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Sex hormone-binding globulin, its membrane receptor, and breast cancer: a new approach to the modulation of estradiol action in neoplastic cells ☆

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Abstract

The role of human Sex Hormone-Binding Globulin (SHBG), the plasma carrier of sex steroids, and its membrane receptor, SHBG-R, in estrogen-dependent breast cancer has been investigated in our laboratory in the past few years. SHBG-R is expressed in MCF-10 A cells (not neoplastic mammary cells), MCF-7 cells (breast cancer, ER positive) and in tissue samples from patients affected with ER positive breast cancer, but not in estrogen-insensitive MDA-MB 231 cells. The SHBG/SHBG-R interaction, followed by the binding of estradiol to the complex protein/receptor, causes a significant increase of the intracellular levels of cAMP, but does not modify the amount of estradiol entering MCF-7 cells. The estradiol-induced proliferation of MCF-7 cells is inhibited by SHBG, through SHBG-R, cAMP and PKA. Similarly, the proliferation rate of tissue samples positive for SHBG-R was significantly lower than the proliferation rate of negative samples. SHBG and SHBG-R could thus trigger a 'biologic' anti-estrogenic pathway. In order to get a more detailed knowledge of this system, we first examined the frequence of the reported mutated form of SHBG in 255 breast cancer patients. The mutated SHBG is characterized by a point mutation (Asp 327 → Asn) causing an additional *N*-glycosylation site, which does not affect the binding of steroids to SHBG. The frequence of the mutation was significantly higher (24.5%) in estrogen-dependent breast cancers than in healthy control subjects (11.6%). This observation confirms the close relationship between SHBG and estrogen-dependent breast cancer and suggests that the mutation could modify SHBG activity at cell site. Lastly, the possibility of using SHBG to modulate the estradiol action in breast cancer was further studied by transfecting MCF-7 cells with an expression vector carrying the SHBG cDNA (study in collaboration with G.L. Hammond). Transfected cells are able to produce significant amount of SHBG in their medium, but their SHBG-R is reduced to undetectable levels. The SHBG produced by transfected MCF-7 cells is, however, able to inhibit estradiol-induced proliferation of MCF-7 cells expressing a functional receptor. Thus, the local production of SHBG obtained with transfection could be a useful tool to control cell growth in estrogen-dependent breast cancer.

1. Introduction

The relationship between plasma SHBG and breast cancer has been widely investigated for the last twenty years. The estrogen-dependence of some breast tumours and their singular behaviour as well as the ability of SHBG to bind circulating estradiol at high affinity induced many investigators to search for a possible SHBG involvement in the pathogenesis of the neoplasm.

Plasma SHBG levels in breast cancer patients had been evaluated by many authors and several attempts to find a clinical application had been made. The plasma SHBG levels had been reported to be reduced

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Table 1
Binding characteristics of the interaction SHBG/SHBG-R in mammary cultured cells (MCF-10A: not neoplastic cells; MDA-MB 231: estrogen insensitive breast cancer cells; MCF-7: estrogen-sensitive breast cancer cells) and estrogen-dependent breast cancer tissue samples

	Number of sites	B_{max} fmol/10^6 cells	*Kd* (M)
MCF-10A cells ($n=3$)	1	44.2 ± 2.8	$1.90 \pm 0.6 \times 10^{-11}$
MDA-MB 231 cells ($n=3$)	0	–	–
MCF-7 cells ($n=3$)	2	Site no. 1, 0.65 ± 0.07	Site no. 1, $1.09 \pm 0.6 \times 10^{-12}$
		Site no. 2, $2{,}500 \pm 360$	Site no. 2, $1.87 \pm 0.9 \times 10^{-8}$
Breast cancer tissue samples ($n=3$)	2	Site no. 1, 6.24 ± 0.2	Site no. 1, $1.44 \pm 0.2 \times 10^{-12}$
		Site no. 2, 46.0 ± 6.0	Site no. 2, $3.12 \pm 0.4 \times 10^{-11}$

in postmenopausal breast cancer [1–6] or at least at the lower end of the normal range. In any case, the free fraction of estradiol was increased in these patients and this was related to an absolute or relative reduction of SHBG [7]. The estradiol and SHBG pattern of premenopausal breast cancer patients, on the other hand, is much more debated. In fact, SHBG levels had been reported to be not modified [2,7,8], while no unique opinion about estradiol concentrations was reached.

It is also noteworthy that few reports in the late 70 s observed increased plasma SHBG levels in ER + breast cancer patients with respect to healthy controls [9–11].

It is obvious that our knowledge about the link between plasma SHBG and breast cancer is still quite poor. Nevertheless, the current opinion seems to be that in postmenopausal breast cancer patients SHBG plasma levels are reduced (causing, especially together with central obesity [12] an increased bioavailable estradiol fraction). What Bulbrook [13] wrote on this issue in 1988 sounds really interesting and actual: ''women whose tumours arise in an environment characterized by SHBG concentrations at the lower end of normal range...will tend to have an earlier age at diagnosis, a lower frequency of estrogen receptor positive tumours and a lower proportion of hormone responsive tumours than women with SHBG levels at the top of the range''.

In addition, the ability of menopausal ERT taken by mouth to increase SHBG and decrease IGF-1 plasma levels has been recently proposed as a protective factor against the risk of developing breast cancer in these patients [14–17].

Almost in the same years, the presence of SHBG in the cytoplasm of breast cancer cells was reported. SHBG was first detected with specific binding assays [18] and successively with immunocytochemistry staining both in breast cultured cells [19] and in tissue samples [20,21]. In one of these studies, it was observed that highly invasive tumours do not stain for SHBG and, thus, the intracellular presence of SHBG seems to be related to a better prognosis [21].

Lastly, a couple of attempts to identify SHBG mRNA in breast cancer cells were done, but results are totally opposite, having been reported either the absence of SHBG message in MCF-7 cells [22] or its presence in MCF-7, ZR-75-2, MDA-MB 231 cells and some tissue samples [23].

Even after so many years, few clear points about the link between SHBG and breast cancer have been reached and, especially, knowledge about the mechanism of action of SHBG in breast cancer is still obscure.

2. The expression of SHBG membrane receptor (SHBG-R) in breast cancer

In the mid 1980 s it was reported that plasma SHBG could bind on membranes of endometrium [24] and prostate [25]. The binding characteristics in both tissues suggested the existence of a specific membrane receptor for SHBG (SHBG-R).

We had to wait until 1992 to have the first report about the presence of SHBG-R in breast cancer MCF-7 cells [26], where SHBG recognized two binding sites at different affinity.

A similar observation was done in our laboratory [27]. The estrogen-dependent MCF-7 cells specifically bound SHBG at two sites at different affinity. No specific binding for SHBG was observed in estrogen-insensitive MDA-MB 231 cells [28]. MCF-10A normal breast cells also specifically recognized SHBG, but only one binding site was detectable [29] (Table 1).

In addition, SHBG-R was also characterized on membranes obtained from breast cancer tissue samples [30]. As in MCF-7 cells, SHBG bound at two sites at different affinity, and SHBG-R was expressed in a higher percentage of ER + tumours (75%, $n=12$) than in ER− tumours (37%, $n=8$).

Even though the SHBG binding to cell membrane shows all the characteristics of the receptor binding and we strongly believe to the existence of SHBG-R,

Table 2
Intracellular levels of cAMP in MCF-7 cells cultured in either serum free medium (SFM), 5% or 10% of foetal calf serum without steroids and bovine sex hormone binding globulin (SHBG-FREE/DCC-FCS), treated with 1 nM estradiol alone or 50 nM human SHBG followed by 1 nM estradiol

	cAMP (fmol/10^6 cells)		
	A) 1nM estradiol	B) 50 nM SHBG + 1 nM estradiol	A vs B
SFM	247 ± 5 ($n = 3$)	325 ± 75 ($n = 3$)	$P < 0.05$
5% SHBG-FREE/ DCC-FCS	165 ± 34 ($n = 3$)	254 ± 8 ($n = 3$)	$P < 0.05$
10% SHBG-FREE/ DCC-FCS	211 ± 22 ($n = 3$)	290 ± 7 ($n = 3$)	$P < 0.05$

until now the receptor has not yet been purified or cloned.

3. The post-receptor events following the interaction between SHBG and SHBG-R in breast cancer cells

Rosner and coworkers [31–33] have repeatedly demonstrated that the interaction of SHBG with SHBG-R in prostate cells, followed by the binding of estradiol or androgens to the preformed protein-receptor complex, caused a significant increase of intracellular cAMP levels. It was, thus, hypothesized that SHBG-R belonged to the G-protein coupled receptor family, but no direct evidence of it is available at present.

We investigated the ability of SHBG to increase cAMP also in breast cancer cells. While no modifications of basal cAMP levels were observed in MDA-MB 231 cells (that do not bind SHBG!), the binding of estradiol to the preformed SHBG/SHBG-R complex in MCF-7 cells determined a significant increase of intracellular cAMP levels [28]. The most relevant effect was exerted by 1 nM estradiol, while androgens were significantly less effective. The induction of cAMP in MCF-7 cells strictly followed the sequence of binding described by Rosner and coworkers: firstly SHBG bound to SHBG-R; secondly estradiol bound to the complex SHBG/SHBG-R, and only at this point cAMP levels went up. In our model in which cells were maintained in serum free medium, SHBG in the absence of estradiol had no effect on cAMP as well as estradiol in the absence of SHBG.

Almost at the same time, Aronica et al. [34] reported that estradiol on its own was able to induce cAMP in MCF-7 cells, suggesting a possible direct membrane action of estrogens in both breast and uterine cells. This observation prompted us to go back to our initial results and to re-evaluate the functionality of the system SHBG/SHBG-R/estradiol in MCF-7 cells either in serum free medium (where there is no interference by bovine sex hormone binding globulin), in DCC-FCS (foetal calf serum treated with dextran-charcoal to remove steroids, but not bovine sex hormone-binding globulin; the same culture conditions used by Aronica and coworkers), or in SHBG-FREE/DCC-FCS (foetal calf serum treated with DCC to remove steroids and with testosterone-agarose to remove bovine sex hormone-binding globulin).

Much to our surprise, estradiol on its own was able to increase MCF-7 cells cAMP content only in the presence of 10% DCC-FCS. The use of either lower DCC-FCS concentrations (e.g. 5%), of serum free medium, or of SHBG-FREE/DCC-FCS did not allow estradiol to modify the basal levels of cAMP. Moreover, the addition of 50 nM purified human SHBG to SHBG-FREE/DCC-FCS (both at 5 and 10%) as well as to serum free medium caused estradiol to induce significantly cAMP. We, thus, believe that the ability of estradiol to induce cAMP in MCF-7 cells depends on the presence of serum factors at appropriate concentrations and that human SHBG (upon the binding to SHBG-R) is one of the critical factors contained in FCS allowing estradiol to induce cAMP [35] (Table 2).

Beside the ability of increasing cAMP, SHBG could also sequester estradiol at cell surface, reducing the amount of the hormone reaching ER inside MCF-7 cells. To address this issue, we studied the amount of radiolabelled estradiol entering MCF-7 cells in the absence and in the presence of SHBG [27]. We observed that the presence of SHBG did not modify significantly the amount of estradiol entering cells, but it determined a significant amount of estradiol to be bound at the outer side of cell membrane. It is, thus, likely that SHBG does not reduce the amount of estradiol entering MCF-7, but any possible effect exerted in MCF-7 cells has to be related to triggering of the cAMP cascade.

4. The effect of SHBG and SHBG-R on breast cancer cell proliferation

The existence of SHBG-R and the ability of the SHBG/SHBG-R system to induce cAMP suggested that the protein could have some effects on the function of MCF-7 cells. Since our first observations, we

Table 3
Proliferation, expressed as cell number, of MCF-7 cells treated with: serum free medium (A), 1 nM estradiol (B), 1 nM SHBG (C), 1 nM SHBG followed by 1 nM estradiol (D), 1 nM DES (E), 1 nM SHBG followed by 1 nM DES (F), 1 nM SHBG complexed with the anti-SHBG antibody followed by 1 nM estradiol (G); 1 nM SHBG followed by 1 nM estradiol in the presence of 100 nM of PKI (6-22) amide[a]

		Cell number ($\times 10^3$)	
Basal	A	96.6 ± 13.3	
1 nM estradiol	B	263.0 ± 47.0	A vs B, $P < 0.01$
1 nM SHBG	C	92.3 ± 13.3	A vs C, NS
1 nM SHBG + 1 nM estradiol	D	97.2 ± 10.0	A vs D, NS: B vs D, $P < 0.01$
1 nM DES	E	258.0 ± 12.0	
1 nM SHBG + 1 nM DES	F	247.0 ± 20.0	E vs F, NS
Complex SHBG.Ab + 1 nM estradiol	G	238.0 ± 10.0	B vs G, NS; D vs G, $P < 0.01$
100 nM PKI(6-22) amide + 1 nM SHBG + 1 nM estradiol	H	192.0 ± 15.0	B vs H, NS; D vs F, $P < 0.01$

[a] Data are expressed as mean± SEM, $n = 3$.

have been mainly interested in the evaluation of the effect of SHBG on the proliferation of mammary cells.

The proliferation of the estrogen-insensitive MDA-MB 231 cells [28] was totally unaffected by both SHBG and estradiol, while the not neoplastic MCF-10A cells slightly increased their growth under estradiol treatment, but were totally insensitive to SHBG used alone or together with estradiol.

The most striking observations we had made are on the behaviour of estrogen-dependent MCF-7 cells. In fact, as expected, MCF-7 cells increased significantly their proliferation when treated with estradiol and did not show a growth different from basal levels when treated with SHBG alone. Treatment of MCF-7 cells with SHBG and estradiol (following the correct sequence of binding to SHBG-R; SHBG first, then estradiol) determined a complete inhibition of the estradiol-induced proliferation [27,36]. This effect was highly specific for the interaction SHBG/SHBG-R/estradiol: (1), the estrogenic compound DES, which does not bind to SHBG, increased significantly the cell growth in the absence and in the presence of SHBG; (2) when SHBG was blocked with a specific antiserum against human SHBG, the antiproliferative effect was completely lost. In addition, in MCF-7 cells treated with PKI (6-22) amide, a specific inhibitor of PKA, the target of cAMP, SHBG was no more able to block estradiol action [36] (Table 3).

Even if undirectly, we obtained also evidences that SHBG-R was linked to a reduction of proliferation in estrogen-dependent breast cancer tissue samples [30]. In fact, samples ER + expressing SHBG-R were characterized by lower thymidine kinase (proliferation index) levels than tumours negative for SHBG-R (SHBG-R + samples, $TK = 17.82 \pm 4.7$ mIU/mg protein, $n = 16$; SHBG-R- samples, $TK = 33.48 \pm 9.77$ mIU/mg protein, $n = 12$; $P < 0.001$).

We concluded that: (1) SHBG inhibits the estradiol-induced proliferation of estrogen-sensitive breast cancer cells (but it has no effect on normal mammary cells and estrogen-insensitive cells); (2) the action of SHBG at cell site is, thus, strictly dependent on the estrogen sensitivity of cells and on the levels of expression of SHBG-R; (3) SHBG exerts its antiproliferative effect in estrogen-dependent breast cancer cells through the interaction with SHBG-R, the induction of cAMP, and the phosphorylation of unknown target proteins by PKA.

5. The additionally glycosylated SHBG variant and breast cancer

In recent years, a variant form of the plasma SHBG was described [37,38]. The variant SHBG results from a point mutation in exon 8 (TAG → CAG), causing the substitution of Asp 327 with Asn and, thus, an additional *N*-Glycosylation of the mature protein [39]. The variant SHBG, that is present with quite a low frequency in the general population [37], shows the same immunological and steroid binding characteristics [39], but a reduced clearance [40], with respect to the wild type protein.

No information is available about the interaction of the variant SHBG with SHBG-R, but in the past it was reported that the glycidic residues of the wild type SHBG are of critical importance for a correct binding [41]. It was, thus, suggested that the interaction of the variant SHBG with SHBG-R could be somehow modified.

We evaluated in 255 breast cancer patients and 120 healthy women the presence of the variant SHBG [42,43]. The SHBG gene exon 8 was amplified from genomic DNA with PCR (Polymerase Chain Reaction) and the amplification products were analyzed with Hinf*I* RFLP (Restriction Fragment Length Polymorphism) to detect the mutation. The frequency of the SHBG variant was 21.2% in breast cancer patients and 11.6% in healthy controls (statistical significance was not attained). When breast cancer

patients were subgrouped on the basis of the hystology of the tumour, the age at diagnosis (before or after 50 years), and the ER/PR pattern of the tumour, the frequency of the variant SHBG was significantly higher in postmenopausal patients (23.6%) and in ER+/PR+tumours (24.5%) than in the controls, while no significant difference was observed in the different hystological types.

These observations are still preliminary and undirect, but they demonstrate clearly and for the first time that the variant SHBG could be a positive prognostic factor in the work-out of breast cancer.

6. The transfection of SHBG cDNA in breast cancer cells

The data obtained on MCF-7 cells suggest that SHBG and SHBG-R could function as an anti-estrogenic system able to modulate the estradiol action in estrogen-dependent breast cancer. Therefore, together with Dr. G.L. Hammond (London Cancer Research Laboratories., London Cancer Centre, London Ontario, Canada), we have manipulated this system by transfecting MCF-7 cells with a human SHBG expression vector to determine how locally produced SHBG might affect both SHBG-R and estrogen-induced cell proliferation [44].

MCF-7 cells transfected with an expression vector carrying the human SHBG cDNA, the Neo resistence gene, and the CMV enhancer/promoter sequence [45], that will be called S-MCF-7 cells, were shown to secrete in their medium significant amount of SHBG (3.0–5.7 nM), while no SHBG was detectable in the medium of parental MCF-7 cells (P-MCF-7 cells).

SHBG-R was evaluated with a specific binding assay. In P-MCF-7 cells, SHBG recognized two binding sites ($B_{max1} = 6.8$ fmol/10^6 cells; $Kd_1 = 2.1 \times 10^{-12}$ M, $B_{max2} = 77$ fmol/10^6 cells; $Kd_2 = 8.3 \times 10^{-10}$ M), while no binding for SHBG was detectable in S-MCF-7 cells. The transfection of the SHBG cDNA thus caused a disappearance of SHBG-R, through unknown mechanisms.

Both P-MCF-7 and S-MCF-7 cells under estradiol treatment increased significantly their proliferation (P-MCF-7 cells +136% with respect to basal, S-MCF-7 cells +109% with respect to basal). As far as S-MCF-7 cells are concerned, even in the presence of high SHBG concentrations (produced by cells themselves), the estradiol-induced proliferation was unaffected because of the absence of SHBG-R.

Interestingly, the treatment of P-MCF-7 cells, which express a normal SHBG-R, with medium obtained from S-MCF-7 cells (containing SHBG), inhibits completely the estradiol-induced proliferation (+10% with respect to basal), while no anti-proliferative effect was exerted by medium obtained from P-MCF-7 cells, lacking SHBG (+174% with respect to basal). Therefore, the SHBG secreted by transfected cells is able to inhibit estradiol action in the presence of a functioning SHBG-R as it occurs in P-MCF-7 cells.

Studies on manipulated S-MCF-7 cells are still in progress, but these preliminary observations suggest that the local production of SHBG obtained with transfection may represent a means of controlling the estradiol-induced proliferation of breast cancer cells.

7. Conclusions and future perspectives

Plasma SHBG, through SHBG-R, cAMP, and PKA, acts as an antiproliferative agent in estrogen-dependent breast cancer, by inhibiting the estradiol-induction of cell growth. Many aspects of the involved mechanisms are still unclear, but potential applications of the SHBG/SHBG-R system in the managing of estrogen-dependent breast cancer are suggested. Moreover, the preliminary data we obtained on biotechnological manipulated MCF-7 cells open up new and interesting perspectives.

The development of new strategies to control the estrogen-dependent breast cancer growth, involving SHBG and SHBG-R, as well as a better understanding of the molecular mechanisms involved in the SHBG action at cell site will be the future in which we hope to be deeply involved.

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