Tamoxifen-induced Increase in the Potential Doubling Time of MCF-7 Xenografts as Determined by Bromodeoxyuridine Labeling and Flow Cytometry

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ABSTRACT

The anti-estrogen tamoxifen (TAM) is widely used in the therapy of human breast cancer. Shown to induce a G1 transition delay in vitro, the kinetic effects of TAM on breast carcinoma cells growing as tumor xenografts in nude mice have been less well characterized. In this study, we demonstrate a significant increase in the tumor potential doubling time (Tpot) and decrease in the labeling index (%LI) of estradiol (E2)-stimulated MCF-7 xenografts following TAM treatment or E2 deprivation. MCF-7 tumor pieces were transplanted s.c. into nude mice supplemented with Silastic capsules containing E2. After 2–4 weeks, animals were randomized to continued E2 treatment, E2/TAM treatment, or E2 deprivation. At times ranging from 0 to 23 days after treatment, animals were given injections of bromodeoxyuridine and tumors excised for kinetic analysis. Using flow-cytometric techniques, the Tpot and %LI were estimated for all tumors. Seven independent experiments were performed and data pooled for statistical analysis.

At the time of hormonal manipulation, E2-stimulated tumors had a volume doubling time of 5 days, a Tpot of 2.3 days, and a %LI of 23%. Continued E2 treatment resulted in only minimal changes in Tpot and %LI over the remainder of the observation period. Treatment with TAM resulted in a slowing of tumor growth (tumor doubling time, 12 days), a significant (P < 0.001) increase in Tpot to 6.6 days, and a decrease in %LI to 8% by 23 days posttreatment. E2 deprivation resulted in a cessation of tumor growth and similar changes in Tpot and %LI to 5.3 days and 10%, respectively (P < 0.001). In contrast to previous reports, these data demonstrate that TAM treatment and E2 deprivation both significantly decrease tumor cell proliferation in MCF-7 xenografts.

INTRODUCTION

The antiestrogen TAM, has been extensively studied both in the laboratory and in clinical trials. Initially used to treat advanced and metastatic breast cancer, TAM has been demonstrated to provide a clear survival benefit to postmenopausal women with early stage node-negative disease (1–3). Furthermore, most women with early stage node-negative breast cancer may also benefit from TAM administration (2, 4, 5). While initially tested in clinical trials of TAM administration for 6–24 months, several recent trials have clearly demonstrated a survival benefit for continuous TAM administration for up to 5 years (2, 5–7).

In vitro evidence suggests that TAM induces an early to mid-G1 transition delay with a corresponding decrease in the S-phase fraction and reduction in growth fraction (8–10). In contrast, to the data in vitro, there is less consensus on the effects of TAM on the cell cycle kinetics of human breast carcinoma cells in vivo. The administration of TAM to rapidly growing, E2-stimulated xenografts results in a dose-dependent retardation or cessation of tumor growth (11–15).

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3 The abbreviations used are: TAM, tamoxifen; Tpot, potential doubling time; TD, tumor doubling time; %LI, percent labeling index; CI, cell loss factor; 95% confidence intervals; E2, estradiol; BrdUrd, bromodeoxyuridine.
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RESULTS

Tumor Growth and Flow Cytometric Analysis. Tumor growth curves for MCF-7 xenografts maintained in ovariectomized nude mice from each of the 3 experimental groups are shown for a representative experiment in Fig. 1. In animals supplemented with E2, tumor growth rate was exponential with a Tp value of 5 days. As expected, tumor growth rate was decreased by the addition of capsules containing TAM, and was completely arrested by removal of the E2 capsules. The changes in tumor growth resulting from hormonal manipulation were accompanied by alterations in cell proliferation kinetics as measured with BrdUrd labeling and flow cytometric analysis (Fig. 2). The bivariate contour plot of red fluorescence (DNA content) versus green fluorescence (BrdUrd content) for an E2-treated tumor labeled with BrdUrd and excised on Day 0 demonstrates a large proportion of cells with increased green fluorescence corresponding to BrdUrd incorporation during the DNA synthesis phase of the cell cycle. Since BrdUrd is only available for incorporation immediately after injection, %LI can be estimated from the proportion of cells with elevated green fluorescence (19). In this example, the %LI was approximately 25%. Bivariate plots for tumors labeled 7 to 11 days after hormonal manipulation demonstrate that there is minimal change in the green fluorescence distribution with continued E2 treatment, but a marked decrease with TAM treatment. An even larger decrease was seen in E2-deprived tumors.

Changes in Tp. The labeling index and Tp were calculated from the flow cytometry data collected for tumors excised at time points from 0 to 23 days after hormonal manipulation (data not shown) to establish the magnitude and temporal pattern of change in Tp and %LI indices induced by such treatment. The measured points for Tp as a function of time are shown in Fig. 3 for each of the 3 experimental groups. Comparisons among the 3 treatment groups were based on the curves shown, which were fitted to the raw data as described in "Statistical Methods." In Fig. 3, CI are represented by the dashed curves. At the time the tumor bearing animals were randomized to their respective treatment groups (Day 0), a mean baseline Tp value of 2.3 days (95% CI: 1.9, 2.8) was measured. Continued treatment with E2 alone resulted in a gradual increase in Tp over time, such that 23 days later, the mean Tp value had increased to 3.4 days (95% CI: 2.7, 4.2). The addition of TAM resulted in a more pronounced increase in Tp, which reached a value of 6.6 days (95% CI: 5.3, 8.3) 23 days after TAM addition. The Tp values after TAM treatment became significantly greater than the Tp values for E2-stimulated tumors within 4 days of implantation of the TAM capsules and gradually increased over the remainder of the observation period. In contrast to TAM treatment, E2 deprivation resulted in a steep initial increase in Tp and rapid establishment of a new steady state Tp value of approximately 5.3 days (95% CI: 4.4, 6.4). The mean Tp value for tumors in the E2-deprived group was significantly different (P < 0.01) from those of both the E2- and E2/TAM-treated groups within 4 days of hormonal treatment and remained significantly different from the E2 group at all later time points analyzed (P < 0.001). However, beyond 7 days posttreatment, the Tp values calculated for the E2-deprived and TAM-treated groups were not significantly different. The major difference between these 2 treatment groups is the rapid initial rate of increase in the Tp values observed following E2 deprivation.

Changes in Labeling Index. Corresponding changes in labeling index were observed for all 3 groups as shown in Fig. 4. The mean %LI for E2-stimulated tumors at the start of treatment was calculated to be 23% (95% CI: 20%, 27%) on the basis of the proportion of BrdUrd-labeled cells. Consistent with the tumor growth data, E2-treated tumors exhibited a gradual decrease in %LI with time to 16% (95% CI: 13%, 21%) by Day 23. Over a similar time period, TAM treatment resulted in a more rapid decline in %LI to 8% (95% CI: 6%, 11%) . E2 deprivation produced a steep decline in %LI with rapid establishment of a new steady state of 10% (95% CI: 9%, 12%) at 23 days after removal of E2. Again, %LI associated with E2 deprivation or TAM treatment were significantly different from those for E2-stimulated tumors for all time points beyond 4 days (P < 0.005). The curves describing the change in %LI over time for all 3 groups were significantly different from each other (P < 0.001).

Cell Loss. An approximate cell loss factor (\( \phi \)) can be calculated for treated tumors from the relationship: \( \phi = 1 - T_{\text{pot}}/T_D \) (22). Based on a minimum of 20,000 ungated events were recorded. In the analysis, doublets and clumps were excluded by gating on the DNA pulse width versus pulse area displays. Analysis of the data was performed with Lysis II Software (Beckton-Dickinson).

Four populations of nuclei were gated on the bivariate distribution of green height (BrdUrd-fluorescein isothiocyanate) versus red area (propidium iodide) fluorescence (see Fig. 2): unlabeled G1, unlabeled G2/M, BrdUrd-labeled cells that had passed through mitosis during the postlabel interval, and BrdUrd-labeled cells that had not (18). The mean fluorescence channels for the G1, G2/M, and BrdUrd-labeled, undivided populations and the fraction of divided and undivided BrdUrd-labeled cells were then determined.

\( T_{\text{pot}} \) measurements were made following calculation of the relative movement parameter for each sample as described by Begg et al. (19) and modified by Carlton et al. (20). Details of the methods utilized for the determination of \( T_{\text{pot}} \) have been described in detail by others (18-20). The validity of this method for the determination of \( T_{\text{pot}} \) in the MCF-7 xenograft model has been reported by Ritter et al. (18). The %LI was approximated as the fraction of BrdUrd-labeled cells divided by the total population of cells.

Statistical Methods. \( T_{\text{pot}} \) and %LI values from 7 different experiments were fit to a nonlinear function of the general form: \( \log(\text{response}) = \log(a(1 + b \exp(-kD))) \), where \( D \) is the number of days after each animal was exposed to treatment (i.e., addition of TAM or removal of E2), \( k \) is a rate constant, and \( a \) and \( b \) are constants. A weighted nonlinear regression was used with weights proportional to the average number of observations at each time point. Overall differences between treatments were compared using a likelihood ratio test for the improvement of a model containing separate curves for the 2 treatments over a model in which one curve was fit to both treatments. Tests for differences at a particular number of days of exposure to treatment were calculated using linear approximate estimates for the variability of the parameter estimates (21).
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Fig. 2. Bivariate contours of log green fluorescence (BrdUrd content) versus red fluorescence (DNA content) for individual tumors exposed to continuous E2 treatment and analyzed on Day 0 and Day 7 following hormonal manipulation (top left and right), E2 and TAM treatment analyzed on Day 11 (bottom left), and E2 deprivation analyzed on Day 7 (bottom right). All contours plotted at 13% probability.

upon $T_D$ and $T_{pot}$ values measured in these experiments, $\phi$ was estimated as 0.32 and 0.45 for E2-stimulated and E2/TAM-treated tumors, respectively, at 23 days after hormonal manipulation. The decreased growth rate in E2/TAM-treated tumors relative to E2-stimulated tumors, therefore, reflects a decrease in the proportion of cells actively cycling as well as an increase in the rate of cell death. Despite similar $T_{pot}$ values measured in xenografts maintained in E2-deprived or E2/TAM-treated animals, TAM-treated tumors continued to grow while tumors in the E2-deprived group actually decreased in volume. These data suggest that E2 deprivation results in an even higher rate of cell loss than E2/TAM treatment. $\phi$ for regressing, E2-deprived tumors is by definition in excess of 100%.

DISCUSSION

The present study demonstrates a significant effect of TAM treatment or E2 deprivation on tumor growth kinetics in MCF-7 human breast carcinoma xenografts. Consistent with previous experience, E2 stimulation supported exponential tumor growth, while the addition of TAM or the withdrawal of E2 resulted in significant reductions in the tumor growth rate. Treatment with E2/TAM resulted in an approximate doubling of the $T_{pot}$ and a corresponding decrease in the $%LI$ as compared to E2-stimulated tumors. E2-deprived tumors demonstrated similar changes in $T_{pot}$ and $%LI$. While both groups were significantly different from E2-stimulated tumors at all time points beyond 4 days, E2-deprived and E2/TAM-treated tumors differed from each other only for the first 4 to 7 days after hormonal manipulation.

Although cell cycle data derived from studies in vitro demonstrate a consistent reduction in the proportion of cycling cells after incubation of MCF-7 cells with TAM, analogous xenograft studies have not enjoyed a similar consensus. Kute et al. (16) examined the cell cycle distribution in MCF-7 xenografts treated with estradiol or estradiol followed by TAM. Tumors were grown from a cell suspension injected s.c. in nude mice implanted with 0.5 mg E2 pellets. Using flow cytometry, the percentage of cells in $G_1$, $S$, and $G_2/M$ were estimated.

For E2-treated animals, the distribution was 73, 11.3, and 15.0%, respectively. Removal of the E2 pellet and treatment with TAM resulted in an increase in $G_1$ to 84.6% and a corresponding decrease in $S$ and $G_2/M$ to 7.5 and 7.9%, respectively. In a series of experiments using identically treated mice, Osborne et al. (11) demonstrated a significant decrease in mitotic index in E2-deprived and E2-deprived/TAM-treated animals. The increase in $T_{pot}$ and the reduction in $%LI$ observed in the present study are consistent with the results of these 2 studies.

Contrasting results were reported by Brunner et al. (17), who examined the effect of TAM treatment on MCF-7 kinetics by flow cytometry and the percent labeled mitosis technique. $T_{pot}$ and $%LI$ were determined for the 3 treatment groups: 1.0 mg E2, 0.1 mg E2, and 0.1 mg E2 followed by 0.1 mg TAM daily. There was no significant difference in $T_{pot}$ or $%LI$ among these 3 groups—$T_{pot}$: 4.5, 4.3, and 4.0 days; and $%LI$: 14, 12, and 15%, respectively. Likewise, flow-cytometric analysis failed to reveal any significant differences in the distribution of cells through the cell cycle. Consequently, Brunner et al. (17) argued that an increase in cell loss was the predominant tumor effect of TAM in the MCF-7 xenograft model and was solely responsible for the changes in tumor growth observed in TAM-treated animals.

The discrepancies between the results reported by Brunner et al. (17) and the other studies in vivo, including our own, are attributable to differences in E2 delivery and also to the different growth charac-

![Diagram](image_url)
The Tpnl values for the E2-depot-treated tumors reported by Brunner et al. (11) and is, in all likelihood, the tumor growth rates observed in the E2 treatment groups of this study for E2-deprived MCF-7 xenografts. Collectively, the data suggest that the failure to detect an effect of TAM treatment on the cell production rate and Tpnl measurements in the Brunner study reflect the already slow growth of the tumors in animals treated with low dose E2-depot injections.

In addition to slowing the cell production rate, TAM treatment also resulted in an increase in cell loss rate in the present series of experiments. By comparison to E2-stimulated tumors, E2/TAM treatment resulted in an approximate 41% increase in cell loss rate. An even greater increase in cell loss was detected in E2-deprived tumors. This dual effect of hormonal manipulation on tumor proliferation and cell death has also been observed in other hormonally dependent tumor systems. Kyprianou et al. (23) demonstrated a complete suppression of mitotic division and a 9-fold increase in apoptosis in PC-82 prostate adenocarcinoma xenografts following androgen ablation. Kyprianou et al. (24) recently examined a similar effect of hormonal manipulation on cell loss in MCF-7 xenografts when they demonstrated that E2 deprivation in MCF-7 xenografts resulted in a doubling of the daily percentage of cells undergoing programmed cell death, or apoptosis, as compared to E2-stimulated tumors. These findings are in agreement with the data presented in the present report, suggestive that TAM treatment or E2 deprivation significantly affects both cell birth rate and cell loss.

The demonstration that TAM can increase the potential doubling time of an estrogen receptor-positive breast tumor in vivo has potential clinical implications. TAM is commonly administered during fractionated radiotherapy and chemotherapy regimens for early stage breast cancer. There is some concern that by slowing tumor proliferation and decreasing the growth fraction, TAM may potentially reduce the cytotoxic effectiveness of these modalities. Although TAM has been shown to antagonize the cytotoxic effects of several chemotherapeutic agents in vitro, there is no direct evidence that this antagonism is due to alterations in cell kinetics (25, 26). Furthermore, there is no conclusive clinical evidence that concomitant TAM administration during non-melphalan-containing chemotherapy regimens or during fractionated radiotherapy has deleterious effects (27–33). Thus, at present, there appears to be no evidence that TAM administration during chemotherapy or radiation therapy has significant deleterious effects.

In conclusion, we have demonstrated that the tumor cell kinetics of MCF-7 human breast carcinoma xenografts grown in ovariectomized female nude mice can be significantly altered by hormonal manipulation. TAM treatment or E2 deprivation resulted in an approximate doubling of the Tpnl and an approximately 40% reduction in labeling index as compared to E2-stimulated tumors. An increase in cell loss rate was calculated for both TAM treatment and E2 deprivation.

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REFERENCES


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