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Original article

Levels of sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) messenger ribonucleic acid (mRNAs) in ovarian endometriosis

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Summary — Recently, much evidence has indicated that sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) play a role in the intracellular action of sex steroids in target cells. In the present work, expression of SHBG mRNA and CBG mRNA was demonstrated in tissues of human normal endometrium and pelvic endometriosis, using the reverse transcription-polymerase chain reaction (RT-PCR). SHBG mRNA levels were higher in pelvic endometriosis than in normal endometrium ($P < 0.02$), while CBG mRNA levels were lower than in normal endometrium ($P < 0.05$). The SHBG mRNA/CBG mRNA ratio was significantly higher in pelvic endometriosis than in normal endometrium ($P < 0.01$). These findings suggest that overexpression of intercellular SHBG in endometriotic tissues results in the formation of the estrogen-predominant milieu, since SHBG-bound estrogen is considered to be protected from the metabolism in liver and available in endometrial cells, thereby assisting the development of the pelvic endometriosis.

sex hormone-binding globulin / corticosteroid-binding globulin / mRNA / endometriosis

Résumé — Niveaux des ARN messagers de la globuline liant la testostérone (ARNm SHBG) et de la transcortine (CBG) dans l'endométriose ovarienne. *Récemment, plusieurs travaux ont montré que la SHBG et la CBG jouent un rôle dans l'interaction intracellulaire de stéroïdes sexuels dans les cellules cibles. Dans cette étude, l'expression des ARNm de la SHBG et de la CBG a été mise en évidence dans des biopsies d'endomètre humain normal et des prélèvements d'endométriose pelvienne par transcription réverse puis amplification en chaîne par la polymérase. La concentration de l'ARNm SHBG est plus élevée dans l'endométriose pelvienne que dans l'endomètre normal ($P < 0,02$), les résultats inverses sont observés pour l'ARNm CBG ($P < 0,05$). Le rapport ARNm SHBG/ARNm CBG est significativement plus élevé dans l'endométriose pelvienne que dans l'endomètre normal ($P < 0,01$). Étant donné que l'œstrogène lié à la SHBG semble être protégé du métabolisme hépatique et rendu disponible dans les cellules endométriales, toutes ces constatations suggèrent que, dans les tissus d'endométriose, l'augmentation de l'expression de la SHBG intracellulaire produisant un milieu où l'œstrogène est prédominant accentuerait un développement de l'endométriose pelvienne.*

globuline liant les hormones stéroïdes sexuels / transcortine / ARNm / endométriose

INTRODUCTION

The proliferation, differentiation, and development of normal uterine endometrium is regulated by sex steroids. In some cases, when endometrial tissue exhibits aberrant growth, which occurs ectopically in various locations in the pelvic cavity (endometriosis), it exhibits the cyclic functional responsiveness of normal endometrium. In other cases an immature or unripe variety develops, which is only responsive to the estrogenic stimulus and not to progesterone (Dizerega *et al,* 1980).

Estrogen-induced growth of endometriosis might be partly explained by the following evidence. Most endometriosis is less responsive to progestogens, due to the low level of progesterone receptor relative to that of estrogen receptor (Tamaya *et al,* 1979), while progestogen has an antiestrogenic effect (Dorfman *et al,* 1961).

Recently, a great deal of evidence has indicated that sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) play a role in the intracellular action of sex steroid hormones in target cells. The expressions of SHBG and CBG have been demonstrated immunohistochemically in human endometrium, prostate (Mercier-Bodard *et al,* 1987) and breast tissue (Sinnecker *et al,* 1990), and in pituitary (Perrot-Applanat *et al,* 1984) and thyroid glands (Kuhn *et al,* 1986). Moreover, mRNA expressions of SHBG and CBG have been analyzed in human endometrial cancer cell lines (Mercier-Bodard *et al,* 1991) and rhesus monkey testis (Hammond *et al,* 1987a) by Northern blot hybridization.

These results prompted us to investigate the expression of SHBG and CBG mRNA in order to know the mechanism of estrogen-induced growth of endometriosis.

MATERIALS AND METHODS

Materials

Human uterine endometria were obtained by endometrial biopsy from 35 patients (aged from 25 to 39 years) with a regular menstrual cycle at the Department of Obstetrics and Gynecology, Gifu University School of Medicine from July 1990 to June 1993. Part of the specimen was submitted for histological dating (Noyes *et al,* 1950). Agreements for the study were obtained from patients and from the Research Committee on Human Subject of Gifu University School of Medicine. Pelvic endometriosis tissues were obtained from 6 patients who underwent conservative or definitive surgery for ovarian endometriosis. These specimens were immediately frozen in liquid nitrogen and prepared for the following procedures, such as RNA isolation, Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR).

Poly(A)$^+$RNA isolation

Frozen tissue (100 mg) was ground, transferred to a Teflon-glass homogenizer containing 1 ml lysis/binding buffer (100 mM Tris-HCl pH 8.0, 500 mM LiCl, 10 mM EDTA (ethylenediaminetetraacetic acid) pH 8.0 1% SDS (sodium dodecyl sulfate), 5 mM DTT (dithiothreitol) and homogenized manually at 4°C. After spinning at 20 000 *g* for 30 s, the supernatant was transferred to an Eppendorf tube. Polyadenylated mRNA (poly(A)$^+$RNA) was isolated from tissue with magnetic beads, Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) (Jacobsen *et al,* 1990). The poly(A)$^+$RNA concentration was determined by UV absorption at 260 and 280 nm.

Preparation of labeled probe

SHBG cDNA (1 143 bp) and CBG cDNA (1 215 bp) were synthesized from human liver poly(A)$^+$RNA (Clontech Laboratories, Palo Alto, CA) using the PCR (as detailed below for the PCR). The primers used for SHBG cDNA synthesis using PCR were

5'-CAGCACACCCGCCAGGGATGGGCC-3' (SHBG-5':1-24, Exon I) and 3'-CCGTTACCGT-GACTGCGAAGGGTA-5' (SHBG-3':1120-1143, Exon VIII) (Hammond *et al*, 1987b). The primers used for CBG cDNA synthesis were 5'-ATGC-CACTCCTCCTGTACAC-3' (CBG-5': 1-20, Exon II) and 3'-TGAACCCAGTGTAAGAGACC-5' (CBG-3':1205-1224, Exon V) (Hammond *et al*, 1987a). The DNA probe was labeled with biotinylated dUTP using a Polar Plex Random Primer Biotin Labelling Kit (Millipore, Burlington, MA).

Northern blot analysis

Poly(A)+RNA (45 μg) was denatured at 65°C for 15 min, size-fractionated by electrophoresis through 1% agarose-formaldehyde gel, and blotted onto a nylon membrane (Immobilon-S; Millipore) by capillary transfer for 20 h using 10 x standard saline citrate (SSC: 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). After blotting, the membrane was dried at 75°C for 20 min and then cross-linked by ultraviolet irradiation (33 000 μJ/cm^2 at 254 nm). Prehybridization was performed at 42°C for 4 h in a mixture containing 5 x SSC, 50% formamide, 2 x Denhardt's reagent, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. The same solution was used for the hybridization but included the biotin-labeled probe (26 ng/ml). After hybridization at 42°C for 24 h, the membrane was washed for 20 min at room temperature and then finally washed twice with 0.1 x SSC for 30 min at 65°C. The detection reaction using a Plex Chemiluminescent Kit (New England BioLabs, Beverly, MA). The membrane was exposed to Kodak XAR-5 films (Eastman Kodak, Rochester, NY) for 15 min.

Reverse transcription

Poly(A)+RNA (10 ng) was reverse transcribed for 1 h at 42°C with a mixture of 600 units of M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) and the following reagents: 50 mM Tris-HCl buffer pH 8.3; 75 mM KCl; 15 mM $MgCl_2$; 40 units of RNAsin (Promega, Madison, WI); 10 mM DTT; 0.5 mM dNTP mix; 1.5 μg oligo $d(T)_{12-18}$ (Pharmacia, Uppsala, Sweden); and 3 μg acetylated bovine serum albumin in 50 μl volume. The reaction mixture was incubated for 5 min at 95°C to inactivate M-MLV reverse transcriptase.

Polymerase chain reaction

The primers used to amplify SHBG DNA fragments were: 5'-TGTAGAATCAAATCCCGGGA-3' (SHBG-5'; 591-610, Exon V) and 3'-TTCCAC-CACAAGAGAAGACC-5' (SHBG-3'; 790-809, Exon VII) (Hammond *et al*, 1987b). The size of PCR products for SHBG mRNA was 219 bp (synthesized by Ricaken Co Ltd). The primers for CBG DNA fragments were synthesized: 5'-ATGACCTTGGAGATGTGCTG-3' (CBG-5'; 929-948, Exon IV) and 3'-TGAACCCAGTGTAAGA-GAAC-5' (CBG-3'; 1205-1224, Exon V) (Hammond *et al*, 1987a). The size of PCR products for CBG mRNA was 296 bp. The primers to amplify glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were 5'-TGAAGGTCGGAGT-CAACGGATTTGGT-3' (G3PDH-5'; 71-96, Exon I) and 3'-CACCACCTG GAGTACCGGGTGTAC-5' (G3PDH-3'; 1053-1030, Exon VIII) (Arcali *et al*, 1984) (Clontech Laboratories, Palo Alto, CA). The size of the PCR product for G3PDH mRNA was 983 bp.

PCR with reverse transcribed poly(A)+RNAs as templates (1 μl) and 5 pmol of each specifc primer was carried out using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) with 0.5 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus) in a buffer containing 50 mM KCl, 10 mM Tris-HCl buffer pH 8.3, 1.5 mM $MgCl_2$ and 0.2 mM dNTPs in 20 μl volume.

Each PCR cycle consisted of 1 min at 94°C for denaturation, 2 min at 60°C for annealing and 3 min at 72°C for extension with a DNA Thermal Cycler (Perkin-Elmer Cetus). Thirty-eight cycles of PCR for SHBG mRNA, 31 cycles for CBG mRNA and 23 cycles for G3PDH were performed after a serial dilution of cDNAs reverse transcribed to obtain the appropriate range of linear amplification of each PCR product.

Gel electrophoresis

An aliquot of amplified PCR products (8 μl) after addition of 2 μl of loading dye mix (0.25% bromophenol blue and 30% glycerol in distilled water) was electrophoresed on 2% NuSieve 3:1 agarose (FMC BioProducts, Rockland, ME) gels in Tris-borate/EDTA buffer (45 mM Tris-borate, pH 8.0, 1 mM EDTA) in a 100 V constant-voltage field for 50 min. The strength of photographed ethidium bromide-staining PCR products was analyzed densitometrically by calculating the area with total

integrated optical density (IOD) using Bio Image (Millipore Corporation, Bedfold, MA). IOD shows arbitrary units calculated by Bio Image.

DNA sequence

Amplified PCR products were electrophoresed on 2% agarose gels. The SHBG and CBG cDNA fragments were isolated from excised gel slices using a QIAEX agarose gel extraction kit (Qiagen, Hilden, Germany) and inserted in pT7 Blue T-vector (Novergen, Madison, WI). After transformation of pT7 Blue T-vector with insertion into Nova Blue competent cells (Novergen) and amplification of the cells, double-stranded plasmid DNA was isolated. Both strands of PCR fragments were treated with a Circum Vent Thermal Cycle Deoxy DNA-sequencing Kit (New England Bio Labs, Beverly, MA) with biotinylated M13/pUC reverse sequencing primer and biotinylated T7 promoter primer, and were sequenced by electrophoresis on denaturing polyacrylamide gels (5% Hydrolink Long Ranger gel; AT Biochem, Malvern PA) at a constant power of 75 W for 3 h. After transfer of sequencing DNA fragments to a nylon membrane (Immobilon-S; Millipore), the membrane was dried, and UV cross-linked. The sequencing DNA bands were detected with a Plex 5 Chemiluminescent Subkit (New England BioLabs).

Statistics

The levels of mRNA was compared by a Student's *t*-test. Correlation coefficients were determined by Spearman's rank test. Differences were considered to be significant at $P < 0.05$. All data were expressed as mean ± SD.

RESULTS

SHBG mRNA and CBG mRNA in uterine endometrium

A single dominant form of SHBG mRNA of 1.6 kb was detected by the biotinylated

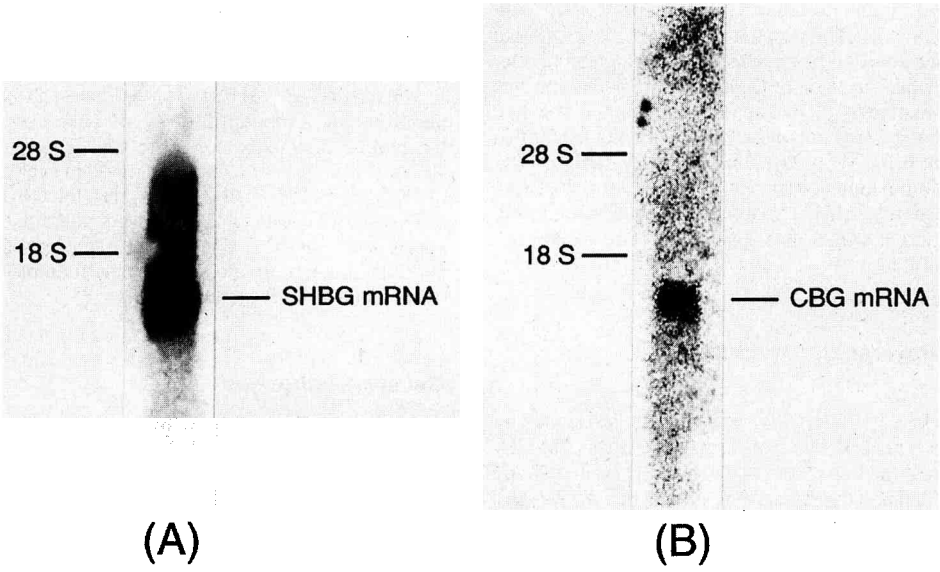

Fig 1. Northern blot analysis of SHBG and CBG mRNA in human endometrium. (A) SHBG mRNA, (B) CBG mRNA. Poly(A)+RNA was extracted from human endometrial tissues in the secretory phase, analyzed by the Northern blotting technique, and hybridized with SHBG and CBG cDNAs. The biotin-labeled probe was used as described in *Materials and methods.*

SHBG DNA probe in human endometrium and was the same in size as SHBG mRNA in the endometrial carcinoma cells (Mercier-Bodard *et al,* 1991) (fig 1A). A single dominant form of CBG mRNA of approximately 1.6 kb was also detected in human endometrium and was the same size as CBG mRNA in rhesus monkey liver (Hammond *et al,* 1987a) (fig 1B). Lower copies of SHBG and CBG mRNAs in endometrium were present at too low concentrations for evaluation by Northern blot analysis, so we performed RT-PCR. Amplified SHBG and CBG mRNAs were detected with the expected size in the uterine endometrium and the pelvic endometriosis in all samples (fig 2), and DNA sequences of both PCR products were identical to those of SHBG and CBG cDNAs (fig 3). In other words, SHBG and CBG mRNA were detected in endometriotic tissues in addition to normal endometrium.

SHBG mRNA and CBG mRNA levels in endometriosis

IOD for CBG, SHBG and G3PDH mRNAs levels obtained by RT-PCR were plotted on a log–log scale against the serial dilution of SHBG, CBG and G3PDH cDNA. There is a good linear relationship between the

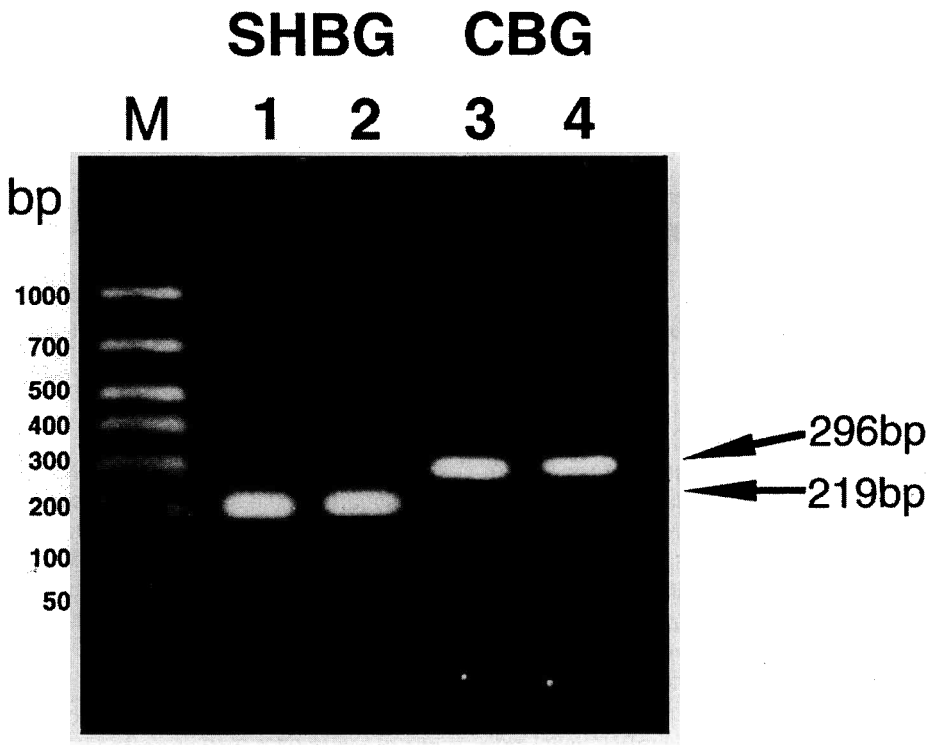

Fig 2. Analysis of PCR products by gel electrophoresis. SHBG and CBG PCR products are visualized with ethidium bromide staining. Each cDNA was diluted 1:1 in sterile water. Lane 1 shows SHBG PCR product of human uterine endometrium in the secretory phase. Lane 2 shows SHBG PCR product of endometriotic tissue. Lane 3 shows CBG PCR product of human uterine endometrium in the secretory phase. Lane 4 shows CBG PCR product of endometriotic tissue. M represents the molecular marker BioMarker Low (Bioventures Inc, Murfreeboro, TN).

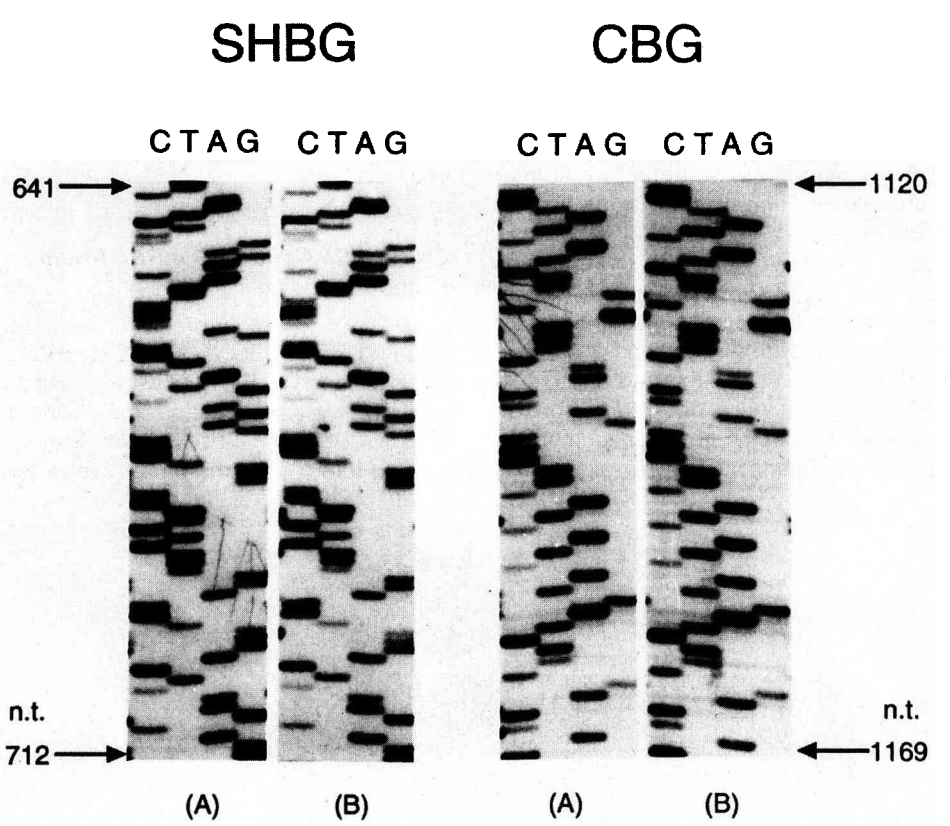

Fig 3. Partial DNA sequencing of SHBG and CBG cDNA in human endometrium **(A)** and endometriosis **(B)**.

amount of input template and the output measurement (fig 4). Relative quantification of mRNA was obtained from the IOD on the graph of SHBG and CBG, based on the individual dilution of cDNA, giving an IOD equivalent to the IOD 0.5 on G3PDH graph. After standardization, the mRNA level was assigned as a corrected IOD value. The relative amount of SHBG mRNA after standardization to the G3PDH mRNA level indicated that the endometrial SHBG mRNA level was significantly higher in the secretory phase (1.46 ± 0.37 corrected IOD) than ($P <$ 0.02) in the proliferative phase (0.38 ± 0.26 corrected IOD) of the mentrual cycle (fig 5).

The relative amount of CBG mRNA was investigated in the same way as described above (fig 4) and the endometrial CBG mRNA level was also significantly higher in the secretory phase (0.45 ± 0.15 corrected IOD) than ($P < 0.05$) in the proliferative phase (1.26 ± 0.81 corrected IOD) of the menstrual cycle (fig 5).

The level of SHBG mRNA in endometriotic tissue (4.07 ± 2.40 corrected IOD) was higher than that in normal secretory phase endometrium. On the other hand, the level of CBG mRNA in the endometriotic tissues (0.27 ± 0.17 corrected IOD) was lower than that in secretory phase endometrium (fig 5).

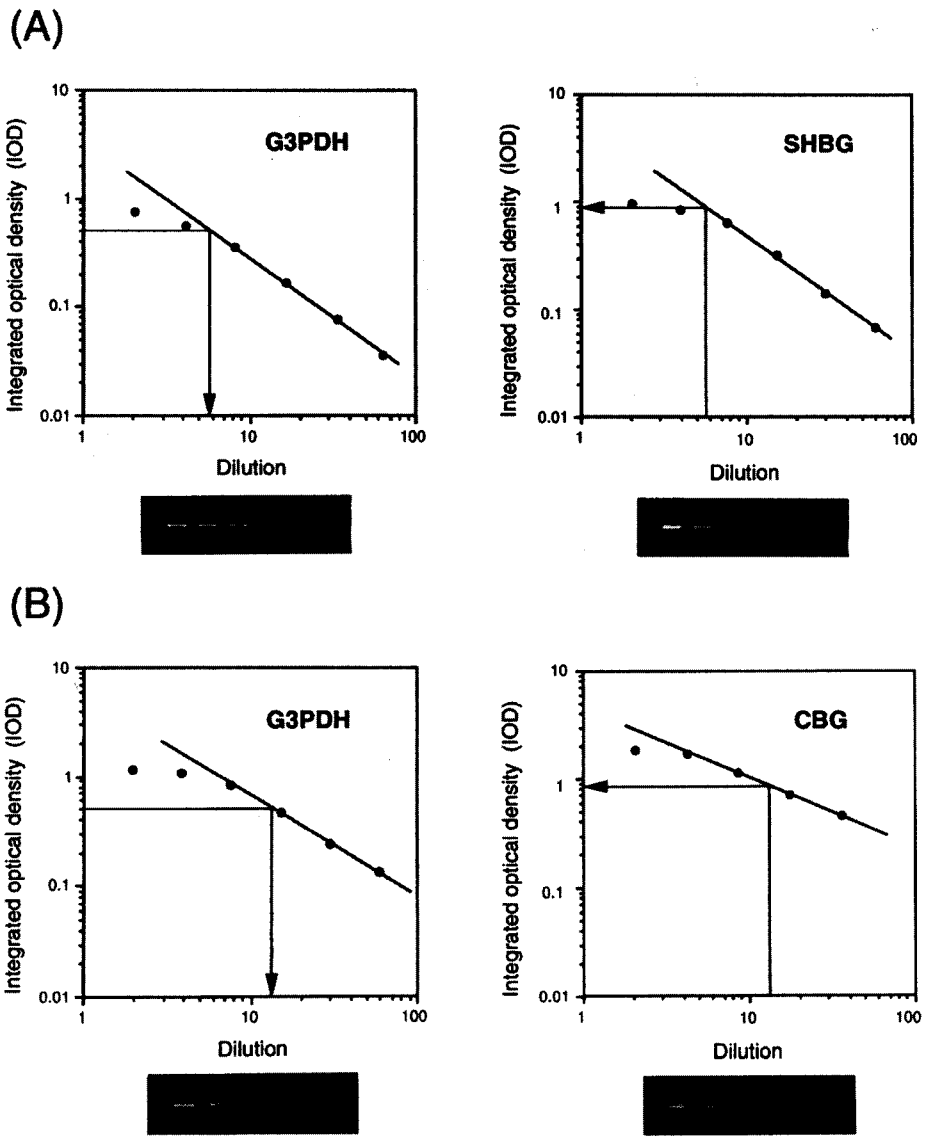

Fig 4. Quantitative analysis of SHBG and CBG mRNA levels in human endometrium. (**A**) In the upper panel, the results from analysis of G3PDH cDNA and SHBG cDNA with serial dilutions are shown in the left and right panels, respectively. In the lower panel, the photograph of the ethidium bromide stained gel demonstrates the linearity of amplification by serial dilution. (**B**) The results of G3PDH cDNA and CBG cDNA are presented in the same manner as **A**.

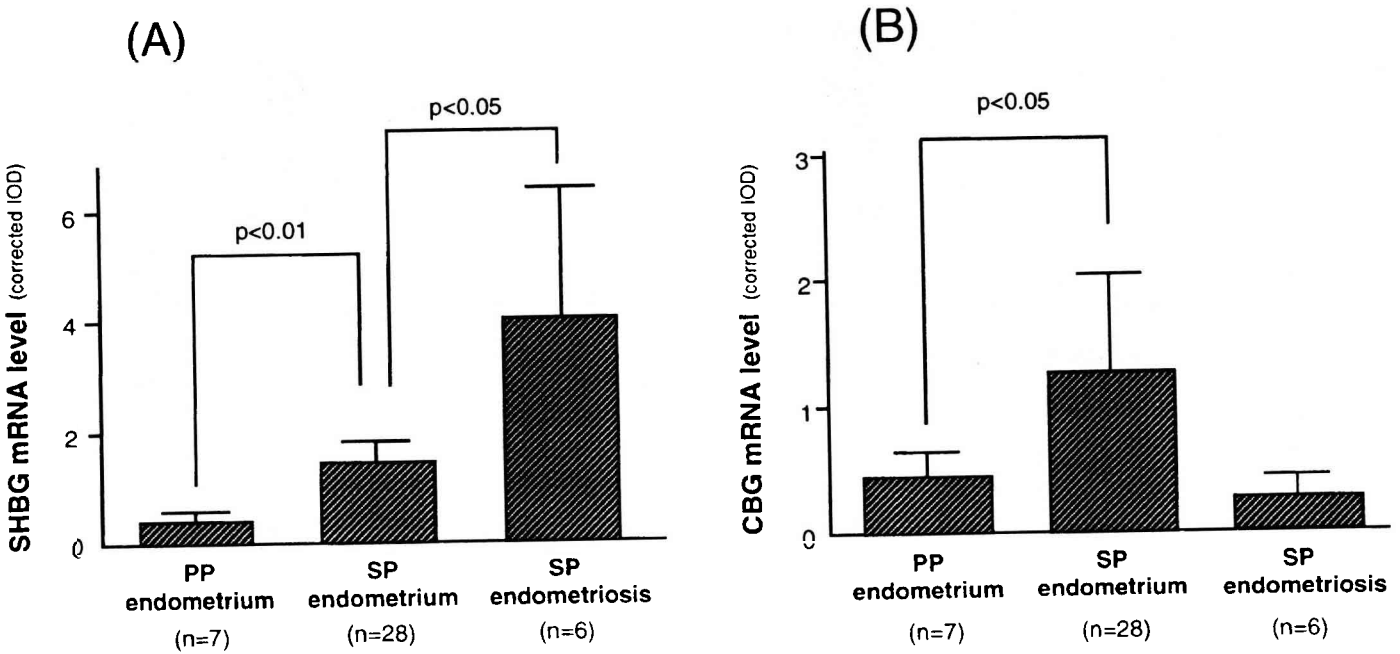

Fig 5. Comparative study on relative quantitation of SHBG **(A)** and CBG **(B)** mRNA levels in the normal endometrium and endometriosis. These mRNA levels were expressed as a value of corrected integrated optical density (corrected IOD). PP: proliferative phase, SP: secretory phase.

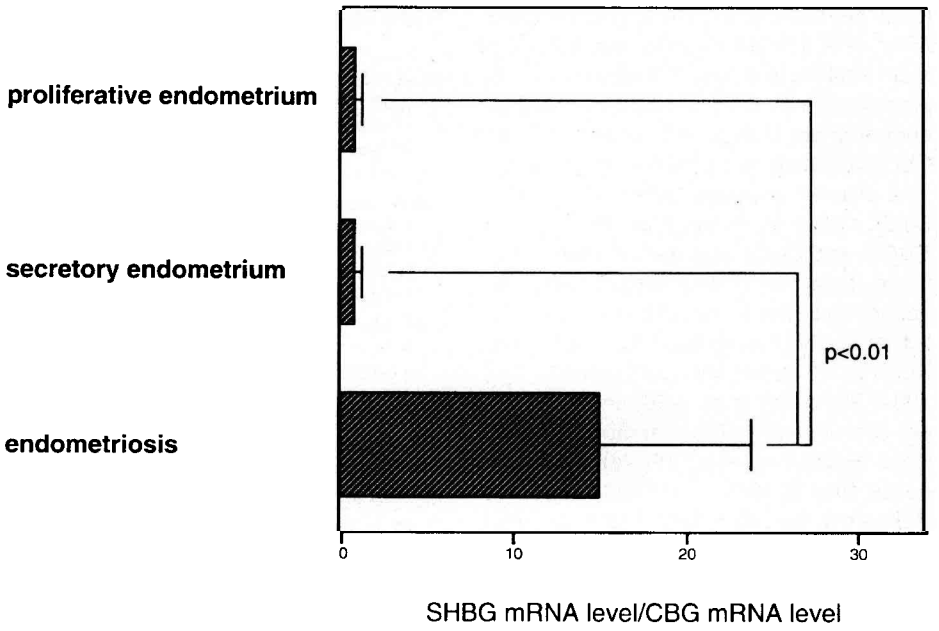

Fig 6. SHBG mRNA/CBG mRNA ratio in the normal uterine endometrium and the endometriosis.

Moreover, the ratio of SHBG mRNA level to CBG mRNA level was derived as the average of the individual ratio from each tissue. The ratio of SHBG mRNA/CBG mRNA in endometriosis was approximately 20 times higher than that in the normal endometrium (fig 6).

DISCUSSION

Estrogen-induced growth of endometriotic lesions has been documented even under the influence of progesterone during the regular menstrual cycle. In the female reproductive tract, the cooperative interaction of estrogen and progesterone (progestogen) plays an important role in biological events. In particular, progestogen has antiestrogenic effects, inhibiting cellular proliferation and bringing about cellular differentiation. Therefore, the substances binding estrogen and progestogen should be investigated in the study of steroid action mechanisms in reproduction.

SHBG and CBG are plasma glycoproteins that bind steroid hormones such as estrogen and androgen (SHBG, Mercier-Bodard *et al,* 1970) and corticosteroid and progesterone (CBG, Seal and Doe, 1966) with relatively high affinity. It has been assumed that the steroid–glycoprotein complex is inactive and only the free steroids are biologically active at the target cells (Hoffmann *et al,* 1969; Vermeulen and Ando, 1979). However, in recent studies, SHBG and CBG coupling to membrane-binding sites, which activate adenylate cyclase and lead to cellular accumulation of cAMP (Nakhla *et al,* 1988; Nakhla *et al,* 1990), has been demonstrated in human decidual endometrium

(Strel'chyonok *et al,* 1984) and prostate (Hryb *et al,* 1985) for SHBG, and in human liver (Strel'chyonok and Avvakumov, 1983), prostate (Hryb *et al,* 1986) and decidual endometrium (Avvakumov *et al,* 1988) for CBG indicating that protein-bound steroid may also be available (Siiteri *et al,* 1982; Selby, 1990). Moreover, the expression of SHBG and CBG, and their mRNAs, has been detected in the target cells (as described in the *Introduction*) immunohistochemically (Perrot-Applanat *et al,* 1984; Kuhn *et al,* 1986; Mercier-Bodard *et al,* 1987; Sinnecker *et al,* 1990) and by Northern blot analysis (Mercier-Bodard *et al,* 1991; Hammond *et al,* 1987a). This suggests that SHBG and CBG might be involved in the regulatory system of some steroid actions as an intracellular reservoir or buffer which regulates the free fraction of steroid hormones in their target cells (Mercier-Bodard *et al,* 1991). In addition, the synthesis of endometrial SHBG and CBG might be complexly regulated by steroid hormones such as estrogen and progesterone, in a manner different from that in the liver (Misao *et al,* 1994a, b).

The expression of SHBG and CBG mRNAs has been documented in the normal endometrium and the endometriosis, indicating that SHBG and CBG are synthesized in target tissue cells.

The expression of SHBG mRNA appears to be higher in endometriosis than in normal endometrium, indicating that SHBG is synthesized more in the endometriosis than CBG. In addition, it is assumed that intracellular SHBG is more involved as a store of estrogen in the regulatory system of steroidal action in the endometriotic cells, while the ratio of the SHBG mRNA level to that of CBG mRNA is much higher in endometriosis than in the endometrium. Therefore, estrogen might be more stored intracellularly due to the abundance of SHBG, thus providing the cellular estrogen-predominant milieu.

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