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Sex hormone binding globulin expression and colocalization with estrogen receptor in the human Fallopian tube

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Abstract

The detection of sex hormone binding globulin (SHBG) or SHBG mRNA in several sex steroid target tissues, has raised the possibility that SHBG modulates the action of sex steroids outside the vascular compartment. The presence of SHBG mRNA was investigated by RT-PCR in the poly (A^+) RNA fraction of the human Fallopian tube. Human and rat liver were used as positive and negative control tissues, respectively. The electrophoretic analysis of the amplified PCR products showed bands at 219 bp, corresponding to the expected size of the SHBG cDNA, in the Fallopian tube and human liver but not in rat liver, indicating that SHBG might be sinthezised by the Fallopian tube. The cellular localization of SHBG and of estrogen receptor (ER) was examined by immunohystochemistry in consecutive sections of Fallopian tube tissues for individual staining or double immunostaining in the same section. Specific immunostaining of SHBG was present in the epithelial, vascular and muscle cells of the ampullary and isthmic region. In epithelial cells, immunoreactive SHBG was present in the apical end with the highest concentration close to the luminal membrane. The ER was localized in the nuclei of epithelial, stromal and muscle cells of the ampulla and isthmus. Double immunostaining showed that SHBG and ER are colocalized principally in epithelial cells of the ampulla and in muscle cells of the isthmus. In conclusion, the detection of SHBG and SHBG mRNA and the localization of SHBG in estrogen target cells was shown. These findings support the hypothesis that SHBG might regulate sex steroid action at the tissue level.

1. Introduction

Sex hormone-binding globulin (SHBG) in human blood binds testosterone and estradiol with high affinity [1]. Changes in plasma concentrations of SHBG regulate the amounts of these sex-steroids that are not bound to plasma proteins and which are generally considered to be available to target cells [2]. This does not preclude that SHBG may play also an active role in influencing sex-steroid hormone action since it is localized in reproductive tissues and was shown to be internalized by a breast cancer cell line [3]. This has been confirmed independently by several other groups [4–6] and receptors for SHBG have been shown to exist in the plasma membrane of steroid-responsive tissues and cell types [7–10]. However the physiological significance of these observations remains obscure.

Like most other plasma proteins, SHBG is synthesized by hepatocytes [11] and its production appears to be regulated by several hormones, including its own ligands [12]. The SHBG gene is also expressed in Sertoli cells where it leads to the androgen-binding protein that is thought to play an important role in sperm maturation in the seminiferous tubules of the testis [13]. More recently, the detection of SHBG mRNA in several other tissues including placenta [6], endometrium [14] and ovary [15], has raised the possibility of local production of SHBG. Thus, SHBG may have the dual function of insuring availability of its ligands to the ligand target cells through its presence in the circulation and its presence within the target cells themselves. If the target cells synthesize SHBG for their own use, this would allow them to have some control of the intracellular availability of its ligands. A

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necessary requirement to hold this thinking is that SHBG synthesis takes place in the same cells that express receptors for its ligands.

The aims of this study were to determine whether SHBG is synthesized by an estrogen-responsive tissue and whether or not the protein is localized in its estrogen target cells. The human Fallopian tube was selected for this purpose since it is one of the estrogen target tissues which contains estrogen receptor (ER) in its epithelial, stromal and muscle cells [16,17] and normal tissue can be readily obtained from hospitals in Santiago.

2. Material and methods

2.1. Samples

Fallopian tubes were obtained from fifteen women undergoing salpingectomy for sterilization or for therapeutic reasons. Cross-sections (5 mm) were cut from nine of them and were quickly frozen in liquid nitrogen after immersion in embedding matrix (TISSUE-TEK® from MILES). They were stored at −80°C until processed for immunohistochemistry. Six were cut in pieces (100–500 mg) and immediately frozen in liquid nitrogen and stored at −80°C until processed for RNA extraction. Pieces of human liver were obtained from surgical specimens. Human and rat liver samples were also frozen and stored at −80°C for RNA extraction.

2.2. RNA extraction and cDNA synthesis

Frozen tissues were homogenized with a Polytron homogenizer and Poly (A^+) RNA was extracted using the Poly A Tract® System from Promega. Aliquots of Poly (A^+) RNA 1 μg were preheated at 65°C for 10 min and then incubated at 42°C for 1 h with the reverse transcription mixture (Promega) that contained: Avian Myeloblastosis Virus reverse transcriptase 0.5 U/μl, RNasin 1 U/μl, deoxyribonucleoside triphosphate mix (dNTP) 1 mM, and random sequence oligonucleotide primers 6 ng/μl in buffer 50 mM TRIS–HCl pH 8.3, 10 mM $MgCl_2$, 50 mM KCl and 10 mM DTT.

2.3. Polymerase chain reaction (PCR)

Oligonucleotide primers (5′-TGTAGAATCAAATCCCGGGA and 3′-TTCCACCACAAGAGAAGACC) which corresponded to sequences in exons 5 and 7 of human SHBG gene [20], respectively, were obtained from Bios Chile IGSA for PCR amplification of a 219 portion of the SHBG cDNA. These primers have been used by others to identify SHBG–mRNA in human endometrium [14] and will not detect differentially spliced SHBG transcripts that lack exon 7 coding sequences [18]. The PCR consisted of 2% of cDNA products, 25 pmol of each primer, 0.63 U of Taq–DNA polymerase (Promega), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$ in 10 mM buffer TRIS–HCl, pH 9.0 with 50 mM KCl and 0.1% Triton X-100. A 40 cycle PCR was performed in a thermal cycler at 94°C for 1 min, 60°C for 2 min and 72°C for 3 min, per cycles. Amplification of β actin [19] was used as internal control of mRNA integrity, cDNA from human liver was used as positive control and cDNA from rat liver were used as negative control. The PCR products were analyzed by electrophoresis in 1 to 2% agarose gels containing 5 μg/ml of ethidium bromide and a 1 kb DNA ladder (GIBCO BRL as molecular marker.

2.4. Immunohistochemistry

Serial 4–6 μm thick cryostat sections were mounted on silanized glass slides and fixed in 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) at 4°C for 15 min and in acetone at −20°C for 10 min. After blocking for nonspecific binding, sections were incubated overnight with primary antibodies diluted in PBS containing 1% BSA. Two antibodies were used for the immunodetection of SHBG: a mouse monoclonal S1B5 at 10 μg/ml [20] and a rabbit polyclonal anti-SHBG antiserum diluted 1:100 [21]. The tissue bound antibody was reacted at room temperature with an appropriate biotinylated second antibody and a Streptavidin–Biotinylated peroxidase complex (Amersham International pcl UK) for 30–60 min/each. Freshly prepared 3,3′-diaminobezidine tetrahydrochloride (DAB) 0,05% (wt:vol) in 50 mM TRIS–HCl, pH 7.6 and H_2O_2 0,03% (vol:vol) was then added for 8±2 min. After dehydration in graded ethanols and xylene the slides were mounted in a permanent mounting medium.

Omission of primary antibody was used for negative control and the specificity of immunostaining was ascertained by pre-adsorption of the primary antibody with a recombinant human SHBG. The recombinant human SHBG was expressed in yeast (*Pishia pastoris*) using a kit provided by Invitrogen, by inserting the coding sequence for the mature human SHBG polypeptide in frame with a yeast secretion sequence and then selecting for transformants that secrete SHBG into the culture medium. For antibody pre-adsorption, a solution containing approximately equimolar concentration of the monoclonal antibody (66 pmol/ml) and the recombinant SHBG (75 pmol/ml) was pre incubated at 4°C overnight, before it was incubated with tissue sections. In parallel, monoclonal antibody preincubated with PBS–BSA 1% instead of the recombi-

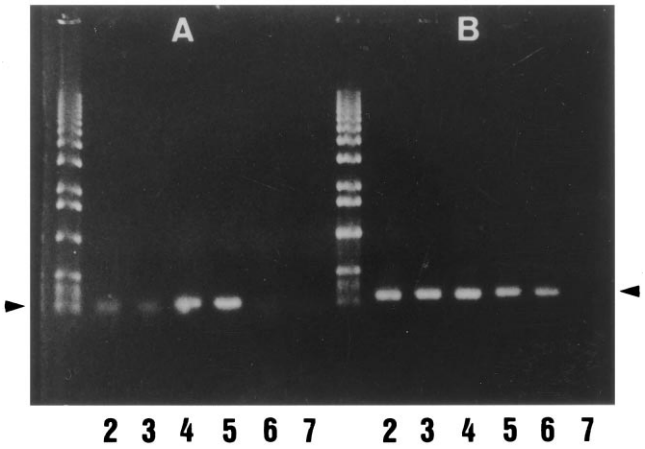

Fig. 1. Gel electrophoresis showing the ethidium bromide staining of RT–PCR products using primers for SHBG (A, lanes 2–7) and for β actin (B, lanes 9–14). Lanes 1 and 8 correspond to molecular marker 1 kb DNA Ladder. The arrows indicate the DNA size expected: at 219 bp for SHBG cDNA and 238 bp for β actin cDNA. Lanes marked 2–4 correspond each to a different Fallopian tube, lanes 5 correspond to human liver and lanes 6 to rat liver, used as positive and negative control of SHBG expression, respectively. Lanes 7 correspond to the negative control of the reaction. The three Fallopian tubes and the human liver showed both, SHBG and β actin transcripts. Rat liver showed β actin but not SHBG transcript and the negative control of the reaction did not show any product.

nant SHBG was used as positive control of the immunoreaction.

Immunohistochemical localization of the ER was performed using the ER–ICA kit from Abbott Laboratories. For SHBG plus ER in the same tissue sections, an equal mixture of mouse monoclonal S1B5 10 μg/ml and the rat monoclonal antibody against ER (H222) from the ER–ICA kit was applied overnight at 4°C. Subsequently, the two antigens were detected separately by incubation with specific second antibody–enzyme complexes. First the SHBG bound by S1B5 was stained using DAB to give a brown color and then ER bound by H222 was detected using True-Blue solution that contains tetramethylbenzidine (TMB) to give a blue staining.

3. Results

3.1. Detection of SHBG mRNA in the Fallopian tube

To determine whether SHBG might be synthesized by the Fallopian tube, cDNA transcripts from poly (A^+) RNA extracts of this tissue were subjected to a PCR using primers that correspond to a region of the coding sequence for human SHBG mRNA. In total six Fallopian tubes were examined in this way. Fig. 1 shows the electrophoretic analysis of the PCR products from three periovulatory Fallopian tubes, human liver, rat liver and in the absence of cDNA, using primers for SHBG (A) and for β actin (B). In A, a DNA fragment corresponding to a 219 bp region of the SHBG cDNA was observed in the Fallopian tubes, lanes 2 to 4 and in human liver, lane 5. This product was not present in rat liver nor in the absence of cDNA, lanes 6 and 7, respectively. Lighter bands were found in the Fallopian tubes in comparison to the human liver and differences in staining intensity among the Fallopian tubes were also observed. In B, the quality of the experimental and control extracts used are illustrated by the presence of the expected DNA fragment of the β actin cDNA, at 238 bp, in equal quantity of poly (A^+) RNA from the same samples: Fallopian tubes lanes 2–4, human liver lane 5 and rat liver lane 6 but not in lane 7, without cDNA.

3.2. Immunocytochemical localization of SHBG

In the nine Fallopian tubes examined, SHBG immunoreactivity was found in the ampullary and isthmic regions. The immunostaining was localized to the apical side of the luminal epithelium, in blood vessels and muscle cells. Both, polyclonal and monoclonal antibodies against human SHBG gave a similar distribution of immunostaining, supporting the specificity of the immunoreaction. Fig. 2 shows polyclonal (A and B) and monoclonal (C) antibodies staining in successive tissue sections of the ampullary region of a Fallopian tube obtained during the periovulatory period of the menstrual cycle; (B) is a magnification of (A) showing the localization of SHBG at the apical end of epithelial cells. The immunoreaction shown in (C) was abolished by the competitive adsorption of the antibodies with the recombinant SHBG (D). In the muscle layer, staining was particularly strong in the isthmic segment (E and F); F being a magnification of E.

Fig. 2 (A, B and C) clearly show that epithelial cells positively stained for SHBG are interspersed between unstained epithelial cells. This pattern differs from the one shown in Fig. 3 (B and C) where all or almost all epithelial cells appears positively stained for SHBG. The intensity of staining also differed from one specimen to another. Although the precise stage of the menstrual cycle of most donors was unknown, the tissues removed from four who were in the periovulatory period of the menstrual cycle, showed the strongest staining.

3.3. Immunocytochemical colocalization of SHBG and ER

The ER was detected in the nuclei of luminal epithelial, stromal and muscle cells. It was not found in blood vessel cells, although in some samples a strong staining of stromal cells surrounding blood vessels was

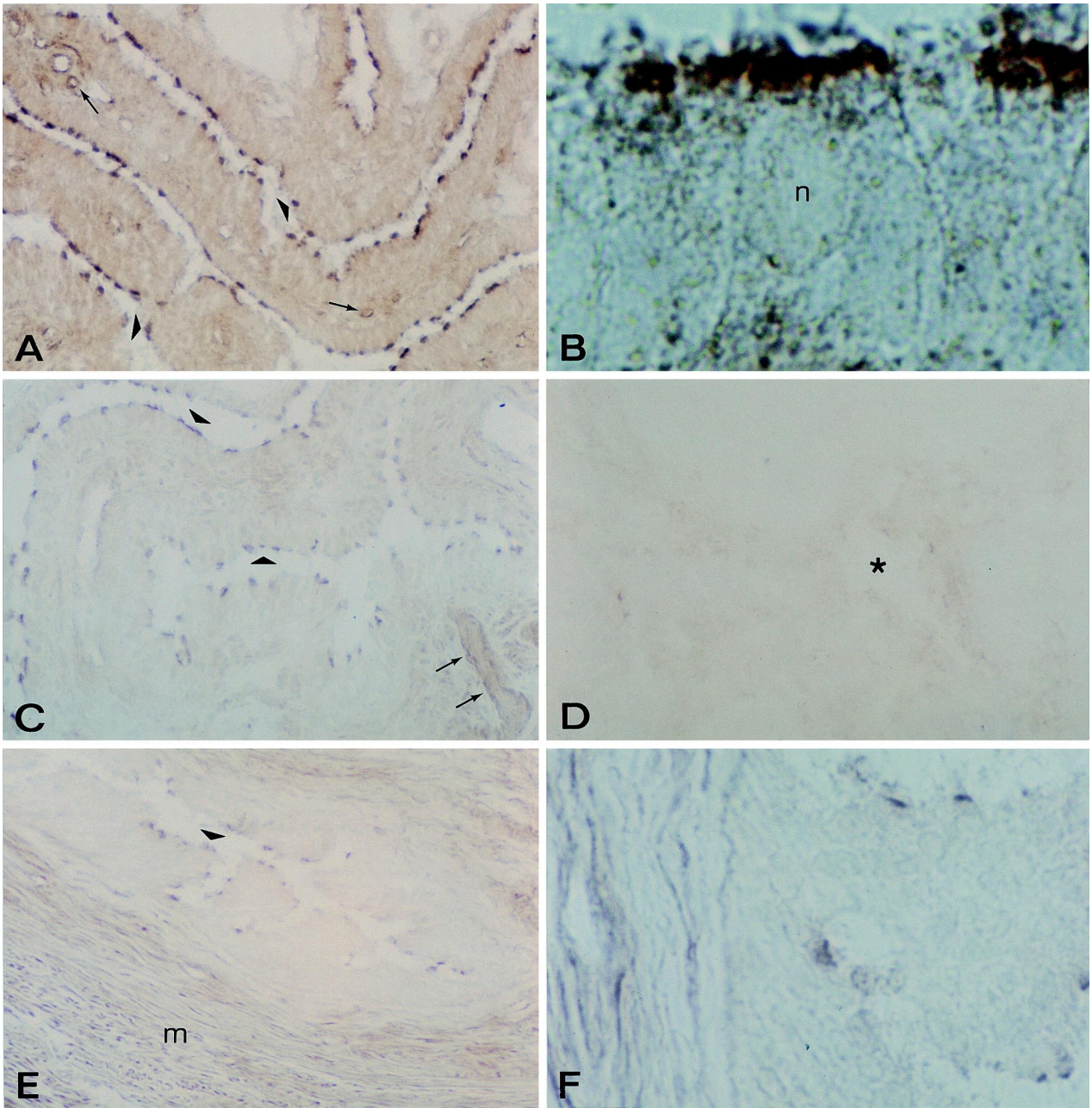

Fig. 2. Light micrographs showing immunocytochemical localization of SHBG in Fallopian tubes. (A) Shows the immunoreaction of SHBG with the polyclonal anti-SHBG antiserurn in blood vessels (arrow) and epithelial cells (arrow head), 250×; (B) is a magnification of A showing the intense staining in the apical end of epithelial cells, n is the cell nucleus, 3000×. (C) Shows the immunoreaction with the monoclonal antibody S1B5 in an adjacent section to A, the distribution of immunoreaction is similar to that observed in A, 250×. (D) Shows the neutralization of the immunostaining by pre-adsorbing the monoclonal antibody with a recombinant human SHBG, the asterisk indicates the lumen, 250×. (E) Shows the immunoreaction with antibody S1B5 in the isthmic segment; note the strong staining of muscle cells (m), 250×; (F) is a magnification of E, 3000×.

observed. Fig. 3(A) is a section of ampullary mucosa showing nuclei of the epithelial and stromal cells immunostained with anti-ER antibody.

When SHBG and ER were examined in successive tissue sections of the ampullary segment, many epithelial cells contained both antigens; this distribution was clearly visualized in experiments of double immunostaining, where SHBG and ER were colocalized in most epithelial cells (Fig. 3(B)) and clearly occupied different sub-cellular compartments. Immunoreactive ER was present in the nucleus while SHBG was localized between the nucleus and the apical membrane or at the apical membrane itself, (Fig. 3(C)). The large quantity of epithelial cells showing both, ER and SHBG suggests that colocalization occurs in secretory and ciliated cells however this needs to be confirmed

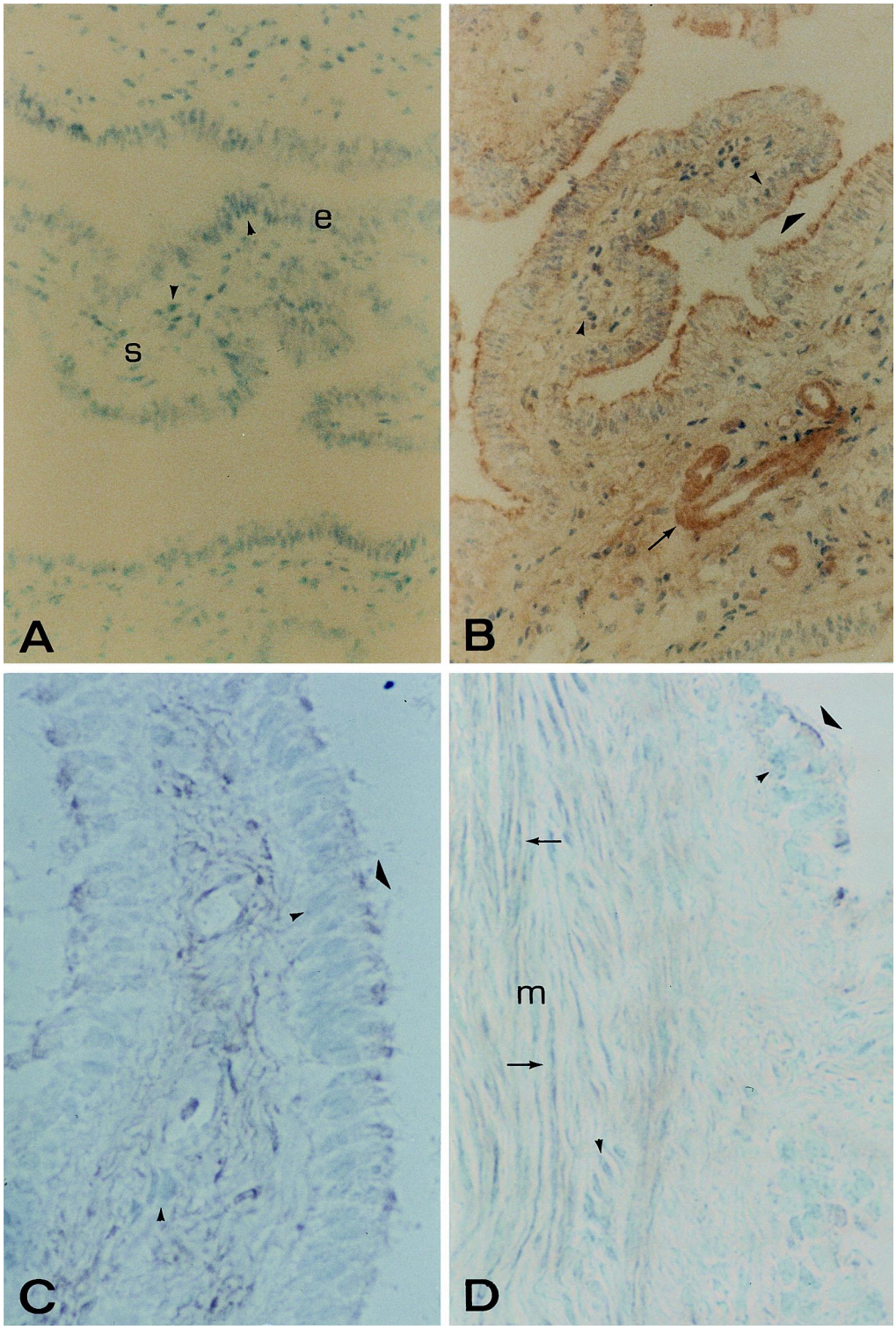

Fig. 3. (A) Shows the immuno detection of estradiol receptor (ER) (little arrow head) in the nuclei of epithelial (e) and stromal (s) cells of an ampullary section, 250×. (B) Shows the double immunostaining for ER (little arrow head) and SHBG using antibody S1B5 (large arrow head), most epithelial cells contain both proteins; blood vessels reacted only with antibody S1B5 (arrow), 250×; (C) is a magnification of B showing the colocalization of SHBG and ER on different compartments of the same epithelial cells, 850×. (D) Shows the colocalization of SHBG and ER in some muscle cells (arrow) in the isthmic segment of a Fallopian tube, 850×.

utilizing electron-microscopy to clearly identify the stained cells. In the isthmic segment, colocalization of SHBG and ER was also observed in the muscle cells (Fig. 3(D)).

4. Discussion

Immunoreactive SHBG was found in the epithelial, vascular and muscle cells of the ampullary and isthmic region of the Fallopian tube. The protein could reach this location as a result of local synthesis or by sequestration from the blood circulation. Both possibilities seem likely since injection of human SHBG into the blood circulation in adult female rats results in its localization in the luminal cells of the oviduct and uterus [22] and the finding of SHBG mRNA in the Fallopian tube proves that SHBG gene is expressed in this tissue and supports that SHBG is synthesized by Fallopian tube cells. Thus Fallopian tube cells can have SHBG of both, intra- and extra-cellular origin.

In spite of the qualitative techniques used in this study, the expression of SHBG mRNA was apparently more intense in the liver than in the Fallopian tube. This difference is to be expected considering that hepatic cells maintain circulating SHBG levels in the order of nM whereas oviductal SHBG synthesis is likely to be only for local use. In addition to the liver and the testis, several other tissues all of which, including the Fallopian tube, are estrogen responsive have been shown to contain SHBG gene transcripts [6,14,15]. A type of intracellular, non secreted or alternate SHBG, has been reported to occur in human liver and testis [18,23] and in rat testis [24]. Human alternate SHBG mRNA transcripts may contain or lack exon VII which codes information for the steroid-binding domain of the protein, (Dr. G.L. Hammond personal communication). Thus alternate SHBG mRNA may be present in two forms, one of which can be translated to an intracellular SHBG with conserved steroid-binding affinity.

Variations in the intensity of staining with the SHBG antibodies, as well as in the proportion of stained epithelial cells, from one Fallopian tube specimen to another suggest that either expression or uptake of SHBG by these cells is subject to regulation and therefore must have important implications for the function of this organ.

The role of SHBG in the Fallopian tube, as in other organs, remains to be elucidated. A large number of studies deal with the role of circulating SHBG on the action of its ligands, but few deal with the functions of the SHBG present in steroid target tissues. To approach the possibility that SHBG might control the bioavailability of its ligand in the target cells, the distribution of SHBG in cells containing ER, as marker of estrogen target cells, was examined. Estrogen receptors have been localized in the nucleus of epithelial, stromal and muscle cells in every segment of the human Fallopian tube [16,17]. By immunoelectron–microscopy, ER was found in both secretory and ciliated cells of the epithelium [16]. A semiquantitative estimation of ER concentration indicated that immunostaining increased during midcycle in the ampulla, but in the other segments of the Fallopian tube, ER score was unrelated to the stage of the cycle or to serum E2 levels [17]. The present study confirms the distribution of ER in the Fallopian tube and shows that in many epithelial and muscle cells SHBG is localized in estrogen target cells as judged by the presence of ER. This colocalization suggests that estrogen target cells may use locally synthesized SHBG to regulate intracellular availability of estradiol. A non exclusive alternative is that estrogens may regulate the expression of SHBG in the Fallopian tube as they do in the liver. Further studies will be required to find out the biological meaning of this coexistence in the same cells.

In conclusion, this study shows that SHBG mRNA and SHBG are present in the Fallopian tube and that in epithelial and muscle cells SHBG is colocalized with ER, supporting that intracellular SHBG may also regulate the availability or action of estrogens within estrogen target cells.

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