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Adaptation of Estrogen-dependent MCF-7 Cells to Low Estrogen (Phenol Red-free) Culture*

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Abstract—*When human breast cancer-derived MCF-7 cells were maintained in low estrogen medium (phenol red-free), the cells adapted to grow without added estrogen, but growth could be inhibited by antiestrogen in the medium. Estrogen-stimulated progesterone receptor levels remained basal but could be stimulated by estradiol. Estrogen receptor content increased steadily during adaptation, which may model the increasing levels of estrogen receptor observed in breast cancer with increasing patient age. The mechanism of the adaptation to low estrogen medium is unclear; however, cell lines such as MCF-7 may need to be cultured in the presence of an estrogen such as phenol red in order to maintain a stable estrogen-sensitive phenotype. On the other hand, maintenance of estrogen-dependent cells in low estrogen media may convert them to dependence on factors which are not currently understood. This may ultimately increase their value as models of hormone action.*

INTRODUCTION

THE MCF-7 cell line is derived from hormone-dependent human breast cancer [1]. The growth of these cells in tissue culture shows stimulation by estrogens and inhibition by antiestrogens [2]. These cells have been used extensively to study both hormone-dependent breast cancer and the basic mechanism of estrogen action. A number of additional breast cancer-derived cell lines have been established subsequent to MCF-7, and which exhibit a spectrum of estrogen dependence [3].

The study of estrogen action in tissue culture has been affected by the recent observation that the phenol red indicator present in most media is a weak estrogen [4]. The structure of phenol red resembles that of a number of non-steroidal estrogens [5], and it is clear that removal of this source of estrogen from the medium alters the growth rate and steroid receptor content of the breast cancer cells. The influence that phenol red may have on estrogen-dependent cells in tissue culture is of significance because virtually all such cell lines have been established and maintained in the presence of this indicator.

We cultured two sublines of MCF-7 cells for extended periods in low estrogen, phenol red-free medium and found that the cells adapted to grow without the estrogenic stimulation provided by phenol red, which the original stock cells used in order to grow maximally. Interestingly, these adapted cells did not shed the estrogen-dependent phenotype, but rather expanded their levels of estrogen receptors, and continued to show estrogen stimulation of progesterone receptor [6], a marker of estrogen action. The growth of the adapted cells, which no longer required the presence of known estrogens such as estradiol or phenol red, was nonetheless inhibited by antiestrogen.

METHODS

Stock MCF-7 cells were maintained in Minimum Essential Medium (MEM; Gibco) with phenol red containing non-essential amino acids, 10 mM hepes, insulin 6 ng/ml, penicillin 100 units/ml, streptomycin 100 μg/ml, and 5% calf serum (Gibco) that was charcoal stripped. Phenol red-free medium was made using Sigma phenol red-free MEM. To measure steroid receptors or growth, cells were plated at 25,000 per well in 16-mm multiwell plates, and after attachment for 1–3 days, the cells were treated with compounds in medium containing 0.1% solvent ethanol with daily medium

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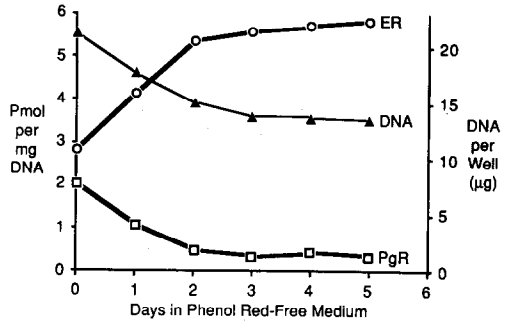

Fig. 1. MCF-7 cells were plated on day 0 at 25,000 per well in phenol red-containing medium. Cells were fed daily with either phenol red-containing or phenol red-free medium, so that at the end of 6 days, cells had been cultured in phenol red free-medium for up to 5 days. The 0-day group was cultured in phenol red-containing medium throughout the experiment. On day 6, progesterone receptor (PgR), estrogen receptor (ER) and DNA were determined in all cells as described and shown plotted against the number of days since transfer to phenol red-free medium.

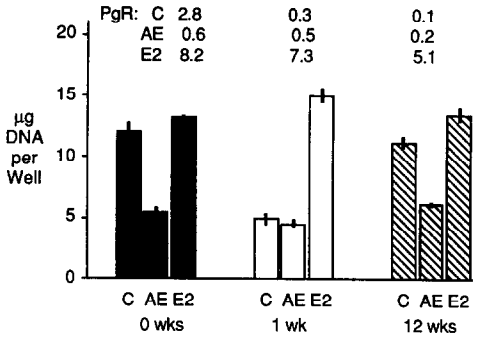

Fig. 2. Stimulation of proliferation (DNA) and progesterone receptor (PgR), in phenol red-containing medium, and after short- and long-term adaptation to phenol red-free medium. Cells were plated at 25,000 per well on day 0 and fed again on day 1. Beginning on day 3, the cells were treated for 4 days with compounds in the appropriate medium (containing 0.1% solvent ethanol), with daily medium changes. On day 7, progesterone receptor and DNA were measured as described. '0 Weeks': stock MCF-7 cells were plated and treated in phenol red-containing medium throughout the experiment. '1 Week': stock MCF-7 cells were plated and treated in phenol red-free medium; the cells were therefore adapted to phenol red-free medium for 3 days before treatment with compounds (in phenol red-free medium, 4 days), and were a total of 1 week in phenol red-free medium by the end of the experiment. '12 Weeks': cells had been cultured for 11 weeks in phenol red-free medium, and were plated and treated in phenol red-free medium; therefore, by the end of the experiment, the cells had been in phenol red-free medium a total of 12 weeks. C: control. AE: antiestrogen LY156758, 10 nM. E2: estradiol 0.1 nM. Error bars are the standard error, n = 4 wells per measurement. Progesterone receptor values are given above the growth data, in pmol/mg DNA. Estrogen-stimulated PgR values were essentially indistinguishable across the course of many experiments, despite variations of 8.2–6.5 pmol/mg DNA in this particular experiment.

changes 1 ml per well. The antiestrogen used in these experiments was LY156758 (keoxifene), an estrogen antagonist that shows minimum agonist activity *in vivo* and *in vitro* [7].

Estrogen receptor was measured in the attached cells by whole cell uptake of [^{3}H]estradiol (New England Nuclear), 5 nM in Hanks' balanced salt solution with 25 mM hepes (HBSS), 0.5% ethanol and bovine serum albumin (BSA) 2 mg/ml. For progesterone receptor, [^{3}H]R5020 (New England Nuclear), was used in the same buffer, to which 25 nM dexamethasone was added to reduce binding of R5020 to glucocorticoid receptor. One hundred-fold excess of non-radioactive ligand was added to measure non-specific binding in separate wells. After incubation with 0.5 ml of the tritiated ligand for 45 min at 37° C, the wells were washed four times with 1 ml of HBSS containing BSA 2 mg/ml. The washed cells were sonicated in 1 ml of the wash buffer diluted 1:9 with water, and samples were taken for radioactivity and DNA. DNA was measured fluorometrically using Hoechst 33258 (Calbiochem-Behring) according to Labarca and Paigen [8].

RESULTS

Stock MCF-7 cells were maintained in phenol red-containing medium, which stimulated the cells (Fig. 1, 0 days). When cells were transferred into phenol red-free medium, estrogenic responses (progesterone receptor and growth rate) reached baseline in the new medium after 3–4 days (Fig. 1). Estrogen receptor content was also measured and increased to a plateau at also 3–4 days (Fig. 1). This increase in estrogen receptor content is reverse to the net reduction (processing) or down regulation of receptor that is observed during estrogenic stimulation in these cells [6]. For estrogen response experiments, cells were used after 3 or more days adaptation to phenol red-free medium.

The responses of MCF-7 cells in phenol red-containing and phenol red-free media are shown in Fig. 2. In the presence of conventional medium containing phenol red (Fig. 2, 0 weeks), control growth rate was high and was reduced in the presence of the antiestrogen, LY156758. The addition of estradiol had little effect on the growth rate compared to control; growth was already near maximal. After short-term culture in phenol red-free medium (Fig. 2, 1 week), the control growth rate was low, only slight reduction was observed in the presence of an antiestrogen, but the growth was stimulated 3-fold by the addition of estradiol to the medium for 4 days (Fig. 2, 1 week). The same pattern was observed for estrogen-stimulated progesterone receptor levels (Fig. 2), except that the stimulation by phenol red-containing medium was a smaller proportion of maximal than was the case for the growth response. This is not surprising since the growth response is 10-fold more sensitive to estrogen than is the progesterone receptor response (see dose-response studies below).

When the cell line was maintained in phenol red-free medium for longer than a month (Fig. 2, 12 weeks); however, the growth response came to adapt

to resemble the response originally seen in phenol red-containing medium. The control growth rate increased, estradiol produced decreasing proportional increases in proliferation, and the control growth rate in the adapted cells was reduced by antiestrogen (Fig. 2, 12 weeks). Inhibition of growth by antiestrogen could be competitively 'rescued' by an excess of estradiol (100 nM, not shown) indicating that the inhibition was receptor-mediated, not due to development of toxicity to the antiestrogen.

Interestingly, while the growth response adapted to become like that of the stock cells stimulated by medium with phenol red, the other two indications of estrogenic stimulation were not affected. Progesterone receptor response remained at baseline, although it could be stimulated fully by estradiol (Fig. 2), and the estrogen receptor content remained high. The low baseline progesterone receptor in adapted cells was paradoxical, because sufficient estrogen ($\geq$ 10 pM estradiol; see dose responses, below) to stimulate growth to the degree that was observed in the adapted cells would also stimulate the progesterone receptor level to approx. 50% of the maximal response.

Timecourse of the adaptation over 15 weeks is shown in Fig. 3 for two separate sublines of MCF-7 cells maintained in phenol red-free medium. After 30 days in the low estrogen medium, the estrogenic stimulation of growth compared to control began to decline (Fig. 3A), as the control growth rate increased (Fig. 3B). During this time, the estrogen receptor content of the cells was steadily expanded (Fig. 3C), doubling over the course of the experiment.

The dose–responses to estradiol of the stock cell line and of the adapted cell lines are shown in Fig. 4. In stock cells, the growth response was approximately 10-fold more sensitive than was the progesterone receptor response (half-maximal stimulation at 2.6 pM vs. 15 pM; Fig. 4). For the adapted cells, the progesterone receptor response (50%) was slightly more sensitive than that observed in the stock cells; 7.0 and 8.4 pM compared to 15 pM in the original stock. This 2-fold greater sensitivity would be consistent with the 2-fold greater concentration of estrogen receptors in these adapted cells. The growth response by the adapted cells was slight, but the small stimulation of growth by added estradiol was similar in dose-response to that in the control cells (half-maximal stimulation at around 1 pM; data not shown). Overall, adaptation involved little change in the sensitivity of the cells to estradiol.

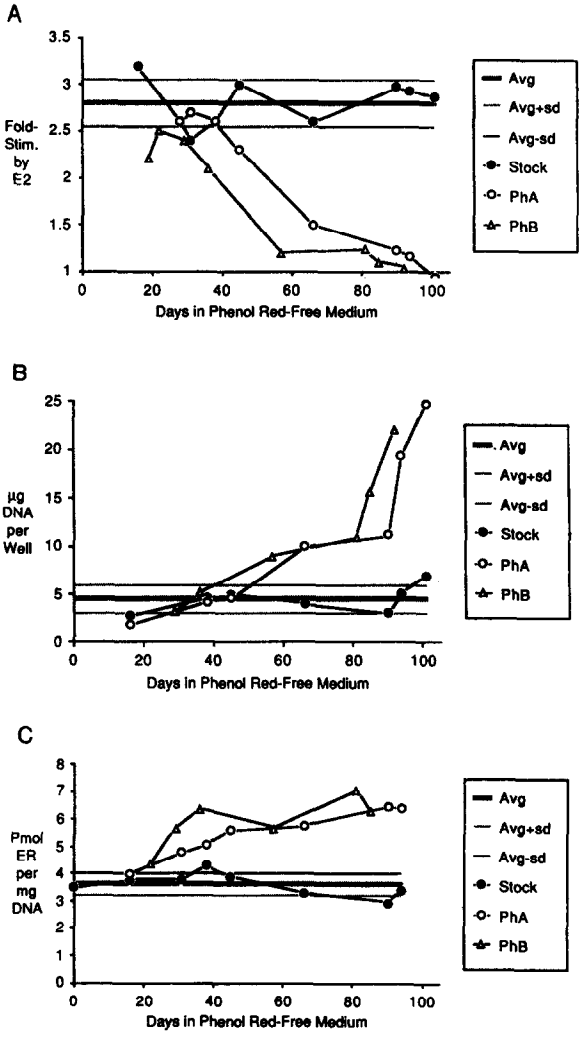

Fig. 3. Adapted cell sublines PhA and PhB were derived from stock MCF-7 cells maintained in phenol red-free medium, beginning 9 days apart and maintained separately throughout the experiments. The total time in phenol red-free medium for the adapted cells is shown on the horizontal axis. At intervals during adaptation, the two adapted cell lines, and the stock cell line for comparison, were plated at 25,000 cells per well in phenol red-free medium and tested for estrogen stimulation of proliferation and progesterone receptor as described in Fig. 2, as for '1 week' and '12 weeks'. The response to E_2 is shown in A and the change in DNA in control wells shown in B. Estrogen receptor content was also measured in phenol red-free medium (C). The average and the range of the standard deviation is for the responses in the stock cells; solid symbols show the series of individual measurements during the experiment. There was no apparent drift in the responses of the stock cells during the course of the experiment.

DISCUSSION

We have documented changes in the growth of MCF-7 cells that occur when the cell line is cultured in the absence of known estrogens, in particular when phenol red is removed from the growth media. The adaptation of these cells to grow in the absence of the indicator diminishes the degree to which estrogen-responsive growth can be used to study estrogen action in these cells. A stable estrogen-responsive phenotype is observed if the phenol red is left in the maintenance medium of these cells, removing it only from cells just before use in growth assays. This step-down protocol for the study of

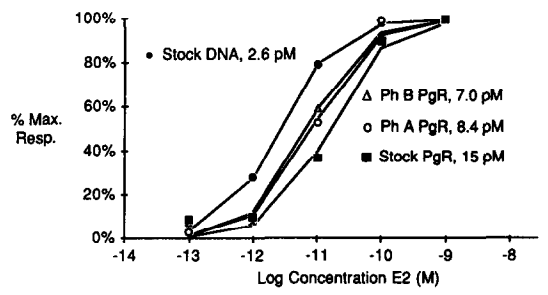

Fig. 4. Dose–responses to estradiol by control stock cells (closed symbols) and by adapted cells (open symbols). Growth as DNA/well, and progesterone receptor stimulation as pmol/mg *DNA were measured as described after 4 days exposure to estradiol at the indicated concentrations. Response is shown as a fraction of maximal stimulation observed at highest estradiol concentration. Growth and progesterone receptor are shown for the stock MCF-7 cells. Progesterone receptor is shown for the two cell lines adapted to phenol red-free culture for 13–15 weeks; growth was not shown for the adapted cells because the growth rates were already near maximal. The estradiol concentration of half-maximal response is given for each of the four dose–response curves.*

estrogen-stimulated growth may apply to other estrogen-responsive cell lines.

A steady increase in estrogen-receptor content with time in low estrogen medium was observed in our experiments. A relationship of increasing estrogen receptor level with patient age has been described for breast cancer. This observation has been variously attributed to menopausal status, occupation of receptors by endogenous hormone, effects of progesterone or other correlations (summarized in [9]). However, others [10] have suggested that a 'homeostatic' increase in receptors with increasing estrogen deficiency was more likely, and our observations would tend to support this hypothesis.

There are several mechanisms that may be considered for the adaptation of estrogen-dependent cells to growth in low-estrogen medium. The development of a classical estrogen-independent (estrogen-receptor negative) cell line did not occur since the adapted cells had high levels of the estrogen receptor and showed normal estrogenic stimulation of progesterone receptor.

It is possible that the adapted cell line develops an increased sensitivity to utilize the next most active (weak) estrogen present in the medium after the phenol red was removed. This possibility is suggested by the antiestrogen-sensitive growth pattern of the adapted cells. However, the progesterone and estrogen receptor levels in the cells did not show evidence of reaction to the presence of an estrogen, as would be expected if the estrogen receptor were occupied by some estrogenic ligand. On the other hand, a novel ligand may selectively stimulate growth without increasing progesterone receptor.

Growth may be stimulated by the unoccupied receptor. Both the unoccupied and occupied forms of steroid receptors appear to be nuclear in the intact cell [11–13], and therefore both could be in a position to affect transcription. Since our observations are not fully consistent with an *occupied* estrogen receptor mechanism, and since the estrogen receptor levels are high in these adapted cells, it may be that the high *unoccupied* estrogen receptor level is itself stimulating the growth response. This would explain why the sensitivity to antiestrogen is retained: the conformation of the receptor would still be altered by antiestrogen binding to prevent active nuclear interactions [14, 15].

A related possibility is the development of estrogen receptor-independent growth combined with a separate antiestrogen mechanism for inhibiting growth. This is suggested by recent reports of antiestrogen-stimulated proteins, secreted by MCF-7 cells, which may have the ability to inhibit cell growth in an autocrine or paracrine fashion [16, 17]. If, however, these proteins are estrogen inhibited [18] rather than antiestrogen stimulated, an estrogen receptor-dependent mechanism would still be required.

In summary, we have found that estrogen-dependent MCF-7 cells, after a period of adaptation in a low estrogen (phenol red-free) environment, continue to grow and progress through a series of changes in the estrogen dependence of the line. It is clear that for preservation of the growth phenotype in a form that can be used to study estrogen stimulation of growth, it is important that the cell line be maintained in the presence of an estrogen, such as the phenol red.

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