Maturitas 77 (2014) 336–343

Contents lists available at ScienceDirect

Maturitas

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

Effects of estriol on growth, gene expression and estrogen response element activation in human breast cancer cell lines

Magnus Diller, Susanne Schüler, Stefan Buchholz, Claus Lattrich, Oliver Treeck*, Olaf Ortmann

Department of Obstetrics and Gynecology, University Medical Center Regensburg, Landshuter Str. 65, 93053 Regensburg, Germany

ARTICLE INFO

Article history:
Received 14 October 2013
Received in revised form
23 December 2013
Accepted 14 January 2014

Keywords:
Estriol
Estradiol
Breast cancer
Growth

ABSTRACT

Objective: Local application of estradiol (E2) to treat vulvovaginal atrophy in postmenopausal breast cancer patients receiving aromatase inhibitors is known to elevate serum estradiol levels and thereby might counteract breast cancer therapy. Thus, vaginal application of estriol (E3) has been recommended for these patients. However, it is unclear to what extent E3 stimulates breast cancer cell growth. In this study, we examined the effect of E3 on growth and gene expression of two human breast cancer cell lines.
Methods: We used an established in vitro cell culture assay and compared the effect of E2 and E3 on growth of the estrogen receptor alpha-positive breast cancer cell lines MCF-7 and T-47D testing a wide range of hormone concentrations of 10^{-12}–10^{-7} M. E3 effects on gene expression were examined by means of reporter gene assays, RT-qPCR and Western blot analysis.
Results: E3 acted as a potent estrogen and exerted a mitogenic effect on T-47D and MCF-7 cells at concentrations of 10^{-9} M (288 pg/ml) and higher. With regard to activation of an estrogen response element (ERE) in breast cancer cells, effects of E3 were visible at 10^{-10} M. The same concentrations of E3 activated expression of the estrogen-responsive gene PR and of the proliferation genes cyclin A2, cyclin B1, Ki-67, c-myc and b-myb, providing molecular mechanisms underlying the observed growth increase.
Conclusions: Like E2, low levels of E3 were able to trigger a robust estrogenic response in breast cancer cells. Thus, our data suggest caution regarding use of E3 by breast cancer survivors.

1. Introduction

It has been suggested that about 70% of all breast cancer patients suffer from menopausal symptoms, either due to their age or as a result from breast cancer therapy [1]. Chemotherapy and ovarian suppression can result in premature ovarian failure and thus can lead to menopausal symptoms [2,3]. Endocrine therapy of breast cancer patients, particularly with aromatase inhibitors, has been reported to enhance menopause-related symptoms, especially vulvovaginal atrophy, a term which describes a complex of symptoms consisting of vaginal dryness, vaginal or vulvar irritation/soreness and dyspareunia [4,5]. Since vulvovaginal atrophy results from lowered estrogen levels, local application of estrogens has been recommended for treatment [6]. Given that local estradiol (E2) application has been reported to elevate serum E2 levels and thus might counteract endocrine breast cancer therapy, usage of estriol (E3) has been suggested as an alternative [7,8]. However, the safety of vaginal E3 application is still discussed, especially for treatment of breast cancer patients [9]. A dose of 0.5 mg E3 applied as vaginal suppository has been reported to rise plasma levels of unconjugated E3 up to peak values about 5.1×10^{-10} M [10]. Even after application of 0.03 mg, plasma levels reach peak values between 45 pg/ml (1.6×10^{-10} M) after single dose (day 1/2) and 13 pg/ml (0.5×10^{-10} M) after multiple doses (day 21/22) [11]. In another study, vaginal E3 application was reported to decrease LH and FSH levels [12]. In conclusion, even application of low doses of E3 might elevate plasma E3 levels.

The role of E3 in the development of breast cancer and its effect on breast cancer cells has been discussed since 1969. In this year, Cole and MacMahon hypothesized that the relative levels of the estrogen fraction let draw conclusions to the breast cancer risk: high levels of E3 or low levels of E2 produced between puberty and about the age of 25 years were suggested to protect women against breast cancer [13]. This possibly pivotal role of E3 in prevention of breast cancer was supported by several studies on rats, which suggested that its application could lower the incidence of induced

* Corresponding author. Tel.: +49 941 782 7520; fax: +49 941 782 7515.
E-mail address: otreeck@caritasstjosef.de (O. Treeck).

0378-5122/$ – see front matter © 2014 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.maturitas.2014.01.004

Table 1
Sequences of PCR primers used for real time PCR.

Gene	Forward primer	Reverse primer	Size (bp)
PR	5′-CAACTACCTGAGGCCGGATT-3′	5′-CATTGCCCTCTTAAAGAAGACCT-3′	160
c-myc	5′-AGTGGAAAACCAGCAGCCTC-3′	5′-CCTCCTCGTCGCAGTAGAAA-3′	113
B-MYB	5′-GGAAGACCAAAAAGTCATCGAG-3′	5′-CTCAGGGTTGAGGTGGTTGT-3′	130
KI-67	5′-CAGTTCCACAAATCCAACACA-3′	5′-CTTTCATTTTCATACCTGAAGGAAC-3′	114
Cyclin A2	5′-CTGCTGCTATGCTGTTAGCC-3′	5′-TGTTGGAGCAGCTAAGTCAAAA-3′	158
Cyclin B1	5′-GACAACTTGAGGAAGAGCAAGC-3′	5′-ATGGTCTCCTGCAACAACCT-3′	133
Cyclin D1	5′-CTGGAGGTCTGCGAGGAA-3′	5′-GGGGATGGTCTCCTTCATCT-3′	162
p21/WAF	5′-GCATGACAGATTTCTACCACTCC-3′	5′-AAGATGTAGAGCGGGCCTTT-3′	122
GAPDH	5′-CGTCGCCAGCCGAGCCAC-3′	5′-GGTGACCAGGCGCCCAATACG-3′	89

mammary breast neoplasm [14–16]. Other studies came to contradictory results, and suggested that E3 might be able to accelerate breast cancer cell growth [17–20].

To clarify the role of E3 in breast cancer cell growth, unlike the majority of previous studies employing single cell lines, we compared the effect of E3 and E2 in a wide range of concentrations (10^{-12} M to 10^{-6} M) on growth of two human estrogen-sensitive cell lines, MCF-7 and T-47D [21,22]. In contrast to previous studies, we additionally examined the specific effects of both estrogens in regulation of seven growth regulatory genes.

2. Materials and methods

2.1. Materials

Phenol red-free Dulbecco's modified Eagle's medium culture medium (DMEM), Insulin from bovine pancreas, 17-β-estradiol (estradiol, E2), 16α-hydroxy-17β-estradiol (estriol, E3), Tris and Serum replacement 2 (SR2) (50×) were obtained from Sigma–Aldrich (St. Louis, USA). Fetal calf serum (FCS) and accutase were purchased from PAA Laboratories GmbH (Pasching, Austria); PBS powder was obtained from Biochrom AG (Berlin, Germany). MCF-7, T-47D and T-47D (KBluc) cells were obtained from American Type Culture Collection (Manassas, VA, USA) and the PCR primers were synthesized from MWG Biotech AG (Ebersberg, Germany).

2.2. Methods

2.2.1. Cell culture and growth assays

T-47D and MCF-7 breast cancer cells (ATCC, Manassas, USA) were cultured in DMEM/F12 medium containing 10% fetal calf serum (FCS), supplemented with 0.01 mg ml^{-1} insulin and 1 mM sodium pyruvate. The cells were maintained in a humidified incubator with an atmosphere of 5% CO_2 and a temperature of 37 °C. Before seeding the cells in 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany), they were serum-starved first by culture for 8 h in 5% FCS, 0.01 mg ml^{-1} insulin and 1 mM sodium pyruvate and later on for 16 h with 1% FCS, 0.01 mg ml^{-1} insulin, 1 mM sodium pyruvate and 0.5× SR2 (serum replacement 2). Afterwards, the cells were removed with accutase, resolved in 1× SR2, counted, and 1500 T-47D breast cancer cells respective 1600 MCF-7 cells were seeded in each well of a 96-well plate. The next day, cells were treated with E2 or E3 in triplicates in concentrations of 10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M and 10^{-7} M. After 0, 3, 4, 5 and 6 days, relative numbers of viable cells were measured in comparison to the untreated control using the fluorimetrical, resazurin-based CellTiter-Blue® Cell Viability Assay (Promega, Mannheim, Germany) according to the manufacturer's instructions at $560_{Ex}/590_{Em}$ nm in a VICTOR^{3TM} multilabel counter (PerkinElmer, Massachusetts, USA). Cell growth was expressed as a percentage of the vehicle control. Statistical analysis of the growth effects was performed by one-way repeated measures ANOVA and Dunnett tests. The differences between E2 and E3 in stimulation of cell growth were analyzed by a paired *t*-test. The used statistical software was GraphPad InStat 3.10 (GraphPad Software, San Diego, USA) and the charts were created with Microsoft® Office Excel 2010® (Microsoft Corporation, Redmond, WA, USA).

2.2.2. Reporter gene assay

For examination of activation of estrogen response elements (ERE) by E3, the commercially available cell line T-47D (KBluc) (ATCC, Manassas, USA) has been employed [23]. T-47D (KBluc) cells are stably transfected with a luciferase reporter gene construct regulated by a triplet of EREs. After serum starvation as described above, cells were removed with accutase, resolved in 1x SR2, counted, and 1500 T-47D-KBluc cells were seeded in each well of a 96-well plate. The next day, cells were treated with E2 or E3 in triplicates in concentrations of 10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M and 10^{-8} M. After 24 h, cells were washed with phosphate buffered saline at room temperature, then harvested in 25 μl lysis buffer (Ligand Pharmaceuticals, La Jolla, USA) per well. Alternatively, estrogenic stimulation medium was replaced by normal medium after 2 h, and incubated for further 22 h. Luciferase activity was determined using a VICTOR^{3TM} multilabel counter (PerkinElmer, Massachusetts, USA) and quantified in relative light units (RLU) and in percent of the untreated control. For normalization, viable cell numbers were measured at the same time point using the fluorimetrical, resazurin-based CellTiter-Blue® Cell Viability Assay (Promega, Mannheim, Germany) as described above.

2.2.3. Real-time PCR

For gene expression analysis, MCF-7 and T-47D cells were treated in the same manner as in the growth assays, but were seeded in 250 ml cap cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) with a confluence of 20%. After stimulation with E3 and E2, the treated cells were frozen after 2 or 5 days. Total RNA and protein was isolated by NucleoSpin® (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The cDNA was synthesized with the AffinityScript Multiple Temperature cDNA Synthesis Kit 50 rxn (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions and afterwards diluted 1:5. For the qPCR analysis, 4 μl of this solution were analyzed by using the SYBR Green I and the LightCyler 2.0 PCR device (Roche Diagnostics, Mannheim, Germany). The PCR program was 95 °C for 15 min, followed by 45 PCR cycles (95 °C for 10 s, 60 °C for 5 s, and 72 °C for 12 s) and followed by a standard melting curve analysis. In all RT-qPCR experiments, a 89 bp GAPDH fragment was amplified as reference gene by using the PCR program above with the exception of the annealing temperature being 66 °C. All sequences of the used PCR primers are listed in Table 1. After performing dilution experiments with sample cDNA over a 100-fold range confirming the PCR efficiencies of all primer pairs to be approximately equal [24], data were analyzed using the comparative $\Delta\Delta C_T$ method [25] calculating the difference between the threshold cycle (C_T) values of the target and