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ESTROGEN INDUCED EXPANSION OF THE GROWTH FRACTION IN RECEPTOR NEGATIVE HUMAN BREAST CANCER

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Summary—The feasibility of a cytokinetic chemotherapy based on estrogenic recruitment has been evaluated in 5 patients, affected by locally advanced breast cancer with low or absent receptor content. Tumor proliferative activity was evaluated by the thymidine labeling index (TLI) and the primer-dependent αDNA polymerase assay (PDP-LI) which gives an *in vitro* estimation of tumor growth fraction. The patients have been treated with diethylstilbestrol (DES) 1 mg/die. for 3 days, followed by FAC (5-Fluorouracil 600 mg/m^2, Adriamycin 50 mg/m^2, Cytoxan 600 mg/m^2) i.v. on day 4 q. 21 days. Radical surgery was performed after 3 DES–FAC regimens. Tumor biopsies for evaluation of tumor proliferative activity were performed immediately before and after DES and 24 h after chemotherapy. Our results demonstrate that DES was able to induce an increase in TLI in $\frac{3}{5}$ of the patients while the PDP-LI was significantly increased in $\frac{5}{5}$ of the patients; subsequent chemotherapy induced a sharp decrease in tumor proliferation. These results provide the rationale for the design of cytokinetic regimens where chemotherapy is administered at the time of estrogen induced tumor cell recruitment.

INTRODUCTION

Combined chemo-hormone therapy has enabled higher response rates to be reached in breast cancer but complete remissions remain relatively infrequent and improvement in survival rate is still questionable [1–5]. In fact, in hormone dependent cases, survival of patients treated with combined modalities is similar to that observed in patients first treated with endocrine therapy followed by chemotherapy at the time of relapse.

This lack of true synergism might be due to a mutually exclusive mechanism of action: anti-oestrogens inhibit the proliferative activity of mammary carcinoma cells [6, 7] whereas cytotoxic drugs kill cells predominantly engaged in the mitotic cycle [8, 9]. Therefore an alternative more rational way to combine hormone and chemotherapy, should be based on the capacity of oestrogens to recruit the G_0 hormone responsive cells into the cell cycle. Oestrogens in fact are able to induce a transient and synchronous increase of thymidine labeling index in mammary tumours [10–12]. Interestingly, this oestrogenic recruitment can be obtained even in receptor negative tumors [13]. If oestrogens are really able to recruit previously resting cells into the cell cycle, combination chemotherapy should be administered at the time of maximally induced tumor proliferation.

In the present paper, we report our preliminary data on the induction of an oestrogenic recruitment in 5 cases of receptor negative human breast cancer. Tumor proliferative activity has been evaluated with the thymidine labeling index (which expresses the percentage of cells synthetising DNA) and the PDP index. The PDP index expresses the percentage of cells containing an active α DNA polymerase. This polymerase is present in actively cycling cells, independent of their position along the cycle [14]. There fore the PDP index gives an *in vitro* estimation of the growth fraction [15].

EXPERIMENTAL

Patients and treatment

Five patients with locally advanced inoperable breast cancer entered this study on oestrogenic recruitment. The criteria for inclusion were: histologically proven diagnosis of breast cancer, no previous therapy, no controindication to chemotherapy, informed consent.

The treatment plan was the following: diethylstilbestrol (DES) 1 mg total dose p.o. daily for 3 days followed by FAC (5-Fluorouracil 600 mg/m^2, Adriamycin 50 mg/m^2, Cytoxan 600 mg/m^2) i.v. on day 4 q. 21 days. The 3 day oestrogenic stimulation was chosen on the basis of data from literature [13].

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Only in 1 patient was the DES administration prolonged for 7 days and CT was administered on day 8. Radical surgery was delayed after 3 DES + FAC treatments if progression did not occur. In order to monitor tumor proliferative activity, serial tru-cut tumor biopsies were performed before DES, after DES administration and after chemotherapy.

Thymidine labeling index (TLI)

Tumor fragments were minced mechanically and single cell suspensions were obtained with serial passage through needles of different size. The cells were suspended in RPMI and checked for viability with the trypan blue exclusion test. If viability was too low a Ficoll–Hypaque gradient was employed at the end of incubation period in order to recover more viable cells. A quota of the cells was processed for the PDP index (see below) and the remaining cells were utilized or the determination of the TLI. Briefly, 2–3 10^6 cells suspended in RPMI with 10% FCS were incubated for 30 min at 37°C in 5% CO_2 with 10 μc/ml of [^{3}H] dThd (sp. act. 5 C/mM); radiolabeling was stopped with cold saline, the cells were cytocentrifuged onto slides and fixed in methanol–acetic (9:1, v/v) for 15 min. The slides were than dipped in Kodak NTB_2 nuclear track emulsion (Eastern Kodak, Rochester, NY), exposed for 24 h at 4°C and stained with hematoxylin-eosin after gold activated autoradiography (GAG) [16].

The fraction of tumor cells having more than 5 nuclear grains is referred to as the TLI and represents the fraction of cells in DNA synthesis. A minimum of 1000 tumor cells were scored for each specimen.

PDP index

The PDP assay is an autoradiographic method to measure the simultaneous presence of nuclear α DNA polymerase and nuclear DNA primer template activity in individual, unfixed cell nuclei [14, 15]. Cells, suspended in 0.9% NaCl solution, were cytocentrifuged onto acid-cleaned slides; slides were air dried, dipped in 0.25% agar solution at 41–42°C and air dried again. This process disrupts the cytoplasm while leaving the intact nuclei on the slide. In this situation the unfixed nuclei are able to synthesize DNA if α DNA polymerase, DNA template and the necessary materials for DNA synthesis are present. The incubation mixture was added to chambers made by affixing a glass ring to each slide. The incubation mixture consisted of 0.02 M Tris, pH 7.7; 0.089 mM each deoxyadenosine-5′-triphosphate (dATP), deoxycytidine-5′-triphosphate (dCTP) and deoxyguanosine-5′-triphosphate [dGTP] (Sigma Chemical Co., St Louis, MO); 10 μc/ml of [^{3}H]deoxythymidine 5′-triphosphate ([^{3}H]dTTP, sp. act. 60–80 C/mmol, New England Nuclear), 12.77 mM KCl, 4.68 mM $MgCl_2$ and Ficoll 400 mg/ml (Sigma).

Slides were incubated for 45 min at 37°C in 5% CO_2 with 0.5 ml of the mixture. The radiolabeling was then stopped in cold saline after removing the rings and the cells were fixed for 30 min in three changes of acidic formaldehyde (100 ml formalin, 25 ml 1 Na HCl, 875 ml water). After rinsing in tap water the fixed smears were dehydrated through ethanol–xylene–acetone series (2′ethanol 70%, twice 2′absolute ethanol, twice 5′xylene, 5′acetone) air dried, dipped in NTB-2 nuclear track emulsion, exposed 7 days at 4°C, submitted to GAG and stained with hematoxylin eosin. At least 500 nuclei were scored for determination of the PDP-LI. Each nucleus with 5 or more grains above the back ground (usually 0–1 grain for nucleus area) was considered labeled.

RESULTS

The 5 patients in the study had large unresectable primary breast cancer which allowed repeated biopsies. The first biopsy was surgical in order to establish the histology and the receptor-status beside the proliferative activity. Subsequent samples (after DES and after CT) were obtained with tru-cut biopsies of the residual tumor. Presence of tumor cells in these biopsies were verified cytologically. Four patients were postmenopausal, one (patient no. 3) premenopausal. Receptor status were determined with the charcoal–dextran technique in all the patients; in 3 patient the sucrose gradient test was utilized too. Three patients were receptor negative (both oestrogen and progesterone receptors), 2 patients had borderline values (patients Nos 3 and 5). The TLI-LI and PDP-LI at diagnosis, after 3 day DES and 24 h after CT are reported in Table 1.

In $\frac{3}{5}$ patients the TLI showed a significant increase after DES and returned to basal values (or lower) after CT. The PDP-LI increased in all the five patients after DES and again lowered to basal values after CT. Interestingly in the patient who received DES for 7 days both TLI and PDP-LI peaked on day 3; on day 7 the TLI was lowering despite continuous DES administration but the PDP-LI increase was still present (Fig. 1). Here again, CT

Table 1. Estrogenic recruitment in receptor negative human breast cancer

	Before DES		During DES		After CT		ER	PR
Patients	LI%	PDP%	LI%	PDP%	LI%	PDP%	fm/mg	fm/mg
1	1.8	2.0	11.8	20.7	0.7	0.9	neg	neg
2	0.9	1.7	2.1	4.4	0.8	2.4	neg	neg
3	1.2	3.5	1.1	6.7	1.4	1.5	11.0	neg
4	2.6	4.8	2.1	6.4	not done		neg	neg
5	2.9	8.1	7.0	14.3	3.1	9.5	7.2	neg

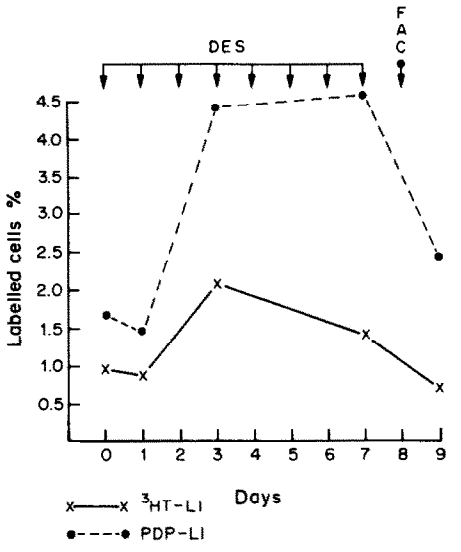

Fig. 1. Proliferative pattern in a human breast cancer during 7 day DES stimulation.

induced a sharp decrease in proliferative activity (both TLI and PDP-LI) after 24 h. It must be mentioned that even if the PDP assay disrupted the cell cytoplasm, differentiation between cancer cells and normal inflammatory cells (mainly granulocytes and lymphocytes) was easily accomplishable. All the slides were reviewed by two independent observers and, for each experiment, a morphologic sample was prepared to verify the percentage of cancer cells.

DISCUSSION

Both endocrine therapy and chemotherapy are effective for human breast cancer, but the optimal way to associate these two treatments has still to be established [1–5]. In fact the results obtained herein with hormone-chemotherapy do not show a synergistic effect and even suggest a competitive mechanism. Theoretically the hormone therapy could affect the hormone responsive clones while chemotherapy could control the receptor negative cells. On the other hand one of the effects of antioestrogens seems to be the inhibition of the cell cycle progession, thus increasing the percentage of quiecent cells more resistant to the anticancer drugs [7, 8, 9]. An alternative method for combining hormone and chemotherapy is therefore the attempt to utilize the oestrogens as a "trigger" to start cancer cell proliferation, with the aim of increasing the susceptibility to a subsequently administered CT. In this paper we present our preliminary results on the induction of an oestrogenic recruitment in locally advanced human breast cancer. The following points must be mentioned:

(1) Locally advanced breast cancers have proliferative activity lower than metastatic breast cancer (unpublished personal data). This low proliferative activity can explain the paradox of a large primary tumor without hematogenous metastasis and could imply a high degree of chemoresistance.

(2) We have utilized the PDP-LI as an *in vitro* estimation of tumor growth fraction. Many data, both from human and experimental tumors, lend support to the view that PDP positive cells are actively moving along the cell cycle and the PDP index gives a reliable measure of the GF [14, 15].

The TLI alone can be a fallacious index of tumor proliferation for the study of oestrogenic recruitment. Oestrogens in fact can induce a transient and synchronous entry in S-phase and this short lasting phenomenon can go unobserved if daily repeated samples are not obtained. In fact in human breast cancer, T_s values are about 16–18 h [17]. Moreover most anticancer drugs, while cell cycle specific, are not phase specific and therefore the GF is a more reliable index of kinetic related chemosensitivity than the percentage of S-phase cells. In our series we have in fact observed an increase in TLI after DES only in $\frac{3}{5}$ of the patients, while the PDP-LI peaked in all the patients. These data strongly suggest that in patients Nos 2 and 4 the S-phase entry had occurred earlier than day 3 and therefore was missed to observation. In all cases the CT induced a quick and profound decrease in tumor proliferation; this fact means that the recruited cancer cells have been inhibited and possibly killed by CT and that the size of cell killing was related to the size of cell recruitment. In one case the oestrogen administration was prolonged for 7 days but tumor proliferative activity did not show a further increase after the peak on day 3; however, while the TLI showed a decline after day 3, the PDP-LI remained constantly high. This discrepancy can be explained with a synchronous recruitment into the S-phase induced by oestrogens, followed by an asynchronous proceeding along the mitotic cycle of the proliferating cancer cells. These data further outline the importance of the GF to evaluate tumor proliferation because a single S wave of synchronously recruited cells can be easily missed. The reasons why oestrogens can stimulate receptor negative cells are not yet clear; two hypothesis can be suggested: (1) Oestrogens can selectively recruit a subset of receptor positive cells in a receptor negative tumor or (2) oestrogens do not exert their kinetic stimulation through a direct interaction with cell receptors, but through the production of some growth factor in the host [18]. In conclusion, our results confirm that mammary cancer cells can be recruited into the mitotic cycle following a low dose of oestrogens, even in the case of receptor negative tumors. These recruited cancer cells can be inhibited by subsequently administered CT. Further extended clinical trials are necessary in order to establish the therapeutic effectiveness of oestrogenic recruitment.

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