Differential Regulation of Growth and Invasiveness of MCF-7 Breast Cancer Cells by Antiestrogens

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ABSTRACT

Estrogen increases the ability of the estrogen-dependent MCF-7 human breast cancer cell line to both proliferate and invade through an artificial basement membrane. In studying the response of MCF-7 cells to various antiestrogens, we found that 4-hydroxytamoxifen and tamoxifen inhibited cell proliferation but increased their invasiveness. In contrast, the structurally unrelated benzothiophene antiestrogens, LY117018 and LY156758, were potent antiproliferative agents which did not stimulate invasiveness. The differential effects of these antiestrogenic agents on invasion correlated with changes in production of collagenase IV, while no significant change was seen in the chemotactic activity of the cells. Invasiveness was increased by 17β-estradiol or 4-hydroxytamoxifen after a few hours of treatment and was rapidly lost when 17β-estradiol was withdrawn. Stimulation of invasiveness with 17β-estradiol was blocked by the antiestrogen, LY117018. Cells from the MDA-MB-231 line which lacks estrogen receptors were not affected by estrogen or antiestrogen in terms of proliferation or invasion. These studies indicate that the invasiveness of MCF-7 cells is regulated by antiestrogens through the estrogen receptor and may be mediated by collagenase IV activity. Antiestrogens which reduce both the proliferation and invasiveness of these cells may be interesting new candidates for clinical application.

INTRODUCTION

The MCF-7 cell line, derived from a pleural effusion of a malignant breast cancer (1), is a widely studied model for hormone-dependent human breast cancer. These cells contain functional estrogen receptors and show a pleiotropic response to estrogen (2-5). Estrogen induces MCF-7 cells to form tumors in the nude mouse (6, 7), and it stimulates the proliferation of these cells in vitro (2, 8-10). In contrast, the MDA-MB-231 cell line provides a model for human breast cancer which exhibits an estrogen-independent state and does not express estrogen receptors (11). These latter characteristics are associated with advanced disease.

An appreciation of the necessity of estrogen for the growth of some breast cancers led to the clinical use of antiestrogens, particularly tamoxifen, to suppress the growth of these tumors (12-15). However, a number of studies in model systems attribute partial estrogenic activity to tamoxifen and to its active metabolite OHT. For example, under certain conditions, tamoxifen stimulates the proliferation of estrogen-dependent human breast cancer cell lines in culture (10, 16-18) as well as in the nude mouse (7, 19, 20), and tamoxifen induces an estrogen-like morphological change in cultured MCF-7 cells (21). Agonistic activities of tamoxifen and OHT on MCF-7 cell proliferation have previously been masked by the estrogenic nature of phenol red, the pH indicator dye commonly used in cell culture medium (10, 22). Like estrogen, tamoxifen induces uterine growth in ovariectomized rats and mice (23-25), and both tamoxifen and 4-hydroxytamoxifen show partial estrogenic activity in inducing progesterone receptors in cultured MCF-7 cells (3, 26-30). May and Westley (31) have recently reported that tamoxifen and 4-hydroxytamoxifen show some ability to stimulate two mRNA species which are also estrogen inducible. Thus, a variety of estrogen-regulated functions exist which are similarly regulated by tamoxifen.

Recent studies have demonstrated that estrogen-treated MCF-7 cells are more capable of traversing a reconstituted basement membrane barrier in vitro than their estrogen-deprived counterparts (32). Such activity is thought to relate to the invasive and metastatic potential of tumor cells (33). The aim of this study was to examine the effect of tamoxifen and 4-hydroxytamoxifen, as well as the structurally distinct benzothiophene antiestrogens LY 117018 and LY 156758 (25, 34-36) on the invasiveness of breast cancer cells. The results indicate that, while tamoxifen and hydroxytamoxifen stimulate the invasiveness of estrogen-dependent MCF-7 breast cancer cells at concentrations which are growth inhibitory, benzothiophene antagonists inhibit both the invasiveness and proliferation of these cells.

MATERIALS AND METHODS

Cell Lines. MCF-7 cells and MDA-MB-231 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in T75 flasks (Costar) in IMEM (Biofluids, Rockville, MD) supplemented with 2 mM glutamine and 10% fetal bovine serum (GIBCO, New York, NY; FBS/IMEM). To deplete estrogen, the cells were passaged for at least 2 wk in IMEM supplemented with 5% calf serum (GIBCO) which had been treated sequentially with sulfatase (Sigma, St. Louis, MO) and DCC (Sigma) to remove endogenous estrogen (DCS/MEM; Ref. 2).

Estrogen and Antiestrogens. 17β-Estradiol was obtained from Sigma; tamoxifen and OHT were obtained from ICI America, Inc., Wilmington, DE; LY 117018 and LY 156758 were gifts from Eli Lilly Co., Indianapolis, IN. Stock solutions (1000x) in absolute ethanol (Midwest Solvents Co., Perkin, IL) were stored at −20°C and applied directly to the culture dish.

Estrogen and Antiestrogen Treatment. Cells were trypsinized, reseeded into tissue culture dishes (1 × 10⁶ cells/10-cm-diameter Falcon dish), and allowed to adhere overnight in a humidified incubator (37°C, 5% CO₂/95% air). The cells were treated the next day with either 17β-estradiol (10⁻⁶ M), one of the antiestrogens (10⁻⁷ M hydroxytamoxifen, 10⁻⁷ M tamoxifen, 10⁻⁷ M LY 117018, 10⁻⁷ M LY 156758), or ethanol alone (0.1% final concentration). Four days later, the cells were harvested with trypsin (GIBCO), washed twice in IMEM containing 0.1% bovine serum albumin (BSA/IMEM), counted with a Coulter Cell Counter, and tested for chemotaxis and chemoinvasion activities. In some experiments, confluent MCF-7 cells were harvested from culture with trypsin, washed twice in BSA/IMEM, counted, and treated directly in the chemoinvasion assay.

Chemoinvasion Assay. The Boyden chamber chemoinvasion assay was performed as described previously (32, 37). Matrigel, a mixture of basement membrane components (38), was kindly provided by Dr. Hynda Kleinman, NIH. Polycarbonate filters (12-μm pore, polycryl-
pyrrolidone-free, Nucleopore) were coated with matrigel (25 μg/filter) which was dried and then reconstituted at 37°C into a solid, even layer over the surface of the filter. Fibroblast-conditioned medium, obtained by incubating confluent NIH-3T3 cells for 24 h with IMEM, was used as the chemoattractant. Cells were harvested with trypsin, washed twice with BSA/IMEM, and added to the top chamber (300,000 cells/chamber). Chambers were incubated in a humidified incubator at 37°C in 5% CO₂ in air for 6, 9, or 12 h. The cells which had traversed the matrigel and attached to the lower surface of the filter were stained with Diff-Quick (American Scientific Products) and quantitated electronically with the Optomax V image analyzer.

Chemotaxis Assay. Chemotaxis assays were performed as described for the chemoinvasion studies with the single exception that the filter surfaces were coated with 5 μg of collagen IV (kindly provided by Dr. Roy Ogle, NIH) instead of the layer of matrigel. This coats the interstices of the filter but does not form a barrier over the surface. Chemotaxis assays were performed in parallel to the chemoinvasion assays using the same cells and conditioned medium. Duplicate or triplicate filters were used in each experiment. One-way analysis of variance was performed on the data from each experiment using the Stat Works Package with the Apple Macintosh II computer.

Type IV Collagenase Assay. Collagenase IV activity was measured using a modified solid phase radioimmunoassay (39). Briefly, collagen IV isolated from EHS tumor tissue (40) was iodinated by the Bolton-Hunter method, and a solution of the labeled collagen (10,000 to 20,000 cpm) was allowed to bind to microtiter plates (Removawell; Dynatech) overnight. Serial dilutions of medium from the Boyden chambers were then incubated in the microtiter plates for 24 h at 37°C and the collagenase activity was determined from the dilution within the linear range which gave half-maximal degradation of 125I-collagen IV from the solid phase in the presence of serine proteinase inhibitors.

RESULTS

Studies were carried out comparing the effects of estrogen with various antiestrogens on the proliferation, invasiveness, chemotactic responsiveness, and collagenase production of human breast cancer cells.

Hormonal Effects on Invasion and Proliferation. Fig. 1 compares the invasiveness and chemotactic activity of MCF-7 cells and MDA-MB-231 cells after culture in either complete serum-containing medium (FBS/IMEM) or estrogen-depleted medium (DCS/IMEM). In confirmation of our previous studies, the MCF-7 cells harvested from medium supplemented with complete serum (containing endogenous estrogen) were more invasive than those cultured in estrogen-depleted medium. MDA-MB-231 cells, which lack estrogen receptors, were considerably more invasive than the MCF-7 cells regardless of which medium the cells were cultured in prior to testing. No differences was noted in the treated cells' ability to show directed movement (chemotaxis) towards fibroblast-conditioned medium as a source of chemoattractants.

Since estrogen increases the invasiveness of the MCF-7 cells, it was of interest to study the response of these cells to various antiestrogens. Subconfluent cultures of MCF-7 cells grown in FBS/IMEM were treated with 17β-estradiol, an antiestrogen, or the ethanol vehicle (Fig. 2). Under these conditions, 17β-estradiol had no effect on the proliferation of the cells or on invasiveness. Treatment with tamoxifen or 4-hydroxytamoxifen markedly reduced proliferation but did not significantly increase invasiveness. The benzothiophene antiestrogens LY117018 and LY156758 also reduced proliferation but did not stimulate the invasiveness of the cells. No significant differences were seen in the chemotactic activities of these cells after the different treatments.

We further examined the effects of 17β-estradiol and these antiestrogens on the invasive, chemotactic, and proliferative activities of the MDA-MB-231 cell line, which lacks estrogen receptors (Fig. 3). In these studies, MDA-MB-231 cells were cultured in FBS/IMEM and treated for 4 days with estrogen or antiestrogen as described for MCF-7 cells in Fig. 2. As
expected, their proliferation was not affected by either 17β-estradiol or 4-hydroxytamoxifen. Furthermore, neither their invasiveness nor chemotactic responsiveness (data not shown) was altered by these treatments.

The effects of 17β-estradiol and antiestrogens were seen even more clearly with MCF-7 cells which had been cultured in estrogen-depleted medium (Fig. 4). Under these conditions, added 17β-estradiol caused a much greater stimulation of growth while the antiproliferative effects of the antiestrogens were less evident (data not shown). 17β-Estradiol and 4-hydroxytamoxifen dramatically stimulated MCF-7 cell invasiveness (Fig. 4), while the benzothiophene compounds LY117018 and LY156758 did not (not shown). The effects of 17β-estradiol (10⁻⁹ M) on both proliferation and invasion under these conditions were blocked by LY117018 at 10⁻⁷ M (data not shown). The same trends were seen in experiments performed with MCF-7 cells cultured in the absence of phenol red (10, 22) for 1 to 2 wk prior to treatment and assay (data not shown). Preliminary results from a similar experiment using the estrogen-dependent human mammary carcinoma T47D cell line showed similar effects of 17β-estradiol and OHT on proliferation, invasiveness, and chemotaxis. These data suggest that the effects of 17β-estradiol and 4-hydroxytamoxifen on the invasiveness and proliferation of MCF-7 cells are mediated via the estrogen receptor.

In some studies, the cells were pretreated with 17β-estradiol or 4-hydroxytamoxifen in culture, but the treatment was omitted from the subsequent chemoinvasion assay (Fig. 4, withdrawn group). In this case, the increased invasiveness caused by 17β-estradiol was lost during the 9-h assay period. In contrast, cells treated with 4-hydroxytamoxifen under the same conditions maintained a high level of invasiveness. Such observations suggest that the stimulation of invasiveness by 17β-estradiol involves a rapidly reversible process. Perhaps differences in the retention of 4-hydroxytamoxifen account for its longer duration of action. These effects also have a rapid onset, and a kinetic analysis showed that MCF-7 cells exposed to 4-hydroxytamoxifen in the assay chamber for as little as 9 h showed increased invasiveness (Fig. 5). Under these conditions, the maximal effect of hydroxytamoxifen was observed at 10⁻⁸ M (Fig. 6).

Effects on Collagenase IV Production and the Chemotactic Responsiveness of the Cells. To explore possible mechanisms by which estrogen and certain antiestrogens induced invasiveness, we measured the chemotactic response of the cells and their production of type IV collagenase (Table 1). These agents did not cause any significant change in the chemotactic response of the cells, suggesting that the increased invasiveness was not due to an enhanced migratory capacity. Since the only difference between the invasion and chemotactic assays was the presence of a layer of basement membrane components on top of the porous filter in the Boyden chamber, we also assayed for collagenase IV, the enzyme activity that degrades basement membranes. These studies showed that both 17β-estradiol and 4-hydroxytamoxifen significantly increased collagenase IV levels in the medium collected from the upper compartment of the Boyden chamber at the conclusion of the assay. These data indicate that an increased production of collagenase IV in cells treated with either 17β-estradiol or 4-hydroxytamoxifen could mediate their increased ability to invade through basement membrane.

**DISCUSSION**

As noted previously, 17β-estradiol enhances the ability of MCF-7 cells to invade through a reconstituted basement membrane gel as well as to proliferate (32). We have extended these results here to show that the effects of estrogen on invasiveness are rapidly achieved and also rapidly lost when estrogen is removed. In addition, the invasive responses of the MCF-7 cells to antiestrogens were striking. The antiestrogens studied are...
separable into two classes, substituted triphenylethylenes (tamoxifen and 4-hydroxytamoxifen, which have appreciable agonist activity) and the benzothiophenes (LY117018 and somewhat smaller agonist activity) and the benzothiophenes (LY117018 and the similar responsiveness seen with the T47D cell line suggest a role for the estrogen receptor in mediating these effects. Consistently, no effects of 17β-estradiol or antiestrogens were seen on the invasiveness of the MDA-MB-231 breast cancer cell line or on its proliferation. This particular cell line lacks estrogen receptors and would not be expected to respond to signals mediated in this way. The MDA-MB-231 cell line is more invasive and may exhibit a constitutively elevated expression of malignant parameters, attained during its progression. A similar situation may also exist with v-ras<sup>69</sup> transfected MCF-7 cells, which form hormone-independent tumors (41, 42) and show increased invasiveness in this assay (32). While the estrogen receptor is clearly implicated in the increased invasiveness caused by OHT, the fact that OHT effects were sometimes stronger than those of estrogen and their relative persistence suggest something more complex than partial agonism. Distinct antiestrogen binding sites are known to exist in the cytosol of these cells (43-45), and these could make some contribution.

Recent studies suggest that the degradation of the collagen IV network in the reconstituted basement membrane represents the critical step in the passage of the tumor cells (46). Laminin increases collagenase IV production by malignant tumor cells (47), and 17β-estradiol as well as v-ras<sup>69</sup> transfection appear to regulate laminin receptor expression in the breast cancer cells (32). The effects of antiestrogens on invasion, mediated via the estrogen receptor, could also involve the laminin receptor. A proteolytic cascade, including plasminogen activator, plasmin, and collagenases I and IV, is also required for the degradation of various extracellular matrix barriers by tumor cells (46, 48). Type I collagenase production by another estrogen-dependent human breast cancer cell line (ZR75-1) is not stimulated by estrogen (49), but the enzyme is activated by an estrogen-sensitive plasminogen activator (50, 51). The possibility exists that increased production of plasminogen activator, or some other protease cascade component, may result in the generation of active proteases which facilitate the increased invasiveness observed in response to estrogen. Although the production of plasminogen activator by MCF-7 cells is stimulated by estrogen (52, 53), it is not stimulated by tamoxifen or hydroxytamoxifen (54). It should also be noted that many cells secrete inhibitors of collagenases, plasminogen activator, and other proteases, and a reduction in the production of such inhibitors could also increase the degradation of matrix barriers. Taken together, the data suggest that alterations at some level of this protease cascade might account for the increased invasiveness seen in this study.

The relevance of our observations on the enhancement of the in vitro invasive activity of the breast cancer cells by tamoxifen and 4-hydroxytamoxifen to the in vivo behavior of estrogen-dependent breast cancer is unclear. Antiestrogens, including tamoxifen and 4-hydroxytamoxifen, have been extraordinarily useful in the treatment of breast cancer (55, 56). There are, however, some epiphenomena associated with tamoxifen treatment, particularly “tumor flare,” which involves a transient increase in tumor size (57). In addition, estrogenic effects of tamoxifen are thought to cause partial reduction in gonadotropin levels in postmenopausal women, changes in vaginal cytology, increased circulatory levels of sex hormone binding globulin, and decreased antithrombin II levels (58-61). It is possible that the estrogen agonism of tamoxifen and hydroxytamoxifen could increase a variety of activities of breast cancer cells in vivo and could eventually contribute to the escape of the tumor from antihormonal control.

Antiestrogens from the benzothiophene class appear to lack such activity, and for this reason they may be more effective in inhibiting both the growth and invasive spread of breast cancer. It should be noted, however, that LY 156758 compared poorly to tamoxifen in terms of its capacity to reduce tumor appearance in the nitrosoglutethimide-induced rat mammary tumor model (62), apparently due to poor pharmacokinetics. Tamoxifen is generally tumorstatic to MCF-7 tumors in the athymic mouse (63), but prolonged exposure can lead to antiestrogen-resistant tumors (19, 20), perhaps analogous to the escape from antihormonal control often seen in human treatment. Interestingly, progesterone receptor expression, another tamoxifen-sensitive, estrogen-dependent function, was found to be elevated in MCF-7 cells from these tumors, indicating that tamoxifen recognition persists in these cells for certain functions. Tamoxifen-stimulated invasiveness could thus figure prominently in the subsequent progression of these tumors, since proliferation and invasiveness are shown in the current study to be independently regulated by antiestrogens. The extension of these studies into in vivo models of human breast cancer cell invasion and metastasis, perhaps using antiestrogen-resistant clones, should help to shed light on the relevance of our findings to the clinical situation.

REFERENCES

ANTIESTrogens and MCF-7 Cell Invasiveness


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