Journal of Steroid Biochemistry & Molecular Biology 138 (2013) 54–62

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Effect of androgens on different breast cancer cells co-cultured with or without breast adipose fibroblasts

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ARTICLE INFO

Article history:
Received 24 December 2012
Received in revised form 25 March 2013
Accepted 26 March 2013

Keywords:
Breast adipose fibroblasts
Breast tumor cells
Co-culture
Estrogen receptor
Androgens
Cell proliferation
pS2
Ki-67
Aromatase

ABSTRACT

About 70% of breast tumors express androgen receptors. In addition, there is clinical evidence suggesting that androgens can inhibit mammary epithelial proliferation. *Vice versa*, there is also significant evidence indicating that androgens can increase the risk of breast cancer *via* multiple mechanisms, *e.g.* direct conversion to estrogens that can bind to the estrogen receptor and thereby stimulate cell proliferation. We examined the effect of testosterone (T) and dihydroxytestosterone (DHT) on cell proliferation, *pS2* and *Ki-67* expression in three different breast cancer cell lines alone or in co-culture with primary human breast adipose fibroblasts (BAFs) obtained from breast cancer patients. In the co-cultures, T induced cell proliferation, *pS2* and *Ki-67* expression in the estrogen receptor positive (ER^+) MCF-7 and T47D cells. This was not observed in the (ER^-) MDA-MB-231 cells. The differences might be explained by the high expression of aromatase, which converts androgens to estrogens in BAFs followed by ER-mediated cell proliferation. In line with this absence of increased cell proliferation, *pS2* and *Ki-67* expression was observed in the presence of DHT, which is not a substrate for aromatase. In contrast, DHT caused a significant suppression of cell proliferation (68% and 38%), *pS2* and *Ki-67* expression in the (ER^+) MCF-7 and T47D cells. More importantly, DHT decreased cell proliferation in (ER^-) MDA-MB-231 cells by 38%. The results suggest that androgens that cannot be aromatized, like DHT, may provide a perspective for treatment of breast cancer patients, especially those with triple negative breast cancer.

1. Introduction

Breast cancer is one of the most frequent cancers among women. In 2008, approximately 1.38 million new cases were diagnosed worldwide. Of those 1.38 million cases, the number of fatalities by breast cancer was about one third (approximately 458,000 women) [1]. There is general consensus that treatment of breast cancer needs a multimodality approach to eradicate residual cancer cells and prevent recurrent disease. In this respect, targeting cell growth and invasion pathways of the breast tumor is critical for an effective treatment of breast cancer [2–4].

One of the well-known pathways for development and progression of breast cancer is mediated through interaction with the estrogen receptor alpha (ERα) leading to enhanced proliferation of the tumor cell [5–8]. In addition, breast cancer is more frequently diagnosed in postmenopausal women and the ERα positive tumor (ER^+) incidence is higher in this group (60%) than in premenopausal women [9,10]. Consequently, many selective estrogen receptor modulators (SERMs) that compete with endogenous estrogens on the ERα in tumor cells or selective estrogen enzyme modulators (SEEMs) that target enzymes involved in the biosynthesis of steroid hormones have been developed for breast cancer treatment, thereby significantly decreasing the risk of recurrence and mortality [11–14]. However, there is a group of breast cancer patients who do not respond to hormonal therapies. Often, these breast tumors do not express the hormonal receptors ERα, progesterone receptor (PR) and human epidermal growth factor receptor (HER-2), the so-called triple negative breast tumors [15,16]. Not only are these patients resistant to various hormonal treatments, but these tumors are also often resistant to chemotherapy, resulting in a high morbidity and mortality rate [17–20].

Recent studies have found that up to seventy percent of all breast tumors, including triple negative breast tumors, express

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http://dx.doi.org/10.1016/j.jsbmb.2013.03.007

the androgen receptor (AR) in the malignant tissue [21–23]. However, limited and conflicting information is available regarding the role of androgens or the AR in the etiology of breast cancer. Some studies report an association between a low affinity androgen receptor that displays a CAG repeated polymorphism and breast tumor formation [24]. Furthermore, *in vitro* experiments have shown both proliferative and anti-proliferative actions of androgens in breast cancer cells. Preclinical data suggested that dehydroepiandosterone (DHEA) can stimulate growth in AR positive breast cancer cells [25], but other studies indicated that this weak androgen can inhibit the growth of human ER negative (ER^-) breast cancer tumor cells, if there is a strong expression of the AR [26]. In addition, there is clinical evidence that suggests that androgens normally inhibit mammary epithelial proliferation and breast growth [27,28]. In contrast, there is also evidence suggesting that androgens may increase the risk of breast cancer [29,30]. Also, the peripheral conversion of androgens to estradiol by aromatase (Cytochrome P450 19A1, CYP19A1) has been proposed to contribute to breast tumor cell growth [24,26]. Despite the contradictory results obtained so far, androgens and the AR are often suggested as possible targets for hormonal treatment in breast cancer patients, especially in the case of triple negative breast cancer [26,31,32].

Most breast tumors are classified as invasive ductal carcinomas, which are the malignant growth of the ductal epithelial cells of breast tissue [33–35]. The remaining breast stroma comprises fibroblasts, adipocytes and endothelial cells. There is increasing evidence that tumor-associated stromal cells promote the development of epithelial malignancies. For example, *in vitro* premalignant mammary epithelial cells adopt a malignant growth pattern if cultured with carcinoma derived adipose fibroblasts (CAFs) [36]. In addition, a study by Heneweer et al. found that primary breast adipose fibroblasts (BAFs) could have an indirect effect on the proliferation of breast tumor cells in a co-culture system *via* modulation of cytokine-regulated estrogen receptors [37].

The main objective of the present study was to demonstrate the effects of testosterone (T) and dihydrotestosterone (DHT) on cell proliferation and *pS2* expression of three different breast tumor cell lines MCF-7 (ER^+, PR^+, $HER2^+$), T47D (ER^+, PR^+, $HER2^-$), and MDA-MB-231 (ER^-, PR^-, $HER2^-$). DHT was chosen because it is a potent androgen, but unlike T cannot be converted to estradiol by aromatase. Experiments were performed either as mono-cultures of these three breast tumor cell lines or in co-cultures with primary human BAFs. The latter *in vitro* model has been shown to better reflect the *in vivo* situation, as it takes into consideration intra-endocrine effects between the breast tumor cells and surrounding fibroblasts (Fig. 1).

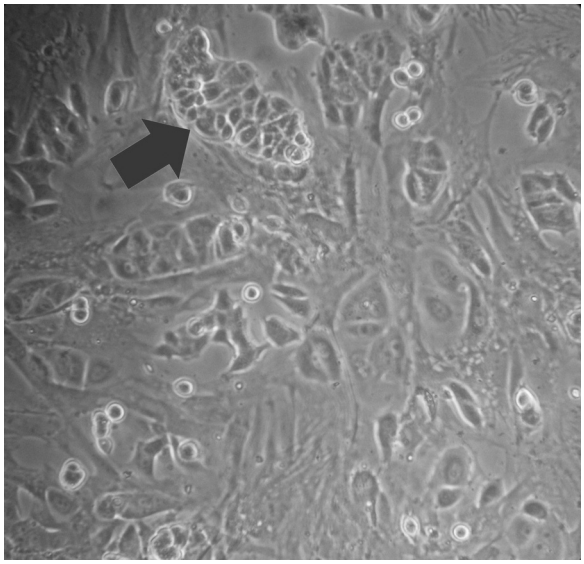

Fig. 1. The ductal formation of MCF-7 cells with surrounding human breast adipose fibroblasts in a co-culture (arrow).

2. Materials and methods

2.1. Breast cancer cell culture and incubation

The three human breast cancer cell lines MCF-7, T47D and MDA-MB-231 were obtained from ATCC (Rockville, MD, USA). MCF-7 cells were grown in culture medium comprising Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine at seeding concentration of 5×10^4 cells/ml. T47D cells were grown in culture medium comprising RPMI 1640, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1% glucose, 1% sodium pyruvate, and 0.08% insulin solution at seeding concentration of 5×10^4 cells/ml. MDA-MB-231 cells were grown in culture medium comprising Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine, and 1% non-essential amino acids (NEAA) at 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 from three breast cancer patients who attended the Bamrasnaradura Infectious Disease Institute (Nonthaburi, Thailand) for modified radical mastectomy, after informed consent. The research protocol was approved by the Medical Ethical Committee, the Bamrasnaradura Infectious Disease Institute, Thailand. About 5–10 g of macroscopically normal breast tissue was collected for this study and the remaining tissue was sent for routine pathological examination. After the tissue was obtained, it was stored in 4 °C saline solution (0.9% NaCl) directly after surgery and immediately transported to the laboratory. BAFs were isolated from breast tissue following the method described earlier [37,38]. Human BAFs were 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 at 37 °C with 5% CO_2. The BAFs were sub-cultured when the bottles were confluent, which was usually once a week.

2.3. Breast cancer cells in co-culture with BAFs

Approximately three weeks after isolation, fibroblasts were used to establish a co-culture together with the human mammary carcinoma MCF-7, T47D or MDA-MB-231 cells [37,39]. On day 1, BAFs were plated at a density of approximately 4×10^3 cells/well in a 96-well plate (for proliferation) or 5×10^4 cells/ml in a 25 cm^2 flask (for gene expression) in culture medium. The day after, mono-cultures of breast cancer cells and plated primary BAFs were washed with phosphate buffered saline (PBS) and placed on the 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 4×10^3 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