Effect of medroxyprogesterone acetate on sex hormone-binding globulin mRNA expression in the human endometrial cancer cell line Ishikawa

Ryou Misao, Yoshihito Nakanishi, Jiro Fujimoto and Teruhiko Tamaya

Department of Obstetrics and Gynecology, Gifu University School of Medicine, Gifu, Japan

(Correspondence should be addressed to Ryou Misao, Department of Obstetrics and Gynecology, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500, Japan)

Abstract

To understand the rationale of high-dose medroxyprogesterone acetate (MPA) in the treatment of well-differentiated uterine endometrial cancers, the effect of MPA on intracellular sex hormone-binding globulin (SHBG) mRNA expression in well-differentiated uterine endometrial cancer cell line Ishikawa was determined by competitive reverse transcription-polymerase chain reaction-Southern blot analysis. Estradiol-17 β (E2, 10⁻⁸ mol/l) did not alter SHBG mRNA expression, but the addition of 10⁻¹⁰ mol/l MPA increased it, while a high concentration of MPA (10⁻⁶ to 10⁻⁵ mol/l) with or without E2 suppressed it. Furthermore, a high dose (10⁻⁶ mol/l) of chlormadinone acetate or danazol with or without E2 significantly suppressed its expression, while MPA was the most effective among the hormones tested. The effect of MPA and the other steroid hormone analogs on SHBG expression was not mediated via the progesterone receptor.

These findings suggest that intracellular SHBG suppression might partly contribute to the abolition of the intracellular estrogen-dominant milieu, and may be involved as one of the mechanisms of the antitumoral effects of high-dose MPA on the development and growth of some well-differentiated endometrial cancer cells.

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Introduction

Generally speaking, sex hormone-binding globulin (SHBG), a plasma glycoprotein, binds estrogen and androgen with high affinity, playing a role in regulating their free concentration in serum. However, SHBG binding sites have been demonstrated in cell membranes of human decidual endometrium (1) and prostate (2). Immunocytochemical detection of SHBG has been reported in cells of human endometrium, prostate (3) and breast tissue (4). The expression of SHBG mRNA has been detected in human normal endometrium (5), ovarian endometriosis (6), prostatic carcinoma cell lines (7), placenta (8) and ovarian tumors (9). Additionally, the expression of SHBG mRNA has been demonstrated in human endometrial cancer cell lines (10) and uterine endometrial cancers, especially in cases of well-differentiated adenocarcinoma (11). These studies indicate that SHBG synthesized within uterine endometrial cancer cells plays a direct role in intracellular steroidal actions.

Some endometrial cancers develop and grow under an estrogen-dominant milieu and regress in response to progestin therapy (12). High-dose medroxyprogesterone acetate (MPA) treatment has been widely used for

advanced or recurrent endometrial cancers. The cellular immunohistochemical staining intensity of estradiol in endometrial cancers appears to decrease after MPA treatment (13).

These data suggest that MPA might be involved in steroidal actions, at least in part, via intracellular SHBG cascades in endometrial cancer tissues. To understand the rationale of MPA treatment for endometrial cancers, we investigated the effect of MPA and other steroids on intracellular SHBG expression (mRNA) in well-differentiated endometrial cancer cell line Ishikawa using competitive reverse transcription-polymerase chain reaction (RT-PCR) under the influence of sex steroids.

Materials and methods

Chemicals

Estradiol-17 β (E2), progesterone, MPA, chlormadinone acetate, cortisol and norethindrone (ENT, 17 α -ethynyl-17 β -hydroxyestra-4-en-3-one) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Danazol (17 α -pregn-4-en-20-yno [2, 3-*b*] isoxazol-17-ol) was obtained from Sterling-Winthrop (New York, NY, USA).

Onapristone (ZK 98,299 [11 β -(4-dimethylaminophenyl) 17-hydroxy-17 β -(3-hydroxy-propyl)-13-methyl-4,9-gonadien-3-one]) was obtained from Schering AG (Berlin, Germany). Promegestone (R5020, 17 α ,21-dimethyl-19-nor-pregnane-4,9-diene-3,20-dione-[17 α -methyl-³H], 86.3 Ci/mmol) was purchased from New England Nuclear Corporation (Boston, MA, USA). All other chemicals and reagents were of experimental grade.

Cell cultures

Ishikawa cells, derived from well-differentiated adenocarcinoma of the endometrium (14), were cultured in Eagle's minimum essential medium (EMEM, Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, NY, USA) treated with 1% dextran-coated charcoal, then with 1% penicillin (Banyu Pharmaceutical Co. Ltd, Tokyo, Japan) in a humidified atmosphere with 5% CO₂. Afterwards, the media were switched to phenol red-free EMEM without FBS. Forty-eight hours later, subconfluent Ishikawa cells were incubated with sex steroids for 1, 3, 6, 12, 24, and 48 h in the steroid concentrations indicated in the figures for each experiment. These hormones in 0.05% ethanol were solubilized in ethanol and added every 12 h in order to maintain hormone levels. Solvent alone was added to the control cells. The cell number/well was approximately 1×10^5 and the cell viability was confirmed at over 95% by trypan blue stain before the experiment and after 48 h.

Progesterin binding assay

All subsequent steps were performed at 4°C. Cells (2×10^6) were homogenized with 2 ml TEG buffer (10 mmol/l Tris-HCl pH 7.4, 1.5 mmol/l ethylenediaminetetraacetic acid (EDTA), 10% glycerol). The amount of DNA was determined in the 100 μ l aliquot of the suspension before the first centrifugation by the method of Burton (15). The suspension was centrifuged at 800 *g* for 10 min and the supernatant was centrifuged at 160 500 *g* for 60 min. This supernatant, designated the cytosol fraction, was treated with the same volume of 0.3% dextran-coated charcoal (DCC) for 15 min to remove all free steroids.

The 800 *g* pellet was resuspended in 0.5 ml KCl buffer (10 mmol/l Tris-HCl pH 8.5, 1.5 mmol/l EDTA, 10% glycerol, 0.6 mol/l KCl) for 60 min with vigorous mixing every 15 min, and centrifuged at 90 300 *g* for 30 min. This supernatant, designated the nuclear KCl extract fraction, was treated with 0.3% DCC for 15 min. The 90 300 *g* pellet was homogenized in 1 ml TEG buffer and designated the nuclear KCl insoluble fraction.

Each soluble fraction was diluted with an adequate volume of TEG buffer at a concentration of at least 1 mg protein/ml to avoid interference by the various proteins

of each sample. Protein levels in the samples were measured by the method of Bradford (16).

Each fraction (500 μ l) was incubated with 0.2 to 5.0 nmol/l [³H]R5020 plus a 200-fold molar excess of cortisol with or without a 200-fold excess of ENT for 1 h at 20°C. The bound form of the soluble fractions (cytosol and nuclear KCl extract fractions) was determined according to the 0.3% DCC absorption method, and that of the insoluble fraction (nuclear KCl insoluble fraction) by washing and pelleting (17). Scatchard plot analysis (18) was carried out. Progesterone receptor concentrations were expressed as fmol/ μ g DNA.

Preparation of internal standard recombinant RNA (rcRNA)

Following the procedures described in our previous study (9), the synthesis of internal standard rcRNA was performed. DNA construction of the internal standard was originated and synthesized by PCR from the BamH/EcoRI fragment of v-erbB (Clontech Laboratories, Palo Alto, CA, USA) with two sets of oligonucleotide primers containing T7 promoter and SHBG specific primer sequences. The sequences of the first set of primers for the first PCR were as follows: 5'-TG TAGAATCAAA TCCCGGGACGCAAGTGAAATCTCTCCG-3', and 3'-GATGTTTGACGTAAGTGTCTTTCCACCACAAGAGAAG ACC-5'. The sequences of the second set of primers for secondary PCR were as follows: 5'-TAATACGACTCACT ATAGGTGTAGAATCAAATCCCGGGA-3', and 3'-TTCCA CCACAAGAGAAGACC-5' (19, 20). The two sets of primers were synthesized by Rikaken Co. Ltd (Nagoya, Japan).

The first PCR was conducted in a final volume of 50 μ l containing PCR buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl pH 8.3, 1.5 mmol/l MgCl₂), 0.2 mmol/l deoxyribonucleoside triphosphates (dNTPs), 2 ng BamH/EcoRI DNA fragment of v-erbB, 10 pmol each of the first set of PCR primers and 2.5 units Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). The second PCR was conducted in a final volume of 100 μ l containing PCR buffer, 0.2 mmol/l dNTPs, 20 pmol each of the second set of PCR primers and 5 units Amplitaq DNA polymerase. The mixtures were amplified for 28 cycles of PCR at 95°C for 45 s for denaturing, 60°C for 45 s for annealing, and 72°C for 90 s for extension in a DNA Thermal Cycler (Perkin-Elmer Cetus).

The second PCR product was purified with a Gene Clean II Kit (BIO 101 Inc, La Jolla, CA, USA), and transcribed using 100 units T7 RNA polymerase (Gibco BRL, Gaithersburg, MD, USA) in a final volume of 100 μ l containing T3/T7 buffer (40 mmol/l Tris-HCl pH 8.0, 8 mmol/l MgCl₂, 2 mmol/l spermidine-(HCl)₃, 25 mmol/l NaCl), 0.1 mol/l dithiothreitol (DTT), 10 mmol/l ribonucleoside triphosphates, 40 units RNase inhibitor (Promega, Madison, WI, USA), 20 nmol/l template DNA, and 10 μ Ci [α -³²P]UTP (New

England Nuclear Corp.) as a tracer. The reaction was incubated at 37 °C for 1 h, and then treated with 70 units RNase-free DNase (Takara Shuzo Co. Ltd, Kyoto, Japan) at 37 °C for 5 min to remove the DNA template. Subsequently, products were extracted with water-saturated phenol/chloroform, and passed through a Sephadex G50 column (Boehringer Mannheim, Mannheim, Germany). The amount of transcribed internal marker RNA was calculated from the total radioactivity of the transcribed RNA.

Competitive RT-PCR

Total RNA was isolated from the cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (21). To obtain a standard curve each time, the total RNA (3 µg) and a series of diluted recombinant RNA (10^{-1} to 10^{-3} pg) were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RT, 200 units, Gibco BRL) in 50 mmol/l Tris-HCl pH 8.3, 75 mmol/l KCl, 3 mmol/l MgCl₂, 40 units RNasin (Toyobo, Osaka, Japan), 10 mmol/l DTT, 0.5 mmol/l dNTPs, and 30 pmol 3'-end specific primer (SHBG-3', as detailed below) at 37 °C for 1 h. The reaction was incubated for 5 min at 95 °C to inactivate MMLV-RT.

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 Amplitaq DNA polymerase (Perkin-Elmer Cetus) in a buffer containing 50 mmol/l KCl, 10 mmol/l Tris-HCl pH 8.3, 1.5 mmol/l MgCl₂, and 0.2 mmol/l dNTPs. Amplification was performed for 38 cycles 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 (SBA)

Amplified PCR products were applied to 1.2% agarose gel for electrophoresis performed at 100 V, and capillary-transferred to a nylon membrane (Immobilon-S; Millipore, Burlington, MA, USA) for 20 h using 10× standard sodium citrate solution (SSC; 1.5 mol/l NaCl, 0.15 mol/l sodium citrate, pH 7.0). After blotting, the membrane was dried at 75 °C and then cross-linked by ultraviolet irradiation (33 000 µJ/cm² at 254 nm). The membrane was prehybridized in hybridization buffer (1 mol/l NaCl, 50 mmol/l Tris-HCl pH 7.6, 1% sodium dodecyl sulfate) at 42 °C for 2 h, then in the same solution with the biotinylated SHBG gene-specific oligonucleotide probe and biotinylated internal standard gene-specific oligonucleotide probe simultaneously to detect specific genes, or hybridized with biotinylated SHBG-5' probe (10 pmol/µl, Rikaken Co. Ltd) to detect their precise intensities at 42 °C for 24 h (Fig. 1). 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 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, USA). Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA) was exposed to the membrane for 15 min. The strength of the recorded signal on film was analyzed densitometrically using Bio Image (Millipore Corporation, Bedford, MA, USA).

Western blotting

Cells (1×10^6) were washed three times with phosphate-buffered saline, pH 7.4. After removal of the supernatant, 500 µl extraction buffer (10 mmol/l Tris-HCl

Figure 1 Strategy for Southern blot analysis to detect two specific genes and analyze the intensity of two bands.

pH 7.4, 150 mmol/l NaCl, 0.5% Triton X-100, 0.2 mmol/l phenylmethylsulfonyl fluoride) were added to the pellet, and the cells were incubated on ice for 45 min, and then centrifuged. The supernatants were used for Western blot experiments. All samples were reduced in the presence of mercaptoethanol and subsequently separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes for 2.5 h at 60 V in buffer containing 25 mmol/l Tris-HCl pH 8.3, 192 mmol/l glycine and 20% methanol. The membrane was incubated successively with mouse anti-human SHBG (Biogenesis, UK), followed by incubation with horseradish peroxidase-conjugated donkey anti-mouse IgG at room temperature for 1 h. The detection reaction was performed using enhanced chemiluminescence detection reagent (Amersham International plc, Amersham, Bucks, UK). Kodak XAR-5 film (Eastman Kodak) was exposed to the membrane for 5 min. The strength of the recorded signal on film was analyzed densitometrically using Bio Image (Millipore Corporation). Moreover, Western blotting with mouse anti-human actin (Biogenesis) as an internal marker was performed using same sample-detected SHBG protein.

Statistics

Statistical analysis was performed with one-way analysis of variance (ANOVA), followed by Fisher's test for multiple comparisons. Differences were considered significant when P was less than 0.05. Data were expressed as the mean $\pm$ s.d.

Results

In the competitive RT-PCR-SBA, only two predicted PCR products (219 and 440 bp) were detected without non-specific products in Ishikawa cells. The amount of SHBG mRNA was determined using a standard curve of a serial dilution of rcRNA by competitive RT-PCR-SBA, as shown in Fig. 2.

In the dose-response curve showing the effect of sex steroids on SHBG mRNA expression in Ishikawa cells, E2 did not alter SHBG mRNA levels after incubation for 12 h. On the other hand, MPA dose-dependently up to 10^{-5} mol/l significantly ($P < 0.05$) suppressed the SHBG mRNA levels (Fig. 3). MPA (10^{-10} mol/l) with E2 (10^{-8} mol/l) increased SHBG mRNA levels after incubation for 12 h (Fig. 4). In contrast, a high concentration of MPA (10^{-6} to 10^{-5} mol/l) with E2 (10^{-8} mol/l) significantly ($P < 0.05$) suppressed the levels (Fig. 4).

Even in postmenopausal women, extraglandular estrogen is synthesized and has some effect on the uterine endometrium. Therefore, the concentration of E2 was set at 10^{-8} mol/l to work estrogen receptor cascades, although E2 did not alter the SHBG mRNA level. The experimental concentrations of MPA, 10^{-8} mol/l or 10^{-6} mol/l, were set to mimic, respectively, the

Figure 2 Quantitative analysis of SHBG mRNAs in Ishikawa cells by competitive RT-PCR-SBA. The RT-PCR reactions for total RNA and serial-diluted SHBG rcRNA in the range 10^{-4} to 10^{-1} pg were carried out. The precise intensities of amplified PCR products in Southern blot were determined. In the upper panel, data to calculate the SHBG mRNA levels are plotted as the log ratio of rcRNA/SHBG mRNA in total RNA isolated from samples. The lower panel shows the Southern blot after competitive RT-PCR.

physiological and therapeutic status of MPA for the following experiments.

The effect of E2 and MPA on progesterone receptor concentration in Ishikawa cells was investigated. E2

Figure 3 Dose-response curve showing the effect of E2 (●) or MPA (○) on expression of SHBG mRNA in Ishikawa cells. Ishikawa cells were incubated in MEM medium with increasing concentrations (10^{-10} to 10^{-5} mol/l) of E2 or MPA for 12 h. The SHBG mRNA levels are expressed as pg internal standard recombinant RNA (rcRNA)/mg total RNA. Values are means $\pm$ s.d. of 5 experiments. * $P < 0.05$ compared with the respective control (untreated) group.

Figure 4 Dose-response curve showing the effect of MPA with E2 on expression of SHBG mRNA in Ishikawa cells. Ishikawa cells were incubated in the medium with 10^{-8} mol/l E2 plus increasing concentrations (10^{-10} to 10^{-5} mol/l) of MPA for 12 h. The SHBG mRNA levels are expressed as pg rcRNA/mg total RNA. Values are means $\pm$ s.d. of 5 experiments. * $P < 0.05$ compared with the control (untreated) group.

(10^{-8} mol/l) induced an increase in progesterone receptor concentration after incubation for 24 h (Fig. 5). On the other hand, neither the high nor the low dose of MPA had an effect on progesterone receptor concentration.

In the time-course study of the effect of sex steroids on SHBG mRNA expression in Ishikawa cells, E2 (10^{-8} mol/l) alone did not alter SHBG mRNA levels, but MPA (10^{-6} mol/l) alone significantly suppressed SHBG mRNA levels time-dependently from 3 h to 12 h (Fig. 6). MPA (10^{-10} mol/l) with E2 (10^{-8} mol/l) increased SHBG mRNA levels up to 12 h. In contrast, the high concentration of MPA (10^{-6} mol/l) with E2 (10^{-8} mol/l) induced a temporary increase in the levels up to 3 h, followed by a decrease to a level lower than that of the control from 6 h to 48 h.

To investigate the correlation between SHBG mRNA levels and SHBG protein levels in Ishikawa cells, Western blot analysis was carried out. Ishikawa cells were incubated in MEM medium with 10^{-8} mol/l E2, 10^{-6} mol/l MPA, and 10^{-8} mol/l E2 plus 10^{-6} mol/l MPA or 10^{-10} mol/l MPA for 72 h. Polyclonal anti-SHBG antibodies recognized a protein of 44 kDa in the protein extracts prepared from Ishikawa cells. This result demonstrated that the changes in SHBG protein levels paralleled the changes in mRNA levels (10^{-8} mol/l E2, $89.5 \pm 17.1\%$ of control; 10^{-6} mol/l MPA, $53.9 \pm 9.8\%$; 10^{-8} mol/l E2 plus 10^{-6} mol/l MPA, $59.2 \pm 11.8\%$; 10^{-8} mol/l E2 plus 10^{-10} mol/l MPA,

Figure 5 The effect of E2 and MPA on progesterone receptor (PR) concentration in Ishikawa cells. Ishikawa cells were incubated in MEM medium with 10^{-8} mol/l E2, 10^{-6} mol/l MPA or 10^{-10} mol/l MPA for 24 h. PR concentrations are expressed as fmol/ μ g DNA. Values are means $\pm$ s.d. of 5 experiments. ** $P < 0.01$ compared with the control group.

$157.9 \pm 21.1\%$) (Fig. 7). On the other hand, polyclonal anti-actin antibodies used as an internal marker recognized a protein of 43 kDa in the protein extracts and their bands show identical intensity.

The effects of other hormones on SHBG mRNA expression in Ishikawa cells were as follows: a high concentration (10^{-6} mol/l) of MPA ($38.1 \pm 10.4\%$ of control), progesterone ($46.3 \pm 6.7\%$), chlormadinone acetate ($61.2 \pm 6.7\%$), or danazol ($56.0 \pm 7.5\%$) with 10^{-8} mol/l E2, and MPA ($40.3 \pm 4.7\%$), progesterone ($47.8 \pm 7.4\%$), chlormadinone acetate ($70.1 \pm 10.3\%$), or danazol ($55.2 \pm 3.7\%$) without 10^{-8} mol/l E2 suppressed SHBG mRNA levels after incubation for 24 h. MPA suppressed SHBG mRNA levels to a significantly ($P < 0.05$, Student's *t*-test) greater extent than chlormadinone acetate and danazol, and to a slightly greater extent than progesterone, independently of the presence of E2 (Fig. 8). The effect of MPA, progesterone, chlormadinone acetate and danazol on SHBG mRNA expression was not antagonized by onapristone.

Discussion

We investigated the rationale of using high-dose MPA treatment of endometrial cancers from the standpoint of the mechanism of SHBG action. Progestin treatment produces a relatively high response rate among well-differentiated endometrial adenocarcinomas (12, 22), which express a large amount of steroid receptors (23) and SHBG (11). Well-differentiated endometrial adenocarcinoma cell line Ishikawa, containing estrogen and progesterone receptors (14), was the most likely candidate for this study. SHBG mRNA in the normal

Figure 6 Time course for the effects of E2, MPA, or MPA plus E2 on SHBG mRNA expression in Ishikawa cells. (A) Ishikawa cells were incubated in MEM medium with 10^{-8} mol/l E2 or 10^{-6} mol/l MPA for 48 h. (B) Ishikawa cells were incubated in MEM medium with 10^{-8} mol/l E2 plus 10^{-6} mol/l MPA or 10^{-8} mol/l E2 plus 10^{-10} mol/l MPA for 48 h. SHBG mRNA levels are expressed as pg rcRNA/mg total RNA. Values are means $\pm$ s.d. of 5 experiments. * $P < 0.05$ compared with the respective control group.

uterine endometrium as well as in Ishikawa cells (10) is expressed in a copy number too low to be evaluated by Northern blot analysis (5). Competitive RT-PCR-SBA gives us an accurate quantitation of mRNA expression, in which the target RNA in the sample and the synthesized rcRNA as the internal control are reverse-transcribed and amplified simultaneously with the same efficiency. In the competitive RT-PCR-SBA, we confirmed SHBG gene specificity using inter-primer probes (data not shown), and detected their signal intensity using SHBG-5' probe as shown in Fig. 1.

Colony formation in Ishikawa cells treated with high-dose MPA alone was not significantly different from that in those treated with high-dose MPA combined with norethindrone as a luteohormone or RU-486 as an antiprogesterone (24). Therefore, the antitumoral effects of high-dose MPA might be less derived from the progesterone receptor cascades than from cytotoxic effects on the tumor cell (12). Increased oxidative activity of 17β -hydroxysteroid dehydrogenase (25) inducing the conversion of estradiol to estrone (26), suppression of adrenal androgen synthesis and reduction in estrogens as the metabolites of the peripheral conversion (27, 28), or inhibition of angiogenesis (29, 30) might be relevant. The effect of MPA on intracellular SHBG could be another mechanism of action.

Ishikawa cell growth has been demonstrated in athymic nude mice, but it is maintained in an estrogen-free medium (14). It appears that the cells have no estrogen dependency *in vitro*. Moreover, MPA does not show a significant antitumor effect in Ishikawa cells *in vitro* (31). It is suggested that the antitumor effect of MPA is not a direct action on the tumor cells, but an indirect action through the interstitial cells of the target organ. On the other hand, the effects of MPA on SHBG mRNA expression are considered to be direct action in Ishikawa cells. Actually, the antitumor effect of MPA in Ishikawa cells appears to be indirect and direct actions as stated above.

E2 slightly increases SHBG mRNA levels in human hepatoma cells (10, 32, 33). The suppressive effect of high-dose MPA on serum SHBG has been noted in patients with breast cancer (34) and endometriosis (35). On the other hand, progesterone has no effect on SHBG mRNA levels in hepatoma cells (10). SHBG mRNA in uterine endometria might be regulated by estrogen and progesterone in a way which differs from that in the liver (5). In Ishikawa cells, E2 alone does not affect the induction of SHBG mRNA (10). Similarly, E2 did not exert any effect on the expression of SHBG mRNA in the data presented herein.

While high-dose MPA suppressed SHBG mRNA expression in Ishikawa cells, low-dose MPA (10^{-10} mol/l) increased its expression, probably via

Figure 7 Western blot analysis of SHBG in Ishikawa cells. Ishikawa cells were incubated in MEM medium with 10^{-8} mol/l E2, 10^{-6} mol/l MPA, or 10^{-8} mol/l E2 plus 10^{-6} mol/l MPA or 10^{-10} mol/l MPA for 72 h. Proteins were separated in denaturing polyacrylamide gels and electrotransferred to nitrocellulose membranes as reported in Materials and methods. The upper panel shows the results of Western blot analysis. The lower panel shows intracellular SHBG levels in Ishikawa cells by densitometrical analysis. Values are means $\pm$ s.d. of 3 experiments. * $P < 0.05$ compared with the control group. Samples are (1) control, (2) 10^{-8} mol/l E2, (3) 10^{-6} mol/l MPA, (4) 10^{-8} mol/l E2 plus 10^{-6} mol/l MPA and (5) 10^{-8} mol/l E2 plus 10^{-10} mol/l MPA treatment. M represents the molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA).

progesterone receptors which are present at physiological concentrations. This rise in SHBG mRNA levels seems to be related to the estrogen induction of progesterone receptors. In fact, E2 (10^{-8} mol/l) induced progesterin binding sites after incubation for 24 h. A high concentration of MPA (10^{-6} to 10^{-5} mol/l) with E2 (10^{-8} mol/l) suppressed SHBG mRNA expression in Ishikawa cells, which might be associated with the antitumoral effect of MPA on endometrial cancers, because SHBG suppression might contribute to a defect in the estrogen-dominant milieu (5). This evidence reflects the fact that the immunohistochemical staining intensity of estradiol in endometrial cancers appears to decrease after MPA treatment (13).

Danazol and MPA in high doses decrease serum SHBG concentrations (35). Conversely, danazol increases SHBG secretion in human hepatocarcinoma cells (36). In Ishikawa cells, danazol and chlormadinone acetate suppressed SHBG mRNA expression. It is suggested that a tissue difference in steroidal induction of SHBG synthesis is demonstrated. MPA suppressed the expression of SHBG mRNA to a greater extent than chlormadinone acetate and danazol. High-dose MPA seems to be one of the most effective inhibitors of the transcription of the SHBG gene in endometrial cancer cells. Onapristone, an anti-progestin, did not affect the SHBG mRNA suppression of these agents. In addition, MPA in high and low doses showed no effect on progesterone receptor concentration. These findings support the view that the effect of MPA and the other steroid hormone analogs on SHBG expression is not mediated via the progesterone receptor.

Figure 8 Effects of steroids on SHBG mRNA expression in Ishikawa cells. Ishikawa cells were grown for 24 h in serum-free medium in the absence (control) or in the presence of 10^{-6} mol/l MPA, progesterone, chlormadinone acetate, and danazol with (+) or without (–) E2 (10^{-8} mol/l) and in the presence or absence of onapristone. The level of SHBG mRNA was determined by competitive RT-PCR-SBA, and expressed as a percentage of the control value. Values are means $\pm$ s.d. of 5 experiments. * $P < 0.05$ compared with the respective control group; ** $P < 0.05$ compared with the respective control and MPA treatment groups.

In conclusion, these findings suggest that sex steroids induce alterations in the transcription of the SHBG gene in Ishikawa cells and that intracellular SHBG suppression might contribute to a defect in the intracellular estrogen-dominant milieu. Suppression of intracellular SHBG might be involved as one of the mechanisms of the antitumoral effects of high-dose MPA against the development and growth of some well-differentiated endometrial cancers.

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