Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 723–727. Pergamon Press. Printed in Great Britain

STEROIDS AND HUMAN BREAST CANCER

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SUMMARY

Estrogen receptor is present in the rat in target tissues, including mammary gland and hormone dependent mammary tumors, but is absent in non-target tissues and in many autonomous tumors. In human tissues, estrogen receptor behaves much like that of the rat. Its absence in a breast tumor specimen predicts the failure of hormone therapy, as shown by data collected from many sources, and this fact will be useful to clinicians in selecting therapy for metastatic breast cancer patients. The receptor is present, however, in a substantial number of nonresponsive tumors. These tumors may contain defects in the normal nuclear localization or action of the receptor–estrogen complex or even in the functions of other hormones which affect the mammary gland. These defects will have to be identified for a complete biochemical prognosis for endocrine therapy in breast cancer.

INTRODUCTION

The human mammary gland is exquisitely sensitive to a number of hormones, among which estrogen and prolactin exert perhaps the most dramatic effects. It would be reasonable to predict that tumors arising by malignant transformation of mammary gland cells would retain these hormone controls. Indeed, the first demonstration of the regression of metastatic breast cancer in response to ovariectomy was made 78 years ago [1]. Unfortunately, only 30% of such tumors are responsive, and most of the same tumors will respond equally to adrenalectomy or hypophysectomy [2]; experiments to discover which hormones are actually controlling have not resolved the question. These tumors are therefore classified simply as "hormone dependent".

Target tissues for any hormone have been found to contain specific receptors for that hormone–cytoplasmic proteins for the steroids, and surface membrane molecules for polypeptides and some others. Hormone dependent tumors likewise contain receptors, but it now appears that independent, or autonomous, tumors often may not [3].

These findings, which will be discussed in detail, have led to the following hypotheses:

1. Normal mammary cells contain cytoplasmic or membrane receptor sites for each of the hormones known to influence the growth and function of the mammary gland. These receptor sites are responsible for the initial interaction between the hormone and the cell, and function to trigger the biochemical chain of events characteristic for the particular hormone.

2. When malignant transformation occurs, the cell may retain all or only part of the normal population of receptor sites. If the cell retains the receptor sites, its growth and function is potentially capable of being regulated by the hormonal environment as in a normal cell; however, if the receptors are lost from the cell as a consequence of malignant transformation, the cell is no longer recognized as a target cell by circulating hormones and endocrine control is absent.

3. The absence of specific receptors in mammary tumor tissue may therefore indicate hormonal autonomy, thus aiding in selecting the 30% of breast cancer patients who will actually benefit from endocrine therapy.

Only estrogen receptors have thus far been studied with respect to all three hypotheses. The preferential uptake of radioactive estrogen by target tissues and dependent tumors was demonstrated *in vivo* and *in vitro* in the years following 1959; autonomous tumors were less active [4–10]. The discovery of the specific receptor for estrogen in these active tissues explained the preferential uptake of the hormone and also suggested assaying tumors for receptor to predict hormone dependence. Aspects of the mechanism of action of the receptor–estrogen complex have also been studied.

It is the purpose of this review to examine some of these studies, in human mammary tumors where possible but also in induced rat mammary tumor models [11] and in other estrogen target tissues. Finally, the third hypothesis will be strongly confirmed by 380 collected cases from several centers, and other implications of these cases will be discussed.

Estrogen effects on breast tumors

Estrogen has been shown to act directly on the normal mammary gland to promote growth and differentiation. However, estrogen also stimulates the release of pituitary prolactin, and prolactin also acts upon the mammary cell. Since estrogen cannot support mammary tumor growth in the absence of a pituitary, whereas prolactin reportedly supports normal mammary gland and mammary tumor growth in the absence of ovaries and adrenals, estrogen is considered by many to play only a secondary role

in breast tumor growth and regression [12]. In this regard, however, it may be significant that experiments showing prolactin stimulation of tumor growth in the absence of ovarian steroids were of brief duration; if DMBA tumor bearing rats are ovariectomized and simultaneous lesions are placed in the median eminence to increase prolactin release, these tumors grow at an accelerated pace for only 10–12 days and then regress, even though prolactin levels remain elevated [13]. Furthermore, it has been reported that the transplantation survival of the MTW9 rat mammary tumor requires ovarian hormones, and MTW9 tumors transplanted to rats immunized with estradiol-BSA conjugates will grow less well than in untreated controls [14]. One might summarize the evidence bearing on the role of physiological estrogen levels as follows: estrogens are probably essential but not sufficient for growth of certain mammary tumors.

Another important effect of estrogens is the regression of mammary tumors following pharmacologic doses. This seemingly paradoxical effect of estrogen appears to act by interfering with the prolactin stimulation of growth, since the effect can be overcome by increasing endogenous or exogenous prolactin [15].

Estrogen receptors

Study of the nuclear binding of estrogen receptor, its presence in both rat and human mammary tumors, and its role in hormone dependence of tumor growth were all stimulated by the emergence of the findings just discussed. In the following four sections, we describe primarily our own part in these studies.

Hormone dependent rat mammary tumors

We first demonstrated estrogen receptor (R) in the cytoplasm of DMBA induced hormone dependent tumors by incubating the cytosol fraction with tritiated estradiol (^{3}H-E) and applying it to a Sephadex G-100 column; a majority of the ^{3}H-E was eluted bound to the macromolecular fraction. Sucrose gradient centrifugation of these cytosols usually revealed two peaks of protein bound radioactivity—one at 8 S and another at 4 S. Whereas the 8 S binding peak always represented specific R–E interaction, the 4 S peak contained both specific and nonspecific binding components. In 0·3 M KCl, specifically bound ^{3}H-E in either uterus or tumor cytosol migrated exclusively at 4 S, suggesting that the 8 S binding molecules dissociate into subunits at high ionic strength. Physiological ionic strength (0·15 M salt) caused the receptor to sediment at 6 S. Although the 8 S species obtained in low salt gradients is therefore probably a nonphysiological form, it is a fortuitous one where separating specific receptor is concerned since all proteins which bind estradiol nonspecifically sediment at 4·6 S or less. The significance of these sedimentation forms is still in question, especially in the light of the finding that polyanions such as heparin can cause receptor to sediment at any rate between 8 S and 4 S, depending on the polyanion concentration [16].

Evaluation of the affinity of receptor for E requires a method for separating bound from free estradiol. The dextran coated charcoal method as recently modified [17] has proved both accurate and convenient for this purpose. We find a single class of high affinity binding sites with a Kd of the order of 2×10^{-10} M in hormone dependent DMBA induced tumors. The binding of ^{3}H-E is estrogen specific, since it is inhibited by low concentrations of unlabeled E but not by 1000- to 10,000-fold excesses of hydrocortisone, progesterone, or testosterone [18].

The properties found for receptor in hormone dependent rat mammary tumors are essentially those described for other estrogen target tissues. And like other target tissues, these tumors accumulate injected E in their cell nuclei *in vivo*, apparently still bound to a 4–5 S form the the receptor. We will consider the nuclear binding of E in more detail later.

Autonomous rat mammary carcinoma

In this laboratory we have used the R3230AC transplantable mammary carcinoma, described in detail by Hilf *et al.* [19], as one example of a breast tumor that does not regress after ovariectomy.

We first found that nuclei from this tumor do not appreciably concentrate ^{3}H-E injected *in vivo* [20]. This was attributed to the very low level of cytoplasmic receptor after we demonstrated a tenfold lower E binding capacity in R3230AC cytosol than in a representative hormone dependent DMBA tumor cytosol. The affinity constants were equal. The R3230AC 8 S receptor as revealed by sucrose gradient sedimentation was also very low [21].

We also considered the possibility that R3230AC might have lost the ability to bind R–E to chromatin, providing another reason for its autonomy. We therefore prepared chromatin from purified R3230AC nuclei and measured the ability of cytosols containing various amounts of R to bind E to chromatin. We found that cytoplasm from R3230AC tumor, muscle, or brain failed to bind E to the tumor chromatin. This failure is due to the paucity or total lack of R in these tissues. However, the complex of E with rat uterine cytosol which contains abundant R, demonstrated remarkable binding to R3230AC chromatin. These results indicated that chromatin of these autonomous breast tumor cells possesses the capacity for extensive interaction with R–E. Consequently, the failure of R3230AC nuclei to accumulate E *in vivo* can apparently be attributed to a deficiency of R in the cytoplasm [22, 23].

Nuclear acceptor activity

The finding that chromatin from the autonomous R3230AC tumor binds R–E led to an investigation of R–E binding by nuclei of other tissues [24]. As indicated above, only target cells for estrogen possess

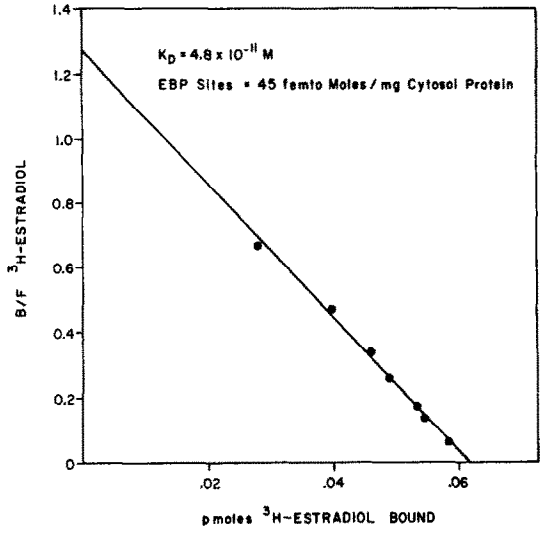

Fig. 1. Scatchard plot of the data from a dextran coated charcoal assay for estrogen receptor in a human breast tumor cytosol.

the specific cytoplasmic R, and there have been suggestions that nuclear acceptor sites for R–E are likewise found only in target cells. In questioning these latter suggestions, we determined quantitative R–E binding to nuclei prepared from several different tissues, and also examined the recovered receptor qualitatively by sucrose sedimentation.

We found that cytoplasmic R–E bound equally well to nuclei prepared from target and nontarget tissues and furthermore that nuclear binding *in vitro* was strictly proportional to the available R–E with no evidence of saturation of a special class of binding sites even up to many times the level present *in vivo* [25]. Experiments *in vivo* or with intact tissue have led to similar conclusions [26, 27].

It seems likely, then, that some mechanism other than binding to a small number of specific operator-like sites must be found to explain the action of R–E in target cell and tumor nuclei. In this regard, it is important to recall that binding of R–E in the nucleus has not yet been proved to be directly involved in mediating all responses to the hormone. It may be possible that other activities of R–E in the nucleus or even in the cytoplasm, undetected by present techniques, have major roles in determining the response.

Estrogen-receptor in human breast tumors

The properties of the estrogen receptor as determined in induced hormone dependent rat tumors have now been found in human mammary tumor cytosols as well [28]. Two of these properties are employed in our laboratory to quantitate R in human breast cancer specimens obtained at surgery [29]. The first is the high affinity binding of ^{3}H-E, evaluated by equilibrating cytosol with various low concentrations of labeled hormone and then removing the unbound hormone with dextran coated charcoal as described earlier. Scatchard plots of the binding data reveal the receptor, if present, as a very high affinity binding component (Kd $< 1 \times 10^{-10}$ M), and permit direct extrapolation to determine the amount of this component (Fig. 1). The second property, the sedimentation of receptor primarily at 8 S in low salt sucrose gradients, is employed to confirm the above determination by an independent method. Because part of the 4 S binding peak may also be due to specific receptor, a parallel gradient is always run with a 100-fold excess of unlabeled estradiol to demonstrate the nonspecific binding components alone (Fig. 2).

With these techniques, we were in a position to explore Jensen's original suggestion that the presence of R in a human breast tumor might indicate that the tumor was hormone dependent and could be made to regress by appropriate endocrine manipulation. To this date this laboratory has assayed R in 154 primary tumors and 72 metastatic tumors from surgery for correlation with response to endocrine therapy [30].

In Fig. 3 we see that the values in primary tumors range from 0 to almost 1000 femtomol/mg of cytosol protein. (The level of sensitivity in the two methods is such that a value of less than 3 is essentially equivalent to 0 and is considered a negative assay.) Positive R values (>3) are found in 70% of primary specimens and 58% of metastatic specimens. We have previously speculated that the wide range of values apparent in our results is due to a combination of factors, including: (a) variations in epithelial vs stromal content of the tumor; (b) the degree of dedifferentiation of the tumor; and (c) the amount of endogenous estradiol secreted by the patient (since

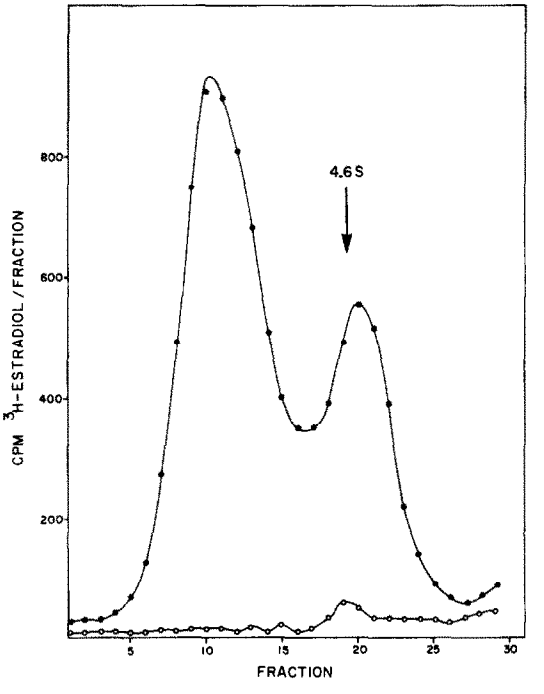

Fig. 2. Sucrose density gradient centrifugation of a human breast tumor cytosol with O——O and without ●——● nonradioactive estradiol competitor.

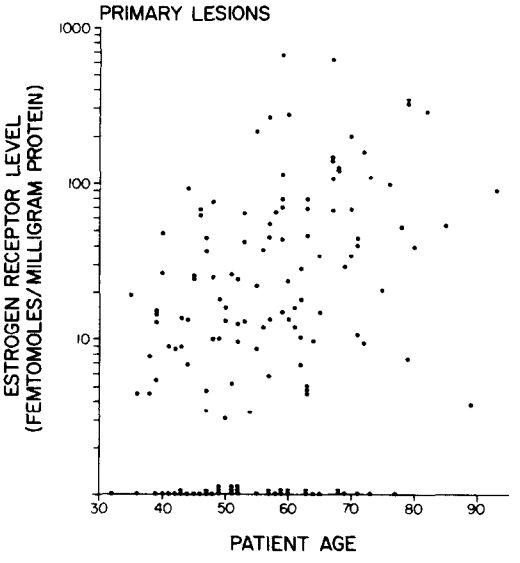

Fig. 3. Estrogen receptor values in primary human breast cancer tissues listed according to the age of the patient.

endogenous estradiol would occupy E–R sites and make them unavailable for assay) [20]. This last point may at least partially explain why the highest values for tumor R are seen in postmenopausal patients.

Clinical correlation

A number of other laboratories have likewise assayed R in breast tumor specimens using a variety of techniques. Data on clinical response to endocrine therapy is now available in many of these cases. On 18–19 July 1974, an international workshop sponsored by the Breast Cancer Task Force of the National Cancer Institute was held in Bethesda, Maryland, for the correlation of this data. Details of both R assay procedures and clinical evaluation criteria were examined, and 436 treatment trials in 380 patients were ultimately accepted. We here provide a brief overview of the data presented at that meeting, indicating the current status of R assays in predicting response to endocrine therapies in patients with metastatic breast cancer. For details, the reader should consult the specific manuscripts and summary chapter published in the conference proceedings [31].

Endocrine responsiveness

Since the organizing committee for this conference felt that clinical response data for endocrine responsiveness was as critical to the correlations as the assay data itself, it was arranged that participating institutions could request an extramural review of their case material. Prior to the conference, eight institutions were visited by two oncologists who evaluated a total of 453 cases by exacting criteria for objective remission. Cases of doubtful response and those not fully documented were removed, and 380 were finally accepted. The data, presented in Table 1, can be summarized as follows:

Ablative therapy—33% of 211 treatment trials yielded objective tumor regressions. Of the 94 trials in patients with negative tumor R values, only 8 (8%) were successful, whereas 59 (55%) of the 107 trials in patients with positive tumor R values succeeded. Patients with borderline tumor R values had a 30% response rate.

Additive therapy—34% of 170 trials yielded objective tumor regressions. Of the 82 trials in patients with negative tumor R values, 7 (8%) were successful, whereas 51 (60%) of the 85 trials in patients with positive tumor R values succeeded.

Miscellaneous therapy—27% of 55 trials yielded responses to a variety of endocrine therapies including antiestrogens, aminoglutethimide, etc. Of 32 trials in patients with negative tumor R values, 5 (16%) were successful, whereas 10 (43%) of 23 trials in patients with positive R values succeeded.

COMMENTARY

The data presented leaves little doubt that estrogen receptor assays can be helpful to predict the results of endocrine therapy for metastatic breast cancer. It is clear that if a patient has a negative tumor R value, the chances of tumor regression in response to endocrine therapy are minimal. It seems that a large number of patients can be thus spared unrewarding major endocrine ablative therapy if R assays are performed routinely. If the tumor R value is positive, the response to endocrine therapy is 55–60%. This single piece of data when coupled with available clinical prognostic factors such as menopausal status, disease free interval, site of dominant

Table 1. Objective breast tumor regressions according to R–E assay and type of therapy as judged by extramural review

Therapy	R–E+	R–E−	R–E±
Adrenalectomy	32/66	4/33	3/8
Castration	25/33	4/53	0/2
Hypophysectomy	2/8	0/8	—
Total	59/107 = 55%	8/94 = 8%	3/10 = 30%
Androgen	12/26	2/24	0/1
Estrogen	37/57	5/58	0/2
Glucocorticoid	2/2	—	—
Total	51/85 = 60%	7/82 = 8%	0/3 = 0%
Antiestrogens	8/20	5/27	—
Other	2/3	0/5	—
Total	10/23 = 43%	5/32 = 16%	

lesion, and especially response to a previous hormonal therapies should permit the practicing oncologist to select or reject endocrine therapy with considerable confidence.

The R data should also be considered in light of current theories of hormone dependence in breast cancer. When malignant transformation occurs, the cell may retain all or only part of the normal population of receptor sites. If the cell retains the receptor sites, its growth and function is potentially capable of being regulated by the hormonal environment as in a normal cell; however, if the receptors are lost from the cell as a consequence of malignant transformation, the cell is no longer recognized as a target cell by circulating hormones and endocrine control is absent. This could explain why patients fail to respond to endocrine therapy if their tumors lack R.

Why then did not all of the patients with positive tumor R values respond to endocrine therapy? It should be emphasized that R is only a part of the complex hormonal control system known to regulate mammary tumor growth. The biochemical mechanism by which prolactin recognizes a breast tumor as a target cell must be equally important [32], and other hormones are undoubtedly involved as well. Nevertheless, the R data suggests that loss of endocrine control is closely related to the loss of R. Does this mean that receptors for other hormones are lost in conjunction with R and will also be absent in the autonomous mammary tumor cell? Or does estrogen–receptor somewhow occupy a unique position in determining hormone dependence? Until we have the answers to these questions it may be useful to consider simply that the absence of R in a tumor cell is one indication of the relative departure from normality that has occurred in the process of malignant transformation and may therefore be useful in predicting the loss of hormone dependence. Because other biochemical events involving estrogen itself as well as prolactin and other hormones are probably prerequisite for complete endocrine regulation, we can predict that other biochemical lesions have occurred in patients in whom endocrine therapy failed despite positive tumor R levels. We still have a great deal to learn about the subcellular biochemistry of hormone action in breast cancer tissue, and future investigation will undoubtedly lead to further improvements in therapy for patients with breast cancer.

Acknowledgements—These studies were supported in part by the USPHS CA-11378, CB-23862, and the American Cancer Society, BC-23C.

The clinical workshop data were contributed by the following participating institutions:
Alton Ochsner Medical Foundation, New Orleans, Louisiana;
Ben May Laboratory for Cancer Research, Chicago, Illinois;
Case Western Reserve University, Cleveland, Ohio;
Institut Jules Bordet, Brussels, Belgium;
Michigan Cancer Foundation, Detroit, Michigan;
Middlesex Hospital & Medical School, London, England;
Netherlands Cancer Institute, Amsterdam, The Netherlands;
Norsk Hydro's Institute for Cancer Research, Oslo, Norway;
St. Mary's Hospital Medical School, London, England;
The Imperial Cancer Research Fund, London, England;
University of Hamburg, Hamburg, Germany;
University of Oregon Medical School, Portland, Oregon;
University of Rochester School of Medicine & Dentistry, Rochester, New York;
University of Texas Health Science Center, San Antonio, Texas;
University of Uppsala, Uppsala, Sweden;
University of Western Australia, Perth, Western Australia.

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