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IDENTIFICATION OF IMMUNOASSAYABLE ESTROGEN RECEPTOR LACKING HORMONE BINDING ABILITY IN TAMOXIFEN-TREATED RAT UTERUS

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Summary : Using two different monoclonal antibodies to human estrogen receptor (ER), the enzymeimmunoassay was performed. The values of ER contents in human breast cancer and untreated rat uteri obtained by this procedure were correlated well with those by [^{3}H] estradiol binding assay. When estradiol was injected to immature rats, the enzymeimmunoassay showed the uterine receptor dynamic pattern similar to those analyzed by exchange assays. In contrast, tamoxifen administration induced the immunoassayable but non-steroid binding form of ER. This ER-like antigen was the heat-labile molecule with the sedimentation constant of 7 S while ER in untreated rat uterine cytosol sedimented at 9 S. These results suggest the presence of unique molecular state of ER induced by tamoxifen.

Estrogen actions are believed to be mediated through estrogen receptor (ER). Although recent studies involving immunohistochemical techniques have implied that unoccupied ER resides exclusively in the nuclei (1), the use of conventional biochemical procedures would support a concept that nonactivated ER is extracted into the cytosol fraction and activated ER complexed with estrogens is tightly associated with nuclei (2). Using these biochemical techniques, the subsequent phenomena in the fate of estrogen-ER complexes have been extensively studied to clarify the molecular events associated with the initiation of estrogen action. In this relation, it should be pointed out that ER contents in the subcellular fractions have been mainly

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Abbreviations used here are: ER, estrogen receptor; EIA enzymeimmunoassay; E_2, 17β-estradiol; DES, diethylstilbestrol; TEM-Mo buffer, 10 mM Tris, 1.5 mM EDTA, 2 mM mercaptoethanol, 10 mM Na_2MoO_4, pH 7.4 at 20°C; Buffer A, 0.8 M KCl, 5 mg/ml bovine serum albumin, 2% (v/v) rat serum, 140 nM E_2, 20 mM phosphate, 100 μg/ml garamycin, 40 mM Na_2MoO_4, pH 8.0 at 20°C.

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measured by so-called exchange assays (3, 4). These procedures require an exposure of ER to relatively high temperatures. The possibility is not completely eliminated that ER is destroyed by heating. More importantly, exchange assays are unable to pursue non-steroid binding form of ER. The molecular event must be seruously taken into consideration that binding of ER to chromatin is followed by a loss of its hormone binding ability (5). Recently, enzymeimmunoassay (EIA) for ER using two different monoclonal antibodies has been developed (6). In this study, therefore, we asked the possibility that EIA is able to identify the molecular form of ER which can not be measured by [^{3}H] estradiol (E_2) binding assays.

MATERIALS AND METHODS

The immature rats from the Sprague-Dawley strain (20-22 days old) were ovariectomized 2 days before experiments. E_2(1.5 μg/rat) or tamoxifen (Tam) (300 μg/rat) was injected intraperitoneally. The rats were sacrificed at the indicated time of periods after injection. Each uterus was homogenized in 1 ml of 10 mM Tris, 1.5 mM EDTA, 2 mM mercaptoethanol, 10 mM Na_2MoO_4, pH 7.4 at 20°C (TEM-Mo buffer), followed by centrifugation at 800 x g for 5 min. The supernatant was subjected to further centrifugation at 105,000 x g for 60 min to obtain the cytosol. The precipitate from low-speed centrifugation was washed four times with TEM-Mo buffer and then extracted with 1 ml of 0.4 M KCl in TEM-Mo buffer at 0°C for 60 min. The nuclear extract was obtained by re-centrifugation at 800 x g for 10 min. Human breast cancer specimen, which had been stored at -80°C, were also homogenized in 7 vol of TEM-Mo buffer and centrifuged at 105,000 x g for 60 min to obtain the cytosol.

Saturation binding curves were constructed by incubating samples with the increasing amounts of [^{3}H] E_2 (0.15-10 nM) for 18 h at 0°C. Nonspecific binding was assessed by adding a 100-fold excess of unlabeled diethylstilbestrol (DES). The exchange assays for ER are modifications of the protocoals reported before (3, 4). The samples were incubated with 10 nM [^{3}H] E_2 ± 1 μM unlabeled E_2 at 0°C for 1 h, followed by an exposure to 30°C for 1 h unless stated otherwise. The bound [^{3}H] E_2 was determined by a hydroxylappatite method (7). In order to perform EIA for ER (6), the aliquots (0.1 ml) of the cytosol or the nuclear extract were mixed with 0.1 ml of Buffer A (0.8 M KCl, 5 mg/ml bovine serum albumin, 2.0% (v/v) rat serum, 140 nM E_2, 20 mN phosphate, 100 μg/ml garamycin, 40 mM Na_2MoO_4, pH 8.0) and incubated with a polysterene bead coated with one monoclonal anti-ER antibody (D547 Spr) at 4°C for 18 h. Then, these beads were washed five times with 0.4 ml H_2O, followed by second incubation at 37°C for 1 h with 0.2 ml of the solution containing the other monoclonal antibody (H-222) conjugated with peroxidase. After incubations, these beads were again washed with H_2O, followed by third incubation at the room temperature for 30 min with 0.3 ml citrate buffer, pH 5.6 at 20°C, containing 0.02% (w/v) H_2O_2 and 0.768 mg O-phenylenediamine-2HCl. The reaction was terminated by adding 1 ml of 1 N H_2SO_4. ER values were determined spectrophotometrically by reading optical density at 492 nm. Desicated ER standard for EIA was also provided by the supplier (Abbott Laboratories).

Sucrose density gradient analyses were performed in 5-20% (w/v) sucrose in TEM-Mo buffer as published before (8). [2,4,6,7-^{3}H] E_2 (SA, 95 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). Tam was supplied by ICI Pharma (Osaka, Japan). The reagents used here were of analytical grade.

RESULTS AND DISCUSSION

The initial experiments were designed to confirm the assay validity of EIA. As shown in Fig. 1a, the levels of ER in human breast cancer cytosols determined by EIA were found to be well correlated with those by saturation binding curves, although EIA gave approximately 2-fold higher values in comparison with [^{3}H] E_2 binding assay. This assay procedure was applied to measure the ER content in the uterine cytosol form ovariectomized rats, the results indicating that this EIA system is able to be used for qualitative analyses of the rat uterine ER (Fig. 1b). These results seem to be consistent with the reported results that antibodies used here can react with ERs from various species (6, 9).

Using this new assay procedure, ER dynamics elicited by E_2 or Tam injection were investigated. When ER levels were determined by [^{3}H] E_2 exchange assays, E_2 injection produced a rapid decrease in the cytosolic ER level with its augumented replenishment at 24 h. EIA was observed to give the slightly elevated values but the pattern similar to that determined by exchange assays. This was also the case in the nuclear ER in E_2 treated rats. A relatively minor increase in nuclear ER is probably due to the fact that KCl (0.4 M)-extractable, but not a total, nuclear ER was measured in

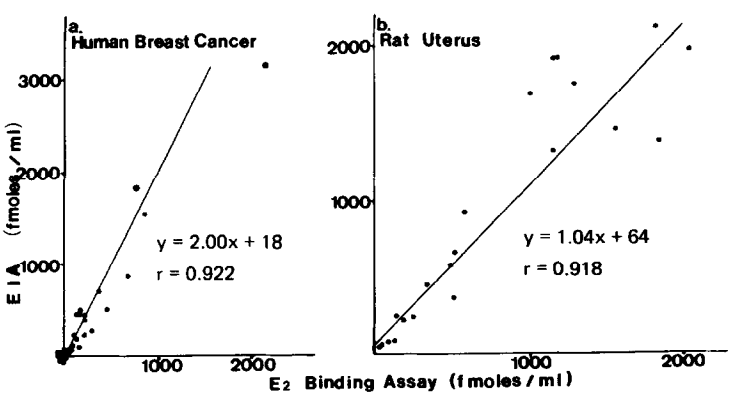

Fig. 1. Correlation between ER contents determined by [^{3}H] E_2 binding assay and EIA.

Human breast cancer specimen (a) and rat uteri (b) were homogenized to obtain the cytosol as described in Materials and Methods. The aliquots of the undiluted (a) or the diluted (b) cytosols were used to measure ER contents by [^{3}H] E_2 binding assay or EIA.

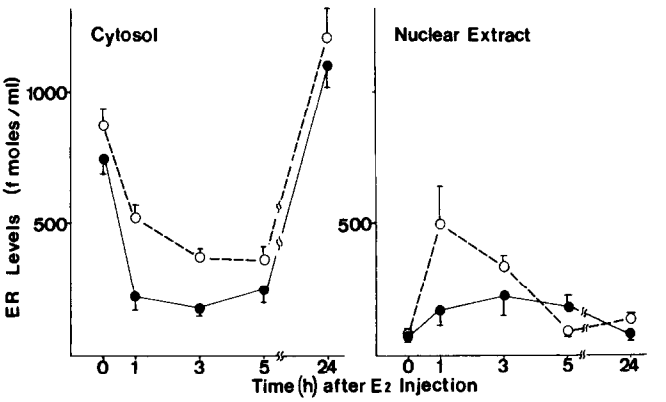

Fig. 2. Effects of E_2 injection on ER contents in the cytosol or the nuclear extract.
E_2 (1.5 μg/rat) was intraperitoneally injected and the rats were sacrificed by cervical dislocation at the indicated time of periods after injection. ER contents in the cytosol or the nuclear extract were determined by exchange assays (●——●) and EIA (o----o) as described in Materials and Methods. The data (mean ± S.E.) were obtianed by four separate experiments.

this study (Fig. 2). In contrast, Tam administration provoked marked difference in uterine ER values between exchange assays and EIA (Fig. 3). When ER levels were measured by the exchange assay, Tam injection was accompanied by a sustained decrease in the cytosolic ER. However, EIA gave much higher levels throughout 24 h of the experiment without significant depletion of the cytosolic ER. The similar difference was also observed in the nuclear extract. Tam injection resulted in a prolonged accumulation of ER

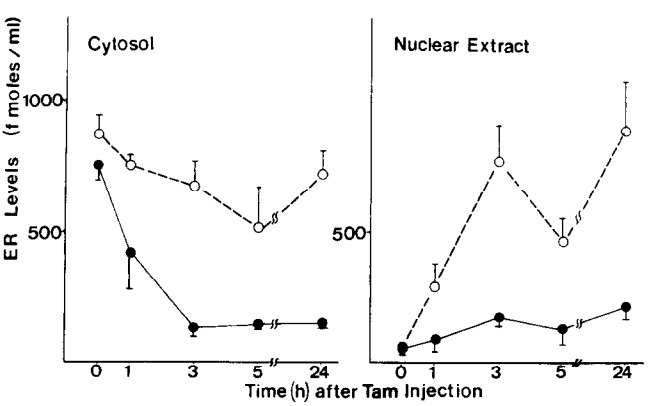

Fig. 3. Determination of ER contents in the uteri of Tam-treated rats by exchange assays and EIA.
The rats recieved a intraperitoneal injection of Tam (300 μg/rat) and were sacrificed at the indicated time of periods after injection. The uterine ER contents were determined as described in the legend of Fig. 3.
exchange assays (●——●); EIA (o----o)

in the nuclear extract with both methods. But, EIA gave much higher values for ER in the nuclear extract.

To explain Tam-dependent appearance of immunoassayable but non-steroid binding form of ER, several possibilities were adressed. Since exchange assays were carried out at the relatively high temperatures, heat stability of uterine ER from Tam-treated rats was examined. The uterine cytosol from rats which were sacriced 24 h after Tam injection was incubated with [^{3}H] E_2 at 0°C for 1 h and then subjected to heating at 30°C for the indicated time of periods (Fig. 4). Under these conditions, the values of the cytosol ER reached to a plateau at 1 h and retained at their levels up to 5 h when measured by the exchange assay. These observations suggest that marked difference in ER values between exchange assays and EIA can not be explained by its instability of E_2 binding ability. Conversely, immunoassayable ER identified in uteri of rats treated with Tam was found to be heat-labile. Interestingly, ER-immunoreactive materials in the untreated rat uterine cytosol was heat-stable (Fig. 4). Collectively, these data would imply that Tam induces ER-immunoreactive material different from putative ER in terms of hormone binding ability as well as heat stability. To further ascertain the

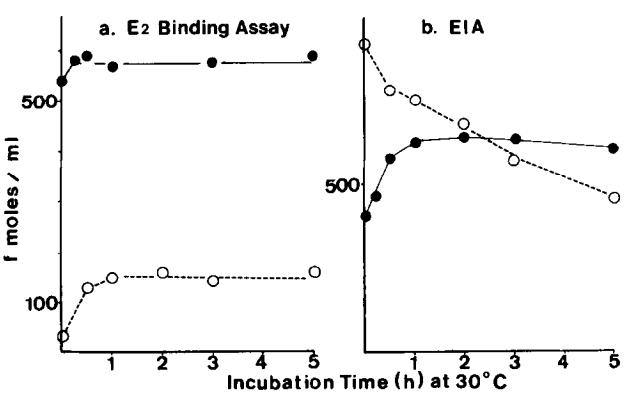

Fig. 4. Heat-stability of ER-like antigenicity and [^{3}H] E_2 binding ability in the uteri of rats untreated or treated with Tam.
The rats were sacrificed 24 h after Tam (300 µg/rat) injection. As a control, the untreated rats were also killed. The cytosols were prepared from the uteri of these rats. After incubation with 10 nM [^{3}H] E_2 ± 1 µM unlabeled E_2 at 0°C for 1 h, these samples were heated at 30°C for the indicated time of periods. The amounts (a) of [^{3}H] E_2 specifically bound to ER were determined by exchange assays. The levels of ER-like antigen (b) were determined by EIA.
Tam-treated rats o----o ; untreated rats ●——●

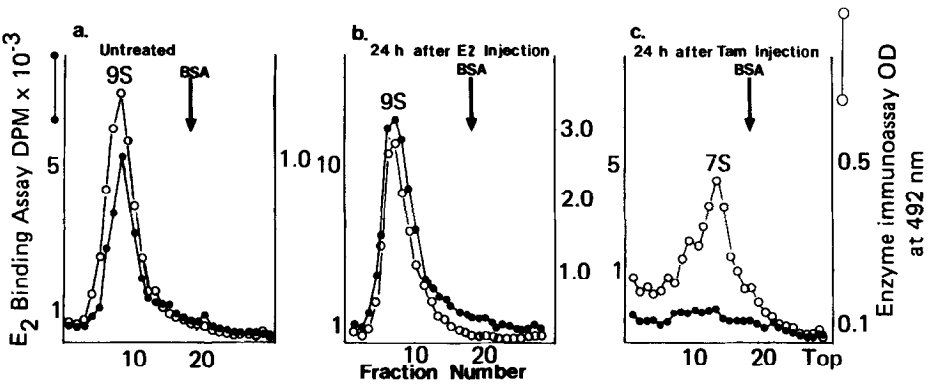

Fig. 5. Sucrose density gradient analyses of ER-like antigen in the uteri of the rat.

The rats were sacrificed 24 h after E_2 (b) or Tam (c) injection. As a control, the untreated rat (a) was also killed. The aliquots (0.2 ml) of the uterine cytosols from these rats were applied to sucrose density gradient analyses as described in Materials and Methods. After centrifugation and fractionation, each fraction was subjected to exchange assays (●——●) and EIA (o——o). The arrow indicates the position of fluorescent bovine serum albumin (4.6 S).

qualitative difference between usual ER and Tam-induced molecule reacting with anti-ER antibodies, sucrose density gradient analyses were conducted. When the uterine cytosol from the untreated rat was analyzed in the low salt condition, EIA-positive molecule cosedimented with E_2 binding protein at 9 S (FIg. 5a). The cytosol from rats sacrificed 24 h after E_2 injection showed the pattern similar to that of the untreated rat uterine cytosol (Fig. 5b). On the other hand, preinjection of Tam into the rat 24 h before sacrifice resulted in an appearance of EIA-positive molecule lacking hormone binding ability at 7 S region (Fig. 5c). These results strongly suggest that Tam induces a new class of ER-related molecule which is definitely different from putative ER. The possibility seems to be quite unlikely that this molecule is entirely unrelated to ER. This consideration emerged from the observation that the values obtained by EIA are correlated well with [^{3}H] E_2 binding assay of ER from human breast cancer and untreated rat uteri (Fig. 1). In addition, non-hormone binding form of steroid receptors has been reported in various systems (10-12). Some of these receptor forms were found to be successfully converted to steroid binding form (10, 11). Furthermore, non-steroid binding form of steroid receptor has been identified in a transformed

cell line (12), although their biological function remains to be elucidated. It would be fascinating future project whether or not non-steroid binding form of ER identified in the present study is related to antiestrogenic properties of Tam.

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