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Expression of Sex Hormone-binding Globulin mRNA in Human Endometrial Cancers

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To more fully understand the role of sex hormone-binding globulin (SHBG) on the intracellular steroidal action in endometrial cancers, we investigated the expression of SHBG mRNA as the substitute of SHBG expression in human endometrial cancers. In the present study, the levels of SHBG mRNA were analyzed using competitive reverse transcription-polymerase chain reaction (RT-PCR)–Southern-blot analysis. The higher level of SHBG mRNA tended to be expressed in the normal secretory and late proliferative phase endometrium > early proliferative phase endometrium > well differentiated adenocarcinoma of the endometrium (G1) > moderately differentiated adenocarcinoma (G2) > poorly differentiated adenocarcinoma (G3), in the order shown. These studies indicate that endometrial cancer cells might synthesize intracellular SHBG to conserve their estrogen-dependent properties. Further, it indicates that endometrial cancer cell synthesis of SHBG mRNA is lost as these cells undergo de-differentiation.

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INTRODUCTION

Most endometrial cancers occur during the postmenopausal period when the production of absolute estrogen is low, while evidence for an important role for estrogens in the development and growth of endometrial and breast cancers has accumulated clinically [1]. Therefore, it is necessary to know the steroidal environments, including steroid binding proteins, and sources of estrogen in the tumors.

Previously it was assumed that the free fraction of serum steroid hormones was biologically active, and the sex hormone-binding globulin(SHBG)-bound fraction was not available in the target cells. During the last decade, the SHBG binding sites were detected in the cell membrane of human decidual endometrium [2] and prostate [3]. Immunocytochemical detection of SHBG has been reported in human endometrium, prostate [4], and breast tissue [5] in addition to the hepatic cells [6, 7]. Moreover, the expression of SHBG mRNA has been demonstrated in human endometrial and prostatic cancer cell lines [8, 9] and human normal endometrium [10]. This evidence has indicated that SHBG might be synthesized in the target cells, and directly play, in part, a role in intracellular steroidal actions. The aim of the present study was to demonstrate the SHBG mRNA expression in human endometrial cancers by competitive reverse transcription-polymerase chain reaction (RT-PCR), and discuss the manner of intracellular steroidal action in estrogen dependent growth of endometrial cancers from its mRNA expression.

EXPERIMENTAL

Materials

Normal endometria were obtained by hysterectomy from 18 patients with uterine leiomyoma, (aged 25–39 years, with regular menstrual cycle) from December 1993 to June 1994. Part of each specimen was submitted for endometrial dating histologically [11]. Agreements for the study were obtained from the patients and the Research Committee for human subjects, Gifu University School of Medicine. Endometrial cancer tissues were obtained from 21 postmenopausal patients (aged 50–56 years) who underwent hysterectomy. Part of these tissues were submitted for histological grading [12], and part immediately frozen in liquid nitrogen and later on prepared for the following sequence: RNA isolation and RT-PCR–Southern-blot analysis.

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Preparation of internal standard recombinant RNA (rcRNA)

A scheme for synthesis of internal standard recombinant RNA (rcRNA) is shown in Fig. 1. DNA construction of the internal standard was originated and synthesized by PCR from BamH/EcoRI fragment of v-*erb*B (Clontech Laboratories, Palo Alto, CA) with two sets of oligonucleotide primers containing T7 promoter and SHBG specific primer sequences. The sequence of the first set of primers for the first PCR was as follows: 5′-TGTAGAATCAAATCCCGGGACGCAAGTGAAATCTCCTCCG-3′, and 3′-GATGTTTGACGTAACTGTCTTTCCACCACAAGAGAAGACC-5′. The sequences of second sets of primers for secondary PCR were as follows: 5′-TAATACGACTCACTATAGGTGTAGAATCAAATCCCGGGA-3′, and 3′-TTCCACCACAAGAGAAGACC-5′ [13, 14]. The described two sets of primers were synthesized by Rikaken Co. Ltd (Nagoya, Japan).

The first PCR reaction was conducted in a final vol of 50 μl containing PCR buffer [50 mM, KCl; 10 mM, Tris–HCl, pH 8.3; 1.5 mM, $MgCl_2$], 0.2 mM deoxyribonucleoside triphosphates (dNTPs), 2 ng Bam H/Eco RI DNA fragment of v-*erb*B, 10 pmol each of the first set of PCR primers and 2.5 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The second PCR reaction was conducted in a final vol of 100 μl containing PCR buffer, 0.2 mM dNTPs, each 20 pmol of the second set of PCR primers and 5 units of Amplitaq DNA polymerase. The mixture was amplified in 28 cycles of PCR at 95°C for 45 s for denature, 60°C for 45 s for annealing, 72°C for 90 s for extension in a DNA Thermal Cycler (Perkin-Elmer Cetus).

The second PCR product was purified by Gene Clean II Kit (BIO 101 Inc, La Jolla, CA), and transcribed using 100 units of T7 RNA polymerase (Gibco BRL, Gaithersburg, MD) containing T3/T7 buffer [40 mM, Tris–HCl, pH 8.0; 8 mM, $MgCl_2$; 2 mM, spermidine-$(HCl)_3$; 25 mM, NaCl], 0.1 M dithiothrei-

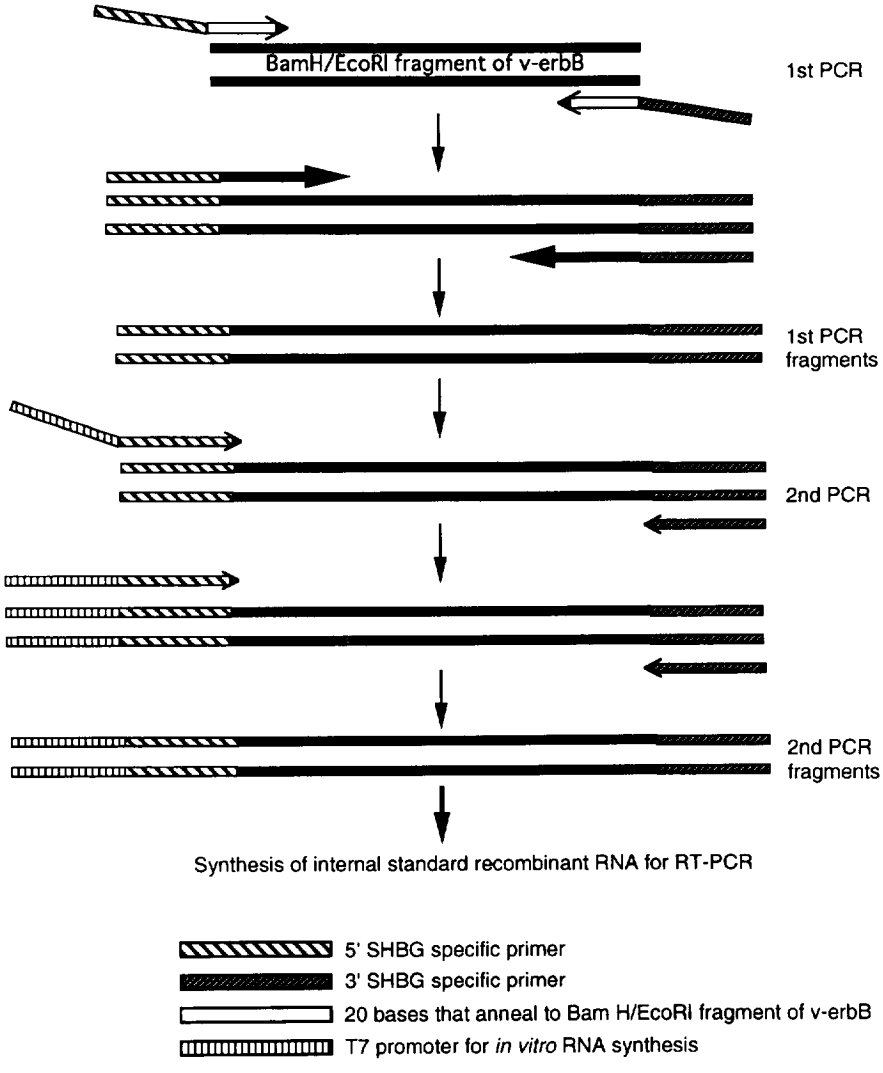

Fig. 1. Scheme for synthesis of internal standard recombinant RNA.

tol (DTT), 10 mM ribonucleoside triphosphates, 40 units of RNase inhibitor (Promega, Madison, WI), 20 nM template DNA, and 10 μ Ci of [α-^{32}P] UTP (New England Nuclear Co., Boston, MA) as a tracer in 100 μl vol. The reaction was incubated at 37°C for 1 h, treated with 70 units of RNase-free DNase (Takara Shuzo Co. Ltd, Kyoto, Japan) at 37°C for 5 min to remove the DNA template. Subsequently, the products were extracted with water-saturated phenol–chloroform, and passed through sephadex G50 column (Boehringer Mannheim, Germany). The amount of transcribed internal marker RNA was calculated with the total radioactivity of the transcribed RNA.

Competitive RT-PCR

Total RNA was isolated from tissues by the acid guanidium–phenol–chloroform extraction method as previously described [15].

Total RNA (3 μg) and a series of diluted recombinant RNA (10 pg) to obtain a standard curve every time were reverse-transcribed with a mixture of 200 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco BRL) and the following reagents: 50 mM, Tris–HCl, pH 8.3; 75 mM, KCl; 3 mM, $MgCl_2$; 40 units of RNAsin (Toyobo, Osaka, Japan); 10 mM, DTT (0.1 M); 0.5 mM, dNTPs; and 30 pmol 3′-end specific primer (SHBG-3′, as detailed below) in 20 μl vol for 1 h at 37°C to obtain a standard curve every time. The reaction was incubated for 5 min at 95°C to inactivate M-MLV reverse transcriptase. The sequences of primers to amplify SHBG gene were as follows: 5′-TGTAGAATCAAATCCCGGGA-3′ (SHBG-5′; 591–610, exon VII), 3′-TTCCACCA-CAAGAGAAGACC-5′ (SHBG-3′; 790–809, exon V) [13] (synthesized by Rikaken Co. Ltd). The sizes of PCR products for SHBG mRNA and internal standard rcRNA are 219 and 440 bp, respectively. PCR with reverse-transcribed RNAs as templates (1 μl) and 5 pmol of each specific primer was carried out using a DNA thermal cycler (Perkin-Elmer Cetus) with 0.5 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus) in a buffer containing 50 mM KCl, 10 mM, Tris–HCl, pH 8.3; 1.5 mM $MgCl_2$, and 0.2 mM dNTPs, in 20 μl vol. Amplification was performed in 38 cycles of PCR at 94°C for 45 s for denaturing, 55°C for 45 s for annealing and 72°C for 90 s for extension.

Southern-blot analysis

Amplified PCR products (8 μl) with 2 μl of loading dye mix were electrophoresed with 1.2% GTG agarose gels (FMC BioProducts, Rockland, ME) in an 100 V constant voltage field for 50 min, and capillary-transferred to a nylon membrane (Immobilon-S; Millipore, Burlington, MA) for 20 h using 10 × standard sodium citrate solution (SSC; 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). After blotting, the membrane was dried at 75°C and then cross-linked by ultraviolet irradiation (33,000 μ J/cm^2 at 254 nm). The membrane was prehybridized in hybridization buffer (1 M NaCl, 50 mM Tris–HCl, pH 7.6, 1%SDS) at 42°C for 2 h, then in the same solution with biotinylated SHBG gene specific oligonucleotide probe (5′-TTCTCTTT-GGACCTGGGACT-3′) and biotinylated internal standard gene specific oligonucleotide probe (5′-TGT-TATACAGGGAGATGAAA-3′) simultaneously, and then hybridized with biotinylated SHBG-5′ (10 pmol/μl, synthesized by Rikaken Co. Ltd) at 42°C for 24 h. The membrane was washed with 2 × SSC for 15 min at room temperature, then with 2 × SSC for 15 min at 42°C, and finally washed with 0.5 × SSC for 15 min at 42°C. The detection reaction for hybridized biotin was performed using the Plex Chemiluminescent Kit (New England BioLabs, Beverly, MA). The membrane was exposed to Kodak XAR-5 films (Eastman Kodak, Rochester, NY) for 15 min. The strength of the recorded signal on film was analyzed densitometrically using Bio Image (Millipore Corporation, Bedford, MA).

Statistics

Statistical analysis was performed with one-way ANOVA for SHBG mRNA levels in normal endometria, and Student's t-test for those in endometrial cancers. Differences were considered significant when P was less than 0.05. All data were expressed as mean $\pm$ SD.

RESULTS

SHBG mRNA expression level in human endometria and endometrial cancers

In the competitive RT-PCR and Southern-blot analysis for SHBG mRNA, only two predicted bands were detected without non-specific products. The amount of SHBG mRNA was measured using a standard curve, a series of rcRNA dilutions, in competitive RT-PCR as shown in Fig. 2.

In normal uterine endometria, SHBG mRNA was detected in all cases and the level of SHBG mRNA at early proliferative phase (3.3 $\pm$ 1.9 pg rcRNA/mg total RNA) tended to be lower than at late proliferative phase (5.7 $\pm$ 3.4) and secretory phase (6.3 $\pm$ 1.9) of the menstrual cycle [no significant difference by one-way ANOVA ($P > 0.05$)] (Fig. 3).

In endometrial cancers, SHBG mRNA was detected in all cases of well-differentiated adenocarcinoma (G1, 10/10), moderately differentiated adenocarcinoma (G2, 5/5) and poorly differentiated adenocarcinoma (G3, 4/6) in 38 cycles of PCR. The higher level of SHBG mRNA was expressed in G1 (1.34 $\pm$ 0.80) > G2 (0.47 $\pm$ 0.27) > G3 (0.01 $\pm$ 0.01), in turn. These expression differences between G1 and G3, and between G2 and G3 were significant ($P < 0.01$ and $P < 0.02$, respectively) (Fig. 4). However, that between G1 and G2 was not ($P = 0.06$). On the other hand, the SHBG

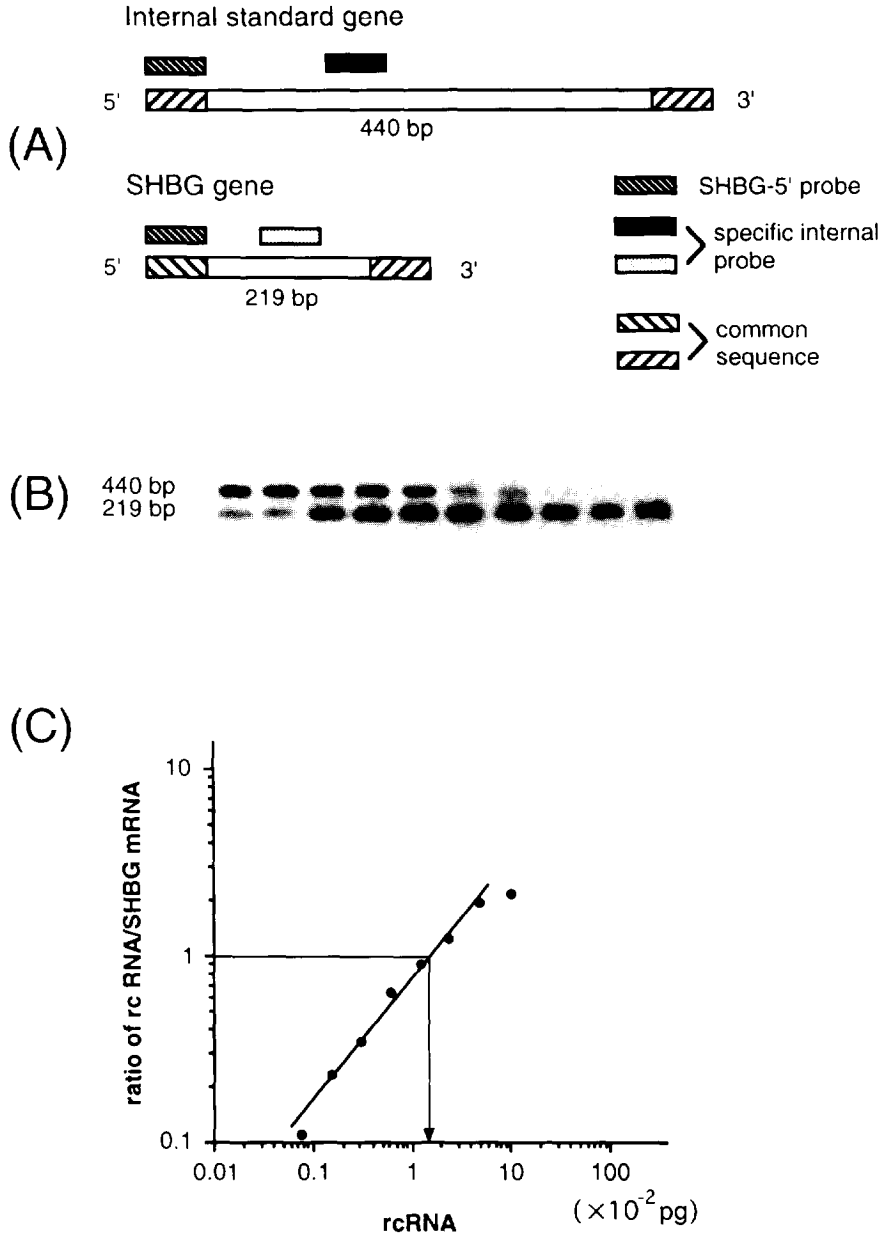

Fig. 2. Quantitative analysis of SHBG mRNA in the normal endometrium by competitive RT-PCR–Southern-blot analysis. Strategy for Southern-blot to detect two specific genes and analyze the intensity of two bands (A) and result of Southern-blot hybridization (B). The RT-PCR reactions containing SHBG specific primers, in the presence of total RNA and serial diluted rcRNA at the range of 10^{-3} to 10×10^{-2} pg were carried out. The signal intensity in Southern-blot was determined. Data are plotted to calculate the level as log ratio of rcRNA/SHBG mRNA vs. log rcRNA (C).

mRNA level in G1 was significantly ($P < 0.02$) lower than that in the early proliferative endometrium.

DISCUSSION

Some endometrial cancers develop under estrogen dominant milieu. Although decreasing ovarian estrogen secretion during the postmenopausal period results in low concentrations of circulating estradiol-17β (E_2), the incidence of endometrial cancers rises. In menopausal women, estrone sulfate (E_1S) is the major estrogen in the circulation [16–18], although biologically inactive. As a result of over-hydrolysis with the conversion of E_1S to E_1 and E_2 in tumors, intracellular estrogens may increase and stimulate the growth of endometrial cancer cells [19]. Moreover, it has been reported that the E_2 concentration in endometrial cancers is higher than that in normal endometria [20].

Generally, SHBG binds estrogen with high affinity as a transporter for steroid hormones in the circulation. However, in the last 10 years, several studies have suggested that SHBG–steroid complex also plays a direct role for the intracellular steroidal interaction in

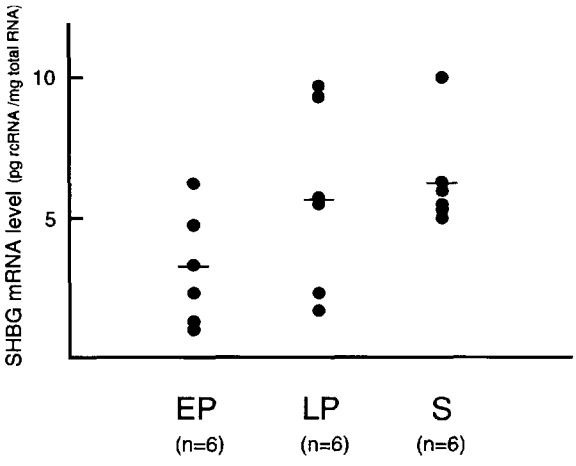

Fig. 3. The level of SHBG mRNA in normal uterine endometria during the menstrual cycle. SHBG mRNA levels were expressed as pg rcRNA/mg total RNA. EP, early proliferative phase endometrium; LP, late proliferative phase endometrium; S, secretory phase endometrium.

the steroid targeted cells, as mentioned previously [2–9]. Therefore, it is logical that cell-associated SHBG might influence the development and growth of endometrial cancers.

Formerly, the SHBG mRNA in normal uterine endometrium was present in a copy concentration too low to be evaluated by Northern-blot analysis [10]. Therefore we necessarily performed RT-PCR. In the present study, we used RT-PCR–Southern-blot analysis to detect the SHBG mRNA in endometrial cancers, which had a lower copy number of SHBG mRNA than normal uterine endometria. In endometrial cancers, the subclone or mutation of SHBG mRNA might be present, which was not determined in the present study. However, such abnormal SHBG mRNA is unlikely to contribute to the normal function of SHBG. Therefore, the recognition of normal SHBG mRNA expression in the present study might be considered useful to evaluate normal SHBG regulation and function in endometrial cancers. The accurate quantitation of mRNA expression can be achieved by competitive RT-PCR–Southern-blot analysis, in which the target RNA in the sample and the internal control rcRNA could be reverse-transcribed and amplified with the same time and efficiency. In the competitive RT-PCR–Southern-blot analysis, only two predicted bands have been detected without non-specific products. We confirmed that two such bands were identified with the signals from SHBG mRNA and rcRNA using each internal probe (data not shown). We used the biotinylated SHBG-5′ as the oligonucleotide probe to evaluate two signal interactions in the Southern-blot analysis with exactly the same efficiency, because the PCR products from rcRNA and target tissues have only the SHBG primer sequence in common.

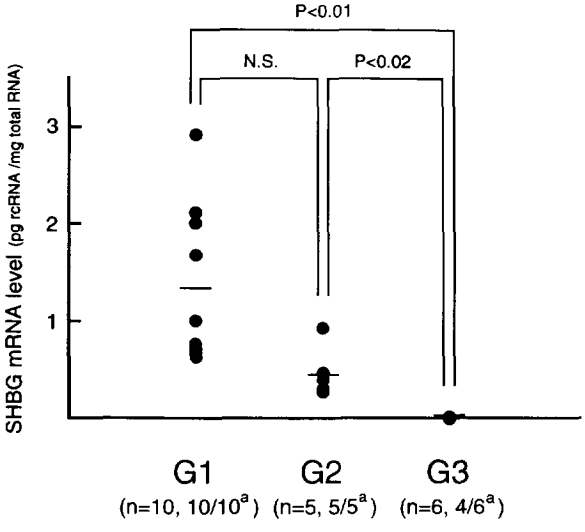

Fig. 4. The level of SHBG mRNA in uterine endometrial cancers. SHBG mRNA levels were expressed as pg rcRNA/mg total RNA. G1, well differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma; a, detective rate.

It has been reported that the concentration and detective rate of steroid receptors tend to decrease as the tumor de-differentiates from G1 to G3 in the endometrial cancers [21, 22]. This tendency was similar to that of SHBG mRNA level. This evidence indicates that the normal steroid responsiveness related to the intracellular interactions with steroid binding proteins gradually decreases with the advance of de-differentiation.

The intracellular interaction of estrogen and endometrial SHBG has been observed in premenopausal endometrium [10]. In the postmenopausal period, normal uterine endometrium becomes atrophic with decrease of serum estrogen and subsequent depletion of estrogen receptor. Whether it is premenopausal or not, G1 endometrial cancer still conserves the mechanism of intracellular estrogenic action, with the estrogen receptor levels being preserved approximately within normal range [23] and SHBG expression to a lesser extent in the present study. Therefore, locally synthesized estrogen can exert an effect on the growth of cancer cells as paracrine control.

While the endometrial SHBG might be synthesized by estrogen [10] and involved as a storehouse of estrogen in the regulatory system of intracellular steroidal action, it is plausible that the synthesis of SHBG in endometrial cancers might be regulated by estrogen, which is locally synthesized to a certain extent even in a small amount of serum estrogen in the postmenopausal period, and SHBG might be involved in the proliferation and development of some estrogen-dependent endometrial cancers.

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