Constitutive Overexpression of Cyclin D1 Does Not Prevent Inhibition of Hormone-responsive Human Breast Cancer Cell Growth by Antiestrogens

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Abstract

Cyclin D1 is a target for positive regulation by estrogens in growth-responsive cells, in which it mediates their mitogenic effects. Amplification and overexpression of the cyclin D1 gene (CCND1) might thus represent a genetic lesion inducing hormone-independent growth of transformed cells. Indeed, cyclin D1 overexpression has been found in up to 50% of primary breast cancers, and in about one-third of these cases, this is linked to amplification of the 11q13 chromosomal region, which also includes the CCND1 gene. These tumors are predominantly estrogen receptor-positive, and for this reason, these patients are often selected for adjuvant antiestrogen therapy. No information is available, however, as to whether cyclin D1 overexpression due to gene amplification might interfere with and reduce antiestrogen efficacy. This was investigated here by taking advantage of an experimental model that reproduces cyclin D1 overexpression resulting from increased CCND1 gene dosage in hormone-responsive human breast cancer cells. For this, MCF-7 cells stably transfected with a tet-inducible cyclin D1 expression vector were tested for their in vitro response to steroidal (ICI 182,780) and nonsteroidal (trans-4-hydroxystyramoxifen) antiestrogens under condition of low (endogenous only) or high (exogenous) cyclin D1 levels. Results show that although cyclin D1 overexpression seems to interfere with the early cell cycle effects of antiestrogens, it does not prevent their cytostatic actions, so that growth of cyclin-overexpressing MCF-7 cells is still efficiently inhibited in vitro by these drugs.

Introduction

The quest for genetic lesions that might help explain the biological properties of breast cancer cells and improve the available means for the clinical management of these diseases led to the identification of multiple chromosomal aberrations and gene mutations in mammary cancers. Among these lesions, one of the best characterized and more frequently found in primary breast cancers is the amplification of the 11q13 chromosomal region that, in the vast majority of the cases, also involves the CCND1 gene, encoding cyclin D1 (1). This gene, whose product carries out a central role in cell cycle regulation (2), is of particular interest, because it is under direct transcriptional regulation by estrogens and progesterins in breast cancer cells in culture (3–5), where its constitutive expression induces significant acceleration of cell cycle kinetics and triggers autonomous growth in vitro (6–7).

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Cyclin D1 overexpression is found in up to 50% of primary breast cancers (8–20), in which it shows a strong correlation with the histological grade of the tumor, going up to 83–87% of the cases in more malignant lesions (21–23). The vast majority of tumors with 11q13 amplification also show high cyclin D1 levels, suggesting that CCND1 gene amplification might result in constitutive and/or dysregulated expression of this cyclin in tumor cells, and are most often ER+ (11–13, 17–18, 24–27). Because the presence of steroid receptors is considered to indicate that tumor cells are still responsive to estrogens, patients with receptor-positive neoplasms are often subject to adjuvant therapy with antiestrogens. However, it is conceivable that overexpression of this estrogen-regulated gene, as a consequence of its amplification, might be a condition that renders the growth of tumor cells autonomous from the estrogenic stimulus and, as a consequence, less responsive to the growth-inhibitory effects of antiestrogens. This could thus result in failure of the antiestrogen therapy, which is indeed clinically observed in about one-third of the cases in ER+ tumors (28).

Using estrogen-responsive human breast cancer MCF-7 cells transfected with a tet-inducible human cyclin D1 cDNA expression vector (6), we tested whether overexpression of this cyclin, due to artificial gene amplification, interferes with the cell cycle and cytostatic effects of the nonsteroidal antiestrogen Tam, a mixed agonist-antagonist, or of the pure antiestrogen ICI 182,780. Results show that although MCF-7 cells expressing elevated levels of this cyclin are less readily arrested in G1 by both drugs during the first 72 h of treatment, when compared to control cells, their growth rate is still efficiently inhibited by antiestrogens. In all cases, ICI was found to be far more potent than Tam in inducing cell cycle arrest in G1, and in inhibiting cell proliferation in vitro.

These data demonstrate for the first time that ER+ breast tumor cells overexpressing cyclin D1 as a consequence of a genetic mutation that renders the CCND1 gene estrogen independent, such as, for example, gene amplification, are likely to still be responsive in vivo to the cytostatic and cytotoxic effects of antiestrogens.

Materials and Methods

Materials. ICI 182,780 and Tam were a kind gift of Dr. A. De Pasqua (Zeneca S.p.A., Milan, Italy) and D. Salin-Drouin (Laboratoires Besins Isovesco, Paris, France), respectively. Tet (chlorotetracycline-HCl) was from Life Technologies, Inc. (Milan, Italy). All other reagents were of analytical grade and were provided by major suppliers.

Cell Culture Conditions. MCF-7/c13 cells, stably transfected with a tet-responsive human cyclin D1 expression vector (6), were propagated as monolayer cultures in DMEM with phenol red, supplemented with l-glutamine (2

The abbreviations used are: ER, estrogen receptor; ICI, ICI 182,780; Tam, trans-4-hydroxystyramoxifen; tet, tetracycline; ERE, estrogen response element; ß-gal, ß-galactosidase.

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hygromycin for 15 days, and surviving clones were pooled for further use. Where indicated (+tet), 10 µg/ml let were also present in the medium. The use described here, stable transfecants were reselected in the presence of 400 µg/ml neomycin (G418) and 100 µg/ml hygromycin for 15 days, and surviving clones were pooled for further use. Estrogen-free medium (CTS) included phenol red-free DMEM and dextrancoated charcoal-treated FCS.

Cell Cycle Analysis. Flow cytometry was performed with a FACS Analyzer (Becton Dickinson, Inc.) according to standard protocols suggested by the manufacturer, as described previously (29). The analysis was performed with a CellFIT cell cycle analysis program (Version 2.0.2; Becton Dickinson, Inc.).

Preparation of Cell Extracts and Immunoblotting. Whole cell protein extracts were prepared and analyzed (20 µg of proteins) by Western blotting as described previously (3). p36D1 was immunodetected in the blots with a mouse monoclonal antibody raised against full-length human cyclin D1 (sc-6281; Santa Cruz Biotechnology, Inc.).

Growth Rate Analysis. Cells were seeded at a density of about 5–6 × 10⁴ cells/35-mm-diameter dish in medium with or without tet. After 12 h, the medium was changed to include the indicated compounds; every 48 h, the medium was replaced with fresh medium. Cells were collected at the indicated times by trypsinization, stained with trypan blue, and counted with a hemocytometer. Each experiment was performed in triplicate, and the count was repeated twice for each sample. Data represent the average of results obtained in two to three triplicate experiments (± SE).

Transient Transfections. Triplicate plates were transfected by liposome-mediated (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate) gene transfer with 5 µg of ERE-luc (from P. Chambón, Strasbourg, France) and 1 µg of pSV-nlsLacZ (30) DNAs according to the manufacturer’s instructions (Boehringer Mannheim). Six h after the addition of the liposome-DNA mixture, cells were washed twice with PBS and further incubated for 40 h in complete medium with or without 10 µg/ml tet and antiestrogens as indicated. Cells were washed and harvested by scraping in PBS before lysis in 0.25 M Tris-HCl by three cycles of rapid freeze-thawing. After clearing the cell lysates with a brief centrifugation, the protein concentration was determined with a colorimetric assay (Bio-Rad); luciferase and ß-gal activities were assayed in 100 µg of protein extract as described previously (3, 30). Luciferase activity data are reported as Relative Light Units (RLU)/µg ß-gal/100 µg of proteins.

Results and Discussion

Effects of Antiestrogens on the Transcriptional Activity of the ER in Cyclin D1-overexpressing MCF-7/c13 Cells. Expression of the tet-responsive hybrid gene encoding human cyclin D1 present in MCF-7/c13 cells is activated on removal of tet from the culture medium, resulting in an overexpression of p36D1 that reaches values up to 5-fold higher than those of the endogenous protein (6). Overexpression of the exogenous gene (Fig. 1A) in the absence of tet (−tet), already shown to be independent from the presence of serum mitogens (6), is also maintained in the absence of estrogen, because p36D1 levels were still relatively elevated, when compared to control cells, in cells cultivated in estrogen-depleted medium (CTS) for 3 days. When cells were exposed to 50 nm ICI 182,780 (+ICI) or 500 nm Tam (+Tam) for up to 72 h, endogenous p36D1 expression rapidly dropped to undetectable levels (Fig. 1A, +tet), whereas expression of the exogenous protein (−tet) remained elevated. It is worth noting that the additional protein band marked with asterisks in Fig. 1A was detectable only with the monoclonal antibody used in this study, but not with other anti-D1 monoclonal or polyclonal antibodies (data not shown), and thus represents a nonspecific protein species unrelated to p36D1.

The ER complex is a transcription enhancer factor that binds to cis-acting DNA elements (EREs) located in estrogen target genes and thereby modulates the activity of the corresponding gene promoters (31). Antiestrogens inhibit the transcriptional effects of ER by interfering at multiple steps in the cascade that lead to receptor activation by its cognate hormones, including dimerization, DNA binding, and interaction with transcriptional coregulators (32). To determine whether cyclin D1 overexpression interferes with antiestrogen inhibition of ER-mediated transcription under the conditions selected for this study, MCF-7/c13 cells overexpressing (−tet) or not (+tet) exogenous cyclin D1 were transiently transfected with an ER-responsive reporter gene comprising a perfectly palindromic ERE sequence cloned upstream of a rabbit ß-globin promoter-firefly luciferase reporter gene (ERE-luc). Luciferase expression in the cells was then measured in the absence (0) or after a 40-h exposure to various concentrations of antiestrogens, all within or above the expected affinity of ER for each of these molecules. Results, reported in Fig. 1B, show that although cyclin D1 overexpression induces a significant increase of reporter gene expression per se (compare 0 in +tet and −tet conditions), profoundly reflecting activation of the ER complex by this cyclin as reported previously (33), both antiestrogens can significantly reduce transcription of this ER-responsive hybrid gene under all conditions tested, with ICI being more effective than Tam. Because
no significant changes in ER levels could be detected during these few
hours of treatment (data not shown), this result indicates that antiestrogens are capable of inhibiting the transcriptional activity of ER even in the presence of high levels of cyclin D1. A similar result was obtained by Neuman et al. (34) that showed how stimulation of the transcriptional activity of the ER by ectopic expression of cyclin D1 is inhibited by both ICI 182,780 and Tam, in apparent contradiction with results reported earlier by Zwijsen et al. (33). It is worth mentioning, however, that in the presence of D1 overexpression (−tet cells), ERE-luc activity in Tam-treated cells was still relatively high, so that luciferase activity under these conditions was about 50% of that detectable in the absence of antiestrogens in cells with normal cyclin D1 levels (0−tet). This is comparable to what was observed by Zwijsen et al. (33) in T47D cells, with the exception that in the presence of very high intracellular levels of p36D1, such as those achieved in that study after transient transfection of a D1 cDNA expression vector, ER was able to activate transcription of ERE-containing reporter genes even in the complete absence of hormone, and this was Tam-resistant.

**Influence of Cyclin D1 Overexpression on the Cell Cycle Effects of Antiestrogens in MCF-7/c13 Cells.** Estrogens show dramatic effects on cell cycle kinetics of MCF-7 and other hormone growth-responsive cells, in which they induce recruitment of quiescent cells in cycle and accelerate cell cycle kinetics in cycling cells, mostly by shortening G1 transition time and by facilitating cell entry into S phase (35). These effects of estrogens are all inhibited by estrogen antagonists, in particular, pure antiestrogens such as ICI 182,780.4 A correlation between the activity of ER on gene transcription and its ability to stimulate cell proliferation has been postulated and is sustained by several lines of evidence (3, 29, 35), although at present, no univocal demonstration of a direct link between these two actions of ER is available. Because a substantial cytostatic action is one of the expected prerequisites for the efficacy of estrogen antagonists against hormone-dependent breast cancers, lack of cell cycle effects of antiestrogens in cells overexpressing cyclin D1 would indicate that genetic lesions inducing constitutive expression of this protein should be considered as a potential mechanism for resistance of ER+ breast cancer cells to adjuvant antiestrogen therapies. This question was addressed using MCF-7/c13 cells as follows: cells were maintained in complete medium with or without tet to reproduce conditions of basal or high expression of p36D1 in an identical cellular background; and they were then exposed to increasing concentrations of antiestrogens for various times (up to 72 h) before analysis of cell cycle parameters by flow cytometry. As shown in Fig. 2A, cyclin D1 overexpression was accompanied by reduction of G0 + G1 (61.1% in +tet versus 47.9% in −tet) and G2 + M-phase (15.5 versus 11.6%) cells in the cultures in favor of S-phase cells that increased from 23.4 (in +tet) to 40.5% (in −tet). Because it has been shown that cyclin D1 overexpression does not affect the length of S phase and G2-M phases in MCF-7/c13 cells (6), these data indicate that the increase in S-phase cells observed here results from an increase of the number of cycling cells in the cultures, due to a reduction of G0-arrested cells and/or a shortening of the G1 transition time. The effects of antiestrogens on cell cycle parameters differed substantially between the two compounds tested. In +tet cultures, ICI 182,780 very rapidly induced accumulation of G0 + G1 cells even at the lowest concentration tested (5 nM), so that 87.4−90% of cells accumulated in these cell cycle compartments after 24−48 h. On the contrary, in −tet, this antiestrogen was much less efficient in inducing the redistribution of cells in cycle (only 70.3−75.8% of the cells were in G0 + G1 after 24−48 h treatment with up to 500 nM ICI). Cells overexpressing cyclin D1 thus seem to be less readily responsive to the cell cycle effects of the ICI compound, even when the data are analyzed after normalization for the initial differences in cell cycle phase distribution due to the elevated intracellular levels of cyclin (Fig. 2B, +ICI). It is worth noting, however, that because G0 + G1 cells increased significantly in the cultures between 48 and 72 h of treatment with ICI under both culture conditions (+tet and −tet), −tet cells are indeed not fully resistant to the cell cycle effects of this antiestrogen. Because ICI 182,780 induces cell cycle arrest in G0 or early G1 in MCF-7 cells (5, 36), and it has been shown that cyclin D1 overexpression inhibits cell cycle exit in MCF-7/c13 cells (6), slower G0 + G1 cell accumulation in the −tet cultures probably indicates that in the presence of cyclin D1, overexpression ICI-induced cell cycle exit occurs less efficiently. Indeed, Wilcken et al. (37) reported that expression of cyclin D1 from an inducible transfected vector reverses the growth-inhibitory effects of steroidal and nonsteroidal antiestrogens on hormone-responsive human breast cancer cells in culture when analyzed during the first 24−48 h of cyclin overexpression.

Contrary to that observed for ICI 182,780, Tam is much less effective in interfering with cell cycle kinetics in MCF-7/c13 cells (Fig. 3A). In +tet, only 79.3% of the cells after 48 h or 83.5% of the cells after 72 h can be found in G0 + G1, in accordance with the lower efficiency of this molecule as a growth inhibitor for MCF-7 cells, which is probably related to its mixed agonist-antagonist action in breast cancer cells (32). This was even less marked in −tet cells (67−67.7% of G0 + G1 cells after 48 h and 79.6−80.3% of G0 + G1 cells after 72 h). Little differences could be observed in cell cycle phase redistribution efficiency between control (+tet) and D1-overexpressing (−tet) cells exposed to Tam (+Tam in Fig. 2B), with the latter being slightly more responsive to cell cycle inhibition by this antiestrogen. This was observed in multiple experiments and at different time points and antiestrogen concentrations and might be related to the mechanism of action of nonsteroidal antiestrogens, which in estrogen-responsive human breast cancer cells also show pleiotropic inhibitory effects on ER-independent pathways (32), which thus could also include cyclin D1-dependent regulatory cascades.

**Growth of MCF-7/c13 Cells Overexpressing Cyclin D1 Is Inhibited by Antiestrogens.** MCF-7 cells respond to the mitogenic stimulus exerted by estrogens both in vitro and in vivo. This is also true for clone 3 cells, because their growth rate is significantly reduced in steroid-depleted medium (Fig. 3A, CTS), and this can be reversed by stimulation with 17β-estradiol.5 Interestingly, cyclin D1 overexpression reduced this hormone requirement for sustained cell growth, because in CTS medium, cells seem to grow more efficiently in the absence of tet than in the presence of tet (Fig. 3A). Because the CCND1 gene is a target for transcriptional regulation by estrogens in breast cancer cells (3), and cyclin D1 has been shown to mediate the mitogenic action of estrogens in these cells (3, 5, 36), growth of MCF-7/c13 cells in estrogen-depleted medium could be related to a condition of hormone independence due to constitutive expression of the product of a gene located downstream of ER in the estrogen-activated mitogenic cascade. However, this cyclin can induce also ER activation in the absence of hormone (33−34), and for this reason, increased proliferation of −tet cells in CTS medium could be mediated by constitutive ER activity. In any case, this result indicates that D1-overexpressing

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Fig. 2. Influence of cyclin D1 overexpression on short-term antiestrogen effects on cell cycle kinetics of MCF-7/c13 cells. A, exponentially growing cells were exposed to the indicated concentrations of ICI 182,780 (ICI) or Tam for up to 72 h; at each time point, cells from duplicate plates were collected and analyzed by flow cytometry. The data represent the average of results obtained in two independent experiments; bars indicate the range of the results obtained. B, direct comparison of antiestrogens effects on S-phase cell concentration in cultures maintained in the presence (● and □, control) or absence (○ and △, D1 overexpression) of tet. The average of results obtained in three experiments performed in duplicate is reported ± SE (bars).

MCF-7 cells, in analogy to their loss of serum requirement (6), lost part of their hormone requirement for growth. Both antiestrogens inhibited hormone-stimulated and -independent growth of MCF-7/c13 cells either in the presence or in the absence of tet (Fig. 3A), with Tam (500 nM) being less effective than ICI 182,780 (50 nM). However, as for its effects on cell cycle kinetics (Fig. 2), the nonsteroidal antiestrogen seemed to be relatively more efficient in inhibiting the growth of D1-overexpressing cells than that of control cells (see Fig. 3B, +Tam), whereas the steroidal compound was equally effective under both conditions tested (Fig. 3B, +ICI).
cause tamoxifen and its derivatives can act on multiple molecular targets in breast cancer cells, it is possible that these pleiotropic effects might also include direct interference with cyclin D1 actions in the cell.

The growth-inhibitory effects of antiestrogens toward D1-overexpressing MCF-7 cells could be due to a combination of one of the following actions: (a) an interference with cyclin D1-independent intracellular pathways, because it has been shown that ICI interferes with the cell cycle effects of estrogens in breast cancer cells by acting at multiple regulatory points throughout G1 and in S phase;4 (b) a reduction in ER levels in the cell after prolonged exposure to antiestrogens, as reported in other cases (32), assuming, however, that the presence of ER is required for full growth of the cells under these conditions; and (c) cytotoxic effects that have been documented for these drugs toward human breast cancer cells (32) and could also be clearly detected in clone 3 cells, in both +tet and in −tet conditions, after prolonged (>9 days) exposure to antiestrogens and were more evident in estrogen-depleted medium (data not shown). A molecular analysis of the mechanisms that lead to growth inhibition of D1-overexpressing ER+ breast cancer cells by antiestrogens, in particular, identification of cytostatic and/or apoptotic pathways activated by these compounds that might interfere with cyclin actions, as well as a better knowledge of the ER-dependent mitogenic pathways that they inhibit, will be necessary to discriminate between these different possibilities.

In conclusion, the results of this study, showing that the growth of ER-positive hormone growth-responsive human breast cancer cells overexpressing cyclin D1 is still inhibited by antiestrogens in vitro, indicate the possibility that ER+ primary breast neoplasms with the 11q13 amplification coupled to p36D1 overexpression are likely to be responsive to antiestrogens in vivo. This hypothesis now needs to be verified clinically, where it will also be important to correlate the clinical response to antiestrogens to the degree of cyclin D1 overexpression in tumors to further confirm the usefulness of these compounds for treatment of a large spectrum of ER+ neoplasms.

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