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Estrogen receptor activation by tobacco smoke condensate in hormonal therapy-resistant breast cancer cells

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ABSTRACT

The relationship between tobacco smoke and breast cancer incidence has been studied for many years, but the effect of smoking on hormonal therapy has not been previously reported. We investigated the effect of smoking on hormonal therapy by performing in vitro experiments. We first prepared tobacco smoke condensate (TSC) and examined its effect on estrogen receptor (ER) activity. The ER activity was analyzed using MCF-7-E10 cells into which the estrogen-responsive element (ERE)-green fluorescent protein (GFP) reporter gene had been stably introduced (GFP assay) and performing an ERE-luciferase assay. TSC significantly activated ERs, and upregulated its endogenous target genes. This activation was inhibited by fulvestrant but more weakly by tamoxifen. These results suggest that the activation mechanism may be different from that for estrogen. Furthermore, using E10 estrogen depletion-resistant cells (EDR cells) established as a hormonal therapy-resistant model showing estrogen-independent ER activity, ER activation and induction of ER target genes were significantly higher following TSC treatment than by estradiol (E2). These responses were much higher than those of the parental E10 cells. In addition, the phosphorylation status of signaling factors (ERK1/2, Akt) and ER in the E10-EDR cells treated with TSC increased. The gene expression profile induced by estrogenic effects of TSC was characterized by microarray analysis. The findings suggested that TSC activates ER by both ligand-dependent and -independent mechanisms. Although TSC constituents will be metabolized in vivo, breast cancer tissues might be exposed for a long period along with hormonal therapy. Tobacco smoke may have a possibility to interfere with hormonal therapy for breast cancer, which may have important implications for the management of therapy.

1. Introduction

Breast cancer is a hormone-dependent tumor, many of which are known to occur and progress in response to estrogen. This effect is exerted by the binding of estrogen to an estrogen receptor α (ERα). The complex then binds to an estrogen-responsive element (ERE) to promote transcription of the growth-related genes [1]. Thus, hormonal therapy is an important strategy for the inhibition of ER activity and blocking the estrogen signal. Hormonal therapy is widely used as a primary adjuvant therapy [2]; the agents used are luteinizing hormone-releasing hormone agonists, anti-estrogen agents, and aromatase inhibitors (AIs). AIs, especially, are widely used in advanced cancer and adjuvant therapy. These drugs are administered in oral or intravenous formulations. The effective period of treatment is 2–10 years depending on the agent, and both administration methods can provide outpatient treatment with few side effects. On the other hand, because all drugs require a long treatment period, patients must manage treatment as part of their daily life. Therefore, it is important that patients receive support with respect to self-care, including maintenance and improvement of self-care ability; lifestyle habits; and management of adverse symptoms during the treatment. To date, there is insufficient data to determine whether

Abbreviations: TSC, tobacco smoke condensate; ER, estrogen receptor; EDR cells, estrogen depletion-resistant cells; E2, estradiol; ERE, estrogen-responsive element; GFP, green fluorescent protein; AI, aromatase inhibitors; Tam, 4-hydroxytamoxifen; Ful, fulvestrant; B[a]A, benz[a]anthracene; B[a]P, benzo[a]pyrene; NNK, 4-(methylnitrosoamoino)-1-(3-pyridyl)-1-butanone; NNN, *N*'-nitrosonornicotine.

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the association of breast cancer with lifestyle factors, such as smoking, which is considered to increase the risk of breast cancer.

Smoking is a risk factor for developing lung, esophageal, and gastric cancer and many other diseases. In recent years, there has been a trend towards increasing habitual smoking among women. It has been recognized that there is some association between smoking and breast cancer. Therefore, it is important to provide self-care support based on the evidence to women with breast cancer. However, most studies on the risk for breast cancer have focused on epidemiological data or have been cohort or case-control studies [3–5]. Little is known about the effect of smoking on patients after they have developed breast cancer and begun therapy. Thus, in vitro studies are needed to provide data. In this study, we examined the effect of tobacco smoke condensate (TSC) on breast cancer cells to assess the impact of smoking on the progression of breast cancer and on therapeutic efficacy. Although AIs have shown substantial clinical benefit in postmenopausal women with ER-positive breast cancer, some patients have relapsed and experienced poor outcomes. In recent years, we have studied the mechanisms of AI resistance and established several estrogen depletion-resistant (EDR) cell lines with different mechanisms of resistance [6–8]. The effects of TSC on hormonal therapy-resistant model cells were also studied.

2. Materials and methods

2.1. Reagents

Estradiol (E2) and 4-hydroxytamoxifen (Tam) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fulvestrant (Ful) was kindly provided by AstraZeneca Pharmaceuticals (London, UK). Benzo[a]pyrene (B[a]P), (−)-nicotine, and (−)-cotinine were obtained from Sigma-Aldrich, benz[a]anthracene (B[a]A) was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan), and 4-(methylnitrosoamoino)-1-(3-pyridyl)-1-butanone (NNK) and *rac N*'-nitrosonornicotine (NNN) were obtained from Toronto Research Chemicals (Toronto, ON, Canada). The sources of antibodies for western blotting were as follows: total ERα (H-184) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); phospho-ERα (Ser118), phospho-ERα (Ser167) from Cell Signaling Technology Inc. (Danvers, MA, USA); and Akt (pan) (C67E7), phospho-Akt (Thr308) (C31E5E), phospho-Akt (Ser473) (D9E), total p44/42 MAPK (Erk1/2) (137F5), phospho-p44/42 MAPK (Erk1/2), (Thr202/Tyr204) (D13,14,4E), and β-tubulin from Cell Signaling Technology. The PI3K inhibitor, LY294002, and MEK1/2 inhibitor, U0126, were from Cell Signaling Technology and CALBIOCHEM (San Diego, CA, USA), respectively.

2.2. Tobacco smoke condensate

TSC was prepared by turning the mainstream smoke of 20 cigarettes (*Peace 20*; Japan Tobacco Inc., Tokyo, Japan; tar, 21 mg; nicotine, 1.9 mg/cigarette) in 50 ml dimethyl sulfoxide (DMSO) by aspiration. Each cigarette was burned until just before the filter portion for 3 min and 40 s. The extract was arbitrarily taken to be 100% TSC concentration and was added into the cell culture in dilution.

2.3. Cell and cell culture

MCF-7-E10 cells were established from human breast cancer MCF-7 cells into which the ERE-green fluorescent protein (GFP) reporter gene had been stably introduced [1,9]. These cells expressed GFP in the presence of estrogen under fluorescence. The ERE-GFP reporter plasmid has an ubiquitination site, so that it has a short half-life of about 2 h [9]. Thus GFP does not accumulate in the cells and we can quantitatively assess the ER activity over time.

A1 and A2 cells are EDR cells and were established in our laboratory by culturing MCF-7-E10 cells under estrogen-depletion conditions for 3 months [7]. These cells can grow in the absence of estrogen and maintain their ER activity [GFP(+)].

MCF-7-E10 cells were routinely cultured in RPMI-1640 medium (Sigma-Aldrich) containing 10% fetal calf serum (FCS; Tissue Culture Biologicals, Turale, CA, USA) and 1% penicillin/streptomycin (Sigma-Aldrich). EDR cells were maintained in phenol red-free RPMI-1640 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% dextran-coated charcoal-treated FCS (DCC-FCS) and 1% penicillin/streptomycin (estrogen-depleted medium). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.4. Cell growth assay

MCF-7-E10 cells (which had previously been stripped of steroids for 3 days by DCC-FCS-containing medium) and EDR cells were seeded at a density of 10,000 cells, per well, in a 24-well culture plate. The cells were incubated for 4 days in the absence or presence of TSC, E2, and compounds to be tested. The cells in each well were washed and harvested, then counted by using a Sysmex CDA-500 automated cell counter (Sysmex Corp., Kobe, Japan). Data are indicated as values relative to the cell numbers of the vehicle-treated control.

2.5. ERE-GFP assay

We assessed ER activation by estimating GFP expression levels as previously reported [7,10,11]. In brief, the number of cells expressing GFP was counted under fluorescence microscopy after the cells had been harvested by trypsin treatment and were suspended. ERE activities were expressed as the percentage of GFP-positive cells among 100 randomly selected cells.

2.6. ERE-Luciferase reporter assay

ER activity in each cell line was measured by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The estrogen reporter plasmid used, ERE-tk-Luci, has been previously described [12]. The vector pRL-TK-Luci (Promega) was used as an internal control of transfection efficiency in the reporter assays. Transient transfection was performed as previously described [12]. After culturing the cells for an additional 24 h in the absence or presence of the TSC or tested compounds, luciferase activity was measured by using the Dual-Luciferase Reporter Assay System and a Glo Max Luminometer (Promega), according to the manufacturer's instructions.

2.7. GAL-4 reporter assay (mammalian one-hybrid assay)

The transcriptional activity of the A/B and E domains in ER was analyzed by luciferase assays using pCMX-GAL4-N-ER.A/B and pCMX-GAL4-N-ER.E expression plasmids, which contained a sequence encoding either the ER.A/B or ER.E region of ERα fused to the GAL4 DNA binding domain [13]. EDR cells were seeded at a density of 5×10^4 or 7.5×10^4 cells per 6-cm dish in estrogen-deprived medium and cultured for 24 h. After co-transfection with 0.05 mg of pCMX-GAL4-N-ER.A/B, 0.5 mg of tk-GALpx3-Luc, or 0.05 mg of the pRL-TK-Luc control plasmid with 5 ml of TransIT LT-1 Transfection Reagent (Mirus, Madison, WI, USA) for 3 h, the cells were treated with TSC (0.5%) or E2 (1 nM) and cultured for another 24 h. Luciferase activity was measured in the same manner as in the ERE-luciferase assay. The results were normalized to pRL-TK-