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Estrogens promote cell–cell adhesion of normal and malignant mammary cells through increased desmosome formation

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ABSTRACT

The association of estrogen receptor alpha (ERα) expression with differentiated breast tumors presenting a lower metastasis risk could be explained by the estrogen modulation of cell adhesion, motility and invasiveness. Since desmosomes play a crucial role in cell–cell adhesion and may interfere in tumor progression, we studied their regulation by estrogens in human breast cancer and normal mammary cells. Estrogens increased the formation of desmosomes in normal and malignant cells. Furthermore, four desmosomal proteins (desmocollin, γ-catenin, plakophilin and desmoplakin) appeared significantly upregulated by estrogens in three ERα-expressing cancer cell lines and this effect was reversed by a pure antiestrogen. Finally, silencing of ERα or desmoplakin expression by specific siRNA revealed that estrogen-modulated desmosomal proteins are essential for the estrogenic control of intercellular adhesion. This estrogen modulation of desmosome formation could contribute to the lower invasiveness of ERα-positive tumors and to the integrity of epithelial layers in estrogen target tissues.

1. Introduction

Estrogens are involved in the development of the mammary gland and in the etiology of human breast cancer (Medina, 2005). Most of their effects are mediated by two specific nuclear receptors, ERα and ERβ (Dahlman-Wright et al., 2006; Osborne and Schiff, 2011; Thomas and Gustafsson, 2011). ERα assessment in primary tumors has been established as a way to predict the efficacy of endocrine therapies based on anti-estrogens or aromatase inhibitors, which are widely used as first-line adjuvant therapy (Fisher et al., 2008). It has been demonstrated that ERα mediate an estrogen mitogenic action in human breast cancer cells (Schiff et al., 2005) but, paradoxically, the presence of ERα is associated with more differentiated and less invasive tumors that have, therefore, a more favorable clinical prognosis (Fisher et al., 2008; Hanrahan et al., 2006; Mirza et al., 2002). However, the role of these hormones in cancer progression, including invasion and metastasis, is more difficult to define given the various pathways involved. Several studies described the *in vitro* regulation of breast cancer cell invasion by estrogens and antiestrogens. ERα-positive cell lines are less invasive and do not metastasize in the same way as ERα-negative cells (Platet et al., 2004; Rochefort et al., 1998). The unliganded and estradiol-activated estrogen receptors decrease cancer cell invasion *in vitro* through distinct mechanisms corresponding to protein–protein interaction with still uncharacterized partner(s) and to a mechanism involving the functional receptor domains required for transcriptional activation of target genes respectively (Platet et al., 2000, 2004; Rochefort et al., 1998; Sisci et al., 2004).

Cell-matrix and the cell–cell adhesion constitute initial elements of resistance to the release of cancer cells from primary tumor and subsequent cell migration (Mareel and Leroy, 2003; Zhang et al., 2010). Several studies have already demonstrated that breast cancer cell adhesion is estrogen dependent, notably, by involving adherens junctions and particularly of proteins such as E-cadherin (Behrens et al., 1992; Fujita et al., 2003; Hartsock and Nelson, 2008; Van Roy and Berx, 2008; Vleminckx et al., 1991).

In the present study, ultrastructural and biochemical approaches were used to study the effects of estrogens on other structures known for their importance in intercellular adhesion: the desmosomes. We have analyzed the effects of prolonged estrogen treatment on cell–cell adhesion of both breast and

Abbreviations: ERα, estrogen receptor alpha; E2, estrogen; FBS-DCC, dextran-coated charcoal stripped fetal bovine serum; SEM and TEM, scanning and transmission electron microscopy; HRP, horse radish peroxydase; PVDF, polyvinylidene fluoride; ICI_{182780}, fulvestrant; siRNA, small-interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SD, standard deviation.

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ovarian cancer cell lines and on primary cultures of normal mammary cells.

2. Materials and methods

2.1. Materials

Stock solutions of the anti-estrogen $ICI_{182,780}$ (gift of A. Wakeling, AstraZeneca, Cheshire, UK) and 17β-estradiol (E2) (Sigma–Aldrich Chimie, St. Quentin Fallavier, France) were prepared in ethanol. Desmocollin 2/3 (7G6), plakophilin 3 (651114), glyceraldehyde 3-phosphate dehydrogenase (abbreviated as GAPDH) (ab8245), β-catenin (610153) and γ-catenin (15F11) and cytokeratin-18 monoclonal antibodies were respectively purchased from Zymed (Clinisciences, Montrouge, France), Tebu-bio (Le Perray en Yvelines, France), Abcam (Paris, France), and BD Bioscience (Le Pont de Claix, France) for the two catenins and Beckman Coulter (France) for the last one. Rabbit polyclonal antibodies against desmoplakin I/II (sc-33555) and E-cadherin (sc-7870) were obtained from Santa Cruz (Heidelberg, Germany). Mouse monoclonal ERα (SRA-1000) was obtained from Stressgen (Ann Arbor, USA). For immunofluorescence staining, Alexa Fluor 488 anti-rabbit and/or Alexa Fluor 568 anti-mouse (Fisher Bioblock scientific, Illkirch, France) were used as secondary antibodies. A non-relevant IgG1 mouse monoclonal antibody (MOPC21), used as control, was purchased from Letton Bionetics Inc. (Kensington, CA, USA) or a purified rabbit IgG1 (Sigma Chemical Co., St. Louis, MO) were used as negative controls for antibodies. Mouse and rabbit peroxydase-conjugated secondary antibodies for Western blotting were purchased from GE Healthcare (Orsay, France) and Jackson Immunoresearch (Cambridge, UK).

2.2. Cell cultures

Two breast cancer cell lines (MCF7 and T47D) and one ovarian cell line (BG1) were maintained in monolayer cultures in phenol red-free Dulbecco's modified Eagle's/Ham's F-12 medium (DMEM) supplemented with 10% of dextran-coated charcoal stripped fetal bovine serum (FBS-DCC) and 50 μg/ml gentamycin. For collagen I outgrowth, MCF7 cells were embedded in collagen I gel at 4 °C (0.3 ml, 0.2 mg/ml) and then added on a pre-set collagen I layer in 24-well plates. For steroid stripping, cells were grown for 5 days in phenol red-free DMEM supplemented with 10% FBS-DCC. The initial seeding concentration was corrected as a function of the respective hormone dependent effects on cell growth in order to obtain the same number of cells after 5 days of culture ($1.5\ 10^6$ - cells per 6-well). The cell seeding ratios were 0.5 for E2-treated cells, 1 for control cells and 1.5 for $ICI_{182,780}$-treated cells. By this way, growth effect was avoided.

However, we assume that these corrected seeding could decrease the observed differences between the treatments since a high number of cells could interact for a longer period in control and $ICI_{182,780}$ treatment.

For primary cultures, epithelial cell clusters with polarized ductal and acinar structures (organoids) were isolated from reduction mammoplasties and maintained in 3D collagen I scaffold as described (Chambon et al., 1984). All patients gave their written informed consent and this study was reviewed and approved by our Institutional Review Board. For scanning (SEM) and transmission (TEM) electron microscopy studies, floating collagen membranes were used as supports. Organoids were first attached to collagen I (from Flow SA, UK) layer and then harvested to obtain floating collagen membranes that retracted after 2 days of culture. Organoids were then grown for 4 days in the presence of 10% FBS-DCC for estrogen withdrawal and finally treated or not with 1 nM estradiol (E2) for 7–12 days.

2.3. Cell–cell adhesion

A first dissociation assay involves subconfluent cells subjected to a moderate incubation with trypsin–EDTA (Gibco). MCF7 cells deprived of steroids for 5 days were differently seeded in order to obtain the same confluence 5 days later, according to treatment effect. After attachment, cells were treated for 5 days with 1 nM E2 or 1 nM E2 + 1 μM $ICI_{182,780}$. For the release of the entire cell monolayer as a single cell suspension, MCF7 cells were usually incubated for 15 min with a solution of 0.5 mg/ml trypsin–EDTA diluted in Dulbelcco's phosphate buffer saline without calcium and magnesium (DPBS) (Gibco). Here, to investigate the adhesive strength in cells pretreated with hormones, adherent cells were simply washed with DPBS and the 0.5 mg/ml trypsin treatment was shortened to only 7 min. Thereby cells were released as aggregates of several cells or as single cells whose number was counted by two investigators using Malassez's chambers. A SD < 5% was observed between duplicates.

The second dissociation assay was performed with dispase as described previously (Kimura et al., 2007) to analyze the presence of calcium-independent desmosomes on confluent cells. MCF7 cells were deprived of steroid and treated for 5 days with E2 or E2 + $ICI_{182,780}$ to obtain the same confluence (98%) as described above. Cell sheets were detached from wells by 45 min incubation with dispase (2.4 U/ml; Gibco) diluted in DPBS. At the end of the treatment, dispase was inactivated by dilution with low calcium medium. The cell suspension was incubated for 90 min in this low calcium medium and then subjected to 20 rotation cycles. Then, single cells and fragments were counted under an inverted microscope.

2.4. Immunohistochemistry

For immunoperoxydase detection, cells were fixed with 4% paraformaldehyde (PFA) in phosphate saline buffer (PBS) for 1 h. They were then permeabilized by 25 min incubation with 0.05% Saponin in 0.05% Tween-PBS (PBS-Ts) and blocked with 2.5% horse serum in PBS-Ts. Cells were incubated for 1 h with anti-cytokeratin-18 (1 μg/ml), desmocollin 2/3 (12 μg/ml) or γ-catenin (1:2000) antibodies. Cells were extensively washed and incubated for 30 min with a biotin-conjugated horse anti-IgG antibody, then washed again and incubated with streptavidin–biotin peroxydase complex (Vectastain ABC kit) as described by the manufacturer (Vector Laboratories, Burlingame, CA, USA). All incubations with antibodies were performed in PBS supplemented with 0.05% Saponin and 1% bovine γ-globulin and all washes were done in PBS-Ts. A non-relevant mouse monoclonal antibody MOPC21 was used as negative control.

For immunofluorescence, cells grown on coverslips were fixed with 4% PFA for 12 min, cold methanol for 4 min, cold acetone for 2 min, and then saturated overnight at 4 °C with 2.5% goat serum in phosphate saline buffer (PBS) containing 4% bovine γ-globulin. For immunostaining, cells were incubated with desmoplakin I/II (2 μg/ml) antibody for 2 h. Cells were extensively washed and incubated for 1 h with Alexa fluor antibody, then washed again and incubated with DAPI (0.5 μg/ml, Sigma–Aldrich Chimie) for nuclear staining. All incubations were performed in PBS supplemented with 1% bovine γ-globulin and all washes were done with PBS. Negative controls were performed with a purified rabbit IgG1.

2.5. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

MCF7 cells grown on glass coverslips and organoids of normal cells grown on floating collagen membranes were fixed with glutaraldehyde (3.75%) for 2 h and post-fixed with 1% osmium tetroxide for 1 h in Millonig buffer at pH 7.3. Coverslips and floating collagen membranes were rinsed in Millonig buffer and dehydrated through a graded series of alcohol and isoamyl acetate