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EFFECTS OF TAMOXIFEN, STILBESTROL AND METYRAPON ON BINDING OF VARIOUS STEROIDS TO CYTOSOL RECEPTORS IN HUMAN BREAST CANCER CYTOSOLS

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INDEXING WORDS
tamoxifen; stilbestrol; cytosol receptors; human breast cancer

SYNOPSIS

Tamoxifen, an antiestrogen drug, and stilbestrol, a synthetic estrogenic substance, exert their effects on progressive and recurrent breast cancers, comparing favorably in efficacy with surgical endocrine therapy. The present study was undertaken to investigate the influences of these drugs on steroid receptors in breast cancer tissues in an attempt to elucidate their mode of action.

Cancer tissues were homogenized and ultracentrifuged at 105,000 x G for 60 minutes. The supernatant was used as a cytosol fraction. Estradiol, dexamethasone, cortisol, dihydrotestosterone, synthesized progesterone (R-5020) and synthesized dihydrotestosterone (R-1881) were used. The drugs used were tamoxifen, stilbestrol and metyrapon. The charcoal-treated supernatant was incubated with 3H-steroid hormones in the presence or absence of unlabeled steroids, stilbestrol, metyrapon or tamoxifen. Hormone-receptor complexes were isolated by Sephadex G-25 gel filtration or by the charcoal method. Radio-activity was measured in a liquid scintillation counter.

Tamoxifen inhibited the binding of estradiol (E), R-1881, R-5020 and dexamethasone (DX) to the corresponding receptors. The binding capacities of serum for steroid hormones were also reduced.

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Tamoxifen had more potent inhibitory effect on binding of endogenous substances in blood and in cells to their receptors.

The inhibitory effect shared by tamoxifen, stilbestrol and metyrapon despite their widely divergent chemical structures and the apparent specificity of steroid-receptor binding reactions suggest that the receptors for the said hormones are either identical in molecular structure, or at least, their receptors have a certain reactive group or moiety in common.

INTRODUCTION

The presence of receptors in the breast cancer tissue for various steroid hormones such as estradiol, dihydrotestosterone, progesterone and dexamethasone has been well documented and a parallelism had been demonstrated between receptor content and hormone dependency. The percentage of positive response to adrenalectomy and oophorectomy has been estimated to be about 70% in patients selected with estradiol-receptors as the sole criterion, while a higher response is expected when several hormone receptors are taken together into consideration. Both tamoxifen, an anti-estrogen drug, and stilbestrol, a synthetic estrogenic substance, are known to produce clinical responses comparable to those achieved by adrenalectomy and oophorectomy, though their mechanism of action has not been fully elucidated. It is, therefore, worthwhile to study the influence of these substances on steroid receptors in breast cancer in order to clarify their mode of action on cancer tissues. Metyrapon, an inhibitor of adrenal corticosteroid synthesis, was used as control.

MATERIALS AND METHODS

Human breast cancer tissues obtained during surgical operations were frozen at -70°C, and stored until assay. These samples were assayed for the presence of hormone receptors. Breast cancer tissues were homogenized in 10 mM Tris buffer, pH 7.4, containing 1 mM EDTA and 250 mM sucrose. The resulting homogenate was ultracentrifuged at 105,000 x g for 60 minutes, and the supernatant was used as test cytosol. Tritiated and nonradioactive steroid hormones of six kinds, i.e. E, DX, cortisol, dihydrotestosterone (DHT), R-1881 and R-5020 (New England Nuclear) were used. The specific activities were 43.0, 22.6, 80.6, 44, 87, and 86 Ci/mMol, respectively. The drugs used were stilbestrol (Sigma), metyrapon (Ciba-Geigy) and tamoxifen (ICI). In an attempt to determine the binding of the steroids to their receptors in cytosol, cytosol fractions were stirred.
for 30 minutes with a suspension containing charcoal (3% w/v) and dextran 500 (0.3% w/v) at a ratio of cytosol:charcoal suspension of 10:1. The mixture was centrifuged and the second supernatant was used in the subsequent steps. Aliquots of the charcoal-treated supernatant were incubated with $^3$H-steroid hormones (2 x $10^{-9}$ M) in the presence or absence of unlabeled steroid, stilbestrol, metyrapon or tamoxifen. Hormone-receptor complexes were isolated by gel filtration on a Sephadex G-25 column (1 x 25 cm) or by the charcoal method (using 2% charcoal and 0.2% dextran in Tris-EDTA buffer). Hormone-receptor complexes isolated by the above gel filtration on Sephadex G-25 were subjected to gel filtration on Sephadex G-200.

The details of the individual experiments are given in the legends of the corresponding figures. The value of each point in Figs. 2 - 8 was the average of three breast cancer cytosols.

Radioactivity of $^3$H-estradiol-17 was measured in a liquid-scintillation counter after mixing aliquots of the individual samples with 10 ml of toluene solution (PPO 5g, 20 ml methyl-alcohol in 1000 ml toluene). The protein content was determined by the method of Lowry et al. using bovine serum albumin as standard.

RESULTS

As shown in Fig. 1, Sephadex G-200 gel filtration demonstrated that E and DHT had two peaks on binding to their respective receptors which were considered to be present in human breast cancer cytosols, while one peak was seen on binding of DX, R-1881 and R-5020 to their respective receptors. (For R-1881, another peak may appear due to its-globulin fraction.) The effects of tamoxifen, stilbestrol and metyrapon on E-receptor binding in human breast cancer cytosol are shown in Fig. 2. E-receptor binding was inhibited by tamoxifen and stilbestrol, while metyrapon did not influence this binding. Fig. 3 shows the results of an experiment in which serum was used instead of cytosol. The same experimental procedures were followed. E-receptor binding was inhibited by tamoxifen, and not by stilbestrol and metyrapon. The effects of the drugs on DHT-receptor binding in breast cancer cytosol were also investigated (Fig. 4). In an attempt to exclude the effect of blood present, R-1881 was used instead of DHT. The results are shown in Fig. 5. Tomoxifen showed the most marked inhibitory effect, followed by stilbestrol, but no effect was obtained with metyrapon. In the experiment shown in Fig. 6, R-5020 was substituted for progesterone to exclude the effect of blood present. The
Fig. 1
Sephadex G-200 gel filtration of human breast cancer cytosol labeled with \(^{3}H\)-estradiol, \(^{3}H\)-dihydrotestosterone, \(^{3}H\)-dexamethasone, \(^{3}H\)-R-5020 and \(^{3}H\)-R-1881. Breast cancer cytosols were incubated with \(^{3}H\)-steroids (2 x 10\(^{-6}\)M) for 5 hours at 4°C. The binding fractions were obtained by Sephadex G-25 column chromatography, and applied to a Sephadex G-200 column (2.5 x 100 cm) equilibrated with the standard buffer. 5 ml fractions were collected and the radioactivity was measured after mixing with 10 ml of toluene solution overnight.

Fig. 2
Inhibitory effects of tamoxifen, stilbestrol and metyrapon on estradiol binding in human breast cancer cytosol. Cytosols (1 mg, 0.5 cc) were incubated with \(^{3}H\)-estradiol (2 x 10\(^{-6}\)M), 0.1 mg, in the presence or absence of unlabeled estradiol, tamoxifen, stilbestrol or metyrapon in quantities of 0.25, 0.5, 1 or 10 mg, respectively. Cytosol protein concentration was 1 mg/0.5 cc. Incubation time was 6 - 8 hours at 0 - 4°C.

Fig. 3
Inhibitory effects of tamoxifen, stilbestrol and metyrapon on estradiol binding in human serum. The same procedures as described in the legend of Fig. 2 were carried out. Serum was substituted for cytosol.

Fig. 4
Inhibitory effects of tamoxifen, stilbestrol and metyrapon on dihydrotestosterone binding in human breast cancer cytosol.
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Fig. 5
Inhibitory effects of tamoxifen, stilbestrol and metyrapon on R-1881 binding in human breast cancer cytosol.

Fig. 6
Inhibitory effects of tamoxifen, stilbestrol and metyrapon on R-5020 binding in human breast cancer cytosol.

Fig. 7
Inhibitory effects of tamoxifen, stilbestrol and metyrapon on dexamethasone binding in human breast cancer cytosol.

Fig. 8
Inhibitory effects of tamoxifen, stilbestrol and metyrapon on cortisol binding in human serum. Serum protein (1 mg, 0.5 cc) was substituted for cytosol and the same procedures as described in the legend of Fig. 2 were carried out.
inhibitory effect on hormone-receptor binding was the greatest with tamoxifen, followed by stilbestrol. Some inhibition was also seen with metyrapon. Fig. 7 shows the effects of the drugs on DX-receptor binding. The inhibitory effect of metyrapon was the most profound, followed by stilbestrol and tamoxifen in that order. Fig. 8 shows the inhibitory effects of the drugs on cortisol binding to serum protein, i.e. cortisol-bound-globulin (C.B.G.). The inhibitory effect of tamoxifen was striking. Both stilbestrol and metyrapon slightly inhibited the binding.

**DISCUSSION**

Thirty to forty percent of breast cancer is hormone dependent and such type of cancer well responds to adrenalectomy and oophorectomy or to hormone administration. Prediction of hormone dependency is essential in selecting appropriate therapy for each patient. Since recent studies have indicated that the effects of hormone therapy on breast cancer might be predicted by assaying estrogen receptors in human breast cancer tissues, extensive studies on this subject have been carried out. Possibilities of the involvement of DHT receptors, progesterone receptors and glucocorticoid receptors have also been suggested, and thus, many investigators have attempted to clarify the role of these hormone receptors in clinical improvement of breast cancer.\(^3, 7, 10, 11, 14, 17\) We also have studied breast cancer in relation to hormone receptors using about 350 human breast tumor cytosols and found that 51% of tumors were estrogen receptor-positive, 38% progesterone receptor-positive, 17% DX receptor-positive, 32% DHT receptor-positive and 28% dehydroandrosterone receptor-positive. The data were analyzed according to the method of Scatchard.\(^13\) A tumor was considered receptor-positive if it contained more than 5 fmoles of receptor per milligram of cytosol protein. Although the relationship between hormone receptors and the effect of adrenalectomy or oophorectomy has not been fully clarified, hormone receptor-positive tumors tended to show favorable responses to adrenalectomy and oophorectomy. It has been reported that about 60 to 70% of estrogen receptor-positive patients respond to tamoxifen, an antiestrogen agent. The effect of this drug is, therefore, comparable to that of adrenalectomy and oophorectomy. In addition, oral administration of the drug is effective at the same efficacy rate even in patients at risk who cannot tolerate such surgical procedures. The mode of action of tamoxifen has been the subject of many published papers.\(^1, 6, 18\) In the present study, we investigated the effect of tamoxifen on
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steroid hormone-receptor binding affinities in comparison with that of stilbestrol and metyrapon. The isolation of steroid hormone-receptor complex by Sephadex G-200 gel filtration shows that there are two types of steroid receptors: One is the substances with presumed molecular weights of more than 200,000 (reported as 240,000) and the other is C.B.G. or sex hormone-binding components present in the serum. 2, 4, 19

When buffer containing 0.3 M NaCl ran through a Sephadex G-200 column previously equilibrated, macromolecules (eluted in void volume) were partially retained in the gel and eluted in a region corresponding to molecular weights of 60,000 to 80,000. (The data are not shown in this paper.) Synthetic hormones such as R-1881, R-5020 and DX are frequently used instead of DHT, progesterone and cortisol, respectively, as these synthetic hormones are thought to be unaffected by binding proteins in the serum. In our study, however, R-1881 was slightly bound to serum. The present study demonstrated that tamoxifen inhibited binding of all the steroids tested to their receptors. As shown in Figs. 2 and 3, the inhibitory effects of tamoxifen and stilbestrol were influenced by the level of hormone receptors in cytosols. In our opinion, however, the difference noted between these two drugs in the inhibitory action was probably due to blood present in the cytosol. Data supporting this are available. When the inhibitory effects on substances with molecular weights of more than 200,000, which were isolated by gel filtration on Sephadex G-200, were examined, tamoxifen was comparable to stilbestrol. (The data are not presented in this paper.) However, since tamoxifen inhibits both endogenous substances in the blood and those in cells, it may exert a stronger action on breast cancer. The ability of the drug to inhibit the binding of DHT to its receptors, as shown in this study, suggests that tamoxifen will possibly work in growth inhibition of prostatic cancer as is the case with stilbestrol. Inhibitory effects of tamoxifen, stilbestrol and metyrapon on steroid hormone binding at receptor sites are seen also in other tissues. Tamoxifen showed a similar inhibitory effect on hormone-receptor binding in human brain cytosols. (The data are not shown in this paper.) This suggests that tamoxifen may also act on other tissues.

In clinical practice, metyrapon is used in a daily dose of 1.5 g, while a daily dose of tamoxifen is as low as 20 mg. Therefore, it is difficult to compare the potency of tamoxifen with that of metyrapon. Since tamoxifen inhibits the binding of steroid other than estrogen to their receptors, there is a possibility that the drug exerts its action, in part, through some effects on the pituitary.
The specificity of receptors was lower than that of antibodies which were used for radioimmunoassay for the blood concentration of each steroid. In addition, estrogen receptor-rich cytosols contain other steroid hormone receptors as well. As demonstrated by the present study, tamoxifen, stilbestrol and metyrapon showed similar inhibitory effects on steroid hormone-receptor binding despite their widely divergent chemical structures. All the findings described above suggest that the receptors for the steroid hormones are identical in molecular structure and have various binding sites. Another possibility is that their receptors have a certain reactive group or moiety in common. Suther et al.\(^{15}\) have presented a similar hypothesis: When a certain steroid is bound to a receptor, the properties of this receptor are changed, and as a result, its binding capacity to other steroids may be increased or decreased. Other reports (Lippman,\(^8\) Tanaka\(^{16}\)) have suggested that the inhibitory effect of tamoxifen may arise from differences in binding affinities between estradiol and tamoxifen. In the present study, the inhibitory effect of tamoxifen on estrogen receptors was different from that reported previously, probably because of differences in experimental conditions such as incubation time and the doses of the drug and steroids. It is quite probable that tamoxifen, stilbestrol and metyrapon may influence the binding of steroids at their receptor sites.

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reserve and binding affinity to estrogen receptor.

