ABSTRACT

The effect of the antiestrogens tamoxifen and nafoxidine on the growth of the human breast cancer cell line MCF-7 is modified by both serum and insulin. Tamoxifen inhibition of the growth of MCF-7 cells in culture is reduced as the concentration of serum in the medium is increased from 0.1% to 10%. Estradiol does not stimulate cell growth over the same range of serum levels.

Insulin changes the sensitivity of MCF-7 cells to both estrogen and antiestrogens. Cells growing in media containing insulin are less sensitive to inhibition by either tamoxifen or nafoxidine than are cells growing in its absence. In addition, higher concentrations of estradiol are required to stimulate the production of plasminogen activator when cells are grown in media containing insulin.

This effect of insulin can be accounted for by the finding that insulin lowers the level of estrogen receptor in MCF-7 cells without altering the binding constant for the hormone. Cells grown with insulin have an average of 21,000 ± 4,700 (S.D.) estrogen binding sites/cell compared to 62,000 ± 9,700 sites/cell in cells grown in the absence of insulin. This difference in receptor level is sufficient to account for the difference in the concentration of estradiol needed for equivalent induction of plasminogen activator in cultures with or without insulin.

These results indicate that the level of estrogen receptor in breast cancer cells can be changed and that the sensitivity of such cells, both to estrogen and to antiestrogens, is altered by changes in the level of estrogen receptor. They also have implications concerning the mechanism by which antiestrogens act to inhibit the growth of mammary tumor cells.

INTRODUCTION

The role of estrogen in the development and growth of mammary carcinomas in humans and animals has been studied in a variety of systems. Some recent work has also focused on the role that the peptide hormone insulin may play, not only in affecting the growth of such tumors, but also in modifying their responsiveness to estrogen (11, 15, 32).

The human breast cancer cell line MCF-7 was derived from the pleural effusion of a patient with a metastatic mammary carcinoma (33). In recent years, it has been used extensively as a model system for studying the effects of various hormones on breast cancer (29). It is well suited to these studies since it contains receptors for several hormones including estrogen (7), androgens, glucocorticoids, progesterone (17), and insulin (30).

The antiestrogens tamoxifen and nafoxidine inhibit the growth of MCF-7 cells, leading eventually to cell death (19). We have noted that the inhibitory effect of tamoxifen is modified by serum and also by insulin (9) and in this paper show that insulin alters the level of estrogen receptor in MCF-7 cells in a way which can account for their reduced sensitivity to tamoxifen and to estrogen.

MATERIALS AND METHODS

Cell Culture. The human breast cancer cell line MCF-7 (33) was obtained from Dr. H. D. Soule. Cells were grown in Falcon plastic T-flasks (25 and 75 sq cm) at 37° in Eagle's minimum essential medium, Hanks' base, supplemented with nonessential amino acids and L-glutamine from KC Biological, Inc., Lenexa, Kans. The medium contained 10% calf serum (KC Biological, Inc.), 12.5 µg bovine insulin per ml (Sigma Chemical Co., St. Louis, Mo.), and 100 units penicillin and 100 µg streptomycin per ml (Grand Island Biological Co., Grand Island, N. Y.). Cells were passed weekly using trypsin:EDTA (Grand Island Biological Co.) when near confluence. Cultures were fed on Days 3 and 6 following transfer.

Most of the experiments reported here were performed on cells growing on the bottoms of sterile glass scintillation vials (2) in 2 ml of the same medium with Earle's base in a humidified atmosphere of 5% CO2:95% air. Serum and insulin were added as indicated for each experiment. Cells were plated at between 50,000 and 100,000 cells/vial. Under these conditions, they reach confluence at approximately 1,000,000 cells/vial.

The serum used in many of these experiments has been stripped of endogenous steroids by two 30-min incubations at 55° with a pellet of dextran-coated charcoal (1) (1% activated charcoal; 0.1% dextran; Sigma Chemical Co.; prepared at one-half of this concentration by stirring overnight at 4° in 0.9% NaCl and pelleted by centrifugation prior to use). Steroids were added as ethanol solutions to a final concentration of not more than 0.2% ethanol.

To obtain counts of cells growing in vials, the medium was removed, and the cells were rinsed gently with 0.9% NaCl. After removing the 0.9% NaCl solution, 0.5 ml of buffer [0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4); 1.5 mM MgCl2] was added followed by one or 2 drops of Zapsloton II (Coulter Diagnostics, Inc., Hialeah, Fla.). This lysed the cells, leaving a suspension of nuclei which were suspended to 10 ml in 0.9% NaCl and pelleted by centrifugation prior to use). Steroids were added as ethanol solutions to a final concentration of not more than 0.2% ethanol.

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Antiestrogens. Tamoxifen (trans-p-β-dimethylaminoethoxy-phenyl-1,2-diphenyl-but-1-en), I.C.I. 469474, was a gift from Lois Trench, I.C.I. United States, Inc., Wilmington, Del., and nafoxidine [1-(2-p-tolyl-3,4-dihydro-6-methoxy-2-phenyl-1-naph-
Thymidine Incorporation. Cells growing in glass scintillation vials were labeled as described with [3H]thymidine (New England Nuclear, Boston, Mass.). At the end of the incubation period, the vials were treated for 5 min once with ice-cold 0.9% NaCl and then 3 times with 5% trichloroacetic acid and once with ethanol. Ten ml of counting solution (toluene, POP, POP) were added, and the radioactivity was counted in a Searle Analytic 81 liquid scintillation counter.

Plasminogen Activator Induction and Assay. Cells were plated in 25-sq cm T-flasks at a density of 500,000 cells/flask in medium containing 10% calf serum. The next day, the medium was changed to a chemically defined serum-free medium described by Higuchi and Robinson (14) but lacking hydrocortisone and thyroxine and containing 5 μg insulin per ml as indicated. Growth in this medium is as rapid as it is in medium with serum. Cells were grown for 5 days with a medium change on Day 2 or 3. They were then approximately 50% confluent and were changed to fresh medium with estradiol added, as indicated, in a final concentration of 0.1% ethanol. Two days later, the medium was collected, centrifuged, and stored frozen for subsequent assay.

Plasminogen activator was assayed by measuring the release of 3H-labeled peptides from [3H]fibrin-coated plates (3). A solution containing 100 μg of [3H]fibrinogen (200,000 cpm) was spread on 35-mm plastic Petri dishes and dried for 24 hr at 45°. The fibrinogen was then clotted by a 2-hr treatment with purified bovine thrombin (plasminogen free; Miles Research Products, Miles Laboratories, Inc., Elkhart, Ind.). The final assayed was carried out at 37° for 4 hr in a volume of 1 ml which contained 1% acid-treated dog serum as a source of plasminogen. In addition to assaying an aliquot of the cell culture medium, usually 0.1 ml, a series of urokinase standards (0.01 to 0.1 units, Calbiochem-Behring Corp., LaJolla, Calif.) was assayed, and the resulting standard curve was used to standardize the assay. The final results are expressed as number of urokinase units per 1,000,000 cells after correcting for the volume of medium assayed and the number of cells in the flask.

Measurement of Estrogen Binding Capacity. The procedure used for measuring estrogen binding capacity in intact cells was similar to that described by Shafie and Brooks (31). We also found that the binding reaches equilibrium by 30 to 60 min at 37° and have used an incubation time of 60 min as they did.

RESULTS

Effects of Serum on the Growth and Tamoxifen Sensitivity of MCF-7 Cells. The sensitivity of MCF-7 cells to the antiestrogen tamoxifen is altered by several factors. Depending on the conditions used, cells may be relatively unaffected, grow at a reduced rate, or die when incubated with tamoxifen. One factor affecting the sensitivity to tamoxifen is the concentration of serum in the medium. MCF-7 cells grow well at serum concentrations between 0.5% and 10%. At lower concentrations of serum, they grow at a reduced rate and to a lower density (Chart 1A). Their sensitivity to 10^{-8} M tamoxifen is much greater in media containing lower concentrations of serum (Chart 1B).

Growth is prevented by tamoxifen at serum concentrations below 1% and inhibited at higher serum concentrations. As has been reported by others (24, 29), we have also seen that tamoxifen inhibition can be overcome by estradiol.

It has been reported that the growth of MCF-7 cells is stimulated by estradiol (24, 29). While we have consistently been able to demonstrate growth inhibition of MCF-7 cells by tamoxifen, we have seldom seen a significant stimulation of growth by estradiol. Estradiol did not stimulate growth at any concentration of serum tested between 0.1% and 10%, although there was some inhibition at low serum levels (Chart 2B). In an attempt to get a consistent and substantial stimulation of growth with estradiol, we have varied the concentration of estradiol and the concentration, type, and treatment of serum. We have also used MCF-7 cells which had been grown first for several passages in medium with estradiol in order to enrich the culture for cells with a requirement for estradiol. Finally, since MCF-7 is an uncloned population containing cells of a variety of phenotypes based on appearance (36), we selected several clones of various types at random and tested them. None of the clones tested was more responsive than the general culture. Others have also reported that growth of their MCF-7 cultures was not stimulated by estrogen2 (16, 18, 23).

For experiments with tamoxifen, we have used both calf and fetal calf serum either untreated or charcoal treated. While results differ from lot to lot, they follow the same pattern. The sensitivity to tamoxifen always decreases with increasing serum concentration, and charcoal treatment of the serum usually results in somewhat greater sensitivity to tamoxifen.

Effects of Insulin on Tamoxifen Inhibition. At serum levels above 1%, the addition of insulin reduces the inhibition pro-

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Effect of serum concentration on the growth of MCF-7 cells ± estradiol. The procedure was the same as described in Chart 1. No insulin was used, and the serum was fetal calf serum.

Effect of insulin on the sensitivity of MCF-7 cells to tamoxifen. The procedure was the same as that described in Chart 1 except that cells were plated directly in the test media. Charcoal-treated fetal calf serum was used. A, no insulin ± 10^{-6} M tamoxifen; B, 12.5 μg insulin per ml ± 10^{-6} M tamoxifen.

Effect of insulin on the induction of plasminogen activator by estrogen. While we have been unable to demonstrate much, if any, stimulation of the growth of MCF-7 cells by estradiol, we have shown that estradiol stimulates the production of the serine protease plasminogen activator in a chemically defined serum-free medium.

Effect of insulin on the inhibition of [3H]thymidine incorporation by tamoxifen. Cells were grown in vials for 5 days in 2% charcoal-treated calf serum ± 12.5 μg insulin per ml. The medium was changed on Day 3. Tamoxifen at the indicated concentrations was then added to the vials in fresh medium ± insulin, and 2 days later, the cultures were labeled for 1 hr with [3H]thymidine (0.2 μCi/ml) and the incorporation into trichloroacetic acid-insoluble material was measured. Point, average of triplicate samples; bars, S.D.

Effect of insulin on the inhibition of [3H]thymidine incorporation by tamoxifen. As shown in Chart 5, the effect of insulin is not to make the cells completely resistant to tamoxifen but, rather, to increase the level of tamoxifen required to give the same degree of inhibition.

Effect of insulin on the induction of plasminogen activator by estrogen. While we have been unable to demonstrate much, if any, stimulation of the growth of MCF-7 cells by estradiol, we have shown that estradiol stimulates the production of the serine protease plasminogen activator in a chemically defined serum-free medium. The stimulatory effect of estradiol on

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the production of this enzyme is also modified by insulin. As is shown in Chart 6, cells grown in the presence of insulin are less sensitive to estradiol than are those grown in its absence and require 5- to 10-fold higher concentrations of estradiol to achieve comparable levels of induction. Thus, the sensitivity of MCF-7 cells to both this stimulatory effect of estradiol and the inhibitory effects of tamoxifen and nafoxidine is reduced by insulin.

Effect of Insulin on the Level of Estrogen Receptor in MCF-7 Cells. In an attempt to explain the effect of insulin on the sensitivity of MCF-7 cells to estrogen and antiestrogens, we measured the level of estrogen receptor in intact MCF-7 cells growing attached to the bottom of glass scintillation vials. We used this assay in order to measure the total binding capacity and binding constant of live cells under conditions similar to those in which their sensitivity to estrogen and tamoxifen has been measured. Cells were grown in 2% charcoal-treated calf serum plus or minus insulin and then incubated with several concentrations of [3H]estradiol for 1 hr at 37° in the same medium lacking serum. Nonspecific binding was measured in a second set of vials which received a 200-fold excess of unlabeled estradiol. The results of one such experiment are shown in Chart 7. The receptor levels and binding constants were determined from Scatchard plots of the specific binding. Cells grown with insulin averaged 21,000 ± 4,700 (S.D.) estradiol binding sites/cell compared to 62,000 ± 9,700 sites/cell in cells grown in its absence. MCF-7 cells contain 10 pg DNA per cell (31), and thus these levels of bound estradiol are equivalent to 3.5 and 10.5 pmol/mg DNA, respectively. The binding constants were not significantly different and averaged 2.6 ± 0.8 × 10^{-10} M and 2.3 ± 1 × 10^{-10} M for cells grown in the presence or absence of insulin, respectively. The estrogen receptor levels reported here are in the range reported previously for total binding in MCF-7 cells; from 3 to 4 pmol (19) to 14 to 18 pmol/mg DNA (31). Thus, cells grown in the presence of insulin have one-third as much receptor as do those grown in its absence. This difference is sufficient to account for their reduced sensitivity to both estrogen and antiestrogens, as is discussed below.

DISCUSSION

In addition to increased growth, a number of other responses to estrogen have been reported for MCF-7 cells. We have shown that estrogen stimulates the production of plasminogen activator (10). Others have reported that estrogen increases the level of progesterone receptor in the cells (18), elevates the intracellular levels of lactate dehydrogenase (8) and thymidine kinase (29), stimulates amino acid incorporation (25), and induces the synthesis of proteins of various molecular weights which are released into the culture medium (35).

In the first report that estrogen stimulated the growth of MCF-7 cells (24), the stimulation was less than 2-fold, and even in the presence of estradiol, growth was slow. McGuire reported that the growth of MCF-7 cells was stimulated either only slightly (27) or not at all (18) by estrogen. More recently, 3 other laboratories have reported that estrogen did not stimulate the growth of MCF-7 cells in culture (16, 23). Our results are in agreement with these later reports. Even under conditions where we can demonstrate the stimulation of plasminogen activator production by estradiol (Chart 6), we see no effect on growth rate (10). Thus, the lack of growth response to estradiol seems unlikely to be due to the presence of residual estradiol in the cells or media.

We have described 2 factors which modify the response of the human breast cancer cell line MCF-7 to antiestrogens. The first, serum concentration, is of concern primarily to those working with MCF-7 and other steroid-sensitive cell lines. The effect of increasing concentrations of serum in reducing tamoxifen inhibition could be due to estradiol in the serum. The concentration of estradiol in calf and fetal calf serum ranges between 10^{-10} and 10^{-9} M (13), and thus the concentrations present in 10% serum (10^{-11} to 10^{-10} M) would be in the range over which estradiol stimulates these cells in serum-free medium (Chart 6). It is less likely, however, that the level of
estradiol present in 2% charcoal-treated serum would still be sufficient to protect against tamoxifen. In our hands, charcoal treatment removes over 90% of an added dose of \(^{3}H\)estradiol, although it is reported to be less effective in removing conjugated steroids such as estrogen sulfates (34). It is also possible that steroid binding proteins in serum reduce the effective concentration of tamoxifen or that factors in serum act, as does insulin, to alter the level of estrogen receptor in the cells. Such a change, as discussed below, could account for the altered sensitivity to tamoxifen. What is important is that the conditions used will affect the response observed and that this will vary with the type, treatment, and level of serum.

The action of insulin in altering the level of estrogen receptor in MCF-7 cells and reducing their sensitivity to both estrogen and antiestrogens demonstrates 2 important points: (a) that the level of estrogen receptor need not be a fixed property of a cell but can be modified by other factors, in this case, the peptide hormone insulin; and (b) that the level of estrogen receptor in cells can be related to their sensitivity to estrogen and antiestrogens.

Insulin has been reported to stimulate the growth of MCF-7 cells when they are grown in the absence of serum (28, 31). Our growth experiments were carried out in medium with serum, and while we occasionally observed some insulin stimulation of growth, we usually did not. The effect of insulin in lowering the estrogen receptor level and reducing the sensitivity of the cells to antiestrogens was seen in either case. We reported previously (9) that cells grown for many passages in the absence of added insulin contained the same level of estrogen receptor as cells maintained with insulin. The present results do not contradict those since the receptor level was not measured in the media in which the cells had been growing. In those experiments, cells from both cultures were grown under the same conditions (without insulin) for the receptor measurement. Similarly, in the experiments reported here, all cells are taken from cultures which have been maintained for many passages in the presence of added insulin. The increased receptor level is seen when they are grown in media without insulin.

The effect of insulin and diabetes on mammary tumors induced in rats by 7,12-dimethylbenz(a)anthracene has been studied extensively by Hilf et al. (15) and Shafie and Hilf (32). About 60% of these tumors regress in diabetic animals (15). When animals are made diabetic, the level of estrogen receptor declines in tumors which regress but increases in tumors which continue to grow (15, 32). By the criteria of growth and estrogen receptor level, MCF-7 cells in culture are similar to the rat mammary tumors which continue to grow in diabetic animals, that is, their growth is not dependent on insulin and the level of estrogen receptor is higher in the absence of insulin. Whether other hormones can also alter the level of estrogen receptor in MCF-7 cells is not known, although it has been reported that the level is increased by prolactin (31).

The mechanism by which insulin lowers the level of estrogen receptor in MCF-7 cells is not known, although preliminary experiments have indicated that the effect may be rapid. It has been suggested recently that phosphorylation and dephosphorylation of the receptor may play a role in the binding of estrogen to the cytosolic receptor and the translocation of the receptor to the nucleus (4). If this proves to be the case, insulin might act by influencing such a system.

Most importantly, the results presented here provide evidence that the level of estrogen receptor in cells can be correlated with their responsiveness both to estradiol and to antiestrogens. The 3-fold decrease in estrogen receptor level, produced by growing cells in the presence of insulin, is sufficient to account for their reduced sensitivity to estradiol, as measured by the induction of plasminogen activator (Chart 6). This is shown in Chart 8 where data on the induction of plasminogen activator (derived from Chart 6) have been combined with a curve showing the number of sites per cell with bound estradiol at different concentrations of estradiol. Binding curves were calculated from the average levels of receptor per cell and the binding constants measured in experiments of the type shown in Chart 7. In both cultures (plus or minus insulin), 50% induction of plasminogen activator occurs at concentrations of estradiol which give similar levels of receptor-bound estradiol (10,000 molecules/cell). However, it requires over 4 times as high a concentration of estradiol to achieve this level in the cells with a lower receptor level (plus insulin).

If receptors, activated by bound estrogen, achieve their effects by binding to specific sites in the nucleus, then their binding to any one site should be dependent on the level of the receptor-estrogen complex rather than directly on the level of estrogen. The relationship described in Chart 8 is consistent with such a model.

The possibility that the action of steroids on cells or tissues is modified by altering the level of steroid receptors has been suggested in other systems. In several tissues, progesterone antagonizes the action of estradiol and appears to do this by lowering the level of estrogen receptor (6, 20, 21). Reducing the estrogen receptor level in a tissue may prevent estrogen action even when the level of circulating hormone is unchanged (6). Another example is cell lines sensitive to dexamethasone in which lines with decreasing levels of receptor are increasingly resistant to dexamethasone (5).

It has been suggested that the level and not just the presence or absence of estrogen receptor in human breast tumors should
be considered in designing therapy (26). The results presented here lend support to that suggestion by providing evidence that the sensitivity of responsive cells to both estrogen and antiestrogens changes as a function of the estrogen receptor level.

The effect of insulin on tamoxifen inhibition, shown in Chart 5, can also be explained by the lower level of estrogen receptor in cells grown with insulin. This observation has implications concerning the mode of action of the antiestrogens tamoxifen and nafoxidine in inhibiting growth and thymidine incorporation in these cells. While the mechanism by which antiestrogens inhibit the growth of mammary tumors is unclear (19), at least 3 models can be suggested whereby antiestrogens, by binding to the estrogen receptor, could inhibit the growth of mammary tumor cells such as MCF-7. (a) If estrogen acts through its receptor to stimulate growth, then antiestrogens, by competing with estrogen for receptor, would block the stimulatory effect of estrogen. (b) It was reported that estrogen-free receptors are present in the nucleus of MCF-7 cells (37), though a more recent report from the same laboratory indicates that may not be so (12). If, in MCF-7 cells, estrogen-free receptors can stimulate the cells, as was suggested (37), then antiestrogens, by binding to free receptors, could make them unavailable and prevent their stimulatory activity. And (c) the receptor, with antiestrogen bound to it, could act in the nucleus to alter gene expression in a way which inhibits cell division or tumor growth. In any of these 3 models, added estrogen would be expected to overcome the inhibition caused by antiestrogens, as it does in MCF-7 cells. However, the effect of lowering the level of estrogen receptor in the cells on their sensitivity to antiestrogens would not be the same in all models. In either of the first 2 models, growth is stimulated by estrogen receptors, and antiestrogens, by binding to receptors, make them unavailable to the cells. Thus, lowering the total level of receptor in the cells should increase their sensitivity to antiestrogens. In the third model, however, lowering the level of receptor would make the cells less sensitive to the action of antiestrogens, just as it makes them less sensitive to the induction of plasminogen activator by estradiol (Charts 6 and 8). The increased resistance to these antiestrogens produced by lowering the receptor level is most easily explained if receptor with bound antiestrogens is actively inhibitory. If antiestrogens only act by competing with estrogen or by titrating out free receptor, then cells with a lower level of receptor should be more sensitive to them, not less sensitive as seen here.

Such an active model of antiestrogen action has a further implication. Tumors containing estrogen receptor might lose either their requirement for estrogen or their sensitivity to antiestrogens independently. It might be expected, therefore, that not all tumors would be equally responsive to procedures such as ovariectomy or adenectomy, which are designed to lower the level of estrogens on one hand and to therapy with antiestrogens on the other. There is some evidence that this is indeed the case. In one report on patients with metastatic breast cancer who had previously undergone adenectomy and were then treated with nafoxidine, the type of response to adenectomy did not necessarily correlate with the type of response to nafoxidine (22). Some responded to both, some to neither, and some to only one or the other, a finding consistent with the model proposed above in which antiestrogens can actively inhibit the growth of mammary tumors cells rather than or in addition to interfering with the action of estrogen.

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