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Tamoxifen Stimulation of Human Breast Cancer Cell Proliferation *in Vitro*: a Possible Model for Tamoxifen Tumour Flare

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Abstract—*When T-47D human breast cancer cells were grown in medium supplemented with foetal calf serum (FCS) which had been depleted of endogenous oestradiol (E_2) by dextran-charcoal treatment (CS-FCS) tamoxifen and E_2 had biphasic effects on cellular proliferation. At lower concentrations E_2 and, to a lesser extent, tamoxifen stimulated cell proliferation, with maximal effect at 10^{-9} M, and with corresponding increases in the % S phase cells; this was not seen with medium containing untreated FCS. At higher doses tamoxifen ($1–1.5 \times 10^{-5}$ M) and E_2 (1.5×10^{-5} M) in the presence of CS-FCS inhibited cell proliferation. These observations may explain features of the clinical phenomenon of tamoxifen-induced tumour flare: tamoxifen may temporarily exert a weak oestrogen agonist activity on the tumour as its concentration gradually increases after initiation of treatment; further, patients with tamoxifen-induced tumour flare often experience a remission with continued administration of the drug, presumably as the intratumour concentration of tamoxifen continues to accumulate to an inhibitory level.*

INTRODUCTION

THE ANTIOESTROGEN tamoxifen has been used in the treatment of human breast cancer for more than a decade [1]. In the mid-1970s the phenomenon of 'tamoxifen flare' was first described [2–4]. This term was used to describe a sudden, and at times severe, increase in skeletal pain usually seen shortly after the initiation of therapy and/or an increase in the size of existing lesions or the appearance of new lesions, especially in the skin. In a number of patients a sudden increase in the level of carcinoembryonic antigen was also seen [3]. In cases where tamoxifen therapy was continued despite the occurrence of tumour flare, subsequent tumour regression often occurred [3, 4]. In a recent study of tamoxifen with or without fluoxymesterone in postmenopausal women it was found that approximately one patient in five on both regimens experienced tumour flare [5]. The authors commented that the clinical severity and potential for life-threatening progression of these reactions presented a difficult problem in deciding whether tamoxifen treatment should be continued. It has also been reported that tumours progressing during tamoxifen treatment may regress following discontinuation of treatment [6]. No mechanism has ever been documented to explain either of these phenomena.

Studies with established breast carcinoma cell lines in many laboratories including our own have shown inhibition of cellular proliferation due to tamoxifen and other antioestrogens [7–16]. This inhibition was accompanied by a dose-dependent decrease in the percentage of S-phase cells and a concomitant increase in the percentage of G_0/G_1 phase cells [13–16]. In a recent study from this laboratory a range of factors affecting the response of the oestrogen receptor (ER)-containing T-47D human breast cancer cell line [17] to tamoxifen were investigated and it was observed that a small but significant increase in proliferation rate occurred when cells grown in medium containing 10% charcoal-treated foetal calf serum (CS-FCS) were treated with 100 nM tamoxifen [18]. In the present study this observation was investigated further.

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It is reported here that when T-47D cells were grown in medium containing 10% CS-FCS both tamoxifen and 17β-oestradiol (E_2) had a biphasic effect on cellular proliferation, with stimulation at lower doses and inhibition at higher doses, and it is proposed that these findings may provide an explanation for the hitherto unexplained phenomenon of 'tamoxifen flare,' and tamoxifen withdrawal response.

MATERIALS AND METHODS

Cells

T-47D cells in their 74th passage were supplied by E.G. and G. Mason Research Institute, Worcester, MA, U.S.A., for the National Cancer Institute Breast Cancer Program Cell Culture Bank. Stock cultures were maintained as previously described [18] in Roswell Park Memorial Institute 1640 medium supplemented with 20 mM HEPES buffer, 13 mM sodium bicarbonate, 5 mM L-glutamine, 20 μg/ml gentamicin, 10 μg/ml insulin and 10% foetal calf serum (FCS). Cells used in the experiments described in this paper were from passages 109–120. Experiments were carried out using either the medium described above or medium in which CS-FCS (prepared as described previously [18]) was substituted for FCS. In the latter case, unless otherwise specified, the cells were maintained in medium containing 5% CS-FCS for two passages prior to the experiment, in view of evidence of prolonged retention of E_2 by breast cancer cells in culture [19]. One batch of CS-FCS was used throughout.

Tamoxifen and estradiol treatment

Tamoxifen (*trans*-1-(4β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene) and E_2 were obtained and prepared as stock solutions in ethanol as described previously [14]. The drugs were added to medium containing 10% serum (either FCS or CS-FCS) to give a final ethanol concentration of 0.1%. Cells (5×10^4) were inoculated into 25-cm^2 flasks in 8 ml medium containing tamoxifen, E_2 or ethanol vehicle. The flasks were harvested by trypsinization after 3–9 days, counted using either a haemocytometer with phase-contrast microscopy or a Coulter counter model ZBI (Coulter Electronics, Harpenden, Herts., U.K.) and analysed by DNA flow cytometry.

Flow cytometry

Cells were stained for DNA with ethidium bromide/mithramycin, subjected to flow cytometry as previously described [20] and the resulting DNA histograms analysed planimetrically [21].

RESULTS

After an initial lag, control cells grew exponentially in medium containing 10% CS-FCS between days 3 and 9 with a mean doubling time of 45 hr (Fig. 1), but in medium containing 10% FCS there was no appreciable lag and the mean doubling time was 33 hr (data not shown). After 9 days of treatment 10^{-8} M E_2 caused a 1.8-fold increase in cell numbers over control ($P < 0.005$) and 10^{-8} M tamoxifen caused a lesser increase ($P = 0.05$). At 10^{-5} M tamoxifen caused marked inhibition of cellular proliferation, and 1.5×10^{-5} M tamoxifen caused almost complete cytostasis (Fig. 1).

The dose-relationship of these effects was investigated further (Fig. 2A), and it was observed that maximal stimulation of T-47D cell growth occurred at a concentration of 10^{-9} M for both tamoxifen and E_2, with E_2-induced stimulation of growth always tending to be greater than that seen with tamoxifen. The inhibition seen with higher doses of these drugs, however, was significantly greater with tamoxifen than E_2 (Fig. 2A).

In contrast, cells grown in medium with untreated FCS were not significantly stimulated by either tamoxifen or E_2, but tamoxifen $\geqslant 10^{-6}$ M caused a significant decline in cell numbers relative to control, as did $E_2 \geqslant 10^{-5}$ M (Fig. 2B).

Cell cycle kinetic analysis of cells grown in medium with CS-FCS yielded results which mostly paralleled the effects on cell proliferation, with maximal stimulation of % S phase cells being seen with 10^{-9} M tamoxifen and 10^{-9} M–10^{-6} M E_2 (Fig. 3).

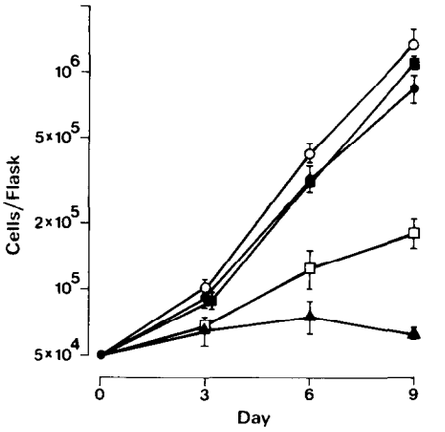

Fig. 1. Effects of tamoxifen and E_2 on the proliferation of T-47D cells in medium containing 10% CS-FCS. 5×10^4 cells from exponentially growing stock cultures were inoculated into 25-cm^2 flasks in 8 ml medium containing 10% CS-FCS and tamoxifen or E_2. Flasks were harvested and counted at days 3, 6 and 9. Data points, mean ±S.E.M. (n = 3) for control (●), 10^{-8} M E_2 (○), and 10^{-8} M (■), 10^{-5} M (□) and 1.5×10^{-5} M tamoxifen (▲).

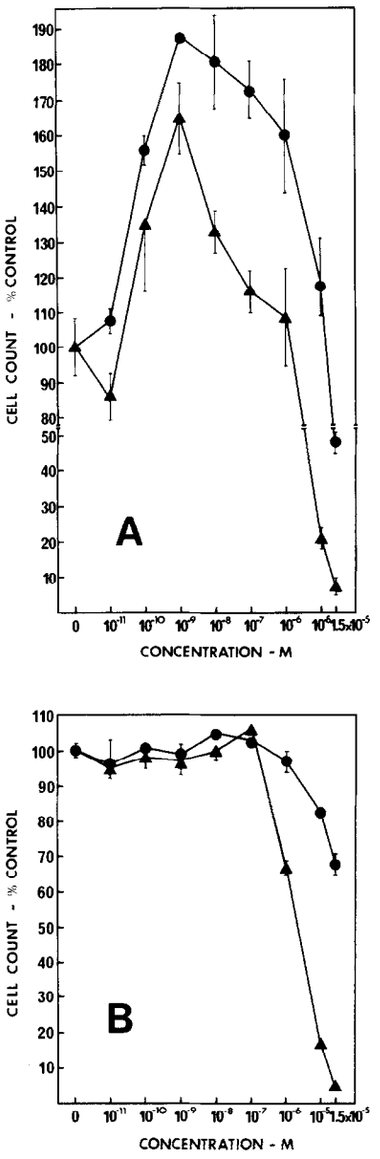

Fig. 2. Effects of tamoxifen and E_2 on the proliferation of T-47D cells in medium containing 10% CS-FCS (A) or 10% FCS (B). Cells were grown as described for Fig. 1 in medium containing 10^{-11} M–1.5 × 10^{-5} M tamoxifen (▲) or E_2 (●). Flasks were harvested and counted after 9 (A) or 7 (B) days. Data points, mean ± S.E.M. [n = 3 (A); n = 4 (B)]. Where error bars are not shown S.E.M. was less than the size of the symbol.

There was, however, some dissociation between the effects on cell proliferation and % S phase at higher doses, since 10^{-5} M tamoxifen and 1.5 × 10^{-5} M E_2 both caused significant decreases in cell numbers accompanied by no significant change (10^{-5} M tamoxifen) or a significant increase (1.5 × 10^{-5} M E_2) in % S phase cells. At the highest tamoxifen dose tested (1.5 × 10^{-5} M) there was a marked decrease in both cell number and % S phase cells (Fig. 3).

Stimulation of T-47D proliferation by tamoxifen in E_2-depleted medium was reproducible in that it was seen in 12 separate experiments, but the maximal increase in cell numbers varied from 10% to more than 100%. One source of this variability in the extent of stimulation is shown in Fig. 4, where it is demonstrated that the length of exposure to CS-FCS affected the stimulatory response to both E_2 and tamoxifen. Cultures passaged weekly for 3 weeks in medium containing CS-FCS were stimulated to a significantly greater extent than those passaged for 5 or 7 weeks under these conditions.

DISCUSSION

When ER-containing T-47D human mammary carcinoma cells were grown *in vitro* in medium depleted of E_2 by dextran–charcoal treatment of the FCS, stimulation of cell proliferation by added E_2 and, to a lesser extent, tamoxifen occurred. The presence of FCS with its endogenous E_2 abolished this response to both E_2 and tamoxifen (Fig. 2A and B). These results confirm a recent report from this laboratory that E_2 is mitogenic for this cell line [18], and are in agreement with the oestrogen stimulation seen in one of two cloned T-47D sublines [22] and in contrast with the oestrogen resistance seen in a variant T-47D line [23]. The results are consistent with tamoxifen having oestrogen agonist activity which tended to be weaker than that of E_2 under the conditions of this study.

Maximal stimulation of MCF-7, ZR-75-1 and CAMA-1 ER-positive breast cancer cells has also been seen to occur with 10^{-10} M–10^{-8} M E_2 [12, 24, 25]. Interestingly, the maximal stimulation of proliferation seen with both tamoxifen and E_2 in T-47D cells occurred at the same concentration,

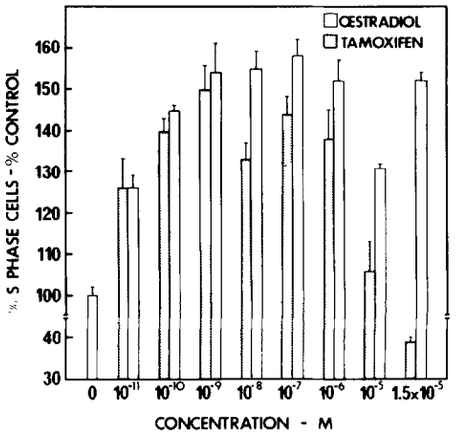

Fig. 3. Effects of tamoxifen and E_2 on the cell cycle kinetics of T-47D cells grown in medium containing 10% CS-FCS. Cells were grown as described for Fig. 1, harvested after 7 days and then subjected to DNA flow cytometry as described in Materials and Methods. Data points, % S phase cells as % control, mean ± S.E.M. (n = 3).

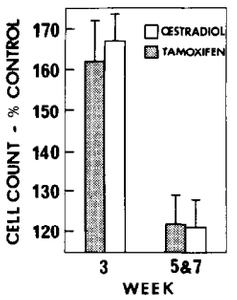

Fig. 4. Effects on E_2- and tamoxifen-induced T-47D cell proliferation of length of prior exposure to medium containing CS-FCS. Cells were passaged weekly for 3, 5 or 7 weeks in medium containing 5% CS-FCS then inoculated at 5 × 10^4 cells/25 cm^2 flask in 8 ml medium containing 10% CS-FCS and 10^{-8} M tamoxifen or E_2, or 0.1% ethanol vehicle. Flasks were harvested and counted after 8 days. Results were pooled for cells passaged for 5 or 7 weeks. Columns, mean ± S.E.M. (n = 3–7).

viz. 10^{-9} M (Fig. 2A), although the affinity of E_2 for ER has been shown to be more than 10-fold greater than that of tamoxifen [13, 26]. It is possible that this may be explained by different extents of metabolism of E_2 and tamoxifen; no data to confirm this are available for T-47D cells, but MCF-7 cells have been shown to metabolize tamoxifen to a negligible extent [10, 27] and to metabolize E_2 rapidly [28]. Another possible explanation would be the different extents of binding of E_2 and tamoxifen to intracellular or serum proteins.

The magnitude of the mitogenic effects of tamoxifen and E_2 on T-47D cells showed similar decreases with increasing time of culture in medium containing CS-FCS (Fig. 4), consistent with both agents acting through a common mechanism. Presumably dextran-charcoal treatment of FCS removes a factor responsible for maintenance of the ability of these cells to proliferate in response to E_2 and tamoxifen. E_2-induced increases in proliferation of CAMA-1, ZR-75-1 and MCF-7 cells have recently been found to diminish with increasing time of prior exposure to E_2-depleted media [24, 25, 29, 30]. Page and co-workers interpreted their results with MCF-7 cells as indicating that serum contains a factor (or factors) which can influence the expression of the growth response to E_2 and tentatively identified it as having an apparent molecular weight of 300–1000 by gel filtration [30].

The observation that antioestrogens appeared to stimulate the growth of some breast cancers *in vivo* in humans [2, 3] and in animals [31, 32] raised the possibility that antioestrogens might have oestrogen agonist activity in such tumours under certain circumstances. This appeared to be partially confirmed by studies in which biopsies of human breast tumours were obtained before and after treatment with tamoxifen and analysed for progesterone receptor and ER content [33, 34]. Waseda *et al.* interpreted their results as indicating that tamoxifen had short-term oestrogenic effects, e.g. in inducing progesterone receptors, but long-term antioestrogenic effects [33]. Studies with human breast cancer *in vitro*, however, had shown that tamoxifen effects on progesterone receptor induction were dissociated from effects on cellular proliferation: in MCF 7 cells, tamoxifen had a biphasic effect on progesterone receptor but was only inhibitory to cell growth throughout the tested concentration range [10].

In the present study both E_2 and tamoxifen had biphasic effects, with stimulation of growth occurring at lower doses and inhibition of growth at higher doses. Such a biphasic effect of oestrogen has been documented previously for the MCF 7 cell line [12, 35, 36], but to our knowledge a biphasic effect of tamoxifen on cell proliferation *in vitro* has not been described before. In ER-containing human breast cancer cell lines tamoxifen and its metabolites have been found to be either inhibitory only [8–10, 12–16] or stimulatory only [37] to cell proliferation.

Such a biphasic action of tamoxifen is not confined to T-47D cells as it has also been seen in MCF 7 cells grown in medium containing CS-FCS [Reddel and Sutherland, unpublished observations]. It is proposed that this observation may provide an *in vitro* model of the clinical phenomenon of tamoxifen tumour flare [2–5], and tamoxifen withdrawal response [6].

It has been shown that with standard clinical tamoxifen dosage regimens the serum level of tamoxifen takes about 4 weeks to reach plateau levels and that of the quantitatively most important metabolite, *N*-desmethyltamoxifen, takes about 8 weeks [38]. The pharmacokinetics of tamoxifen accumulation within tumours have not been reported. All of the reported cases of tamoxifen tumour flare which we have been able to identify where the menopausal status was specified were in postmenopausal patients [2–5], a parallel, perhaps, to the E_2-depleted conditions in which tamoxifen stimulation of breast cancer cell growth occurred *in vitro*. In the situation of clinical tamoxifen tumour flare, presumably as the tamoxifen level is slowly rising the tumour is exposed initially to tamoxifen concentrations which are mitogenic. In patients where tamoxifen treatment was continued despite the occurrence of tumour flare, tumour regression often occurred subsequently [3, 4] as, we would suggest, the drug accumulated to an inhibitory concentration. It may be predicted that the rapid

attainment of inhibitory concentrations of tamoxifen within the tumour, e.g. with tamoxifen loading doses [39, 40], might abolish or decrease the likelihood of flare.

The ability of tamoxifen to stimulate breast cancer cell proliferation under certain circumstances may also explain the clinical phenomenon of tamoxifen withdrawal response [6]. Tumours stimulated by the oestrogen agonist properties of tamoxifen may undergo slower growth or regression on withdrawal of the drug.

Inhibitory doses of E_2 and tamoxifen had differing effects on T-47D cell cycle kinetics (Fig. 3). Previous studies in this laboratory showed that tamoxifen causes a decrease in % S phase and, to a lesser extent, G_2 + M phase cells, and a corresponding increase in % G_0/G_1 cells in ER-containing breast cancer lines [13–16, 18]. In contrast, 1.5×10^{-5} M E_2 caused more than 50% reduction in cell numbers together with a significant increase in % S phase cells (Fig. 2A and 3). It appears, therefore, that the mechanism of action of high-dose E_2 may be distinct from that of tamoxifen; this is currently being investigated in our laboratory.

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