Expression of Estrogen Sulfotransferase in MCF-7 Cells by cDNA Transfection Suppresses the Estrogen Response: Potential Role of the Enzyme in Regulating Estrogen-Dependent Growth of Breast Epithelial Cells

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ABSTRACT
Estrogen sulfotransferase (EST) is a cytosolic enzyme that catalyzes the sulfonation of estrogens at the 3-hydroxyl position by use of 3'-phosphoadenosine-5'-phosphosulfate as an activated sulfate donor. Although largely known and studied as a phase II metabolic enzyme with prominent expression in the liver, the high substrate specificity of EST (with a high \( V_{\text{max}}/K_{\text{m}} \) value for estrogen) suggests that expression of the enzyme in extrahepatic, estrogen target tissues, such as the breast epithelium, may constitute an effective mechanism for local estrogen regulation as well. In this study, we have evaluated the physiological significance of EST expression by cDNA transfection studies with use of the estrogen-dependent MCF-7 breast cancer cell line as a model system. We show that expression of EST in MCF-7 cells effectively reduces the cells' response to physiological concentrations of estradiol (10 nM) by up to 70% as determined in an estrogen-responsive reporter gene assay. In addition, we demonstrate that expression of EST similarly inhibits estrogen-stimulated DNA synthesis and cell proliferation by 21% and 46%, respectively. (The thymidine incorporation rate was measured 3 days after and the cell numbers were counted 8 days after transfection.) These results provide direct evidence for the functional significance of \( \text{in situ} \) EST expression in the breast epithelium and suggest that abnormal regulation of the enzyme may have pathological implications in the development and maintenance of hormone-dependent breast carcinomas.

Estrogens play an essential role in the growth and development of hormone-dependent breast carcinomas as well as the normal mammary gland (Dickson and Lippman, 1988; Topper and Freedman, 1980). One third to one half of all human mammary gland carcinomas are hormone-dependent, and control of estrogen activity is a central objective in the treatment of these malignancies (McGuire \textit{et al.}, 1975; Megdelenat and Pouillart, 1983). Accordingly, understanding the mechanism and regulation of estrogenic activity in both normal and malignant breast epithelial cells is of fundamental importance.

Although recent work has identified a receptor-independent pathway of estrogen signaling in the mouse uterus (Das \textit{et al.}, 1997), it is well accepted that the activity of estrogens in many tissues including the breast generally depends on their interaction with specific nuclear receptors, of which two isoforms, \( \text{ER}_a \) and \( \text{ER}_b \), are now known (Kuiper \textit{et al.}, 1996). In addition to the estrogen receptors, the significance of estrogen biosynthetic and metabolic enzymes in the etiology and treatment of breast cancer also has been recognized. Thus, there has been a high and continued interest in the estrogen biosynthetic enzyme P450 aromatase and the development of a specific inhibitor for this enzyme which might be used clinically for the attenuation of estrogen activity (Simpson \textit{et al.}, 1994; Brueggemeier, 1994; Goss and Gwyn, 1994). Another set of potentially important enzymes in this regard are the estrogen sulfotransferase (EC 2.8.2.4) and sulfatase (EC 3.1.6.2) (Hobkirk, 1985; Strott, 1997). These enzymes catalyze opposing reactions, adding or removing, respectively, a sulfonyl group from the 3-hydroxyl position of estrogens. Because sulfated estrogens do not bind to the estrogen receptor and are therefore hormonally inactive (Brooks \textit{et al.}, 1978), the balance between estrogen sulfotransferase and sulfatase expression in estrogen target tissues such as the mammary gland could be a major factor in determining its estrogen sensitivity.

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ABBREVIATIONS: CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; EST, estrogen sulfotransferase; FBS, fetal bovine serum; HME, human mammary epithelial; HSST, hydroxysteroid sulfotransferase; MEM, minimum essential medium; PBS, phosphate-buffered saline; PST, phenol sulfotransferase; TLC, thin-layer chromatography.
Although activities of steroid sulfotransferase and sulfatase in breast cancer tissues (Tseng et al., 1983; Godefroi et al., 1975; Raju et al., 1980; Pewnim et al., 1984) or cell lines (Pasqualini et al., 1992; Rozhin et al., 1986; Pasqualini, 1992) have been documented extensively, the exact role and regulation of estrogen sulfotransferase in normal and malignant breast epithelial cells remains an issue of uncertainty. Earlier studies have shown that some but not all breast cancer biopsy tissues or cell lines contained estrogen-sulfonating activity (Tseng et al., 1983; Godefroi et al., 1975; Raju et al., 1980; Pewnim et al., 1984; Pasqualini et al., 1992; Rozhin et al., 1986; Pasqualini, 1992). Several early investigations also attempted to correlate the level of estrogen-sulfonating activity in primary breast carcinomas with their estrogen receptor status with the hope that the sulfotransferase activity might serve as a useful independent marker for breast cancer prognosis (Tseng et al., 1983; Pewnim et al., 1984; Adams et al., 1979; Braunsberg et al., 1974; Leung et al., 1973). However, results of these studies were not very consistent and no definitive conclusions could be drawn. Likewise, estrogen-sulfonating activities had been detected both in estrogen receptor negative and positive breast cancer cell lines (Pasqualini et al., 1992; Rozhin et al., 1986; Pasqualini, 1992).

Recent progress in the molecular cloning of cytosolic sulfotransferases has provided new tools with which the role and regulation of estrogen sulfotransferase in mammary gland physiology and carcinogenesis can be addressed in a more precise manner. It is now known that three groups of cytosolic sulfotransferases are expressed in human tissues which are distinguishable based on their primary sequence structures and catalytic properties (Falany, 1997; Weinshilboum et al., 1997). These are the phenol sulfotransferases, the hydroxysteroid sulfotransferase and the estrogen-specific sulfotransferase (Weinshilboum et al., 1997). Although both cloned PST and HSST have been shown to display a certain degree of estrogen-sulfonating activity (Hernandez et al., 1992; Falany et al., 1994), it is clear that only the estrogen sulfotransferase uses estrogens as its natural substrates (Falany et al., 1994, 1995; Song et al., 1995). For example, the \( V_{\text{max}}/K_m \) value of the mouse EST is 23 times that of the mouse HSST with estradiol as a substrate (Kakuta et al., 1998). Similarly, when tested with the synthetic estrogen diethylstilbestrol as a substrate, the \( V_{\text{max}}/K_m \) value of the mouse EST is five times that of the mouse PST (Kakuta et al., 1998). Thus, among these enzymes, one would expect EST to be a more relevant enzyme in the modulation of estrogen activity under normal physiological conditions.

With use of specific antibodies, Falany and Falany (1996) recently examined the isoforms of cytosolic sulfotransferases expressed in normal HME cells and several breast cancer cell lines, both estrogen receptor positive and negative. They showed that EST was expressed in normal HME cells but was absent in all the breast cancer cell lines examined (Falany and Falany, 1996). It is possible that the estrogen-sulfonating activity detected in earlier biochemical studies in some of the same cell lines (Tseng et al., 1983; Godefroi et al., 1985; Raju et al., 1980; Pewnim et al., 1984; Pasqualini et al., 1992; Rozhin et al., 1986; Pasqualini, 1992) was indicative of nonspecific reaction by other sulfotransferases. The differential expression of EST in normal HME and breast cancer cell lines is an important observation and has raised the possibility that down-regulation of EST may lead to unchecked estrogen stimulation and contribute to the neoplastic transformation of the breast epithelium. Two highly intriguing questions are derived from this initial observation. The first relates to the functional significance of EST in breast epithelial cells, i.e., whether the expression of EST in these cells is indeed capable of modulating the activity of physiological concentrations of estrogen. The second is whether the observed differential expression of EST in normal HME and breast cancer cell lines could be extended to primary breast tissues and carcinomas. In this report, we describe the results of our study, which was designed specifically to address the first of these two questions.

### Materials and Methods

**Cell cultures.** MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD). For normal passage and plating, the cells were grown in MEM containing 10% FBS, 10 μg bovine insulin/ml, 1 mM nonessential amino acids and 1 mM sodium pyruvate. Cell number and plating density for each experiment are as specified (see below). Cells were passed routinely at 1:8 to 1:10 dilutions when they became confluent. For the study on the effect of estrogen, the base MEM medium was substituted by phenol red-free α medium (Life Technologies, Grand Island, NY) which contained charcoal-treated FBS (Sigma, St. Louis, MO) in place of the regular serum. HME cells were from the Aging Cell Culture Repository at the Coriell Institute for Medical Research (Camden, NJ, Repository no. AG1132). They were cultured by a mammary epithelial cell growth medium kit (MEGM BulletKit) from Clonetics Corporation (San Diego, CA, Catalog no. CC-3150).

**Activity assay and Western blot analysis of estrogen sulfotransferase.** Cells were harvested and sonicated in 10 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 10% glycerol. Total cell homogenate was centrifuged at 15,000 × g for 10 min, and the supernatant was collected and used for enzyme activity assay or immunoblot analysis. Protein concentration was determined by the Bradford method with a colorimetric assay kit from Bio-Rad (Richmond, CA).

Sulfotransferase activity was measured with \(^3\)H-labeled estradiol (2,4,6,7-T(H(N))estradiol, 87.6 Ci/mmol, Du Pont NEN; final concentration, 1.25 nM) in 200 μl assay buffer consisting of 200 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 1.25% Triton X-100, 100 μM sulfate donor 3′-phosphoadenosine-5′-phosphosulfate and the appropriate amount of cell lysate (100–200 μg total protein). Reaction was initiated by the addition of substrate and continued for 30 min at 37°C. The reaction mixture was extracted with 2 volumes of dichloromethane, and aliquot of the aqueous phase was counted and taken as a measure of the sulfated product (Song et al., 1995). For Western blot analysis, a polyclonal antiserum originally developed with purified bacterially expressed mouse EST as an antigen, was used (Song et al., 1998). Cell homogenate was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (20 μg per lane or otherwise as stated in the figure legends), transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH; BA85, 0.45 μm) and probed with the purified EST antiserum. Immunodetection was performed with the enhanced chemiluminescence Western blotting detection system from Amersham (Arlington Heights, IL).

**cDNA transfection procedures.** The full coding region of the mouse EST cDNA was cloned previously into the bacterial expression vector pGEX-4T-3 for fusion protein production (Song et al., 1995). The cDNA was excised from pGEX-4T-3 vector at BamHI and XhoI sites and cloned at the same sites into the eukaryotic expression vector pCDNA3 (Invitrogen, San Diego, CA). An estrogen responsive reporter gene construct, Vit-A2-CAT, was kindly provided by Dr. C.T. Teng from the National Institute of Environmental Health Sciences. In this reporter gene Plasmid, the *Xenopus* vitel-
logenin gene promoter containing a perfect palindromic estrogen response element was placed upstream of the CAT reporter gene (Liu et al., 1993). To study the effect of EST expression on estrogen-stimulated CAT reporter gene activity, 2 to \(5 \times 10^5\) cells were seeded into each 35-mm plate in complete MEM. Transfection of the mouse EST cDNA with Lipofectamine (Life Technologies, Grand Island, NY) was carried out 24 h later. Two micrograms of pCDNA3-EST or control pCDNA3 vector, together with 2 \(\mu\)g of Vit-A2-CAT plasmid, was mixed with 24 \(\mu\)l Lipofectamine and added to the cells in 1 ml phenol red- and serum-free \(\alpha\) medium. After 6 h incubation, an equal volume of \(\alpha\) medium containing 20% charcoal-treated FBS was added and incubation continued overnight. On the next morning, cells were washed with PBS, and fresh \(\alpha\) medium containing 10% charcoal-treated FBS and estradiol (final concentration, 10 nM) or solvent vehicle (ethanol) was added. For the study of the effect of EST expression on estrogen-stimulated cell growth and proliferation, cells were grown in 12-well plates and transfected with 1 \(\mu\)g of pCDNA3-EST or pCDNA3 plasmid per well with the volumes of Lipofectamine and media appropriately adjusted. Chorolamphenicol acetyltransferase reporter gene assay. Three days after transfection, cells were scraped off the plate in PBS containing 1 mM ethylenediaminetetraacetic acid, washed and resuspended in 100 \(\mu\)l 250 mM Tris-HCl, pH 7.5. They were lysed by repeated freezing and thawing (three cycles) with dry ice-cooled ethanol and a 37°C water bath. The lysate was centrifuged at 15,000 \(\times\) \(g\), and the supernatant was recovered and treated at 70°C for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity. After recenterifugation to remove the denatured proteins, CAT activity was measured for 10 min to inactivate any deacetylase activity.

Results

To detect the expression of endogenous and transfected estrogen sulfotransferase in the cells under study, we used a polyclonal antibody previously developed for the mouse EST (Song et al., 1995). The cross-reactivity of this antibody with the human enzyme has been established previously (Song et al., 1998). With use of this antibody, we could detect endogenous EST expression in normal human mammary epithelial cells (fig. 1A, indicated as HME), but not in the MCF-7 breast cancer cell line (fig. 1B). This result corroborated the earlier finding of Falany and Falany (1996). After transfection with the mouse EST cDNA, a protein band corresponding to the mouse EST in size (35 kDa) was detected in MCF-7 cells on Western blot (fig. 1B). Enzyme activity assays established that the expressed enzyme was catalytically active. Homogenate of cells transfected with EST cDNA but not that of control cells was found to contain EST activity (fig. 1C). To examine the time course of EST expression after cDNA transfection, the level of EST protein in MCF-7 cells was monitored daily by Western blot analysis for 5 consecutive days. As shown in figure 1D, although the level decreased gradually, EST protein expression persisted and still could be detected 5 days after transfection.

To evaluate the regulatory effect of EST expression on the estrogen response in MCF-7 cells, initial experiments were carried out to confirm and define the conditions for the estrogen responsiveness of these cells. Results of these experiments are shown in figure 2 which demonstrate clearly that estrogen (10 nM) stimulated DNA synthesis and increased cell number in MCF-7 cells. We next used the estrogen-dependent reporter gene, Vit-A2-CAT, to assess the effect of EST expression on the estrogen response in these cells. In this reporter gene construct, the Xenopus vitellogenin gene promoter containing a perfect estrogen response element was coupled to the CAT gene, and estrogen response in Vit-A2-CAT transfected cells can be detected readily by measuring the CAT activity (Liu et al., 1993). As expected, in MCF-7 cells co-transfected with Vit-A2-CAT and the control pCDNA3 plasmid, treatment with 10 nM estradiol resulted in a high level of CAT activity (fig. 3). In clear comparison, the estrogen-stimulated CAT activity was significantly lower in cells into which Vit-A2-CAT was co-transfected with pCDNA3-EST. The CAT activity in EST-expressing cells was calculated to be reduced by more than 70% from that attained in cells that did not express EST (fig. 3).

To determine whether the suppressive effect of EST on estrogen activity, as determined in the reporter gene assay above, could be replicated in estrogen-stimulated MCF-7 cell growth and proliferation, the rate of DNA synthesis and increases in cell number were compared between empty vector and EST cDNA transfected cells. The rate of DNA synthesis was compared at day 3 after cDNA transfection, which represented a middle point for the apparent course of EST protein expression in this transient transfection procedure (fig. 1D). Because the increase in cell number caused by estrogen stimulation is accumulative (fig. 2A), to maximize the sensitivity for detecting a difference, total cell numbers in the control vector and EST cDNA transfected cell cultures (both with estrogen supplementation) were determined and compared on day 8 after transfection. Figure 4 shows that under estrogen-supplemented culture conditions, expression
of EST in MCF-7 cells also inhibited DNA synthesis and slowed cell proliferation by 21% and 46%, respectively (P < .05 for both). Finally, to address the mechanism for the observed attenuating effect of EST on the estrogen response, we investigated the catalytic efficiency of the expressed enzyme in MCF-7 cells in culture by determining whether and to what degree sulfated estradiol is released into the culture medium. As shown in figure 5, we found that during 72 h after EST cDNA transfection a significant amount of the added estradiol (>60%) could be recovered from the culture medium in the form of estradiol sulfate. This suggested that there is efficient movement of the sulfated estrogen across the cell membrane in MCF-7 cells.

**Discussion**

Steroid transformation enzymes are recognized increasingly as important modulators of target tissue sensitivity to steroid hormones (Roy, 1992; Penning, 1997). This concept was demonstrated adequately by the expression and activity of 11β-hydroxysteroid dehydrogenase in the kidney (Funder et al., 1988) and the 5α-reductase in the prostate (Roy, 1992). In both of these cases, steroid metabolic enzymes serve as molecular switches to control the occupancy of the concerned steroid receptor, the glucocorticoid receptor and the androgen receptor, respectively. A similar role for steroid sulfotransferases in regulating target tissue sensitivity to steroid hormones has been suggested frequently (Hobkirk, 1985; Roy, 1992). However, up to now no direct experimental data exist to support such a conclusion. The recent cloning and molecular characterization of cytosolic sulfotransferases have provided the necessary tools for this hypothesis to be tested directly. In this study, we showed that the estrogen-specific sulfotransferase was expressed in normal mammary epithelial cells but was absent from the MCF-7 breast cancer cell line. This result serves as an independent confirmation of the earlier observation made by Falany and Falany (1996). In
addition, we demonstrated that restoration of EST expression in MCF-7 cells by cDNA transfection could attenuate the estrogen response significantly, both in a reporter gene activity assay and when DNA synthesis and cell number were used as markers for estrogen-stimulated cell growth and proliferation.

Estrogen is required for the normal growth and development of the mammary gland (Topper and Freedman, 1980). The differential expression of EST between normal HME and breast cancer cell lines and our demonstration that EST can act as an effective modulator of local estrogen activity in the MCF-7 cell model suggest that loss or down-regulation of EST may enhance the growth-stimulating effect of estrogen and contribute to the process of tumor initiation and/or promotion in the breast epithelium. An important question in this regard which remains to be addressed is whether the observed down-regulation of EST in breast cancer cell lines could be extended to primary breast carcinomas. Additionally, the kinetics and pharmacology of EST expression in normal mammary epithelial cells is likely to differ from the EST transfected MCF-7 cells and further studies, such as the reverse experiment using the antisense RNA technology to block EST expression in normal mammary epithelial cells, are required. Nevertheless, our study, for the first time, has provided direct evidence for the importance of EST as a local estrogen modulator at physiological concentrations of the hormone and hopefully will stimulate a renewed interest and serve as a foundation for future investigations on this important aspect of estrogen regulation.

The fact that sulfated estradiol was secreted freely into the culture medium implies that part of the estrogen-attenuating effect of EST in cultured MCF-7 cells may have been
achieved through a reduction in the effective estrogen concentration. Thus, expression of EST in the mammary epithelial cells may not only decrease the intracellular level of receptor active estrogens but also may act to limit the availability of the active hormone in the extracellular local microenvironments within the mammary gland. Conversely, it suggested that estrogen sulfates from systemic circulation can be taken up efficiently by the breast epithelial cells and serve as a substrate for sulfatase to generate the free and active form of the hormone. In either case, the mechanism by which the charged and hydrophilic estrogen sulfates traverse across the cell membrane presently is not understood.

The improved understanding on the expression and function of EST in breast epithelial cells has highlighted the need to further increase our knowledge on the opposing enzyme estrogen sulfatase in these cells. In a recent report describing the development of estrogen sulfatase inhibitors, Selcer et al. (1997) showed that estrogen sulfatase activity was present in MCF-7 cells. However, only at micromolar concentrations was estrone sulfate found to stimulate MCF-7 cell proliferation (Selcer et al., 1997). This result contrasted with our finding on the apparent low $K_m$ of the cDNA expressed EST in MCF-7 cells and suggested that, at least in this model system, EST would be a far more effective and relevant enzyme than the sulfatase at physiological concentrations of the hormone. It is not known whether the estrogen sulfatase activity in MCF-7 cells (Selcer et al., 1997) was reflective of that found in normal mammary epithelial cells, nor do we know, as a matter of fact, if there is an estrogen-specific form of the enzyme. However, should the $K_m$ of the estrogen sulfatase in primary breast carcinomas be similar to that of EST, then we might need to reevaluate the rationale for developing estrogen sulfatase inhibitors which are often aimed at estrogen sulfatase activities with $K_m$ values in the micromolar range as their targets (Selcer et al., 1997; Duncan et al., 1993; Purohit et al., 1995). Considering the overlap in their substrate specificity (particularly at high concentrations of substrate), it is necessary to characterize the individual isoforms of sulfotransferase and sulfatase in tissues such as the breast epithelium and make a distinction between physiological and nonphysiological activities of these enzymes. Finally, given the remarkable efficiency of EST to suppress local estrogen activity and the apparent paracrine effect of its catalytic function as demonstrated here in MCF-7 cells, it is tempting to envision EST as a potential candidate gene for developing a gene therapy strategy in the treatment of hormone-dependent breast carcinomas.


