ORIGINAL ARTICLE

Interleukin-6 Induces Epithelial-Mesenchymal Transition in Breast Cancer Cells

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Abstract Background: Breast cancer is the leading cause of cancer-related death in Mexico, with most deaths being related to locally advanced or metastatic disease at diagnosis. Epithelial-mesenchymal transition (EMT) is one of the steps that are indispensable for metastasis. Different factors trigger EMT, like TGF-β, EGF and interleukin 6 (IL-6), among others. EMT is characterized by E-cadherin expression loss and N-cadherin and vimentin expression. In this study, we investigated the role of IL-6 on EMT induction. Methods: MBCDF and MBCD17 primary breast cancer cell cultures were used. E-cadherin expression was measured using Western Blot. Cells were stimulated with IL-6 to induce EMT. STAT3 activation was measured using phospho-specific antibodies, and E-cadherin expression was measured as EMT marker. Results: MBCDF and MBCD17 primary breast cancer cell cultures stimulation with IL-6 induced STAT3-Tyr705 phosphorylation without its total levels being altered; in addition, IL-6 cell-stimulation was shown to induce EMT, as evidenced by E-cadherin loss. Conclusions: The results of the present work suggest that IL-6 induces EMT in primary breast cancer cell cultures through STAT3 phosphorylation. (creativecommons.org/licenses/by-nc-nd/4.0/).

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INTRODUCTION

Globally, breast cancer accounts for 25% of all malignancies in women, which places it as the most common cancer in this group. In Mexico, it is the leading cause of cancer-related death in women since 2006. In 2012, an increase in breast cancer incidence was reported, from 2% in 1980 to 5% being reported in 2010. In 2009, the Mexican epidemiological surveillance system published a nation-wide incidence of 15 cases per 100,000 population, with the highest incidences being noted in Distrito Federal (now Mexico City), Coahuila and Nuevo León, with 17, 18 and 14 cases per 100,000 population, respectively, while the state with the lowest documented incidence was Chiapas, with 1.5 cases per 100,000 population. With regard to these data, it should be noted that in Mexico there is no national cancer registry, and the reported figures are therefore only an estimate of breast cancer actual situation in the country.

Breast cancer molecular study has enabled to classify the disease in different subtypes, with the purpose to translate this information into targeted therapies and define prognostic groups. In the past few decades, this classification has undergone modifications that represent research advances and adaptations for global classification criteria, with a specific value of Ki67 being eliminated and clinical parameters and multi-parametric molecular markers being added, as main modifications. Hence, luminal tumors, characterized by hormone receptor expression and HER2 non-expression, which are subdivided in luminal A-type, such as those tumors where immunohistochemistry analysis reveals estrogen receptor (ER) and progesterone receptor (PR) high expression, a clearly low Ki67 determination, and tumors classified by size as T1 and T2 and involvement of 0 to 3 lymph nodes; if access to multi-parametric molecular markers determination is available (Oncotype DX®, MammaPrint®), the result should be a favorable risk assessment. This subtype accounts for 40% of breast cancer cases and is associated with favorable prognosis. Luminal B subtype is characterized by hormone receptor low expression, clearly high Ki67, nodular involvement higher than 3 lymph nodes, histological grade 3 (poorly differentiated tumor), extended lymph-vascular invasion and bulky tumors (T3); this type accounts for 20% of breast cancer cases and is associated with a mortality risk of 1.96 (95% CI: 1.08-3.54). HER2 overexpression (25% of tumors) or the lack of estrogen receptor (ER), progesterone receptor (PR) and HER2 expression is defined as “triple-negative” (15 to 20% of breast cancer cases), these two groups considered of poor prognosis, with a mortality risk of 7.39 (95% CI: 1.72-31.77) and 12.41 (95% CI: 5.82-26.49), respectively. It should be noted that, in women younger than 40 years, the factors that more negatively influence on overall survival are lymph node infiltration or triple-negative molecular subtype.

The introduction of targeted therapies to specific molecules such as the epidermal growth factor receptor (Herceptin®, TDM-1, Pertuzumab®), have achieved objective tumor responses of up to 70% when combined with chemotherapy, and have improved overall survival. This is only effective for a selected group of patients; however, the disease has been observed to be able to progress over time and to acquire the capability to generate metastasis to other sites.
that is produced by hematopoietic and epithelial cells. Since its identification in mononuclear cell cultures supernatant, its role in biological functions such as B cell differentiation and T cell proliferation has been described. In breast, kidney and prostate cancer, as well as in myeloma multiple, it has been correlated with poor prognostic and tumor aggressiveness. Recent studies characterize IL-6 as a VEGF positive regulator. IL-6 circulating levels have been found to be 10-fold higher in patients with breast cancer than in healthy women, with a correlation existing between higher levels of IL-6 and breast cancer more advanced stages. The study of IL-6 in breast cancer cells in vivo has yielded controversial results: on one hand, its implication in doxorubicin resistance and in the promotion of the motility required for metastasis have been demonstrated, and on the other, treatment with low-dose IL-6 for 6 days has been shown to inhibit ER-expressing cells proliferation in vitro via apoptosis activation by DNA fragmentation. IL-6 has also been implicated as an EMT promoter by inducing E-cadherin expression repression.

IL-6 signaling occurs through interaction with its receptor (IL-6R), and membrane-binding glycoprotein gp130, which is bound to JAK1,2. JAKs are in charge to phosphorylate gp130 distal cytoplasmic domain, with this phosphorylation serving to recruit SH2 domain-containing proteins, such as STAT3, which is subsequently activated by tyrosine residue 705 (Tyr705) phosphorylation, thus inducing its dimerization. STAT3 dimer translocates to the nucleus, which results in the transcription of its target genes. In IL-6-mediated EMT, STAT3 induces the expression of other transcription factors such as Snail, ZEB, Twist and Slug, which together generate E-cadherin repression and n-cadherin and vimentin mesenchymal genes transcription, which generated cell bounds disintegration and epithelial polarity loss.

Recent reports highlight the oncogenic importance of persistent STAT3 activation, and propose IL-6 autocrine production and paracrine stimulation as STAT3 constitutive activation mechanism. In tumor microenvironment, as well as in tumor invasive fronts, presence of inflammatory cells has been observed, which suggests IL-6 might be a tumorigenic agent through JAK/STAT pathway activation. This places EMT as being indispensable for the generation of metastasis, with this process also being observed to be reversible, which allows the cell to return to an epithelial phenotype once the site of metastasis is reached.

On the other hand, in our laboratory, EGF was found to induce EMT measured as E-cadherin decrease and vimentin expression through transcription factor Snail induction. Given these backgrounds, in the present work we analyze IL-6 effect on EMT induction. A model of primary breast cancer cell cultures was used, where EMT was induced, and changes in epithelial markers (E-cadherin) were examined. The results showed that IL-6 induces EMT with the characteristic E-cadherin loss.

MATERIALS AND METHODS

Reagents

Primary antibodies against E-cadherin, tubulin, pSTAT3 (Tyr705) and STAT3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and E-cadherin was obtained from Cell Signaling Technology (Cambridge, MA). Anti-mouse or anti-rabbit secondary antibodies were acquired from Jackson ImmunoResearch (West Grove, PA). Interleukin-6 (IL-6) was obtained from PeproTech (Rocky Hill, NJ).

Cell culture

MBCDF and MBCD17 primary breast cancer cells, which were derived from a biopsy of the specimen resulting from a mastectomy performed in a patient with breast cancer (protocol approved by the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán Ethics Committee, ref. 1549, BQ0-008-06/9-1). The cell cultures were maintained in RPMI-1640 medium supplemented with 10% bovine fetal serum, antibiotic and antimycotic (Invitrogen Corporation, Carlsbad, CA) at 37 °C in a moisturized atmosphere with 5% CO₂.

Cell stimulation

IL-6-stimulation assays were carried out in order to assess STAT3 phosphorylation by Western blot, using a phospho-specific antibody against pSTAT3 Y705 in MBCDF and MBCD17 cells; for this, 1 X 10⁴ cells were seeded in 60-mm culture plates, maintained in RPMI-1640 medium supplemented with 0.1% bovine fetal serum. The cells were allowed to adhere overnight at 37 °C and 5% CO₂. Stimulation was applied with IL-6 1 ng/mL for the following time intervals: 0, 5, 15, 30 and 60 minutes.

For the EMT induction and reversion assays, MBCD17 epithelial cells, two experimental models were designed: for the first one, short times of EMT IL-6-mediated induction were used of 0, 4, 8, 12 and 24 h.

Immunoblot assay (Western Blot)

Stimulated cells were lysated with a lysis buffer containing: HEPES 50 mM (pH 7.4), EDTA 1 mM, NaCl 250 mM, 1% Nonidet, NaF 10 mM and 1 x protease inhibitors (Complete, EDTA-free, Roche). 25 µg total protein underwent denaturation: polyacrylamide gel electrophoresis and were transferred to Immobilon-P PVDF membranes (Millipore Corp, Bedford, MA), which were blocked for 60 minutes in 5% skim milk in 0.05% PBS-Tween. Then, they were incubated with the respective antibodies overnight at 4 °C with agitation. Subsequently, the membranes were incubated with the anti-mouse or anti-rabbit-HRP antibodies, as appropriate, for 45 minutes. The signal was visualized by chemiluminescence using the Super Signal West Pico kit (Thermo, Rockford, IL) and was finally exposed to a Kodak radiographic film.

RESULTS

IL-6 induces STAT3 phosphorylation in breast cancer cells

It is well established that IL-6 signals through STAT3 activation. To demonstrate that IL-6 was able to induce STAT3 activation in the primary breast cancer cell cultures, we stimulated the MBCDF and MBCD17 cells with 5 ng/mL of IL-6 for

RESULTS
different time intervals. STAT3 activation was measured as STAT3 Tyr705 residue phosphorylation using phospho-specific antibodies. The results demonstrate that IL-6 induces STAT3 phosphorylation. Phosphorylation of STAT3 had an activation peak in MBCDF cells at between 15 and 30 min, whereas in MBCD17 cells, pSTAT3-Tyr705 activation peak occurred at 15 minutes (Fig. 1). These results confirm that, in these cultures, IL-6 signaling is mediated by STAT3 activation.

IL-6 induces epithelial-mesenchymal transition

Once we demonstrated that IL-6 induces STAT3 phosphorylation in primary breast cancer cell cultures, we investigated whether IL-6 stimulation elicits a decrease in E-cadherin expression as an EMT marker. IL-6 was found to induce a slight drop in E-cadherin expression from 2 h, which becomes more pronounced from 12 h and onwards. An anti-tubulin antibody was used as loading control. These results suggest that IL-6 induces rapid changes in E-cadherin expression, as a marker indicating that the tumor cell enters in EMT process (Fig. 2).

DISCUSSION

EMT is one of the most critical steps in the development of metastasis, and the description of the molecular mechanisms of which becomes activate and how it can be inhibited or reverted are therefore highly relevant to the development of new treatment strategies. In this work, we present evidence that IL-6 induces EMT through E-cadherin decrease in primary breast cancer cells. These data suggest that IL-6 is an important cytokine within the tumor microenvironment, which participates in breast cancer process of metastasis.

One of the main causes of mortality in breast cancer patients is metastasis. The process of metastasis includes several steps that a transformed cell has to complete to migrate to a distant site. Crucial steps of this process include EMT, which is characterized by cell polarity loss and invasive properties acquisition. In order to be able to study metastatic cells properties, we developed an EMT in vitro model based on primary breast cancer cell cultures. EMT is a dynamical process, and it is activated by different stimuli of tumor microenvironment, including growth factors, tumor cell-stroma interactions and hypoxia. EMT-activating signals include growth factors such as transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), insulin-like growth factors 1 and 2 (IGF 1 and 2) and epidermal growth factor (EGF). IL-6 is an inflammatory cytokine that has been associated with EMT. In our primary breast cancer cell culture model, we stimulated epithelial marker-bearing cells (MBCDF and MBCD17) with IL-6 trying to induce EMT, demonstrating that IL-6 induces a decrease in E-cadherin expression in hours through STAT3 phosphorylation. These results demonstrate that, in our in vitro model of primary breast cancer cell culture, treatment with IL-6 is able to induce EMT, as demonstrated by the loss of E-cadherin expression.

CONCLUSIONS

In summary, our work demonstrates that IL-6 is a potent EMT inducer through STAT3 activation in primary breast cancer cell cultures. The implications of this suggest that the presence of IL-6 in the tumor microenvironment confers high metastatic potential. These data support the development of new therapeutic strategies for breast cancer treatment, as IL-6 inhibition could be, as an attractive approach to intervene with EMT.

CONFLICT OF INTERESTS

The authors declare no conflicts of interests.

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