Nuclear Estrogen Receptor and Nonhistone Chromosomal Proteins in Hormonal Independency of Murine Breast Cancers

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ABSTRACT

The capability of nuclear binding of cytosol estrogen receptors (ER$_c$) was studied in GR mouse mammary tumors during their alteration of hormonal dependency through serial transplantations. Nuclei from GR mouse mammary tumors were incubated with uterine cytosol receptor complexes labeled with $^{125}$I-estradiol, and the amount of receptor binding in the 0.4 M KCI nuclear extracts was determined. The originally ER$_c$-positive-hormone-dependent (type I) tumors were capable of nuclear receptor binding, while this function was markedly reduced in the evolved hormone-independent (type II) tumors, although the ER$_c$ content in the latter was still positive. The originally hormone-independent (ER$_c$-negative, type III) tumors, however, retained the nuclear binding capability. It appears that the hormonal independency in type III tumors is due to a lack of ER, while in type II tumors it may be attributed to the loss of nuclear binding capability for receptor complexes. Nonhistone chromosomal proteins (NHCP) were analyzed by the 2-dimensional gel electrophoretic technique. A M, 31,000 NHCP was present in 11 of 12 type I, and four of four type III tumors. Following serial transplantation of the type I tumors, this NHCP was either markedly diminished or not observed in all 14 type II tumors examined. Although it coincides with the capability of nuclear receptor binding, the biological function of this NHCP is still undefined and warrants further investigation.

INTRODUCTION

Biological behavior of human breast cancers not only varies, but also constantly evolves during the clinical course. Progression of a tumor toward autonomous status has frequently been observed in animal mammary tumor models and in human breast cancer (8, 10–12, 18). In GR mouse mammary tumors, hormonal independency develops following serial transplantation. We observed that the independency could emerge in ER$_c$-positive tumors, and persist during serial transplantations regardless of the cyclic surge of the level of progesterone and estrogen receptors (10). These observations have suggested a presence of dissociation between steroid receptor status and hormonal dependency. A similar phenomenon has been observed in the treatment of advanced human breast cancer; approximately 30 to 40% of ER$_c$-positive tumors are HI and fail to respond to hormonal therapy (14).

The failure of ER$_c$-positive tumors to be hormonally dependent may be due to a postreceptor defect (15). NHCPs have been known to be involved in the genetic regulation (21) and in the expression of steroid hormone function (19, 23). In the study of progesterone action on chick oviducts, a subtraction of the acidic chromatin proteins has been found to contain the nuclear acceptor molecules responsible for the binding of progesterone receptor complexes (20).

The GR mouse mammary tumor is an ideal animal model for studying the mechanism of hormonal independency. The gradual transition from HD to HI can be experimentally controlled by serial transplantation of these tumors (10, 17). We, therefore, examined the capability of nuclear binding of estrogen receptors and the NHCP profiles in GR mouse mammary tumors which expressed various forms of hormonal independency.

MATERIALS AND METHODS

GR Mammary Tumors. GR mammary tumors were induced with estrone (0.5 mg/ml in drinking water) and progesterone pellets (10 mg) in ovariectomized 3-month-old female GR inbred mice as described by Sluyser and Van Nie (18). When tumors reached approximately 1 cm in diameter, they were removed under sterile technique, minced and mechanically disrupted, filtered through silk screen, and resuspended in normal 0.9% NaCl solution (saline). Approximately $10^6$ viable cells were grafted into the flank region of each of the BALB/c x GR F1 mice which had been castrated 1 week previously. The castrated recipients were divided into 2 groups: (a) one maintained with estrone-progesterone supplement; and (b) the other without hormonal maintenance. Tumor transplantations from generation to generation in castrated recipients were performed in 5 series, namely, Series A, B, D, E, and F.

Tumors of the A, B, E, and F series were HD in the first 4 transplant generations, judging by the marked difference in latent periods (over 30 days) between hormonal supplement and hormonal depletion. They were designated as type I tumors (Charts 1 and 2). Without hormonal supplement, the tumors might ultimately develop after a prolonged latent period. These tumors were autonomous and HI under hormonally deprived conditions, and were designated as type IV (early HI) tumors.

Following serial transplantation, the type I HD tumors became HI by the sixth transplantation generation and thereafter. The tumors at this stage were type II (evolved HI) and had a shorter latent period than their original type I HD tumors. With or without a hormonal supplement, these tumors developed at a similarly short latent period (Charts 1 and 2).

Tumors in the D series were originally HI (type III tumors). They were lacking ER$_c$ and grew at a rapid rate.

For receptor and NHCP studies, all tumors except type IV were removed from a host receiving hormonal (estrone and progesterone) supplements. Only type IV tumors were removed from hormonally deprived (castrated) hosts.

ER$_c$ Assay. Crude nuclei were prepared by homogenizing the tumor tissue in 4 volumes of buffer (10 mM Tris, 0.32 M sucrose, pH 7.4). After the initial centrifugation at 800 x g for 10 min, the nuclear pellet was washed 3 times. The final nuclear pellet was resuspended in buffer containing 0.4% Triton X-100 and immediately washed twice with buffer without Triton X-100. The ER$_c$ assay was carried out by the hydroxyapatite method as described by Garola and McGuire (24).

Nuclear Binding. The study was performed by the method described...
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by Jensen et al. (7). Cytosol from immature rat or mouse uterus was incubated with 5 nm [125I]estradiol in Tris-sucrose buffer for 2 hr at 0-4°C. After dextran-coated charcoal treatment, the supernatant containing E-R complexes were used for nuclear binding study. The amount of E-R complexes was from type II-evolved HD tumors (Chart 3).

Nuclear Protein Extraction. The nuclear proteins were extracted according to the method of Durban et al. (3) and Takami et al. (22). Tumor tissues were homogenized in 0.5% Nonidet P-40, 0.01 M NaCl, 1.5 mM MgCl2, and 0.01 M Tris-HCl (pH 7.6). The homogenate was centrifuged at 2,300 × g for 10 min. The crude nuclear pellet was homogenized in 2.2 M sucrose, 10 mM MgCl2, and centrifuged at 53,000 × g for 60 min, followed by resuspension in 1 mM sucrose, 10 mM MgCl2, and centrifugation at 500 × g for 10 min. The pellet was extracted twice with 10 volumes of the following solutions sequentially: (a) 0.075 M NaCl, 0.025 mM EDTA, pH 8.0; (b) 10 mM Tris-HCl, pH 8.0; and (c) 0.35 M NaCl, 10 mM Tris-HCl, pH 8.0. Phenylmethylsulfonyl fluoride (0.5 mM) was added fresh to all solutions in minimizing proteolysis by proteases. The 0.35 M NaCl extracts were combined, dialyzed against 40 mM acetic acid, lyophilized, and then subjected to 2-dimensional gel electrophoresis.

Extraction of Nonhistone Chromatin Proteins by 0.35 M NaCl. To improve the purity of nuclear preparation and certainty of the NHCP nature, we adopted 2 additional methods in those tumors that could provide sufficient quantity for purification of nuclei and chromatin (1, 5). The purified chromatin preparation was then subjected to the extraction by 0.35 M NaCl, 10 mM Tris-HCl, pH 8.0, for analysis by 2-dimensional gel electrophoresis.

Two-Dimensional Gel Electrophoresis. The 0.35 M NaCl fractions were used for 2-dimensional gel electrophoresis according to the method of O'Farrell et al. (15), using nonequilibrium, pH-gradient electrophoresis for the first dimension and sodium dodecyl sulfate-10% polyacrylamide as the second dimension. The 0.35 M extraction (containing 0.5 to 0.7 mg of protein) was added at the acidic end of the tube gel (inside diameter, 2.5 mm x 130 mm) and run at 2000 V-hr for optimal resolution under our conditions. The tube gels contained 16 g of pH 5 to 8 Ampholines, and 4 g of pH 3.5 to 10 Ampholines (LKB)/liter. Histone-DNA tends to remain in the origin, resulting in a better separation of NHCP with less smearing artifacts. The second dimension run in slab gels (155 mm x 140 mm x 0.75 mm) at 20 mA for 3.5 hr. Silver staining of the gels was performed by the method of Wray et al. (24).

Tumor ERα content was determined by the sucrose gradient method (9). Protein assay was performed by the method of Lowry et al. (13), and DNA was assayed by the method of Burton (2).

RESULTS

Growth Pattern, Steroid Receptor Content, and Hormonal Dependency of GR Mouse Mammary Tumors. Following serial transplantation, the latent period of type I HD tumors shortened when they evolved to type II HD tumors (Chart 2). The mean ERα content in the first 3 transplant generations of type I HD tumors was higher than, but not significantly different from, that of type II-evolved HD tumors in the later transplant generations (Table 1). The ERα content, however, was significantly higher in type I tumors than type II tumors. The assays were performed in samples from the same hormonal condition of the hosts; i.e., they all received estrone and progesterone supplement.

"D" series HI tumors were originally lacking in ERα and ERβ, and were rapidly growing (type III). These characteristics were maintained throughout the serial transplantations.

Nuclear Binding of ERα. Two concentrations of cytosol E-R complexes were used for nuclear binding study. The amount of nuclear binding was dose dependent in rat uteri and GR mammary tumors; a higher magnitude was observed when a high concentration of cytosol E-R complexes (213 fmol/mg protein) was used than that with a low concentration of E-R complexes (70 fmol/mg protein). For comparison, the capabilities of nuclear binding in various tumor tissues are expressed as a percentage of the control, using the rat uterine nuclei as 100%, regardless of the concentration of E-R complexes used. The cytosol ER complexes from rat uterine nuclei were able to bind into the nuclei of the following tissues: (a) rat and mouse uterus; (b) type I HD tumors; (c) type II HD tumors, and (d) mouse splenic cells. The only type of nuclei with a markedly depressed capability in binding E-R complexes was from type II-evolved HD tumors (Chart 3).

This lack of nuclear ER binding in type II tumors cannot be explained by the transspecies experimental design; i.e., mouse nuclei were incubated with receptors from rat cytosol. No difference in receptor binding across the species was observed when rat uterine cytosol was cross-matched with mouse uterine nuclei or vice versa (data not shown).

NHCP Pattern. Over 200 protein spots were identified by the

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[Table 1]

<table>
<thead>
<tr>
<th>Mammary tumors</th>
<th>Transplant generations</th>
<th>ERα (fmol/mg protein)</th>
<th>ERα (fmol/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>1-3</td>
<td>26.9 ± 8.0 (6)</td>
<td>3.6 ± 0.70 (6)</td>
</tr>
<tr>
<td>6-12</td>
<td></td>
<td>15.2 ± 9.8 (17)</td>
<td>1.0 ± 0.35 (4)</td>
</tr>
</tbody>
</table>

a From tumor Series B, E, and F.

b Mean ± S.D.

c Not significant.

d Numbers in parentheses, number of tumors studied.

* p < 0.05.
Type I tumors (HD) | Type II tumors (Evolved HI) | Type III tumors (original HI) | spleen
---|---|---|---
200 | 300 | 50 | 300

Chart 3. Capabilities of nuclear receptor binding of various tissues. The uterine ERα complexes labeled with 125I-estradiol were used as the donor. The results of nuclear binding of each tissue were calculated as fmol of receptor complexes per mg DNA, and were compared with that of uterine nuclei, using the latter as 100%. •, one sample in triplicate determinations.

silver staining method on the 2-dimensional electrophoretic gels. The protein distribution was grossly compatible among GR mammary tumors, and some protein spots were consistently present and arranged in a special pattern that could be used as a landmark. There were numerous spots varying in “quantity,” judging by a gross change in staining intensity in comparison with the nearby “landmark” spots. However, most of the changes were not consistent in the same group of tumors, suggesting that these changes may associate with biological activities other than hormonal dependency. A M, 31,000 protein, with a grayish stain, was noticeably present in 11 of 12 originally HD type I tumors (Table 2; Fig. 1A). Following serial transplantations of these HD tumors, this specific M, 31,000 protein was markedly reduced in 5 and undetectable in 9 “evolved HI” type II tumors (Fig. 1B).

Due to a lack of standardization of the staining technique, accurate quantitation of protein was not feasible. We, therefore, applied a stringent definition to examine the changes in protein spots. The M, 31,000 protein was considered as a “presence” when a discrete spot was darkly stained with a horizontal diameter of over 5 mm. Reduction of this M, 31,000 protein was only assigned to those with either a total disappearance of this spot (or was undetectable), or those with a reduction of the diameter to less than 2 mm (trace).

In those “originally HI” type III tumors, the M, 31,000 protein was persistently present in the early and late transplant generations (Fig. 2, A and B). This protein was also present in “early HI” type IV tumors that were developed in mice without hormonal supplement, and were lacking ERα (not shown).

In order to ascertain that this M, 31,000 protein was not a contaminant from cellular elements other than chromatin protein, we repeated the assay on chromatin that was purified by the method of Garrard and Hancock (5) in those tumors where a large tumor volume was available. Again, this M, 31,000 protein was present, confirming that it was a NHCP in nature. The pl of this M, 31,000 NHCP could only be estimated to be around pH 6, since the technique of nonequilibrium pH gradient electrophoresis was used for the first dimension.

**DISCUSSION**

There were at least 2 types of hormonal independency due to different loci of defect. The first type is the lack of ERα and ERβ in tissues such as those of type III tumors or the spleen, but they retain the nuclear capability of “accepting” E-R complexes. The presence of nuclear receptor sites for progesterone receptors was previously reported in splenic tissue by Spelsberg et al. (20). The second type of defect is the failure of receptor binding in nuclei, as seen in type II-evolved HI mammary tumors. This phenomenon of postreceptor defect was previously described in other experimental animal models (16).

The postreceptor defect may explain why GR mammary tumors became HI after the fifth transplant generation, even though...
they persistently contained cytosol receptors and at times when a cyclic high steroid receptor level was observed in the late transplant generations (10). The loss of the capability in nuclear receptor binding may be one of the mechanisms by which receptor-rich human breast cancers fail to respond to hormonal therapy.

Our results (data not presented) also suggested that the defect in nuclear binding is not due to a change of the characteristics of ERc. There was no decrease of binding affinity (Kd) of ERc during the transition from HD to HI. Furthermore, by the sucrose gradient study and isoelectric focusing study, no demonstrable changes on the characteristics of ERc are accountable for the postreceptor defect. It is, therefore, most likely a result from nuclear alteration which affects the nuclear binding.

Although there are variations of NHCP spots on the gel, it is remarkable that the general pattern of NHCP was highly reproducible among different tumors in origin and at their different transplant generations. In this report, our aim is to search for reducible among different tumors in origin and at their different nuclear alteration which affects the nuclear binding. The M, 31,000 NHCP appeared in both well-differentiated and poorly differentiated tumors (Figs. 1A and 2A). Disappearance of this protein is not related to transplantation procedure, since in the originally HI tumors (type III) this protein has persisted in the tumors of late transplant generations (Fig. 2, A and B). The M, 31,000 NHCP is also not associated with the growth rate of the tumor, since the "evolved" type II HI tumors grew as rapidly as the "original" type III tumors (Chart 2), while only one of these 2 types of tumors has the conspicuous phenotypic expression of the M, 31,000 NHCP (Fig. 2A versus Fig. 1B).

Our results appear to support that type I HD tumors demonstrate the presence of ERc, the capability of nuclear receptor binding, and the presence of nuclear M, 31,000 NHCP; while the type II-evolved HI tumors, even possessing ERc, had lost both the nuclear binding capability and M, 31,000 NHCP. The type III HI tumors, on the contrary, lacked ERc, while their nuclei retained the capability of receptor binding, as well as the M, 31,000 NHCP.

It appears that the evolved HI (type II) tumors may involve genetic alterations. In addition to the change of M, 31,000 NHCP reported here, we have observed the appearance of trisomal chromosome 13 and a marker chromosome in type II tumors (10). Genetic instability coupled to clonal selection was postulated as the mechanism for tumor progression in rat prostatic cancers (6). Characterization of this M, 31,000 NHCP may further unveil its specific biological function in relationship to hormonal dependency of the mammary tumors.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. A, NHCP profile of a HD (type I) well-differentiated tumor; the focus of interest is a M, 31,000 protein (arrow). Other proteins (boxes) are used as "landmarks" because of their persistent presence and unique feature. B, NHCP profile of an evolved HI (type II) tumor with a disappearance of the specific M, 31,000 protein, as compared with the "landmark" proteins (boxes). NEPHGE, nonequilibrium, pH gradient electrophoresis.
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Fig. 2. A, NHCP profile of an original HI (type III) poorly differentiated tumor in its early transplant generation (TG1). Notice the presence of the M, 31,000 protein at the same position as the one shown in Fig. 1A. B, persistent presence of M, 31,000 NHCP in the late transplant generation (TG7) of an original HI (type III) tumor. NEPHGE, nonequilibrium, pH gradient electrophoresis.
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