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Role of estrogens and their receptors in adhesion and invasiveness of breast cancer cells

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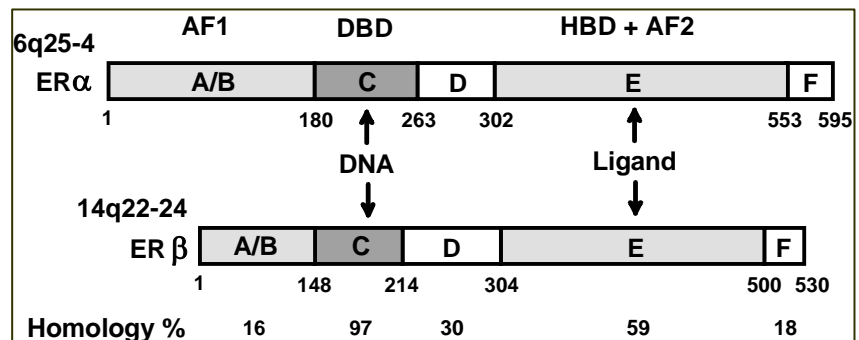
Summary

Estrogen receptors are overexpressed in human breast cancers and associated with differentiated tumors with a more favorable prognosis. Paradoxically, these receptors mediated the mitogenic action of estrogens in human breast cancer cells and the efficacy of antiestrogens in adjuvant therapy of primary tumors. The exact mechanism underlying the ER protection against cancer progression to metastasis, remains to be investigated. Herein, we show that estrogen receptors decrease invasiveness of breast cancer cells. Detailed studies revealed that the unliganded and the estradiol-activated estrogen receptors decrease cancer cell invasion *in vitro* through two distinct mechanisms. In the presence of ligand, ER α inhibits invasion through a mechanism requiring the functional ER α domains involved in the transcriptional activation of target genes. Moreover, we found using different approaches that cell-cell contacts were markedly increased by estradiol treatment and decreased by the pure antiestrogen, ICI 162,780. This cell-cell adhesion was associated with an increase of the major intercellular junctions, desmosomes. Conversely, in the absence of ligand, ER α also inhibits invasion through a distinct mechanism involving protein-protein interaction with the region of the first zinc finger of ER α . The relationship of these data with clinical studies and their potential therapeutic consequences will be discussed.

Introduction

Breast cancer is the most frequent malignancy diagnosed in women in western countries and estrogen receptor (ER) is overexpressed in about two thirds of breast tumors. Estrogens are the major sex steroid hormones involved in the development of normal mammary gland and in the etiology of human breast cancer. The promoter role of estrogens in breast cancer has been evidenced by epidemiological studies indicating an increased incidence in women with prolonged exposure to estrogens and a drastic decrease of incidence in women having non functional ovaries (1). Most of the estrogen effects are mediated by two specific receptors ER α and ER β that have a structure characteristic of members of the large nuclear receptor superfamily (2-3). These receptors are subdivided into several functional domains (Figure 1).

Figure 1: Structures and functions of estrogen receptors.



Functional domains were defined as a DNA binding domain (DBD, C) containing two zinc fingers involved in DNA binding and receptor dimerization, a ligand binding domain (LBD, E) important for ligand binding, receptor dimerization and interaction with transcriptional coactivators and corepressors (4). The ligand binding domain contains a ligand activable transactivation function AF2 whereas the N-terminal A/B region contains a ligand independent transactivating function AF1 (5). The hinge domain (D) contains the nuclear localization signal of the receptor and contributes to the flexibility of the two moieties containing the DNA and the ligand binding. The F domain participates to the transactivation capacity.

In malignant mammary cells, the effects of estrogens are more complex since estrogens affect directly the growth, motility and invasiveness of cancer cells but, could also influence some biological responses of the host such as neo-angiogenesis and immune response. The presence of ER α is associated with more differentiated and less invasive tumors and, several clinical studies have demonstrated the favorable prognostic value of ER in primary breast tumors (6). The routine practice of ER α assay in primary tumors has been established to predict the efficacy of antiestrogens, widely used as first-line adjuvant therapy (7). The second receptor, ER β , has likewise been detected in primary breast cancers by immunohistochemistry but its own clinical relevance in prognosis and tumor progression remains to be established (8, 9).

In this report, we will focus on the effects of estrogens and ER α on the invasiveness of breast cancer cells. Estrogens are classically known to favor growth of cancer cells and, estrogen antagonists are clinically important in the treatment of hormone-dependent breast cancers. However, a series of evidences have demonstrated that ER could protect against cancer cell invasion of basement membranes, an important step of the metastatic process required for cancer dissemination. This last observation proposes a plausible explanation for the good prognosis value associated with the presence of ER α in primary tumors.

Results

Estrogen receptors inhibit invasion through two distinct mechanisms in the presence or absence of hormone.

Estrogens inhibit invasion via ERE-regulated genes. The effects of estrogens on cell invasiveness have been studied *in vitro* using a two-chamber culture model and Matrigel, a reconstituted basement membrane. The initial studies indicated that the

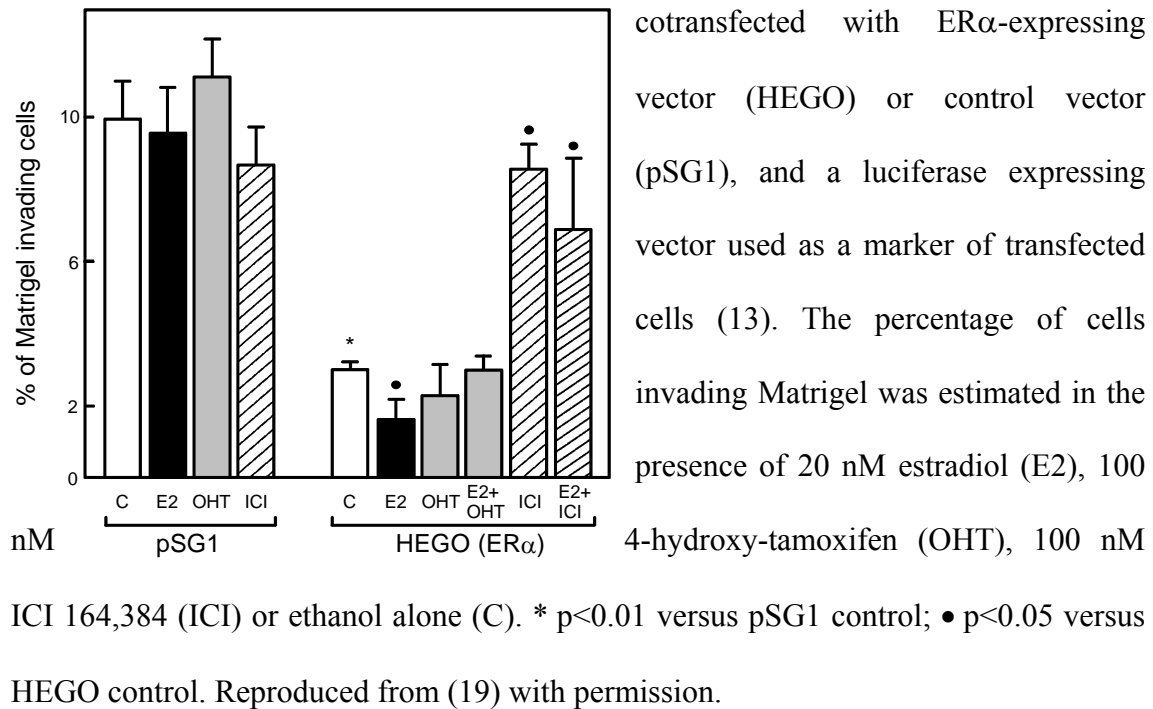
invasiveness of MCF7 breast cancer cells was increased by antiestrogens (10, 11). More recent studies have demonstrated that estradiol significantly reduces invasiveness which is reversed by antiestrogens (12-16). This conclusion was noted in several ER α -positive cancer cell lines established from breast (12) or ovary (14), and in different ER α -negative cancer cells constitutively expressing ER α after stable transfection (13, 16, 17). Similar results were also obtained on the migration of normal cells from vascular smooth muscle (15). These *in vitro* data were confirmed in nude mice, since the formation of experimental lung metastases from metastatic ER α -negative MDA-MB-231 breast cancer cells was inhibited by estradiol after ER α expression by transfection (16, 17).

The mechanism by which estradiol inhibits invasion was studied using a new invasion assay based on the transient expression of ER α in the ER α -negative MDA-MB-231 cell line (18, 19). Estradiol treatment decreased by 2-fold the invasiveness in ER α -transfectant (Figure 2). The inhibitory effect of estradiol is reversed by both types of antiestrogens, OH-tamoxifen (4-hydroxytamoxifen, the active metabolite of tamoxifen) and ICI 164,384, a pure antiestrogen.

By contrast, estradiol or antiestrogen treatments did not significantly affect invasion of control ER-negative cells. Moreover, the analysis of different ER α deletion mutants strongly suggested that some estrogen-regulated genes negatively control invasion since the integrity of the hormone-binding domain, the DNA-binding domain and activating function 2 (AF2) of ER α was required (19). In contrast, the N-terminal domain containing the AF1 function is not involved since a deletion Δ AB mutant was as efficient as the wild-type receptor. As possible candidates among estrogen-regulated proteins, those that increase cell-cell adhesion, such as E-cadherin, or that decrease

matrix degradation, such as α 1-antichymotrypsin, should be considered (reviewed in 12, 20)

Figure 2: Effect of ER α transient transfection and estradiol treatment on MDA-MB-231 cell invasion. ER α -negative MDA-MB-231 cells were transiently



More recently we studied the effects of estrogens on the adhesiveness of breast cancer cells. Using different approaches, we found that cell-cell contacts were markedly increased by estradiol treatment and decreased by the pure antiestrogen, ICI 182,780 (Table 1). Estradiol-treated cells formed aggregates in standard culture conditions or on a collagen layer and, were more resistant to trypsin treatment. In suspension culture conditions, only estrogen-treated cells constituted large spherical multilayered cell structures. Using electron microscope analysis, the increase in cell-cell adhesion was associated with the formation of the major intercellular junctions, desmosomes. Estradiol increased both the number and the shape of desmosomes with enlarged dense plates in MCF7 cancer cells but also in normal mammary cells isolated from reduction

mammosplastics. The estrogen-induced proteins involved in desmosome production are currently investigated.

Table I. Effects of estradiol and ICI_{182,780} on cell-cell interactions. Steroid-stripped MCF7 cells were treated for 5 days with 1nM estradiol (E2), 1nM E2 + 1μM ICI_{182,780} or treated with vehicle alone (control) and the cell-cell interactions studied in the indicated cultures conditions.

| Treatments | Control | E ₂ | E ₂ + ICI _{182,780} |
|---------------------------------------|---------|----------------|---|
| Effects on cell-cell interactions | | | |
| Trypsin resistance | - | ++ | + |
| Cell aggregation in collagen gel | - | ++ | - |
| Spheroid structures in liquid culture | - | + | - |
| Number of desmosomes | + | ++ | + |
| Enlarged dense plates | ± | +++ | ± |

Overall, these data indicated that the up-regulation of cell-cell adhesion by estrogen found in normal mammary cells is a characteristic maintained in ERα -positive breast cancer cells, and could participate to the low invasive potential of ERα -positive tumors.

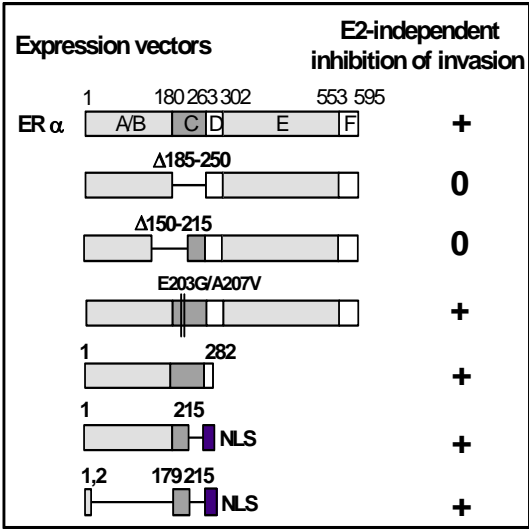
Estrogen receptors inhibit invasion independently of ligand binding: involvement of protein-protein interactions Using the transfection/invasion method described above, we also demonstrated that expression of unliganded ERα and several mutants deleted in the hormone-binding domain drastically reduced MDA-MB-231 cell invasiveness in Matrigel tests. As shown in Figure 2, in estrogen-deprived conditions, transient wild type ERα expression induced a 3-fold decrease in the invasiveness of transfected cells and estradiol treatment reinforced the ligand independent effect by an additional 2-fold reduction. The strong inhibition due to the unliganded ERα is reversed by the pure antiestrogen ICI 164,384 which is known to decrease receptor concentration

but not by the partial antiestrogen, OH-tamoxifen. In breast cancer cells, OH-tamoxifen and other tamoxifen derivatives were shown to up-regulate the receptor by decreasing its degradation (21). This increase of ER α concentration could explain the anti-invasive properties of tamoxifen in certain models *in vitro* (22) and could participate to its beneficial effects *in vivo*.

The domain involved in ligand-independent inhibition of invasion has been further characterized by progressive deletions in the ER α sequence.

As shown in Figure 3, the first zinc finger of the DNA-binding domain (i.e., amino acids 179-215) is responsible for the anti-invasive activity. This activity is independent of the two key aminoacids which are essential for ERE binding and the estrogen specificity of the responses (23). Among the different nuclear receptors, invasion was

Figure 3: E2-independent inhibition of cancer cell invasion requires the first zinc



finger of the DNA binding domain. Effects of ER α mutants on MDA-MB-231 cell invasiveness were determined in transfection/invasion assay in Matrigel (13). A nuclear localization signal (NLS) was added to maintain nuclear targeting of mutants deleted of the endogenous signals. Reproduced from (19) with permission.

specifically decreased by the expression of ER α (3-fold) and to a lesser extent by ER α (2-fold), but was not affected by thyroid hormone receptor α 1, vitamin D receptor, retinoid acid receptor α , or glucocorticoid and androgen receptors. On the basis of these

data, it was proposed that unliganded ER decreases invasiveness *via* interaction of the first zinc finger region with an unknown nuclear factor.

Moreover, immunocytochemical studies of ER α -positive breast cancer cell lines (MCF7, ZR75.1, T47D) indicated that in hormone-deprived conditions, ER α expression was inversely correlated with cell motility (19). Migrating cells had lower ER levels than non-migrating cells. Finally, treatments such as phorbol ester or pure antiestrogen, known to decrease ER α levels in MCF7 breast cancer cells, significantly increased *in vitro* invasiveness (19, 24). Taken together, these *in vitro* data indicate a protective role of ER α against the invasiveness of breast cancer cells.

These data obtained on cell cultures cannot be extrapolated to the *in vivo* situation, where the endocrine and paracrine effects of estrogens may have major consequences on the invasiveness of cancer cells. However, their possible implications in the monitoring of breast cancer should be discussed. Particularly, negative effects should be anticipated in the clinical use of pure antiestrogens such as ICI 164,384 or ICI 182,780, since these drugs increased *in vitro* cancer cell invasiveness by inhibiting the protective effect of both estrogens and ER α by decreasing its content. ER α expression should perhaps also be preserved in cancer cells during adjuvant therapies in order to maintain differentiation and a low invasive potential. In addition, these results suggest new therapeutic strategies based on ER α re-expression to prevent the proliferation, invasiveness and metastatic potency of ER α -negative breast cancer cells (20).

Discussion

Our data demonstrated that estradiol and ER α expression inhibit cancer cell invasiveness in breast cancer cells through different mechanisms. Inhibition by estrogens is dependent to transcriptional activation of specific target genes that are probably involved in an increase of cell-cell adhesion. The unliganded \square inhibits ER α

invasion via protein-protein interactions within the first zinc finger region of the receptor. These data could explain the protective role of ER α against tumor invasion and metastasis previously found in these cell lines. ER α expression has been associated with a low invasiveness and low motility in culture tests (24, 25). Moreover, when ER α -positive cells were implanted in nude mice, tumors appeared only in the presence of estrogens and are poorly metastatic as compared to those developed from ER α -negative breast cancer cell lines (20).

Clinical data supporting that estrogens prevent invasion. There is a great deal of evidence to support the hypothesis that estrogens are important because they are potent mitogens for normal breast epithelial cells, and it is believed that the duration of breast epithelium exposure to estrogens is a significant risk factor for breast cancer development. However, in mammary carcinogenesis, even though the mitogenic effect of estrogens is well demonstrated, the presence of ER α is associated with more differentiated and less invasive tumors and a more favorable prognosis. Moreover, there is some clinical evidences indicating that estrogens and their receptors protect against invasion. Epidemiological studies have evaluated the breast cancer risk in women using hormone replacement therapy (HRT) where 80% were taking preparations containing estrogen alone (26, 27). Among the women using HRT, the risk of breast cancer slightly increased, but the tumors under estradiol treatment were confined to localized disease with more favorable prognosis. Tumors in HRT-users were less invasive to axillary lymph nodes and to more distant sites. Other studies of tamoxifen therapy of primary breast cancer suggest that tamoxifen increased the spreading of ER α -positive primary tumor cells to contralateral sites. Tamoxifen use decreased (0.8 fold) the risk of ER α -positive tumors, but it appeared to increase (4.9-fold) the risk of ER α -negative

contralateral tumors (28). All together, these clinical data are in agreement with an anti-invasive effect of estrogens.

Role of estrogen receptor variants in cancer progression? Numerous studies have identified ER α and ER α variants from differential splicing of their mRNAs in both neoplastic breast tissue and cell lines (29, 30). These mRNA variants lack one or several exons and are usually coexpressed with the wild-type ER message. However, their pathophysiological significance is unclear. Several studies using transient transfection have shown that individual ER α variant proteins can have positive or negative effects on the wild-type ER α activity (29, 30). The presence of one or more variant proteins in variable levels in normal breast epithelium and neoplastic tissue could influence the wild-type receptor (31). The variants ER α Δ 3 (lacking the second zinc finger) and ER α Δ 4 (deleted in the hormone-binding domain) are overexpressed in normal cells rather than in breast cancer cells. These variants, which contain the first zinc finger domain, could have an invasion-suppression activity independent of the hormone action. This was verified by expression of the ER α Δ 3 variant (31). A more detailed clinical evaluation of the ER variants is required to determine their influence in mammary carcinogenesis and the response to therapy.

Missense mutants of the ER α gene with point mutation have been found in only 1 % of primary breast tumors (32). Even the functionality of only few mutations has been studied; initial data indicate that these mutations could affect normal ER α function and alter the evolution of individual tumors. This suggests that these somatic mutations, although infrequent, may significantly alter the evolution of individual tumors.

In conclusion, we present evidences that estrogens inhibit invasiveness of breast cancer cells *via* a classical activation of ERE-regulated genes leading to an increase in cell-cell adhesion. In the absence of ligand, the receptor could also prevent invasion through

interaction with an unknown protein. Non-classical mechanisms of action, in which the receptor may bind to other transcription factors instead of DNA or to the proteins involved in pathways such as motility and invasion, requires further investigation. The identification of the factors that inhibit the invasiveness of ER α positive cells would be a useful step in the development of new therapeutic targets to cure the most aggressive ER α -negative tumors.

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