Reduction of Estrogen Receptor Concentration in MCF-7 Human Breast Carcinoma Cells following Exposure to Chemotherapeutic Drugs

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ABSTRACT

To study the effects of commonly used chemotherapeutic drugs on estrogen receptor (ER) of human breast cancer, we investigated the specific binding of \(^{3}H\)estradiol within intact MCF-7 human breast cancer cells after 1 to 4 hr of exposure to methotrexate (0.5 to 50 \(\mu\)g/ml), 5-fluorouracil, and vincristine in serum- and hormone-free medium. Intracellular \(^{3}H\)estradiol binding was either slightly increased (methotrexate and 5-fluorouracil) or not changed (vincristine) during the first 2 hr of drug exposures at 37°, but slightly decreased at the third hr. After 4 hr of drug treatments, \(^{3}H\)estradiol binding within MCF-7 cells was reduced by 30 to 70%; the response was dose dependent. Most (80 to 83%) of the intracellularly bound \(^{3}H\)estradiol was found within the nuclei, and the drug-induced reduction of ER was reflected by a depleted nuclear uptake of \(^{3}H\)estradiol. The Scatchard plot showed a large decrease of receptor number per cell with no apparent alteration in the binding affinity. The reduction of ER was reversible; regeneration of receptors to the control level occurred at either 4 hr (methotrexate and 5-fluorouracil) or 8 hr (vincristine) after removal of these drugs. The restoration was followed by an increase of ER beyond the control level. The dose-dependent depletion of ER by these cytotoxic drugs was also detectable in a second ER-positive cell line, MDA-MB-134. These data indicate that the cytotoxic drugs may cause a dose-dependent, reversible depletion of ER in human breast cancer, and the effect seems to be due to inhibition of receptor synthesis rather than inhibition of the binding of estradiol to its receptors.

INTRODUCTION

The significance of ER\(^1\) in predicting the response to hormonal therapy in patients with breast cancer has been generally agreed upon. In patients with advanced breast cancer, cytotoxic chemotherapy, alone or in combination with hormonal therapy, is often the choice for treatment. In hormonal therapy, antiestrogens, either tamoxifen or nafoxidine, induced an objective response at a rate of 30 to 40% in patients with advanced breast cancer (11, 17). Tamoxifen had been shown to depress DMA synthesis and was followed by an increase of ER beyond the control level. The restoration was accompanied by an increase of ER beyond the control level. The dose-dependent depletion of ER by these cytotoxic drugs was also detectable in a second ER-positive cell line, MDA-MB-134. These data indicate that the cytotoxic drugs may cause a dose-dependent, reversible depletion of ER in human breast cancer, and the effect seems to be due to inhibition of receptor synthesis rather than inhibition of the binding of estradiol to its receptors.

MATERIALS AND METHODS

Cell Cultures. MCF-7 cells, passages 140 to 170, were maintained as monolayer cultures in minimal essential medium supplemented with 10% calf serum, glutamine, insulin, and antibiotics. At confluency, cells were harvested with 0.1% trypsin in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hanks' balanced salt solution, were washed once, and were resuspended in regular growth medium supplemented with 10% calf serum. Three ml of cell suspension containing 5 \(\times\) \(10^6\) cells were added to sterile glass scintillation vials with foil liner caps and incubated at 37°. ER in the cells was measured after incubation for 72 hr. At that time, cells were detached from the bottom of the vials and were in the log phase of growth.

MDA-MB-134 and MDA-MB-231 cells (4), were grown in Leibovitz's L-15 medium supplemented with 10% calf serum, glutamine, insulin, and antibiotics.

Whole-Cell ER Assay. The specific binding of \(^{3}H\)estradiol within the intact cells was determined by the method of Shafie and Brooks (25) with slight modification. MCF-7 cells were washed once with 1 ml of serum- and hormone-free minimal essential medium containing Hanks' MEM balanced salt solution. The cells in each vial were then added to 0.5 ml serum- and hormone-free Hanks' MEM containing 0.45 to 7.2 \(\mu\)M \(17\beta\)-\(2,4,6,7-^{3}H\)estradiol (107 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.). Nonspecific binding of \(^{3}H\)estradiol was determined by parallel incubations with \(^{1}H\)estradiol plus a 200-fold excess of DES (Calbiochem, San Diego, Calif.). After incubation at 37° for 60 min, the cells were washed twice with ice-cold 0.9% NaCl solution and extracted with 1 ml of ethanol at room temperature. The radioactivity in the extract was counted directly in 5 ml of scintillation fluid (ScintiVerse II; Fisher Scientific Co.). In this report, all results of \(^{3}H\)estradiol binding were expressed as specific binding unless otherwise stated. Specific binding is the difference between the total binding in the absence of DES and the nonspecific binding in the presence of a 200-fold excess of DES.
experiments on the effects of the cytotoxic drugs on \([^\text{3}H]\)estradiol binding, MTX, 5-FUra, or VCR were dissolved in or diluted with plain Hanks' MEM to obtain concentrations of 0.5 to 50 \(\mu g/ml\), and the pH was adjusted to 7.4, if necessary. Representative cell counts for the control and drug-treated groups in each assay were determined by parallel incubation of separate sets of triplicate vials containing cells and incubation media. Cells were counted with an electronic particle counter (Model ZBI, Coulter Electronics, Inc.).

**Nuclear Receptor Assay.** MCF-7 cells were incubated with \([^\text{3}H]\)estradiol and washed as described above. The cells were removed from vials with a rubber policeman into 1 ml of ice-cold 0.01 m Tris buffer containing TMM buffer. The cells were incubated at 4° for 15 min and then centrifuged at 100 \(\times\) g for 5 min. The nuclei were sedimented by centrifugation at 600 \(\times\) g for 5 min, washed once, and resuspended in TMM buffer. The nuclei obtained from this procedure appeared intact and were consistently free of cytoplasmic debris as observed by phase microscopy. An aliquot of the nuclear suspension was transferred into a counting vial. Radioactivity in the nuclear pellets was extracted with ethanol, and disintegrations were counted in the scintillation fluid described above. Another aliquot of the nuclear suspension was taken for DMA determination by the diphenylamine method (3).

**Receptor Regeneration Study.** MCF-7 cells were first incubated for 4 hr at 37° in serum- and hormone-free Hanks' MEM with 0.5 ml (5 \(\mu g/ml\)) of each of the drugs. At the end of the incubation, the drugs were removed, and cells were washed 3 times with Hanks' MEM to remove residual drugs. Three ml of Earle's minimum essential medium containing 10% bovine serum was added to the cells in each vial which was then incubated at 37° under 5% CO2-air atmosphere for various periods of time. At the times indicated, medium was removed and the cells washed once with Hanks' MEM. ER in the intact MCF-7 cells was then measured according to the method described above.

**RESULTS**

**Measurement of ER in Intact Cells.** \([^\text{3}H]\)Estradiol was rapidly taken up by MCF-7 cells during the incubation at 37° (Chart 1). Specific uptake of \([^\text{3}H]\)estradiol reached its maximal level in 60 min. Therefore, 60 min was chosen for further experiments. The specific uptake of \([^\text{3}H]\)estradiol increased as the concentration of \([^\text{3}H]\)estradiol in the medium increased, but it seemed to be saturated in concentrations exceeding 7.2 \(\times\) 10\(^{-9}\) M. Thus, 7.2 \(\times\) 10\(^{-9}\) M of \([^\text{3}H]\)estradiol was used throughout these studies unless otherwise specified.

To determine whether our assay system would be able to quantify the ER content in different cell lines, an ER-positive and an ER-negative cell line were examined next. The MDA-MB-134 is a human breast cancer cell line that was previously determined to be ER-positive, but its ER level is lower than that of the MCF-7 cells (30). The MDA-MB-231 is a human breast cancer cell line that was previously determined to be ER-negative and does not respond to estrogen with respect to thymidine incorporation (14). Chart 1 shows that the specific binding of \([^\text{3}H]\)estradiol was readily detectable with MDA-MB-134 cells, and the ratio of specific binding between MCF-7 and MDA-MB-134 cells was comparable to that measured by the broken cells assays (30). On the other hand, no significant binding of \([^\text{3}H]\)estradiol was detected within the MDA-MB-231 cells by the whole-cell assay (Chart 1).

**Effects of Cytotoxic Drugs on the ER Level in MCF-7 Cells.** To study the effects of the cytotoxic drugs on the specific binding of \([^\text{3}H]\)estradiol within MCF-7 cells, the cells were incubated in 0.5 ml serum- and hormone-free medium with 2.5 \(\mu g\) of each drug for 0 to 5 hr at 37°. \([^\text{3}H]\)Estradiol was then added, and the incubation was continued for an additional hr. Chart 2 shows that, with all the drugs examined, no significant reduction of \([^\text{3}H]\)estradiol-specific binding within MCF-7 cells was detected during the first 2 hr of exposure to the drugs. A slight decrease of \([^\text{3}H]\)estradiol binding occurred at the third hr of drug exposure. After 4 hr of drug treatment, however, there was a 45, 40, and 63% reduction of specific \([^\text{3}H]\)estradiol binding caused by MTX, 5-FUra, and VCR, respectively. No significant decrease of specific binding was detected between the fourth and sixth hr of drug exposure. However, further reduction of \([^\text{3}H]\)estradiol binding beyond what was detected at 4 hr could be achieved by treatments of the cells with higher concentrations of the drugs. Chart 3 shows that MTX, 5-FUra, and VCR (50 \(\mu g/ml\)) reduced the specific binding further to 36, 47, and 21%, respectively, of the non-drug-treated control; the effect of these drugs appeared to be dose dependent. It should be noted that, in pilot experiments which were carried out to ascertain if there was a change on cell proliferation due to the exposure to each of the drugs, we found that there was no significant change of cell number in MCF-7 over the 1- to 4-hr incubation periods when cells were incubated in either the serum-free medium alone or the medium plus the drug. Thus, the reduction of specific \([^\text{3}H]\)estradiol binding caused by these drugs was not resulted from alteration of cellular population density.

It is generally accepted that binding of estradiol to cytoplasmic receptors would be followed by the translocation of hormone-receptor complex to the nucleus (8, 21, 29). Also, MCF-7 cells have been found to contain unoccupied nuclear receptors (30). Therefore, binding activities in nuclei of the control and drug-treated MCF-7 cells were next compared. MCF-7 cells were equilibrated with \([^\text{3}H]\)estradiol and \([^\text{3}H]\)estradiol plus excess DES at 37°, the cells were washed, and the nuclei were then isolated to determine the radioactivity. Chart 4 shows that more than 80% of the specific \([^\text{3}H]\)estradiol binding in the intact control MCF-7 cells were associated with the nuclei. About 90% of the nuclear \([^\text{3}H]\)estradiol binding were specific. The radioactivities in...
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Chart 2. Effects of cytotoxic drugs on the specific binding of [3H]estradiol within intact MCF-7 cells as a function of time of treatment. Cells in the log phase of growth were either preincubated (time points 2 to 6 hr) with each drug (5 ng/ml) in serum- and hormone-free medium for 1 to 5 hr or without the preincubation. 7.2 nm [3H]estradiol and [3H]estradiol plus DES were then added, and the incubation was continued for one additional hr. MTX and VCR, 4 determinations in 2 experiments; 5-FU, 2 determinations in one experiment. Bars, S.E.

Chart 3. Dose-response relationships for the effects of cytotoxic drugs on the specific binding of [3H]estradiol within intact MCF-7 cells. Effects of cytotoxic drugs on [3H]estradiol binding were measured as described in the legend to Chart 2. Various concentrations of each drug were used. Effects of the drugs at only one time point (4 hr) were measured. Values are the means of 8 or more determinations in 4 experiments. 5-FU, 5-FUra; bars, S.E.

Chart 4. Effects of cytotoxic drugs on the ER concentrations in MCF-7 cells and MCF-7 nuclei after incubation of intact cells at 37°C for 1 hr with a saturating concentration (7.2 nm) of [3H]estradiol. Cells were preincubated with each drug (5 ng/ml) for 3 hr at 37°C before a second incubation with [3H]estradiol and [3H]estradiol plus DES for one additional hr. Total drug treatment time was 4 hr. See "Materials and Methods" for the detailed procedures. Values are the means of 6 or more determinations in 3 experiments. 5-FU, 5-FUra; bars, S.E.

the values (3.0 × 10^{-8} M) obtained by others using cell-free systems (15, 30) but agrees reasonably with that (1.5 × 10^{-8} M) obtained from a similar whole-cell method (25). It may be that K_d is altered by intracellular factor and by the loss of the estradiol ligand due to conversion to its metabolites by the whole

Chart 5. Scatchard plots of the specified binding of [3H]estradiol in non-drug-treated control and drug-treated intact MCF-7 cells. Concentration of each drug was 5 μg/ml; treatment time was 4 hr. Data presented are representative of results from 2 separate experiments. □, non-drug-treated control MCF-7; △, O, □, drug-treated cells; △, MTX; O, 5-FU, 5-FUra; □, VCR.
cells. Shafie and Brooks (25) found that about 15% of estradiol was converted to estrone during the incubation of intact MCF-7 cells with [$^3$H]estradiol at 37°.

**Time Course of Regeneration of ER.** To investigate whether the loss of ER from MCF-7 cells after drug treatment is reversible, MCF-7 cells were washed after 4 hr of exposure to the drugs and incubated at 37° in fresh culture medium containing calf serum for periods of time as indicated, and ER in these cells was then measured. Chart 6 shows the time course of the regeneration of ER in drug-treated MCF-7 cells after incubation in drug-free medium. Immediately after the 4-hr drug treatment (i.e., Chart 6, Time 0), specific [$^3$H]estradiol binding within MCF-7 cells, which was measured in drug-free medium, was either the same as or lower than that measured in the cells exposed to the drugs for 4 hr, but in which the [$^3$H]estradiol binding was measured in the presence of these drugs (i.e., Chart 2, Time 4 hr). ER increased gradually during the incubation in drug-free medium and was restored to the non-drug-treated control level at either 4 hr (MTX- and 5-FUra-treated) or 8 hr (VCR-treated) after the incubation. The recoveries of ER concentrations to the control level were followed by "overshooting" increases of receptor concentrations above the control level.

**Effects of Cytotoxic Drugs on the ER Concentration of Another ER-positive Human Breast Cancer Cell Line.** To ascertain whether the reduction of ER in response to the treatments of these cytotoxic drugs is a unique property of MCF-7 cells, a second ER-positive human breast cancer cell line, MDA-MB-134, was also investigated for the drug effect on ER. Chart 7 shows that the cytotoxic drugs, which caused a reduction of the ER concentration in MCF-7 cells, also similarly reduced the ER concentration in the MDA-MB-134 cells.

**DISCUSSION**

Using intact cells to measure binding of steroids to specific receptors has been applied to studies of various types of cells (2, 19, 25, 27, 28). With MCF-7 cells, it has been shown that the specific uptake of [$^3$H]estradiol by the whole cell was directly related to the ER concentration in the cells (25). Recently, using a whole-cell suspension assay technique, Macindoe et al. (16) demonstrated the specific binding of testosterone and dihydrotestosterone to androgen receptors within intact MCF-7 cells. In our study, the correlation between cellular [$^3$H]estradiol binding capacity and ER concentration was indicated in the results from 2 experiments: (a) specific binding of [$^3$H]estradiol was detectable only in ER-positive cells, the MCF-7 and MDA-MB-134 cell lines; but not in ER-negative cells, the MDA-MB-231 cell line. Furthermore, the ratio of [$^3$H]estradiol binding capacities within the MCF-7 and MDA-MB-134 cells, measured by the whole-cell method in our study, was comparable to the ratio estimated from other cell-free systems (30); (b) most (>80%) of [$^3$H]estradiol that specifically bound within MCF-7 cells was associated with the nuclei after a 30-min incubation of the whole cells at 37°.

Our results clearly indicate that the cytotoxic drugs, MTX, 5-FUra, and VCR, reduced ER concentrations in 2 ER-positive human breast cancer cell lines examined thus far. The reduction of ER was not likely due to blocking the formation of [$^3$H]estradiol-receptor complex by the drugs. This conclusion is indicated by 2 pieces of evidence: (a) The amounts of the specific [$^3$H]estradiol binding within the drug-treated MCF-7 cells measured in drug-free medium (i.e., Chart 6, Time 0) were either the same as or lower than those measured in the presence of the drugs (i.e., Chart 2, Time 4 hr); (b) Scatchard analysis indicated a reduction of receptor sites per cell with no change in the apparent binding affinity. Our findings agree with the results of Muller et al. (18), who found that certain cytotoxic drugs, including MTX and VCR, did not affect the specific binding of [$^3$H]estradiol to the cytosols extracted from human breast tumors. Di Carlo et al. (6), on the other hand, studied uterine cytoplasmic ER and concluded that Adriamycin and MTX decreased rat uterine ER by inhibiting both protein synthesis and formation of the [$^3$H]estradiol-receptor complex. Since Muller et al. investigated the drug effect in a cell-free system, and Di Carlo et al. studied it in vivo, both of their methods are quite different from ours; thus, comparison of the results would be complicated. In our study, protein synthesis in the treated MCF-7 cells was inhibited 54, 45, and 64% by MTX, 5-FUra, and VCR (5 μg/ml), respectively, after 4-hr drug incubation. Thus, our results support the notion that the reduction of ER by the cytotoxic drugs is due to...
to the direct action of these drugs on the de novo synthesis of ER but not to the inhibition of formation of estradiol-receptor complexes. However, whether these drugs affect the rate of receptor degradation in addition to the inhibition of receptor synthesis still remains to be studied.

The loss of ER in MCF-7 cells that was induced by the cytotoxic drugs appeared to be reversible when the cells were incubated in drug-free medium after drug treatments. The apparent recovery of ER to the control level occurred after 4 hr and was followed by an increase of ER above the control level. A similar "overshooting" phenomenon has been reported in studies of estrogen-induced replenishment of cytoplasmic ER (5, 6, 23). It is possible that these cytotoxic drugs act by a mechanism similar to estrogen in this respect. It should be noted that there was no significant reduction of number of MCF-7 cells immediately after the drug treatment and also during the 12-hr period shown in Chart 6. However, prolonged incubation of the drug-treated MCF-7 cells in drug-free culture medium containing 10% calf serum resulted in considerable cell death (e.g., 35 to 55% cell death occurred after a 21-day incubation as determined by the colony formation technique). It is possible that a subpopulation of the treated cells that did not survive treatment might not have restored their ER, but that this deficiency was overshadowed by the overshooting increase of ER in other subpopulations that survived the prolonged post-drug-treatment incubation. This speculation is supported by preliminary results showing that the cell-killing activities of these drugs at doses of 0.5 to 50 µg/ml, as determined by the colony formation technique, were proportional to the degree of ER reduction caused by the corresponding doses of each drug. Experiments are now in progress to determine the ER concentration in the cells that do not survive the prolonged incubation after drug treatment and to compare those concentrations with the ER concentration in cells that do survive.

We conclude that the cytotoxic drugs may cause a dose-dependent depletion of ER in human breast cancer, and the effect seems to be due to inhibition of receptor synthesis rather than inhibition of the binding of estradiol to its receptors.

ACKNOWLEDGMENTS

We would like to thank Dr. Charles McGrath for the MCF-7 cell line and Dr. Rekda Cailleau for the MDA-MB-134 and MDA-MB-231 cell lines.

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