromatase in TAM-resistant human breast cancer (TAMR-MCF-7) cells are increased compared to control MCF-7 cells. And, we further tried to find out the signaling pathway(s) mediating aromatase up-regulation in TAMR-MCF-7 cells.

Materials and methods

Materials

Antibodies against aromatase were purchased from Abcam (Cambridge, UK, Cat#: ab18995). Antibodies recognizing CREB, P-CREB, P-p38 kinase, p38 kinase, P-extracellular signal-regulated kinase (ERK) (p-ERK T202/Y204), ERK, p-Akt (p-Akt S473) and Akt were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against phosphatase and tensin homolog (PTEN) and glucocorticoid receptor (GR) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-rabbit, anti-goat IgG, and alkaline phosphatase-conjugated donkey anti-mouse IgG were acquired from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-actin antibody and most of the reagents used for molecular studies were obtained from Sigma (St. Louis, MO). pGRE-Luc plasmid and pcDNA-PTEN, a PTEN overexpressing vector were donated from Dr. Lee KY (Chonnam National University, Gwangju, South Korea). pCRE-Luc was purchased from Stratagene (La Jolla, CA, USA). The aromatase-Luc reporter plasmid was kindly provided from Dr. Jeong HG (Chungnam National University, Daejeon, South Korea). Myc-p85 (a dominant negative form of PI3K) and p110-myc (constitutive active form of PI3K) overexpression vectors were provided by Dr. A. Toker (The Boston Biomedical Research Institute, Boston, MA) and Dr. J. Downward (Imperial Cancer Research Fund, London) [19].

Cell culture and establishment of TAMR-MCF-7 cell

MCF-7, Raw 264.7 (mouse monocyte cell line) and mouse embryonic fibroblast (MEF) cells were cultured at 37 °C in 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Hormone-dependent T47D:A18/Neo cells and hormone-independent T47D:A18/PKCα cells were cultured in RPMI-1640 medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. TAMR-MCF-7 cells were established as previously reported [20–22].

Immunoblot analysis

After washing with sterile PBS, cells were lysed in lysis buffer containing 20 mM Tris–Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerolphosphate, 2 mM sodium inorganic pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin. Total cell lysates were centrifuged at 10,000*g* for 10 min to remove cell debris, and proteins in the supernatant were fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted with specific antibodies. Antibody dilution ratio used for western blotting was 1:1000. Protein concentration in samples was measured by using Protein measurement kit (iNtron biotechnology, Seongnam, Korea).

Preparation of nuclear extracts

Cells in the dishes were washed with ice-cold PBS. The cells were then scraped, transferred to microtubes, and allowed to swell after adding 100 μl of a hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mMKCl, 0.1mMEDTA, 0.5% Nonidet P-40, 1mMdithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride. The lysates were incubated for 10 min on ice and centrifuged at 7200*g* for 5 min at 4 °C. Pellets containing the crude nuclei were resuspended in 50 μl of an extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride and incubated for 30 min on ice. The samples were centrifuged at 15,800*g* for 10 min to obtain supernatants containing the nuclear fractions. The nuclear fractions were stored at −80 °C until needed.

Reporter gene analysis

A dual-luciferase reporter gene assay system (Promega, Madison, WI) was used to determine promoter activity. Briefly, cells were plated in 12-well plates and transiently transfected with 1 μg/ml reporter plasmids and phRL-SV plasmid (hRenilla luciferase expression for normalization) using Hillymax® reagent (Dojindo Molecular Technologies, Gaithersburg, MD). The cells were then incubated in culture medium without serum for 18 h. Firefly and hRenilla luciferase activities in the cell lysates were measured using a luminometer (LB941, Berthold Technologies, Bad Wild, Germany). Relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to the hRenilla luciferase.

Determination of aromatase activity

Aromatase activity was measured in both MCF-7 and TAMR-MCF-7 cells with a tritiated water release assay [23]. 80% confluent MCF-7 and TAMR-MCF-7 cells were incubated in serum-free medium for 36 h and the cells were treated with [1β-^{3}H] androst-4-ene-3,17-dione (100 nM) for an additional 3 h. The medium was then mixed thoroughly with 5% charcoal/0.5% dextran for 12 h and centrifuged at 10,000*g* for 30 min at 4 °C to remove any residual androst-4-ene-3,17-dione. One ml of the supernatants were added in a scintillation vial containing 3 ml scintillation cocktail, and radioactivity was measured as disintegrations per minute using a liquid scintillation counter (LS 6500, Beckman Coulter Inc., Fullerton, CA).

Immunohistochemistry for human cancer tissues

Blocks for all the samples were consecutively cut in 4 μm sections and mounted on poly-l-lysine coated glass slides. Xylene was used to remove the paraffin from the sections, and the samples were rehydrated. Antigen retrieval was performed by boiling sections for 5 min in 1 μM sodium citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 10 min, followed by three times washing with PBS. Sections were then incubated overnight with anti-aromatase antibody (1:200, Abcam, Cambridge, UK) at 4 °C. After washing with PBS, sections were incubated with HRP-conjugated anti-rabbit IgG for 30 min and washed with PBS. The color was developed by incubation with DAB solution. Finally, sections were counterstained with hematoxylin, dehydrated, mounted, and observed. When we determined immunoreactivity in rabbit IgG-incubated breast cancer tissue samples (negative control), we could not detect any positive staining.

Statistical analysis

Student's *t* test was used to examine between group differences. Statistical significance was accepted at either $P < 0.05$ or $P < 0.01$.

Results

Up-regulation of aromatase expression in TAM-resistant human breast cancer

As aromatase activity plays an important role in breast cancer development through estrogen synthesis [11], we compared changes in aromatase protein expression and activity in MCF-7 and TAMR-MCF-7 cells. Western blotting revealed that the protein expression of aromatase was significantly increased in TAMR-MCF-7 cells compared to MCF-7 cells (Fig. 1A). Reporter gene analysis using an aromatase-luciferase (luc) reporter plasmid containing the −294/+20 bp promoter region of rat aromatase gene [24] showed that reporter activity was higher in TAMR-MCF-7 cells than in MCF-7 cells (Fig. 1B). We also noted that basal aromatase activity was consistently enhanced in TAM-MCF-7 cells (Fig. 1C). To confirm these results in human cancer cases, tumor tissues were obtained from two groups of patients, which differed in terms of the occurrence of relapse after TAM therapy. Four cases included in "Non-recurrence group after TAM therapy" experienced no recurrence for at least 6 years of follow-up after mastectomy with adjuvant TAM therapy. The other four cases in "Recurrence group after TAM therapy" relapsed within 3–4 years after mastectomy with adjuvant TAM therapy. Immunohistochemical analyses showed that aromatase-positive cell number was significantly higher in recurrence group after TAM therapy than in non-recurrence group after TAM therapy (Fig. 1D and E). To confirm the results and aromatase band size, we determined the basal level of aromatase expression in diverse cell types, including Raw264.7 cells that have no expression of aromatase (negative control), and MEF cells where aromatase expression can be detected by western blot [25], and another tamoxifen resistant breast cancer cell line T47D:A18/PKCα that is stably overexpressing PKCα and