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IL-1β induced methylation of the estrogen receptor ERα gene correlates with EMT and chemoresistance in breast cancer cells

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ABSTRACT

Inflammation has been recently acknowledged as a key participant in the physiopathology of oncogenesis and tumor progression. The inflammatory cytokine IL-1β has been reported to induce the expression of markers associated with malignancy in breast cancerous cells through Epithelial-Mesenchymal Transition (EMT). Aggressive breast cancer tumors classified as Triple Negative do not respond to hormonal treatment because they lack three crucial receptors, one of which is the estrogen receptor alpha (ERα). Expression of ERα is then considered a good prognostic marker for tamoxifen treatment of this type of cancer, as the binding of this drug to the receptor blocks the transcriptional activity of the latter. Although it has been suggested that inflammatory cytokines in the tumor microenvironment could regulate ERα expression, the mechanism(s) involved in this process have not yet been established. We show here that, in a cell model of breast cancer cells (6D cells), in which the inflammatory cytokine IL-1β induces EMT by activation of the IL-1β/IL-1RI/β-catenin pathway, the up regulation of TWIST1 leads to methylation of the *ESR1* gene promoter. This epigenetic modification produced significant decrease of the ERα receptor levels and increased resistance to tamoxifen. The direct participation of IL-1β in these processes was validated by blockage of the cytokine-induced signaling pathway by wortmannin inactivation of the effectors PI3K/AKT. These results support our previous reports that have suggested direct participation of the inflammatory cytokine IL-1β in the transition to malignancy of breast cancer cells.

1. Introduction

Inflammation has been recently recognized as an important factor in the physiopathology of cancer [1]. Pro-inflammatory cytokines normally released by macrophages can induce an abnormal phenotype when released for long periods due to chronic inflammation. Within the inflammatory microenvironment of tumors, high levels of IL-1β have been reported in breast and other cancers [2,3]. IL-1β is an up-stream signaling molecule that initiates the production of other inflammatory mediators in cancer genesis, tumor growth and invasiveness. The molecular effects of IL-1β on each cancerous tissue are very diverse and could include oncogene expression, anti-apoptotic signaling and the transition of epithelial cells to a mesenchymal-invasive phenotype (EMT) [4–7]. An EMT has been shown to occur in a breast cancer cell model by stimulation with IL-1β [6]. This and further reports, have shown that IL-1β induces the nuclear translocation of β-catenin and the sequential expression of genes *c-MYC*, *CCDN1*, *SNAIL1*, *MMP2*, *and BIRC3*, known as EMT markers, and the acquisition of an aggressive invasive phenotype, including resistance to doxorubicin [6,7].

A recent report proposes a relationship between the expression of markers of EMT and the triple-negative phenotype of breast cancer tumors (TNBC) [8]. These cells show malignancy features and resistance to hormone therapy and are characterized by the

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loss of receptors for estrogens, progesterone and epidermal growth factor [9]. Other studies have highlighted that the lack of the estrogen receptor alpha (ERα) correlates with an increased production of inflammatory cytokines by tumor cells, including IL-1β, suggesting that the receptor expression is determinant for the pattern of cytokine secretion [10]. However, the direct participation of IL-1β in the regulation of ERα expression and the cell resistance to hormonal treatment has not been established.

Estrogen receptor alpha is a transcription factor encoded in the *ESR1* gene. In response to binding of estrogens, ERα translocates to the nucleus where activates the estrogen-responsive genes [11]. From a clinical point of view, ERα presence is considered a good prognostic marker in the treatment for mammary cancer, as this receptor is a very effective target of anti-estrogen treatments, with a positive response rate over 70% [12]. Tamoxifen is the most commonly used chemotherapeutic agent for patients diagnosed with ERα positive breast cancer. This drug competes with estrogens for binding to ERα, inhibiting the growth of mammary epithelial cells [13]. However, several patients with loss or modification of ERα expression show resistance to tamoxifen, suggesting that alternative mechanisms could be involved in the cell response to the drug effect [14,15].

It is known that epigenetic modifications such as methylation, phosphorylation and deacetylation can regulate the function and stability of ERα and could be the cause of the different degrees of resistance to hormone therapy [12]. Although, it has been reported that 25% of the ERα negative tumors showed methylation of the *ESR1* promoter [16,17], it is also reported that in breast cancer cells positive for ERα, phosphorylation of the receptor in its serine167 is associated with chemoresistance to tamoxifen [18].

In this work, we show the specific effect of the inflammatory cytokine IL-1β in the induction of the *ESR1* gene promoter methylation that leads to loss of ERα expression in the breast cancer cells stimulated by the cytokine. Moreover, our results indicate that these modifications occur by IL-1β activation of the PI3K/AKT/TWIST1 signaling pathway and lead to tamoxifen resistance and survival of the cancerous cells.

2. Materials and methods

2.1. Cell culture

MCF-7 parental cell line (ATCC, Manasas, VA, USA) and its IL-1β-highly responsive clone, 6D cells [6], were cultured in DMEM-F12 supplemented with 10% fetal bovine serum, penicillin (5000 U/ml) and streptomycin (5000 μg/ml) from Gibco BRL (Grand Island, NY, USA). Cultures were incubated at 37 °C with 5% CO_2. For all the experiments, 6D cells were re-stimulated with 20 ng/ml of human recombinant IL-1β (Peprotech, Rocky Hill, NJ, USA) for 48 h to ensure the expression of the IL-1β-induced phenotype [6].

2.2. Inhibitors

The TWIST1 expression inhibitor, harmine (30 μM, Sigma-Aldrich, St Louis, MO, USA) was added to cultured cells 48 h prior to Methylation Specific PCR (MSP) analysis. To determine that TWIST1 expression is mediated IL-1β/IL-1RI/PI3K/AKT pathway, 250 μM wortmannin (Sigma-Aldrich), inhibitor of PI3K signaling, was added for 48 h. For all experiments, inhibitors were added to the culture, 1 h prior to IL-1β re-stimulation.

2.3. Gene expression

Total RNA was extracted from the cells using Trizol® (Invitrogen, Carlsbad, CA, USA). One microgram of RNA was used for reverse transcriptase reactions. Gene expression was carried out using the Fast Start SYBR Green Master kit (Applied Biosystems, Foster City CA, USA), using a 7500 Real Time Thermal Cycler (Applied Biosystems). Relative gene expression values were normalized to the constitutive expression of *RPLP0*. Values were determined using the $2^{-\Delta\Delta CT}$ method [19]. The specific primers for target genes are shown in the Supplementary Table S1.

2.4. SDS-PAGE and western blot

Protein extracts were obtained by cell lysis with RIPA 1X buffer supplemented with Complete®. Proteins were separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes and blocked. The membranes were then exposed to mouse anti-human ERα antibody (1:750), anti-TWIST1 (1:750) antibodies (GeneTex, Irvine, CA, USA), anti-pSer473-AKT (1:1000) or anti-AKT (1:1000) antibodies (both from Cell Signaling Technology, Danvers, MA, USA). Anti-β-actin monoclonal antibody [kindly donated by Dr. JM Hernández (CINVESTAV-IPN)] was utilized to detect cell actin as protein load control. As secondary antibody HRP-labeled anti-mouse IgG (1:5000, Sigma-Aldrich) was used and bands revealed with a chemiluminescence kit (GE Healthcare Life Sciences, Little Chalfont, UK). Densitometry analyses were performed with ImageJ software.

2.5. Bisulfite treatment of ESR1 DNA and promoter methylation status

DNA methylation patterns in the CpG island of *ESR1* promoter were determined by MSP, as indicated [20]. One microliter of bisulfite-modified DNA was added to a Taq platinum PCR mix (Invitrogen), following the manufacturer's indications. MSP was carried out using the primers indicated in Supplementary Table S1. PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide and documented.

2.6. Tamoxifen treatment and viability assays

For all assays, 4×10^5 MCF-7 or 6D cells were plated in 60-mm wells and cultured in DMEM-F12 supplemented with 10% FBS for 24 h, then switched to DMEM-F12 supplemented with only 1% FBS for 18 h. Tamoxifen (Sigma-Aldrich) was added to a final concentration of 10 μM for 48 h. Cell viability was assessed using the LIVE/DEAD® Fixable Red Stain kit (Invitrogen), according to the manufacturer's protocol. Flow cytometry determinations were performed with a FACSort flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with FlowJo software. At least 10,000 events were acquired in three replicates from three independent experiments for each condition.

2.7. Statistical analysis

Data are presented as mean plus/minus standard deviation. Statistical analyses were performed by Mann–Whitney test or Kruskal–Wallis test. P values ≤ 0.05 were considered significant.

3. Results

3.1. IL-1β induces hemi-methylation of ESR1 promoter in breast cancer cells

Our previous work has shown that IL-1β-stimulated breast cancer cells acquire an invasive phenotype through this cytokine-induced EMT [6,21]. Previous reports in the literature have described DNA methylation as a mechanism related to the

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