Molecular Heterogeneity of Cytosolic Forms of Estrogen Receptors from Human Breast Tumors

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Abstract

Molecular heterogeneity of estrogen receptors in cytosols from human breast carcinomas was demonstrated with regard to size and surface ionic charge. Following separation of the molecular forms of estrogen receptors into either the 8S or the 4S species by sedimentation on linear gradients of sucrose, diethylaminoethyl cellulose chromatography was used to fractionate the various components in these receptors. Separation of the 8S species by diethylaminoethyl cellulose chromatography revealed a number of specific binding components, each eluting at a different ionic strength. The number of components as well as their relative specific estrogen-binding capacities were highly variable. At least five different species were identified on the basis of their elution at different ionic strengths. Estrogen-binding components eluted either in the wash buffer or at a KCl concentration of 0.02 to 0.05, 0.13 to 0.17, 0.18 to 0.21, or >0.3 M. The 4S form of the estrogen receptor separated into at least two components, each eluting at a different ionic strength. Clinical correlations of objective remissions in breast cancer patients given hormonal therapy with the molecular species of estrogen receptors indicated that women with tumors containing the 8S form showed a greater likelihood of responding. Our findings with diethylaminoethyl cellulose chromatography suggest that the molecular composition of the 8S species of estrogen receptors from hormonally responsive tumors is different from that of certain unresponsive neoplasms.

Introduction

Currently, it is accepted that analyses of estrogen receptors in tumor biopsies should be included with other clinical criteria used in the management of the patient with advanced breast cancer (e.g., see Refs. 5, 9, and 18). Since the original report of Folca et al. (2) indicating a greater uptake of labeled hexestrol by breast tumors of patients showing a response to endocrine-ablative surgery, numerous studies (5, 9, 13, 14, 17) have shown that approximately one-half of all biopsies of malignant breast tumors contain estrogen receptors. Furthermore, 55 to 60% of the patients exhibiting estrogen receptors are responsive to hormone therapies of the additive or ablative types.

While the absence of estrogen receptors predicted a failure of response in all but a few patients, the presence of estrogen receptors in a tumor predicted a response in only one-half of the cases. Several reasons have been proposed for the failure of receptor to act as a true predictive test. Firstly, breast tumors are heterogeneous tissues likely to contain both endocrine-dependent and -independent cells. If a tumor mass has enough estrogen receptor-containing cells to yield a positive laboratory result but contains a preponderance of cells without the binding proteins, the response to hormonal therapy may be dominated by the latter type cells. Thus, little or no measurable effect may be seen with regard to the size of the tumor. On the other hand, unresponsive cells that contain the estrogen receptor may occur because of the failure of some step in the cascade of events that follow the interaction of estradiol with its receptor in a target cell.

Thus, it appears that there are at least 2 possible reasons for the lack of a response in a patient with advanced breast cancer whose tumor contained estrogen receptors. On the other hand there may be insufficient quantities of intact estrogen receptors to initiate a response. In contrast to this quantitative argument, there is the possibility of a qualitative difference in the estrogen receptors of a responsive tumor compared to those of an unresponsive one. Earlier we proposed that the "activation" step for estrogen receptors in breast tumor cells of hormonally responsive patients requires at least 2 subunits, which combine to form an 8S aggregate observed upon sucrose gradient analysis (17, 18). Each of these subunits sedimented at 4 to 5S when detected by sucrose gradient centrifugation. Preliminary investigations suggested that the presence of 4S components alone in a breast tumor biopsy did not predict responsiveness to endocrine therapy (18, 19). The elimination of patients with tumors containing only the 4S species from the group whose tumors were positive for estrogen receptors increased the accuracy in predicting a response to approximately 75%. These data suggested that the molecular properties of estrogen receptors in human breast tumor biopsies may be related to the clinical responsiveness of patients treated by hormonal manipulations.

In this study we characterized further the 8S and 4S forms of estrogen receptors from human breast tumors by first purifying them using sucrose gradient centrifugation and then separating them on columns of DEAE-cellulose. Our results indicate that these receptors contain a number of different specific estrogen-binding components, each eluting at a different ionic strength.

Materials and Methods

The radioactive ligand 17β-[2,4,6,7-3H]-estradiol (105 Ci/
mmol) was obtained from New England Nuclear, Boston, Mass. Purity was checked periodically by thin-layer chromatography. Unlabeled DES \(^6\) was purchased from Steraloids, Pawling, N. Y. Schwarz/Mann, Orangeburg, N. Y., supplied RNase-free sucrose, while Norit A was obtained from Matheson Coleman and Bell, Cincinnati, Ohio. Omnifluor was obtained from New England Nuclear, and Triton X-100 was obtained from Beckman Instruments, Inc., Palo Alto, Calif. DEAE-cellulose was purchased from Whatman Laboratories, Clifton, N. J.

**Preparation of Intracellular Estrogen Receptor Complexes.** Human breast tumors were obtained through cooperation with surgeons and pathologists of hospitals associated with the School of Medicine, University of Louisville, and from hospitals outside Louisville. The tissues were stored at \(-86^\circ\) as described earlier (21). Cytosol was prepared by homogenizing either individual frozen tumors or a powder of 70 frozen tumor tissues in 10 mm Tris-HCl buffer, pH 7.4 or 8.0, containing 1.5 mm EDTA, 10 mm monothioglycerol, and 10% glycerol unless noted otherwise. The homogenates were centrifuged for 30 min at 105,000 \(\times g\) in a fixed-angle rotor to prepare the cytosol fraction. The resulting cytosol was incubated with 5 to 10 \(\mathrm{nm}\) \(^{3}H\) estradiol alone (total binding) or in the presence of a 100- to 250-fold excess of unlabeled DES (nonspecific binding) for 3 to 6 hr at 0\(^\circ\) unless noted otherwise. After the incubation period bound and free steroids were separated by dextran-coated charcoal method described earlier (4, 21), and the resulting supernatant containing labeled estrogen receptors was separated by sucrose density gradient centrifugation and/or by DEAE-cellulose chromatography.

**Sucrose Density Gradient Centrifugation.** Labeled estrogen receptor complexes (200-\(\mu\)l aliquots) were layered onto linear sucrose (10 to 35%, w/w) gradients, which were prepared in 10 mm Tris-HCl buffer, pH 7.4, containing 1.5 mm EDTA. In some experiments the gradients contained 0.4 m KCl. The gradients were centrifuged at the speeds and times described in the chart legends with the use of either a Beckman SW60 Ti or a VTÌ65 rotor. Each was fractionated into 5-drop portions with a Beckman fraction recovery system, and the radioactivity was measured in a Beckman LS-9000 liquid scintillation counting system with a digital integration program.

**DEAE-Cellulose Chromatography.** Two DEAE-cellulose columns (1 x 30 cm), each with a bed height of 15 cm, were prepared from Whatman DE-52 and were extensively washed with TET buffer until the conductivity and pH were identical with those of the elution buffer. One to 2 ml of cytosol containing the labeled estrogen receptor complexes were added to each of the ion-exchange columns. One column was developed to access total binding (\(^{3}H\)-ligand alone), while the second provided a profile of the nonspecific binding components measured in the presence of DES. Estrogen receptors were eluted with a wash of TET buffer followed by a 200-ml linear gradient of 0.0 to 0.4 m KCl. The protein-bound radioactivity was determined on each fraction with the use of liquid scintillation counting procedures. The protein elution profile of the columns was determined either by relative protein fluorescence or by the absorbance at 280 nm. The conductivity that indicated the [KCl] was determined with a Yellow Springs conductivity meter, Model 31. This procedure is useful for the study of progesterone and glucocorticoid receptors also (20).

**Calculation of Results.** Specific estrogen-binding capacity was estimated in the various elution or sedimentation peaks as the difference in radioactivity bound in the presence and absence of the unlabeled competitor, DES. These results are expressed as fmol of bound estradiol per mg of cytosol protein where the concentration of protein was determined by the method of Waddell (16). The description and distribution of the various forms of estrogen receptors in human breast tumors has been described (18).

**Results and Discussion**

**Influence of Ionic Strength, Temperature, and Protease Inhibitors on Sedimentation Profiles of Estrogen Receptors.** A number of molecular forms of estrogen receptors have been described in normal and neoplastic breast tissues by us (3, 21) and by others (5, 8, 13, 14). In general the normal mammary gland of lactating rats contains estrogen receptors that sediment at approximately 8S under conditions of low ionic strength and at \(\sim 4S\) in sucrose gradients containing 0.4 m KCl (3). Human breast carcinomas exhibited at least 4 types of estrogen receptor profiles, those that contain either the 8S species only, the 4S species alone, a combination of 8S and 4S species, or no specific binding components (e.g., see Refs. 5, 18, and 21) when sucrose gradient centrifugation was used under low-ionic-strength conditions. Both the 8S and the 4S species exhibited high affinities and ligand specificities (17, 18).

It has been reported that estrogen receptors in the immature rat uterus undergo a shift in their sedimentation behavior from approximately 4S to 5S when warmed to 25-28\(^\circ\) when sucrose gradients containing urea and KCl are used (6). In contrast with those of the uterus, estrogen receptors from lactating mammary gland, activated under identical conditions, did not exhibit an alteration in their sedimentation characteristics (10). Estrogen receptors of rat mammary gland that had been either charged only or charged with ligand and activated at 28\(^\circ\) sedimented as 4S species in gradients containing 0.4 m KCl (10).

The influence of ionic strength and temperature was studied with regard to the sedimentation profiles of estrogen receptors in human breast carcinomas (Chart 1). For these experiments a tissue powder composed of numerous human breast carcinomas was used. Three different sedimentation peaks, a major estrogen-binding component at \(-4.5S\) with a shoulder in the \(<4S\) region of the gradient and a defined 8S component, were separated by density gradient centrifugation under conditions of low ionic strength (Chart 1A). We have observed similar profiles in a few individual breast carcinomas under the same conditions. In a sucrose gradient containing 0.4 m KCl, the same cytosol revealed only 2 peaks; again a major peak at 4.5S and a shoulder at \(<4S\); however, there was no detectable 8S component (Chart 1B). Upon warming (activation) at 30\(^\circ\) for 45 min, the 8S species disappeared, while there was an increase in the amount of binding by the 4S and 4.5S

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\(^{6}\) The abbreviations used are: DES, diethylstilbestrol; TET buffer, 10 mm Tris-HCl buffer containing 1.5 mm EDTA and 10 mm monothioglycerol.
The total binding capacities of the 2 reactions were similar within 10%. Similar results were observed in other experiments conducted under identical conditions. Again, these data indicate that, although proteolytic cleavage of the 8S estrogen receptor may occur under low-ionic-strength conditions, there is no change in the specific binding capacities. It appears that, if the lower-molecular-weight forms of estrogen receptors are cleavage products, they retain the full capacity to associate with [\(^3\)H]estradiol.

Characterization of the Ionic Properties of Estrogen Receptors. Further characterization of the estrogen-binding components in the cytosol of breast tissue was accomplished with DEAE-cellulose chromatography. With tissue from lactating mammary glands of rats, estradiol receptor complexes sedimented primarily as 8S components and bound quantitatively to DEAE-cellulose columns at pH 7.4. Two estrogen-binding components were eluted; one with 0.02 to 0.05 M KCl and another at 0.18 to 0.20 M KCl (19). In similar experiments with human breast carcinomas, the elution profiles of estrogen receptors were highly variable.

For the determination of whether the 8S form of estrogen receptors from human breast tumors exhibited elution profiles similar to those observed for these components in normal mammary gland, estrogen-binding proteins were separated simultaneously by sucrose gradient centrifugation and by DEAE-cellulose chromatography. Chart 3A illustrates the sedimentation profile of the estrogen receptors from an infiltrating ductal carcinoma. This tumor contained primarily the 8S form of the receptor, which was assessed quickly by gradient centrifugation in the Beckman VTi65 rotor.

When an aliquot of this cytosol (Chart 3A) was separated by DEAE-cellulose chromatography, 3 components were eluted, each at a different ionic strength (Chart 4B). One was eluted with 0.03 M KCl, while the other 2 were eluted at low-salt conditions, as seen in Chart 1, C and D, respectively. Subjecting these estrogen receptor complexes to the activation conditions described in Chart 1C resulted in a greater affinity for DNA-cellulose (19). The specific binding capacities were similar under all of the conditions examined. Therefore, if proteolysis of the estrogen receptor was occurring under conditions of warming, only a change in receptor profile was observed without destruction of specific estrogen-binding capacity. These data suggest that estrogen receptors of human breast tumors exhibit activation properties similar to those of lactating mammary gland of rat, i.e., no shift from a 4S \(\rightarrow\) 5S species but an increase in binding of the 4S species to DNA-cellulose (10).

Leupeptin, a peptide isolated from actinomycetes (1), has been used as a protease inhibitor for the study of receptors from a number of sources (11). For determination of whether the 4S or the 4.5S components observed under low-ionic-strength conditions resulted from proteolytic cleavage of the 8S binding component, homogenates of the tumor powder described in Chart 1 were prepared either with 1 mM leupeptin or in buffer alone (Chart 2).

As seen in Chart 2, the sucrose gradient profile of the estrogen-binding components in the tumor powder from this experiment was similar to that observed in an earlier experiment, illustrated in Chart 1A. The data suggest the consistency of profile appearance. In addition to the 8S species, both the 4S and 4.5S entities were identified under low-salt conditions. In the presence of leupeptin, a small increase was observed in the quantity of the 8S component; however, no higher-molecular-weight species was observed, as has been reported by Sherman et al. (11). The extent of binding by the 4.5S component was not altered significantly, although the quantity of steroid bound by the 4S component was diminished considerably. In this experi-
Chart 3. Sedimentation profiles of estrogen receptors in cytosol from 2 human breast tumors. Cytosol prepared in TET buffer, pH 7.4, was incubated with 5 nM \(^{3}H\)estradiol in the presence \((O)\) or absence \((\bullet)\) of 1.25 x 10\(^{-6}\) M DES for 3 hr at 3°. Estrogen receptors were separated on linear gradients of 20 to 30% (w/w) sucrose with a VTi-65 rotor at 365,000 x g for 3.5 hr at 0°.

Chart 4. DEAE-cellulose chromatography of estrogen receptors in cytosol from a human breast carcinoma. A sample of cytosol from the breast tumor described in Chart 3A was incubated with 5 nM \(^{3}H\)estradiol either alone \((\bullet)\) or in the presence of an excess of DES \((O)\), and each was separated on an identical column of DEAE-cellulose \((B)\). A, protein profile represented by relative fluorescence \((\bullet)\) and KCl gradient measured by conductivity \((O)\).

Chart 5. DEAE-cellulose chromatography of cytosol from a human breast carcinoma containing no estrogen receptors. A sample of cytosol from the breast tumor described in Chart 3B was analyzed by the same procedure described in the legend of Chart 4.

0.09 and 0.22 M KCl. Another peak eluted during the wash step contained radioactivity that was not displaced in the presence of DES; this binding was classified as nonspecific. These data demonstrate that the 8S form of the receptor of this tumor contained several specific estrogen-binding components, each with different ionic properties. As a control cytosol from a tumor that did not show specific binding on sucrose gradients (Chart 3B) also did not exhibit specific estrogen-binding components with DEAE-cellulose chromatography (Chart 5).

To ascertain whether the presence of ligand during the incubation altered the elution profile of estrogen-binding components, we mixed tumor cytosol with 5 nM \(^{3}H\)estradiol for 20 hr at 0° or incubated it first without ligand for 17 hr at 0°, after which \(^{3}H\)estradiol was added for 3 hr. If lower-molecular-weight forms of the estrogen receptors were generated by proteolysis in the absence of ligand, they would be observed by sucrose gradient centrifugation. In fact there was no difference in either the sedimentation profiles or the ion-exchange elution patterns of estrogen-binding proteins whether or not estradiol was present during the entire incubation (data not shown).

As a further means of purifying the estrogen receptors as well as demonstrating the heterogeneity of components in the various species separated on sucrose gradients, molecular forms \((i.e., 8S or 4S)\) were collected and applied to columns of DEAE-cellulose. These experiments required considerable quantities of the individual species of estrogen receptors, such that as many as 12 sucrose gradients were used in each study.
Chart 6 illustrates the profile of estrogen receptors in a single primary breast tumor, W112B, as well as that of a tissue powder from 70 human breast carcinomas (HBC-105). Both samples contained considerable quantities of the 8S species. The fractions from the sucrose gradients containing only the 8S forms were collected, pooled, and applied to a DEAE-cellulose column. After being washed, the individual estrogen-binding components of the 8S species were eluted with a linear gradient of KCl (Charts 7B and 8B). For the primary tumor, at least 3 entities were separated, each at a different ionic strength. The first eluted with TET buffer just prior to the salt gradient (Chart 7). The other specific binding components eluted at 0.09 and 0.23 M KCl. The estrogen receptors from the mixture of human tumors separated into 3 specific binding components, which were eluted at [KCl] of 0.04, 0.15, and <0.3 M. Another component was eluted with the buffer wash (Chart 8B). Nonspecific binding was not assessed in this sample because there was little contribution of these components in the 8S region (Chart 6B). In general, recoveries were 65 to 75%. Thus far, only a few tumors with estrogen receptors of the 4S type have been separated by these procedures. Preliminary data also suggest that these 4S forms contain several specific binding components, each with different ionic properties.

These data suggest that both the 8S and the 4S species of estrogen receptors from human breast tumors contain a number of components that associate specifically with \([\text{H}]\text{estradiol}\). Whether these represent subunits or cleavage products due to proteolytic digestion of the estrogen receptors is unknown.

**Clinical Significance of Estrogen Receptors.** Although we do not have data on clinical response to endocrine manipulation on a large number of those patients on whom we have measured estrogen-binding components in tumor biopsies, our summary results are presented in Table 1. We have not observed an objective remission in a patient with advanced breast carcinoma who has had an estrogen receptor-negative tumor regardless of the type of hormone therapy administered. This correlation is far better than that reported for the collective results presented at the international workshop (9). Thirty-three remissions were observed in 44 patients administered various types of hormone therapies in whom the biopsies contained either the 8S species or both the 8S and the 4S forms of estrogen receptors as identified by sucrose gradient centrifugation (Table 1). The mean ± S.E. of the estrogen-binding capacities was 104 ± 21 fmol/mg cytosol protein, with a range of 14 to 354. As shown in the footnote to Table 1, 9 additional patients whose tumors contained specific estrogen-binding capacity exhibited remissions to endocrine therapy. However, because these tumors were examined by titration analysis, we were unable to distinguish the types of estrogen receptors in these specimens. The mean ± S.E. of the estrogen-bind-
Chart 8. DEAE-cellulose chromatography of 8S estrogen-binding components in cytosol from a mixture of human breast carcinomas. Following incubation of cytosol from a tissue power (HBC-105) with [3H]estradiol as described in Chart 6, the reactions were layered onto 10 identical sucrose gradients (10 to 35%, w/w). One gradient was fractionated, and the profile was determined as shown in Chart 6B. The 8S region (Fractions 22 to 40) of the remaining 9 gradients was pooled and layered onto a DEAE-cellulose column. This column was developed as described in "Materials and Methods." The 8S components contained little nonspecific binding, as shown in Chart 6B.

Chart 9. Proposed explanation of unresponsiveness to hormone therapy by breast cancer patients exhibiting estrogen receptor-positive tumors. The subunits of the estrogen receptor in normal and responsive breast cells (B) are depicted as separate entities that sediment at 4S. In the presence of estradiol under physiological conditions, it is proposed that the subunits combine to form a 6S dimer, which then translocates into the nucleus and stimulates a cellular response. In unresponsive cells (A and C), 2 types of defects (X) are postulated, either of which results in detection of 4S components. (Taken from Ref. 17.)

Table 1

| Objective remissions according to estrogen receptor species in tumor |
|-----------------------------|----------------|----------------|----------------|
| Therapy                      | 8-9S, 8-9S and 4-5S | 4-5S | Undetectable |
| Oophorectomy                | 7/10 | 0/3 | 0/4 |
| Adrenalectomy                | 10/11 | 1/4 | 0/7 |
| Hypophysectomy               | 0/1 | 1/8 | 0/17 |
| Estrogen                     | 11/15 | 2/5 | 0/10 |
| Androgen                     | 3/5 | 2/5 | 0/10 |
| Tamoxifen                    | 0/1 | 0/2 | 0/6 |
| Elipten                      | 2/2 | 0/2 | 0/6 |
| Total                        | 33/44 | 42/33 | 0/44 |

* Nine additional patients exhibited remissions but had unclassified estrogen receptor-positive tumors; 5 responded to estrogen, 3 responded to androgen therapy, and one responded to oophorectomy.

Molecular Basis of Unresponsiveness. The ligand-binding properties of the 8S and 4S forms of estrogen receptors in human breast tumors appear similar. What then is the molecular basis for unresponsiveness in tumors containing estrogen receptors? Earlier we proposed (17, 18) that the estrogen receptor of normal mammary cells and hormonally responsive breast tumor cells is composed of subunits that combine in the presence of estradiol-17β under physiological ionic strength and temperatures to form a 6S dimer (Chart 9). We suggested that these subunits are synthesized as separate entities in breast cells and that both are required for translocation into the nucleus and stimulation of a response. Our proposal was supported by the observation that only breast tumors containing the 8S form of estrogen receptors under low-ionic-strength conditions yielded a 5.5S species when extracted with buffers of physiological ionic strength. Furthermore, the majority of patients with breast tumors exhibiting 8S estrogen receptors responded to hormonal therapy. Thus, certain unresponsive breast tumor cells apparently do not contain a full complement of the estrogen receptors necessary for activation and nuclear translocation. Presumably, these cells have a defective mechanism whereby a single estrogen-binding subunit of the 4S type is synthesized by these cells (17, 18). The other subunit, which we have suggested also sediments at 4S, is either not synthesized or does not combine properly with the second 4S component. Thus, only 4S species of estrogen receptors are detected in cytosol when these breast tumors are examined by sucrose gradient centrifugation. Other types of defective receptor mechanisms have been proposed (7, 12, 15).
Our results show clearly that the 8S estrogen receptor of lactating mammary gland is composed of at least 2 components, each with different ionic properties as measured by DEAE-cellulose chromatography (19). Furthermore, the 8S estrogen receptor of human breast tumors separated into a variable number of components, each with different ionic properties. We have also found that the 4S estrogen receptor of human breast tumors separated into at least 2 components, each with different ionic properties. These data indicate that the estrogen receptors of human breast cancers exhibit molecular heterogeneity. Our results also suggest that the molecular properties of estrogen receptors in human breast tumor biopsies may be related to the clinical responsiveness of patients treated by hormonal manipulations.

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