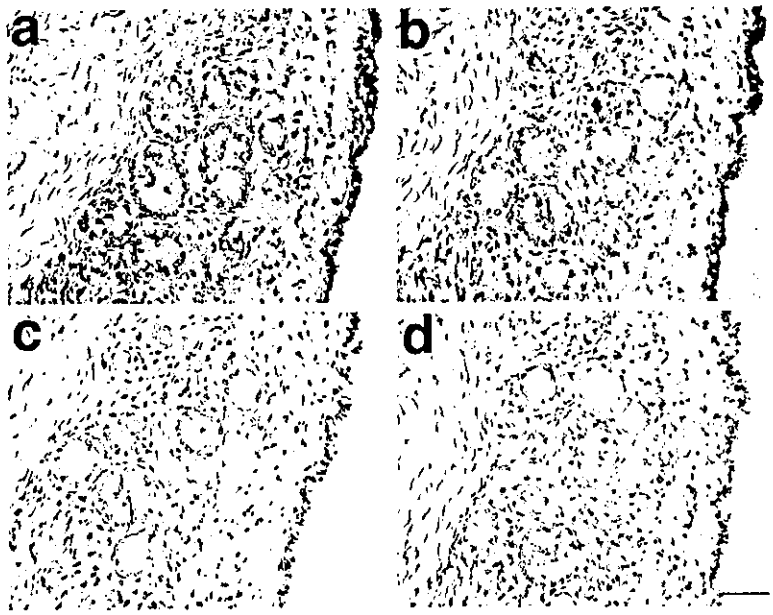
FIGURE 3

In situ localization of ER-α and ER-β mRNA in an ovarian endometriotic cyst obtained from a patient (case no. 6 in Table 3). The ISH was performed on adjacent sections of an ovarian endometriotic cyst with an antisense probe to ER-α (a) and an antisense probe to ER-β (b). Both ER-α and ER-β mRNA hybridization signals, appearing red as a result of fast red, were detected in glandular epithelial and stromal cells. Negative controls with a sense probe to ER-α (c) or a sense probe to ER-β (d) showed no detectable specific mRNA hybridization signals. Bar = 50 μm.



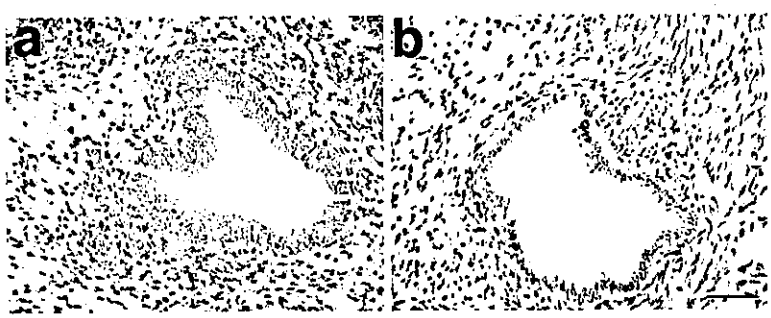
Matsuzaki. ER-α and -β in endometriosis. Fertil Steril 2000.

press both ER-α and ER-β mRNA, regardless of the phase of the menstrual cycle during which the samples were obtained. In ovarian endometriotic cyst tissues, both ER-α and ER-β mRNA hybridization signals were detected in glandular epithelial and stromal cells. In samples that express both ER-α and ER-β mRNA, the localization patterns were similar in glandular epithelial and stromal cells, although ER-α mRNA expression was more prominent than that of ER-β.

Positive signals for the mRNA of ER-α and ER-β were

FIGURE 4

In situ localization of ER-α and ER-β mRNA in an ovarian endometriotic cyst obtained from a patient (case no. 19 in Table 3). The ISH was performed on adjacent sections of an ovarian endometriotic cyst with an antisense probe to ER-α (a) and an antisense probe to ER-β (b). The ER-α hybridization signals, appearing red as a result of fast red, were detected in glandular epithelial and stromal cells. No ER-β hybridization signals were detected. Bar = 50 μm.



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found in 15 of 32 (47%) of the ovarian endometriotic cyst tissue samples of the patients. The remaining 17 samples (53%) displayed positive signals for ER-α mRNA but no signals for ER-β. We did not observe any systematic variation in the expression of mRNA for ER-β during the different phases of the menstrual cycle (7 cases of positive expression and 8 cases of negative expression in follicular-phase samples compared with 8 cases of positive expression and 9 cases of negative expression in the luteal-phase samples).

DISCUSSION

Using the RT-PCR technique, we successfully detected mRNA of ER-α and ER-β in tissue samples obtained from ovarian endometriotic cysts. The localization of each mRNA expression of both receptor types was confirmed and further analyzed with use of the ISH technique. We found that the expression of ER-β mRNA did not parallel that of ER-α mRNA. Messenger RNA for ER-α was detected in all of the samples analyzed, regardless of the phase of the menstrual cycle during which the samples were obtained. In contrast, mRNA for ER-β was apparently more restricted. Using the RT-PCR method, we could only detect ER-β mRNA in 63% of the samples. This figure was reduced to 47% when the ISH technique was used (the difference in the values obtained from these two techniques probably results from the greater sensitivity of the RT-PCR technique, which in turn may arise from the amplification inherent in the PCR method.

We detected ER-α and ER-β mRNA expression in all samples of eutopic endometrium and normal ovaries. In addition, a previous report showed ER-α and ER-β mRNA expression in the eutopic endometrium from patients with endometriosis (20). Therefore, even if we were to suppose that the apparent absence of mRNA from ER-β was due to the mRNA expression levels being below the detection threshold for the RT-PCR, the differences we observed in the mRNA expression may be of pathophysiological significance in ovarian endometriosis.

A recent study using a semiquantitative RT-PCR technique demonstrated that stromal cells derived from ovarian endometriotic cysts predominantly express ER-α mRNA (21). Thus, we speculate that predominant expression of ER-α in glandular epithelial and stromal cells may be essential to the development and growth of ovarian endometriosis. Brandenberger et al. (22) reported the different expression of ER-α and ER-β mRNA in various fetal organs, including the uterus and ovaries, and suggested that the two receptors play different, organ-specific roles. Moreover, studies have demonstrated putative functional differences between ER-α and ER-β in various organs, and the two receptors seem to display cooperative and contradictory effects, depending on the precise conditions in which they are found (23–25). Future studies to clarify functional roles of ER-β in ovarian endometriosis would provide further insight into the pathophysiology of the disease.

We found no changes in the expression of ER-α and ER-β mRNA in ovarian endometriotic cysts in any of the phases of the menstrual cycle. Although further studies using a quantitative evaluation of mRNA expression of the two receptor types are required, this result suggests that hormonal changes do not significantly affect mRNA expression for the two ER types in ovarian endometriotic cysts.

Some suggestions of the pathogenesis of ovarian endometriosis were obtained from the present study. Previous reports suggested that ovarian endometriosis may develop from a single precursor cell (26, 27). The endometrial cell and the celomic cell (including an ovarian surface epithelial cell) have been suggested as candidates for the precursor cell; a precursor cell progresses to form a cyst, becoming transplanted outside the uterine cavity, undergoing metaplasia, or combining these two processes (28).

On the basis of these previous reports and the present result that some endometriotic samples did not have ER-β mRNA expression, we address questions concerning the pathogenesis of ovarian endometriosis. First, some glandular epithelial and stromal cells expressed only ER-α mRNA in eutopic endometrium, whereas control ovarian surface epithelial cells expressed both ER-α and ER-β mRNA. Therefore, we may speculate that an endometrial cell with or without ER-β mRNA expression is a candidate of the precursor cell. Second, expression of ER-β mRNA decreased in breast and ovarian carcinogenesis (15, 29). It is possible, therefore, that a transcriptional alternation may occur on the ER-β gene in the process underlying metaplasia that leads to endometriotic cyst formation.

Considering possible metaplasia, we speculate that an ovarian surface epithelial cell is a candidate of the precursor cell. Furthermore, no response to hormonal changes was observed in the ovarian surface epithelium, but there was a response in the eutopic endometrium. From the viewpoint of hormonal response, endometriotic cysts expressing ER-β mRNA resemble ovarian surface epithelium. Finally, it is also possible that an endometrial cell that originally expressed both ER-α and ER-β mRNA diminished its expression of ER-β mRNA through metaplasia.

In summary, we examined the expression of mRNA for both the α and β forms of the estrogen receptor in tissue samples of ovarian endometriotic cysts. Our results suggest that predominant expression of ER-α in both glandular epithelial and stromal cells may be essential to the development and growth of ovarian endometriosis. However, because of the qualitative nature of the analysis methods used, further studies, preferably using more quantitative methods, are needed.

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Expression of estrogen receptor alpha and beta in peritoneal and ovarian endometriosis

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Objective: To quantify and compare messenger RNA (mRNA) levels of ER-α and ER-β among ovarian endometriotic cysts and red and black peritoneal endometriotic lesions.

Design: Prospective study.

Setting: University hospital.

Patient(s): Patients with or without endometriosis.

Intervention(s): Samples of peritoneal (n = 33) and ovarian endometriotic lesions (n = 37) were obtained during laparoscopic surgery. Normal eutopic endometrial tissues and macroscopically normal peritoneal tissues were obtained as controls during or just after surgery.

Main Outcome Measure(s): Expression of mRNA for ER-α and ER-β, using a real-time reverse transcription (RT)-PCR assay, TaqMan RT-PCR, and nonradioactive in situ hybridization (ISH) techniques.

Result(s): Both eutopic endometrium and endometriotic tissues showed predominantly higher levels of ER-α than ER-β mRNA. Relative ratio of ER-α to ER-β (ER-α/ER-β) mRNA in red peritoneal lesions was significantly higher than in black lesions and ovarian endometriotic cysts. There was no significant difference in ER-α/ER-β between proliferative eutopic endometrium and red peritoneal lesions. These results were confirmed by ISH analysis, which also revealed that the two estrogen receptors were localized in both epithelial and stromal cells of endometriotic tissues.

Conclusion(s): The predominant expression of ER-α in both glandular epithelial and stromal cells may be essential for the development and growth of peritoneal and ovarian endometriosis. (Fertil Steril® 2001;75: 1198–1205. ©2001 by American Society for Reproductive Medicine.)

Key Words: Estrogen receptor isoforms, endometriosis, in situ hybridization, TaqMan reverse transcription-polymerase chain reaction

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Endometriosis, a common gynecological disorder accounting for infertility and pelvic pain, occurs almost exclusively in menstruating women of reproductive age. Although little is known about its etiology and pathogenesis, it is very likely that estrogen stimulates the growth of endometriotic tissue. Pathophysiological actions of estrogen are mediated within target cells by two estrogen receptor isoforms: estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β; see Reference 1, 2). Previous studies have demonstrated a differential characterization of the two ER isoforms between the eutopic endometrium and ovarian endometriotic cyst (3–5). It is thus very important to investigate the possible biological roles of the two ER isoforms to estimate disease progression and use of therapeutic agents in treatment of endometriosis.

Pelvic endometriosis includes three different types: peritoneal, ovarian, and rectovaginal (6). Furthermore, according to their macroscopic appearance, peritoneal endometriosis can be further classified into three lesion types: red, black, and white (6–8). It is postulated that these three types of peritoneal lesions may represent different stages of the spontaneous evolution of endometriotic implants, the first-stage red lesions being more active than black and white lesions (6–9) However, there have been no reports characterizing ER-α and ER-β expression according to the types of endometri-

otic lesions. Therefore, in the present study, we investigated the pathophysiological differences of those types by quantifying and comparing messenger RNA (mRNA) levels of ER-α and ER-β among ovarian endometriotic cysts and red and black peritoneal endometriotic lesions. For this purpose, we used a real-time reverse transcription–polymerase chain reaction (RT-PCR) assay, TaqMan RT-PCR (10, 11). On the basis of results of the RT-PCR analysis, we evaluated the localizations and expression levels of the ER-α and ER-β mRNAs in individual cells using in situ hybridization (ISH) techniques. In addition, we compared ER-α and ER-β mRNA expression of endometriotic tissues with normal eutopic endometrium and normal peritoneum.

MATERIALS AND METHODS

Experimental Subjects

During laparoscopic surgery, samples of peritoneal endometriotic lesions (n = 33) and ovarian endometriotic lesions (n = 37) were obtained from a total of 62 patients (20 to 42 years of age) at Tohoku University Hospital, Sendai, Japan and Polyclinique de l'Hotel-Dieu, Clermont-Ferrand, France. Both peritoneal and ovarian lesions were derived from seven patients, but either peritoneal or ovarian tissues were from 55 patients. All patients received no hormonal treatments, such as GnRH agonist (GnRHa) or sex steroids, during the minimum 6-month period preceding surgery, and had regular menstrual cycles, confirmed by their menstrual history and measurement of serum 17β-estradiol and progesterone levels.

Thirty-three peritoneal endometriotic lesions were divided into red, flamelike lesions (n = 15: seven in proliferative phase, eight in secretory phase) and typical black lesions (n = 18: 8 in proliferative phase, 10 in secretory phase), as proposed in the latest revision of the American Society for Reproductive Medicine classification (12). Of the 37 samples from ovarian endometriotic tissues, 18 samples were obtained during the follicular phase, and 19 during the luteal phase. All samples contained a glandular epithelium surrounded by stromal tissue. The presence of these features was sufficient to meet the criteria for histopathological diagnosis of endometriosis.

Control samples were obtained from normal eutopic endometrial tissues of 24 patients (12 in proliferative phase, 12 in secretory phase), determined using the criteria of Noyes et al. (13) and from macroscopically normal peritoneal tissues of 10 patients with endometriosis (five in proliferative phase, five in secretory phase) and 10 patients without endometriosis (five in proliferative phase, five in secretory phase). All of the patients underwent an operation because of uterine leiomyoma, carcinoma in situ of the cervix, or benign ovarian diseases. We microscopically confirmed that normal peritoneal tissues were not contaminated endometriotic tissues. Each of the fresh tissue samples was divided into two portions. One portion was fixed in 4% paraformaldehyde (pH 7.4) and embedded in paraffin for routine histopathological examinations and ISH analysis, whereas the second portion was immediately frozen in liquid nitrogen and stored at −80°C until it was used in the RT-PCR analysis.

Out of the 33 samples of peritoneal endometriotic tissues, nine samples were used only for the ISH analysis. Out of 37 ovarian endometriotic tissue samples, 13 samples were excluded from the TaqMan RT-PCR analysis because they appeared to be contaminated by normal ovarian tissue. All of the tissue samples were obtained with the patients' fully informed consent. The research protocol was approved by the human research board of the ethical committees of Tohoku University School of Medicine and the Polyclinique de l'Hotel-Dieu.

Oligodeoxynucleotide Primers and Probes

Oligodeoxynucleotide primers and TaqMan probe for the TaqMan RT-PCR analysis and oligodeoxynucleotide probes with a 3′-biotinylated tail (Brigati tail; 5′-probe-biotin-biotin-biotin-TAG-TAG-biotin-biotin-biotin-3′) for ISH analysis were designed using published sequences, as we reported elsewhere (5, 14, 15). The primer and probe sequences are shown in Table 1, as well as the locations of cDNA corresponding to the human ER-α (16) and ER-β (2).

Extraction of RNA and TaqMan RT-PCR Analysis

As previously reported, we have developed a rapid, accurate, and highly sensitive method to determine levels of ER-α and ER-β mRNA using a real-time RT-PCR assay with the TaqMan detection system (15). This technique can generate yes-or-no quantitative results much faster than Northern blotting, ribonuclease protection assay, and several other quantitative RT-PCR techniques (10, 11). Briefly, each of the frozen tissue samples subjected to the RT-PCR analysis was homogenized, and total RNA was extracted with ISOGEN (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. Using a single-tube, single-enzyme system, reverse transcription and DNA polymerization are conducted without the addition of subsequent enzymes or buffers. Each RT-PCR experiment included a standard curve assay with five RNA concentrations in duplicate (10-fold serially diluted recombinant RNA; ER-α: 10^{-10}–10^{-14} g, ER-β: 10^{-12}–10^{-16} g) and a blank assay without RNA template. The synthesized recombinant ER-α and ER-β RNAs for generation of a standard curve were as previously reported (15). A strong linear relationship between the threshold cycle and the log of the starting RNA copy number was always demonstrated ($R^2 \geq 0.99$).

Determination of Levels of ER-α and ER-β mRNA and the Relative Ratios of ER-α to ER-β

ER-α and ER-β mRNA levels were finally determined as the copy number per microgram of total RNA extracted from each tissue sample. Copy numbers for standard curves of

TABLE 1

Sequences of primers, TaqMan probes, and hybridization probes for ER-α and ER-β mRNA.

mRNA	Sequence		Sense/antisense
ER-α	5′-TGATTGGTCTCGTCTGGCG-3′[a]	1455–1463	Sense
	5′-CATGCCCTCTACACATTTTCCC-3′[b]	1555–1583	Antisense
	5′-TGCTCCTAACTTGCTCTTGGACAGGAACC-3′[c]	1504–1525	Sense
	5′-CAGCTCGTTCCCTTGGATCTGATGCAGTAG-3′[d]	332–361	Antisense
ER-β	5′-GGTCCATCGCCAGTTATCACAT-3′[a]	129–150	Sense
	5′-GATGCGTAATCGCTGCAGACAG-3′[b]	329–356	Antisense
	5′-TGTGAAGCAAGATCGCTAGAACACACCT-3′[c]	181–202	Sense
	5′-TGTTGGCCACAACACATTTGGGCTTGTGGT-3′[d]	76–105	Antisense

Note: TaqMan probe consists of an oligonucleotide with 5′-reporter dye (6-carboxy-fluorescein) and 3′-quencher dye (6-carboxy-tetramethyl rhodamine). GenBank accession numbers: ER-α, M12674; ER-β, X99101.

[a] Sequences of forward primers.

[b] Sequences of reverse primers.

[c] Sequences of TaqMan probes.

[d] Sequences of hybridization probes.

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ER-α and ER-β were calculated using the mean molecular weight of recombinant RNA of ER-α and ER-β as previously reported (15). It can be presumed that mRNAs levels of the two isoforms are affected by the heterogeneity of distributions of the glandular epithelium and the stroma in each endometriotic sample. Therefore, to compare mRNA levels between different types of endometriotic lesions, we used the relative ratios of ER-α to ER-β mRNA levels (ER-α/ER-β) in samples in which both isoforms could be detected.

TABLE 2

Summary of results of ER-α and ER-β mRNA levels.

		ER-α		ER-β		
Samples		No. of positive samples	mRNA levels ($\times 10^5$)[a]	No. of positive samples	mRNA levels ($\times 10^5$)[a]	ER-α/ER-β mRNA levels
Eutopic endometrium	(n = 24)	24		24		
Proliferative phase	(n = 12)	12	874.5 ± 233.5	12	4.0 ± 0.9	376.6 ± 60.1
Secretory phase	(n = 12)	12	111.4 ± 41.2	12	1.7 ± 0.5	71.3 ± 12.5
Ovarian endometriosis	(n = 24)	24		20		
Proliferative phase	(n = 12)	12	36.8 ± 11.5	9	3.3 ± 0.8	132.1 ± 53.3
Secretory phase	(n = 12)	12	49.1 ± 18.7	11	3.1 ± 1.0	106.2 ± 66.2
Peritoneal endometriosis	(n = 12)					
Red lesions		12		12		
Proliferative phase	(n = 6)	6	29.2 ± 7.8	6	0.13 ± 0.03	316.6 ± 104.9
Secretory phase	(n = 6)	6	25.7 ± 6.3	6	0.11 ± 0.03	319.5 ± 128.1
Black lesions		12		12		
Proliferative phase	(n = 6)	6	13.6 ± 6.8	6	1.1 ± 0.42	38.6 ± 19.7
Secretory phase	(n = 6)	6	11.3 ± 5.2	6	0.46 ± 0.10	51.6 ± 18.7
Normal peritoneum	(n = 10)					
from patients with endometriosis		10		10		
Proliferative phase	(n = 5)	5	1.7 ±0.34	1	—	—
Secretory phase	(n = 5)	5	0.35 ± 0.11	1	—	—
from patients without endometriosis		10		10		
Proliferative phase	(n = 5)	5	2.3 ± 0.72	2	—	—
Secretory phase	(n = 5)	5	0.34 ± 0.07	1	—	—

Note: mRNA levels and ER-α/ER-β mRNA levels were represented as mean ± SE.

[a] mRNA levels are expressed as copy number per microgram of total RNA.

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In Situ Hybridization

Briefly, as described elsewhere (5, 14, 17), serial thin sections (3 μm thick) of paraffin-embedded tissues were cut and mounted on Probe-On glass slides (Fisher Scientific, Pittsburgh, PA). ISH was performed with a manual capillary action system (MicroProbe staining system, Fisher Scientific). Fetal kidney tissues, in which moderate expression of ER-β mRNA has been proven by RT-PCR (18), were used as positive controls. Negative control experiments were performed using sense probes that had complementary sequences to one of the antisense probes and showed no detectable specific mRNA hybridization signals.

The results are shown in terms of the relative intensity of mRNA hybridization signals, classified as *no signal over background, equivocally positive signal,* or *positive signal.*

Statistical Analysis

Statistical analysis was performed with the Stat View 4.5 program (Abacus Concepts, Inc., Berkeley, CA). Mann-Whitney *U*-test was applied to compare results from different groups. Statistical significance was defined as $P<.05$.

RESULTS

ER-α and ER-β mRNA Levels Measured With TaqMan RT-PCR

ER-α and ER-β mRNA levels in different tissues are summarized in Table 2. All analyzed samples, which included eutopic endometrial tissues, normal peritoneal tissues, and endometriotic lesions, showed predominantly higher levels of ER-α than of ER-β mRNA.

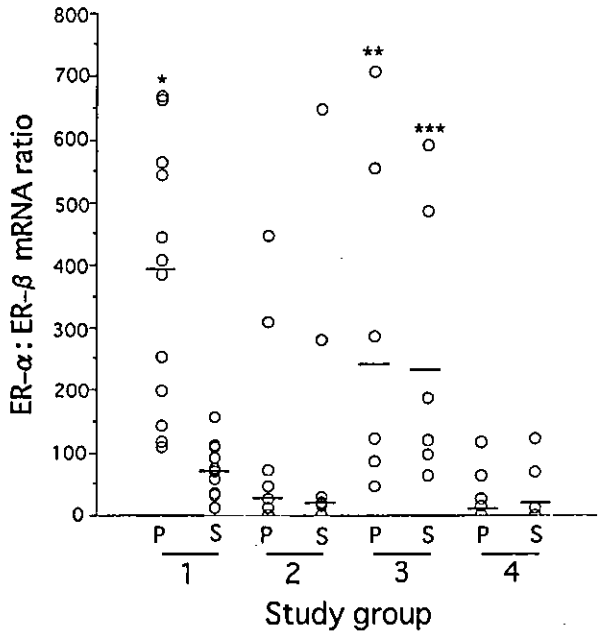
In the 24 eutopic endometrium samples, both ER-α and ER-β mRNA levels were detected throughout the menstrual cycle, but the mean levels of both the isoforms were significantly higher in the proliferative phase than in the secretory phase (ER-α; $P<.003$, ER-β; $P<.04$).

In the 20 normal peritoneal samples, ER-α mRNA was detected in all the samples, whereas ER-β mRNA was detected in only five (25%) of the samples. The mean ER-α mRNA level during the proliferative phase was significantly higher than during the secretory phase (with endometriosis; $P<.01$; without endometriosis, $P<.01$). There was no significant difference in the mean ER-α mRNA levels between samples from patients with and without endometriosis.

In ovarian endometriotic lesions, ER-α mRNA expression was detected in all 24 samples analyzed, whereas ER-β mRNA was detected in 20 (83%) samples. In peritoneal endometriotic lesions, ER-α mRNA and ER-β mRNA were detected in all analyzed samples of red ($n = 12$) and black ($n = 12$) lesions. Comparison of mean ER-α and ER-β mRNA levels in ovarian and peritoneal endometriotic lesions showed no significant differences between the proliferative and the secretory phases.

FIGURE 1

ER-α:ER-β mRNA ratios in the eutopic endometrium and endometriotic tissues. Values represent type of samples and their conditions: 1, eutopic endometrium (P: $n = 12$, S: $n = 12$); 2, ovarian endometriotic lesions (P: $n = 9$, S: $n = 11$); 3, red peritoneal lesions (P: $n = 6$, S: $n = 6$); and 4, black peritoneal lesions (P: $n = 6$, S: $n = 6$). The horizontal lines represent median value. P = proliferative phase; S = secretory phase. *Significantly different from the values observed in secretory endometrium ($P<.001$). **Significantly different from the values observed in ovarian endometriotic lesions and black peritoneal lesions during the proliferative phase ($P<.04$ and $P<.01$, respectively). ***Significantly different from the values observed in ovarian endometriotic lesions and black peritoneal lesions during the secretory phase ($P<.05$ and $P<.01$, respectively).



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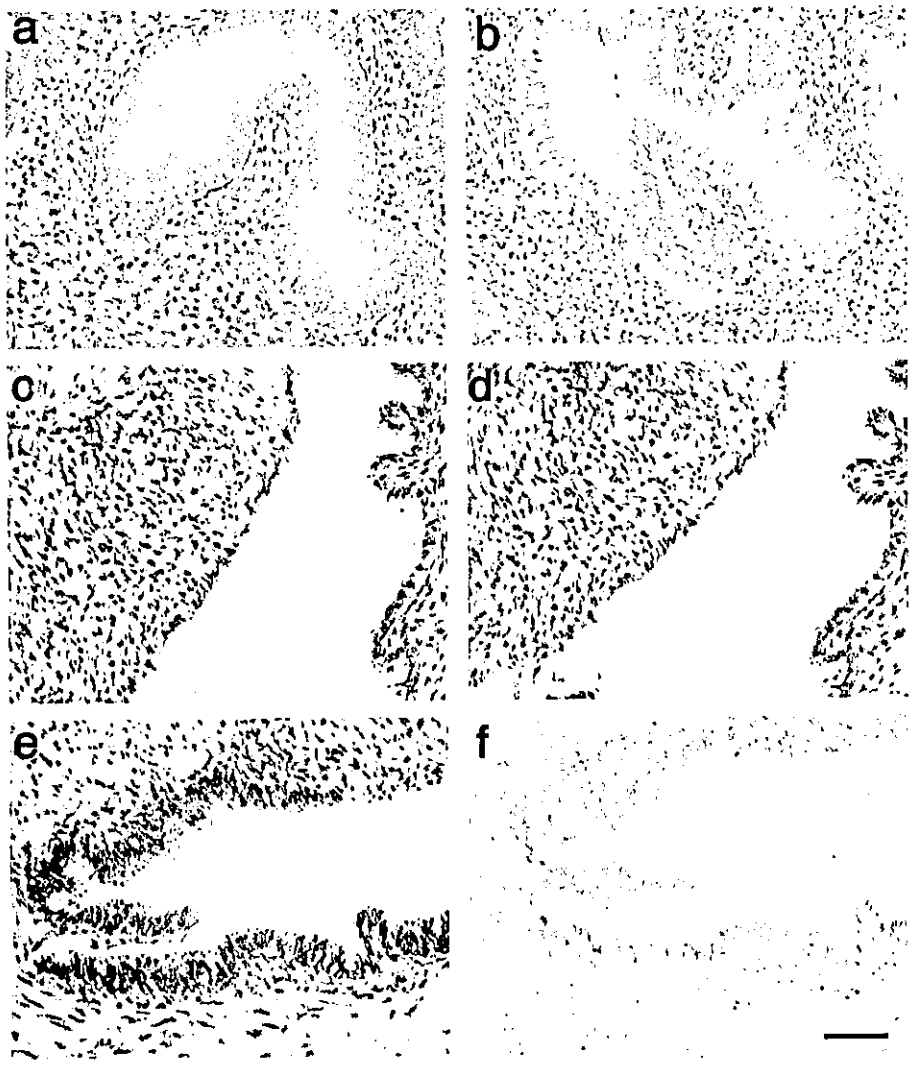
Relative Ratios of ER-α:ER-β mRNA Levels

The mean ER-α/ER-β mRNA in different tissues are summarized in Table 2, and their distributions are shown in Figure 1. The mean ER-α/ER-β mRNA in the eutopic endometrium differed significantly between proliferative and secretory phases ($P<.001$). However, no significant differences were detected in any endometriotic tissue samples between the phases. Comparisons among the red and black peritoneal lesions and ER-β–positive ovarian lesions showed that the mean ER-α/ER-β mRNA of red lesions was significantly higher than that of black lesions and ovarian lesions throughout the different phases of the cycle (red lesions vs. black lesions; proliferative phase: $P<.01$, secretory phase: $P<.01$; red lesions vs. ovarian lesions; proliferative phase: $P<.04$, secretory phase: $P<.05$). There were no significant

FIGURE 2

In situ localization of ER-α and ER-β mRNA in peritoneal and ovarian endometriosis. ISH was performed on adjacent sections with an antisense probe to ER-α (**A, C, E**) and an antisense probe to ER-β (**B, D, F**). Red peritoneal lesion (**A, B**): ER-α hybridization signals, appearing red as a result of fast-red staining, were detected in glandular epithelial and stromal cels. No ER-β hybridization signals were detected. Black peritoneal lesion (**C, D**): both ER-α and ER-β mRNA hybridization signals, appearing red as a result of fast-red staining, were detected in stromal cells. Ovarian endometriotic cyst (**E, F**): ER-α hybridization signals, appearing red as a result of fast-red staining, were detected in glandular epithelial and stromal cells. No ER-β hybridization signals were detected. For all parts, bar = 50 μm.



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differences in the mean ER-α/ER-β mRNA between black and ovarian lesions. Comparisons between red lesions and the eutopic endometrium revealed that the difference in the mean ER-α/ER-β mRNA was not significant in the proliferative phase but was significant in the secretory phase ($P<.03$).

In Situ Hybridization

The results of the ISH analysis are shown in Figure 2 and in Tables 3–5. In the eutopic endometrium, ER-α mRNA signals were more prominent than those of ER-β. In the eutopic endometrium of the proliferative phase, ER-α mRNA was expressed in both glandular epithelial and stromal cells, whereas ER-β mRNA was expressed predominantly in glandular epithelial cells. Although the same expression pattern was observed in the secretory phase, expression levels of both ER-α and ER-β mRNA in glandular epithelial and stromal cells were markedly decreased in this phase. In normal peritoneal tissues from patients with or

TABLE 3

Results of in situ hybridization study for ER-α and ER-β mRNA in endometriotic tissues.

	ER-α						ER-β					
	G			S			G			S		
Result	+	±	−	+	±	−	+	±	−	+	±	−
Red (n = 15)	14	1	0	12	2	0	2	2	11	4	2	9
Black (n = 18)	14	4	0	12	6	0	6	7	5	13	2	3
Ov (n = 32)	25	7	0	26	6	0	11	4	17	9	6	17

Note: Signal intensity was classified into three categories: −, no signal over background; ±, equivocally positive signal; +, positive signal. Red = red peritoneal lesions; Black = black peritoneal lesions; Ov = ovarian endometriotic cysts; G = glandular epithelium, S = stroma.

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without endometriosis, neither ER-α nor ER-β mRNA signals were detected.

In peritoneal and ovarian endometriotic lesions, positive signals for ER-α mRNA were detected in all samples. In contrast, positive signals for the ER-β mRNA were detected in 6 (40%, four in follicular phase, two in secretory phase) of 15 red peritoneal samples, 15 (83%, seven in follicular phase, eight in secretory phase) of 18 black peritoneal samples and 15 (47%, seven in follicular phase, eight in secretory phase) out of 32 ovarian samples. We did not observe any systematic variation in the expression of ER-β mRNA during the different phases of the menstrual cycle. In both ER-α and ER-β mRNA–positive endometriotic tissues, signals were detected in glandular epithelial and stromal cells, but ER-α mRNA signals were more prominent than those of ER-β mRNA. When comparing signal expression between red and black peritoneal lesions, ER-α mRNA signals in red peritoneal lesions were more prominent than those in black lesions in both glandular epithelial and stromal cells. ER-β mRNA signals in glandular epithelial cells were not apparently different, whereas those in stromal cells were more prominent in black peritoneal lesions than in red peritoneal lesions. Neither ER-α nor ER-β mRNA signals were detected in normal peritoneal tissues immediately adjacent to endometriotic lesions.

DISCUSSION

Using TaqMan RT-PCR and ISH analyses, we demonstrated that ER-α mRNA was predominantly expressed both in control (the eutopic endometrium and the normal peritoneum) and all (peritoneal and ovarian) endometriotic samples analyzed. Moreover, cyclical changes in ovarian hormones had apparently different effects on ER-α and ER-β mRNA expression on normal control and endometriotic tissues. However, further studies in vivo and in vitro are required to confirm the effects of ovarian hormones on ER-α and ER-β in endometriotic tissues.

Although mRNA levels do not directly indicate bioactivity of the protein, we propose that the principal and regulatory effects of estrogens might be mediated mainly via ER-α, rather than ER-β, in both control and endometriotic tissues.

TaqMan RT-PCR analyses revealed significant differences in ER-α/ER-β mRNA among the eutopic endometrium and peritoneal and ovarian endometriosis. The results derived through ISH analyses demonstrated that there were some samples that were negative for ER-β mRNA signals in endometriotic lesions; more than half of the samples were negative, especially in the red peritoneal and ovarian lesions. On the basis of these results, we postulate that estrogen bioaction through ER-β might have no direct effect on

TABLE 4

Results of in situ hybridization study for ER-α and ER-β mRNA in eutopic endometrium.

	ER-α						ER-β					
	G			S			G			S		
Group	+	±	−	+	±	−	+	±	−	+	±	−
Proliferative (n = 12)	12	0	0	12	0	0	12	0	0	12	0	0
Secretory (n = 12)	6	4	2	6	4	2	2	5	5	1	4	5

Signal intensity was classified into three categories: −, no signal over background; ±, equivocally positive signal; +, positive signal. G = glandular epithelium; S = stroma.

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TABLE 5

Results of in situ hybridization study for ER-α and ER-β mRNA in normal peritoneum.

	ER-α						ER-β					
	M			C			M			C		
Group	+	±	−	+	±	−	+	±	−	+	±	−
E(+) (n = 10)	0	0	10	0	0	10	0	0	10	0	0	10
E(−) (n = 10)	0	0	10	0	0	10	0	0	10	0	0	10

Signal intensity was classified into three categories: −, no signal over background; ±, equivocally positive signal; +, positive signal. E(+) = normal peritoneum from patients with endometriosis; E(−) = normal peritoneum from patients without endometriosis; M = mesothelium; C = connective tissue.

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progression of endometriosis. Because it has been recently reported that one possible role of ER-β is to modulate ER-α transcriptional activity, the relative expression levels of the two isoforms are an important determinant of cellular sensitivity to estrogens and anti-estrogens (19). Therefore, it is also possible that cellular sensitivities to estrogen might vary under the different distributions of ER-α and ER-β among these endometriotic tissues.

Results of ISH analyses demonstrated that ER-α mRNA signals in both glandular epithelial and stromal cells were more prominent in red than in black peritoneal lesions. In contrast, ER-β mRNA signals (especially in stromal cells) were more prominent in black peritoneal lesions than in red peritoneal lesions. These results were equivalent to those obtained by quantitative TaqMan RT-PCR, which revealed lower ER-α/ER-β mRNA in black, compared with red, peritoneal lesions.

Recent studies of endometriosis demonstrated aberrant aromatase expression in stromal cells (20) and the deficiency of 17β-hydroxysteroid dehydrogenase type 2 in glandular epithelial cells (21, 22). These enzymatic alterations might suggest accumulation of local estradiol in endometriosis and support the hypothesis that local estradiol strongly affects proliferation of endometriotic cells (21, 22). Moreover, the local estradiol might also cause the loss of effects of cyclical changes in ovarian estrogen in endometriotic lesions. Because there is strong evidence that red peritoneal lesions are more active than black lesions (6–9), we postulate that the higher expression of ER-α than ER-β may be responsible for cellular proliferation in endometriotic lesions, especially in red peritoneal lesions. Studies of ER-α in knockout mice demonstrated that the mitogenic effects of estradiol on uterine epithelial cells could be either partially or totally mediated by ER-α–positive stromal cells (23). Studies suggest that stromal cells probably play primordial roles in the development and growth of the endometriotic lesions (6–9, 24, 25). Therefore, the relatively higher levels of ER-α mRNA in stromal cells of red peritoneal lesions may have biological significance in the growth of peritoneal endometriosis. However, the in situ hybridization approach used in the present study has some unavoidable limitations in terms of quantitation. Further studies are required to confirm this possibility by determining levels of ER-α and ER-β mRNA in glandular epithelial or stromal cells in conjunction with sensitive analytical techniques, such as quantitative TaqMan RT-PCR and laser-capture microdissection (26, 27).

A recent study demonstrated that human endometrial tissues obtained during the late secretory phase could be successfully transplanted into nude mice and that all the grafts revealed histological characteristics of the proliferative phase (25). In the present study, proliferative eutopic endometrium and red peritoneal lesions showed similar ER-α/ER-β mRNA values, whereas the majority of normal peritoneum analyzed expressed only ER-α mRNA. These findings support the premise that peritoneal endometriotic lesions result from endometrial transplantation to peritoneal surface and that red peritoneal lesions may be the first stage of the spontaneous evolution of endometriotic implants. Furthermore, differences in ER-α/ER-β mRNA values between red and black peritoneal lesions indicate that a transcriptional alternation may act on the ER isoform genes during progression of peritoneal endometriosis (28, 29).

In conclusion, our results suggest that the predominant expression of ER-α in both glandular epithelial and stromal cells may be essential for the development and growth of peritoneal and ovarian endometriosis. Furthermore, the predominant expression of ER-α in endometriosis indicates a potential use of ER subtype–selective compounds as therapeutic agents. Further studies are required to delineate the precise roles of ER isoforms in endometriosis.

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Systemic Distribution of Steroid Sulfatase and Estrogen Sulfotransferase in Human Adult and Fetal Tissues

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Estrogens play a key role in various target tissues. Enzymes involved in the biosynthesis and metabolism of these sex steroids also regulate estrogenic actions in these tissues. Estrone sulfate (E1S) is a major circulating plasma estrogen that is converted into the biologically active estrogen, estrone (E1), by steroid sulfatase (STS). E1 is also sulfated and reverted into E1S by estrogen sulfotransferase (EST). These two enzymes have recently been shown to play important roles in the *in situ* estrogen actions of various sex steroid-dependent human tumors. However, the distribution of STS and EST in normal adult and fetal human tissues remains largely unknown. Therefore, in this study, in addition to examining the tissue distribution of both STS and EST mRNA in human adult and fetal tissues using RT followed by quantitative PCR, we studied the activity of these enzymes using ^{3}H-labeled E1/E1S as substrates in the homogenates of various human adult tissues. We also examined the localization of STS and EST protein in human adult and fetal tissues using immunohistochemistry, and that of EST mRNA in the adult kidney using laser dissection microscopy and PCR. STS mRNA, enzyme activity, and immunoreactivity were either absent or detected at very low levels in all adult and fetal tissues examined in this study. EST mRNA expression, however, was detected in all of the tissues examined, except for adult spleen and pancreas. EST enzyme activities were consistent with those of mRNA expression in the great majority of the tissues examined. Marked EST immunoreactivity was detected in hepatocytes, adrenal gland (adult, zona fasciculate to the reticularis; fetus, fetal zone), and epithelial cells of the gastrointestinal tract, smooth muscle cells of the tunica media in aorta, Leydig cells of the testis, and syncytiotrophoblast of the placenta. Patterns of EST immunolocalization were similar between adult and fetal human tissues, but EST immunoreactivity was detected in the urinary tubules of adult kidney, whereas in the fetal kidney, it was localized in the interstitial cells surrounding the urinary tubules. In the adult kidney, the presence of EST mRNA was also confirmed in the cells of urinary tubules using laser dissection microscopy and RT-PCR.

Although the number of human tissues available for examination in this study was limited, our results suggest that between the enzymes involved in estrogen activation or inactivation, EST and not STS is the more widely expressed enzyme in various peripheral tissues in humans. We speculate that EST may play an important role in protecting peripheral tissues from possible excessive estrogenic effects. (*J Clin Endocrinol Metab* 87: 5760–5768, 2002)

ESTROGEN HAS TRADITIONALLY been considered to be a female sex steroid because it is mainly synthesized in the ovary and plays a critical role in female reproduction. However, estrogens have also been reported to have important roles in male reproduction organs and numerous other tissues including bone, liver, the central nervous system, and the vascular system. The biological activity of estrogen is well known to be mediated through an initial interaction with estrogen receptors (ERs). Results of recent studies have demonstrated that ERs are widely expressed in a variety of tissues. In addition to those tissues described above, ERs have been shown to be expressed in the mammary gland, lung, and intestine (1, 2). Estrogen is able to enter the fetal circulation via the umbilical vein (3), whereby it binds to ER, which has also been reported in various human fetal tissues (4, 5). Estrogens are therefore postulated to play important roles in human fetal development.

A major circulating form of plasma estrogen is estrone sulfate (E1S), a biologically inactive form of estrogen. E1S has a relatively long half-life in the peripheral blood (6), where serum levels of E1S are known to be 10-fold higher than those of unconjugated estrone (E1) or estradiol (E2; Ref. 7). In addition, E1S has been shown to be a predominant form of estrogen in the urine and milk (8, 9). E1S is transformed into a biologically active form, E1, by steroid (estrone) sulfatase (STS; Refs. 10–12). E1 is sulfated into E1S by cytosolic enzymes, phenol sulfotransferase and estrogen sulfotransferase (EST; Ref. 13). In both human breast cancer and endometrial carcinoma, *in situ* synthesized estrogen is considered to play very important roles in the pathogenesis of these cancers. STS has been postulated to be involved in the process of *in situ* production of estrogens in these neoplastic tissues (10–12). EST, *SULT 1E1* or *STE* gene, is a member of the superfamily of cytosolic steroid sulfotransferases (13), and its enzyme activity has been reported in both male and female tissues including liver, kidney, brain, adrenal gland, *etc.* (14). It is also well known that marked

Abbreviations: DAB, Diaminobenzidine; E1, estrone; E1S, E1 sulfate; E2, estradiol; ER, estrogen receptor; EST, estrogen sulfotransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; gpEST, guinea pig adrenal cortical EST; HuH7, human hepatocellular carcinoma; 17β-HSD2, 17β-hydroxysteroid dehydrogenase type 2; LCM, laser capture microdissection; P450arom, cytochrome P450 aromatase; STS, steroid sulfatase.

differences of EST expression and/or activity exist in tissues depending on species, sex, age, development, and physiological status in laboratory animals (15–17). STS expression has been examined in estrogen-dependent neoplasms such as breast cancer and endometrial carcinoma (11–13). However, to date, the expression of STS and EST has not been examined in normal tissues.

Therefore, we examined the expression of mRNA transcripts for STS and EST using real-time PCR in both fetal and adult human tissues. Moreover, we examined the activity of these enzymes using ^{3}H-labeled E1/E1S as substrates in the homogenates obtained from various human adult tissues. Then, we employed immunohistochemistry to study the cellular distribution of STS and EST proteins in these tissues with the aim of further characterizing possible roles of these enzymes in peripheral estrogen metabolism. In addition, localization of EST mRNA was further characterized using laser dissection microscopy and PCR in the human adult kidney.

Materials and Methods

Tissue preparation

Human tissues from seven adults (4 males, 24, 54, 84, and 87 yr old; 3 females, 15, 38, and 86 yr old) were obtained during autopsy at the Department of Pathology, Tohoku University Hospital, within 2 h postmortem. Human fetal tissues (gestational age, 17–21 wk) were obtained following elective termination in normal pregnant women at Nagaike Maternal Clinic (Sendai, Japan). Informed consent was obtained from the pregnant women before elective termination. Normal human endometria, one proliferative phase endometrium (51-yr-old patient) and three secretory phase endometria (37, 43, and 49 yr of age, respectively), were obtained from women who underwent hysterectomy due to carcinoma *in situ* of the uterine cervix at the Department of Obstetrics and Gynecology, Tohoku University Hospital. Informed consent was obtained from these patients before surgery. The Ethics Committee at Tohoku University School of Medicine approved this research protocol.

PCR

Real-time PCR. All specimens were immediately frozen in liquid nitrogen and stored at −80 C until RNA isolation. RNA was extracted from these frozen specimens within 2 wk. Total RNA was extracted by homogenizing frozen tissue samples in 1 ml TRIzol reagent (Life Technologies, Inc., Grand Island, NY) followed by a phenol-chloroform phase extraction and isopropanol precipitation. All RNA samples were quantified by spectrophotometry and stored at −80 C until processed for RT. The SUPERSCRIPT Preamplification system RT kit (Life Technologies, Inc.) was employed in the synthesis and amplification of cDNA. cDNA was synthesized from total RNA (2 μg) using 25 ng/μl Oligo $(dT)_{12-18}$ Primer (Life Technologies, Inc., Gaitherburg, MD) on a PTC-200 Peltier Thermal Cycler DNA Engine (MJ Research, Inc., Watertown, MA). To test for the presence of genomic DNA contamination, we performed the RT step in the absence of SUPERSCRIPT II RNase H$^-$ Reverse Transcriptase (Life Technologies, Inc.) followed by PCR. RT-PCR products lacking reverse transcriptase in the initial RT step were run on an ethidium-bromide stained 2% agarose gel. No band was observed in these samples (data not shown). The resulting cDNA was used as a template for real-time PCR. Real-time PCR was carried out with the Light Cycler System (Roche Diagnostics GmbH, Mannheim, Germany) using the DNA binding dye SYBER Green I (Roche Diagnostics GmbH) for the detection of PCR products. PCR was set up using 2 mM $MgCl_2$, 10 pmol/liter of each primer (Table 1; Refs. 18–20), and 2.5 U *Taq* DNA polymerase (Life Technologies, Inc.). An initial denaturing step of 95 C for 1 min was followed by 40 cycles, respectively, of 95 C for 0 sec; 15 sec annealing at 60 C [STS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], 58 C (EST); and extension for 15 sec at 72 C. The fluorescence intensity of the double-strand specific SYBER Green I, which reflects the amount of formed specific PCR products, was read by the LightCycler at 85 C after the end of each extension step. After PCR, these products were resolved on a 2% agarose ethidium bromide gel. Images were captured with Polaroid film under UV transillumination. In initial experiments, PCR products were purified and subjected to direct sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Genetic Analyzer, Perkin Elmer Corp., PE Applied Biosystems, Foster City, CA) to verify amplification of the correct sequences. As a positive control, frozen tissues of placenta were used for STS (21), and liver (Ref. 22; HuH7, human hepatocellular carcinoma) cells were used for EST. Negative control experiments lacked cDNA substrate to check for the presence of exogenous contaminant DNA. No amplified products were observed under these conditions. The mRNA levels of STS and EST in each case are summarized as a ratio of GAPDH and evaluated as a ratio (%) compared with that of each positive control. Although conventional quantitative PCR requires the use of a purified plasma cDNA in the construction of a standard curve, we found that we were able to semiquantify our PCR products with the LightCycler using purified cDNA of known concentrations. Other studies to date have used a similar protocol to semiquantify PCR products with the LightCycler (23, 24).

Microdissection/PCR. Adult kidney (38-yr-old female) obtained during autopsy was rapidly embedded in Optimal Cutting Temperature medium (SAKURA Finetechnical Co. Ltd., Tokyo, Japan) and frozen-sectioned at a thickness of 8 μm. The specimen was subsequently mounted on membrane-attached glass slides (Cell Robotics, Inc., Albuquerque, NM). All tissue sections were stored at −30 C until laser capture microdissection (LCM). These frozen sections were fixed in 100% methanol for 5 min and then stained with toluidine blue. LCM was performed using a CRI-337 (Cell Robotics, Inc.) with 30–60 μm laser spot size applying default to full-strength pulse power (40–100 W) and extreme strength of the pulse width (50–100 msec). Approximately 100 cells were laser-transferred from the urinary tubules and the interstitium. Total RNA was extracted from laser-transferred cells according to the RNA microisolation protocol reported by Niino *et al.* (25) and Emmert-Buck *et al.* (26). Briefly, after precipitation and a 70% ethanol wash, the pellets were resuspended in 9 μl of RNase-free H_2O. Total RNA from the microdissected kidney tissue was reverse transcribed in a reaction mixture containing 50 mM Tris acetate, pH 8.4, 75 mM potassium acetate, 8 mM magnesium acetate, 0.01 M dithiothreitol, 2 mM dNTP, 25 μM Oligo $(dT)_{12-18}$ Primer, 25 μg/μl random hexamer oligonucleotides, and SUPERSCRIPT II RNase H-Reverse Transcriptase (Life Technologies, Inc.) for 60 min at 50 C. The resulting cDNA was amplified in 25 μl of a PCR mix consisting of GeneAmp, 1× PCR Gold Buffer (PerkinElmer Life Sciences, Inc., Boston, MA), 1.5 mM $MgCl_2$, 200 μM dNTP, and 0.125 U of AmpliTaq Gold (PerkinElmer Life Sciences, Inc.) under the following conditions: initial denaturing at 95 C for 10 min followed by 40 cycles of 1 min at 94 C, 1 min at 55 C, and 1 min at 72 C,

TABLE 1. Primer sequences used in RT-PCR analysis

cDNA		Sequence	Size (bp)	Reference (no.)
STS	Forward	5′-AGGGTCTGGGTGTGTCTGTC-3′	290	Utsumi *et al.* (18)[a]
	Reverse	5′-ACTGCAACGCCTACTTAAATG-3′		
EST	Forward	5′-AGAGGAGCTTGTGGACAGGA-3′	114	Aksoy *et al.* (19)
	Reverse	5′-GGCGACAATTTCTGGTTCAT-3′		
GAPDH	Forward	5′-TGAACGGGAAGCTCACTGG-3′	307	Tokunaga *et al.* (20)[a]
	Reverse	5′-TCCACCACCCTGTTGCTGTA-3′		

[a] Oligonucleotide primers for STS and GAPDH were designed using the previously published cDNA sequence.

after which PCR products were subjected to a final extension step for 7 min at 72 C. Primers used for PCR amplification are described above.

Enzyme assay

Adult tissues (38- and 86-yr-old females) obtained from autopsy were frozen in liquid nitrogen immediately after diagnostic observation and dissection at the time of autopsy, and stored at −80 C until assay. EST was assayed as described previously (27). Briefly, frozen samples were homogenized in a reaction buffer at 4 C and centrifuged for 15 min at 1000 × *g*. The upper layer was used as the enzyme source. About 0.2 mg of protein were added in each assay, and the reaction contained 50 mM Tris-HCl, pH 7.4, 7 mM $MgCl_2$, and E1 contained [^{3}H]E1 at 20 nM. Reactions were started with the addition of 3′-phosphoadenosine-5′-phosphosulfate to a final concentration of 20 μM, in a final volume of 0.125 ml. The reaction mixtures were incubated at 37 C for 30 min, and the reactions were terminated with the addition of 4.0 ml chloroform, followed by the addition of 0.375 ml 0.25 M Tris-HCl, pH 8.7, to alkalinize the solution. The reaction mixtures were centrifuged at 600 × *g* for 5 min to separate the aqueous and organic phases. Synthesis of the tritiated E1S was determined with a liquid scintillation counter (Beckman LS-6500, Beckman Coulter, Fullerton, CA). The STS activity was assayed according to Utaaker *et al.* (28) with slight modifications. Briefly, enzyme solution (~0.2 mg protein) was mixed with E1S containing [6,7-^{3}H] E1S (1.6×10^5 dpm, 0.5 pmol/liter) at 20 μM and added to a reaction volume up to 0.15 ml with PBS (Ca^{2+} free) containing 25 mM sucrose and 4 mM nicotinamide. The reaction mixture was incubated at 37 C for 60 min in a shaking water bath. The enzyme reaction was terminated with the addition of toluene and mixed by vortex mixer for 1 min. The reaction mixtures were centrifuged at 600 × *g* for 5 min to separate the aqueous and organic phases. The toluene layer was collected, and [^{3}H] radioactivity was measured by liquid scintillation counter (Beckman, LC-6500), which is equivalent to E1 formed. Incubation conditions of these assays were designed so that the formation of product was linear.

Immunohistochemistry

Antibodies used in this study are as follows: rabbit polyclonal antibody for EST (PV-P2237) was purchased from Medical Biological Laboratory (Nagoya, Japan) and was raised against the synthetic N-terminal peptide of human EST corresponding to amino acids 1–13. The affinity purified monoclonal antibody for STS, KM1049, was raised against the enzyme purified from human placenta, which recognized the STS peptide corresponding to amino acids 420–428. Use of STS antibody in the evaluation of human breast cancer has been previously reported (29).

All specimens obtained from autopsy were fixed for 18–24 h in 10% formalin. After fixation, the specimens were dehydrated in ethanol and xylene series and embedded in paraffin. Thin sections, 3 μm thick, were mounted on silan-coated glass slides (Matsunami, Tokyo, Japan). These sections were immunostained by a biotin-streptavidin method with EnVision (DAKO Corp., Ltd., Carpinteria, CA) for STS, and Histofine SAB-PO (R) kit (Nichirei Co. Ltd., Tokyo, Japan) for EST. Sections were deparaffinized with xylene and microwaved (500 W) for 15 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0) for antigen retrieval. These reacted sections were incubated with normal goat serum for 30 min. These slides were further incubated with primary antibody for 12–18 h in a moist chamber at 4 C. The dilutions of primary antibodies were as follows: STS, 1:3000; and EST, 1:750. These sections were further reacted with methanol that contained 0.3% H_2O_2 for 30 min to block any endogenous peroxidase. The antigen-antibody complex was then visualized with 3.3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer, pH7.6, and 0.006% H_2O_2) and counterstained with hematoxylin. Normal full-term placenta was used as a positive control for STS, and normal liver was also used for EST. Normal rabbit and mouse IgG was used instead of the primary antibody as a negative control. No specific immunoreactivity was detected in these tissue sections.

Results

PCR

Real-time PCR. Results of quantitative analysis of STS and EST are summarized in Table 2 and Fig. 1. All quantified data are expressed with respect to GAPDH mRNA levels in each specimen. In human adult tissues, a very small amount of STS expression was detected in lung, aorta, liver, thyroid, testis, and uterus (1.0–3.7% of the levels of placenta), but was under the limits of detection in other tissues. STS mRNA transcripts in fetal tissues were, in general, widely detected in all of the tissues examined, albeit at low levels (1.0–2.9%). On the other hand, relatively abundant EST mRNA transcripts were detected in various human tissues (adult, 25.4–215.9%; fetus, 16.4–265.0%) with the exception of adult spleen and pancreas. In the endometrium, during the normal menstrual cycle, EST mRNA expression was low in the follicular and late secretory phases (8.1 and 3.3%, respectively), increased in the early secretory phase (12.4%), and reached a plateau by the mid secretory phase (70.2%). After PCR, amplified products were detected as a specific single band (Fig. 1A, 290 bp for STS, 114 bp for EST, and 307 bp for GAPDH) in all of the tissues examined. Amplified STS mRNA transcripts were shown to be weakly expressed in adult lung, aorta, liver, thyroid, testis, uterus, and all fetal tissues examined. EST mRNA transcripts were also detected as a distinctive band in all adult and fetal tissues examined, with the exception of spleen and pancreas.

Microdissection/PCR. After PCR for EST, amplified products were detected as a specific single band (Fig. 1B, d (*top*), 114 bp) in urinary tubular epithelial cells isolated by LCM as well as in whole kidney tissues. EST mRNA was totally absent in the renal glomerulus and stromal cells isolated by LCM. Expression of GAPDH was detected in all of the specimens examined (Fig. 1B, d (*bottom*), 307 bp).

Enzyme assay

The levels of STS and EST activities are summarized in Table 3. Both STS and EST enzymatic activities were detected in all specimens examined in this study. STS activities were below that of placenta (5%) in all tissues examined (0.2–4.5%). Among these tissues, STS enzyme activity in the adult liver (2.8 and 4.5%) and adrenal gland (3.2%) was relatively high, whereas EST activities in these tissues were nearly equivalent except for spleen, which demonstrated very little EST activity. Results of STS activity were consistent with those of STS mRNA transcripts, but there were discrepancies between mRNA levels and activities for EST in the adrenal gland and pancreas (Table 3). mRNA transcripts for EST were detected in the adrenal glands, despite low enzymatic activity, and vice versa in the pancreas.

Immunohistochemistry

STS immunoreactivity was not detected in all tissues examined, including those in which STS mRNA was identified, except for the placental syncytiotrophoblast (Fig. 2A). EST immunoreactivity was detected in hepatocytes employed as a positive control and in various tissues summarized in Table 4. In adult and fetal gastrointestinal (GI) tracts including stomach, small intestine (Fig. 2G), and large intestine, EST immunoreactivity was detected in surface or absorptive epithelial cells. In the adult kidney (Fig. 2C), EST immunoreactivity was exclusively present in urinary tubules, but in the

TABLE 2. Summary of quantitative PCR and immunohistochemistry (IMC)

Tissues	Age/sex		Adult				Fetus			
			STS		EST		STS		EST	
	Adult (age in yr)	Fetus (age in wk)	IMC	PCR	IMC	PCR	IMC	PCR	IMC	PCR
Brain	54/M	21/F	−	0.0	+	29.9	−	2.4	−	10.7
Trachea	84/M	17/M	−	0.0	+	22.0	−	[a]	+	[a]
Lung	54/M	18/M	−	1.4	+	32.0	−	2.2	+	229.2
Aorta	24/M	[a]	−	1.4	+	68.2	[a]	[a]	[a]	[a]
Heart	84/M	20/F	−	0.2	+	3.7	−	2.0	+	52.0
Esophagus	24/M	20/F	−	0.3	+	8.6	−	[a]	+	[a]
Stomach	24/M	20/F	−	0.2	+	43.6	−	[a]	+	[a]
Small intestine	24/M	20/F	−	0.0	+	50.4	−	1.0	+	147.1
Large intestine	24/M	20/F	−	0.0	+	37.8	−	2.9	+	57.4
Liver	84/M	20/F	−	1.5	+	24.5	−	1.2	+	265.0
Spleen	84/M	20/F	−	0.0	−	0.0	−	[a]	−	[a]
Pancreas	84/M	20/F	−	0.0	−	0.0	−	[a]	−	[a]
Adrenal gland	84/M	21/M	−	0.2	+	104.4	−	1.0	+	78.8
Thyroid	84/M	18/M	−	2.6	+	27.2	−	1.6	+	125.4
Thymus	[a]	20/F	[a]	[a]	[a]	[a]	−	1.4	−	16.4
Kidney	87/M	20/F	−	0.0	+	33.6	−	1.7	+	117.7
Urinary bladder	38/F	20/F	−	0.0	+	12.1	−	[a]	+	[a]
Skin	84/M	19/M	−	0.0	+	11.5	−	[a]	+	[a]
Mammary gland	15/F	[a]	−	0.8	+	134.0				
Muscle	15/F	[a]	−	0.0	+	10.5	[a]	[a]	[a]	[a]
Testis	24/M	19/M	−	2.1	+	25.4	−		+	
Prostate	24/M	[a]	−	0.8	+	9.3	[a]	[a]	[a]	[a]
Uterus profile	37/F	[a]	−	2.3	−	8.1				
Early secretory phase	51/F	[a]	−	2.5	+	12.4				
Middle secretory phase	43/F	[a]	−	3.7	+	70.2				
Late secretory phase	49/F	[a]	−	1.0	+	3.3				
Placenta		21/M	+	100.0	+	210.9				
Umbilical cord		19/M	−	0.2	+	56.0				

Data are expressed on the basis of GAPDH mRNA level (%). Intensity of staining was subjective; − represents no reactivity; + represents reactivity. M, Male; F, female.

[a] No specimens were available for study.

fetus (Fig. 2D), immunoreactivity was detected in the interstitial cells surrounding the urinary tubules, but not in the urinary tubules of adult kidney. EST immunoreactivity in the adult adrenal gland (Fig. 2E) was detected in the zonae fasciculata and reticularis of the adrenal cortex; in the fetal adrenal, positive immunostaining for EST was detected in the fetal cortex, but not in the definitive zone (Fig. 2F). EST immunoreactivity was also detected in the intimae and media of aorta, Leydig cells of testis (Fig. 2H), ductal epithelial cells of mammary gland, epithelial cells of urinary bladder, epithelial cells of endometrium, and placental syncytiotrophoblast (Fig. 2B).

Discussion

In this study, STS mRNA expression, enzyme activity, and immunoreactivity for STS were undetectable or were present at very low levels in both human adult and fetal tissues, whereas those of EST were detected in a wide range of adult and fetal tissues. Adult liver, testis, thyroid, lung, and aorta have all previously been reported to be associated with the expression of estrogen biosynthesis via the enzyme, cytochrome P450 aromatase (P450arom), an enzyme involved in the conversion of androgen into estrogen (30–34). P450arom immunoreactivity has also been reported in intima and media of aorta (34, 35), and Leydig cells in the testis (31). However, STS expression was found to be either absent or expressed at very low levels in those tissues described above. Two major pathways are believed to be involved in providing peripheral sources of E1 in the human adult and fetus. One is through the aromatization of testosterone or androstenedione to E1 or E2, which is considered to be a major pathway for peripheral E1 production. However, conversion of E1S to E1 by STS is also postulated to be present as a source of peripheral estrogen production, albeit in small amounts. STS enzyme in the adult liver and adrenal gland demonstrated relatively high amounts of enzyme expression. Yamamoto *et al.* (36) demonstrated that normal adult liver tissues had relatively high aromatase activity. Phornphutkul *et al.* (37) reported relatively high aromatase activity and expression in an adrenal adenoma with very low activity and expression of aromatase in non-neoplastic adjacent adrenal. In addition, the level of P450arom mRNA in normal adrenal tissue was reported to be below the level of detection by RT-PCR (37). Therefore, E1 produced as a result of STS activity in these tissues may exert some influence on the function of normal human adult liver and adrenal glands, but it awaits further investigations for clarification.

Dooley *et al.* (22) recently reported that EST gene (*1E1*) transcripts are present in normal male skin, intestine, lung, prostate, liver, brain, and ovary, but not in female esophagus, adrenal, kidney, and male trachea or stomach. It is well known that there are differences in plasma concentration of E1S between males and females (6). EST is known to consist of a number of isoenzyme forms (38), but Dooley *et al.* (22),

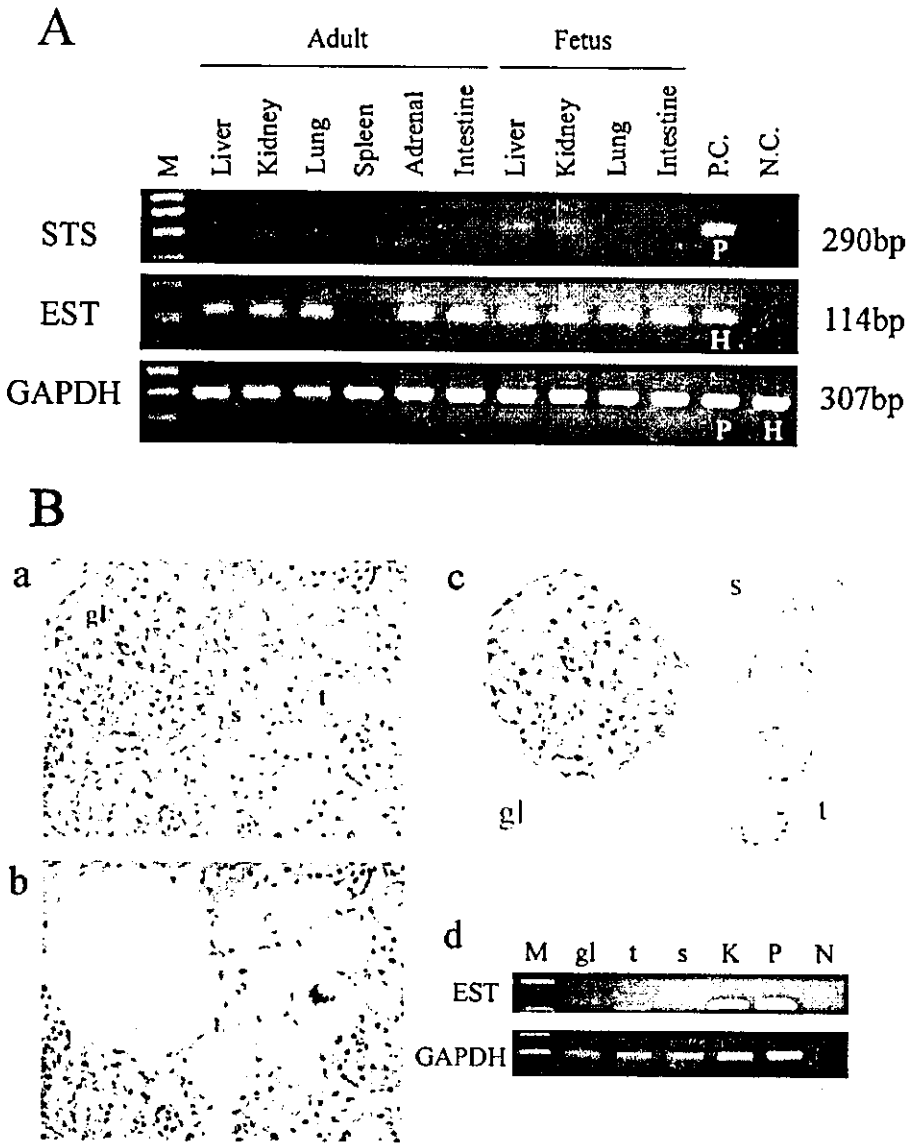

FIG. 1. Results of PCR analysis for STS and EST in human adult and fetal tissues. A, mRNA expression for STS, EST, and GAPDH was detected as a specific single band (290 bp for STS, 114 bp for EST, and 307 bp for GAPDH). In total, six adult tissues and four fetal tissues are shown. P.C., Positive control [placenta (P) for STS, and HuH7 (H) for EST]; N.C., negative control (no cDNA substrate); intestine, small intestine; M, 100-bp ladder. B, In human adult kidney (38-yr-old woman), glomerulus, urinary tubule, and stromal cells surrounding the urinary tubules were microdissected. Specimens are shown before (a) and after (b) microdissection and after laser pressure cell transfer (c). gl, Glomerulus; s, stromal cells; t, tubules. mRNA expression for EST was detected in urinary tubules (d, *top*, 114 bp), but not in the glomerulus and stromal cells. GAPDH was detected as a specific single band (d, *bottom*, 307 bp). K, Whole tissue of this kidney; P, positive control (HuH7); N, negative control (no cDNA substrate); M, 100-bp ladder.

in contrast to our present study, examined only the gene expression of this estrogen-metabolizing enzyme and did not examine enzyme activity. Differences between results of our study and Dooley's study may be due to the combination of these factors above; however, further investigations are required to clarify these discrepancies.

In contrast to STS, EST mRNA, immunohistochemistry, and activity were detected in a wide range of human tissues. These results appear to suggest that EST may play very important roles in the regulation of estrogen metabolism in various peripheral tissues in humans. In addition to observing EST mRNA expression in the adrenal gland, we detected EST immunoreactivity in the zonae fasciculate and reticularis of the adult adrenal and fetal cortex. However, EST activity was markedly low in the adrenal glands. Guinea pig adrenal cortical EST (gpEST) was purified and cloned as a pregnenolone binding protein by Oeda *et al.* (39) and Strott *et al.* (40). gpEST is also known to specifically bind to E2 with a relatively high affinity. Pregnenolone and E2 effectively compete for binding to gpEST, but pregnenolone, which is not sulfonated by gpEST, does not inhibit sulfonation of E2 (41). These results suggest that EST may participate in steroid production as a pregnenolone binding carrier protein in the human adrenal gland as described above. In addition, P450 side-chain cleavage, which catalyzes the conversion of cholesterol to pregnenolone, has been shown to be expressed in the fetal zone but not in the definitive zone in the early stage of gestation (42). Therefore, EST may be involved in functions other than sulfonation of estrogens, such as a pregnenolone binding protein described above in the human adrenal cortex. In the pancreas, expression of EST mRNA was low, but enzyme activity was found to be relatively high. The discrepancy between EST mRNA expression and activity detected in the pancreas suggests that other members of steroid

TABLE 3. Summary of enzyme assay and quantitative PCR

Tissues	Age (yr), sex	STS		EST	
		E1S to E1 (pmol/mg protein·min)	PCR	E1 to E1S (pmol/mg protein·min)	PCR
Trachea	38, F	0.051	0.0	1.606	18.9
Lung	38, F	0.015	1.0	0.132	45.2
Esophagus	38, F	−0.013	0.1	0.425	14.8
Stomach	38, F	0.093	0.1	0.526	29.3
Small intestine	38, F	0.149	0.0	0.287	56.0
	86, F	0.036	0.0	0.500	70.7
Large intestine	38, F	0.185	0.1	0.231	42.9
	86, F	0.307	0.0	1.406	30.9
Liver	38, F	0.244	0.9	0.225	36.3
	86, F	0.394	1.2	0.128	20.4
Spleen	38, F	0.108	0.0	0.061	0.6
Pancreas	38, F	0.302	0.0	1.389	0.1
Adrenal gland	38, F	0.282	0.0	0.215	161.2
Thyroid	38, F	0.034	1.9	0.091	30.0
Kidney	38, F	0.085	0.0	0.106	44.4
Urinary bladder	38, F	0.015	0.0	0.526	12.1
Placenta		8.784	[a]	0.812	[a]

PCR data are summarized as a ratio of GAPDH, and evaluated as a ratio (%) compared with that of positive control (placenta for STS, HuH7 for EST).

[a] No specimens were available for study.

sulfotransferase such as the monoamine sulfating form of phenol sulfotransferase may be expressed in the pancreas and may be functioning in the sulfonation of estrogens in this organ (13, 43, 44). Further investigations are required, however, to clarify the expression pattern and enzymology of steroid sulfotransferases in the pancreas.

In liver, kidney, and GI tract tissues, EST may play an important role in metabolizing excessive amounts of biologically active estrogens to biologically inactive sulfonated forms with marked hydrophilic property. In addition, proximal tubular cells of the kidney are also known to absorb E1S from the blood via multispecific organic anion transport pathways. Organic anion transporter (OAT)3 has recently been reported to be localized in the basolateral membrane of the proximal tubular (45), and very recently, a novel member of the OAT family, OAT4, was isolated in the kidney (46). Therefore, the proximal tubular cells in kidney can excrete E1S metabolized from E1S by EST. Moreover, E1S in human adult urine is known to be high (8). In the fetal kidney, the expression of EST immunoreactive protein was present in interstitial and stromal cells surrounding the urinary tubules, but not in the tubules or glomeruli. 17β-Hydroxysteroid dehydrogenase type 2 (17β-HSD2) has also been localized in the human fetal kidney, with a similar pattern of expression as described for EST above (47). EST and 17β-HSD2 in the fetal kidney may be functioning in the inactivation of estrogens, possibly leaking from the urinary tubules because of the immaturity of intercellular connections in the tubular epithelium.

Marked immunoreactivity for both 17β-HSD2 and EST was also detected in surface epithelial cells of both adult and fetal GI tract (47, 48). Her *et al.* (49) demonstrated that both EST and dehydroepiandrosterone sulfotransferase proteins are expressed in human jejunal mucosa. It is well established that orally administered E2 and testosterone are rapidly inactivated and do not necessarily enter the circulation to any significant degree in adults (50, 51). In addition, in the fetus, large quantities of estrogens are present in the amniotic fluid (52); the estrogens may enter the fetal circulation in an excessive quantity by swallowing and absorption from the GI tract unless a means of degradation and/or inactivation of estrogens as described above is present in epithelial cells of the fetal GI tract. Results from studies to date, including the present investigation, appear to suggest that EST and 17β-HSD2 expression in the GI tract may be involved in the rapid degradation and excretion of sex steroid in the surface epithelial cells of the GI tract in both the human adult and fetus. In our study, the levels of STS and EST mRNA expression in fetal tissues were relatively higher than those of adult tissues. The fetoplacental unit is known to produce and secrete large quantities of biologically active estrogens during pregnancy (3). Estrogens play important roles in fetal development, but excessive exposure to estrogens may cause abnormal development after birth (53). Therefore, peripheral STS/EST is considered to play very important roles in peripheral estrogen metabolism and actions in the human fetus.

EST has been shown to be expressed in the female, but not in the male rat liver (54). In this study, although the number of specimens available for examination was limited, both EST mRNA and protein were shown to be expressed in the human liver. In addition, there were no differences in the expression of EST between male and female livers. Hepatocytes have been reported to be associated with expression of liver-specific OAT and 17β-HSD2 (55, 56). Therefore, human liver is also considered to be involved in the excretion and metabolism of estrogens in a manner similar to that of kidney and GI tract described above. Sulfation of estrogens can indirectly influence the action of estrogens by regulating the levels of unconjugated estrogens that can bind to ERs. In the uterus and mammary gland, there are significant influences on the levels of unconjugated estrogen available for binding to ERs. EST activity in cultured normal cells has been reported to be high and exceeding that of breast cancer cell lines (57). Unconjugated estrogens result in an increased risk

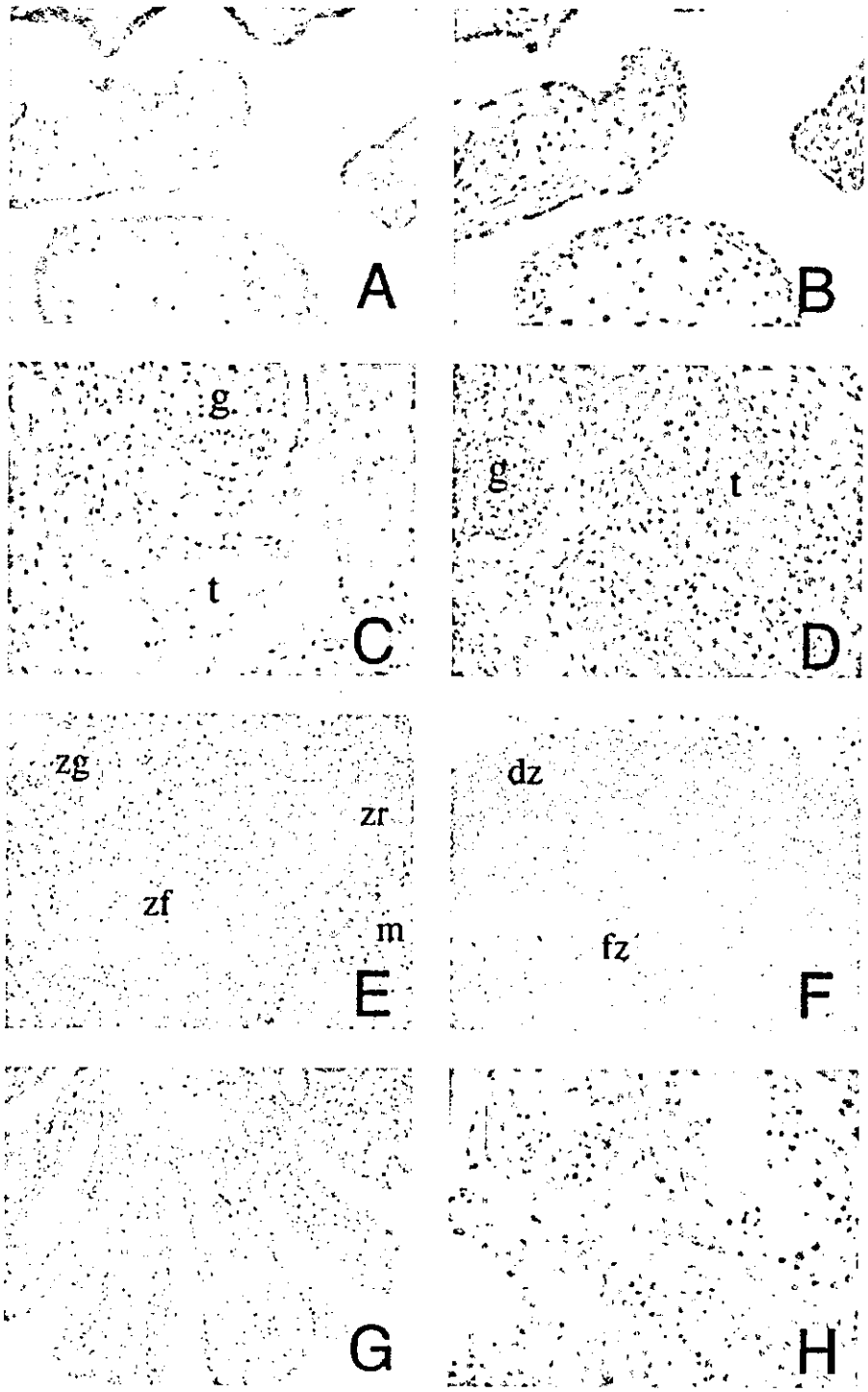

FIG. 2. Immunohistochemistry for STS and EST in adult and fetal tissues. Immunoreactive cells appear *brown* as a result of the DAB colorimetric reaction. Hematoxylin is used as the nuclear stain. STS (A) and EST (B) immunoreactivities were detected in the syncytiotrophoblast of placenta. In adult kidney (C), EST immunoreactivity was exclusively present in urinary tubules; in the fetus (D), EST was present in interstitial cells surrounding the urinary tubules but not in urinary tubules (t) or glomerulus (g). EST in the adult adrenal gland (E) was found to be immunopositive in the zona fasciculate (zf) and zona reticularis (zr) of the adrenal cortex, but not in the medulla (m) or zona glomerulus (zg). In the fetus (F), EST was shown to be expressed in the fetal zone (fz), but not in the definitive zone (dz). In addition, immunoreactive EST was detected in surface epithelial cells of adult small intestine (G) and Leydig cell of testis (H). Original magnification, A, B, C, D, E, F, and H, ×200; G, ×100.

of breast cancer, whereas estrogen sulfonation in normal breast tissues may serve as an important mechanism of inhibiting excessive estrogenic actions (57). Rubin *et al.* (58) reported that EST mRNA expression was lower during the follicular phase than during the secretory phase of the human menstrual cycle, and reached a plateau in the mid-secretory phase. Results from our present study are also consistent with the results described above. EST activity and expression have been reported to be induced by progesterone in the uterus (59–61). Furthermore, in the porcine uterus, EST activities were highest during the secretory phase of the menstrual cycle, suggestive of a possible correlation between

TABLE 4. Immunohistochemical localization of EST

Tissues	Adult	Fetus
Trachea	Surface epithelium	Surface epithelium
Aorta	Media	[a]
Esophagus	Surface epithelium	Surface epithelium
Stomach	Surface epithelium	Surface epithelium
Small intestine	Surface epithelium	Surface epithelium
Large intestine	Surface epithelium	Surface epithelium
Adrenal gland	Zona fasciculate to the reticularis	Fetal zone
Kidney	Urinary tubules	Interstitial
Urinary bladder	Surface epithelium	Surface epithelium
Skin	Epidermis	Epidermis
Mammary gland	Secretory epithelium	[a]
Testis	Leydig cell	Diffuse[b]
Prostate	Secretory epithelium	[a]
Uterus profile	[c]	
Early secretory phase	Endometrium epithelium	
Middle secretory phase	Endometrium epithelium	
Late secretory phase	Endometrium epithelium	
Placenta	Syncytiotrophoblast	

[a] No specimens were available for study.
[b] Immunoreactivity was detected, but no localization.
[c] No immunoreactivity.

uterine EST activity and plasma progesterone levels (62). These results may suggest that uterine EST expression and activity may be regulated by progesterone; however, further investigations are required to clarify the influence of progesterone and/or other sex steroids or hormones on the expression of EST in the human uterus.

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