ABSTRACT

The effects of trans-4-hydroxytamoxifen (OHTam) on proliferation of cells of the Ishikawa human endometrial adenocarcinoma line were studied under serum-free, phenol red-free conditions and compared to those of estradiol. The addition of OHTam (1 μM) to basal medium (BM), consisting of equal parts of Dulbecco's modified Eagle's medium and Ham's F-12 with additional glutamine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, resulted in significant increases in cell numbers relative to controls. These effects were even greater than those obtained with estradiol (10 nM to 1 μM) or 1% charcoal-treated fetal bovine serum (ctFBS). Addition of 1% ctFBS to BM containing 1 μM OHTam further increased cell numbers whereas addition of estradiol (10 nM) did not do so. The stimulation of growth was positively correlated with OHTam concentrations in the range of 10 nM to 1 μM. Dissociation of estradiol and OHTam proliferative effects was observed in a variant of Ishikawa cells in which estradiol did not increase proliferation while OHTam had a strong stimulatory effect.

The growth-promoting effects of OHTam were also observed in BM containing 5% or 15% ctFBS. In contrast, in parallel experiments in which BM was replaced by minimal essential medium (Eagle's) with Earle's salts, OHTam (1 μM) did not stimulate proliferation under these conditions and acted as an antiestrogen, inhibiting the proliferative effects of estradiol. These results illustrate marked effects of medium composition on proliferation and antiestrogenic actions of OHTam.

Alkaline phosphatase activity was strongly stimulated by estradiol (10 nM) but only very weakly affected by OHTam (1 μM); at these concentrations, OHTam inhibited the effect of estradiol, both in serum-free BM and in minimal essential medium plus 15% ctFBS, demonstrating dissociation in its actions on proliferation and on enzymatic activity.

These findings suggest that OHTam may stimulate the proliferation of particular clones of endometrial cancer cells in human tumors. They also suggest that OHTam can exert effects not mediated by the estrogen receptor system, or form OHTam-estrogen receptor agonistic complexes unlike those resulting from estradiol-estrogen receptor interactions. Clearly, Ishikawa cells provide a useful model to investigate mechanisms of action of antiestrogens.

INTRODUCTION

The antiproliferative effects of Tam, the nonsteroidal antiestrogen widely used in the treatment of breast cancer, has been demonstrated by several investigators using human mammary cancer cell lines (1–3). However, the mechanism of its action is not well understood.

Progesterins are the first choice for hormonal therapy in endometrial carcinoma, but Tam has also been proposed as a therapeutic agent for this disease in postmenopausal patients (4, 5). Previous studies have shown that its active 4-hydroxylated metabolite (OHTam) can antagonize the proliferative effect of estradiol on human adenocarcinoma cells of the Ishikawa line (6). This cell line, established by Nishida et al. (7), was shown to respond to estrogen by increasing progesterone receptor levels (6) as well as AlkP and DNA polymerase α activities (8, 9), effects that could also be suppressed by OHTam. Cells used for these previously reported studies were maintained and tested in MEM containing 15% ctFBS and phenol red, a pH indicator with weak estrogenic activity (10). In other studies carried out in this laboratory, it was shown that Ishikawa cells proliferate and respond to estrogens in serum-free, phenol red-free BM, consisting of equal parts of Dulbecco's modified Eagle's medium and Ham's F-12, with additional 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and l-glutamine. Unexpectedly, when the effects of OHTam on Ishikawa cell proliferation were tested under these conditions, a stimulation of proliferation even greater than that of estradiol was noted. This observation led to the study that we are now reporting.

MATERIALS AND METHODS

Ishikawa cells were maintained in MEM plus 15% FBS and, in order to remove estrogenic components, the medium was changed to phenol red-free MEM plus 15% charcoal-treated FBS for at least 1 week before using cells for experiments. Treatment of FBS with dextran-coated charcoal to remove steroid hormones was carried out as described elsewhere (6). Cells were routinely tested for Mycoplasma with the Gen-Probe Mycoplasma T.C.II Rapid Detection System (Gen-Probe, San Diego, CA) and found to be negative. For growth studies, cells were seeded in BM or MEM plus 1% ctFBS (no differences were found in attachment efficiency) and were equally distributed in several dishes at a density of 0.5 million/dish (6-cm diameter). Basal medium was prepared by adding to a phenol red-free mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, 1:1 (specially prepared by Flow Laboratories, McLean, VA), 10 mM l-glutamine, 1% antibiotic-antimycotic mixture (GIBCO), and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. One day after seeding, the medium in each dish was replaced by BM containing test compounds, ctFBS or, to serve as control, 0.1% vehicle (ethanol) and thereafter changed every other day. For each condition, cells from 3 dishes were counted in a Neubauer-Levy hemacytometer under an inverted light microscope. For studies of cell proliferation in MEM with 15% ctFBS, cells were seeded at a density of 0.25 million/dish in the presence of test compounds or vehicle.

Alkaline phosphatase activity was determined by the method involving the measurement of p-nitrophenol formed by hydrolysis of p-nitrophenyl phosphate. p-Nitrophenol levels were measured spectrophotometrically at 400 nm, pH 10.4, as described in detail elsewhere (11).

trans-4-Hydroxytamoxifen was a gift from Stuart Pharmaceuticals, Division of Imperial Chemical Industries, Wilmington, DE.

RESULTS

Effects of OHTam, Estradiol, and ctFBS on Cell Numbers. Fig. 1 shows a growth curve of Ishikawa cells in serum-free BM...
and illustrates the effects of OHTam and estradiol on cell numbers. As reported in more detail separately, estradiol (10 nM) stimulates growth in the absence of serum. Surprisingly, OHTam (1 µM) increased cell numbers to levels higher than those obtained in BM with 10 nM estradiol or 1% ctFBS (Figs. 1 and 2). A similar stimulation of growth by OHTam was observed in all of 10 additional experiments. Evaluation by the Student t test indicated that the effects of both estradiol and OHTam were significant as early as 5 days after addition of the compounds. Cell numbers obtained with mixtures of estradiol (10 nM) and OHTam (1 µM) were not significantly different from those resulting from exposure to OHTam alone (Fig. 1).

As indicated in Fig. 2, the addition of 1% ctFBS also stimulated cell proliferation, but the effect was smaller than that obtained with 1 µM OHTam. In contrast to the lack of additivity of estradiol and OHTam action, the effects of mixtures of ctFBS (1%) and OHTam (1 µM) were significantly greater than their individual effect (Fig. 2).

Fig. 3 shows concentration dependence in the action of OHTam. Cell numbers in cultures containing 100 nM OHTam were significantly higher than those in control dishes beginning 8 days after addition of the drug; in the presence of 1 µM OHTam cell numbers were significantly higher than controls on day 2 and thereafter. The results from parallel experiments carried out with estradiol and OHTam at various concentrations, shown in Fig. 4, indicate that at high concentrations of OHTam (1 µM), the effect was greater than the maximal stimulation obtained with estradiol.

In order to evaluate the influence of medium composition on OHTam actions, we also examined the effects of 1 µM OHTam and 10 nM estradiol in phenol red-free MEM at different concentrations of ctFBS. As shown in Fig. 5, OHTam in BM plus 5% ctFBS stimulated cell proliferation whereas in MEM plus 5% ctFBS it failed to elevate cell numbers and inhibited the stimulatory effects of estradiol. The medium dependence of OHTam effects on cell proliferation was also observed at 15% ctFBS levels (Table 1, Experiments 8 and 9). The antiestrogenic action of OHTam on Ishikawa cell proliferation observed in MEM containing 15% ctFBS agrees with our previously reported findings.

Dissociation of Estradiol and OHTam Effects on Cell Proliferation. A culture of Ishikawa cells maintained in phenol red-free MEM plus 15% ctFBS for 3 months lost the proliferative response to estradiol but, as shown in Fig. 6, the cells retained responsiveness to OHTam, revealing dissociation of the agonistic effects of estradiol and the triphenylethylene compound.

Effects of OHTam and Estradiol on AlkP Activity. The effects of OHTam (1 µM) and estradiol (10 nM) on AlkP activity were tested under culture conditions in which the drug was shown either to increase proliferation (serum-free BM) or be antiestrogenic (MEM plus 15% ctFBS) (Table 1). As shown in Fig. 7, OHTam in serum-free BM plus 5% ctFBS it failed to elevate cell numbers and inhibited the stimulatory effects of estradiol. The medium dependence of OHTam effects on cell proliferation was also observed at 15% ctFBS levels (Table 1). The antiestrogenic action of OHTam on Ishikawa cell proliferation observed in MEM containing 15% ctFBS agrees with our previously reported findings.

DISCUSSION

The results obtained in this study demonstrate that OHTam can stimulate cell proliferation in a human endometrial cancer
The effect of OHTam on proliferation of Ishikawa cells in BM was significantly greater than controls on day 2 and thereafter ($P < 0.05$). Cell numbers in the presence of 100 nM OHTam were significantly greater than controls on days 8 and 10 ($P < 0.05$).

Fig. 3. Concentration dependence of the effects of OHTam on proliferation of Ishikawa cells in BM. Cells were plated and maintained as described in the legend to Fig. 1. Cell densities achieved in the presence of 1 $\mu$M OHTam were significantly greater than controls on day 2 and thereafter ($P < 0.05$). Cell numbers in the presence of 100 nM OHTam were significantly greater than controls on days 8 and 10 ($P < 0.05$).

Fig. 4. Dose response to OHTam and estradiol. Cells were seeded and maintained as described in the legend to Fig. 1. The effects on cell numbers of OHTam and estradiol at concentrations ranging from 1 to 1000 nM in serum-free BM were compared after 7 days of treatment in parallel experiments. Although at a 10 nM concentration estradiol was more potent than OHTam, the opposite was true at 100 and 1000 nM concentrations. It is noteworthy that the effect of OHTam at 1 $\mu$M was twice the maximal effect obtained with estradiol. Bars, SD.

Fig. 5. Influence of medium on the effects of OHTam and estradiol ($E_2$). Cells were seeded in MEM or BM containing 5% ctfBS and maintained as described in the legend to Fig. 1. The effects on cell numbers of OHTam were significant on days 8 and 10 ($P < 0.05$). The differences between control and estradiol values were not significant.

Fig. 6. Effects of estradiol ($E_2$) and OHTam on proliferation of an estradiol-unresponsive variant of Ishikawa cells in BM. Cells were plated in BM and maintained as described in the legend to Fig. 1. OHTam increased cell numbers in the estradiol-unresponsive cells. The effects of OHTam were significant on days 4, 7, and 10 ($P < 0.05$). The differences between control and estradiol values were not significant.

Fig. 7. Effects of estradiol ($E_2$) and OHTam on AlkP activity in Ishikawa cells. Cells (2-3 $\times$ 10$^6$) were plated in 10-cm dishes using either MEM plus 15% ctfBS or serum-free BM containing the compounds indicated. Cells were incubated for 4 days and media were replaced 2 days after seeding. Under both conditions, estradiol markedly stimulated AlkP activity and OHTam antagonized the stimulatory effect of estradiol.

The dissociation of proliferative effects of estradiol and OHTam in an estradiol-unresponsive variant of Ishikawa cells cultured in serum-free BM (Fig. 6) appears to contradict the common assumption that OHTam acts by competition with estradiol for a common ER. Some variants of MCF-7 cells also show discrepancies between estradiol and Tam effects, such as Tam resistance associated with responsiveness to estradiol (18) or inhibition of proliferation by Tam in estradiol-unresponsive cells (12). These observations suggest direct effects of Tam and OHTam on cell proliferation by mechanisms which may not involve the estrogen receptor system. The weak stimulation by OHTam of AlkP activity, a sensitive marker for estrogenic effects, and its antagonistic effect on the action of estradiol on the activity of the enzyme in BM (Fig. 7) also indicate that the effect of OHTam on proliferation occurs by a different mechanism than its action on AlkP activity and may not involve the estrogen receptor.

Another puzzling finding in these studies relates to the influence of the medium on proliferative responses. Namely,
OHTam stimulated cell proliferation in BM whereas in MEM, at the same concentration of ctFBS, it did not do so. These results indicate that medium components influence the action of OHTam.

The possibility that the effects of Tam and OHTam are mediated by interaction with specific antiestrogen binding sites, showing high affinity for Tam and OHTam but not for estradiol (19), remains unproved. It should be considered, however, that Tam or OHTam at 1 µM concentrations interact with other receptors, e.g., histaminergic (20) or dopaminergic (21) receptors, influence calmodulin action (22) and may regulate phosphatidylinositol hydrolysis, consequently affecting intracellular levels and distribution of Ca²⁺, known to modify protein kinase C activity either directly or by altering diacylglycerol levels (23). If OHTam does not exert its action on Ishikawa cell proliferation by interacting with the ER, its mechanism of action remains unidentified. Estradiol and OHTam may even exert different changes on ER due to differences in their interaction with the receptor (24). The activation of receptors, usually considered to involve conformational changes, may be influenced by the medium.

A mediation of transforming growth factor β on the actions of OHTam, suggested by the results obtained with MCF-7 cells (25), is unlikely in our system since OHTam enhances Ishikawa cell proliferation while transforming growth factor β inhibits it.⁵

The relevance of our findings to a suggested treatment of endometrial cancer with Tam is unclear since the results are influenced by the composition of the culture medium. It is also questionable whether the concentrations of OHTam to which the cells were exposed in these experiments (10 nM–1 µM) are reached during administration of Tam to patients. However, Ishikawa cells may be representative of clones present in endometrial tumors which could proliferate more rapidly in the presence of Tam or OHTam in vivo. Certainly Ishikawa cells provide a useful model to study mechanisms of action of estrogens and antiestrogens.

REFERENCES

Stimulatory Effects of 4-Hydroxytamoxifen on Proliferation of Human Endometrial Adenocarcinoma Cells (Ishikawa Line)


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