Interaction of Phenol Red with Estrogenic and Antiestrogenic Action on Growth of Human Breast Cancer Cells ZR-75-1 and T-47-D

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ABSTRACT

Studies reported here confirm that the pH indicator, phenol red, acts as a weak estrogen and reexamine the significance of estrogenic and antiestrogenic effects on growth of the human breast cancer cell lines ZR-75-1 and T-47-D in the absence of phenol red. Removal of phenol red reduces but does not immediately eliminate cell growth in the absence of estradiol. Basal cell growth can be reduced for T-47-D cells and eliminated for ZR-75-1 cells by prior growth in the absence of steroid and phenol red for 3 weeks, demonstrating that estrogens can have long lasting effects on cells in culture (termed "steroid memory") and that there exist both cells which are responsive (T-47-D) and dependent (ZR-75-1) on estradiol for growth. Antiestrogen action in these cell lines is affected by at least four parameters: (a) presence of phenol red; (b) time in culture; (c) cell density; (d) steroid memory effects. At high cell density, antiestrogens suppress phenol red-stimulated activity but have little effect in the absence of phenol red. However, at low cell density in the absence of phenol red, tamoxifen has a biphasic action: initial weak stimulation, later inhibition. trans-Hydroxytamoxifen does not stimulate but inhibition increases with time in culture. Following deprivation for 3 weeks of phenol red and steroid, antiestrogen action on ZR-75-1 cells at low density became much more inhibitory. Such responses to antiestrogens are discussed in relation to possible autocrine/paracrine growth regulation of the cells. Clinical relevance is suggested.

INTRODUCTION

It is generally agreed that steroid hormones regulate growth of both normal mammary cells (1, 2) and a variety of breast cancer cell lines (3-7) in culture, although undoubtedly other components can interact (8-10). Most attention has been paid to estrogen stimulation of the MCF-7 cell line (6) where differences are evident between sublines (11, 12) but it is generally agreed that these cells are estrogen-stimulated in vivo (13). However, proliferation of ZR-75-1 (3) and T-47-D (4) cells is also regulated by steroids. Recently, it has been shown that the pH indicator, phenol red, which is present in all culture media, can act as a weak estrogen, and that at the concentration found in tissue culture media can cause stimulation of both cell proliferation and progesterone receptor levels in estrogen-responsive MCF-7 cells (14). These observations have caused much concern as to the significance of previous studies in tissue culture utilizing estrogen-responsive cell lines and in particular of conclusions about basal cell growth without added estrogens.

Antiestrogens are compounds that inhibit the effects of estrogens although they can have effects in their own right (10, 15) and are clinically important in endocrine therapy of breast cancer (16). Tamoxifen has both agonist and antagonist effects on breast cancer cell growth in vitro when studies are carried out in the presence of phenol red (17, 18). However, it now appears that such compounds act by antagonizing phenol red-stimulated proliferation in MCF-7 cells and have no effect in the absence of phenol red (14, 19). To date, the only inhibitory effects of antiestrogens themselves on MCF-7 cell proliferation in phenol red-free medium has been when growth is stimulated by insulin or epidermal growth factor (16).

Studies reported here confirm that phenol red acts as a weak estrogen and reexamine the significance of estrogenic and antiestrogenic effects on growth of human breast cancer cell lines ZR-75-1 and T-47-D in the absence of phenol red.

MATERIALS AND METHODS

Culture of Stock Cells. The ZR-75-1 human breast cancer cells were kindly provided by Dr. M. Lippman (National Cancer Institute, Bethesda, MD) and were of about the same passage generation as in previous experiments (3, 17). The T-47-D human breast cancer cells were kindly provided by the originators of the cell line to our institute (20). Stock ZR-75-1 and T-47-D cells were grown routinely as monolayer cultures in DMEM2 supplemented with 5% FCS (Gibco-Biocult, Glasgow, Scotland) and 10-9 M estradiol in a humidified atmosphere of 10% carbon dioxide in air at 37°C. Cells were subcultured at weekly intervals by suspension with trypsin (see below).

The S115 +A mouse mammary tumor cells were an androgen/glucocorticoid responsive cell line (21). These cells were grown as monolayer cultures in DMEM containing 2% FCS, 30 mM HEPES buffer (Sigma Chemical Co., Poole, Dorset, England) and 3.5 x 10-8 M testosterone but otherwise as for the human cells above.

Growth Curves in Monolayer Culture. Cells from stock plates were washed in phosphate-buffered saline to remove all phenol red, suspended by treatment in a 0.06% trypsin/0.02% EDTA, pH 7.3, solution lacking phenol red, and then counted on a hemocytometer. Phenol red-free media, either RPMI 1640 (special order from Gibco-Biocult) or DMEM (Sigma Chemical Co., Poole, Dorset, England) were used for all experiments. DCFCFS was used for all studies, unless use of DCF-replaced calf serum is specified. DC treatment of serum has been described previously (3). Cells were added to the overall required volume of phenol red-free medium containing either 5% DCFCFS (ZR-75-1 and T-47-D cells) or 2% DCFCFS/30 mM HEPES buffer (S115 +A cells) in the absence of steroid to achieve the appropriate seeding density. Cells were then plated onto 50-mm diameter plastic tissue culture dishes (Nunc, Roskilde, Denmark) in 5 ml aliquots for 24 h. The medium was then changed to contain the required concentration of phenol red, DCFCFS, steroid, or antiestrogen. Stock 1% phenol red (BDH Chemicals, Ltd., Poole, England) solution was diluted as appropriate. Testosterone and estradiol (Steraloids, Ltd., Croydon, England), tamoxifen and transhydroxytamoxifen (ICI Chemical Co., Macclesfield, England) were added as stock solutions in ethanol and diluted x 10,000 in the culture medium.

Growth Curves in Suspension Culture. Cells were grown as monolayer cultures but in 5-cm diameter plastic bacteriological dishes (Sterilin, Teddington, England).

Growth of Stock Cells without Phenol Red or Steroid. Growth of stock cells in the absence of phenol red or steroid prior to assessing growth was achieved by growing cells in phenol red-free RPMI 1640 with 5% DCFCFS for the required length of time. The cells were subcultured at weekly intervals as for normal stock cells.

Cell Counts. Cells in monolayer cultures in 5-cm diameter tissue culture dishes were washed in phosphate-buffered saline and then lysed in 2 ml 0.01 M HEPES buffer containing 1.5 mM MgCl2 plus 4 drops of Zaponin (Coulter Electronics, Ltd., Harpenden, England).

Received 11/30/87; revised 3/21/88; accepted 3/31/88.

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2 The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; DC, dextran charcoal; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
RESULTS

Effects of Phenol Red on Steroid Regulation of Cell Growth in Culture. Growth of ZR-75-1 cells in phenol red-free DMEM (Fig. 1A) (repeated four times) and phenol red-free RPMI 1640 medium (Fig. 1B) (repeated three times) has been assessed with and without $10^{-8}$ M estradiol and the effects of adding back phenol red at the normal levels present in these media (15 mg/liter DMEM-5 mg/liter RPMI 1640) have been studied. Phenol red did not affect growth in the presence of estradiol (Fig. 1A, Day 7 $P = 0.02$; all other $P_s > 0.10$). In the absence of estradiol, removal of phenol red lowered base-line cell growth more in DMEM than in RPMI 1640 (but in all cases $P_s < 0.001$ were obtained). This difference between the media was solely related to phenol red concentration because when the phenol red concentration in RPMI 1640 was trebled, it then gave similar results to that seen in DMEM (data repeated three times but not shown). Cell growth has been studied in many different batches of DCFCS and DC-stripped calf serum over a 6-month period and cell growth in the absence of estradiol was always reduced on removal of phenol red but on no occasion was growth ever reduced to zero. Analogous experiments have been carried out twice on growth of ZR-75-1 cells in suspension culture with similar results (data not shown).

Similar conclusions were drawn for growth of T-47-D cells in monolayer culture (Fig. 2). Phenol red had no effect in the presence of estradiol ($P_s > 0.10$). In the absence of estradiol, phenol red had a dose-dependent effect on cell growth (Fig. 2) (all $P_s < 0.001$) but growth was never reduced to zero. Interestingly, the effects of removal of phenol red were also greater at lower cell densities (Fig. 2B). Data in Fig. 2A were reproducible over three separate experiments.

S115 +A mouse mammary tumor cells are responsive to androgen and glucocorticoid (21): phenol red had no effect on growth of these cells (data not shown) (all $P_s > 0.01$).

Memory Effects. The possibility was considered that the basal growth seen in the absence of both phenol red and estradiol (Figs. 1 and 2) was due to a "memory effect" from previous growth in stock cultures with phenol red and estradiol. Growth of ZR-75-1 and T-47-D cells immediately after removing phenol red and estradiol (Figs. 1 and 2) has been compared with their behavior after 3 weeks' deprivation (Fig. 3). Growth of ZR-75-1 cells for 3 weeks without phenol red or estradiol resulted in loss of all basal cell growth (Fig. 3A) (repeated six times). Growth of T-47-D cells for 3 weeks without phenol red or estradiol resulted in reduced basal cell growth (Fig. 3B) (repeated four times) but even after 10 weeks (data not shown) it was not lost completely as for the ZR-75-1 cells. Plating efficiencies and cell viability were unaffected by these treatments.

Effects of Phenol Red and Cell Density on Antiestrogen Action. Effects of the two antiestrogens tamoxifen and trans-hydroxytamoxifen on growth of ZR-75-1 (Fig. 4) and T-47-D (Fig. 5) cells in monolayer culture have been studied in the presence and absence of phenol red. All cell culture was carried out using 1% serum since higher serum levels can mask antiestrogen effects (16). In addition, studies were performed to determine...
RESULTS

Effects of Phenol Red on Breast Cancer Cell Growth

- Cells were grown in RPMI 1640 medium in 5% DCFCS with or without 15 mg/liter phenol red (●), 10^{-6} M tamoxifen, 10^{-8} M trans-hydroxytamoxifen as follows:
  - plating density of cells: □, -phenol red-tamoxifen-trans-hydroxytamoxifen; ●, +phenol red-tamoxifen-trans-hydroxytamoxifen; △, -phenol red+tamoxifen; ○, +phenol red+tamoxifen; ■, -phenol red+trans-hydroxytamoxifen; ▪, +phenol red + trans-hydroxytamoxifen. Bars, SE of triplicate dishes. No bars are shown if variation was too low for visual display.

- For ZR-75-1 cells at high cell density, both tamoxifen and trans-hydroxytamoxifen inhibited cell growth in the presence of estradiol and that its removal lowers cell growth in the absence of estradiol. No evidence was found that phenol red could affect growth in the presence of estradiol. It is obvious that from now on, any studies of estrogen effects on cells in culture must be carried out in medium free of phenol red. If such medium is unobtainable, then at least RPMI 1640 should be used in preference to DMEM since the lower levels of phenol red had negligible effects on these cells.

- The question of whether steroids affect the proliferation of breast tumor cells directly on their own (dependent) (9, 24) or indirectly via other agents (responsive) (12) is centered around the ability of cells to grow in tissue culture without added hormones and the effects of phenol red are of importance in this context. Neither our data presented here (Figs. 1 and 2) nor published data for MCF-7 (14, 19, 22) and rat pituitary (23) cells showing that phenol red can act as a weak estrogen and that its removal lowers cell growth in the absence of estradiol. Pituitary (23) cells showed that phenol red can act as a weak estrogen and that its removal lowers cell growth in the presence of estradiol. It is obvious that from now on, any studies of estrogen effects on cells in culture must be carried out in medium free of phenol red. If such medium is unobtainable, then at least RPMI 1640 should be used in preference to DMEM since the lower levels of phenol red had negligible effects on these cells.

DISCUSSION

This report has reexamined the significance of estrogenic and antiestrogenic action on growth of human breast cancer cell lines ZR-75-1 and T-47-D in the absence of phenol red. The results support published data for MCF-7 (14, 19, 22) and rat pituitary (23) cells showing that phenol red can act as a weak estrogen and that its removal lowers cell growth in the absence of estradiol. No evidence was found that phenol red could affect growth in the presence of estradiol. It is obvious that from now on, any studies of estrogen effects on cells in culture must be carried out in medium free of phenol red. If such medium is unobtainable, then at least RPMI 1640 should be used in preference to DMEM since the lower levels of phenol red had negligible effects on these cells.

The question of whether steroids affect the proliferation of breast tumor cells directly on their own (dependent) (9, 24) or indirectly via other agents (responsive) (12) is centered around the ability of cells to grow in tissue culture without added hormones and the effects of phenol red are of importance in this context. Neither our data presented here (Figs. 1 and 2) nor published data for MCF-7 cells (19) suggest that phenol red (all Ps < 0.001) (Fig. 5). Interestingly, at low density in the absence of phenol red, the same biphasic response to tamoxifen was found as for ZR-75-1 cells: a weak stimulation after 7 days but inhibition after 14 days (Fig. 5A). This biphasic response was reproduced in three separate experiments.

Effects of Memory on Antiestrogen Action. To test the effect of memory on antiestrogen response, ZR-75-1 cells were grown for 3 weeks without estradiol or phenol red prior to antiestrogen addition (Fig. 6). Cells thus treated showed only inhibitory effects with antiestrogens at low cell density (all Ps < 0.001) and cell numbers fell even below plating density (Fig. 6A). This contrasted with the effects seen with cells tested immediately after estradiol/phenol red withdrawal (Fig. 4). However, at high cell density, antiestrogens inhibited cells only in the presence of phenol red (Ps in presence of phenol red all < 0.001; Ps in absence of phenol red all > 0.05) and never below plating density (Fig. 6B).
red alone can account for all the cell growth found in the absence of added estradiol. However, we now present data to show that this basal cell growth can be attributed, totally in the case of ZR-75-1 cells, to steroid memory effects. Growth of ZR-75-1 cells for 2 weeks or more in the absence of phenol red and estradiol resulted in loss of all cell growth in the absence of added steroid. The cells remained 100% viable but did not grow at all unless estradiol was added back. Since the only difference between these static cells (Fig. 34) and stock cells (Fig. 1) was removal of phenol red and estradiol, and since serum at 5% remained present at all times, the effects on residual growth must be related to steroid memory and not to any other growth factors in serum or medium. We thus conclude that ZR-75-1 cells are completely dependent on estradiol for growth but that in agreement with previous suggestions, estrogens can have long lasting effects on cells in culture (25) maybe through long half-lives of regulatory factors. The same was not true for the T-47-D cells studied here nor for the MCF-7 cells (19) where limited cell growth remained even after 10 weeks of culture in the absence of phenol red and estradiol. Thus, while these latter two cell lines are much more responsive to estradiol in the absence of phenol red, they are not totally dependent on estradiol for growth. The ability to compare steroid-responsive and -dependent cells should aid us in our search to understand the mechanism of steroid regulation.

The mechanism of agonist/antagonist activity of antiestrogens in vitro remains a subject of much debate and the role of phenol red in this context is of vital importance. Recently, it has been suggested that when phenol red is removed from culture media, antiestrogens are ineffective (14). Our data agree with these conclusions but only when considering cells at high density at early 1-week time points. However, we now suggest that antiestrogen action is affected not only by the presence of phenol red but also by three other parameters: (a) time in culture; (b) cell density; (c) steroid memory effects. As for MCF-7 cells (14), antiestrogen effects on ZR-75-1 cells at high cell density in the presence of phenol red can be accounted for by the suppression of phenol red-stimulated activity and no inhibitory effects were seen in the absence of phenol red. However, the story is different at low cell density. Under these conditions in the absence of phenol red, tamoxifen showed a biphasic action: initial weak stimulation but later inhibitory effects. trans-Hydroxytamoxifen did not have the stimulatory effects but inhibitory action was much greater as time in culture increased. Interestingly, these effects at low cell density occurred with both ZR-75-1 and T-47-D cells. These cell density effects could be due to differential antiestrogen action on exponentially growing and plateau phase cells (4, 26). However, they could also be explained by antiestrogen antagonism of growth stimulating factors (16). At low cell density, the local autocrine growth factor milieu will be lower resulting in stronger inhibitory effects of antiestrogens. Published data for MCF-7 cells refer to antiestrogen effects at fairly high cell densities (14) but it may be that since MCF-7 cells produce relatively more growth factors than do ZR-75-1 or T-47-D cells (27) that the high levels of autocrine stimulation from these cells render antiestrogens less effective. It is interesting to note also that in the absence of any steroid memory effects, antiestrogens become much more inhibitory to ZR-75-1 cells, actually causing cell death.

Finally, these data carry clinical implications: (a) the effects of cell density on antiestrogen action in culture suggest that these agents may be more effective in vivo on tumors of low cellularity and that areas within a tumor packed densely with malignant cells may be difficult to attack with antiestrogens alone; (b) the biphasic effects of tamoxifen at $10^{-4}$ M with time in culture may explain the response sometimes seen in vivo of an early rise in tumor size before regression. This phenomenon of tamoxifen tumor flare is seen in both MCF-7 cells grown in nude mice (28) and in the clinic (29-31). It has been shown previously that tamoxifen is weakly stimulatory at low concentration and inhibitory at high concentration (17, 18) but this is the first report of biphasic effects at a single high concentration. Previous data suggested that tumor flare could result from low blood levels of tamoxifen at the start of therapy while remission results from a higher concentration of tamoxifen achieved after prolonged treatment (18). The data presented here imply that the phenomenon of tumor flare could also occur with time at a single high tamoxifen concentration. However, whether the initial stimulation is due to the fact that tamoxifen effects have to accumulate before they become inhibitory or to an interaction with "estrogen memory" (in culture, tamoxifen is more inhibitory to ZR-75-1 cells in the absence of steroid memory) remains speculative.

ACKNOWLEDGMENTS

We thank Dr. R. J. B. King (Imperial Cancer Research Fund) for his advice and for his critical reading of this manuscript. We are very grateful to Dr. R. Hallowes (Imperial Cancer Research Fund) for his help in obtaining a good and reliable source of phenol red-free medium.

REFERENCES


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