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Report

4-Hydroxytamoxifen, an active metabolite of tamoxifen, does not alter the radiation sensitivity of MCF-7 breast carcinoma cells irradiated *in vitro*

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Summary

The effect of 4-hydroxytamoxifen (4OH-TAM), the potent anti-estrogenic metabolite of tamoxifen, on the radiosensitivity of MCF-7 cells irradiated *in vitro* was determined. Radiation dose response curves were generated for MCF-7 cells maintained and irradiated in phenol red-free medium containing 10^{-10} M estradiol (E_2) with or without 10^{-7} M 4OH-TAM. Immediately after irradiation cells were transferred to medium containing 10^{-10} M E_2 supplemented with bovine serum to stimulate colony formation. Estradiol-stimulated cell proliferation was inhibited by 10^{-7} M 4OH-TAM, but radiation sensitivity was not significantly altered ($p > 0.3$). Continued incubation in the absence of E_2 for an additional 24 hours after irradiation likewise failed to alter the radiosensitivity of 4OH-TAM-treated MCF-7 cells. These studies indicate that growth-inhibitory concentrations of the anti-estrogen 4OH-TAM do not modify the *in vitro* radiation sensitivity of this line of human breast carcinoma cells.

Introduction

The non-steroidal anti-estrogen tamoxifen (TAM) has been demonstrated to provide a definite survival advantage to postmenopausal women with early stage node-positive breast cancer and also provides a similar benefit to women with early stage node-negative disease [1–5]. Extensive experimentation with human breast carcinoma cells maintained in tissue culture or as tumor xenografts in nude mice strongly suggests that TAM acts as a cytostatic agent causing a G_1 transition delay, a decrease in the growth fraction, and a prolongation of the potential doubling time of breast carcinoma xenografts [6–9].

In conventional treatment settings, TAM therapy is initiated shortly after surgery and is frequently continued during and after adjuvant therapy, including systemic chemotherapy and fractionated irradiation. The demonstration that TAM exerts cytostatic effects on estrogen-receptor positive (ER+) breast cancer cells has spawned concerns that a TAM-induced decrease in the rate of cell proliferation may diminish the effectiveness of ionizing radiation by increasing cellular radioresistance. Recent reports by Wazer *et al.* [10, 11] demonstrating that the radiation responsiveness of estrogen-receptor positive MCF-7 breast cancer cells *in vitro* was significantly reduced by TAM exposure have heightened these concerns of possible antagonistic effects of TAM on radiation response. The resultant increase in survival was manifest as a wider shoulder region on the survival curve, a change which

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over the course of a typical thirty fraction treatment regimen could result in a reduction of greater than six logs of cell kill. If true, this would seriously compromise the probability of local control.

However, for those studies MCF-7 cultures were grown, drug-treated, and/or irradiated in medium containing phenol red and fetal bovine serum, two sources of exogenous estrogenic compounds which could complicate interpretation of the resultant radiation survival curves. If TAM-associated radioprotection does indeed occur, it could result in a significant deleterious effect on radiation response. We therefore re-examined the effect of anti-estrogen treatment on the radiosensitivity of MCF-7 cells maintained under hormonally defined conditions and utilizing 4-hydroxytamoxifen (4OH-TAM), a potent metabolite of TAM. This anti-estrogenic agent displays the same pharmacologic activity as TAM, has a higher binding affinity for the estrogen-receptor, and is nearly two-orders of magnitude more potent [12]. For these reasons, it is ubiquitously used as a model compound for laboratory studies examining the anti-estrogenic actions of TAM.

Material and methods

Cell culture

The estrogen receptor-positive MCF-7 cell line was originally obtained from Dr. Dean Edwards (University of Texas Health Science Center at San Antonio, San Antonio, TX) who obtained the stock from the Michigan Cancer Foundation [13]. Stock cultures of MCF-7 breast carcinoma cells were routinely maintained in phenol red-containing minimum essential medium supplemented with 5% (*v/v*) calf serum, L-glutamine (0.29 mg/ml), and bovine insulin (6 ng/ml). The medium was buffered with 0.35 g $NaHCO_3$/l and 25 mM N-hydroxyethylpiperazine-N′-2-ethane sulfonic acid. In the following descriptions this medium is referred to as 'complete medium'. Cultures were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37° C. The cells were negative for *Mycoplasma* contamination.

Drug treatment

Estradiol (E_2; Sigma Chemical Co.) and 4OH-TAM (ICI Pharmaceuticals, Macclesfield, England) were prepared as concentrated stocks in 100% ethanol and diluted 1 : 1000 (v/v) into the culture medium. Final ethanol concentration in the media was 0.1%. At this concentration the vehicle did not modify plating efficiency or radiosensitivity.

Growth response assay

The effects of E_2 and the anti-estrogen 4OH-TAM on the growth of MCF-7 cells were assayed as previously described [14]. Briefly, cells were plated into 24-well dishes at a concentration of 14,000 cells per well in complete medium. Twenty-four hours later, the medium was replaced with phenol red-free medium containing 5% (v/v) dextran-coated charcoal-stripped calf serum (SCS). Cells were maintained in this estrogen-deficient medium for 4 days and then cultured for an additional 5 days in similar medium containing E_2 with or without various concentrations of 4OH-TAM. Cells were then harvested for cell number determination by DNA assay. Each well was treated with 1 ml of hypotonic, 0.1 × calcium-magnesium-free Hank's balanced salt solution and sonicated for 12 sec/well with a Kontes ultrasonic cell disruptor. Samples (50–100 µl) from each well were then removed, incubated with Hoescht 33258 according to LaBarca and Paigen [15], and analyzed on a SLM-Aminco Fluoro-Colorimeter III (SLM Instruments, Inc., American Instrument Company, Urbana, IL).

Radiation treatment

Prior to each experiment, MCF-7 cells were plated into 162 cm^2 flasks in phenol red-free medium supplemented with steroid-stripped calf serum and 10^{-12} M E_2. Twenty-four hours later the medium was removed and replaced with phenol red-free medium supplemented with 5% SCS. Cells were maintained in this estrogen-deprived condition for a total of four days before the addition of E_2 (10^{-10} M)

and 4OH-TAM (10^{-7} M) in fresh medium. After the addition of the compounds, the cultures were maintained for an additional 5 days. Medium was changed every other day. At the conclusion of this incubation period, cells were harvested from the flasks and allocated to individual treatment groups for irradiation.

Cells were exposed at 4° C to X-rays from a Philips 250 kVp source operating with a 0.2 mm Cu filter in place at a dose rate of 2.00 Gy/min. Prior to and after irradiation, flasks containing cells were kept on ice until the cells were counted and plated for survival determination by conventional clonogenic assay. To permit colony formation, cells were plated in phenol red-free medium supplemented with 10^{-10} M E_2 and 5% SCS. Estradiol-stimulated cells and one group of 4OH-TAM-treated cells were plated into this medium immediately after irradiation. A second group of 4OH-TAM-treated cells was maintained in a non-proliferative state in phenol red-free medium with 5% SCS for a 24 hour period after irradiation. At the conclusion of this interval, E_2 was added directly to each plate to a final concentration of 10^{-10} M.

A feeder cell effect was detected in control experiments (data not shown). Consequently, 10^5 lethally-irradiated cells were added to each plate. Two weeks after plating, colonies consisting of > 50 cells were counted. Survival was expressed as the ratio of plating efficiency of individual treatment groups to that of the unirradiated control.

Statistical analysis

Each radiation dose response curve was fitted to a linear quadratic model of the form: $\ln S(D) = \theta - \alpha D - \beta D^2$, where D is radiation dose in grays (Gy), S(D) is the proportion of cells surviving at dose D, and θ is an estimate of lnS at zero dose. The α and β values were then used as data in a multivariate analysis of variance to test for differences among the treatment groups. The reported p-value was calculated by a Wilk's Lambda analysis [16].

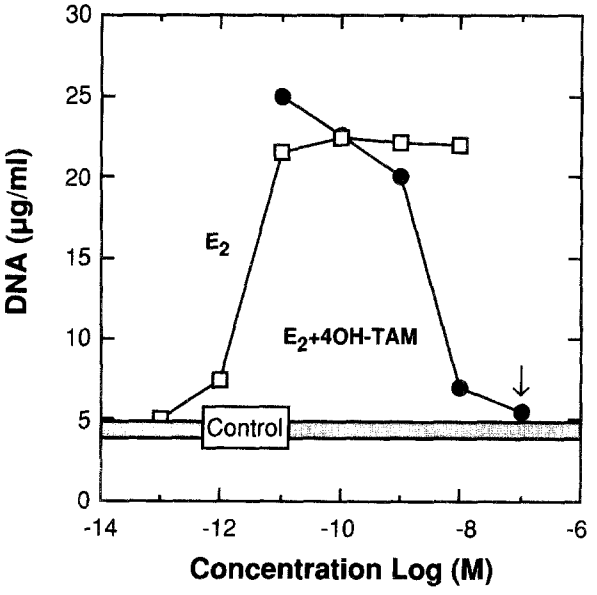

Fig. 1. Results of a representative proliferation experiment illustrating the effects of increased E_2 concentration on proliferation of MCF-7 cells (open squares) and the inhibitory effect of increasing concentrations of 4OH-TAM on E_2 (10^{-10} M) stimulated growth (filled circles). The arrow indicates the concentration of 4OH-TAM used in the radiation experiments.

Results

Prior to initiation of radiation studies, experiments were designed to identify the doses of E_2 and 4OH-TAM required to stimulate cell growth of MCF-7 cells or inhibit E_2-stimulated growth, respectively. As shown in Fig. 1, the addition of increasing concentrations of E_2 resulted in an increase in cell number, manifest in this assay as an increase in amount of DNA recovered per well. Proliferation was maximal at an E_2 concentration of 10^{-10} M. Consequently, this concentration was selected for all subsequent experiments. Also shown in Fig. 1 is the effect of various concentrations of 4OH-TAM on the proliferation of MCF-7 cells maintained in medium containing 10^{-10} M E_2. E_2-stimulated growth was completely inhibited when 10^{-7} M 4OH-TAM was included in the medium. Based upon these studies, 10^{-7} M 4OH-TAM was selected as a growth-inhibitory dose for use in combination with 10^{-10} M E_2 for the radiation experiments.

The radiation dose response curve for E_2-stimulated, exponentially growing MCF-7 cells exposed to X-rays and immediately plated into growth-stim-

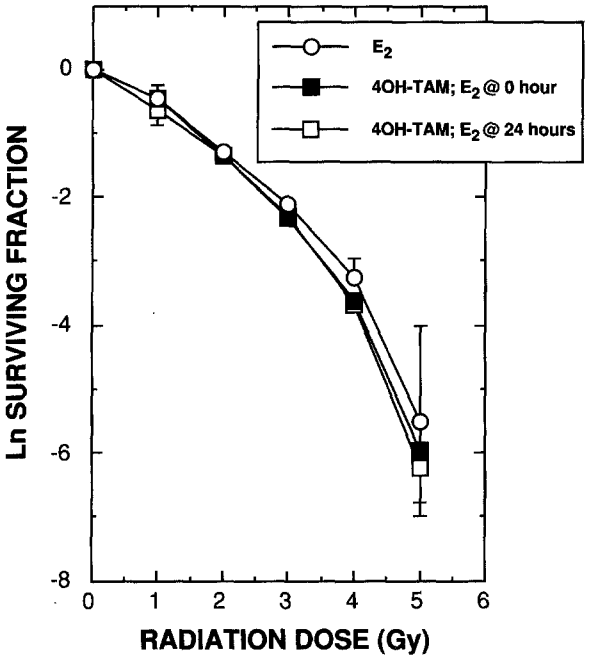

Fig. 2. Radiation dose response curves for MCF-7 cells irradiated in the presence of 10^{-10} M E_2 in the absence (○) or presence of 10^{-7} M 4OH-TAM. Cells treated with 4OH-TAM were plated for survival assay either immediately (■) or 24 hours after (□) irradiation. Error bars represent standard deviations of three independent determinations for each treatment condition.

ulating medium is shown in Fig. 2. Based on survival data from three independent determinations, the best fit to the linear quadratic equation, $\ln S(D) = \theta - \alpha D - \beta D^2$, resulted in values of 0.317 Gy^{-1} (± 0.10; SEM) and 0.143 Gy^{-2} (± 0.03) for α and β, respectively. The corresponding α and β parameters for MCF-7 cells maintained in the presence of 10^{-7} M 4OH-TAM prior to radiation exposure and then plated directly into E_2-containing medium for colony formation were 0.302 Gy^{-1} (± 0.06) and 0.168 (± 0.017) Gy^{-2}, respectively. The radiation response of cells from these two treatment groups did not differ significantly.

Placement of 4OH-TAM-treated MCF-7 cells into medium containing growth-stimulatory concentrations of E_2 immediately after irradiation could potentially negate any protective effect resulting from the cytostatic action of the anti-estrogen by promoting recruitment of the growth-inhibited population into active proliferation. Such recruitment might minimize the time available for damage repair prior to DNA synthesis. In order to evaluate this possibility, one group of MCF-7 cells was cultured and irradiated in the presence of 4OH-TAM but maintained in E_2-free medium for 24 hours after irradiation. Cell proliferation was then stimulated by the addition of E_2 to a final concentration of 10^{-10} M. As shown in Fig. 2, this 24 hour delay between the time of irradiation and the mitogenic stimulus did not significantly alter radiation response. The α value for this treatment group was 0.424 (± 0.083) Gy^{-1} while β was 0.143 (± 0.025) Gy^{-2}. Of particular interest is the clear lack of a difference in survival at 2.0 Gy (Fig. 2), the most commonly used dose per fraction in radiotherapy. In summary, no significant differences in radiation sensitivity were detected by multivariate analysis of α and β values for the three treatment groups ($p > 0.3$).

Discussion

Treatment conditions which inhibit or retard the progress of cells through the cell cycle have been reported to reduce the radiosensitivity of affected populations [17]. It has been hypothesized that this protective effect is attributable to a greater opportunity for damage repair in non-proliferating cells, presumably related to a delay in damage fixation associated with DNA synthesis. Consequently, the use of cytostatic agents might be considered ill-advised in circumstances were maximal radiation response is desirable, as would be the case for the treatment of solid tumors with radiation. The anti-estrogen TAM is an effective cytostatic agent which is commonly administered during the course of fractionated radiation for the treatment of estrogen receptor positive (ER+) human breast cancer [1–5]. If TAM does indeed reduce the radiation sensitivity of ER+ breast carcinoma cell populations, then concommitant administration of TAM during fractionated radiation could adversely affect treatment outcome. Particularly disconcerting in this regard, then, are the reports by Wazer *et al.* that growth-inhibitory concentrations of TAM reduce the radiation sensitivity of hormone responsive MCF-7 breast carcinoma cells *in vitro* [10, 11]. The altered radiation sensitivity was reported to be predomin-

ately due to a change in the shoulder, or low dose region, of the survival curve, at doses typical of those used clinically. According to the survival parameters included in their paper, the change in sensitivity for TAM-treated cells would result in a reduction in excess of 6 logs of cell kill over the course of a thirty fraction treatment. Clearly, then, the alleged TAM-associated radioprotection could have profound implications for breast cancer therapy.

However, several studies published in recent years indicate that not all cytostatic therapies alter the radiation responsiveness of targeted cells. Of particular relevance to the situation with TAM, are studies indicating that certain hormonal manipulations can result in growth inhibition without influencing radiosensitivity [18, 19]. This information, combined with concerns about culture conditions employed in previous studies examining the effect of TAM on radiation sensitivity, prompted us to re-examine the relationship between anti-estrogen induced growth inhibition and radiation response. It should be noted that the experimental conditions employed in the current series of experiments differ considerably from those used by Wazer and colleagues [10, 11]. Paramount among these differences is the fact that in the present experiments MCF-7 cells were maintained in E_2-deficient, phenol red-free medium supplemented with calf serum which had been charcoal-stripped to remove extraneous sources of estrogens. Furthermore, cell proliferation was inhibited by the addition of the potent metabolite 4OH-TAM, as opposed to the parent compound. 4OH-TAM, a more proximate form of the active metabolite of TAM, is approximately two orders of magnitude more potent in terms of growth-inhibitory properties than TAM itself [12] and is commonly used in the laboratory to examine the anti-estrogenic effects of triphenylethylenes, including TAM. The use of this metabolite does not invalidate comparison between the two studies, since in both cases growth-inhibitory concentrations of the respective anti-estrogen were selected for study.

In contrast to previous reports, no significant differences were observed in radiosensitivity for E_2-stimulated or 4OH-TAM-inhibited cultures plated into growth-stimulating conditions immediately after irradiation or following an additional 24 hours in estrogen-free conditions. Clearly, under defined hormonal conditions consistent with the recommended procedure for growing MCF-7 cells *in vitro* [20], no protective effect of the active TAM-metabolite, 4OH-TAM, was observed. Although in their latest report Wazer *et al.* reported a slight survival advantage for TAM-treated MCF-7 cells based upon analysis of single dose radiation survival curves, no differences in the extent of DNA damage, DNA repair, or split-dose recovery was detected between these cells and their E_2-stimulated counterparts. The similarity in response observed for the two treatment groups following split-dose irradiation and the reported difference in survival at low doses would appear to be somewhat contradictory. Judging from the response curves presented, larger recovery ratios would be expected of the TAM-treated cells. However, similar recovery ratios were measured, suggesting that E_2-stimulated and TAM-inhibited cells have quite similar radiosensitivities. In fact, the authors do conclude that the difference in radiosensitivity between the two groups of cells is so small as to be of questionable biological significance. Despite other inherent differences, in this regard our two studies do not disagree.

Our results are also consistent with similar studies with thyroid epithelial cells in culture [19] and proliferating or quiescent rat mammary epithelial cells hormonally manipulated *in vivo* prior to irradiation [18]. In both these other studies, proliferation-inhibiting hormonal manipulations failed to alter the radiosensitivity of the target cell populations. Collectively these data can be interpreted as suggesting that inhibition of proliferation *per se* does not necessarily lead to altered radio-responsiveness. In this regard, it is interesting that Wazer and colleagues recently reported that inhibition of proliferation induced in MCF-7 cells by incubation with high concentrations of E_2 likewise does not alter radiosensitivity [21].

These results have specific implications not only for the combined use of TAM and fractionated radiation for the treatment of breast cancer, but also for the potential application of cytostatic agents to reduce repopulation during radiotherapy in general. In so far as the *in vitro* data with MCF-7 cells main-

tained under hormonally defined growth conditions can be extrapolated to the clinical situation, admittedly a risky exercise, no alteration in responsiveness would be expected following TAM exposure. Although no clinical trials have been designed to specifically address the effect of concurrent TAM on response to conventional radiotherapy, results from clinical trials which included treatment arms with and without TAM suggest that no deleterious consequences accompanied TAM treatment [22–24]. There is, therefore, no compelling evidence to suggest that TAM administration during radiotherapy is contraindicated.

The use of cytostatic agents to inhibit tumor cell proliferation during a course of radiotherapy has been suggested as an approach to minimize the impact of repopulation occurring during treatment [25]. However, simple calculations reveal that the potential benefit of this strategy would be easily negated if the agent(s) being used also decreased radiosensitivity, even modestly. Since it is generally believed that inhibition of proliferation would result in increased radioresistance, the wisdom of this approach has been challenged. However, the current study, as well as several others, indicate that decreased radiosensitivity and reduced proliferative status are not necessarily linked. Therefore, even though reducing proliferation rate is not likely to offer any benefit for the treatment of human breast cancer because of the normally slow proliferation rates typical of these tumors [26], the 4OH-TAM/radiation experiments reported here suggest that this approach may be feasible for rapidly proliferating tumors, such as head and neck malignancies, if effective cytostatic agents can be identified.

In conclusion, the active metabolite of TAM, 4OH-TAM, did not significantly alter the radiation sensitivity of MCF-7 cells maintained *in vitro* under hormonally-defined conditions. These results suggest that inhibition of proliferation by select cytostatic agents may not be accompanied by increased radioresistance, thereby establishing the feasibility of using these agents to inhibit cellular repopulation in rapidly proliferating tumors during the course of fractionated radiotherapy.

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