Cancer Chemother Pharmacol (2001) 48: 305–311
DOI 10.1007/s002800100349

ORIGINAL ARTICLE

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Changes in the expression and binding properties of the estrogen receptor in MCF-7 breast cancer cells during growth inhibition by tamoxifen and cisplatin

Received: 21 November 2000 / Accepted: 18 May 2001 / Published online: 5 July 2001

Abstract Most mammary carcinomas contain estrogen receptors (ER), which are an important factor in diagnosis and prognosis, and in deciding on the type of therapy. ER-positive tumors are most commonly treated with the antiestrogen tamoxifen or with a combination of chemotherapeutic drugs. An important aspect for further treatment and anticipating possible side effects is the fate of the ER during the course of therapy. To study the effect of drug-induced growth inhibition on ER expression and binding properties, human breast cancer MCF-7 cells were treated with tamoxifen and cisplatin, and also estradiol (E_2) for 5 days. Following this incubation, intact cells were incubated with [^{3}H]E_2 to determine the dissociation constant (K_D) and maximal number of binding sites (B_{max}) of the ER. The amount of ER protein per cell was quantified using anti-ER antibodies. For analysis of ER mRNA, total cellular RNA was subjected to Northern blotting. The 5-day treatment with E_2 reduced B_{max} and the amount of ER protein by about 70%, while the cellular level of ER mRNA was reduced by 40%. Treatment with E_2 did not affect the subsequent growth inhibitory response to tamoxifen or cisplatin. In contrast, tamoxifen reduced the capacity for E_2 binding; it caused about a 30-fold increase in the K_D value. At the same time, B_{max} and ER protein content were increased (about 3.5- and 2-fold, respectively), but the cellular level of ER mRNA was again reduced by 40%. The growth of tamoxifen-treated cells remained sensitive to subsequent treatment with estradiol, tamoxifen or cisplatin. Treatment of MCF-7 cells with cisplatin likewise reduced E_2 binding due to a 20-fold increase in K_D value. In this case, both B_{max} and the amount of ER protein were decreased when calculated per milligram of protein, but were increased on a cellular basis due to an increase in cell size. The ER mRNA content was not altered in cisplatin-treated cells. Growth of these cells also remained sensitive to tamoxifen and cisplatin. In conclusion, drug-induced changes in ER expression and binding capacity do not necessarily indicate a loss of sensitivity of breast cancer cells to a subsequent chemotherapeutic treatment.

Keywords Estrogen receptor · Breast cancer cells · Growth inhibition · Tamoxifen · Cisplatin

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Introduction

Estrogen receptors (ER) can be detected in approximately 50–70% of mammary tumors, depending on the detection method and hormonal status [17], and they are an important factor in diagnosis and prognosis, and in deciding on the type of treatment [7, 18]. The level of ER is usually determined by the binding of a single saturating dose or of various concentrations of tritium-labelled estradiol (E_2) to cytosols from tumor tissue [6]. The binding capacity will depend not only on the number of ER, but also on the dissociation constant (K_D) and the estrogen concentration in the tissue. The immunocytochemical assay with ER antibodies avoids this problem. However, neither the measurement of E_2 binding alone nor the immunochemical determination of the ER protein is sufficient to predict the binding capacity for (anti)estrogenic ligands and the functionality of the ER during the course of antitumor treatment.

The level of estrogen binding is affected by multiple parameters including exposure time. For example, a brief exposure of cells to E_2 as short as 1 min is sufficient to reduce the E_2 binding capacity of the ER by almost

20% [19]. This is due to the activation of a small fraction of the ER which bind E_2 with high affinity. A loss of receptor protein, indicative of ER degradation, begins after about 45 min [10, 19]. In contrast, a brief exposure of cells to the antiestrogen tamoxifen reduces E_2 binding by competitive inhibition at the binding site, and there is no reduction in ER protein even after 24 h. The increase in the dissociation constant for E_2 corresponds with clinical observations that ER binding is reduced after hormonal therapy [27].

The majority of ER-positive tumors are initially treated with the antiestrogen tamoxifen. Not all of these tumors are responsive, so that these and ER-negative tumors will be treated with a combination of chemotherapeutic drugs. However, clinical results on ER binding following chemotherapy show great variations [11, 27], and in general the effects of chemotherapeutic drugs on ER binding have not received much experimental attention. Yet, observing changes in the binding capacity and functionality of the ER during the course of treatment could provide an indication of the differentiation state of the tumor tissue and would be an important auxiliary parameter contributing to the decision on further therapeutic regimens. In studies in which breast cancer cells were incubated in vitro with drugs such as methotrexate, melphalan, 5-fluorouracil and vincristine for up to 24 h, E_2 binding has been found to be reduced [5, 29]. However, the experimental protocols did not reflect the situation in which cells have had time to respond to the treatment with growth inhibition. It is evident that growth inhibition, whether the result of cell cycle arrest or the induction of programmed cell death, is accompanied by changes in the biochemical regulation of the cell.

It was thus the aim of this study to investigate how growth inhibition after a 5-day incubation with tamoxifen and cisplatin affects ER expression and ER binding properties. We also sought to determine whether a change in the ER binding capacity in turn has any effect on the growth response towards a subsequent treatment with an antitumor drug using the ER-positive and estrogen-sensitive mammary tumor cell line MCF-7 in culture as a model system. Even though ER expression and binding were markedly changed by drug treatment, this did not affect the cells' sensitivity to subsequent treatment with antiestrogen or cisplatin.

Materials and methods

Cell culture

MCF-7 cells were maintained in Richter's Improved MEM or in Dulbecco's MEM/F12 supplemented with 10% fetal calf serum and without phenol red and antibiotics. For experiments, cells were plated at a titer of 0.2×10^5/ml and incubated with test compounds for 5 days or the times indicated. Stock solutions of E_2 and tamoxifen of 1 m*M* each were prepared in ethanol and diluted in phosphate-buffered saline (PBS) or a 150 m*M* NaCl solution. A 1 m*M* stock solution of cisplatin was prepared and diluted in 150 m*M* NaCl. For a first incubation period, drugs were added directly to the culture 1 day after plating. When replating pretreated cells for a second incubation, drugs were added several hours later on the same day.

Determination of cell number

The cells were allowed to swell in a hypotonic buffer (20 m*M* HEPES, pH 7.4, 1 m*M* $MgCl_2$, 0.5 m*M* $CaCl_2$) on ice for 15 min before the addition of a lysis solution (5% benzalkonium chloride in 10% acetic acid) as described previously [3, 20].

Whole-cell [^{3}H]E_2 binding assay

After the 5-day incubation with the indicated test compounds, cells were washed twice with PBS. [^{3}H]E_2 was added to the cultures at a concentration of 0.4 n*M* (specific activity 3.3 TBq/mmol) alone or with increasing amounts of unlabelled E_2, and incubated at 37°C for 30 min as described previously [19].

Determination of ER protein with antibodies

An enzyme-linked immunoassay kit (Abbott) with two monoclonal antibodies (D547 and H222) directed against two different epitopes on the ER [9] was used according to the instructions of the supplier and as described previously [19]. The procedures were applied to unfractionated cell lysates prepared with hypotonic buffer (as above) containing 5 m*M* MoO_4 and 1 m*M* monothioglycerol.

Northern blot analysis of ER mRNA

Total RNA was isolated from MCF-7 cells following the 5-day treatment. The cells were collected using a rubber policeman and carefully suspended for taking aliquots to determine cell number and protein content. RNA was isolated with using an RNeasy Midi Kit (Qiagen) according to the protocol for animal cells grown in a monolayer. The basic procedure included cell lysis with guanidine isothiocyanate, addition of ethanol and binding of the RNA to a silica-based membrane, from which RNA was eluted with water. The amount of extracted RNA was determined by measuring absorbance at 260 nm.

For RNA separation, 20 μg of total RNA was loaded per lane and subjected to formaldehyde agarose gel electrophoresis. RNA was blotted to Nytran membranes [4]. The ER cDNA-containing plasmid HEG0 [23] (kindly provided by Prof. Dr. E Holler, University of Regensburg) was digested by EcoRI to obtain a 1.8-kb cDNA probe specific for the ER. The fragments were separated by agarose gel electrophoresis and purified with Qiaex spin columns (Qiagen). The probes were ^{32}P-labelled by a random primer DNA labelling system (GIBCO/BRL) and subsequently used for hybridization.

Hybridization was performed at 42°C in a solution containing 50% (v/v) formamide, 5×SSPE, 5× Denhardt's solution, 0.1% SDS, and 0.1 mg/ml herring sperm DNA for about 16 h (20×SSPE comprises 3.6 *M* NaCl, 200 m*M* sodium phosphate, pH 7.7, 20 m*M* Na_2EDTA; 50× Denhardt's solution comprises 1% w/v bovine serum albumin, 1% w/v Ficoll, 1% w/v polyvinylpyrrolidone). The final wash was performed at 60°C in a solution containing 0.5×SSC, 2 m*M* Na_2EDTA and 0.2% SDS (20×SSC comprises 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0).

Photographic images of the ethidium bromide-stained gels and autoradiograms were scanned and the relative intensities of the bands were quantified with Image Quant software. The relative intensities of the 28S and 18S rRNA bands were used as loading controls.

Results

Growth inhibition and binding of $[^3H]E_2$

To test for $[^3H]E_2$ binding capacity upon growth inhibition, MCF-7 cells were incubated for 5 days with the antitumor compounds tamoxifen or cisplatin, or estradiol. As shown in Fig. 1A, the fraction of surviving cells in the monolayer was reduced to about 20% with 1 μM tamoxifen and to 10% with 1 μM cisplatin. Growth inhibition of cisplatin-treated cells was accompanied by an increase in protein content up to twofold (Fig. 1B). In parallel, cells were analyzed for their capacity to bind a physiological concentration of $[^3H]E_2$. The amount of E_2 bound was calculated per cell and is also presented as a function of the cellular protein content, thereby accounting for changes in cell size (Fig. 2A and B, respectively). A 5-day incubation of the cells with E_2 markedly reduced their subsequent binding capacity for $[^3H]E_2$, a well documented effect. Similarly, cells which had been treated with tamoxifen bound $[^3H]E_2$ much less efficiently than untreated cells. This effect paralleled the reduction in cell number. When cells had been treated with different concentrations of cisplatin, which showed no affinity for the ER in a $[^3H]E_2$ competition assay (data not shown), the binding capacity for $[^3H]E_2$ per cell was increased by 30% (Fig. 1A). However, when calculated as a function of the cellular protein content, $[^3H]E_2$ binding was reduced with increasing concentrations of cisplatin (Fig. 2B). This is explained by the fact that cisplatin increased cell size concomitantly with growth inhibition (Fig. 1B). Taken together, these results show that the level of E_2 binding can be altered by growth inhibition evoked not only by tamoxifen, but also by a drug without binding affinity for the ER, namely cisplatin.

ER binding parameters

To analyze the binding parameters responsible for the changes in $[^3H]E_2$ binding, the maximal number of binding sites (B_{max}) and the K_D value were calculated from a Scatchard plot. In the control experiment with cells grown in medium supplemented with 1 nM E_2, the reduction in E_2 binding was mainly the result of a diminished number of binding sites with only a small increase in the K_D value (Fig. 3). In contrast, following a 5-day treatment of the cells with 1 μM tamoxifen, there

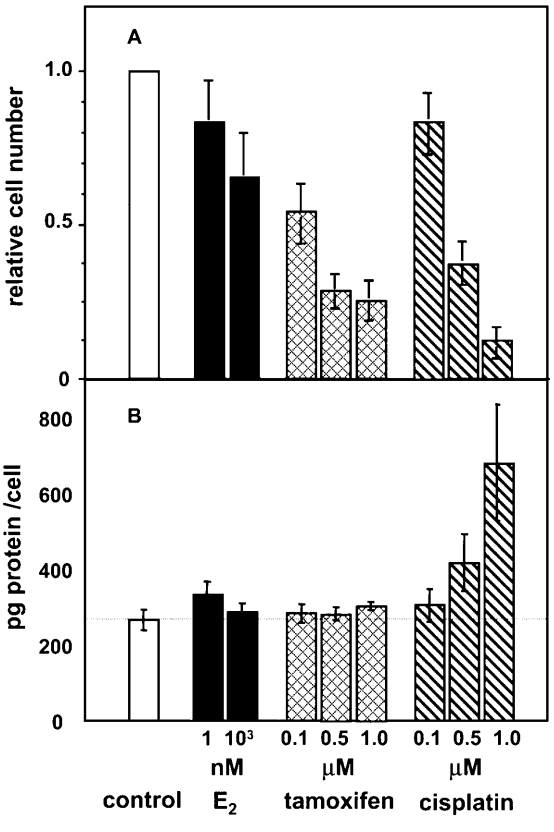

Fig. 1A, B Relative growth inhibition (**A**) and protein content (**B**) of MCF-7 cells incubated for 5 days with E_2, tamoxifen or cisplatin at the indicated concentrations. Results show averages and standard deviations from six experiments

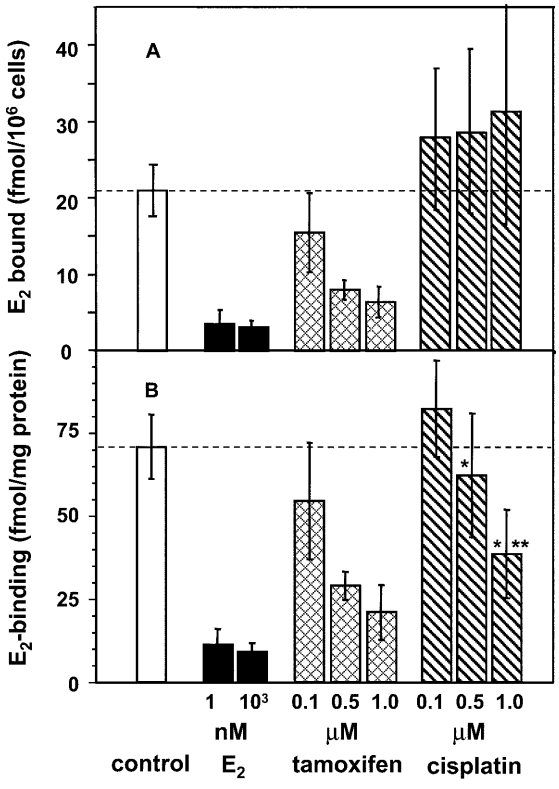

Fig. 2A, B Specific binding of $[^3H]E_2$ in MCF-7 cells after a 5-day incubation with the indicated compounds. Test compounds were removed before the subsequent incubation with 0.4 nM $[^3H]E_2$ alone or with 1 μM unlabelled E_2. Specific binding was calculated as a function of cell number (**A**) and of cellular protein content as given in Fig. 1B (**B**). Results are from the same experiments as Fig. 1. $^*P < 0.001$ vs untreated cells, $^{**}P < 0.01$ vs cells treated with 0.5 μM cisplatin; paired t-test

was a three- to fourfold increase in the calculated B_{max}. However, the K_D value was also markedly increased by almost 30-fold, explaining the reduction in the amount of E_2 bound.

Even though there was no evidence for cisplatin directly competing with [^{3}H]E_2 at the ligand binding site, ER binding properties were nevertheless changed when cells had been exposed for 5 days to a growth-inhibitory concentration of cisplatin. This was manifested by about a twofold increase in B_{max} per cell (Fig. 3A). More pronounced was an approximately 20-fold increase in the K_D value (Fig. 3B), which explains the reduced binding of [^{3}H]E_2. This demonstrates that a growth-inhibitory drug which does not compete for ER binding in untreated cells can evoke changes in the binding parameters of the ER during long-term interaction with cells.

ER protein level

To test whether the number of calculated binding sites reflected the amount of ER protein, unfractionated lysates were prepared from MCF-7 cells following a 5-day incubation with E_2, tamoxifen or cisplatin. Lysates were subjected to two monoclonal antibodies directed to different sites on the human ER, the first close to the DNA-binding domain, the second near the hormone-binding domain [9]. Quantification of receptor-bound antibodies (Fig. 3A) showed that the level of ER protein in untreated cells approximated B_{max}. However, incubating cells with 1 n*M* E_2 reduced the levels of both ER protein and B_{max}, as was expected [10, 12, 16]. In contrast, in tamoxifen-treated cells there was a marked rise in the ER protein level by about twofold compared to untreated cells, confirming the results reported by others [15]. However, the protein level was always found to be approximately 30–50% lower than B_{max}. Following incubation with cisplatin, the amount of ER protein was only slightly higher than in untreated control cells, but again $B_{max}$was found to be about 30% higher.

Expression of ER mRNA

Does the change in ER protein reflect changes in ER gene expression? The average amount of total RNA isolated per MCF-7 cell depended on the drug treatment (Table 1): it was reduced by almost 50% in tamoxifen-treated cells, while incubation with E_2 or cisplatin produced no or little change. For each sample, Northern blots with the ER cDNA generally showed at least two detectable RNA bands (Fig. 4), as has also been reported by others [1]. RNA from tamoxifen-treated cells

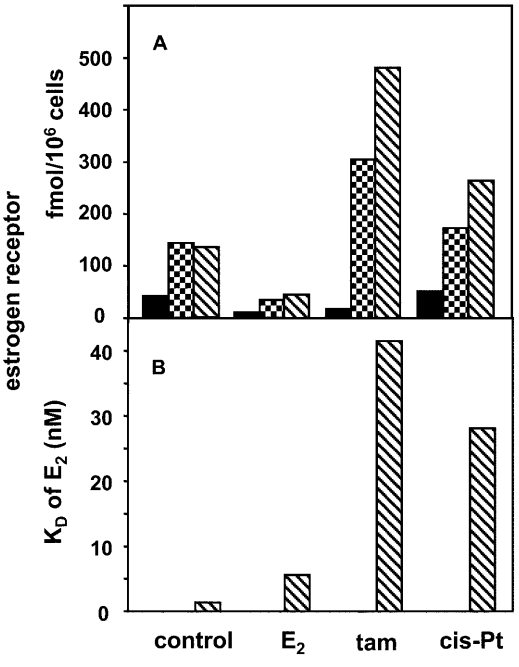

Fig. 3A, B E_2 binding parameters and ER protein content in MCF-7 cells after a 5-day incubation with E_2 (1 n*M*), tamoxifen (1 μ*M*) or cisplatin (1 μ*M*). **A** Comparison of specific E_2 binding obtained upon incubating MCF-7 cells with 1 n*M* [^{3}H]E_2 and 1 μ*M* unlabelled E_2 (*filled bars*), the number ER proteins determined with ER antibodies (*checkered bars*) and the maximal number of binding sites (B_{max}) calculated by Scatchard analysis (*hatched bars*). **B** Dissociation constants calculated from Scatchard analysis. Data shown are from one of two similar immunoassays and of at least three binding assays

Table 1 Changes in the amount of cellular RNA of MCF-7 cells following a 5-day incubation with 1 nM E_2, 1 μ*M* tamoxifen and 2 μ*M* cisplatin. Data are from the three experiments used for the Northern blot analyses shown in Fig. 4

	RNA (pg/cell)
Control	21.33 ± 2.22
E_2	24.00 ± 2.00
Tamoxifen	12.47 ± 0.98
Cisplatin	21.67 ± 0.44

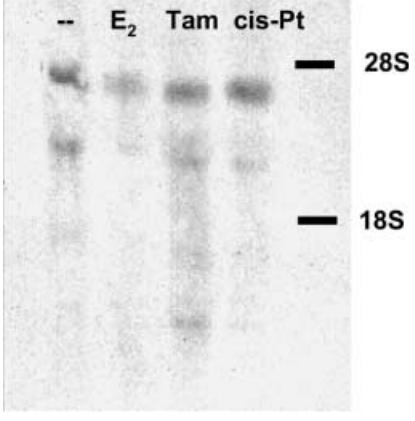

Fig. 4 Northern blot analysis with radioactively labelled ER cDNA of RNA from MCF-7 cells after a 5-day incubation with E_2 (1 n*M*), tamoxifen (1 μ*M*) or cisplatin (1 μ*M*). The migration of 28S and 18S rRNA from the same gel is indicated

showed a third, weaker band of markedly smaller size. The observed quantitative differences between the samples pertained equally to both upper bands in a lane, and these together were used for quantification of ER mRNA by intensity scanning. As expected, E_2 treatment reduced the relative amount of ER mRNA per total RNA by almost 50% (Fig. 5A). This reduction was also observed on a cellular basis (Fig. 5B), and thus corresponded to the reduced level of ER protein (Fig. 3A). In tamoxifen-treated cells the relative amount of ER mRNA per total RNA was at the level of untreated cells or only slightly increased (Fig. 5A), as also reported by others [24]. However, when the relative amount of ER mRNA was calculated per cell, it was actually reduced to a level similar to that of E_2-treated cells. Therefore, neither evaluation corresponded to the marked increase in ER protein in tamoxifen-treated cells. Cisplatin, in contrast, did not alter the ER mRNA level either in relation to the total RNA or on a cellular basis, while the cellular content of ER protein was slightly increased. Taken together, these results demonstrate that changes (or the lack of change) in the level of ER mRNA need not correspond with changes in the amount of ER protein.

Cell growth upon subsequent drug treatment

A key question was how cells which had been treated for 5 days with a drug or hormone would grow when subsequently exposed to the same or another drug. MCF-7 cells were incubated with tamoxifen or cisplatin for 5 days and, after replating, subjected to treatment with the same or a different compound (Fig. 6A–D). To test whether a reduced ER level would have any influence on the sensitivity towards tamoxifen or cisplatin treatment, cells were also first incubated with E_2. Under normal cell culture conditions with sufficient steroids in the FCS-supplemented medium, E_2 had no effect on the subsequent proliferation rate of cells, which continued to grow at the same rate without further E_2 addition (Fig. 6A). E_2 preincubation also had only a marginal effect on the growth-inhibitory activities of the two drugs, i.e. tamoxifen was slightly less, but cisplatin was slightly more inhibitory (Fig. 6B). The estrogen-induced reduction in the level of ER, therefore, had no marked influence on growth behavior.

The growth of MCF-7 cells preincubated with tamoxifen remained at least transiently inhibited after replating without a drug (Fig. 6C). The addition of E_2,

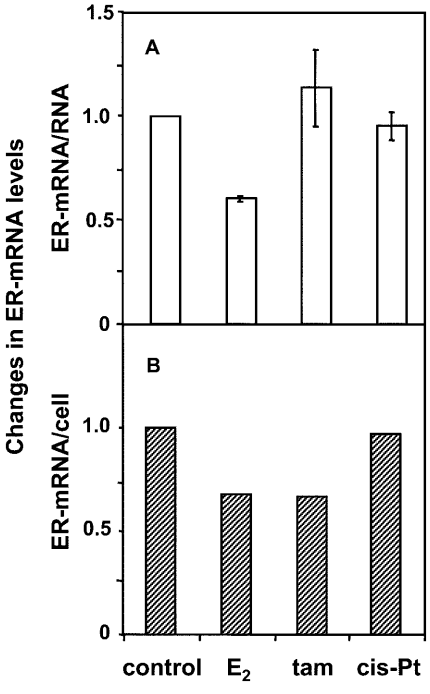

Fig. 5A, B Analysis of ER mRNA from MCF-7 cells after a 5-day incubation with E_2 (1 n*M*), tamoxifen (1 μ*M*) or cisplatin (1 μ*M*). **A** Relative amount of ER mRNA per analyzed amount of total RNA (20 μg). **B** Relative amounts of ER mRNA calculated with reference to the amount of RNA per cell as given in Table 1. The data presented are the averages and ranges from three experiments. The densities of the two upper bands were scanned and pooled, and the values from untreated cells set as 1.0. The scanned densities of ethidium bromide-labelled 28S and 18S RNA in each sample were found to be the same within a 3% error range

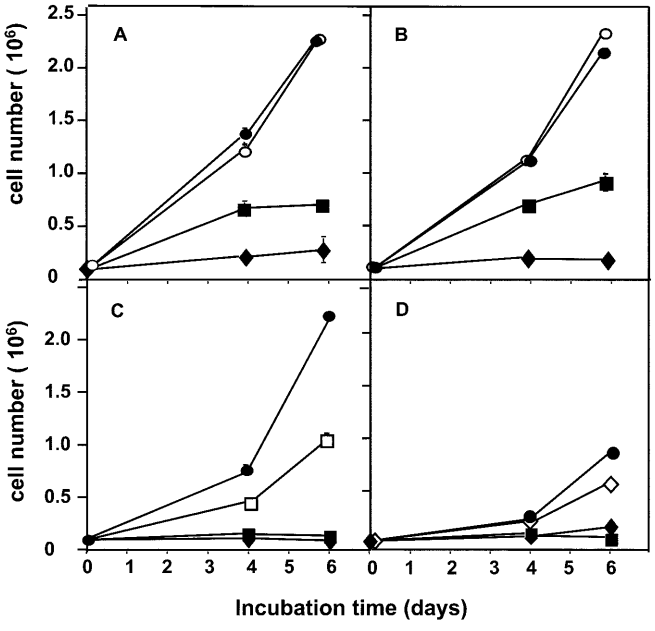

Fig. 6A–D Growth kinetics of MCF-7 cells after a 5-day preincubation with E_2, tamoxifen or cisplatin. Pretreated cells were trypsinized, plated in 48-well plates at 0.1×10^5 cells/well in 0.5 ml culture medium, and incubated a second time without or with the same compounds as during the first incubation. Cell numbers were determined at the indicated times. **A** No preincubation (control). Preincubations were with 1 n*M* E_2 (**B**), 1 μ*M* tamoxifen (**C**) or 1 μ*M* cisplatin (**D**) (*open symbols* no further additions, *closed symbols* addition of test compounds; *open* and *closed circle*, 1 n*M* E_2; *open* and *closed square*, 1 μ*M* tamoxifen; *open* and *closed diamond*, 1 μ*M* cisplatin)

however, first gradually and after 4 days increasingly reversed the inhibitory effect of tamoxifen, even though E_2 binding had been largely curtailed (Fig. 2). On the other hand, the subsequent incubation of tamoxifen-treated cells with either tamoxifen or cisplatin maintained the growth-arrested state for at least the 6-day incubation. Neither the changes in the ER binding properties nor the increase in the ER levels induced by tamoxifen impaired the growth-inhibitory effects of these two drugs.

After replating cisplatin-treated MCF-7 cells, the growth rate remained suppressed for at least 4 days when no drug was added (Fig. 6D). Again, tamoxifen as well as cisplatin maintained the cells in the growth-arrested state. With the addition of E_2, however, cells appeared to recover more quickly from the cisplatin treatment (Fig. 3). Therefore, in spite of impaired E_2 binding, the cells were still sensitive to the growth-promoting effect of E_2 after treatment with cisplatin.

Discussion

The goal of this investigation was to analyze ER binding parameters in conditions under which the cells had had time to respond to the drug treatment with growth inhibition, which is obviously accompanied by numerous changes in the biochemistry of the cell. A 5-day exposure of estrogen-sensitive breast cancer cells to tamoxifen as well as to the DNA-binding drug cisplatin dramatically altered the binding properties of cellular ER in a manner different from the reported short-term exposures.

In previous studies, MCF-7 cells were incubated with methotrexate, 5-fluorouracil and vincristine for up to 6 h [29] or with Adriamycin, melphalan and 5-fluorouracil for 24 h [5], i.e. for times not yet resulting in growth inhibition. The decreased [^{3}H]E_2 binding has been described as the result of a reduced number of binding sites without effects on the K_D [5, 29]. In contrast to these effects, a 5-day incubation with cisplatin, which led to a marked decrease in cell numbers, reduced E_2 binding mainly by increasing the K_D value, while only marginally reducing B_{max} or ER protein levels (with reference to total protein). There is no evidence for cisplatin competing directly with estradiol for receptor binding to explain the increase in K_D value. However, cisplatin not only binds to DNA but also reacts readily with other bionucleophiles in the cell, including cysteine residues [2]. Since cysteine is located in the ligand-binding domain of the ER [14], platination of the sulfhydryl group could be a plausible explanation for reduced E_2 binding.

Another effect of cisplatin after a 5-day incubation is that it leads to a marked increase in the cellular protein content (Fig. 1B). However, the number of ER per cell did not increase in proportion to the protein content (and cell size), thereby leading to an actual reduction in the intracellular ER concentration. The fact that there was no comparable change either in the total amount of RNA per cell or in the level of ER mRNA compared to untreated cells suggests that cisplatin interferes with the synthesis or stability of the ER protein. This shows that while there appears to be a good correlation between the levels of ER mRNA and ER protein in biopsies of different (untreated) breast cancer tissues [1], such a correlation may not hold when the tumor has received chemotherapeutic treatment.

The most dramatic effect on E_2 binding was produced by a 5-day incubation with tamoxifen as manifested by a twofold increase in the immunologically detectable ER protein and in an approximately 3.5-fold increase in B_{max}. This is in accordance with the results of previous studies [15, 16, 25]. The apparent paradox between a high level of ER protein and low E_2 binding is resolved by a 30-fold increase in the K_D value. The high K_D value may have been the result of an intracellular concentration of tamoxifen, which could be retained by other structures, i.e. antiestrogen-specific binding sites [26, 28]. Similar changes in the ER binding parameters can also be observed with tamoxifen with much shorter incubation times [12, 15, 20]. A brief tamoxifen exposure of 20 min changes neither B_{max} nor the level of ER protein, but it does increase the K_D value, indicative of competitive inhibition (Otto, unpublished results). However, after a 20-h incubation with tamoxifen, an increase in the number of E_2 binding sites of almost 30% can be observed [20]. Up to this time ER synthesis may have continued while ER degradation is already blocked. After 5 days tamoxifen treatment led to a reduced level of ER mRNA per cell similar to the level in E_2-treated cells (Fig. 5), suggesting that ER synthesis was then curtailed. This is another example of a discrepancy between changes in the level of ER mRNA and ER protein.

Similar to previous observations by others in various breast tumor tissues as well as MCF-7 cells [1], at least two ER mRNA species were detected in the Northern blots. A third band also found in breast tumor tissue [1] became visible in RNA blots from tamoxifen-treated cells. In view of studies showing that several ER mRNA variants appear in normal and tumor tissues with no correlation with antihormonal sensitivity [21], the functional significance of the different bands is not clear. With respect to interpreting the amount of ER mRNA in treated cells, other authors have reported that ER mRNA (as a single band) remains at control levels during a 2-day tamoxifen incubation [22]. This evaluation, however, was made on the basis of comparing equal amounts of RNA and relating the intensity of the bands to that of actin mRNA. Actin expression (as well as GAPDH expression), however, also changes with drug treatment (Otto et al., submitted for publication). In this study we took into account that tamoxifen-treated MCF-7 cells have a much lower amount of total RNA than untreated or E_2-treated cells (Table 1, Fig. 5). This then led to the conclusion that the cellular level of ER mRNA was actually reduced by about 30% after the 5-day tamoxifen incubation.

While there was a good correspondence between the immunologically detectable ER protein and the calculated B_{max} in unfractionated lysates of untreated or estradiol-treated cells, there was a reproducible difference between these parameters, especially following tamoxifen treatment, where B_{max} was almost twice as high as the amount of ER protein. This disparity may be accounted for by the different parameters assessed by the two methodologies. One possible explanation could be that the first antibody (D547) in the assay, which preferentially recognizes activated ER [8], binds the drug-liganded receptors with reduced efficiency.

An important result of this study is that the sensitivity towards the growth-inhibitory effects of a subsequent drug treatment was not affected by the reduction in E_2 binding capacity after a 5-day estrogen or drug treatment. The fact that ER binding parameters as well as ER protein levels are sensitive to agonists and antagonists indicates that the presence of ER may be more an indicator of differentiation, the hormonal environment of the tumor cells or previous chemotherapy rather than a prognostic marker for chemotherapeutic responsiveness. This is in agreement with the observation that there is no difference between ER-positive and ER-negative breast tumors in their response to chemotherapeutic agents [13].

Acknowledgements This work was supported by a scholarship and grant from the Deutschen Forschungsgemeinschaft to A.M.O.

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