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Estradiol-induced ezrin overexpression in ovarian cancer: a new signaling domain for estrogen

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Abstract

We have for the first time exposed estrogen's role in the epithelial ovarian cancer (OVCA) metastatic cascade and discovered that it is related to the induction of ezrin over-expression. 17β Estradiol (E_2) was administered to SKOV3 (ERα > β) and DOV13 (ERα < ERβ) OVCA cells in serum-and phenol red-free medium fortified with transferrin and insulin. Incubated cells that penetrated Matrigel membranes were counted, immunostained and analyzed for immunoreactive ezrin. E_2 induced an invasive phenotype with translocation of ezrin to cell edges, including pseudopodia and ruffles. A strong correlation was found between ezrin expression and Matrigel penetration induced by E_2. Increases in cell number and ezrin expression were confirmed by flask incubations. E_2 stimulation of OVCA cell proliferation, motility and Matrigel penetration was dose-related and raloxifene or tamoxifen blocked E_2's effect, supporting an ER action. This previously unreported effect of estrogen on ezrin expression may play a role in the clinical course of estrogen-sensitive cancers and other normal or diseased cell actions.
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1. Introduction

Epithelial ovarian carcinoma (OVCA) is the most lethal female genital cancer (1–4). This is because of gaps in knowledge of events in the progression of OVCA. There are presently no adequate means for early detection of these neoplasms and as a result most are metastatic at the time of diagnosis (5,6). Although evidence suggests that steroid hormones might be involved in OVCA genesis and progression (7) and it has long been known that OVCA usually expresses estrogen receptors (ER) (8), the role of estrogen in OVCA has remained unclear (9–12). The only reports of in vitro studies used serum-fortified media that contain estrogen (13); they showed no effect of E_2 or mixed results on proliferation or invasion by OVCA (14–17).

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There is evidence that proliferation and the metastatic phenotype in OVCA depend upon the protein ezrin, a member of the Band 4.1 super-gene family (18–20). The ERM proteins ezrin, moesin and radixin are membrane-cytoskeleton-linking proteins. In general, they regulate cell motility, signal transduction, cell-to-cell and cell-to-matrix recognition, cell growth, and invasion. These functions are of fundamental importance during early development of tissues and organs, wound healing, tumor progression and metastasis (20–26).

We have shown that ezrin is over-expressed in metastatic OVCA (20) and that ezrin levels correlate with invasive behavior of endometrial cancer cells in the Matrigel membrane penetration assay (19). We have also shown that exposure of OVCA cells to epithelial growth factor or interleukin 1α/β (IL1 α/β) quickly induces membrane ruffling, ezrin translocation, proliferation and tyrosine phosphorylation (20). In this report we show for the first time that E_2 induces over-expression of ezrin, Matrigel membrane invasion and proliferation by OVCA cells. These effects were blocked by the selective estrogen receptor modulators (SERMs) raloxifene (RLX) and tamoxifen (TMX).

2. Materials and methods

2.1. Ovarian cancer cell culture

SKOV3 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and Dr Robert Bast provided DOV13 cells. Prior to study, cells were maintained in McCoy medium or minimal essential medium (MEM) supplemented with penicillin, streptomycin, and 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD, USA). DOV13 were cultured with similar medium supplemented with 5 mg/500 ml insulin (Sigma, St Louis, MO, USA). Cells were cultured in 5% CO_2 and humidified air and the medium was changed twice weekly. After flask-incubation to sub-confluence (60–70%) in McCoy medium or MEM containing 10% FBS, the medium was changed to phenol red-free, serum-free OptiMEM 1×, see below.

2.2. Pre-incubation with serum-free OptiMEM

During the pre-treatment period and experimental treatment the cells were cultured in phenol red-free OptiMEM 1× (GIBCO BRL, Gaithersburg, MD, USA). This medium is designed by the manufacturer to allow culturing in serum-free conditions. It contains HEPES buffer, 2400 mg/l sodium bicarbonate, hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and transferrin plus insulin, but no phenol red, serum or sex steroids. Since we were anxious to avoid the presence of the sex steroids that are in serum (13), we initially tried culturing the OVCA cells in the non-serum-containing, glutamine-fortified medium DMEM F12 (GIBCO) without added serum or phenol red, but found that the cells would not survive the 48 h culture period of the study. We then tested OptiMEM 1× for more than 72 h during which time the cells grew to 100% confluence without evidence of cell death (apoptotic bodies, floating cells, etc.). Following 24 h pre-incubation in OptiMEM 1× the cells were exposed to 0.05% trypsin (supplemented with 5.3 nM EDTA-4Na, mycoplasma-free; GIBCO BRL, Gaithersburg, MD, USA) for 5 min, lightly shaken to loosen them from the flask bottom. After detachment the cells were washed with fresh OptiMEM 1× to stop the action of the trypsin.

2.3. Treatment of cultures

Cells were transferred to plastic Falcon tubes (Fisher Scientific, CA, USA) in OptiMEM 1× and gently centrifuged. The cell pellet was re-suspended in culture medium and transferred to six-Boyden chamber-plates for the Matrigel invasion assay (BD Bioscience, Bedford, MA, USA). For treatments the 10^{-3} M ethanol stock solution stock was diluted with OptiMEM 1× furnishing $<0.001\%$ ethanol in the vehicle.

2.4. Preparation for protein and Western blot analysis

Sub-confluent cell cultures were washed twice with PBS, then lysed with lysis buffer (25 mM Tris, pH 7.4, 1% Triton X-100, 5 mM ethylene glycotetra acetic acid, 5 mM $MgCl_2$ plus Protease Inhibitor Cocktail [Roche, Indianapolis, IN]) for 20 min on ice. Cell lysates were centrifuged and the supernatants collected. Pellets were re-extracted with 1× sodium dodecyl sulfate (SDS)-polyacrylamide electrophoretic sample buffer [0.5 DW, 0.13 (0.5 M Tris–HCl), 0.1 Glycerol, 0.2 (10% SDS),

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