ABSTRACT

The proportion of single intact viable mouse mammary tumor cells containing estrogen receptor (ER) as determined by 17-fluoresceinated estrone binding and the proportion labeled with [3H]thymidine (LI) have been assessed in the same cell population after either primary tumor removal, radiation, cyclophosphamide, or tamoxifen administration. Subsequent to tumor removal, the increase in LI occurring in a cell population from a residual tumor focus was associated with a concomitant decrease in the proportion of cells demonstrating 17-fluoresceinated estrone binding (fluorescence). As the level of LI in the tumor focus returned to that observed prior to tumor removal, the proportion of ER-containing cells simultaneously reverted to its original value. Following cyclophosphamide administration, there was a decrease in tumor LI and a concomitant elevation in the proportion of fluorescent cells which were dose related. The prolonged depression in LI following radiation was accompanied by a sustained increase in the proportion of ER-containing cells. Thus, the change in ER-containing cells was related to the alteration of the proliferating cell population by the various therapies. The findings support the thesis that fewer cells in the growth fraction of the tumor studied contain ER than in the nonproliferating cell pool. Following tamoxifen administration, a decrease occurred in the proportion of fluorescing cells due to competitive binding. There was no alteration in LI. This observation is not in conflict with the thesis that there is a correlation between ER and LI since the mechanism for reduction in detectable ER is different.

These studies provide additional support to the credibility of the use of 17-fluoresceinated estrone binding for the determination of ER in individual tumor cells. They also indicate the usefulness of the method for obtaining biological information regarding tumor ER which cannot be obtained with the use of conventional biochemical analyses.

INTRODUCTION

Increasing evidence indicates that there is an association between the ER status of a mammary carcinoma and its growth rate. Tumors which are ER negative are less differentiated, grow more rapidly, and have a poorer prognosis than do those which are ER positive. The relationship between tumor cell proliferation, i.e., LI, and ER has been studied by only a few investigators. Both Meyer et al. (5) and Silvestrini et al. (7) noted that there was a correlation between the ER and LI in primary and metastatic breast cancers removed from untreated patients. Tumors with a low proportion of labeled cells had more ER binding sites than did those with a high LI. In all studies, the ER was determined by using biochemical methods which measure the binding of [3H]estradiol or a close analogue with receptor proteins in the cytosol of tumor homogenates. Such methods preclude the assessment of LI and ER in the same cell population and fail to indicate the proportion of tumor cells which contain ER. We have recently reported our experience with the use of 17-FE for the identification of a marker in intact viable single cells obtained from mouse and human breast cancers (2, 4). The proportion of cells with marker (fluorescence) has been related to the ER content of the same tumors determined biochemically. This technique provides a method for determining the proportion of ER-containing cells and permits assessment of both ER and LI in the same cell population.

To our knowledge, no investigations have assessed both kinetics and ER in the same tumor cell population following the use of treatment interventions which result in an increase or decrease in the proportion of labeled tumor cells. This report presents information from studies carried out to determine whether alterations in the LI of tumors are associated with a predictable modification of ER.

MATERIALS AND METHODS

Animals. Female 8- to 12-week-old C3HeB/FeJ mice from The Jackson Laboratory, Bar Harbor, Maine, were used. All animals were housed in separate cages and fed standard mouse chow and water ad libitum.

Tumor. A spontaneous mammary adenocarcinoma which arose in a C3H/He female and has been carried by transfer in C3HeB mice was used. The donor tumor cell suspensions were prepared by mincing tumor fragments with scissors on an 80-mesh nylon screen and washing the cells through the screen with Medium 199. A suspension of 2 x 10^6 viable tumor cells in 0.1 ml Medium 199 was injected s.c. into the left hind leg distal to the popliteal node. In experiments evaluating the effect of primary tumor removal on a residual tumor focus, those tumors are arbitrarily designated as "primary" and tumors resulting from the inoculation of 3 x 10^6 cells into the right hind leg are designated as a "metastatic" tumor or a residual tumor. Primary tumors were removed by amputation of the left tumor-bearing leg. In experiments evaluating the effects of tamoxifen or CY animals bearing a single tumor were used.

Radiation. Mice bearing a tumor on the left hind leg were anesthetized with pentobarbital sodium and shielded with lead so that only the tumor extended beyond the shielding. They received 5000 rads from a 100-kV GE Maxima R100 unit over 31.3 min. Preliminary evaluation indicated that such therapy effectively retarded tumor growth for almost 2 weeks.

CY. The CY was dissolved in distilled water so that the desired amount, 60, 120, or 240 mg/kg, was contained in 0.01 ml/g body weight.
It was administered i.p.

**Tamoxifen ICI 46,474.** Tamoxifen was dissolved in absolute ethanol and diluted with sesame oil so that the desired amount, 2.5 mg, was contained in 0.1 ml oil. The ethanol was evaporated under N2 at 60°. The tamoxifen was injected s.c. at sites distant from the tumor. In the current experiments, it was administered as a single dose, or 2 weekly doses, or 5 daily doses.

**Preparation of Cells for LI and ER.** Cell suspensions were prepared by mincing the tumor in McCoy’s enriched medium with 20% calf serum. The brei was filtered through a stainless steel sieve. Cells were washed with PBS (Grand Island Biological Co., Grand Island, N. Y.) and resuspended in PBS.

**Incubation of Cells with 17-FE.** The ligand used was 1-(4)-fluoresceinylcineolestrone thiocarbamidocarboxylic which consists of a fluorescein moiety coupled to position 17 of estrone. All ligand was prepared by Dr. W. B. Dandliker, University Research Foundation, San Diego, Calif. The stock solution of 17-FE (2 x 10^-3 m) was diluted to 2 x 10^-7 with PBS just prior to its use. Cells were incubated with ligand for 60 min at 37°.

Following incubation, the cells were washed twice with cold PBS (15 min each time) and were cytospun onto slides. Two drops of PBS were added to the cells under a coverslip. Slides were maintained in a cold moist chamber. They were examined with a Zeiss epifluorescence microscope with a 50-watt high-pressure mercury source and appropriate filters.

**Evaluation.** Two or more slides prepared from each cell suspension were counted. A minimum of 500 cells/slide were counted. All slides were evaluated independently by 2 observers. Tumor cells were identified by morphological characteristics.

**LI.** The cells were incubated for 1 hr at 37° with 2 μCi [3H]thymidine per ml (14 to 17 Ci/mu; New England Nuclear) in 2 ml Roswell Park Memorial Institute medium with 20% calf serum. At the end of the incubation period, labeling was terminated by inserting the flask containing the cells into ice. The cells were washed with cold Ca2+- and Mg2+-free Eagle’s minimal essential medium (Grand Island Biological Co.). After washing, the cells were digested in 0.25% Bacto-Trypsin (Difco Laboratories) plus DNAse (0.1 mg/ml) (Sigma Chemical Co.) and incubated for 10 min at 37°. Viability exceeds 90% when this method is used. The cells were centrifuged at 500 x g for 3 to 5 min and resuspended in fetal bovine serum. The cells were cytospun onto the slides and fixed in methanol, washed in distilled H2O, and autoradiographed.

**Autoradiography.** The slides were dipped in liquid photographic emulsion (Kodak NTB-2) at 42°, air dried for 1 hr, and placed in light-tight boxes containing drierite and stored at 4°. In these studies, gold activation autoradiography was used to shorten the exposure period. To intensify the latent image in the photographic emulsion, slides were incubated at 15° in KAuCu solution for 15 to 20 min prior to development. Slides were developed (15 min at 15°) with Kodak amido developer, fixed, washed, air dried, and finally stained. Utilizing this technique, the exposure times were reduced to 1 day. The percent of cells undergoing DNA synthesis (LI) was determined by counting 500 to 1000 cells.

**RESULTS**

**Primary Tumor Removal.** Following tumor removal, an increase in LI occurred in the residual tumor focus (Chart 1). The LI 1 and 2 days following operation was significantly greater (p<0.001) than in unoperated controls. The increase in LI was accompanied by a decrease in the proportion of cells binding 17-FE. By 7 days, both had returned to control values.

**Tumor Radiation.** Following exposure of tumors to 5000 rads the LI 2 days later was markedly decreased (from 16.0 to 5.3%) (Chart 2). The proportion of cells demonstrating DNA synthesis remained depressed during the subsequent time of observation. This was accompanied by a marked increase in the proportion of fluorescent cells which remained elevated during the same time period.

**CY Administration.** A decrease in LI was observed 4 days after CY administration (Chart 3). The reduction was directly related to the amount of drug. At 7 days, the LI was increasing but was still below the level present prior to CY. Conversely, the proportion of fluorescent cells was elevated at 4 days following CY and began to decrease as the LI was found to increase. To relate the proportion of fluorescent cells in tumors to their LI without reference to time of therapy, the LI values of all 64 animals receiving CY (240 mg/kg) who were followed for as long as 21 days were ranked from lowest to highest, and the values were divided into quartiles. The average LI for each quartile was compared with the average proportion of 17-FE-bound cells in the same tumors (Chart 4). Higher LI levels were associated with a lower proportion of fluorescent cells.

**Tamoxifen Administration.** The administration of tamoxifen, either 1, 2, or 5 doses, had no effect on the LI (Chart 5). That value remained relatively constant during the period of observa-
tion. The percentage of fluorescence-positive cells was found to decrease following each of the dose schedules used. During the first 14 days, the findings were similar whether 1, 2, or 5 doses were used; consequently, they are presented as a single group. By 21 days, the proportion of positive cells had returned to the pretherapy level in those receiving only one dose, whereas it was still depressed when 2 or 5 doses were administered.

DISCUSSION

With the advent of methodology for evaluating tumor cytokinetics, considerable information has been obtained regarding the growth characteristics of both primary and metastatic cancers. Few investigators, however, have determined the effect of removal or manipulation of a primary tumor on the kinetics of metastases (3, 6, 8, 9). Our own investigations in that regard (3) have demonstrated that within 24 hr following removal of a primary C3H mammary tumor changes occurred in the kinetics of cells in a distant tumor focus. There was an increase in the LI which persisted for between 7 and 10 days, an increase in primer-dependent DNA polymerase index, and a decrease in the tumor-doubling time. The kinetic changes were reflected in a measurable increase in tumor size which became apparent about a week following tumor removal (3). Absence of an alteration in DNA synthesis time and in cell cycle time indicated that the tumor growth was probably the result of the conversion of noncycling cells in Go into proliferating cells.

In the present study, the changes in LI observed following primary tumor removal are similar to those previously reported by us. An entirely new finding is the observation that the proportion of cells demonstrating 17-FE binding (fluorescence) decreases as the LI increases but returns to the level present prior to tumor removal at the time that the proportion of labeled cells recedes to its initial level. Conversely, when a decrease in tumor LI is observed following radiation or CY administration, there is a concomitant elevation in the proportion of fluorescent cells. The prolonged depression in LI following radiation is associated with a sustained increase in the proportion of ER-containing cells. Findings following CY administration are dose related. The greater the dose of drug, the greater the decrease in LI and the greater the concomitant increase in ER containing cells.

These findings, revealing that there is an inverse relationship between the proportion of DNA-synthesizing cells and the proportion of cells demonstrating fluorescence, indicate that in the mouse mammary tumor the proliferating population of cells contains fewer (or no) ER-containing cells than does the nonproliferating population. Since cells comprising the growth fraction of tumors are more sensitive to radiation and to alkylating agents (CY), it is likely that in these studies there is a selective destruction of proliferating cells following the use of those therapeutic modalities. Only if the cells destroyed do not express ER to the same extent as do cells in Go and Gi would their deletion result in an increase in the proportion of ER-positive cells. Similarly, a decrease in ER-positive cells would be likely to occur only if the proportion of proliferating cells which increases following primary tumor removal contains fewer cells with ER than does the nonproliferating cell pool. Thus, it would seem that the change in the proportion of ER-containing cells is the result of an alteration of the proliferating cell population by the therapies used.

The findings following the administration of tamoxifen indicating no change in LI but a decrease in the proportion of ER-containing cells are not to be considered in conflict with the thesis formulated from the other observations that there is a correlation between ER and LI values. The tamoxifen when administered in vivo or when incubated with tumor cells in vitro prior to 17-FE incubation competitively binds with ER, resulting in a decrease in the percentage of cells displaying fluorescence. The mechanism of reduction in detected ER is thus entirely different from that occurring following primary tumor removal. It has no relationship to changes in LI.

We have previously addressed ourselves (2) to the criticism by some investigators (1) that nonspecific binding by cytochemical methods limits their worth for ER determination. Our prior studies (2) have indicated that the proportion of fluorescing mouse and human breast cancer cells, as well as uterus and liver cells, decreased following in vitro incubation with estradiol and tamoxifen or following administration of tamoxifen prior to tissue removal, findings indicative of 17-FE binding to ER. In addition, a variety of incubation times and temperatures and the use of ligand concentrations between 10^-16 and 10^-5 M failed to alter the proportion of marker-positive cells present in tumor, indicating that nonspecific binding, should it occur as a consequence of high concentrations of ligand, is limited to cells containing ER. The findings from the current studies provide additional support to the credibility of the use of 17-FE for the determination of ER in individual tumor cells. It is extremely unlikely that the specific relationship between the proportions of LI and fluorescing cells could have been so clearly defined unless the ligand was specifically binding to ER. Since the relationships described are in keeping with current concepts regarding ER
and tumor growth, it is entirely reasonable to conclude that the method is indeed depicting ER in individual tumor cells. Aside from the potential value of the use of 17-FE binding to tumor cells for determining patient prognosis or for predicting response to therapy, the current studies indicate the usefulness of the method for obtaining information regarding ER from laboratory investigations which could not be derived by the use of conventional biochemical methods.

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REFERENCES

Interrelation between Tumor Cell Proliferation and 17-Fluoresceinated Estrone Binding following Primary Tumor Removal, Radiation, Cyclophosphamide, or Tamoxifen

Bernard Fisher, Nurten Gunduz and Elizabeth A. Saffer