Panel Discussion II

Localization of Estrone Sulfatase in Human Breast Carcinomas

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We generated anti-human E1-STS monoclonal antibodies to localize estrone sulfatase (E1-STS) in human breast carcinomas. In particular, we examined the MCF-7 clone E3, ZR-75-1, MDA-MB 231, and MDA-MB-468 breast cancer cell lines and 25 breast carcinomas by either immunohistochemistry or Western blotting analysis. Simultaneously, we analyzed histological data, estrogen receptor (ER) status, progesterone receptor (PgR) status and epidermal growth factor receptor (EGFR) in breast tissue. All were surgical specimens from female patients. Nine of 25 carcinomas were obtained from premenopausal women, and 16 carcinomas were obtained from postmenopausal women. All cell lines demonstrated positive staining for E1-STS. Interestingly, fine granulated staining of E1-STS on the cell membrane was observed. In addition, Western blotting analysis detected a 65 kD protein with an E1-STS specific band in all breast cancer cell lines regardless of the presence or absence of E2. Twenty-two of 25 (88.0%) carcinomas showed positive staining for E1-STS, whereas negative staining was observed in the interstitial tissue surrounding tumors. In the premenopausal patients, 8 of 10 carcinomas (80.0%) showed positive staining for E1-STS, whereas 14 of 15 carcinomas (93.3%) revealed positive staining in the postmenopausal patients. The frequency of E1-STS expression was relatively higher in postmenopausal patients than in premenopausal patients but not statistically significant. The intensity of immunostaining for E1-STS depended upon the size of the tumor (NS). There was no correlation between E1-STS expression and other parameters. This evidence suggests E1-STS expression may be involved in the development of breast cancer. Further studies are necessary to clarify the relationship between E1-STS expression and prognostic factors. Immunoreactive E1-STS may be localized in cancer cells but not in surrounding tissues in breast cancer.
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Key words: Estrone sulfatase, Breast cancer, Immunohistochemistry, Western blotting

Estrogens are key hormones in the regulation of proliferation of breast cancer cells, and a reduction in estrogen concentration may inhibit the growth of breast tumors[1]. Alteration of the hormonal environment in postmenopausal women may effect a unique biological behavior of human breast carcinoma. However, low levels of plasma estrogens in postmenopausal women do not reduce the estrogen concentration in breast cancer tissues, suggesting that intratumoral production of estrogens might exist. Aromatase and estrone sulfatase (E1-STS) are catalytic enzymes that contribute to local estrogen production, and aromatase inhibitors are clinically useful to reduce the progression of breast tumors in postmenopausal women[2-4]. However, even complete regulation of aromatase dose not decrease the estrogen levels of either tumor or plasma in breast cancer patients[5,6]. Estrone sulfate is a potent abundant estrogen in peripheral blood and is catalyzed by E1-STS in cancer tissues, suggesting that the concentration of either estrone sulfate or estradiol depend upon the activity of E1-STS. *In vivo* and *in vitro* E1-STS studies have demonstrated by enzyme assay that E1-STS is more active in malignant than in normal tissue[7,8]. However, immunohistochemical localization of E1-STS in either carcinomas or normal tissues has not been performed. In the present study, to localize E1-STS in human breast cancer, we examined 4 cell lines and 25 carcinomas by either immunohistochemistry or Western blotting, and we simultaneously analyzed the correlation between E1-STS expression and

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Abbreviations:
ER, Estrogen receptor; PgR, Progesterone receptor; EGFR, Epidermal growth factor receptor; E1-STS, Estrone sulfatase; E2, Estradiol; PBS, Phosphate buffer saline; IgG, Immunoglobulin G

other parameters in a small breast cancer panel.

Materials and Methods

Samples

Cell lines; MDA-MB 231, MDA-MB 468 and ZR-75-1 cell lines were procured from ATCC (Rockville, MD, USA). The MCF-7 clone E3 was obtained from Dr Samuel Brooks (Michigan Cancer Foundation, MI, USA). These cell lines were cultured in appropriate mediums containing 5-10% calf serum with phenol red. Then, they were trypsinized and harvested on chamber slides for 7 days[9].

Human breast cancer tissues: All breast cancer tissue samples were obtained surgically in our hospital, frozen immediately after resection and kept at −75℃ in a deep freezer.

E2 Depletion Assay

For E2 depletion, calf serum was incubated with dextran-coated charcoal overnight and treated with sulfatase (Sigma, St Louis, MO, USA). The calf serum was subsequently centrifuged and supernatant was obtained as charcoal-stripped sulfatase-treated calf serum (CCS). For cell culture, the MCF-7 clone E3 and ZR-75-1 were cultured for 7 days in a phenol red-free medium containing 5% CCS[10]. Cells were used for immunohistochemistry and Western blotting.

Immunohistochemistry

Frozen specimens were fixed in 4% formaldehyde for 10 min. Frozen tissue sections (approximately 10 mm) were washed in PBS for 5 min and incubated with 0.3% H_2O_2+NaN_3 in PBS for 10 min to block endogenous peroxidase activity. After washing the sections in PBS, the sections were incubated with 10% goat serum for 30 min. The slides were then incubated with 15 μg/ml of the affinity purified anti-human estrone sulfatase KM1049 mouse antibody or with a control mouse serum for 12 hrs at room temperature. The slides were subsequently washed 3 times in PBS for 5 min and incubated with 1 μg/ml of biotinylated goat anti-mouse IgG (Nichirei, Tokyo, Japan) for 40 min. Following several washes in PBS, the slides were reacted for 40 min with horseradish peroxidase-conjugated streptavidin (Nichirei) and washed in tap water for 10 min. The slides were then incubated for 1 min in 0.05% diaminobenzidine and in 0.01% H_2O_2. Finally, slides were washed in tap water and counterstained with Mayers' hematoxylin.

Evaluation of Immunoperoxidase Staining

Slides were graded for staining intensity and for the percentage of immunopositive cells as previously described[11]. Briefly, specific staining with immune serum was semiquantitated by assigning a score of 0 to 3 based on the color intensity of the brown diaminobenzidine precipitate with +1 representing light brown staining, +2 a moderately brown color, and +3 an intense brown color. Reactivity was considered positive and specific only when the intensity of immunoperoxidase staining with the immune IgG exceeded the staining intensity observed with appropriate control IgG.

Assay for ER, PgR and EGFR

Estrogen receptor (ER) levels were analyzed by DCC methods (positive >5 fmol/mg protein). Progesterone receptor (PgR) was assayed with EIA (positive >10.0 fmol/mg protein). Epidermal growth factor receptor (EGFR) was measured by radioimmune assay. The cut-off value of EGF binding is 1 fmol/mg protein. The analysis of ER, PgR and EGFR was conducted by the Ohtsuka Assay Laboratory (Tokushima, Japan).

Western Blotting Analysis

Approximately 35 μg of protein from the crude enzyme extract prepared from breast tissue extracts was loaded onto 10% SDS-containing polyacrylamide gels and run at a constant power of 2.5 mA/cm. The gels were then transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA) for 18 hrs. The membrane was then washed and blocked with 5% skimmed milk in buffer A containing 20 mM Tris-HCl (pH 7.4), 0.3 M NaCl and 0.1% Triton-X 100 at room temperature for 1 hr. The membrane was sequentially blocked with 10% human serum diluted in buffer A. The membrane was then washed in buffer A and reacted for 12 hrs at room temperature with 20 μg/ml of KM 1049 mouse antibody diluted in buffer A containing 1% bovine serum albumin. After washing the membrane several times with buffer A, the membrane was blocked with 10% sheep serum in buffer A and washed in buffer A. The membrane was reacted with a 1:500 dilution of biotinylated antimouse goat IgG (Nichirei) and then incubated with sheep anti-goat Ig-HRP

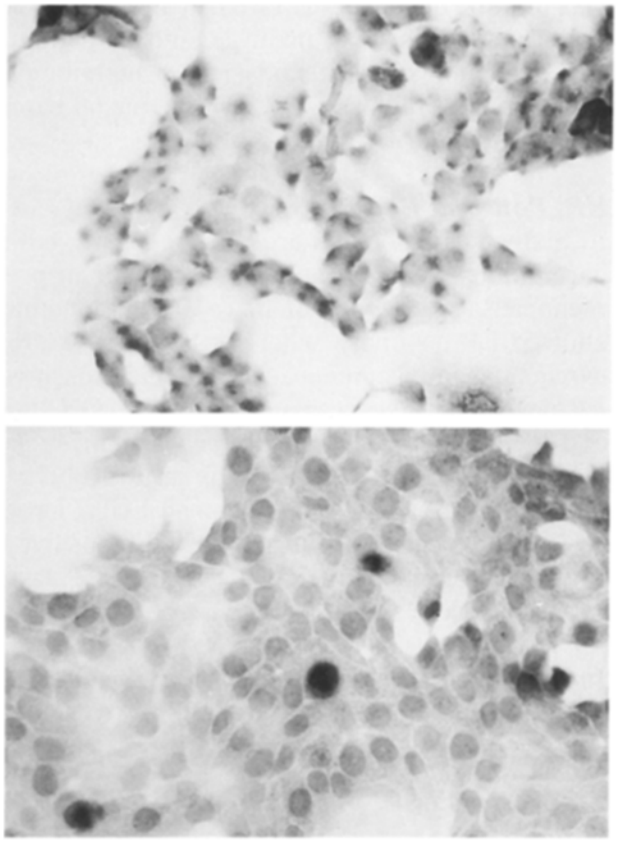

Fig 1. a, Staining for E1-STS was observed on either the membrane or the cytoplasm of the ZR-75-1 breast cancer cell line in the presence of E2. Top, KW 1049 (anti-E1-STS monoclonal antibody); Bottom, Normal mouse IgG.

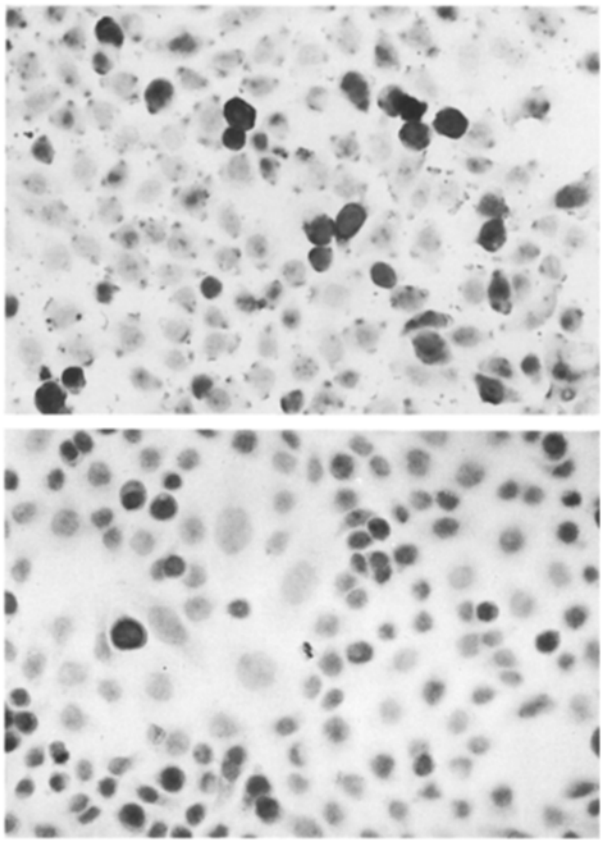

Fig 1. b, Staining for E1-STS was observed on the membrane of the MDA-MB 231 breast cancer cell line. Top, KW 1049 (anti-E1-STS monoclonal antibody); Bottom, Normal mouse IgG.

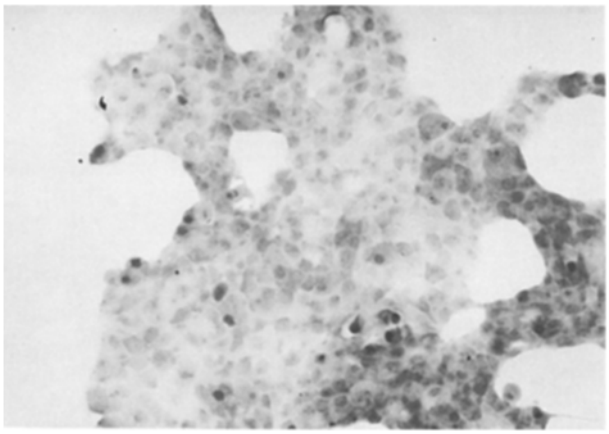

Fig 2. In the absence of E2, E1-STS expression was detected in the cytoplasm of the MCF-7 clone E3.

(Amersham, Arlington Heights, IL, USA) for 0.5 hr at room temperature and washed in buffer B that contained 20 mM Tris-HCl (pH 7.4), 0.6 M NaCl, 0.5% Triton X-100 and 0.1% SDS. Finally, the immunoreactive E1-STS was stained with 0.05% diaminobenzidine (DAB) diluted PBS in the presence of 0.01% H_2O_2.

Results

Specificity of KM 1049 against Human Estrone Sulfatase

Positive immunostaining of E1-STS in MCF-7 clone E3, ZR-75-1, MDA MB 231 and MDA MB 468 was observed by either immunohistochemistry (Figs 1, 2) or Western blotting (Fig 3). A positive correlation between the results of immunohistochemistry and Western blotting analysis was obtained.

Patient Characteristics

All patients were treated with surgery, including breast conserving therapy or mastectomy with level 2 lymph node dissection. Ten women were premenopausal patients, and 15 were postmenopausal patients (Table 1). The average age was 54.9 years and the average tumor size was

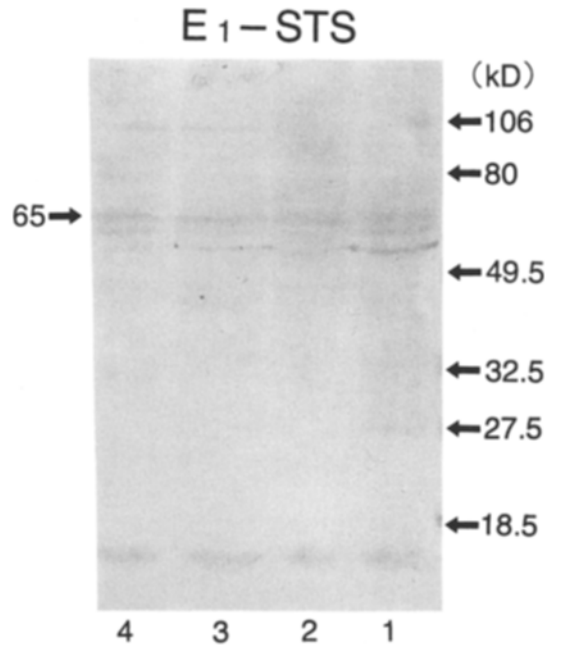

Fig 3. In MCF-7 clone E3 (lane 1), ZR-75-1 (lane 2), MDA-MB 231 (lane 3) and MDA-MB 468 (lane 4), a 65 kD band specific to E1-STS was observed on Western blotting. All cell lines were culutured in medium containing bovine serum.

Table 1. E1-STS Expression in Breast Cancer

Variable	Positive	Negative	*p*-value	Overall (n=25)
Node Status (pN)				
pN0	18	2	0.504[a]	20
pN1-2	4	1		5
ER status				
+	14	2	0.713	16
−	8	1		9
PgR status				
+	13	2	0.654	15
−	9	1		10
Histology				
invasive	22	3		25
Menopausal status				
pre-	8	2	0.345	10
post-	14	1		15
Age at diagnosis (Mean)	54.9	45.3	0.075[b]	
Tumor size (pT)				
pT1 & pT2	18	3	0.578	21
pT3 & pT4	4	0		4
EGFR status				
+	5	0		5
−	6	1	0.583	7
unknown	11	2		13

[a]*p*-values caluculated from Chi-Square test (Fisher's exact probability).
[b]Student-*t* test.

36.2 mm (pT) in diameter. Five patients had lymph node metastasis (pN1 or pN2), and 20 patients had no lymph node metastasis. On histological evaluation, all tumors were invasive ductal carcinomas.

ER, PgR and EGFR Status

Sixteen carcinomas were positive for ER, but 9 carcinomas were ER negative by DCC. In premenopausal patients, 7 of 10 (70.0%) carcinomas showed ER positivity, whereas 11 of 15 (73.3%) carcinomas in postmenopausal patients presented ER positive. Fifteen carcinomas expressed PgR and 10 carcinomas did not. There was no correlation between ER and PgR status in these breast tumors. In 12 samples we examined EGFR levels by RIA and 5 carcinomas showed EGFR positivity. There was no relationship between these parameters (Table 1).

Immunohistochemistry of Breast Tissues

Twenty-two of 25 (88.0%) carcinomas showed

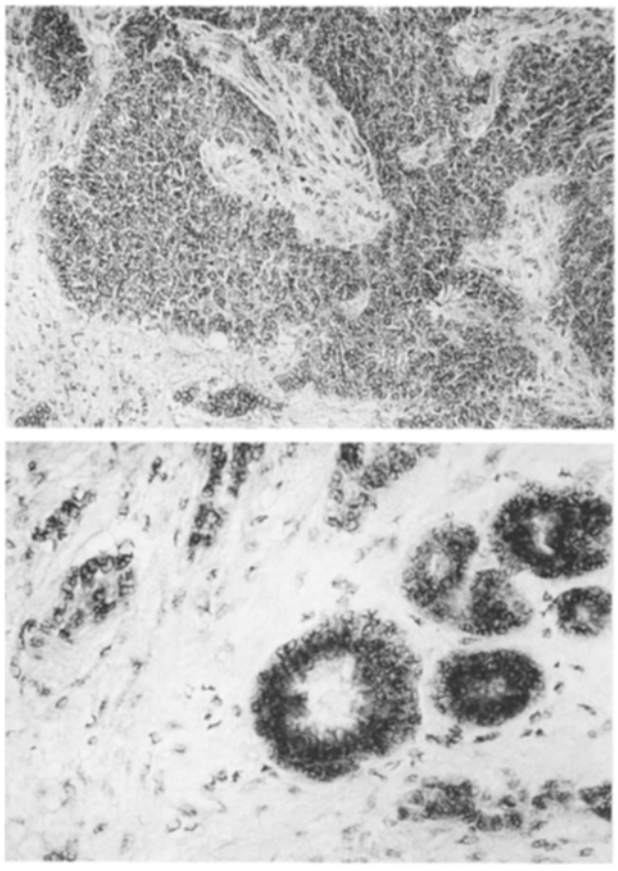

Fig 4. Diffuse and cytoplasmic staining for estrone sulfatase (E1-STS) was observed in human breast cancer tissue (original magnification ×80). Top, Sample 2; Bottom, Sample 3.

positive immunostaining for E1-STS, whereas negative staining was observed in the interstitial tissue adjacent to carcinoma in all 25 cases (Fig 4).

Western Blotting Analysis for Breast Tissues

We observed a 65 kD band representing E1-STS on Western blotting in 2 samples. Samples 2 and 3 also showed positive immunostaining for E1-STS. Otherwise, no specific E1-STS band was observed in either lane 1 or lane 4, which were blotted with protein obtained from normal breast tissue (Fig 5).

Correlation between E1-STS Expression and other Parameters

In the premenopausal patients, 8 of 10 carcinomas (80.0%) showed positive immunostaining for E1-STS, whereas 14 of 15 carcinomas (93.3%) expressed E1-STS in the postmenopausal patients (Table 1). The frequency of E1-STS expression was relatively higher in the postmenopausal patients than in the premenopausal patients. In addition, the average staining score was relatively higher in the postmenopausal patients than in the premenopausal patients (NS). There were no statistically significant associations between E1-STS expression and pN, pT, ER status, PgR status, EGFR status, of other histological parameters.

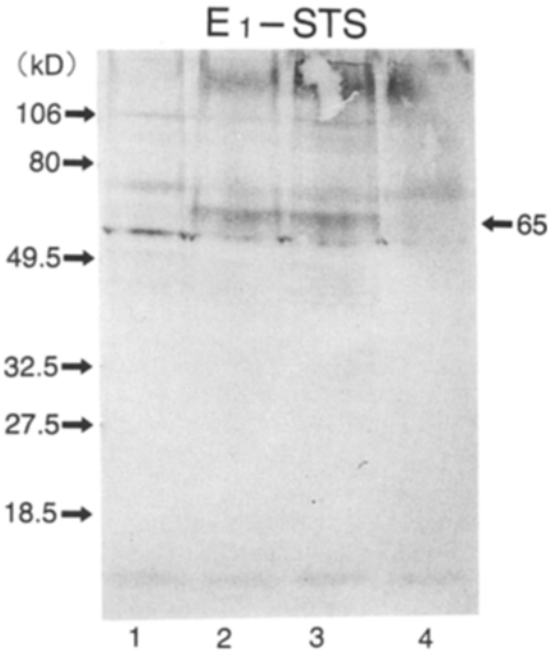

Fig 5. A 65 kD band was detected in both lane 2 and lane 3. In lanes 2 and 3, breast carcinomas showing positive staining of E1-STS (Fig 4) were blotted. In lanes 1 and 4, no band specific to E1-STS was observed. Samples 1 and 4 were normal breast tissue adjacent to carcinomas.

Discussion

The metabolism of steroids is complicated and the synthesis of estrogens is generally related to menopausal status in women[2,12]. However in postmenopausal women the role of hormone producing organs, such as the ovaries, adrenal glands and pituitary, changes. Therefore, peripheral estrogen synthesis in adipose tissue[13] and tumor cells is more significant than the plasma level of estrogens since the peripheral concentration of estrogen is 10-40 fold higher than the circulating estrogen concentration in postmenopausal breast cancer patients[14]. The source of estrogens in postmenopausal women is estrone converted from androgens or estrone sulfate by aromatase or estrone sulfatase. Recently, aromatase activity and estrone sulfatase activity have been investigated. Evans *et al* reported that aromatase activity in breast cancers correlated with histological grade and survival after relapse[15]. Also, aromatase inhibitors are effective in reducing breast cancer progression[16-18]. Santner *et al* demonstrated that the E1-STS pathway might be more important than the aromatase pathway since E1-STS contributed to intratumoral production of estradiol[6]. This evidence suggests that local production of estradiol, catalyzed by E1-STS, may be involved in breast cancer development. Our immunohistochemical findings showed differential E1-STS expression in cancer cells and interstitial tissues adjacent to tumor, suggesting that high levels of intratumoral estrogen are due to expression of E1-STS in tumor cells but not in surrounding tissues. Moreover, an *in vitro* study by Stantner *et al* demonstrated that physiological concentrations of E1-STS could increase the S-phase fraction of MCF-7 cells, and that estrone sulfate promoted nuclear uptake of estradiol converted from estrone in the conditioned medium of MCF-7 cells[8], suggesting that cancer cells might produce E1-STS. In this regard, reduction of E1-STS may be a possible method of inhibiting breast cancer development. In fact, some inhibitors of E1-STS have been reported[19-22]. A relationship between E1-STS expression and ER has been investigated. Chetrite *et al* demonstrated the effect of anti-E1-STS on estrogen-dependent, ER positive, T-47D

cells and estrogen-independent, ER negative, MDA-MB 231 cell[22]. The latter cell line has been shown to highly express E1-STS mRNA[23]. *In vivo*, there was no correlation between E1-STS expression and ER in either the present study or other studies previously reported[2,6,7,14,24]. This suggests that E1-STS expression may be a factor independent of ER status. We also examined the relationship between E1-STS expression and EGFR levels, which could be another biological parameter since in the absence of ER, growth factors such as transforming growth factor alpha and amphiregulin can stimulate the proliferation of breast cancer cells through EGFR *in vitro*, and estrogens may indirectly regulate EGFR expression through autocrine and/or paracrine mechanisms[25,26]. No correlation between E1-STS expression and EGFR was observed in the present study. No correlation between E1-STS and other hormonal parameters was observed. Utsumi *et al* demonstrated a correlation between estrone sulfate mRNA levels and prognosis[24]. Immunoreactive E1-STS is localized in breast cancer cells but not in the surrounding interstitial cells and because intracellular E1-STS may contribute to the local production of estrogens. Further investigation of a large number of tumors is needed to determine the role of E1-STS expression in breast cancer.

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