Chemotherapy following Estrogen-induced Expansion of the Growth Fraction of Human Breast Cancer

P. F. Conte, G. Fraschini, A. Alama, A. Nicolin, E. Corsaro, G. Canavese, R. Rosso, and B. Drewinko

Departments of Medical Oncology [P. F. C., R. R.], Pharmacology [A. A., A. N., E. C.], and Surgery [G. C.], Istituto Nazionale Ricerca Cancro, Genova, Italy, and M. D. Anderson Hospital and Tumor Institute [G. F., B. D.], Houston, Texas 77030

ABSTRACT

We have evaluated the feasibility of a cytokinetically oriented regimen based on the induction of cell recruitment by diethylstilbestrol (DES) in locally advanced human breast cancer. Tumor proliferative activity was evaluated by the thymidine labeling index and the primer-dependent \( \alpha \)-DNA polymerase labeling index, which gives an in vitro estimation of the growth fraction. Sixteen previously untreated patients received DES (1 mg 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 every 21 days. Radical surgery was delayed to allow for three DES-FAC regimens in responsive patients.

Proliferative activity on tumor biopsies was evaluated immediately before and after treatment with DES, 24 h after chemotherapy and, in nine patients, at the time of radical surgery. DES was able to induce a significant increase in thymidine labeling index in 8 of 16 patients, while the primer-dependent \( \alpha \)-DNA polymerase labeling index was significantly increased in 13 of 16 tumors, independently of their estrogen receptor content. Subsequently administered chemotherapy induced an early decrease in tumor proliferation. In the nine patients submitted to surgery after three DES plus FAC courses, the average thymidine labeling index and primer-dependent \( \alpha \)-DNA polymerase labeling index were 27.8 and 73% of the pretreatment values. Our preliminary results provide the rationale for the design of new therapeutic schemes in which antitumor drugs are given at the time of maximally induced tumor cell proliferation. Further extended studies are required to establish whether induction of tumor cell recruitment will actually translate into appreciable improvement of the clinical response to chemotherapy.

INTRODUCTION

Combination chemotherapy has a response rate of about 50–80% in patients with advanced carcinoma of the breast (1–3). Similar response rates are achievable with antihormone therapy alone in the case of ER\(^2\)-positive tumors (4). Because most human tumors of the breast contain various proportions of ER-positive and ER-negative cells, it was conceivable that an association of both therapeutic modalities could increase the overall response rate (5). However, results achieved by such a combination of antihormone and chemotherapy were not superior to those obtained by chemotherapy alone and in fact have been less than additive in terms of complete responses and mean survival times (6–9). These subadditive effects might be explained by mutually excluding mechanisms of action: the endocrine ablation and/or antiestrogen therapy would slow down tumor cell proliferation by inducing accumulation of endocrine-dependent cells in a non-proliferating, quiescent state (10–13), and this maneuver would promote a kinetic sanctuary for the malignant cells that would become refractory to most cytotoxic drugs which exhibit their optimal activity on rapidly growing tumors (14, 15).

Estrogens are known to induce a transient and synchronous increase in the TLI of mammary tumors (16–18) and to induce cell recruitment even in receptor-negative tumors (19). Furthermore, estrogens are capable of reversing the accumulation of human breast carcinoma cells in G\(_1\) phase induced by tamoxifen (20). Therefore, an alternative approach to the unscheduled combination of hormone therapy and chemotherapy might be based on the capacity of estrogen to recruit hormone-responsive cells into the proliferative pool, followed by the administration of chemotherapy at the time of maximally induced tumor cell proliferation.

The present study shows that low-dose DES induces recruitment into cycle of tumor cells of locally advanced human breast cancer, regardless of their estrogen receptor status, and that subsequent inhibition of tumor proliferation can be achieved by properly designed sequential chemotherapy.

MATERIALS AND METHODS

Sixteen patients with locally advanced breast cancer were entered into the study. Criteria for inclusion were: histologically proven diagnosis of breast cancer, no previous therapy, no contraindication to chemotherapy, and informed consent. The experimental plan required at least three biopsies for evaluation of tumor-proliferative activity and the administration of polichemotherapy; therefore, only patients with a large primary tumor (greater than 5 cm in diameter by mammography) and clinically involved axillary nodes were eligible. Four patients had hematogenous metastasis at presentation.

Treatment consisted of DES (1 mg p.o. daily for 3 days) followed by FAC i.v. on day 4 every 21 days. The 3-day period of estrogenic stimulation was selected on the basis of data from literature (19). In one patient (no. 2), DES administration was prolonged for 6 days for the first cycle, and FAC was administered on day 7. Biopsies were performed immediately before and after treatment with DES and 24 h after ending FAC chemotherapy. The first, surgical, biopsy procured enough tumor tissue to also establish histological diagnosis and receptor status; subsequent biopsies were performed with Trucut needles in at least three different areas of the remaining tumor to avoid local variations in proliferative activity (21), and the resultant values were combined. Estrogen-binding capacity was estimated by the dextran-coated charcoal assay (22).
Radical surgery was delayed in 11 patients without distant metastasis and in two patients with bone metastasis who were treated with radiotherapy to their lytic lesions, to allow for three DES+FAC treatments. In nine such patients, histological examination, receptor status, and tumor proliferative activity were also evaluated at the time of surgery. In three additional patients with T4 ductal carcinoma, a surgical biopsy was performed 4 days before radical mastectomy. The TLI and PDP-LI were determined at the time of surgical biopsy and again at radical surgery. No treatment was administered to these patients before surgery.

To determine the TLI, single tumor cell suspensions were obtained by mechanical disaggregation of biopsy specimens followed by serial passages through needles of different bores. Cells were suspended in RPMI, checked for viability with the trypan blue exclusion test, and subdivided into two aliquots. One aliquot was processed for the PDP-LI assay; the remaining cells were suspended in RPMI supplemented with 10% FCS (2 to 3 x 10^6 cells/ml) and incubated for 30 min at 37°C in 5% CO2 with 10 µCi/ml of [3H]thymidine (specific activity, 60-80 Ci/mM; Amersham). The cells were then washed twice in cold saline, cytocentrifuged onto slides, and fixed in methanolic-acetic acid (3:1) for 15 min at room temperature.

Slides were then dipped in Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY), exposed for 24 h at 4°C, and stained with hematoxylin-eosin. At least 500 nuclei were counted for each TLI.

The PDP-LI assay is an autoradiographic method to measure the simultaneous presence of nuclear DNA polymerase and nuclear DNA primer template activity in individual, unfixed cell nuclei (24). This polymerase is present in actively cycling cells independent of their position in the cell cycle and, therefore, the index gives an in vitro estimation of the GF (25, 26). For this purpose, cells were suspended in 0.9% NaCl and cytocentrifuged onto acid-cleaned slides; slides were air dried, dipped vigorously in 0.25% agar solution at 41-42°C, and air dried again. This maneuver disrupts the cytoplasm while leaving the intact nuclei on the slide; the intact nuclei are able to synthesize DNA if α-DNA polymerase, DNA template, and the necessary materials for DNA synthesis are present. A glass ring was affixed to each slide, and the nuclei were incubated for 45 min at 37°C in 5% CO2 with 0.5 ml of a mixture containing: 0.02 M Tris, pH 7.7; 0.081 µM each of deoxycytidine-5'-triphosphate, deoxythymidine-5'-triphosphate, and deoxyguanosine-5'-triphosphate (Sigma Chemical Co., St. Louis, MO); [3H]thymidine (about 4 days later) the corresponding mean values were: TLI, 0.1, 3, 10, and 13) were premenopausal; four patients had distant metastasis to bone or lung. Fourteen tumors were ductal, one was lobular. Estrogen or progestin receptors were low or negative in most patients.

Results of TLI and PDP-LI determinations at diagnosis, after 3 days of DES treatment, 24 h after chemotherapy, and, in nine patients, at the time of radical surgery, are displayed in Table 2. Our initial TLI and PDP-LI values were lower than those reported by others (26), possibly because most of our patients were postmenopausal and had large primary tumors with an anticipated decrease in GF. The PDP-LI was always higher than the TLI, with an average ratio of PDP-TLI of 2.8.

The three "control" patients who did not receive any treatment between biopsy and surgery had a mean basal TLI value of 3.0% (3.1, 1.7, and 4.3, respectively) and a mean basal PDP-LI value of 6.1% (5.0, 5.9, and 7.5, respectively). At the time of surgery (about 4 days later) the corresponding mean values were: TLI, 3.1% (4.1, 1.8, and 3.5); and PDP-LI, 6.1% (6.8, 5.8, and 5.7).

Following DES administration the first ten patients shown in Table 2 showed an increase of both TLI and PDP-LI on day 3 with increments in GF ranging up to 10- and 15-fold, respectively, for patients 1 and 4. In particular, two postmenopausal patients with low ER receptors (patients 2 and 3) had significantly increased TLI and PDP-LI values. Patients 11 and 15 failed to show statistically significant changes in both the TLI and the PDP-LI. For patient 12, only the TLI increased, possibly as an expression of synchronization in S phase without an actual expansion of GF. In the remaining three cases, expansion of the GF was evident by the PDP-LI assay, but the percentage of S-phase cells was not increased. In all patients, chemotherapeutic treatment induced a drop of both TLI and PDP-LI to either close to or markedly below basal values.

In one patient (no. 2), DES was administered continuously for 7 days, and tumor proliferative activity was evaluated on days 0, 1, 3, and 6 and 24 h after chemotherapy given on day 7 (Chart 1). No changes in either TLI or PDP-LI were noted on day 1; on day 3, both the TLI and PDP-LI reached peak values that were maintained until day 6. Chemotherapy induced a rapid drop of both TLI and PDP-LI on day 7.

Thirteen patients, including patients 3 and 11, who achieved a complete remission of their lytic lesions with radiotherapy, underwent radical surgery following 3 cycles of the DES+FAC regimen, and in nine such cases proliferative activity was evaluated at surgery (Chart 2). The average basal TLI and PDP-LI for these patients was 1.8 and 3.7, respectively. After the first course of DES treatment, both values peaked at 4.3 and 8.0 (a

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**RESULTS**

Pertinent clinical characteristics of 16 patients with breast carcinoma are presented in Table 1. Twelve patients were postmenopausal and four (nos. 2, 3, 10, and 13) were premenopausal; four patients had distant metastasis to bone or lung. Fourteen tumors were ductal, one was tubular, and one was lobular. Estrogen or progestin receptors were low or negative in most patients.

Results of TLI and PDP-LI determinations at diagnosis, after...
CHEMOTHERAPY AFTER EXPANSION OF BREAST CANCER GROWTH FRACTION

### Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>TLI (T0)</th>
<th>TLI (T1)</th>
<th>TLI (T2)</th>
<th>TLI (T3)</th>
<th>PDP-LI (T0)</th>
<th>PDP-LI (T1)</th>
<th>PDP-LI (T2)</th>
<th>PDP-LI (T3)</th>
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<td>1</td>
<td>1.8 ± 0.1</td>
<td>11.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
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<td>20.7 ± 0.2</td>
<td>0.9 ± 0.1</td>
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<td>2</td>
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<td>2.1 ± 0.1</td>
<td>0.8 ± 0.4</td>
<td>N/A</td>
<td>1.7 ± 0.1</td>
<td>4.4 ± 0.5</td>
<td>2.4 ± 0.3</td>
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<td>3</td>
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<td>7.0 ± 1.4</td>
<td>3.1 ± 0.7</td>
<td>0.5 ± 0.1</td>
<td>8.1 ± 0.7</td>
<td>14.3 ± 0.6</td>
<td>9.5 ± 0.3</td>
<td>6.3 ± 0.3</td>
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<td>4</td>
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<td>8.8 ± 0.5</td>
<td>3.0 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.8 ± 0.1</td>
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<td>3.7 ± 0.3</td>
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</tr>
<tr>
<td>6</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.1</td>
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<tr>
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<td>1.9 (&gt;0.1)</td>
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<td>3.7 ± 0.3</td>
<td>3.2 ± 0.3</td>
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<tr>
<td>13</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.3</td>
<td>1.4 ± 0.3</td>
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<td>3.5 ± 0.8</td>
<td>6.7 ± 0.5</td>
<td>1.5 ± 0.3</td>
<td>N/A</td>
</tr>
<tr>
<td>14</td>
<td>2.6 ± 0.5</td>
<td>2.1 ± 0.3</td>
<td>N/A</td>
<td>N/A</td>
<td>4.8 ± 0.9</td>
<td>6.4 ± 0.5</td>
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<td>N/A</td>
</tr>
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<td>15</td>
<td>0.5 ± 0.1</td>
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<td>0.8 ± 0.1</td>
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<td>1.9 ± 0.2</td>
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<tr>
<td>16</td>
<td>1.8 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>5.8 ± 0.5</td>
<td>2.6 ± 0.3</td>
<td>7.7 ± 0.5</td>
</tr>
</tbody>
</table>

- *t0*, before therapy; *t1*, after DES; *t2*, after the first cycle of chemotherapy; *t3*, at surgery; N/A, not applicable.
- Mean ± SE.
- Statistically significant against *t0* (range, *P* < 0.05 to *P* < 0.00001).

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**DISCUSSION**

The purpose of our study was to determine the feasibility of a cytokinetic-directed chemotherapeutic regimen with estrogenic recruitment of cells in locally advanced human breast cancer. Experimental evidence to support the feasibility of such cytokinetic-directed chemotherapy in human tumors is very limited, although a few promising results have been reported for acute granulocytic leukemia, multiple myeloma, melanoma, and metastatic breast cancer (21, 27–32).

Dao et al. (19) have shown that physiological doses of estrogen and progesterone increased the TLI in human breast cancer even in the absence of estrogen receptors. However, this observation was not used to design enhanced chemotherapeutic treatments on the basis of such cytokinetic findings. Markaverich greater than 2-fold increase for both the TLI and the PDP-LI). After the first cycle of chemotherapy, the average TLI decreased to 1.7% and the PDP-LI decreased to 3.5%. At the time of radical surgery, performed 3–4 weeks after completion of 3 courses of chemotherapy, both indices were again significantly low (TLI = 0.5 that is, 27.8% of the basal value) and PDP-LI = 2.7 (73% of the basal value).

Early evaluation of clinical responses revealed objective regressions of the bulky breast tumor in all 13 patients (including two patients with distant metastasis). Six patients had less than 50% regressions, five had more than 50% regressions, and two patients demonstrated complete clinical responses. All 13 patients are continuing chemotherapy; 12 are presently disease-free at 2+ to 15+ months after surgery; one patient relapsed at 12 months. Two patients with metastatic disease at diagnosis (nos. 2 and 15) progressed after 3 and 6 months, respectively. These patients were treated also with radiotherapy to the primary breast tumor. One patient (no. 12) responded after 2 courses of DES and FAC. However, she refused further chemotherapy or surgery and was lost to follow-up.

**Chart 1.** Thymidine labeling index (D) and PDP-LI (O) in one breast cancer patient during DES and after chemotherapy (FAC).

**Chart 2.** Average values of TLI (D) and PDP-LI (O) in nine patients with breast cancer before therapy (*t0*), after DES (*t1*), after the first cycle of chemotherapy (*t2*), and at radical surgery (*t3*). Bars, SE. *P*-values of *t2* versus *t0* and *t2* versus *t1* were significant (range, *P* < 0.05 and *P* < 0.001, respectively). *P*-values of *t3* versus *t2* were not significant.
et al. (33) were able to demonstrate that low-dose estrogens can stimulate the growth of the MXT-transplantable mammary tumor and that the combined administration of estrogen and cyclophosphamide resulted in significant inhibition of tumor growth.

To our knowledge, our results represent the first experimental evidence for the possibility of chemotherapy following estrogenic recruitment in human breast cancer. In our series of locally advanced breast cancer, low-dose DES was able to induce a significant increase in TLI in 8 of 16 patients (P ≤ 0.05–≤0.00001), while the GF, as assessed by the PDP-LI, was increased in all but three tumors. The subsequently administered chemotherapy was able to induce an early and sharp decrease in tumor proliferation. The increase in TLI without a parallel rise in PDP-LI, seen for patient 12, could represent a partial synchronization in S phase induced by DES, without an actual expansion of the GF. In patients 13 through 16, an expansion of the GF was evident by the PDP-LI but was not associated with a concordant TLI increase. There are several possible explanations for this discrepancy: (a) the TLI could be a fallacious index of estrogenic recruitment because the hormone would induce a transient and synchronous entry into S phase; such a short-lived phenomenon could be missed if daily samples were not obtained, because the mean duration of the S phase in human breast cancer is reported to be in the order of 15–16 h (26); (b) estrogenic stimulation may induce a simultaneous recruitment of breast cancer cells arrested previously in different phases of the cell cycle; or (c) estrogens may shorten the length of the S phase (34), and the same TLI value might correspond to a higher proportion of rapidly cycling S-phase cells.

It is noteworthy that 13 of 16 tumors showed estrogenic recruitment despite the absence or low content (<10 pmol/g protein) of ER. Thus, it is possible that DES may selectively recruit a subset of ER-positive cells in an ER-negative tumor or that stimulation into cycle is independent of the presence of receptors and perhaps mediated by a putative growth factor produced in the host (35). The first possibility, although quite reasonable, is not supported by our findings, as there was no direct relationship between the level of ER positivity and degree of recruitment. The second possibility is supported by experimental studies showing estrogen dependence on tumor proliferation to be mediated by growth factors in DMBA rat mammary tumors (36) and in cell lines transplanted into animals (37).

Our study suggests that low-dose estrogen induces recruitment of the GF of breast cancer cells independently of their ER receptor content. This observation provides the rationale for the design of new therapeutic schemes in which antitumor drugs given at the time of estrogen-induced tumor cell proliferation might achieve a greater degree of tumor cell kill. Although our preliminary results prove that an expansion of the GF of breast tumors is feasible, the data do not allow definite conclusions on the therapeutic advantage of hormone stimulation, because the number of patients is too small and follow-up is too short. Further studies are required to establish whether induction of tumor cell recruitment will actually translate into an appreciable improvement of the clinical response to chemotherapy and whether similar schemes can be used for ER-positive tumors.

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REFERENCES
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