Effect of Cytotoxic Drugs on Estrogen Receptor Expression and Response to Tamoxifen in MCF-7 Cells

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ABSTRACT

We have examined the effects of a 24-h exposure to clinically achievable concentrations of adriamycin, melphalan, 5-fluorouracil, and vincristine on the estrogen binding capacity of MCF-7 human breast cancer cells using a whole cell binding assay. Adriamycin (0.018 to 1.8 μM), melphalan (0.1 to 5 μM), 5-fluorouracil (0.077 to 15.4 μM), and vincristine (0.01 to 1 nm) reduce the estrogen binding capacity in a dose dependent manner. The rate of protein synthesis is reduced following exposure to 5-fluorouracil but not following exposure to adriamycin, melphalan, or vincristine. The rate of cell proliferation, influx of the ligand, and the KD of remaining estrogen receptor are unaltered following drug exposure. These drugs may, therefore, be inducing a nonspecific reduction in the rate of receptor recycling and/or synthesis. Vincristine (1 nm) abolished estrogen receptor expression but following removal of the drug receptor levels did not reach that expressed in untreated cells for at least 48 h. Prior exposure to vincristine (1 nm) reduced the antiproliferative effects of tamoxifen (2 μM) toward MCF-7 cells.

INTRODUCTION

Chemotherapeutic and endocrine manipulative regimens are of proven value in the management of carcinoma of the breast. However, the results of the few clinical trials which have compared chemoendocrine therapy with single modality treatment have proved equivocal (1–3). This may reflect the lack of prospectively randomized patient selection (4) and poor understanding of the pharmacological interactions of cytotoxic drugs and hormones. The rationale for combining endocrine manipulative and chemotherapeutic regimens is largely based on the hypothesis that ER+ positive tumor cells will be selectively killed by the endocrine therapy while the ER negative cells will be killed by the cytotoxic drugs. Clearly, possible modulation of the action of one class of agent by the other must be considered. Thus, Weichselbaum et al. (5) demonstrated that 17β-estradiol (estradiol) increases the cytotoxicity of 1,2-diaminobenzanthracene in the hormone responsive breast cancer cell line MCF-7. We have also shown that estradiol can increase the cytotoxicity of methotrexate in these cells (6) but reduce its cytotoxicity in the unresponsive MDA-MB-436 cell line (7).

Adjuvant chemotherapeutic regimens induce significant increases in recurrence free survival in some breast cancer patients (8, 9). Since some patients may subsequently undergo endocrine manipulation, an understanding of possible short and long term effects of cytotoxic drugs on ER expression is important. Yang and Samaan (10) reported that clinically achievable concentrations of 5-fluorouracil and methotrexate and suprapharmacological concentrations of vincristine reduce both the ER content of MCF-7 cells and the rate of gross protein synthesis. Thus, a drug induced reduction in receptor expression was attributed to an inhibition of receptor synthesis. While we confirm that exposure of MCF-7 cells to cytotoxic drugs results in a reduction in ER expression, this effect was observed in the absence of any effect on gross protein synthesis following treatment with clinically achievable concentrations of adriamycin, melphalan, or vincristine. However, gross protein synthesis was inhibited following treatment with 5-fluorouracil. We have also investigated the time course of recovery of ER expression following exposure of cells to vincristine and report that a drug induced abolition of estrogen receptor expression may protect cells from the antiproliferative effects of tamoxifen.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells were the gift of Dr. C. D. Green, Liverpool University, and were obtained originally from Dr. M. E. Lippman, Bethesda, MD. The cells were routinely cultured in MEM which consisted of Eagle's modified minimal essential medium with Earle's salts supplemented with 5% fetal calf serum, 1% nonessential amino acids (final concentration), 1 mM sodium pyruvate (final concentration), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Gibco, Ltd., Paisley, Scotland). HFMEM contained MEM supplemented as described but with the calf serum replaced with serum which had been stripped of endogenous steroids using dextran coated charcoal. Fetal calf serum contained <10^-11 M unconjugated estradiol. The cells were regularly tested and were found to be free of Mycoplasma contamination.

Incorporation of Radiolabeled Precursors into DNA and Protein. Cells (5 x 10⁶) were plated out in MEM into each well of a 24-well culture dish (Flow Laboratories, Irvine, Scotland) and allowed to attach for 48 h. MEM was replaced with HFMEM containing the relevant concentrations of adriamycin (Farmitalia Carlo Erba, Ltd., Barnet, United Kingdom), melphalan, 5-fluorouracil (Sigma, London, United Kingdom), or vincristine (Eli Lilly & Co., Ltd., Basingstoke, United Kingdom) for a further 24 h. The influence of cytotoxic drugs on the rate of DNA synthesis was determined by the incorporation of [6^-3H]thymidine (30 Ci/mmol) or deoxy-6^-3H]uridine (17 Ci/mmol) into acid precipitable material (11). The rate of protein synthesis was determined in a manner following exposure to [6^-3H]leucine (1 μCi/ml; 45 Ci/mmol) in Earle's salts for 1 h at 37°C. Radiochemicals were obtained from Amer sham International, Plc. (Amersham, United Kingdom).

Determination of Estradiol Binding Capacity. The estradiol binding capacity of treated and untreated cells was determined using a whole cell binding assay (12, 13). Cells (5 x 10⁶) were plated into microwells as described above. Specific binding was estimated by exposing cells to 0.5 to 6 nM [2,4,6,7,16,17^-3H]estradiol (140 Ci/mmol; Amersham International, Plc.) in HFMEM for 1 h at 37°C in the presence or absence of a 200-fold excess of diethylstilbestrol (Sigma) as the cold competing ligand. Following incubation with [3H]estradiol the medium was removed, the cell monolayer was washed twice with 1 ml ice-cold MEM without supplements, and the radioactivity was extracted by incubating with 1 ml ethanol for 30 min at room temperature. Radioactivity was determined by liquid scintillation counting and the Bmax and KD of estradiol binding were estimated from transformed data using Scatchard and Woolf analyses (14). The dose-response effect of cytotoxic drugs on estradiol binding was determined using a single saturating concentration of [3H]estradiol (4 nM).

Cell Proliferation. Cells (10⁶) were plated into microwells as described previously. After 24 h growth medium was replaced with medium containing vincristine for a further 24 h. Following removal of vincristine, the cells were exposed to 2 μM tamoxifen for 6 days in HFMEM containing 1% stripped serum. Monolayers were refed on day 3. Cell proliferation was determined by estimating cell number following re-
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moval of vincristine (day 0) and 6 days later (day 6). Cell proliferation is expressed as cell number on day 6 as a percentage of cell number on day 0.

RESULTS

Figs. 1 to 4 demonstrate the effects of adriamycin, melphalan, 5-fluorouracil, and vincristine on estradiol binding expressed as a percentage of control values. A dose dependent decrease was observed in each case. The steepest response curve was observed following exposure to adriamycin and melphalan with estradiol binding being virtually reduced to undetectable levels over a 1–1.5-log drug concentration range. The same figures demonstrate that protein synthesis was significantly reduced only following exposure to 5-fluorouracil concentrations ≥3.85 μM, while there was a close correlation between a reduction in ER binding and inhibition of DNA synthesis for all drugs examined.

Table 1 shows $B_{\text{max}}$ and $K_d$ for estradiol binding following drug exposure obtained from full Scatchard and Woolf analyses of estradiol binding in the concentration range 0.5 to 6 nM [3H]estradiol. $B_{\text{max}}$ and $K_d$ were determined for untreated cells in parallel with each drug treatment. Confidence limits for $B_{\text{max}}$ were estimated at the 95% level as described by Davies and Goldsmith (15). While drug treatment markedly reduced the $B_{\text{max}}$, there was no significant alteration in the affinity of the remaining receptor proteins for estradiol.

Following exposure to 1 nM vincristine, ER levels were abolished (Figs. 4 and 5). After removal of the drug, ER levels returned to or exceeded that expressed by untreated cells by 72 h (Fig. 5). However, this response was markedly variable and...
Cells were treated with vincristine for 24 h. ER levels were determined using Scatchard and Woolf analyses on removal of the drug and at 24-h intervals thereafter. Proliferation is expressed as the percentage change in cell number; bars, SE.

Fig. 5. Time course of ER recovery following exposure to 1 nM vincristine. Cells were exposed to (a) 1 nM vincristine for 24 h, (b) 2 μM tamoxifen for 6 days, (a–b) 1 nM vincristine for 24 h followed by 2 μM tamoxifen for a further 6 days, and (c) untreated cells. Cell proliferation is expressed as the percentage change in cell number; bars, SE.

Fig. 6. Effect of a prior exposure to 1 nM vincristine on the ability of cells to respond to subsequent tamoxifen treatment. Cells were exposed to (a) 1 nM vincristine for 24 h, (b) 2 μM tamoxifen for 6 days, (a–b) 1 nM vincristine for 24 h followed by 2 μM tamoxifen for a further 6 days, and (c) untreated cells. Cell proliferation is expressed as the percentage change in cell number; bars, SE.

DISCUSSION

Adriamycin, melphalan, 5-fluorouracil, and vincristine reduce the estradiol binding capacity of MCF-7 cells in a dose-dependent manner (Figs. 1–4). While our results with 5-fluorouracil compare with those of Yang and Samaan (10), we observe an abolition of ER expression following exposure to concentrations of vincristine approximately 1000-fold lower. This may reflect the longer period of exposure to vincristine (24 h versus 4 h) used in this study.

Table 1 shows that, after drug treatment, the $K_a$ of any remaining ER was unaltered (Table 1). This suggests that the drugs are not competitively or allosterically inhibiting the binding of estradiol to its receptor. Since the drugs are structurally different both from each other and from estradiol, such competitive interaction may be considered unlikely. While a report of an adriamycin-induced reduction in the ER content of rat uterine tissue was initially attributed in part to a direct interaction between ER and the drug (16), these results were not subsequently confirmed (17, 18).

We consider that the observed reduction in specific binding capacity is unlikely to be the result of a reduced influx of ligand since the reduction in specific binding is the result of a decrease in total binding without a decrease in the nonspecific component. The rate of cell proliferation has been reported to influence the ER content of MCF-7 cells (19, 20). In this study the rate of cell proliferation was not significantly altered during the period of drug exposure. ER has a half-life of 3–5 h in MCF-7 cells (21). Since cells are unable to maintain receptor levels as a result of drug treatment, cytotoxic drugs appear to be influencing either receptor processing, synthesis, or recycling.

While the precise nature of receptor processing is unclear, Horwitz and McGuire (22) observed that processing occurs subsequent to ligand binding. Thus, following a 10-min exposure to estradiol, processing commences within 1 h and is complete by 5 h. In our culture conditions some ER will be unoccupied and, therefore, unavailable for processing. Thus, modulation of receptor processing could not account for the ability of some cytotoxic drugs to abolish ER expression. Furthermore, Horwitz and McGuire (23) were unable to detect any effect of adriamycin on either ER processing or translocation.

An inhibition of protein synthesis, as a result of drug exposure, might be expected to reduce ER content. There was some correlation between a reduction in estradiol binding capacity and an inhibition of protein synthesis following exposure to 5-fluorouracil (Fig. 3); this is in agreement with the work of Yang and Saaman (10). However, concentrations of adriamycin, melphalan, and vincristine which reduced the ER content of MCF-7 cells failed to influence the apparent rate of gross protein synthesis (Figs. 1, 2, and 4). Since we have not measured synthesis of the ER protein itself, some modulation of receptor synthesis by cytotoxic drugs cannot be precluded.

Receptor recycling may contribute to the maintenance of intracellular receptor levels (24). Butler et al. (25) observed that insulin reduces expression of ER in MCF-7 cells and suggested that this may be mediated by a recycling process. The ability of progesterone to reduce nuclear ER levels is dependent upon the induction of both RNA and protein synthesis (26) and ER is inactivated (and possibly reactivated) in vitro by a phosphorylation/dephosphorylation process (27). Since ER expression, but not protein synthesis, is inhibited by melphalan, adriamycin, and vincristine, an inhibition of receptor recycling could account for the ability of these drugs to inhibit receptor expression. However, 5-fluorouracil reduces both gross protein synthesis and ER expression. Thus, an inhibition of both receptor synthesis and recycling may occur following exposure to 5-fluorouracil. The mechanism by which cytotoxic drugs reduce ER levels clearly requires further study. We are currently using monoclonal antibodies to determine if there are receptor molecules present which are not detectable by ligand exchange.
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There is a close correlation between a reduction in ER content and the rate of DNA synthesis as measured by the incorporation of radiolabeled precursors into acid precipitable material (Figs. 1–4). Brooks et al. (28) reported that in postconfluent monolayers of MCF-7 cells, where the rate of DNA synthesis is also reduced, a reduction in ER levels is observed. Hence, a reduction in the rate of DNA synthesis which is not drug induced also correlates with a reduction in ER expression. This suggests that maintenance of ER levels may be related to the appearance of specific acceptor sites (29–31) on newly synthesized DNA.

Fig. 5 shows that, in contrast to previously published data (10), ER levels may not return to or exceed control levels for up to 72 h following removal of vincristine in vitro. Since cytotoxic drugs may remain in significant concentrations for more than 24 h in some tissues, ER expression may remain suppressed for periods in excess of 72 h in vivo.

Since vincristine and tamoxifen have different mechanisms of action, a combination of these drugs might be expected to be at least additive. However, if the antiproliferative effects of tamoxifen are due to its interaction with ER, a reduction in ER levels could induce a degree of resistance to tamoxifen. Fig. 6 shows that both 1 nM vincristine and 2 μM tamoxifen reduced the rate of cell proliferation. However, a combination of 1 nM vincristine followed by 2 μM tamoxifen was no more inhibitory. While the possibility that tamoxifen is reversing the ability of cells to recover from vincristine treatment cannot be excluded, it is more likely that the vincristine induced abolition in ER content renders the cells insensitive to tamoxifen. Results from this laboratory indicate that prior exposure to 0.5 nM vincristine, which reduces ER levels by approximately 70% (Fig. 4), does not influence the ability of either MCF-7 or ZR-75-1 cells to respond to tamoxifen (32).

The ability of cytotoxic drugs to reduce ER levels and subsequent response to antiestrogens may have important consequences for the combination of chemotherapeutic and endocrine manipulative techniques in the management of carcinoma of the breast.

REFERENCES


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