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Mammary Cancer: Selective Action of the Estrogen Receptor Complex

Abstract. *Progesterone receptors in the autonomous rat mammary tumor MTW-9B are reduced 80 to 90 percent after ovariectomy, but are not reduced if ovariectomized animals are given estrogen. Tumor growth, however, is independent of estrogen status and insensitive to pharmacological doses of estradiol. This represents an unusual system characterized by a selective action of an inducing agent on the genome.*

Approximately 55 percent of breast cancer patients whose tumors contain estrogen receptors respond to endocrine ablative or additive steroid therapy (*1*). The failure of this therapy in patients positive for the estrogen receptor implies that the estrogen receptor is not functionally competent or that the tumor cells or their nuclei no longer respond to hormonal stimulation (*2*). Other possibilities have been summarized by McGuire *et al.* (*3*).

Horwitz *et al.* (*4*) suggested that the simultaneous presence of the progesterone receptor with the estrogen receptor would be a more reliable indicator of hormone responsiveness in breast cancer. This proposal is supported by studies wherein the synthesis of the progesterone receptor was shown to depend on the presence of estradiol in uterus (*5*), oviduct (*6*), the hormone-dependent mammary tumor induced by 9,10-dimethyl-1,2-benzanthracene (DMBA) (*7–9*), and the human breast cancer cell line MCF-7 (*10*). In the last case, progesterone receptor synthesis was shown to be mediated by the estrogen receptor system (*10*). Presumably, synthesis of the progesterone receptor, as a gene product, would be indicative of a functioning estrogen receptor. Indeed, when both receptors are present, an improved clinical response rate is observed, although about 17 percent of patients with both receptors do not respond, and, conversely, some patients whose tumors have receptors for estrogen but not progesterone do respond (*11*). This suggests that there might not always be an association between estrogen-induced tumor growth and progesterone receptor regulation; the results reported herein show that in the estrogen-independent MTW-9B mammary tumor system, these two parameters are completely dissociated.

The transplantable mammary tumor MTW-9B, in Wistar/Furth rats, is estrogen-independent as evidenced by its equal growth rate in syngeneic males and females and also by the lack of effect of ovariectomy or hypophysectomy on its growth rate (*12*). In addition, this tumor does not regress in animals treated with pharmacological doses (1 mg per kilogram of body weight) of estradiol benzoate (*13*). Both estrogen and progesterone receptors are present (Fig. 1) and in amounts comparable to those observed in the hormone-dependent DMBA tumor (*9*, *14*). Specific binding of [^{3}H]estradiol and the synthetic progestin [^{3}H]R5020 to tumor cytosol is saturable and of high affinity. Binding of [^{3}H]-progesterone to tumor cytosol is similar to that observed with [^{3}H]R5020; however, the K_d is about threefold higher.

The presence of the progesterone receptor in this autonomous tumor suggests that the concept advanced by Horwitz *et al.* (*4*) requires further critical evaluation. The data in Table 1 indicate that the synthesis of the progesterone receptor in the MTW-9B tumor is under estrogen regulation. Thus, in tumors obtained from rats ovariectomized 2 weeks earlier, progesterone receptor levels were decreased to 10 to 20 percent of those in the sham-operated controls. This represents a true decrease in binding capacity since the binding affinity, measured by Scatchard analysis (*15*), did not change. That this decrease was due to a reduction in circulating estradiol is suggested by the fact that when estradiol benzoate (25 μg/kg) was given to rats daily for 2 weeks, starting 1 day after ovariectomy, progesterone receptor levels in the tumor remained in the normal range. In fact, when R5020 was used as the tritiated ligand, an apparent "superinduction" was observed. These changes occurred in the absence of any effect on tumor growth. It is not known whether the differences between binding observed with tritiated progesterone and R5020 are real, or whether they simply reflect variation from one group of animals to another. It is also possible that in comparison to progesterone, R5020 binds to additional components, such as serum albumin, although this binding has been reported to be nonspecific (*16*).

The concentration of estrogen receptor showed a slight but not significant increase after ovariectomy (Table 1). Similar results have been reported for the R3230AC mammary adenocarcinoma (*17*) and for hormone-independent DMBA-induced mammary tumors (*8*), although both a decrease (*7*, *8*, *18–20*) and an increase have been noted for the more common hormone-dependent DMBA tumors (*9*). The decrease in estrogen receptors observed after estradiol treatment may not reflect a true decrease in receptor concentration but rather an increase of receptors in the nucleus or of occupied receptors in the cytosol, since the assay used here measured only available cytosol sites. Kelly *et al.* (*18*) also showed a decrease in cytosol estrogen receptor levels in DMBA tumors from intact rats after long-term administration

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of estrogen, and a decrease has been reported after estrogen administration to ovariectomized animals (*9*); conversely, an increase has also been observed (*7*).

Regulation of progesterone receptor by estrogen has been studied only in hormone-dependent or -responsive systems (*5-10*), although a slight loss of progesterone receptor was noted by Horwitz and McGuire in two DMBA tumors which failed to regress after ovariectomy (*14*); similar results were reported in five of seven independent DMBA tumors by Koenders *et al.* (*8*). In neither of these studies was an attempt made to induce the progesterone receptor in ovariectomized rats.

To our knowledge, this is the first report indicating that in an estrogen-independent mammary tumor, progesterone receptor levels are increased after estrogen priming. This is of clinical significance because it demonstrates that the concurrent presence of estrogen receptor and progesterone receptor in human breast cancer does not necessarily indicate that the growth of the tumor will be dependent on estradiol or that tumor growth will be inhibited by endocrine organ ablation. This effect of estradiol is not present in all autonomous tumors; we have observed, for example, that in the estrogen-independent mammary tumor MTW-9D, specific progestin binding sites are absent and cannot be induced by injection of estradiol benzoate (*13*).

Current thought on the mode of action of steroid hormones is that after binding of the steroid to the cytosol receptor, the complex is translocated to the nucleus, binds to chromatin, and initiates a chain of molecular events resulting in a physiological effect (*21*). The MTW-9B tumor system presents an unusual case where there appears to be a selective action of the estrogen receptor complex; thus this complex induces the synthesis of the progesterone receptor, yet does not elicit those biochemical changes required to mediate either tumor dependency on estrogen for growth or tumor regression after estrogen treatment. McGuire *et al.* (*22*) noted the apparent dissociation between growth and estrogen sensitivity in the R3230AC carcinoma; in that case, the results were attributed to the low levels of estrogen receptor that were thought to be insufficient to influence tumor growth but adequate to stimulate some biochemical changes.

The results reported here are consistent with the interpretation that the steroid-receptor complex is acting at more than one site on the genome. The reason for the absence of estrogen dependency or responsiveness in the MTW-9B tumor in the presence of adequate cytosol estrogen receptor is unknown. Several possible explanations for the selective action of the steroid-receptor complex in a target tissue include these: (i) the specific genome site that determines estrogen sensitivity is blocked and thus prevented from interacting with the receptor complex; (ii) the conformation of the estrogen receptor complex is such that it is active only in mediating certain functions of the steroid; (iii) the tumor, while continuing to synthesize estrogen receptor, has circumvented its growth dependence upon the receptor but retains certain specific functions such as the synthesis of progesterone receptor; (iv) the

Table 1. Effect of ovariectomy and estradiol treatment on tumor weight and cytosol receptors in MTW-9B mammary tumors. Animals were ovariectomized or sham-ovariectomized 38 days after tumor implantation, and injections of corn oil or estradiol benzoate in corn oil were started 1 day later. Injections were given daily for 14 days, and animals were killed 24 hours after the last injection. In order to minimize variations in receptor values due to varying stages of the estrus cycle, the sham-operated animals were ovariectomized 18 hours before death; this did not affect the average receptor concentration. The other animals were sham-ovariectomized 18 hours before death. When [^{3}H]progesterone was used to measure the progesterone receptor, cytosol was first incubated for 30 minutes at 4°C with a 1000-fold excess of cortisol to eliminate competition due to the cortisol-binding globulin. [^{3}H]Progesterone or [^{3}H]progesterone plus a 100-fold molar excess of unlabeled progesterone were then added, and incubation was continued an additional 2 hours. In all other respects, the assay was the same as that described in the legend to Fig. 1. Data are means ± standard error of mean; 10 to 24 rats per group were used.

Treatment	Tumor weight (g)	Receptor (fmole per milligram of protein)		
		Estrogen	Progesterone	
			[^{3}H]R5020	[^{3}H]Progesterone
Sham operated	7.21 ± 0.65	71.0 ± 5.5	335 ± 42	269 ± 22
Ovariectomized	7.74 ± 0.95	80.1 ± 7.3	39.5 ± 7.9*	53.8 ± 10.0*
Ovariectomized + estradiol benzoate (25 μg/kg)	6.13 ± 0.88	50.7 ± 4.7*	1064 ± 85*	329 ± 50

*$P < .01$ for comparison to other two groups.

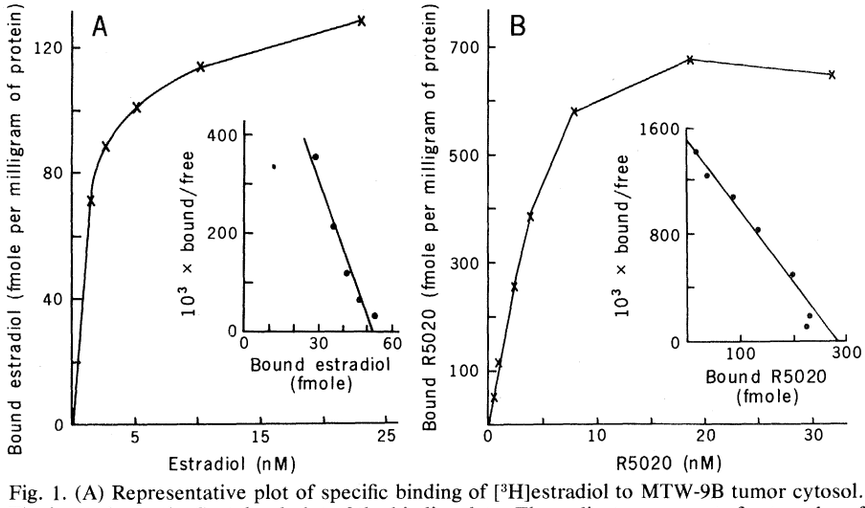

Fig. 1. (A) Representative plot of specific binding of [^{3}H]estradiol to MTW-9B tumor cytosol. The inset shows the Scatchard plot of the binding data. The ordinate represents femtomoles of estradiol bound per portion incubated. In this example, the K_d was calculated to be $8.90 \times 10^{-10} M$ and the total binding capacity was 128 fmole per milligram of protein. To measure estrogen receptors, tumor cytosol was prepared as described (*23*), and binding of [^{3}H]estradiol was measured by incubating 75 μl of charcoal-treated cytosol with 5 μl of [^{3}H]estradiol or 5 μl of [^{3}H]estradiol containing a 100-fold molar excess of unlabeled estradiol for 2 hours at 4°C. For Scatchard analysis, incubations were done for at least five concentrations of [^{3}H]estradiol, varying from 0.5 to 24 n*M*. When only a single point was used, the [^{3}H]estradiol concentration was approximately 10 n*M*. Specific binding was determined as described (*23*). (B) Representative plot of specific binding of [^{3}H]R5020 to MTW-9B tumor cytosol. The inset shows the Scatchard plot of the binding data. In this example, the K_d was calculated to be $2.33 \times 10^{-9} M$ and the total binding capacity was 823 fmole per milligram of protein. Progesterone receptors were measured in the same cytosol used for estrogen receptor determination; 75 μl of charcoal-treated cytosol were incubated with 5 μl of [^{3}H]R5020 or 5 μl of [^{3}H]R5020 containing a 1000-fold molar excess of unlabeled R5020 for 2 hours at 4°C. [^{3}H]R5020 concentrations between 0.5 and 32 n*M* were used for Scatchard analysis; a concentration of 20 n*M* was used for a single point determination.

tissue is heterogeneous with regard to cell type and contains a preponderance of cells inherently insensitive to the steroid; (v) after interaction of the receptor complex with chromatin some event at the translational level, such as new protein synthesis, does not occur; and (vi) there is more than one estrogen receptor in the cell, each with its own function.

MARGOT IP
RICHARD J. MILHOLLAND
FRED ROSEN
Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14263
UNTAE KIM
Department of Pathology, Roswell Park Memorial Institute

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Chitin Synthetase Distribution on the Yeast Plasma Membrane

Abstract. *Purified, intact yeast plasma membranes were allowed to synthesize chitin, and the nascent chains of polysaccharide were observed either by the fluorescence produced with a brightener or by autoradiography. By both methods, it was concluded that the newly formed chitin emerged at many sites on each membrane. Thus, the synthetase that catalyzes chitin formation has a similar distribution. Since chitin synthetase is found mainly in a zymogen form, these results confirm the hypothesis that initiation of the chitinous primary septum of* Saccharomyces *occurs by localized activation of the uniformly distributed zymogen.*

The formation of the chitin primary septum of budding yeast is subject to precise temporal and spatial regulation (*1*). The onset of this event appears to be linked to the transformation of a zymogen, or latent form of chitin synthetase, into active enzyme (*2*). We have successfully solubilized the synthetase and have shown that the latency of the enzyme was preserved after solubilization and therefore was not due to shielding by a membranous structure (*3*).

According to a hypothesis explaining the localization in space of septum initiation (*2*), the chitin synthetase zymogen is uniformly distributed on the plasma membrane, and is activated only in a restricted area by a factor carried inside vesicles. The bulk of the chitin synthetase zymogen is indeed associated with purified, intact plasma membranes (*4*), but its distribution on the membrane remained unexplored. Although the most direct way of ascertaining the location of chitin synthetase would be through the use of a labeled antibody against the enzyme, this procedure is not available because the enzyme has not yet been purified to homogeneity. An indirect approach was suggested by the finding that the reaction product, chitin, remains associated with the particles used to catalyze its formation. It was concluded that the position of the enzyme could be inferred from that of the nascent chitin chains.

Saccharomyces cerevisiae X2180 (ATCC 26109) was grown, and plasma membranes were purified (*4*).

Purified plasma membranes were first activated with trypsin, and then incubated with UDP-*N*-[^{11}C]acetylglucosamine (UDP, uridine diphosphate) in the chitin synthetase reaction mixture; the mixture was finally divided into two portions: in one, the amount of radioactivity incorporated into chitin was determined (*5*). The other portion was subjected to centrifugation in a Renografin gradient identical to that previously used in the isolation of the membranes (*4*). Of the radioactivity incorporated into chitin, 73 percent was found in the reisolated membrane band, an indication that most of the chitin remained attached to the particles on which it was formed.

An attempt to visualize the polysaccharide synthesized by the particulate preparation was made. In order to observe the newly formed chitin, the fluorescence produced by interaction between the polysaccharide and a brightener, Calcofluor White MR2 New (*6*), was used. With this technique, membranes that had synthesized some chitin showed patches of fluorescent material all over their surface (Fig. 1, B and D). The spotty appearance of the fluorescence is more pronounced in Fig. 1D, where individual membranes are shown, than in Fig. 1B, where the membranes are in clumps. Under direct observation the spots appeared to be smaller and more numerous than in the photographs, which are somewhat blurred because of the impossibility of bringing each plane of the membrane into focus at the same time. Importantly, the fluorescent areas were restricted to the location of plasma membranes, thus indicating the absence of smaller particles with the ability to catalyze chitin synthesis. A control (Fig. 1, E and F) in which the chitin synthetase inhibitor polyoxin D was added to the reaction mixture, showed no fluorescence.

These observations were repeated with the electron microscope because of greater resolution. In this case UDP-*N*-[^{3}H]acetylglucosamine was used as substrate, and the product was located by autoradiography. Subsequent to the incubation, some manipulation of the membranes was required in order to eliminate the excess labeled substrate. It was found, however, that centrifugation of the membranes, followed by suspension and washing, resulted in the aggregation of chitin into large clumps, thereby destroying the intitial localization of nascent chains. In order to preserve the configuration of the system, and to immobilize membranes and nascent polysaccharide, the membranes were enrobed in small agar blocks. The agar allowed diffusion in and out of UDP-*N*-

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