7145-4-7-3

Final Rpt

CHARACTERIZATION OF STEROID HORMONE BINDING AND INDUCTION IN ESTROGEN DEPENDANT MAMMARY CARCINOMAS

Presented to

Faculty Research Committee
Morehead State University

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INTRODUCTION

Mechanism of Action

Steroid hormone receptors are soluble proteins which probably play a key role in the mechanism of action of the steroid hormones. The presence of receptor appears to be prerequisite for cellular response to changes in hormonal milieu (1). The mechanism of action of steroids with their target cells is shown in Figure. Unbound steroid enters the cell by passive diffusion and combines with cytoplasmic receptor in an association characterized by high affinity and ligand specificity (2). In the case of estradiol, it has been proposed that binding occurs onto a 4 S binding

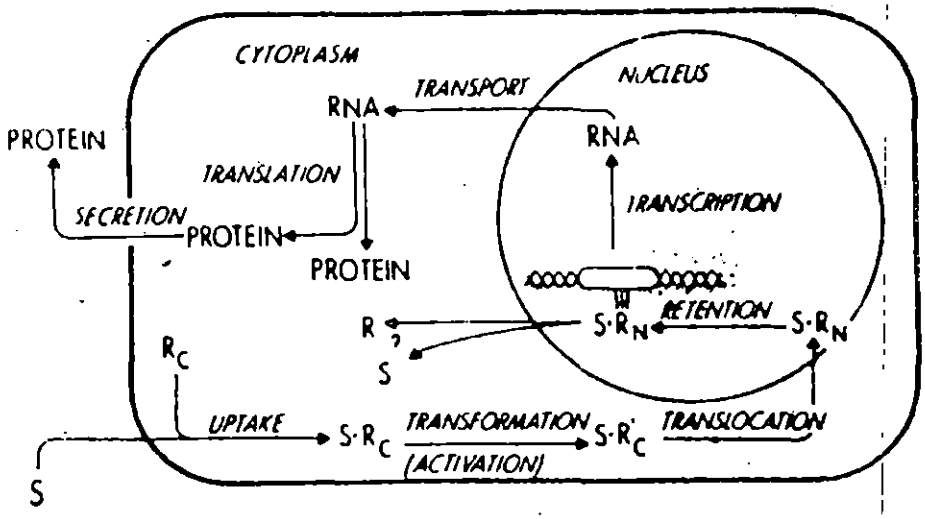

Figure 1. Proposed Steps in Steroid Hormone/Target Cell Interaction. The steroid is designated as S, the cytoplasmic receptor as Rc, and the nuclear form as Rn. Taken from Wittliff.

4 S binding subunit of the 8 S receptor followed by a temperature dependent 4 S to 5 S transformation and subsequent translocation to the nucleus (3). The nuclear hormone receptor complex then associates with chromatin resulting in stimulation of messenger RNA synthesis and the subsequent formation of cellular proteins (4). The nature of the departure and fate of the nuclear hormone receptor complex is unknown (1). Cellular replication and differentiation appear to result from this cascade of events, when intact (5).

Normal breast contains specific binding sites for estrogens, progestins, corticoids, and androgens, prolactin, growth hormone, and insulin (6). Estrogen and prolactin are the two primary hormones in breast growth regulation (7). A model of complete endocrine regulation of breast tissue must include the interaction of these various hormones.

Endocrine Therapy

The concept underlying endocrine therapy is that certain tumor cells retain the ability to respond to the same hormonal perturbations as their normal progenitors (1). That is, certain cancer cells are dependent on one or more hormones and succumb when deprived of supporting hormones or when pharmacologic levels of hormone are administered (8).

Human and experimental breast tumors which regress following endocrine therapy frequently contain a cytoplasmic protein that specifically binds estradiol with high affinity (9).

It is assumed that the presence of estrogen receptor (ER) in breast tumor is due to the expression of a phenotypic characteristic of the normal, hormonally responsive cell line of origin (1). Cells which have undergone malignant transformation may retain all or only part of the normal receptor population, and, in theory at least, hormonal control should be absent in the absence of specific receptor (8).

The observation, as previously noted, that labeled estrogens are localized in highest concentrations in estrogen target organs, led to the suggestion by Jensen et al. in 1965 that the ability of breast tumor to bind estrogen might be predictive of responsive to endocrine therapy (10). This idea was supported by their later findings in which the presence of estrogen receptor in tumor biopsies correlated with favorable response to adrenalectomy (11) and by the observation of Folca et al. (12) that when tritiated hexestrol was administered to breast cancer patients prior to adrenalectomy, metastases which subsequently regressed concentrated a larger fraction of the labeled estrogen than those which failed to respond. A correlation between the presence of estrogen receptor in breast tumor biopsy material and favorable response to endocrine therapy was presently confirmed in the laboratories of Wittliff (13) and McGuire (14).

Endocrine additive and ablative therapies are interventions are without potentially serious complications. Methods of accurate selection could spare critically ill patients in whom endocrine therapy is unlikely to succeed from unnecessary

treatment. An expansion of steroid binding data has followed to the present, and, while it remains true that estrogen receptor negative tumors rarely respond to endocrine therapy, the response of ER positive tumors varies over a wide range (15). It is now generally accepted that selection of patients by estrogen receptor analysis improves the response rate to endocrine therapy by at least two-fold over non-selected cases (2). It has also been suggested that ER negative tumors tend to have higher mitotic rates as estimated by thymidine labeling, and that these more rapidly growing tumors might be expected to respons more favorably to cell cycle phase specific cytotoxic chemotherapy (16).

Specific receptor for progesterone has also been identified in breast tumor cytosols (17). Improved response rates to endocrine therapy in ER positive tumors has been reported if specific progesterone receptor (PR) is also present (18). It has been demonstrated that following oophorectomy, cytosol progesterone receptor levels diminish while cytosol ER levels remain high (19). As the synthesis of progesterone receptor may be estrogen dependent, Heusen et al. has suggested that PR positivity might simply be a reflection of ER concentration. This concept is complicated by the puzzling observation that tumors which are ER negative but PR positive (an enigma in theory) often respons to endocrine manipulation (20). It is currently accepted that steroid receptor analysis in mammary carcinoma allows for prediction of response to endocrine therapy, and that the rate of response is highest when both

ER and PR are present (21).

Failure to Respond

Many tumors which contain specific estrogen binding proteins do not respond to endocrine maneuvers. The capacity to bind estrogen, therefore, does not obligate a biological response to the hormone. Events beyond the level of binding to receptor may be defective, such as receptor transformation (4 S - 5 S), translocation to the nucleus, and binding into chromatin.

Thus, given the multiple subunit model shown in Figure 1, certain unresponsive breast cancers could contain specific estrogen binding protein, but not the full complement of receptor subunits necessary for transforamtion and translocation (2). This may also explain, at least in part, the improved response rates of tumors when both ER and PR are present. The response of progesterone receptor, assuming the dependence of PR synthesis on estrogen stimulation, would rule out a defect in estrogen interaction beyond the level of binding to receptor (22).

Another factor which may enter into the failure of receptor positive tumors to respond to endocrine manipulation is the heterogeneity of tumors with regard to cellular composition. While the receptor concentration by assay represents an integrated value for the entire biopsy, the actual concentration of receotpr may vary widely from cell to cell. Tumor biopsies which are receptor (R) positive are likely to

include a variable number of receptor negative cells. It may be that hormone dependent cells atrophy as a result of milieu alterations, while hormone independent cells continue to proliferate. This theory is complicated by the observation that in tumors which regress following oophorectomy and then resume growth (obstensibly due to proliferation of the hormone independent sub-population) a second remission can be induced by further ablative therapy such as adrenalectomy or hypophysectomy. A possible explanation for this phenomenon is that regrowth included both receptor positive and receptor negative cells, with peripheral conversion of androstenedione to estrone allowing R positive cells to participate. Adrenalectomy or hypophysectomy would eliminate the adrenal source of estrogen precursors, allowing for the second regression of R positive cells. In reality, tumor cell subpopulations may represent a continuum of varying receptor populations in varying concentrations, and different endocrine therapies may be affecting different levels of endocrine regulation. There is no apparent correlation between tumor histology and hormone binding capacity (1, 2, 6).

Carcinogenesis

It is established that the mammary gland is sensitive to the actions of carcinogens and is a major site for the concentration of dimethylbenz (a) - anthracene (DMBA) and trimethylbenz (a) anthracene (TMBA) (23). Both 7, 12, DMBA and 7, 8, 12 TMBA are highly carcinogenic, characterized by

multiplicity of tumors and rapidity of carcinogenesis. Carcinogenicity of aromatic hydrocarbons is associated with their ability to form charge transfer complexes with local acceptors and donate an electron. There is also a direct increase in carcinogenicity as hydrocarbons become sterically similar to steroids. There is a remarkable steric similarity between carcinogenic polynuclear aromatic hydrocarbons, growth promoting steroids, and nucleic acid base pairs.

Specific receptors for estrogen and profesterone have been demonstrated in DMBA induced tumors (24). Receptor of DMBA induced tumors behaves like that of human mammary tumors with regard to range of concentrations, affinity (Kd), and molecular species by gradient centrifugation (6). It has been reported that among tumors induced by DMBA in rats of the Sprague-Dawley strain, 85-90% are estrogen receptor positive and 85% are progesterone receptor positive (25).

DMBA induced carcinoma of the rat shares a number of characteristics with human breast cancer, as well as some differences. The common characteristics in biological behavior and response of cytostatic and endocrine therapy make chemically induced rat mammary carcinoma a suitable model for human breast cancer (26). In this investigation, cytoplasmic and nuclear binding sites for estrogen and progesterone in DMBA induced mammary carcinomas were analyzed by titration assay. Results were compared between two groups of tumors categorized by response to oophorectomy. In one group, termed hormone dependent, tumors regressed completely

following oophorectomy. In the second group, termed hormone independent, tumors continued to grow. It is the purpose of this study to examine the differences in steroid binding parameters between these two biologically defined tumor populations.

MATERIALS AND METHODS

Animal Treatment

Immature female rats of the Sprague-Dawley strain were obtained from Harlan Industries, Indianapolis, Ind., housed in temperature- and light-controlled quarters, and supplied with water and Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) ad libitum.

At age 50 ± 1 days, under light ether anesthesia, animals were administered a single feeding of 20 milligrams of 7,12 dimethylbenz(a) anthracene (DMBA), in 2 milliliters of oil emulsion, by intra-gastric instillation.

Beginning at one month after DMBA treatment, animals were palpated once a week for the appearance of mammary tumors. When the average diameter of a palpable tumor exceeded 2 cm., the animal was anesthetized with pentabarbital (the Butler Co., Columbus, O.) at a dose of 3 - 4 mg per 100 g body weight intraperitoneally, and subjected to bilateral oophorectomy. At the time of surgery, tumors were measured with calipers and mapped. Approximately 1/2 of the tumor was removed for receptor analysis, leaving 1/2 part in the host for observation of its growth pattern.

Tumor biopsies were dissected free of connective

tissue and debris, frozen on dry ice/acetone and stored at -20° C.

Hormone Dependence

If following oophorectomy, a tumor regressed completely it was termed hormone-dependent, and if it continued to grow it was termed hormone-independent, the period of observation being one month. For the hormone dependent group, biopsy material obtained at oophorectomy was utilized for receptor analysis. For the hormone independent group, animals bearing tumors which continued to grow following oophorectomy were stunned and decapitated and tumor tissue was removed and processed as described for tissues obtained at oophorectomy.

Reagents

For binding experiments [2,3,4,5,16,17-^{3}H] estradiol - 17 beta (151 ci/mmol), [17 α methyl - ^{3}H] R5020 (17,21 dimethyl-19-nor-4,9-pregnadiene-3,10-dione; 85 ci/mmol), and radioinert R5020 were obtained from New England Nuclear Corp. Unlabeled diethylstibestrol (DES), Tris preset crystals (pH 7.0 at 27° C), and n,n-dimethyl formamide were purchased from Sigma Chemical Co. ScintiVerse E was purchased from Fischer Scientific, and Analytical grade hydroxylapatite (DNA grade BioGel HTP) was purchased from BioRad.

Determination of Cytosol and Nuclear Binding Sites

Binding site determinations employed and modifications of the hydroxylapatite methods of Chamness et al and Pavlik and Coulson (27, 28). All procedures were carried out on ice or refrigerated at 0 - 4° C, unless otherwise specified.

Frozen tissue was weighed, minced on a cold glass plate and homogenized in buffer containing 10 mM Tris (pH 7.56 at 0°) and 7 1/2 percent n,n dimethylformamide (v/v). Homogenizations were carried out in a seven milliliter TenBroeck pyrex glass-glass tissue grinder at a ratio of approximately 100 mg tissue per ml buffer. The homogenate was centrifuged 10 minutes at 800 x g. The supernatant was further centrifuged for 50 minutes at 45,000 x g to yield the high speed supernatant (cytosol). The first pellet was resuspended in homogenization buffer and washed twice by centrifugation for 10 minutes at 800 x g discarding the supernatants, then resuspended for one hour in homogenization buffer containing 0.4 M potassium chloride for extraction of nuclear receptors. The pellet suspension was then centrifuged for 10 minutes at 3000 x g and the supernatant (nuclear extract) was assayed for nuclear receptors.

Five hundred microliter aliquots of cytosol or nuclear extract were incubated with four final concentrations of either [^{3}H] estradiol - 17 Beta (.15, .5, 3.0, 10.0 nM) or [^{3}H] R5020 (.5, 3.0, 15.0, 45 nM) in homogenization buffer. Reactions were performed in parallel with a 200 - fold excess of radioinert competitor for determination of non-specific binding. DES was used in estrogen receptor assays and unlabeled R5020 was used in progesterone receptor assays. Incubations were for 16-18 hours at 0 - 4^{O} C. The synthetic progestin R5020 is especially useful in receptor studies because it does not bind to corticosteroid binding globulin (CBG) (17). Similarly, an important advantage of DES (a non-steroidal estrogen) is its low affinity for sex steroid binding globulin (SSBG) (29).

Hydroxylapatite (HAP)

At the end of incubation duplicate 200 microliter aliquots from each incubation tube were added to tubes containing one milliliter of 10% hydroxylapatite slurry (prepared by suspending 10 grams of DNA grade BioGel HTP in 100 ml of homogenization buffer). Adsorption to HAP was carried out for 45 minutes, with occasional vortex mixing to maintain suspension. The HAP was then pelleted by centrifugation for 2 minutes at 800 x g. The pellet

was washed three times with 2 ml homogenization buffer by centrifugation for 2 minutes at 800 x g, discarding the supernatants.

Bound radioactive steroid was removed from HTP by extraction into 1.5 ml 100% ethanol (30 minutes, room temperature), and then counted in 5 ml of scintillation fluid (ScintiVerse E, Fischer Scientific). All counts were made in Packard TriCarb Model 300 Liquid Scintillation Spectrometer at Maxey Flats Low-Level Nuclear Waste Disposal Site.

For the calculation of binding capacity (B_{max}) and affinity (dissociation constant, Kd), data were plotted according to the method of Scatchard (46) and subjected to least squares regression analysis. Table 1 and Figure 2 depict representative titration data and Scatchard analysis. Total bound steroid (B_T) is the amount of radioactive steroid bound in the absence of competitor. Non-specifically bound steroid (B_{NS}) represents the amount of tritiated steroid bound in the presence of a 200-fold excess of radioinert competitor. Total radioactive steroid (T) was estimated by direct sampling of incubates prior to the addition of the hydroxylapatite slurry. The quantities of B_T, B_{NS}, and T were determined by scintillation counting while specifically bound steroid (B_S) was estimated by

subtracting B_{NS} from B_T, and total free steroid (F) was estimated by subtracting B_T from T. Specifically bound steroid (B_S), as the x-variable, was plotted against the bound to free ratio (B_S/F) as the y-variable (Figure 2). A line was fitted to the data points by least squares regression analysis and B_{max} and Kd were estimated as the x-intercept and - 1/slope, respectively. B_{max} and Kd were calculated in terms of count rates (disintegrations per minute, dpm) and then converted to molar concentrations. Estimated binding sites (the molar equivalent of B_{max}) were standardized by protein concentration, as determined by the method of Lowry et al (30), with bovine serum albumin (1 mg/ml) as standard. The data were expressed as femptomoles of bound steroid per milligram of protein (fmol/mg) for estimated binding sites and moles per liter (M) for Kd.

Table 1. Representative Titration Data (Cytosolic ^{3}H Estradiol Binding, Tumor Number 5) In Disintegrations Per Minute (dpm). B_T = total bound, B_{NS} = non-specifically bound, B_s = specifically bound ($B_T - B_{NS} = B_s$), T = total radioactive steroid added, and F = total free steroid ($T - B_T = F$).

B_T	B_{NS}	B_s	T	F	B_s/F	^{3}H Estradiol Concentration
66,139	54,691	11,448	656,300	590,161	.019	10.0 nM
30,535	19,115	11,420	189,811	159,276	.072	3.0 nM
11,529	3,819	7,710	45,500	33,971	.227	.5 nM
1,578	472	1,106	4,639	3,061	.361	.05 nM

Table 4. Estrogen Receptor (ER) Distribution and Response to Oophorectomy.

Receptor Status Cytosol	Nuclear	Total Number of Tumors	Number of Regressed Tumors	Number of Growing Tumors
ER+		7	4 (57%)	3
ER-		1	(B) 0	1
	ER+	5	3 (60%)	2
	ER-	3	(C) 1 (33%)	2
ER+	ER+	4	(A) 3 (75%)	(D) 1
ER+	ER-	3	1 (33%)	2
ER-	ER+	1	0	1

Table 5. Progesterone Receptor (PR) Distribution and Response to Oophorectomy.

Receptor Status Cytosol	Nuclear	Total Number of Tumors	Number of Regressed Tumors	Number of Growing Tumors
PR+		4	2 (50%)	2
PR-		4	2 (50%)	2
	PR+	4	2 (50%)	2
	PR-	4	2 (50%)	2
PR+	PR+	1	1 (100%)	0
PR+	PR-	3	1 (33%)	2
PR-	PR+	3	1 (33%)	2
PR-	PR+	1	(A) 1 (100%)	0

Table 6. Distribution and Response To Oophorectomy. Estrogen and Progesterone Receptor.

Receptor Status		Total Number of Tumors	Number of Regressed Tumors	Number of Growing Tumors
Cytosol	Nuclear			
ER+PR+		3	(A) 2 (67%)	(C) 1
ER+PR-		4	2 (50%)	2
ER-PR+		1	0	1
	ER+PR+	1	1 (100%)	0
	ER+PR-	4	2 (50%)	2
	ER-PR+	3	1 (33%)	2
ER+PR+	ER+PR+	1	(B) 1 (100%)	0
ER+PR+	ER+PR+	2	1 (50%)	1
ER+PR-	ER+PR-	1	1 (100%)	0
ER+PR-	ER-PR+	3	(D) 1 (33%)	2
ER-PR+	ER+PR-	1	0	1

independent *in vivo*. Also, one tumor which was negative for progesterone receptors, both cytosolic and nuclear, (A, table 5) and one tumor which was negative for nuclear estrogen receptor and cytosolic progesterone receptor, (D, table 5) were hormone-dependent in vivo. Leung *et al*, (31) has shown that prolactin can stimulate estrogen receptor in mammary tumors, and it is unknown whether progestin receptors are dependent on estrogen action directly or estrogen induced prolactin secretion (32). The presence of prolactin binding in the absence of estrogen binding can be associated with autonomous growth (7). Prolactin dependence may explain the presence of ER and PR in these "hormone-independent" tumors, and conversely, sensitivity to alterations in prolactin concentration may be responsible for regression of the two tumors with minimal estrogen and progesterone receptor populations. The same model of prolactin dependence can account for the regression of tumors which are steroid receptor negative and the continued growth of steroid receptor positive tumors following oophorectomy.

Mean receptor concentration was higher for both hormones in both locations, cytosol and nuclear extract, in the hormone dependent group (table 7). Differences between groups did not attain statistical significance. Inspection of the binding data indicates that mean binding

Table 7. Average Receptor Concentration Expressed As Femptomoles Per Milligram Of Protein (Mean ± Standard Deviation).

Group	^{3}H estradiol-17 cytosol	^{3}H estradiol-17 nuclear	^{3}H R5020 cytosol	^{3}H R5020 nuclear
Hormone Dependent	23.6 ± 28.6	24.7 ± 32.5	267.0 ± 403.7	139.1 ± 223.7
Hormone Independent	12.2 ± 9.0	8.1 ± 14.7	56.8 ± 71.0	36.2 ± 43.2
All	17.9 ± 20.6	16.4 ± 24.9	161.9 ± 290.9	87.7 ± 158.9

site concentrations in the hormone dependent group are shifted upward by the presence in each hormone receptor location of one tumor with a binding capacity several fold larger than the rest, (A,B,C,D; Table 2 . The observation that very high binding capacity in each case is associated with hormone independence is particularly interesting in light of the suggestion by several investigators that hormone dependency among breast tumors may represent a continuous function of receptor concentration (15).

SUMMARY

Normal breast tissue contains specific binding sites for each of the hormones known to influence its growth or development. With malignant transformation, cells may retain all or only part of the normal population of receptor sites. If a cell retains receptors, it remains at least potentially capable of being regulated by the hormonal environment. If receptor sites are lost, the cell may escape from endocrine control.

The results of this investigation suggest a correlation between increasing estrogen and progesterone receptor populations in the qualitative sense and increasing numbers of regressions in response to oophorectomy, and perhaps a quantitative relationship between steroid receptor concentration and hormone-dependence in vivo. The relationship between intermediate receptor populations and intermediate binding capacities is unreliable however, and suggests the need for evaluation of binding parameters for other hormones influencing breast growth and function, particularly prolactin, for a more complete understanding of hormone responsiveness.

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