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Modulation of human breast cancer cell adhesion by estrogens and antiestrogens

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In order to study the effect of estrogens and antiestrogens on the adhesive properties of human breast cancer cells, the attachment on endothelial cells (EC), on subendothelial extracellular matrix (ECM) and on ECM components (collagen I and IV, laminin, fibronectin) of estrogen-dependent (MCF-7, ZR75-1) and estrogen-independent (BT-20) breast cancer cell lines was investigated. The cells were grown under conditions of controlled exposure to estrogen [17β-estradiol (E_2)] and/or antiestrogens [tamoxifen (Tam) or 4-hydroxytamoxifen (OH-Tam]. Treatment by E_2 enhanced the ability of ZR75-1 cells to adhere to the various substrates, which contrasts with the observed absence of effects with the BT-20 cells. Similarly, Tam or OH-Tam induced a reduction of the adhesion of ZR75-1 tumor cell, but not of BT-20 cells. This effect was reversed by competing concentrations of E_2. The effects on MCF-7 cell adhesion were similar to those described for ZR75-1 cells, but could not be reproducibly observed. Adhesion assays carried out with ZR75-1 cells grown in the absence or presence of phenol red, a pH indicator which behaves as a weak estrogen, led to a similar pattern of cell attachment. Conditioned media harvested from E_2- or Tam-treated ZR75-1 cells failed to induce any effect on adhesion of other ZR75-1 cells grown in E_2-deprived medium, suggesting that secretory activities are not required for the control of cell adhesiveness. The results suggest that estrogens and antiestrogens can control the adhesive behavior of breast tumor cells through their hormone responsive structures possibly by regulating expression of cell adhesion proteins and/or their cell surface receptors.

Introduction

Many of the *in vitro* effects of estrogens on breast cancer cells which have been described may modulate breast cancer progression *in vivo*. These effects include growth stimulation through estrogen-induced autostimulatory growth factors (for a review, see ref. 8), and modulations of other activities thought to mediate metastatic events. It is generally agreed that estrogen supplementation is required for the growth of the estrogen receptor-positive cell lines MCF-7 and ZR75-1 in nude mice

[20]. In addition, estrogen treatment increases the frequency of metastasis of MCF-7 cells in nude mice [24]. Estrogens can also enhance the production of proteolytic enzymes such as tissue plasminogen activator [5], collagenases [14], 52K-cathepsin-D-like [19] or cathepsin-D mRNA [30] in breast cancer cells. These enzymes may contribute to tumor progression by allowing the tumor cells to digest and traverse encapsulating basement membranes [17]. It has also been demonstrated that estrogen treatment induces marked rearrangements of cytoskeleton and adhesion structures [23] and an enhanced attachment of MCF-7 cell to laminin, a basement membrane glycoprotein [2].

The metastatic behavior of tumor cells is currently thought to depend partly on the ability of the cells to adhere to host tissue structures such as adjacent stromal tissues and vascular endothelium [26]. Increasing evidence supports the existence of multiple interactions between tumor cells and the various extracellular matrix (ECM) components such as collagens, laminin and fibronectin. These adhesive interactions are mediated through glycosaminoglycans, glycoproteins such as laminin and fibronectin, expressed on tumor cells, and their specific cell surface receptors [18]. The adhesive capacity of the cells and the expression of these cell adhesion proteins and/or their receptors can be modulated by various components [9–13, 15, 27].

Since enhanced production and expression of molecules involved in cell adhesion can be associated with invasive and malignant behavior of tumor cells, it is of interest to study whether estrogens contribute to cellular invasiveness of hormone responsive breast tumor cells. In this study, we investigated the adhesive properties of estrogen-dependent and estrogen-independent breast cancer cell lines grown under controlled conditions of hormone exposure. We found that the interactions between tumor cells and ECM or ECM components can be modulated by estrogens and antiestrogens.

Materials and methods

Materials

Acid-soluble collagens type I (C-7774) and type IV (C-7521) from human placenta, human plasma fibronectin (F-2006), bovine serum albumin (A-8022), laminin (L-8263) and 17β-estradiol (E-2257) were obtained from Sigma (Isle d'Abeau, France). Tamoxifen [trans-(*p*-dimethylaminoethoxyphenol)-1,2-diphenylbut-1-ene] and its 4-hydroxylated metabolite were gifts from ICI Pharmaceuticals Division (Alderley Park, Macclesfield, U.K.). All culture materials were obtained from Falcon (Becton Dickinson, Grenoble, France) and culture media from Gibco (Paisley, U.K.), except phenol red and phenol red-withdrawn media (Seromed, Intermed, Lyon, France). Indium-111 oxinate was supplied by ORIS (France). Other chemicals were of analytical reagent grade.

Cell lines and culture conditions

Human breast carcinoma cell lines MCF-7 (kindly supplied by Pr. Chambon, Strasbourg, France), BT-20 (a gift from Dr Saez, Centre Léon Bérard, Lyon, France) were grown in Eagle's minimum essential medium (EMEM) and ZR75-1 (American Type Culture Collection, Rockville, MD, U.S.A.) was grown in RPMI 1640. All of these media were supplemented routinely with L-glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 μg/ml), non-essential amino acids (1 per

cent for EMEM), insulin (10 μg/ml for MCF-7 cells) and 10 per cent fetal calf serum (FCS). All the cell lines were cultivated in 75 cm^2 flasks at 37°C in a humidified atmosphere of 5 per cent (v/v) CO_2 and subcultured weekly after detachment with 0·05 per cent trypsin–0·02 per cent EDTA. The presence of estrogen (ER) and progesterone receptors (PgR) in MCF-7 and ZR75-1 cell lines and the absence of estrogen receptors on BT-20 tumor cells were confirmed by immunochemical procedures (PgR EIA, ER EIA, Abbott, France).

Prior to experiments, tumor cells were cultured for 6 days in an estrogen-deprived medium composed of their respective media supplemented with 10 per cent charcoal-absorbed fetal calf serum (CCS) [22]. Then 17β-estradiol (E_2) at a final concentration of 10^{-8} or 10^{-10} M, and/or tamoxifen (Tam) (10^{-6} M) or 4-hydroxytamoxifen (OH-Tam) (10^{-8} M) were added for 5 days. The hormones were stored in solution in ethanol at −20°C and added to the growth media to give a final concentration of 0·01 per cent ethanol. In some experiments the cells were grown in phenol red-withdrawn media six days before and during hormone treatments.

Preparation of the adhesion substrates

All the adhesion substrates were coated on 24-well Falcon culture plates. The human umbilical vein endothelial cells (EC) were cultured as described previously [16]. To obtain the subendothelial matrix surfaces (ECM), 3-day post-confluent monolayers of EC were exposed for 30 min at 22°C to 0·5 per cent Triton X-100 followed by a 2–3 min exposure to 25 mM ammonia solution, and then the dishes were thoroughly washed with PBS and stored at 4°C. The other substratum-coated dishes were prepared as follows. The collagens were dissolved in 0·1 M acetic acid (200 μg/ml) and 100 μl were added to each well and allowed to dry for 24 h. Then plates were sterilized by exposure to UV light overnight, stored dry for a maximum of 1 month and desorbed for 2 h in PBS before use. The culture wells were coated immediately prior to use with fibronectin and laminin solutions in PBS (4 μg per well) by incubation for 1 h at room temperature followed by two washes with PBS.

Adhesion assay

The adhesion assay was performed as described previously [1]. Briefly, the tumor cells were harvested from cultures with trypsin–EDTA solution, washed and suspended in Hank's balanced salt solution and then labelled with indium-111. A volume of 0·5 ml of the labelled tumor cell suspension (10^5 cells/ml), in media supplemented with 10 per cent charcoal absorbed fetal calf serum or with 0·2 per cent BSA, was plated on the various substrates. The plates were incubated without agitation at 37°C in a 5 per cent CO_2 atmosphere and after 1 h the non-attached cells were removed for determination of radioactivity. The percentage of adherent cells was calculated as described previously [1].

Results

Effect of E_2, Tam and OH-Tam on tumour cell adhesion

The MCF-7 and ZR75-1 cell lines used in this study showed an enhanced proliferation induced by E_2 concentrations of 10^{-8} and 10^{-10} M for ZR75-1 tumor cells (data not shown). In contrast, the addition of antiestrogens such as Tam (10^{-6} M) or OH-Tam (10^{-8} M) to the E_2-deprived medium resulted in a reduction

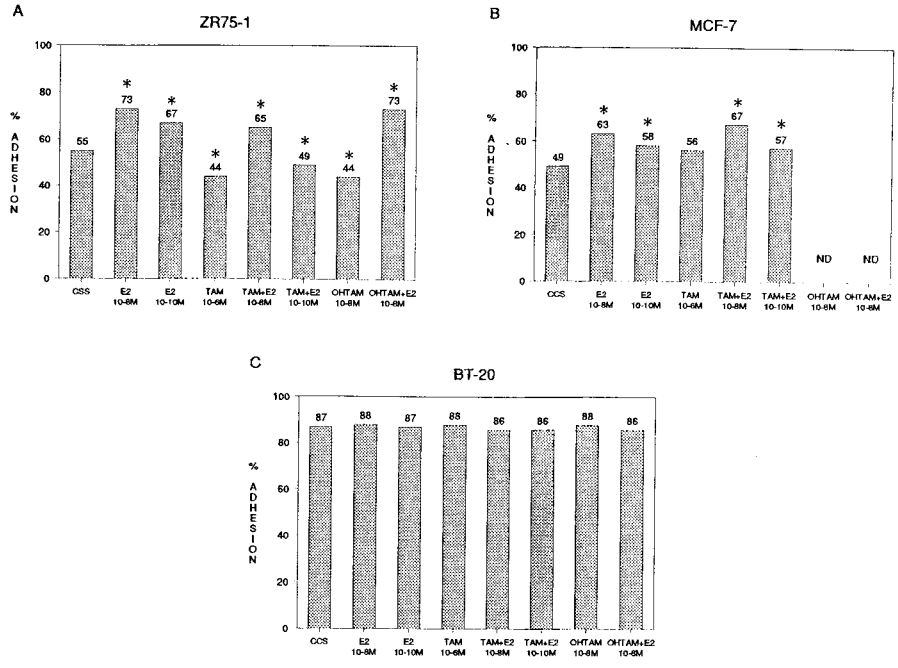

Figure 1. Effect of estrogen and antiestrogen on the adhesion capacity of various mammary tumor cell lines. The tumor cells were grown for 5 days in the presence of E_2 and/or Tam or OH-Tam. Their adhesion capacity to ECM was then determined and expressed as a percentage of total cells that adhered after 1 h (mean values from five separate experiments carried out in triplicate). * Significantly different from the control (CCS), $p < 0{\cdot}05$ by Wilcoxon's test.

of cell growth when compared with the basal growth obtained in E_2-deprived medium. The proliferation of BT-20 cells was not affected either by E_2 or Tam. The various hormone treatments did not alter the cell viability as determined by the trypan blue exclusion test either after trypsin treatment or cell labeling with indium-111.

Adhesion assays were carried out with MCF-7, ZR75-1 and BT-20 mammary tumor cells grown either in E_2-deprived medium alone or medium supplemented with estrogen or antiestrogen alone (10^{-8} or 10^{-10} M E_2, 10^{-6} M Tam, 10^{-8} M OH-Tam) or associated (10^{-8} or 10^{-10} M E_2 plus 10^{-6} M Tam, or 10^{-8} M E_2 plus 10^{-8} M OH-Tam). As indicated in figure 1A, the treatment of ZR75-1 cells with 10^{-8} or 10^{-10} M E_2 increased the proportion of cells adhering to ECM when compared with the adhesion of cells cultured in E_2-deprived medium, from 55 ± 13 to 73 ± 6 or 67 ± 6 per cent, respectively. Conversely, 10^{-6} M Tam or 10^{-8} M OH-Tam decrease the adhesion of ZR75-1 cells to ECM to 44 ± 16 per cent.

The inhibitory effects of the antiestrogens were reversed when either 10^{-6} M Tam or 10^{-8} M OH-Tam treatment was concomitant with 10^{-8} M E_2 treatment, since adhesion levels similar to those of E_2-treated cells were observed (65 and 73 per cent respectively). This reversion of the Tam- or OH-Tam-induced reduction of ZR75-1 cell adhesion did not occur when a non-competitive concentration of E_2 (10^{-10} M) was used. Results of estrogen and antiestrogen modulation of ZR75-1 cell

adhesion on other substrates than ECM such as EC, fibronectin, laminin, collagen I and IV are reported in table 1. These results are expressed as adhesion ratio between E_2- or Tam-treated cells and cells grown in E_2-deprived medium (control). Although the levels of control adhesion were very different depending on the substrate used, the estrogen- and antiestrogen-induced modifications of cell adhesion were reproducibly observed.

Similar adhesion experiments with MCF-7 cells led generally to the same E_2-induced enhancement since the percentage of adherent MCF-7 cells to ECM increased from 49 to 63 per cent (figure 1B). However, the effects of E_2 on attachment were not uniformly observed between experiments. In addition, the Tam-induced inhibition observed for ZR75-1 cells adhesion was not observed with MCF-7 cells.

In contrast, as shown in figure 1C, no significant effect of E_2 or Tam could be observed on the attachment of ER-negative BT-20 tumor cells, which remained at approximately 87 per cent. This absence of adhesion modification was regularly found with all the substrates tested (EC, collagens, laminin, fibronectin), irrespective of the different basal adhesion levels observed on these substrates (data not shown).

Effects of phenol red on tumor cell adhesion

Phenol red (PR), the pH indicator in tissue culture medium, has been described as a weak estrogen which is able to stimulate the proliferation of MCF-7 cells [4]. As shown in table 2, the presence of PR in the culture medium results also in a stimulation of ZR75-1 cell proliferation when compared with cell proliferation in PR-deprived media. However, unlike the results with MCF-7 cells, Tam induced an inhibition of ZR75-1 cell proliferation even when cells were grown in the absence of PR. Under both conditions, a competitive concentration of 10^{-8} M E_2 was able partly to reverse the inhibitory effect of Tam on cell growth.

To determine the effect of PR on tumor cell attachment, we used for adhesion assays ZR75-1 cells grown for 5 days in various conditions of hormone exposure in

Table 1. Influence of estrogen or/and tamoxifen on the attachment of ZR75-1 cells to the various substrates.

Substrate	Control[a]: percentage adhesion	Treated/control adhesion ratio[b]		
		E_2 (10^{-8} M)	TAM (10^{-6} M)	TAM (10^{-6} M)+E_2 (10^{-8} M)
Endothelial cells	45·3	1·19±0·13[c]	0·67±0·22[c]	1·14±0·13[d]
Collagen IV	59·7	1·30±0·38[c]	0·95±0·36[c]	1·30±0·23[d]
Collagen I	60·5	1·26±0·32[c]	0·82±0·14[c]	1·30±0·41[d]
Fibronectin	59·8	1·24±0·22[c]	0·70±0·11[c]	1·03±0·17[d]
Laminin	29·5	1·41±0·54	0·99±0·12	1·28±0·32
Tissue culture grade polystyrene	30·0	1·37	0·64	1·20

[a] The cells were grown in E_2-deprived medium before testing their adhesive capacities. The adhesion test was carried out in the presence of medium supplemented with 0·2 per cent BSA.

[b] The ratio was the mean of 3–5 experiments carried out in triplicate. The differences in the ratio values were examined by Friedman's test [6].

[c] Significantly different values/control.

[d] Significantly different values/Tam-treated cells.

Table 2. Influence of phenol red on the effect of estrogen and tamoxifen on growth and adhesion of ZR75-1 cells.

	Growth[b]				Adhesion[c]	
	Day 6		Day 10			
Hormone treatment[a]	+PR	−PR	+PR	−PR	+PR	−PR
Control (E_2-deprived medium)	11·0(100)[d]	8·2(100)	33·8(100)	20·5(100)	47·2(100)	44·6(100)
E_2 (10^{-8} M)	22·1(200)	15·8(192)	40·9(120)	36·5(177)	67·2(133)	58·6(131)
Tam (10^{-6} M)	7·7(70)	7·3(89)	14·5(43)	12·9(63)	31·7(67)	27·9(63)
E_2 (10^{-8} M)+Tam (10^{-6} M)	17·4(158)	13·8(172)	30·7(91)	30·5(148)	54·1(115)	45·2(101)

[a] The cells were treated for 5 or 9 days with 17β-estradiol (E_2) or/and tamoxifen (Tam) in phenol red (PR) withdrawn medium with or without PR supplement before testing their adhesive capacities on confluent EC.

[b] Number of cells per cm^2 ($\times 10^{-3}$).

[c] Percentage of adherent cells on EC 1 h after seeding.

[d] Values in parentheses represent the percentage of control values.

media with or without PR. The results in table 2 indicate that in the absence of PR, apart from a slight decrease in the adhesion levels, the usual significant modifications induced by E_2 or Tam treatment always occurred. For the ZR75-1 cells grown in the absence or presence of PR, the amplitudes of E_2-induced enhancement of adhesion (131 and 133 per cent, respectively) or Tam-induced decrease of attachment (63 and 67 per cent, respectively) were very similar.

Influence of E_2- and Tam-induced ZR75-1 cell secretory activities on attachment

A variety of secreted protein activities have been identified as markers for estrogen action [8]. It may be asked whether the estrogen and antiestrogen effects on cell adhesiveness could be mediated by these controlled secretory activities. We studied the attachment to ECM of E_2- and/or Tam-treated ZR75-1 cells in adhesion assays using their conditioned media or fresh E_2- and/or Tam-supplemented media, respectively. As shown in figure 2A, the adhesion patterns were similar between treated ZR75-1 cells when the adhesion assays were performed either in fresh or in conditioned media, although the adhesion levels were slightly lower in the latter media. In addition, conditioned media harvested from E_2- and/or Tam-treated ZR75-1 cells were unable to induce any modification of adhesion of other ZR75-1 cells grown in E_2-deprived medium (figure 2B). These data suggest that the estrogen- and antiestrogen-induced adhesion modifications cannot be related to the release of secreted factors.

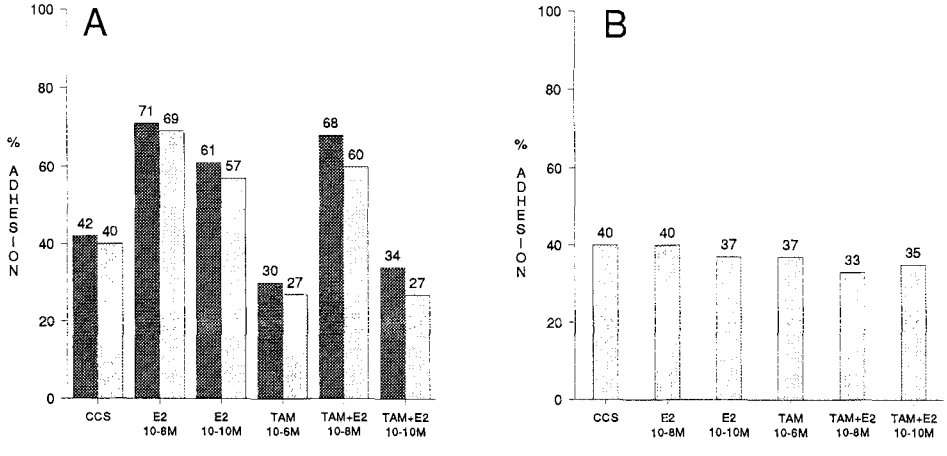

Figure 2. Influence of E_2- or Tam-induced secretory activities on ZR75-1 cell attachment. (A) The adhesion assay of cells grown in various conditions was carried out in their respective conditioned media (light shading) or in fresh medium containing 10 per cent CCS and hormones (dark shading). (B) The adhesion assay of cells grown in estrogen-deprived conditions (CCS) was carried out in the presence of various conditioned media. The numbers represent the mean values ($\pm$S.E.M.) of adhesion percentage in triplicate wells.

Discussion

In our study, the treatment with E_2 induced an enhanced ability of the ER-positive cell line ZR75-1 to adhere to various substrates. This was in contrast to the absence of effects observed for the ER-negative BT-20 cells. Previous ultrastructural investigations have shown that E_2 stimulation of MCF-7 and ZR75-1 cells induced an increase of microvilli at the cell surface which resulted in cell rounding and looser attachment to polystyrene dishes [28]. We have also observed these E_2-induced morphological changes in ZR75-1 cells (data not shown). However, the increased attachment to ECM or ECM components occurred when these cells were grown in the presence of E_2. Moreover, treatment with antiestrogens such as Tam or OH-Tam induced a reduction in the adhesion of ER-positive ZR75-1 cells, but not that of ER-negative BT-20 cells to EC, ECM, collagens I and IV, laminin and fibronectin. This effect could be reversed by a competitive concentration of estrogen (10^{-8} M E_2).

It has been reported that Tam and OH-Tam at the concentrations which were used in this study can induce cytotoxic effects [3]. In our experiments we only used the cells which still adhered to the flasks after estrogen and antiestrogen treatments. In addition, we have also noted that the viability of these cells was not modified by exposure to any of the various hormones tested, as determined by the trypan blue exclusion test, before and after the labeling procedure. Tam or OH-Tam treatment is known to induce a marked decrease in mitotic index and protein turnover [8, 25] in ER-positive cells. This effect is apparently related to a specific binding of antiestrogens to the ER and to an interference with the trophic effects of E_2. The affinity of OH-Tam for the ER was found to be 100-fold higher than that of Tam and a good correlation was observed between the relative binding affinity of these compounds for ER and their biological efficiency in preventing the growth of MCF-7 cells [7]. The greater inhibitory effects of OH-Tam on ZR75-1 cell adhesion seem to be related also to its high affinity for ER, since this metabolite was active on cell adhesive properties at a concentration 100-fold lower than that of Tam.

Although the MCF-7 cell line used in this study showed a proliferation curve dependent on E_2 and Tam concentrations in the culture medium, we observed irregular and less clear effects of estrogen and antiestrogens on MCF-7 tumor cell adhesion. Biological differences have been described among MCF-7 human breast cancer cell lines used in various laboratories [21]. Morphologically similar MCF-7 cell lines display marked differences in their estrogen and progesterone receptor content, growth rate, estrogen and antiestrogen responsiveness, cloning efficiency and tumorigenicity in athymic nude mice. Genetic instability resulting from cell passages may account for the irregular and poorly defined effects of estrogens and antiestrogens on MCF-7 cell adhesive properties.

Thus, in spite of the irregular results observed with MCF-7 cells, the increased cell adhesion of ER-positive ZR75-1 cells induced by E_2 treatment, the efficiency of OH-Tam at a lower concentration than Tam on the inhibition of ZR75-1 cell attachment, the reversal of antiestrogen effects only by a competitive concentration of E_2 and the absence of effects on ER-negative BT-20 cell line suggest that estrogens and antiestrogens control the adhesive behavior of breast tumor cells through their hormone responsive structures.

Berthois *et al.* [4] showed that phenol red, whose structure resembles that of certain non-steroid estrogens, exerted a significant estrogen-like stimulus on MCF-7 cells. They also suggested that the antiestrogens act only by a direct

antagonism of E_2 action. However, recent work has shown that the antiestrogen-induced growth inhibition could occur even in the absence of estrogens, in phenol red-free medium [29]. We also found that antiestrogens have inhibitory effects on ZR75-1 growth, even in the absence of estrogens. Modulation of ZR75-1 cell attachment by estrogens and antiestrogens was similarly observed in either the presence or absence of PR, particularly the inhibitory effect of antiestrogens, thus suggesting an intrinsic effect of Tam and OH-Tam on cell adhesion.

Conditioned medium harvested from E_2- or Tam-treated cells failed to induce any effect on adhesion of other ZR75-1 cells grown in E_2-deprived medium. The secretory activities modulated by estrogens and antiestrogens do not seem to be required to control cell adhesiveness. Consequently, the increased ability of E_2-treated cells to bind to ECM components should be the result of regulated expression of cell adhesion proteins and/or their membrane receptors. As described previously, E_2 could increase the cell surface expression of laminin receptors, since E_2 treatment increases laminin binding to MCF-7 cells and enhances their attachment to laminin-coated membranes [2]. The similar ZR75-1-treated cell adhesion pattern induced by E_2 and/or Tam, irrespective of the substrate used, suggests that E_2 may exert its effect through modulation of a wide range of cell attachment factors. On the other hand, the Tam or OH-Tam treatment which induces membrane modifications and increased cell permeability [3] may also alter the cell-surface expression or functional characteristics of cell attachment factors. These modifications and the decreased synthesis of factors involved in the adhesive process may be related to the reduced antiestrogen-induced cell attachment. Finally, our results suggest that cell adhesiveness regulation of human breast cancer cells, and consequently some aspects of their invasive behavior, are modulated by estrogens and antiestrogens, possibly through their hormone responsive structures.

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