Toxicology in Vitro 28 (2014) 1215–1221

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Anti-aromatase effect of resveratrol and melatonin on hormonal positive breast cancer cells co-cultured with breast adipose fibroblasts

Suthat Chottanapund [a,b,c,e,*], M.B.M. Van Duursen [d], Panida Navasumrit [a,b,c], Potchanee Hunsonti [b], Supatchaya Timtavorn [b], Mathuros Ruchirawat [a,b,c], Martin Van den Berg [d]

[a] Division of Environmental Toxicology, Chulabhorn Graduate Institute, Bangkok, Thailand
[b] Laboratory of Environmental Toxicology, Chulabhorn Research Institute, Bangkok, Thailand
[c] Center of Excellence on Environmental Health, Toxicology and Management of Chemicals, Bangkok, Thailand
[d] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
[e] Bamrasnaradura Infectious Diseases Institute, Ministry of Public Health, Thailand

ARTICLE INFO

Article history:
Received 14 January 2014
Accepted 29 May 2014
Available online 12 June 2014

Keywords:
Co-culture
Human fibroblasts
Breast cancer
Resveratrol
Melatonin

ABSTRACT

Targeting the estrogen pathway has been proven effective in the treatment for estrogen receptor positive breast cancer. There are currently two common groups of anti-estrogenic compounds used in the clinic; Selective Estrogen Receptor Modulators (SERMs, e.g. tamoxifen) and Selective Estrogen Enzyme Modulators (SEEMs e.g. letrozole). Among various naturally occurring, biologically active compounds, resveratrol and melatonin have been suggested to act as aromatase inhibitors, which make them potential candidates in hormonal treatment of breast cancer. Here we used a co-culture model in which we previously demonstrated that primary human breast adipose fibroblasts (BAFs) can convert testosterone to estradiol, which subsequently results in estrogen receptor-mediated breast cancer T47D cell proliferation. In the presence of testosterone in this model, we examined the effect of letrozole, resveratrol and melatonin on cell proliferation, estradiol (E_2) production and gene expression of *CYP19A1*, *pS2* and *Ki-67*. Both melatonin and resveratrol were found to be aromatase inhibitors in this co-culture system, albeit at different concentrations. Our co-culture model did not provide any indications that melatonin is also a selective estrogen receptor modulator. In the T47D-BAF co-culture, a melatonin concentration of 20 nM and resveratrol concentration of 20 μM have an aromatase inhibitory effect as potent as 20 nM letrozole, which is a clinically used anti-aromatase drug in breast cancer treatment. The SEEM mechanism of action of especially melatonin clearly offers potential advantages for breast cancer treatment.

1. Introduction

Adjuvant hormonal therapy is an effective treatment to reduce or prevent the recurrence of hormone receptor positive breast cancer (Bando, 2013; Bell et al., 2013; Higgins et al., 2012; Rao and Cobleigh, 2012; Yoshinami et al., 2013). Here, two common groups of pharmaceuticals are used for clinical hormonal therapy; Selective Estrogen Receptor Modulators (SERMs) e.g. tamoxifen (Balkenende et al., 2013; Yang et al., 2012) and Selective Estrogen Enzyme Modulators (SEEMs) such as the aromatase inhibitor letrozole (Doughty, 2008; Joerger and Thurlimann, 2009; Mouridsen et al., 2009; Thurlimann et al., 2005). SERMs have anti-estrogenic properties because of antagonistic actions on estrogen receptor α (ERα), which reduces the proliferation of estrogen responsive breast tumor cells. SEEMs reduce the synthesis of estrogens from androgens that results in lower circulating levels of estrogens and also reduced estrogen production in peripheral tissues. This subsequently leads to reduced proliferation of estrogen receptor (ER)-positive breast tumor cells. In this approach, the estrogen-stimulated pathways are among the most important targets for adjuvant breast cancer therapy (Lumachi et al., 2013; Yue et al., 2012).

Beside several pharmaceutical SERMs and SEEMs that have been developed over the last decades to treat or prevent breast cancer, a variety of naturally occurring, biologically active compounds have been identified that may be useful as chemopreventive agents for breast cancer. Two biological agents that are often suggested to have such cancer chemopreventive actions are resveratrol and melatonin. Resveratrol is a non-flavonoid phytoestrogen

* Corresponding author at: 126 Bamrasnaradura Infectious Disease Institute, 4th. Floor, 2nd Building, Soi Tiwanon 14, Tiwanon rd., Nontaburi 11000, Thailand. Tel.: +66 863006645; fax: +66 25903411.
E-mail address: suthat.c@bidi.mail.go.th (S. Chottanapund).

http://dx.doi.org/10.1016/j.tiv.2014.05.015
0887-2333/

found for example in grapes. Several epidemiological studies indicated that high resveratrol intake could reduce breast cancer risk (Levi et al., 2005). Multiple mechanisms of action have been identified that could explain the anti-carcinogenic properties of resveratrol, which include its role as a SERMs, aromatase inhibitor and/or anti-oxidant (Alkhalaf et al., 2008; Lee et al., 2012; Leon-Galicia et al., 2012). With respect to its properties as an aromatase (*CYP19A1*) inhibitor, several *in vivo* and *in vitro* studies have confirmed the modularity function of resveratrol in this key step in sex steroidogenesis (Le Corre et al., 2005; Lee and Safe, 2001; Wang et al., 2006; Zhang et al., 2004). It has been suggested that the anti-aromatase activity of resveratrol is its major property with respect to chemoprevention of breast cancer (Le Corre et al., 2005). In a 28-day repeated dose study, the no observed adverse effect level (NOEAL) was 300 mg/kg/day in rats (Crowell et al., 2004). Considering this low toxicity of resveratrol, it is a potential good candidate for adjuvant breast cancer treatment.

Melatonin (N-acetyl-5-methoxytryptamine) is an indolic and endogenous compound that is naturally produced by the pineal gland in the human body. Melatonin plays a primary role in the circadian pattern and is regulated by the hypothalamic suprachiasmatic nucleus (SCN). In addition, melatonin is believed to have oncostatic properties against many forms of cancers such as leukemia, breast, colorectal and prostate cancer (Dai et al., 2008; Mediavilla et al., 2010; Mills et al., 2005; Srinivasan et al., 2008). Multiple mechanisms have been proposed that might explain the breast cancer chemopreventive properties of melatonin (Blask et al., 2005; Grant et al., 2009; Korkmaz et al., 2009; Sanchez-Barcelo et al., 2005) from which two are especially important with respect to our present study. Firstly, melatonin can act as SERM by reducing estrogen binding to ERα receptors and inhibiting binding of the E_2-ERα complex to the DNA. The proposed mechanism of this anti-estrogenic effect of melatonin does not depend on its binding to the ER but depends on the high affinity binding to membrane melatonin receptors (MT1). The melatonin-MT1 complex interferes with the estrogen-binding activity of ERα without changing its affinity and reduces the ligand-receptor transactivation (Sanchez-Barcelo et al., 2005; Treeck et al., 2006; Yuan et al., 2002). Another important mechanism of action of melatonin with respect to prevention of breast cancer may be its role as aromatase inhibitor. Melatonin could decrease cAMP formation and down-regulate expression of promoter regions pII, pI.3 and p1.4-driven aromatase expression in MCF-7 cells (Martinez-Campa et al., 2009).

Previous studies from our laboratories have demonstrated the added value of using co-cultures from breast tumor cells and human primary breast fibroblasts (BAFs) in *in vitro* breast cancer studies (Heneweer et al., 2005a). In contrast to mono-cultures of breast tumor cell lines, these co-cultures have paracrine interactions between both cell types and therefore represent a more realistic approach to the actual *in vivo* situation of a breast tumor (Heneweer et al., 2005b). In our present study we examined the breast cancer chemopreventive properties of resveratrol and melatonin in co-cultures of ERα positive T47D cells and BAFs with an emphasis on the modulating effects on tumor cell proliferation and aromatase activity.

2. Materials and methods

2.1. Breast cancer cell culture and incubation

The human breast cancer cell line T47D was obtained from ATCC (Rockville, MD, USA). T47D cells were grown in culture medium comprising of RPMI 1640, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1% glucose, 1% sodium pyruvate, and 0.08% insulin solution at a seeding concentration of 5×10^4 cells/ml. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Primary breast adipose fibroblasts (BAFs)

Primary breast tissue fibroblasts were obtained after informed consent from three breast cancer patients who attended the Bamrasnaradura Infectious Institute (Nontaburi, Thailand) for modified radical mastectomy. The research protocol was approved by the Medical Ethical Committee of the Bamrasnaradura Infectious Diseases Institute. About 5–10 g of macroscopically normal breast tissue was collected for this study. The remaining tissue was used for routine pathological examination. After the tissue was obtained, it was stored directly at 4 °C in a saline solution (0.9%NaCl) and immediately transported to the laboratory. BAFs were isolated from breast tissue following the method described earlier (Heneweer et al., 2005a) and cultured, as adherent cultures in RPMI 1640-medium w/o phenol red (Gibco/Invitrogen 11835) supplemented with Pen/Strep (invitrogen 15140), FCS (invitrogen 10270) and Insulin 10^{-3} M (144 mg/25 ml) (sigma I-5500) at a seeding concentration of 5×10^4 cells/ml. The cells were maintained in a humidified atmosphere of 37 °C with 5% CO_2. The BAFs were sub-cultured when the bottles were confluent, which was usually after one week (Heneweer et al., 2005a,b). The BAFs were kept for subculture. We isolated three primary BAF cultures from three patients (F1-3). In order to reduce the biological variation in the co-culture experiments, the BAFs from the patient with the highest aromatase expression (F3) (data from our previous experiments) were used for the co-culture in this study (Chottanapund et al., 2013).

2.3. Co-cultures of T47D breast cancer cells with BAFs

Approximately three weeks after isolation, the fibroblasts were used to establish a co-culture with T47D cells. On day 1, BAFs were plated at a density of approximately 5×10^3 cells/well in a 96-well plate (for proliferation) or at 5×10^4 cells/ml in a 25 cm^2 flask (for gene expression) in culture medium. At day 2, mono-cultures of breast cancer cells and plated primary BAFs were washed with phosphate buffered saline (PBS) and placed on assay medium in which heat inactivated FCS was replaced with heat-inactivated, charcoal/dextran-treated FCS (Hyclone, SH30068.03). On day 4, BAFs were washed with PBS and breast cancer cells were trypsinized and seeded on top of the BAFs at a density of 5×10^3 cells/well (for proliferation) or 2.5×10^5 cells/flask (for RNA expression). On day 5, fresh assay medium was added to the co-cultures after which the cells were exposed to the test compounds for 120 h (Birrell et al., 1995; Cops et al., 2008; Sonne-Hansen and Lykkesfeldt, 2005). Final solvent (ethanol) concentration was 0.1% v/v in the medium and concentrations of testing chemicals were respectively, 1 nM, 5 nM and 10 nM for testosterone (Sigma–Aldrich, Saint Louis, USA), 1 pM, 5 pM and 10 pM for 17β-estradiol (E_2) (Sigma–Aldrich, Saint Louis, USA), 10 nM, 20 nM and 30 nM for letrozole (Sigma–Aldrich, Saint Louis, USA), 5 μM, 10 μM and 20 μM for resveratrol (Sigma–Aldrich, Saint Louis, USA), and 1 nM, 5 nM, 10 nM and 20 nM for melatonin (Sigma–Aldrich, Saint Louis, USA).

2.4. Cell proliferation

Cell proliferation was determined after treatment with the test compounds by performing an MTT assay as described earlier (Heneweer et al., 2005a). The optimal cell number was established based on results from a cell viability assay of vehicle-control treated cells and visual inspection of the cells. After exposure to the