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VII. Hormonal Recruitment and Chemotherapy

MANIPULATION OF CELL CYCLE KINETICS: INFLUENCE ON THE CYTOTOXICITY OF DOXORUBICIN IN HUMAN BREAST CANCER CELLS

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Summary—*In vitro* exposure of estrogen receptor-negative (ER−) EVSA-T human breast cancer cells to insulin and/or estradiol had no effect on cell cycle distribution, in contrast to a 3–5-fold increase in the percentages of cells in the S-phase of the cell cycle in the ER+ MCF-7 cell line. Estrogen pretreatment of MCF-7 cells followed by incubation with doxorubicin resulted in an augmented inhibition of cell growth compared to unstimulated controls. This delay in growth was accompanied by a decrease in the percentages of cells actively synthesizing DNA, and by an augmented percentage of cells exhibiting a G_2M-amount of DNA at the end of a 6–9 day period of culture in complete growth medium.

INTRODUCTION

Anti-hormones decrease the growth fraction of hormone-responsive breast tumors, leading to an accumulation of cells in the G_0G_1-phase of the cell cycle. This influence of anti-hormones on cell cycle kinetics could be one of the reasons for the disappointing results of combined endocrine- and chemotherapy, because most chemotherapeutic agents exert their effect mainly on cells in the S- and G_2M-phases of the cell cycle. Even adverse effects of the combination of tamoxifen with chemotherapy have been reported in subsets of patients [1].

In contrast to this growth inhibition, several hormones and growth factors are known to accelerate the growth of slowly proliferating breast tumors, leading to the recruitment of a population of quiescent or G_0G_1-phase cells into the S- and G_2M-phases of the cell cycle. Several clinical studies have already applied the principal of hormonal recruitment of breast cancer cells into the active phases of the cell cycle followed by chemotherapy [2–6]. No uniform conclusions can be made yet due to: (a) the use of different therapeutic schemes; (b) the possibility of inadequate recruitment due to incomplete reversion of the anti-estrogenic effect of tamoxifen, the synchronizing agent in several trials; and (c) the absence of proof of concealed recruitment in most of the studies.

We investigated the following in the ER+ MCF-7 and the ER− EVSA-T breast cancer cell line:

(1) The effects of growth-promoting hormones and of doxorubicin on the kinetics of the tumor cells, as measured by dual-parameter flow cytometry (PI/anti-BrdUrd FITC).
(2) The effects of estrogen pretreatment followed by incubation with doxorubicin on the growth of the cell cultures.

MATERIALS AND METHODS

Cell culture

Culture conditions for both cell lines were identical to those described previously [7]. MCF-7 cells were maintained in RPMI-1640 medium containing 5 μg/ml Phenol Red, supplemented with 10% heat-inactivated (30 min at 56°C) bovine calf serum (BCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml

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gentamycin and 10 μg/ml porcine insulin. EVSA-T cells were maintained in Ham/F12 DMEM medium containing 8.6 μg/ml phenol red, supplemented with 5% heat-inactivated BCS, antibiotics and insulin. For experiments, logarithmical growing cell cultures were trypsinized and seeded in T25-flasks at a density of 0.5×10^6 cells/flask in experimental medium, i.e. RPMI-1640 or Ham/F12 DMEM medium without Phenol Red and insulin, supplemented with antibiotics and 4.5% (MCF-7) or 2.5% (EVSA-T) steroid-hormone depleted fetal calf serum. Cells were precultured for 2 days. Experimental medium without additions (control) or supplemented with 1 nM estradiol (Merck, Darmstad, F.R.G.), 1.7 μM insulin or a combination of both was successively added to the cell cultures for upto 24 h. In hormone–chemotherapy experiments MCF-7 cell cultures pretreated with estradiol (1 nM) for 19 h were incubated for 30 min or 23 h with experimental medium containing, respectively, 3 and 0.06 μM doxorubicin (Adriablastina, Farmitalia, Milan, Italy). After the 23-h incubation period with doxorubicin, cells were washed and cultured further in complete growth medium. After the 30-min doxorubicin incubation and washing, the cells were cultured for another 24 h in experimental medium in the absence of doxorubicin, followed by culturing in complete growth medium. The cell number per flask was set at regular intervals, as indicated in the figures.

Assessment of cell cycle distribution by flow cytometry

Bromodeoxyuridine (BrdUrd, Serva, Heidelberg, F.R.G.) at a final concentration of 10 nM, was added to the cultures 30 min before harvesting. After this incubation cells were washed twice and were harvested by a 5-min incubation at 37°C with 0.5 ml trypsin/EDTA (0.05:0.02%; Biochrom, Berlin, F.R.G.) in 2 ml PBS, and the addition of 1 ml trypsin inhibitor (0.1 mg/ml; Sigma, St Louis, Mo., U.S.A.) in PBS. An aliquot of the cell suspension was collected for assessment of the cell number, using a hemocytometer. The remainder of these cells were stored in 2 ml of 70% ethanol (−20°C) before preparation for analysis by flow cytometry. The labeling and staining procedures, as well as the fluorescence measurement by flow cytometry, were performed as described previously [7].

RESULTS

We have previously shown that the addition of estradiol and/or insulin to a medium of slowly proliferating ER+ MCF-7 cell cultures results, after a lag-period of 6–12 h, in a rapid increase of the percentage of cells in the S-phase of the cell cycle. The maximum of this increase is reached 18–24 h after the addition of the hormones [7].

In the initial experiments of the present study, the effects of estradiol (1 nM), insulin (1.7 μM) or a combination of both were examined in the ER− EVSA-T cell line. In Fig. 1, the effects of a 24-h incubation with estradiol and/or insulin on the percentages of cells in the S-phase of the cell cycle in EVSA-T cells are compared with the effects of the hormones in the MCF-7 cell line. Under steroid-hormone deprived culture conditions the EVSA-T cell line proliferates faster than the MCF-7 cell line, and no growth-stimulating effects of the hormones are observed in EVSA-T cells in contrast to the MCF-7 cells.

The impact of estradiol pretreatment on the ultimate cytotoxicity of doxorubicin was investigated in subsequent experiments. MCF-7 cells were incubated with 1 nM estradiol 19 h prior to incubation with doxorubicin. Doxorubicin was administered to the cultures at a low concentration (60 nM) for 23 h or at a high concentration (3 μM) for 30 min. Figure 2 shows an example of the growth of MCF-7 cells pretreated for 19 h with or without 1 nM extradiol followed by a 23-h incubation with 60 nM doxorubicin. Estradiol pretreatment resulted in an enhanced cytotoxic effect of doxorubicin, as can be seen in the much slower increase in cell number in these cultures from day 5 onwards.

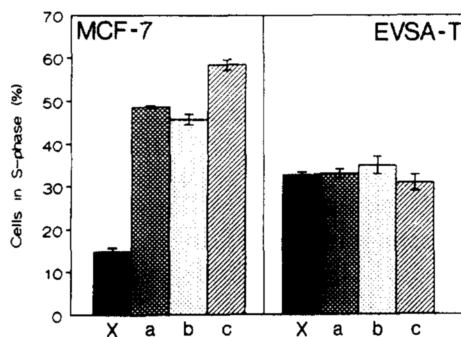

Fig. 1. Effects of a 24-h incubation with 1 nM estradiol (a), 1.7 μM insulin (b), or a combination of both hormones (c) on the increase in the percentage of S-phase cells in MCF-7 and EVSA-T cells, compared to controls (control, ×: 0.01% ethanol). Data are the means ±SD of triplicate incubations.

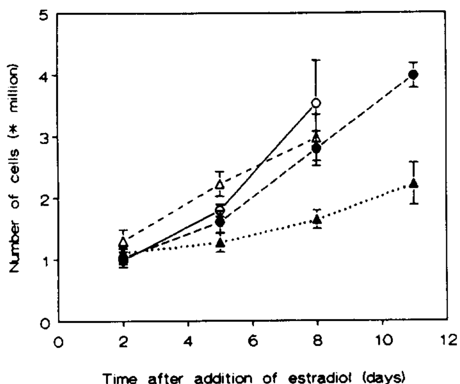

Fig. 2. Effects of a 19-h pretreatment with estradiol (1 nM), followed by a 23-h incubation with doxorubicin (60 nM) (E_2/dox, ▲) on the growth of MCF-7 cells, as compared to unstimulated cultures (dox, ●) and to the control cultures not incubated with doxorubicin (with E_2, △; control without E_2, ○). Data are the means ±SD of triplicate incubations.

The unstimulated cultures incubated with doxorubicin grew almost as rapidly in the presence of doxorubicin as the unstimulated control cultures. Figure 3 shows the growth curves of MCF-7 cells stimulated with 1 nM estradiol for 19 h followed by incubation with doxorubicin (3 μM) for 30 min. Growth in the unstimulated cultures is markedly retarded by doxorubicin compared with the cultures not incubated with doxorubicin, but there is nevertheless a small increase in cell number after 5 and 8 days of culture inhibited in growth by doxorubicin. However, in the cultures pretreated with estradiol followed by doxorubicin even no increase in cell number is observed during this period. In addition to assessment of cell number as a result of hormone–chemotherapy, the cell cycle distribution was

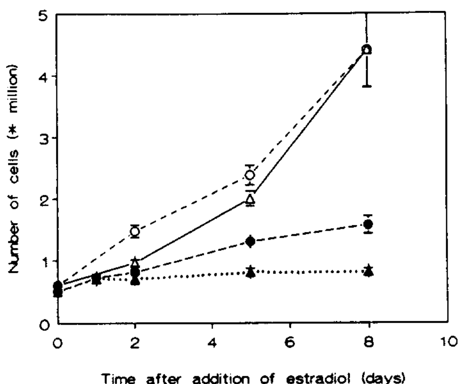

Fig. 3. Effects of a 19-h pretreatment with estradiol (1 nM), followed by a 30-min incubation with doxorubicin (3 μM) (E_2/dox, ▲), on the growth of MCF-7 cells, as compared to unstimulated cultures (dox, ●) and to control cultures not incubated with doxorubicin (with E_2, △; control without E_2, ○). Data are the means ±SD of triplicate incubations.

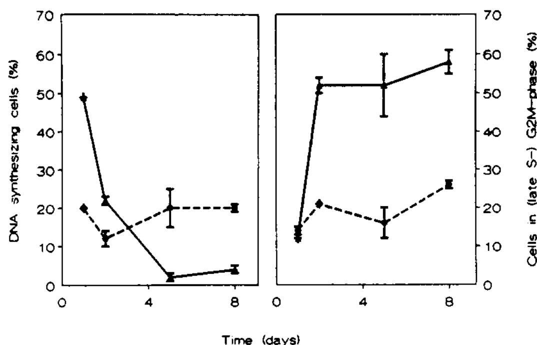

Fig. 4. Effects of estradiol pretreatment followed by a 30-min incubation with doxorubicin (3 μM) on the cell cycle distribution of MCF-7 cells (E_2/dox, ▲; dox, ●). Data are the means ±SD of duplicate incubations.

measured at the same time points as the cell counts were obtained. Figure 4 shows the cell cycle distribution on days 1–8 from the experiment depicted in Fig. 3. A 30-min incubation with doxorubicin has no immediate effect on the percentages of cells in the S-phase of the cell cycle both in the non-stimulated (18 vs 20% S-phase cells) and estradiol-stimulated (46 vs 49% S-phase cells) cultures. Cells present in the S-phase directly after estrogen pretreatment and doxorubicin incubation (= day 1 in Fig. 4) accumulated in the (late) S- and G_2M-phase of the cell cycle at day 2, and remained there for the following days. On the other hand, when doxorubicin was administered in a low concentration for 23 h, after or without estradiol pretreatment (Fig. 2), accumulation of S-phase cells into the late S–G_2M-phase was less pronounced (maximal amount of cells in the late S-G_2M-phase: 30%, data not shown).

DISCUSSION

Endocrine therapy and chemotherapy act in different ways, and possibly exert their effects on different tumor cell populations in heterogenous breast cancer. Therefore, combinations of growth inhibitory anti-hormones with chemotherapy are applied. However, the results of clinical trials are so far not spectacular and even conflicting results have been obtained [1].

On the other hand, evidence was found that estrogens could promote the growth of (slowly proliferating) breast cancers. Weichselbaum *et al.* [8] used this principal of hormonal recruitment in MCF-7 cells, and showed that growth stimulation of MCF-7 cells with 17β-estradiol resulted in a enhanced cytotoxic effect of successively administered 1-β-D-arabinofuranosylcytosine, an S-phase-specific chemotherapeutic

drug. Our results show that estradiol and/or insulin can recruit hormone-responsive MCF-7 breast cancer cells into the proliferative phase of DNA-synthesis of the cell cycle, whereas no growth stimulation of the hormones was found in the ER− EVSA-T cell line. When these growth-stimulated MCF-7 cells were incubated with doxorubicin at the moment that the highest percentages of S-phase cells were found, a significantly improved cytotoxic effect of doxorubicin was observed. Assessment of the cell cycle distribution, as a result of hormone–chemotherapeutic perturbation with the high doxorubicin concentration, shows that the percentage of cells actively synthesizing DNA drops rapidly in the cultures pretreated with estradiol. The drop in the percentage of S-phase cells is accompanied by a long-term accumulation of cells into the (late) S- and G_2M-phase of the cell cycle, an effect which lasts at least until the end of the experiments. The lower concentration of doxorubicin, applied for a prolonged period, has a less pronounced effect on the cell cycle distribution.

These results support findings reported by several investigators. In ER+ breast cancer cells a higher cytotoxic effect of chemotherapy after hormonal priming was observed [2, 8–11]. Paridaens *et al.* [2] found a synergistic cytotoxic effect in MXT mouse mammary tumor when cyclophosphamide was administered 24 h, and not 48 h, after estradiol pretreatment, whereas the synergistic effect was optimal when estradiol was used in the dose which had led to the greatest mitogenic stimulation. This suggests that the improved cytotoxicity of cyclophosphamide on the tumor cells is due to recruitment of the cells into the active phases of the cell cycle, rather than to the simultaneous presence of both drugs in the cell. Epstein *et al.* [12–14] found that estrogen potentiates the cytotoxicity of VP-16 in the T-47D breast cancer cell line, not dependent upon cellular commitment to DNA-synthesis, but by recruiting a clonogenic subpopulation characterized by increased topoisomerase II levels localized to an activated G_1-phase cell subset. On the other hand Shaikh *et al.* [15, 16] reported a synergistic cytotoxic effect in MCF-7 cells pretreated for 48 h with medroxyprogesterone acetate (MPA) followed by incubation with vincristine, methotrexate or doxorubicin. The concentration of MPA used in these experiments had little effect on the cell cycle distribution. Even exposure to doxorubicin before MPA led to an additive, but not synergistic cytotoxic effect. Osborne *et al.* [1] described an improved cytotoxic effect on MCF-7 cells from a combination of tamoxifen pretreatment followed by administration of doxorubicin or hydroxycyclophosphamide, but a decreased cytotoxic effect was found when tamoxifen was followed by melphalan or fluorouracil. This might be caused by the effects of tamoxifen on the intracellular transport of these cytotoxic drugs.

The results reported in most studies show that growth stimulation of slowly proliferative breast cancer with estradiol followed by chemotherapy seems worthwhile. Even specific combinations of anti-hormones and chemotherapy may be of interest. However, little is still known about the effects of combined or sequential treatment of hormones and chemotherapy on tumor cells. Besides the kinetic implications of (anti-hormones on breast cancer cells, combinations and sequences of (anti-)hormones and chemotherapy can lead to changes in: the fluidity of the cellular membrane, enzyme activation, drug-uptake and -metabolism, binding of drugs to molecular targets, or in the repair of drug-induced damage; all with possible consequences for the cytotoxicity. For clinical reasons it seems to be advisable not to combine both treatment modalities, outside controlled trials, until more of the mechanism of action is revealed.

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