Estrogen sulfotransferase and sulfatase: Roles in the regulation of estrogen activity in human uterine endometrial carcinomas

Kyoko Tanaka,[1] Kaneyuki Kubushiro,[1] Yuriko Iwamori,[2] Yuzuru Okairi,[2] Kazushige Kiguchi,[3] Isamu Ishiwata,[4] Katsumi Tsukazaki,[1] Shiro Nozawa[1] and Masao Iwamori[2, 5]

[1]Department of Obstetrics and Gynecology, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582; [2]Department of Biochemistry, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502; [3]Department of Obstetrics and Gynecology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511; and [4]Ishiwata Gynecological Hospital, 1-4-21 Kamimito, Mito, Ibaraki 310-0041

(Received June 30, 2003/Revised August 8, 2003/Accepted August 11, 2003)

The regulation of estrogen activity through the formation and cleavage of sulfoconjugates of estrogens is known to be related to the progression and metastasis of estrogen-dependent breast carcinomas, but the involvement of sulfoconjugates in the steroid stimulation of endometrial functions and the progression of endometrial adenocarcinomas is not clearly understood yet. Estrogen sulfotransferase (EST) in the uterine endometria during the follicular phase was more active than during the luteal phase, but estrogen sulfate (ES) sulfatase exhibited lower activity during the follicular phase than during the luteal phase. However, ES sulfatase activities in cancerous tissues were lower than those in normal endometria and endometrial adenocarcinoma-derived cells, among which the activity was exceedingly high in Ishikawa cells, suggesting that ES sulfatase in Ishikawa cells contributes to the estrogen-dependent growth of these cells. EST activities higher than that in Ishikawa cells were found in only 3 of 24 cancerous tissues. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the *EST* and *ES sulfatase* genes in carcinoma-derived cells demonstrated the extensive expression of both genes in Ishikawa cells. The isolated *EST* gene was transfected into Ishikawa cells with a mammalian expression vector to establish cell clones with enhanced EST activity, and the estrogen-dependent cell growth of the resultant cell clones was found to be abolished, due to the enhanced sulfoconjugation of estrogen. Since ES sulfatase activity in cancerous tissues was significantly lower than that in Ishikawa cells, it might be not involved in the enhancement of estrogen activity associated with the pathogenesis of endometrial adenocarcinoma tissues. (Cancer Sci 2003; 94: 871–876)

Cellular proliferation and differentiation in the human uterine endometrium are mainly regulated by the periodic stimulation with steroid hormones during the menstrual cycle.[1)] Steroid receptors in the endometrial epithelium are known to be involved in the expression of several genes in response to steroid stimulation of endometrial functions related to the implantation of fertilized eggs, and a failure of the steroid-mediated signal transduction pathway is implicated in the progression of endometrial adenocarcinomas,[2–4)] as well as of breast carcinomas.[5–7)] Estrogen is a steroid relevant to the progression of transformed cells, but its active form, 17β-estradiol (E2), is locally generated by several enzymes, such as estrogen sulfotransferase (EST), estrogen sulfate (ES) sulfatase, aromatase, 17β-hydroxysteroid dehydrogenase and so on, since the circulating estrogen in human sera comprises inactive estrone (E1) sulfate.[8)] Among these enzymes, EST and ES sulfatase in breast carcinomas have been shown to be involved in the regulation of estrogen activity by forming and cleaving the sulfoconjugate of estrogen to inhibit and promote the binding of estrogen to its receptor, respectively.[9, 10)] In this connection, inhibition of sulfotransferase by several chlorinated phenol derivatives, such as 6,6-dichloro-4-nitrophenol and hydroxylated polychlorinated biphenyl, has been shown to enhance the activity of endogenous estrogen, and to be probably implicated in human reproductive disorders and an increased incidence of breast carcinomas.[11)] On the contrary, environmental xenoestrogens, such as bisphenol, 4-*n*-octylphenol, 4-*n*-nonylphenol, and 17α-ethynylestradiol, which have became ubiquitous in the environment and have been shown to cause a decrease in sperm quality and progression of estrogen-dependent cancers by interacting with the estrogen receptor, are converted to their sulfoconjugates by cytosolic sulfotransferase, resulting in detoxification of the chemicals through abolition of their binding ability to the receptor and in facilitation of their removal from the body through an increase in water solubility.[12, 13)] Thus, the sulfoconjugation of estrogens and related compounds occurring inside cells plays essential roles in the regulation of estrogen receptor-mediated phenomena. Accordingly, one of the therapeutic strategies for estrogen-dependent breast carcinomas has been the development of an inhibitor of ES sulfatase, whose expression is closely related to the progression and metastasis of breast carcinomas,[14)] and several successful approaches for suppressing the growth of cancer cells with a sulfatase inhibitor *in vitro* and *in vivo* have been reported.[15, 16)] In the case of uterine endometria, ES sulfatase is distributed in the basilar layer of the normal endometrial epithelium, and has been implicated in the estrogenic activity through cleavage of the sulfoconjugate, but its involvement in the cellular events in the endometria, including in adenocarcinomas, is not clearly understood yet.[17)] To clarify the significance of sulfoconjugation in endometrial adenocarcinomas, we examined the activities in cultured cells and tissues of endometrial adenocarcinomas, and the effect of transfection with the *EST* gene on the estrogen-dependent cell growth of endometrial carcinoma-derived cells.

Materials and Methods

Materials. The following reagents were purchased from commercial sources: estrone (1,3,5-estratrien-3-ol-17-one, E1), estradiol (1,3,5-estratriene-3,17β-diol, E2), estriol (1,3,5-estratriene-3,16α,17β-triol), ethynylestradiol (17α-ethynylestra-1,3,5(-10)-triene-3,17β-diol), adenosine 3′-phosphate 5′-phosphosulfate (PAPS), deoxycholic acid, *p*-nitrophenol, 4-nitrocatechol, 4-methylumbelliferone, *p*-hydroxybenzoic acid methyl ester, charcoal and plastic-coated TLC plates from Sigma (St. Louis, MO), E1S from Wako (Tokyo), cDNA syn-

[5]To whom correspondence should be addressed.
E-mail: iwamori@chem.kindai.ac.jp

thesis, micro plasmid prep and Cellphect transfection kits from Pharmacia (Piscataway, NJ), plasmids pcDNA3.1 and pCR2.1-TOPO from Invitrogen (San Diego, CA), Ampli*Taq*-DNA polymerase, a Bigdye Terminator Cycle Sequencing ready reaction kit, and an ABI Genetic Analyzer from Perkin-Elmer/Applied Biosystems (Foster City, CA), and ^{35}S- PAPS (74.0 GBq/mmol) and ^{3}H-E1S (1594.7 GBq/mmol) from New England Nuclear (Boston, MA).

Preparation of the cytosol and microsomal fractions of tissues and cells. Cancer tissues from patients suffering from uterine endometrial adenocarcinomas and normal endometria were obtained from the Department of Pathology, Keio University Hospital, and the National Saitama Hospital after pathological examination, and were used according to the guidelines of the Committee for Informed Consent. The tissues were homogenized with a homogenizer (Polytron; Kinematica, Luzern, Switzerland) in four volumes of 0.25 *M* sucrose, and then centrifuged at 1000*g* for 10 min at 4°C to remove cell debris. The supernatant was centrifuged at 100,000*g* for 60 min to obtain the cytosol and microsomal fractions, and the microsomal fraction was suspended in 0.25 *M* sucrose with the aid of sonication. In a similar way, cytosol and microsomal fractions were prepared from the following cultured cell lines: uterine endometrial adenocarcinoma-derived cells, HEC-108 (poorly differentiated type), SNG-M (moderately differentiated type), SNG-II, HHUA and Ishikawa (well-differentiated type); uterine cervical carcinoma-derived cells, TCS, SKG-II and SKG-IIIa (large cell, non-keratinizing type); and ovarian carcinoma-derived cells, RMG-1 and HAC-2 (clear cell type), HTBOA (undifferentiated type), and HMKOA and MCAS (mucinous cystadenocarcinoma), all of which were cultured on plastic dishes in either Ham's F12 or Dulbecco's modified Eagle's essential medium supplemented with 10% FCS. The protein concentrations were measured by the protein dye binding method with bovine serum albumin as the standard.[18]

Assay of EST activity. E1 (40 nmol), in chloroform/methanol (2/1, v/v), was put in the reaction tube, and then the solvent was removed with a centrifugal evaporator. The assay mixture comprised 10–40 μg of cytosol protein in 100 m*M* tris-HCl buffer (pH 8.5), 10 μg of cyclodextrin, 10 m*M* dithiothreitol, and 7.38 kBq of [^{35}S]PAPS, in a total volume of 30 μl. After incubation of the mixtures at 37°C for 2 h, the reaction was terminated by the addition of 15 μl of ethanol, and then an aliquot (20 μl) of each solution was applied on a plastic-coated TLC plate, which was developed with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, by vol.). To identify the radioactive products, standard ES was chromatographed on the same plate and visualized with cupric acetate-phosphoric acid reagent. After exposure of the plate to an X-ray film (RX-U; Fuji, Tokyo), the area corresponding to E1S was cut out and placed in a scintillation cocktail (ACS-II; Amersham, Piscataway, NJ), and the radioactivity incorporated into E1S was determined with a liquid scintillation counter (Tri-Carb1500; Packard/Perkin-Elmer, Foster City, CA).

Assay of ES sulfatase. The microsomal ES sulfatase activity was determined using [6,7-^{3}H]E1S as the substrate. The standard assay mixture comprised 100 m*M* tris-HCl buffer, pH 7.5, 0.1% Triton X-100, 80.6 μ*M* [6,7-^{3}H]E1S (10.7 nCi/nmol), and 50–100 μg of enzyme protein, in a total volume of 100 μl. After incubation at 37°C for 1 h, the reaction was terminated by the addition of 100 μl of chloroform/methanol (2:1, by vol.), and then the lower organic phase was spotted onto a plastic-coated plate, which was developed with benzene/ethyl acetate (2:1, by vol.), with E1 as an authentic standard. The radioactivity in the area corresponding to that of E1 was determined with a liquid scintillation counter as described above.

Reverse transcriptase-polymerase chain reaction (RT-PCR) cloning of EST. The cDNA of EST was cloned from endometrial adenocarcinoma-derived cell line Ishikawa by means of RT-PCR. Total RNA extracted from Ishikawa cells by the acid guanidine thiocyanate-phenol-chloroform (AGPC) method[19] was reverse-transcribed to cDNA with reverse transcriptase (M-MuLV; Fermentas, Hanover, MD) and an oligo-dT primer, and the PCR reactions were performed with primer pairs based on reported sequences (GenBank Accession Number Y11195) corresponding to the 5′- and 3′-coding regions of the open reading frame of EST.[20] The PCR amplification conditions were 35 cycles of 94°C for 1 min, 46°C for 1 min, and 72°C for 1.5 min, with 0.5 units of *Taq* polymerase (Ampli*Taq* Gold; Perkin-Elmer/Applied Biosystems) and 10 ng of cDNA as the template. The resulting PCR products were applied to a 1.0% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. The gel band containing the PCR product was cut out and ligated into the pcDNA3.1-TOPO vector (TA cloning kit, Invitrogen) (pcDNA3.1-hEST (human EST from Ishikawa cells)). On the other hand, the cDNA of murine EST was cloned from the placenta of mice at 18 days of gestation, and then ligated into the *Bam*HI and *Xba*I sites of pcDNA3.1 (pcDNA3.1-mpEST (murine placental EST)).[21] The DNA sequences were determined by means of the dideoxynucleotide chain-termination method with a BigDye Terminator cycle sequencing ready reaction kit and a DNA sequencer (ABI Genetic Analyzer; Perkin-Elmer/Applied Biosystems).

Transfection of the *EST* gene. The pcDNA3.1-hEST and pcDNA3.1-mpEST constructs prepared as above and the vector alone were transfected into COS-7, Ishikawa, SNG-II and RMG-1 cells using a Cellphect transfection kit, the transfectants being selected based on their resistance to 600 μg/ml G418 (Gibco/Invitrogen, Carlsbad, CA), a neomycin derivative, and the resultant cell colonies were isolated using a cloning chamber (Iwaki, Tokyo). Then, the expression of His-Tag in the individual colonies was determined by SDS-PAGE and western blotting with anti-His-Tag antibodies in order to determine in which colonies the inserted plasmid was activated, and the activity of EST in each of the His-Tag-positive cell lines was examined by using the method described above. Among the transfected cell lines of endometrial adenocarcinoma-derived cells, ones exhibiting relatively higher specific activity of EST were used for the experiment on estrogen-responsive cell growth, which was examined with 1% charcoal-treated fetal calf serum (FCS) in Ham's F12 or Dulbecco's modified Eagle's medium in the presence or absence of E2 (10^{-8} *M*).[22] Cells transfected with the vector alone were used as a control.

RT-PCR analysis. Total RNAs extracted from the cell lines by the AGPC method were reverse-transcribed to cDNA as described above, and then subjected to PCR under the following conditions: EST (GenBank Accession Number Y11195), sense primer, 5′-TTGTCATTGCCACCTACC-3′, antisense primer, 5′-CACAAGCTCCTCTGATGGC-3′, 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. ES sulfatase (GenBank Accession Number J04964), sense primer, 5′-ACTGCAACGCCTACTTAAATG-3′, antisense primer, 5′-AGGGTCTGGGTGTGTCTGTC-3′, 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a control. The resulting PCR products were electrophoresed on a 1.2% agarose gel, stained with ethidium bromide, and then examined under a UV transilluminator. The densities of the bands were measured with a Photoprint system (IP-008-8D; Cosmobio, Tokyo).

Results

ES sulfatase and EST activities in human gynecological carcinoma-derived cells. The properties of EST in cytosol fractions prepared from the HEC-108 and Ishikawa cell lines were found to

Table 1. Activities of EST and ES sulfatase in human gynecological carcinoma-derived cell lines

Cell line		EST (pmol/mg protein/h)	ES sulfatase (nmol/mg protein/h)
Uterine endometrial adenocarcinoma-derived cells	SNG-II	66.9	2.2±0.3
	HHUA	18.0	4.2±0.3
	Ishikawa	62.0	14.7±1.8
	SNG-M	29.1	2.9±0.1
	HEC-108	70.2	3.5±0.2
Uterine cervical carcinoma-derived cells	TCS	33.4	0.7±0.1
	SKG-IIIa	2.9	5.8±1.5
	SKG-II	14.9	0.4±0.1
Ovarian carcinoma-derived cells	RMG-1	10.7	1.6±0.1
	HMKOA	17.2	1.3±0.1
	MCAS	4.8	1.5±0.1
	HTBOA	9.5	2.6±0.1
	HAC-2	6.1	4.9±0.1

be identical with those of the murine placental enzyme as to the optimum pH, K_m, and requirements of cyclodextrin and a reducing agent, and consequently the activities of EST in gynecological carcinoma-derived cells and tissues were determined using the cytosol fractions according to the procedure described previously.[21] All cells exhibited EST activity in the range of 2.9 to 70.2 pmol/mg protein/h, and the activity in the endometrial carcinoma-derived cell lines was relatively higher than that in the cervical and ovarian carcinoma-derived cell lines (Table 1). Meanwhile, the optimum conditions for ES sulfatase were determined with the microsomal fraction of the HEC-108 cell line. The addition of Triton X-100 at 1 mg/ml stimulated the activity three-fold, and the other detergents, namely Nonidet P-40, Tween 20, Tween 80, sodium cholate, sodium deoxycholate and sodium taurocholate, at the same concentration enhanced the activity to a lesser extent. When the activity was determined in the presence of Triton X-100 (1 mg/ml), tris-HCl buffer gave the highest activity among the buffers examined, that is, triethanolamine-NaOH, imidazole-HCl and phosphate buffers, and the optimum pH was 7.5. The activity was linear up to 80 μg of microsomal protein and up to 30 μ*M* E1S, and the enzyme exhibited an apparent K_m of 41.7 μ*M* for E1S. Under the conditions used, the specific activity of ES sulfatase was examined using the microsomes of various cell lines. As shown in Table 1, significantly high specific activity was detected in the Ishikawa cell line (14.7 nmol/mg protein/h), and the other cell lines, established from uterine endometrial, uterine cervical, and ovarian carcinomas, exhibited activities of 0.4 to 5.8 nmol/mg protein/h, which were not correlated with the type of gynecological carcinoma.

***EST* and *ES sulfatase* genes in human gynecological carcinoma-derived cell lines.** In accordance with the distribution of EST activity in human gynecological carcinoma-derived cell lines, *EST* genes in the endometrial adenocarcinoma-derived cell lines tended to be expressed at higher levels than those in the cervical and ovarian carcinomas (Fig. 1), while the *ES sulfatase* gene was demonstrated to be expressed at relatively high levels in the Ishikawa, SNG-II, SKG-IIIa and RMG-1 cell lines. Nevertheless, the relative activities of EST and ES sulfatase in the cell lines were not directly consistent with the levels of their genes (Fig. 1 and Table 1). To compare the sequence of EST in the cell lines with those reported in the literature, its entire coding region was isolated from the cDNA of Ishikawa cells by RT-PCR and then ligated into the pCR2.1-TOPO vector. Three differences in the nucleotide sequence, in comparison to that of GenBank Accession Number Y11195,[20] that is, adenine instead of guanine at position 245, guanine instead of adenine at 403, and cytosine instead of guanine at 840, were observed in the *hEST* gene from Ishikawa, carrying missense mutations G82E, T135A and D280E in the *hEST* gene-encoded enzyme.

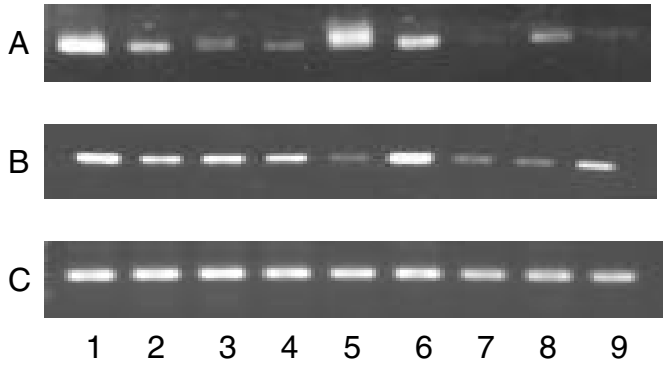

Fig. 1. Expression of the *ES sulfatase* (A), *EST* (B) and *GAPDH* (C) genes in human gynecological carcinoma-derived cells, as judged on RT-PCR. The PCR products obtained under the conditions given in the text were electrophoresed on a 1.2% agarose gel, stained with ethidium bromide, and then examined under a UV transilluminator. 1, Ishikawa; 2, SNG-II; 3, HEC-108; 4, SNG-M; 5, SKG-IIIa; 6, RMG-1; 7, HMKOA; 8, HAC-2; 9, MCAS.

Transfection of *EST* genes into cells. The *human EST* (*hEST*) gene from Ishikawa cells in the pCR2.1-TOPO vector was subcloned into the *Bam*HI and *Xba*I sites of mammalian expression vector pcDNA3.1 (pcDNA3.1-hEST), which was then transfected into COS-7 cells, and the lysates of the resulting transfectants were used to characterize the enzymatic properties. The optimum pH with E1 as the substrate was 8.5, and the phenolic hydroxyl groups in steroids and artificial substrates were found to be sulfonated as acceptors. Among the substrates, E1 and E2 exhibited higher specific activities than those for estriol, *p*-nitrophenol, 4-methylumbelliferone, 4-nitrocatechol and *p*-hydroxybenzoic acid methyl ester, indicating that estrogens are superior to artificial substrates for hEST.

Thus, the enzymatic properties, including the substrate specificity of hEST, were the same as those of mpEST, whose properties were determined using homogenates of COS-7 cells transfected with pcDNA3.1-mpEST,[21] as well as those of cytosolic ESTs prepared from human normal endometria and endometrial adenocarcinoma tissues, and Ishikawa and HEC-108 cells, indicating that the differences in the nucleotide sequence described above do not cause any significant alteration in the essential enzymatic properties. Then, both pcDNA3.1-hEST and pcDNA3.1-mpEST were transfected into SNG-II and Ishikawa cells to establish cell lines with enhanced EST activity.

Table 2. EST activities in Ishikawa and SNG-II cells transfected with pcDNA3.1-hEST and pcDNA3.1-mpEST

			EST (nmol/mg protein/h)	ES sulfatase (nmol/mg protein/h)
Ishikawa cells	pcDNA3.1	clone 1	0.06±0.01	10.6±0.8
		clone 2	0.05±0.01	11.8±0.5
		clone 3	0.05±0.01	—
	pcDNA3.1-hEST	clone 1	2.5±0.3	10.3±0.4
		clone 2	1.9±0.1	13.2±0.3
		clone 3	0.8±0.1	—
	pcDNA3.1-mpEST	clone 1	1.8±0.2	12.7±0.2
		clone 2	0.9±0.1	12.4±0.2
		clone 3	0.8±0.1	—
SNG-II cells	pcDNA3.1	clone 1	0.06±0.01	2.5±0.1
		clone 2	0.06±0.01	2.0±0.1
		clone 3	0.07±0.01	—
	pcDNA3.1-hEST	clone 1	0.9±0.1	2.5±0.2
		clone 2	0.7±0.1	2.5±0.1
		clone 3	0.7±0.1	—
	pcDNA3.1-mpEST	clone 1	0.8±0.1	2.3±0.1
		clone 2	0.7±0.1	2.0±0.2
		clone 3	0.7±0.1	—

—, not determined.

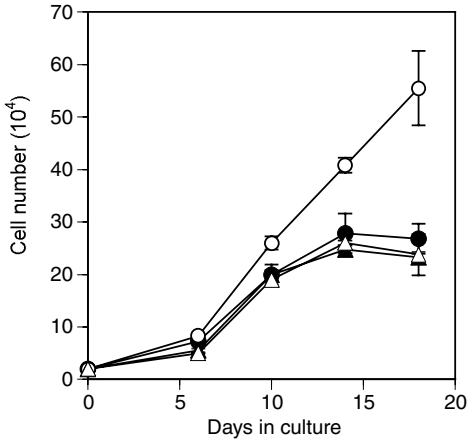

Fig. 2. Change in cell number of Ishikawa cells and their transfectants with pcDNA3.1-hEST in medium with and without estradiol. Ishikawa cells cultured in the presence (○) and absence (●) of estradiol, and Ishikawa cells with pcDNA3.1-hEST cultured in the presence (△) and absence (▲) of estradiol (10^{-8} *M*).

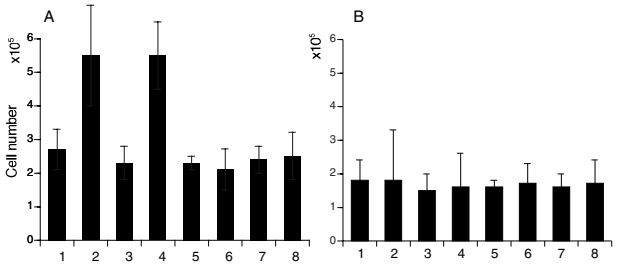

Fig. 3. Cell numbers of Ishikawa and SNG-II cells, and their transfectants with pcDNA3.1-hEST and pcDNA3.1-mpEST cultured in the presence (E+) and absence (E−) of estradiol (10^{-8} *M*) for 18 days. A: 1, Ishikawa (E−); 2, Ishikawa (E+); 3, Ishikawa-pcDNA3.1 (E−); 4, Ishikawa-pcDNA3.1 (E+); 5, Ishikawa-pcDNA3.1-hEST (E−); 6, Ishikawa-pcDNA3.1-hEST (E+); 7, Ishikawa-pcDNA3.1-mpEST (E−); 8, Ishikawa-pcDNA3.1-mpEST (E+). B: 1, SNG-II (E−); 2, SNG-II (E+); 3, SNG-II-pcDNA3.1 (E−); 4, SNG-II-pcDNA3.1 (E+); 5, SNG-II-pcDNA3.1-hEST (E−); 6, SNG-II-pcDNA3.1-hEST (E+); 7, SNG-II-pcDNA3.1-mpEST (E−); 8, SNG-II-pcDNA3.1-mpEST (E+).

After selection with G418, individual colonies isolated in a cloning chamber were monitored as to their reactivity with anti-His Tag antibodies, and their EST activities in cell homogenates and cytosol fractions, respectively. Among the cell clones, ones exhibiting higher EST activities, together with expression of the His Tag, were selected, as shown in Table 2. The resultant cell clones of Ishikawa and SNG-II cells transfected with either pcDNA3.1-hEST or pcDNA3.1-mpEST exhibited significantly higher EST activities than those of cells transfected with the vector alone and the original cells, though their ES sulfatase activities were not affected by the transfection of EST (Table 2).

Effect of transfection of the *EST* gene on estrogen-dependent cell growth. Cell clone 1 of each transfectant was cultured in the presence and absence of 10 n*M* E2, with exchange of the medium every 4 days, and the number of cells was determined after treatment with trypsin. As shown in Fig. 2, the number of Ishikawa cells in the medium with E2 continuously increased till 18 days, whereas that without E2 reached saturation at 14 days after cultivation, indicating that Ishikawa cells have the potential of estrogen-dependent cell growth. However, under the same conditions, no stimulation of cell growth with E2 was observed in Ishikawa cell clone 1 with enhanced EST activity on transfection of pcDNA3.1-hEST, indicating that sulfoconjugation, when it overwhelms desulfation through transfection of the *EST* gene, abolishes the estrogen activity for stimulation of cell growth. Moreover, as shown in Fig. 3, the enhanced activity of EST on transfection of either pcDNA3.1-hEST or pcDNA3.1-mpEST into Ishikawa cells was found to cause a loss of the estrogen-dependency after cultivation in the medium with and without E2 for 18 days. On the other hand, the cell growth of the original SNG-II cells was not stimulated by E2, regardless of estrogen stimulation, probably due to the low activity of ES sulfatase.

EST and ES sulfatase in uterine endometria and endometrial adenocarcinomas. We then examined the activities of EST and ES sulfatase in human uterine endometria and endometrial adenocarcinoma tissues to evaluate the possible involvement of sulfoconjugation in the regulation of the estrogen activity in these tissues. As shown in Table 3, EST activity in the uterine en-

Table 3. Activities of EST and ES sulfatase in the luteal and follicular phases of human uterine endometria

		EST (pmol/mg protein/h)	ES sulfatase (nmol/mg protein/h)
Luteal phase	1	55.5±1.8	8.0±0.5
	2	40.0±4.5	4.6±0.3
	3	9.6±0.7	6.1±0.4
	4	34.8±5.6	2.2±0.3
	5	18.0±3.2	2.9±0.1
Follicular phase	1	108.0±5.8	2.9±0.3
	2	143.0±4.6	1.5±0.4
	3	98.7±3.0	2.1±0.1
	4	144.0±2.1	1.6±0.2
	5	64.3±1.9	1.5±0.1

Table 4. Activities of EST and ES sulfatase in human uterine endometrial adenocarcinomas with different grades of malignancy

Endometrial adenocarcinoma Grade of malignancy		EST (pmol/mg protein/h)	ES sulfatase (nmol/mg protein/h)
Grade 1	1	0.0	0.27±0.02
	2	32.5±2.2	0.13±0.01
	3	12.5±1.5	0.12±0.01
	4	66.5±4.2	0.28±0.01
Grade 2	1	255.0±12.1	0.59±0.03
	2	15.0±2.0	0.42±0.03
Grade 3	1	18.5±1.9	0.30±0.03
	2	20.0±3.0	0.21±0.01
	3	0.0	0.30±0.01
	5	5.0±0.6	0.08±0.01
Undefined	1	25.5±2.2	0.22±0.02
	2	20.0±3.2	0.26±0.02
	3	13.5±1.7	0.19±0.02
	4	31.5±2.8	0.28±0.02
	5	15.0±0.9	0.27±0.01
	6	5.5±2.7	0.35±0.01
	7	37.0±4.5	0.34±0.01
	8	12.5±1.1	0.15±0.01
	9	18.0±1.5	0.22±0.02
	10	27.0±1.5	0.35±0.02
	11	41.5±5.6	0.43±0.03
	12	25.5±3.1	0.40±0.03
	13	40.5±6.1	0.16±0.01
	14	116.5±14.5	0.39±0.02

dometria during the follicular phase was higher than that during the luteal phase, but, in contrast, ES sulfatase in the luteal phase was more active than that in the follicular phase. In the tissues of patients suffering from endometrial adenocarcinomas, the activities of ES sulfatase were lower than those in the normal endometria during both phases, and were not correlated with the grade of malignancy (Table 4). Also, all tissues examined exhibited lower ES sulfatase activities than those in normal endometria and endometrial adenocarcinoma-derived cells. On the other hand, EST in the cancerous tissues from 22 of the 24 patients exhibited similar or lower activities than those in normal endometria during the luteal phase, and no correlation between the specific activities and the grade of malignancy was observed. In addition, the tissues in which EST activities were higher than in Ishikawa cells numbered 3 out of 24, but the activities were significantly lower than those in the transfectants with pcDNA3.1-hEST and pcDNA3.1-mpEST. Thus, sulfoconjugation might be not involved in the regulation of estrogen activity in uterine endometrial adenocarcinoma tissues.

Discussion

Steroids are known to be converted to sulfoconjugates by cytosolic sulfotransferase, and the resultant steroid sulfates exhibit higher solubility in aqueous media than the original steroids, acting as metabolic intermediates in steroidogenesis, and facilitating excretion from the body.[23–25] In addition, endocrine tissues with steroid receptors utilize the sulfation reaction to inhibit the binding of ligands with the respective receptors, though the nature of the involvement of sulfotransferase and sulfatase in the control of hormonal signals is not clearly understood yet. In particular, although the endocrine-disrupting nature of EST has been demonstrated by transfection of EST into endometrial adenocarcinoma-derived Ishikawa cells[22] and breast carcinoma-derived cells,[26, 27] the actual relationship between the activities of the two enzymes and the response to hormonal stimuli is obscure at present. Consequently, the present work was undertaken to explore the contribution of anabolic and catabolic enzymes of ES to the regulation of estrogen activity after determination of the optimum conditions for EST and ES sulfatase in the cytosol and microsomal fractions of uterine endometrial adenocarcinoma-derived cells, respectively. Among 13 gynecological carcinoma-derived cells, Ishikawa cells exhibited the highest ES sulfatase activity, which seemed to contribute to their estrogen-dependent cell growth. One of the reasons why SNG-II cells did not exhibit estrogen-dependent cell growth was thought to be their lower ES sulfatase activity than that in Ishikawa cells. To clarify the involvement of ES sulfatase in the estrogen-dependent cell growth of endometrial carcinoma-derived Ishikawa cells, the *EST* gene was isolated from the cDNA of Ishikawa cells, and its nucleotide sequence was compared with that of human endometrial EST. As described in this paper, among the 885 nucleotides of normal endometrial EST in GenBank Accession Number Y11195,[20] three nucleotides at positions 245, 403 and 840 in the *hEST* gene from Ishikawa cells were found to be different from those of endometrial EST, giving an enzyme protein differing in three amino acids from the 284 amino acids of endometrial EST. However, as determined with enzymes expressed after transfection of pcDNA3.1-hEST and pcDNA3.1-mpEST into COS-7 cells, the substrate specificities of hEST in Ishikawa cells were essentially identical to those of mpEST, showing higher affinity for estrogen than for phenol derivatives, even though the amino acid homology between hEST and mpEST was 78%. Also, the specific activity of hEST was identical with that of mpEST. Consequently, ESTs from different animal species appear to possess essentially the same activities even if only the arrangement of the amino acids in the active site of the enzyme protein is well conserved. Therefore, the mutation of hEST in Ishikawa cells was concluded to be unrelated to the active site, and to have no effect on the substrate specificity and activity of EST. In fact, on transfection of the *hEST* gene, the activity of EST in the cytosol fraction increased dramatically, resulting in inability of exogenous estrogen to inhibit the binding with the estrogen receptor owing to sulfoconjugation. The specific activity of EST in a clone of Ishikawa cells not showing estrogen-dependent cell growth was 30 times that in the original cells, but was still less than one-fourth of the ES sulfatase activity, which was not altered even after the transfection. In the case of Ishikawa cells, ES sulfatase with a specific activity of around 10 nmol/mg protein/h was concluded to be insufficient to eliminate the potential sulfoconjugation by EST with a specific activity of about 1–2 nmol/mg protein/h, but was able to maintain the ability to respond to steroid stimuli in the presence of EST with a specific activity of 0.05 nmol/mg protein/h, suggesting the importance of the EST and ES sulfatase activity ratio. Probably the reason why the exogenous estrogen was inactivated by EST with a lower specific activity

than that of ES sulfatase is the different intracellular distributions of EST and ES sulfatase; they are cytosolic soluble and membranous insoluble enzymes, respectively. Compared with insoluble enzymes, which are reactive on the membrane surface, the soluble EST might readily express its activity in the cytosol, making effective conversion to sulfoconjugates possible in the transfection experiment. Since all clones of Ishikawa cells transfected with either hEST or mpEST exhibited a specific activity of EST of more than 0.7 nmol/mg protein/h and lost the ability of estrogen-dependent cell growth, the role of the marginal activity of EST in maintaining the estrogen-dependency of cell growth could not be clarified in the transfection experiment. Among normal and carcinoma tissues, the uterine endometria during the luteal phase only exhibited relatively high ES sulfatase specific activity. However, as long as the EST activity was at a low level, a sufficiently high level of ES sulfatase seemed not to be necessary for direct regulation of the estrogen binding to the receptor. A comparison of the activities of the two enzymes in the endometria between the luteal and follicular phases indicated that the cleavage and synthetic potentials of ES tended to be predominant, operating in the directions of enhancement and suppression of estrogen activity, in the luteal and follicular phases, respectively. However, both the EST and ES sulfatase activities in the majority of carcinoma tissues were lower than those in normal tissues, irrespective of the grade of malignancy, indicating that neither enzyme is directly involved in the regulation of estrogen activity in endometrial adenocarcinomas.

This work was supported by a grant from the Fujisawa Foundation.

1. Brenner RM, West NB. Hormonal regulation of the reproductive tract in female mammals. *Annu Rev Physiol* 1975; **37**: 273–302.
2. Smith DC, Prenticem R, Thompson DJ, Herrmann WL. Association of exogenous estrogen and endometrial carcinoma. *N Engl J Med* 1975; **293**: 1164–7.
3. Key TJ, Pike MC. The dose-effect relationship between 'unopposed' oestrogens and endometrial mitotic rate: its central role in explaining and predicting endometrial cancer risk. *Br J Cancer* 1988; **7**: 205–12.
4. Parazzini F, La Vecchia C, Negri E, Fedele L, Balotta F. Reproductive factors and risk of endometrial cancer. *Am J Obstet Gynecol* 1991; **164**: 522–7.
5. Lippman ME, Dickson RB. Mechanisms of growth control in normal and malignant breast epithelium. *Recent Prog Horm Res* 1989; **45**: 383–440.
6. van Leeuwen FE, Benraadt J, Coebergh JW, Kiemeney LA, Gimbrere CH, Otter R, Schouten LJ, Damhuis RA, Bontenbal M, Diepenhorst FW, van den Belt-Dusebout AW, van Tinteren H. Risk of endometrial cancer after tamoxifen treatment of breast cancer. *Lancet* 1994; **343**: 448–52.
7. Cook LS, Weiss NS, Schwartz SM, White E, McKnight B, Moore DE, Daling JR. Population-based study of tamoxifen therapy and subsequent ovarian, endometrial, and breast cancers. *J Natl Cancer Inst* 1995; **87**: 1359–64.
8. Bernier F, Leblanc G, Labrie F, Luu-The V. Structure of human estrogen and aryl sulfotransferase gene. Two mRNA species issued from a single gene. *J Biol Chem* 1994; **269**: 28200–5.
9. Seth P, Lunetta KL, Bell DW, Gray H, Nasser SM, Rhei E, Kaelin CM, Iglehart DJ, Marks JR, Garber JE, Haber DA, Polyak K. Phenol sulfotransferases: hormonal regulation, polymorphism, and age of onset of breast cancer. *Cancer Res* 2000; **60**: 6859–63.
10. Santner SJ, Feil PD, Santen RJ. *In situ* estrogen production via the estrone sulfatase pathway in breast tumors: relative importance versus the aromatase pathway. *J Clin Endocrinol Metab* 1984; **59**: 29–33.
11. Song WC. Biochemistry and reproductive endocrinology of estrogen sulfotransferase. *Ann NY Acad Sci* 2001; **948**: 43–50.
12. Wolff MS, Toniolo PG, Lee EW, Rivera M, Dubin N. Blood levels of organochlorine residues and risk of breast cancer. *J Natl Cancer Inst* 1993; **85**: 648–52.
13. Suiko M, Sakakibara Y, Liu MC. Sulfation of environmental estrogen-like chemicals by human cytosolic sulfotransferases. *Biochem Biophys Res Commun* 2000; **267**: 80–4.
14. Utsumi T, Yoshimura N, Takeuchi S, Ando J, Maruta M, Maeda K, Harada N. Steroid sulfatase expression is an independent predictor of recurrence in human breast cancer. *Cancer Res* 1999; **59**: 377–81.
15. de Gooyer ME, Kleynm GT, Smits KC, Ederveen AG, Verheul HA, Kloosterboer HJ. Tibolone: a compound with tissue specific inhibitory effects on sulfatase. *Mol Cell Endocrinol* 2001; **183**: 55–62.
16. Elsadig RE, Reimann K, Yip CH, Lai LC. Inhibition of oestrone sulphatase activity in the MDA-MB-231 breast cancer cell line by breast cyst fluid from Malaysian women. *Anticancer Res* 2001; **21**: 2693–6.
17. Ezaki K, Motoyama H, Sasaki H. Immunohistologic localization of estrone sulfatase in uterine endometrium and adenomyosis. *Obstet Gynecol* 2001; **98**: 815–9.
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–54.
19. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–9.
20. Rubin GL, Harrold AJ, Mills JA, Falany CN, Coughtrie MW. Regulation of sulphotransferase expression in the endometrium during the menstrual cycle, by oral contraceptives and during early pregnancy. *Mol Hum Reprod* 1999; **5**: 995–1002.
21. Takehara K, Kubushiro K, Iwamori Y, Tsukazaki K, Nozawa S, Iwamori M. Expression of an isoform of the testis-specific estrogen sulfotransferase in the murine placenta during the late gestational period. *Arch Biochem Biophys* 2001; **394**: 201–8.
22. Holinka CF, Hata H, Gravanis A, Kuramoto H, Gurpide E. Effects of estradiol on proliferation of endometrial adenocarcinoma cells (Ishikawa line). *J Steroid Biochem* 1986; **25**: 781–6.
23. Hobkirk R. Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles. *Can J Biochem Cell Biol* 1985; **63**: 1127–44.
24. Stein C, Hille A, Seidel J, Rijnbout S, Waheed A, Schmidt B, Geuze H, von Figura K. Cloning and expression of human steroid-sulfatase. Membrane topology, glycosylation, and subcellular distribution in BHK-21 cells. *J Biol Chem* 1989; **264**: 13865–72.
25. Kotov A, Falany JL, Wang J, Falany CN. Regulation of estrogen activity by sulfation in human Ishikawa endometrial adenocarcinoma cells. *J Steroid Biochem Mol Biol* 1999; **68**: 137–44.
26. Falany JL, Macrina N, Falany CN. Regulation of MCF-7 breast cancer cell growth by beta-estradiol sulfation. *Breast Cancer Res Treat* 2002; **74**: 167–76.
27. Shimizu M, Ohta K, Matsumoto Y, Fukuoka M, Ohno Y, Ozawa S. Sulfation of bisphenol A abolished its estrogenicity based on proliferation and gene expression in human breast cancer MCF-7 cells. *Toxicol In Vitro* 2002; **16**: 549–56.
28. Faucher F, Lacoste L, Luu-The V. Human type 1 estrogen sulfotransferase: catecholestrogen metabolism and potential involvement in cancer promotion. *Ann NY Acad Sci* 2002; **963**: 221–8.