Vol. 118, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1984 Pages 488-494

IMMUNOHISTOCHEMICAL DETECTION OF THE SEX STEROID-BINDING PLASMA PROTEIN IN HUMAN MAMMARY CARCINOMA CELLS

J. Tardivel-Lacombe [+], M. Egloff [+], A. Mazabraud [++] and H. Degrelle [+]

+ Laboratoire de Biochimie, Faculté de Médecine,
45 rue des Saints Pères, 75270 PARIS CEDEX 06 (France)
++ Service d'Anatomie Pathologique, Institut Curie,
26 rue d'Ulm, 75005 PARIS (France)

Received December 6, 1983

SUMMARY : A sex steroid-binding plasma protein-like antigen has been detected in human mammary carcinoma cells. A monospecific antiserum was used, and this protein was located mainly on the cytoplasmic membranes. These results are in agreement with a recent hypothesis according to which steroid hormones could be carried into cells by specific binding plasma proteins.

In human plasma, steroid hormones are bound to proteins with high capacity and low affinity, or with low capacity and high affinity (1). The sex steroid-binding protein (SBP) is a glycoprotein, MW ≃ 80.000, which specifically binds androgens (testosterone, dihydrotestosterone) and estradiol (2,3). The plasma concentration of SBP is low (ranging from 3 to 30 mg/l) and may vary in some physiopathological circumstances (4,5,6,7).

The roles of SBP have not yet been clearly elucidated. It may protect hormones from catabolism or conversion (8,9), it may enhance conversion of inactive steroids to active hormones (10) and it may regulate the estrogen-androgen balance (11). However, one of the latest hypotheses is that SBP may participate in the penetration of sexual hormones through the cytoplasmic membrane of their target cells (12). Some authors have reported the presence of an SBP-like protein in steroid hormones target organes (13,14, 15). Other authors have recently reported a possible correlation between the plasma level of SBP and the hormonodependence of human breast cancer (16,17,18,19).

The present work demonstrates, by means of indirect immunofluorescence, the presence in human mammary tumour cells of a protein having common antigenic properties with SBP.

Abbreviations : SBP, sex steroid-binding plasma protein ; DHT, 5α-dihydrotestosterone ; PBS, phosphate buffered saline ; SDS, sodium dodecyl sulphate.

0006-291X/84 $1.50

MATERIAL AND METHODS

Purification of human SBP

Pure SBP was obtained from a pool of late pregnancy human serum. We selected a conventional five steps process previously described (10) : dual ammonium sulfate precipitation, elimination of albumin on Blue Sepharose Cl-6B (Pharmacia), gel filtration on AcA 44 + AcA 54 (IBF) and preparative isoelectrofocusing (LKB). The affinity of fractions for DHT was checked after each step. At this stage of the process, contaminants were detected as faint traces by polyacrylamide gel electrophoresis and immunoelectro - phoresis. The prepurified fraction was incubated with trace amounts of tritiated DHT and submitted to semi-preparative gel electrophoresis. After locating the protein bands with rapid Coomassie blue staining, the gel was sliced and an aliquot of each slice was counted in a β-scintillation counter ; the radioactive slice (containing pure SBP) was crushed in a Potter homogeneizer and re-suspended in phosphate buffered saline (66,7 mM, pH 7,4 with 200 mM NaCl). The purity and the molecular weight of the SBP were checked by SDS polyacrylamide gel electrophoresis (10% acrylamide).

Production and characterization of antisera against human SBP

Prior to immunization of animals, pure SBP was emulsified with complete Freund's adjuvant (Difco). This emulsion was injected into adult male rabbits (Fauve de Bourgogne) as previously described (20). In further experiments, we used antisera without purification. Immunoglobulins were not purified by DEAE chromatography to avoid aggregated material (21). We also avoided affinity purification with immobilized SBP since this method allows mainly the production of low affinity antibodies, high affinity antibodies being too strongly adsorbed to be desorbed in non-denaturing conditions. The antisera were characterized by rocket line immunoelectrophoresis (22) and autoradiography. After migration, rinsed and dried gel plates were either stained with Amido black or left in contact with a photo sensitive film for two weeks (No Screen Film, Ready Pack NS 2T, Kodak, developed with a standard developer).

Immunocytochemistry

Reagents : Immunochemical reagents were obtained from Dako or Nordic : fluorescein-conjugated swine IgG against rabbit IgG (Dako, code F 205) centrifuged before use ; normal rabbit serum (Dako, code X 902) ; swine IgG against rabbit IgG (Dako, code Z 196) ; normal swine serum (Nordic) and swine IgG against rabbit serum proteins (Nordic, code SwARielfo).

Human mammary tissue sections : two carcinomas of different types were studied ; the first was an adenocarcinoma characterized by large cells with acidophilic cytoplasm (apocrine type) ; the cells grew within dilated ducts; the second was a ductal infiltration carcinoma of the breast (common type). Both pieces were obtained by surgery. Semi-thin sections (4 µm) were processed. As the thickness of the sections was less than that of cells, the latter were cut, making their interior readily accessible to antibodies. Three kinds of sections were made in parallel and mounted on glass slides : 1. Cryostat-frozen sections fixed with acetone : distilled water (80 : 20) 15 minutes at -20°C and rinsed with PBS. 2. Sections of samples fixed in Bouin fixative and embedded in paraffin (56°C). Before immunochemical processing, paraffin was removed from these sections (toluene, absolute ethanol 5 mn, ethanol : distilled water 70 : 30, 2 mn, ethanol-distilled water 30 : 70, 2 mn, PBS 5 mn) ; 3. Sections of samples fixed in buffered paraformaldehyde and embedded in paraffin : same process than Bouin-fixed sections. The immunohistochemical procedure is shown in Table 1. After immunochemical processing, sections were mounted in buffered glycerin and sealed with Euckitt. A Leitz Ortoplan microscope was used with an incident UV light system. Photographs were taken with Ektachrome films, 400 ISO (Kodak). In order to check the tissue preservation and the

Table 1. Immunochemical Process.

Reagents	Assays	Controls
normal rabbit serum, 1/2, 10 mn	-	- - - - + + + - + + +
swine IgG to rabbit IgG,1/5,10 mn	-	- - - - - - - + - - - +
swine IgG to rabbit serum proteins 1/5,10 mn	-	- - - - - - + - - - + -
normal swine serum, 1/2, 10 mn	-	- - - + + - - + + - -
rabbit serum to human SBP, 1/5,30 mn	+	- - + - - - - - + + + +
fluoresceine-conjugated swine IgG to rabbit IgG, 1/15, 30 mn in dark	+	- + - + + + + + + + +
Expected results (a)	F	D F

All steps were performed at room temperature ; between each incubation step , sections were rinsed twice 5 mn with PBS.
(a) D = dark, F = fluorescence.

quality of sectioning, some sections were stained with hematoxylin-eosin-safran.

RESULTS

Pure human SBP (Fig. 1)

The advantage of the method of purification used is that SBP is processed under mild conditions thus avoiding partial denaturation. Furthermore, this method does not have the main disadvantage of affinity chromatography used by some authors (24) : it is never necessary to use extremely high concentrations of ligand for elution of SBP. Thus, the antigen injected into animals contains scarcely any DHT and the antibodies obtained do not reveal steroids in the tissue sections.

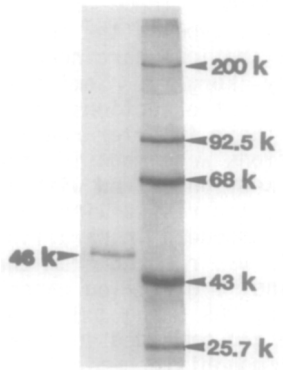

Fig. 1. SDS-polyacrylamide gel electrophoresis. Left : pure SBP denatured 2 mn at 100°C in 3% dithiothréitol and 4% SDS. Right : molecular weight calibration kit. The two identical subunits of SBP are dissociated by denaturation (23) and exhibit one homogeneous band, MW ≃ 46.000.

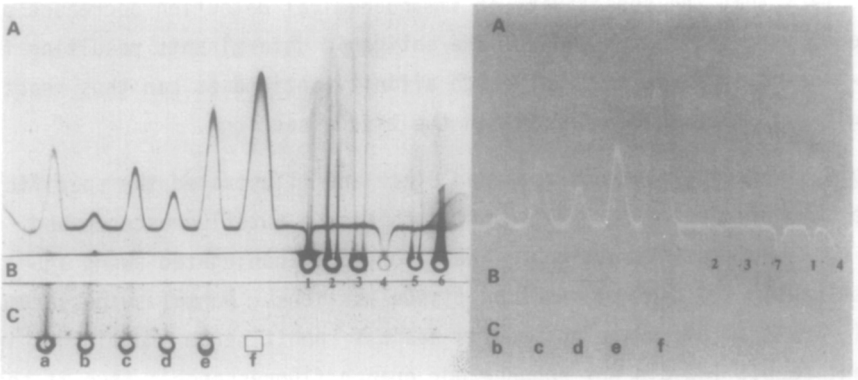

Fig. 2 : Rocket line immunoelectrophoresis. Left : plate stained with Amido black. Right : autoradiography of a similar plate. A : gel containing the immunserum against SBP. B : intermediate gel (1 : polyvalent antiserum against human plasma proteins ; 2 : antiserum against fraction C3 of complement ; 3 : antiserum against β-lipoprotein ; 4 and 7 : antisera against SBP. 5 : antiserum against transferrin ; 6 : antiserum against ceruloplasmin). C : sample of gel section containing 5% late pregnancy woman serum previously incubated with tritiated-DHT. a to f : wells containing samples with various quantities of SBP (a : pregnant Macaca mulata serum; b : human male serum ; c and d : human female sera ; e : pregnancy human serum ; f : purified SBP).

Immunoelectrophoretic characterization of rabbit antiserum against human SBP. (Fig. 2).

After staining with Amidoblack, rocket line immunoelectrophoresis showed only one continuous precipitate line, rocket shaped in front of SBP-containing wells and hollow-shaped only in front of the wells containing antibodies against SBP or total human serum proteins. No other precipitate line could be seen ; the continuity of the antigen-antibody precipitate line (resulting from rabbit antiserum and the antigen present in the late pregnancy human serum in strip C) with the rockets corresponding to deposits of the purified protein and human sera containing various quantities of antigen, implies that the protein which was used for antibody production had the same immunological properties as native SBP.

Another argument in favor of the specificity of our antiserum against SBP is the autoradiography of rocket line immunoelectrophoresis, which shows the same features as the stained plate. Human pregnancy serum mixed in agarose of the strip C was previously incubated for 2 hours with tritiated-DHT (6 µCi/100 µl). After migration, the radioactivity of the tritiated DHT/SBP complex was located only on the antigen-antibody precipitate line. We thus had a ternary complex : tritiated-DHT/SBP/antibody. These results demonstrate that the rabbit antiserum produced was monospecific and directed against SBP.

Immunohistochemistry

Whatever the fixing of the tissues, the results were identical. The image was often better on cryostat-frozen sections : this can be explained

by the fact that the sensitivity of immunochemical detection decreases proportionally to the destruction of the antigenic determinants resulting from cross-linking in fixation. Only high affinity antibodies can thus react strongly with the antigen present in the tissue sections.

Controls : Negative controls appeared black and illustrated the specificity of the immunofluorescence, ruling out both tissue autofluorescence and other non-specific interactions. The fluorescein-conjugated swine IgG against rabbit IgG did not react on tissue sections. Normal swine serum, normal rabbit serum, swine antibodies against rabbit serum proteins or irrelevant rabbit IgG did not enhance nor quench fluorescence ; thus it seems that the observed fluorescence was not artefactual.

Assays : Fluorescence appeared only when antiserum directed against SBP was used ; as demonstrated by the controls, the fluorescence was only found with sections incubated with this immunserum. As shown on photographs (Fig. 3), the aspect was different for each type of carcinoma. The apocrine type carcinoma had well-delimited cells with cytoplasm fluoresence ringing the nuclei. The common type carcinoma exhibited a sharp fluorescence indicating that the antigen was present mainly on the cell membranes. But these results do not allow us to assert that there is a significant difference of localization between the two types of carcinomas.

CONCLUSION

The immunohistochemical detection of an antigen requires the use of a strictly monospecific antiserum. Our results demonstrate that the antiserum we prepared was highly specific for SBP.

In the observed tissue (human mammary carcinomas), which is a target tissue for estrogens, we found a membrane protein whose antigenic determinants are common with those of SBP, which is known to bind DHT, testosterone and estradiol with high affinities (K_A = 2.4 10^9, 1.1 10^9 and 0.6 10^9 respectively). But the immunofluorescence data revealing the presence of the SBP-like protein do not indicate the origin of this antigen. The localization of SBP in the target cells of sex steroids leads us to consider a further physiological role for this protein. It might have a more extensive role than a simple control of the unbound concentration of circulating sex steroids in the plasma (12,15) and it might be involved in estradiol targeting, maybe via a membrane SBP-receptor, allowing this hormone to reach its cytosolic receptors, as previously proposed by others (19). Nevertheless, the complete identity of the membrane protein with SBP has not yet been demonstrated.

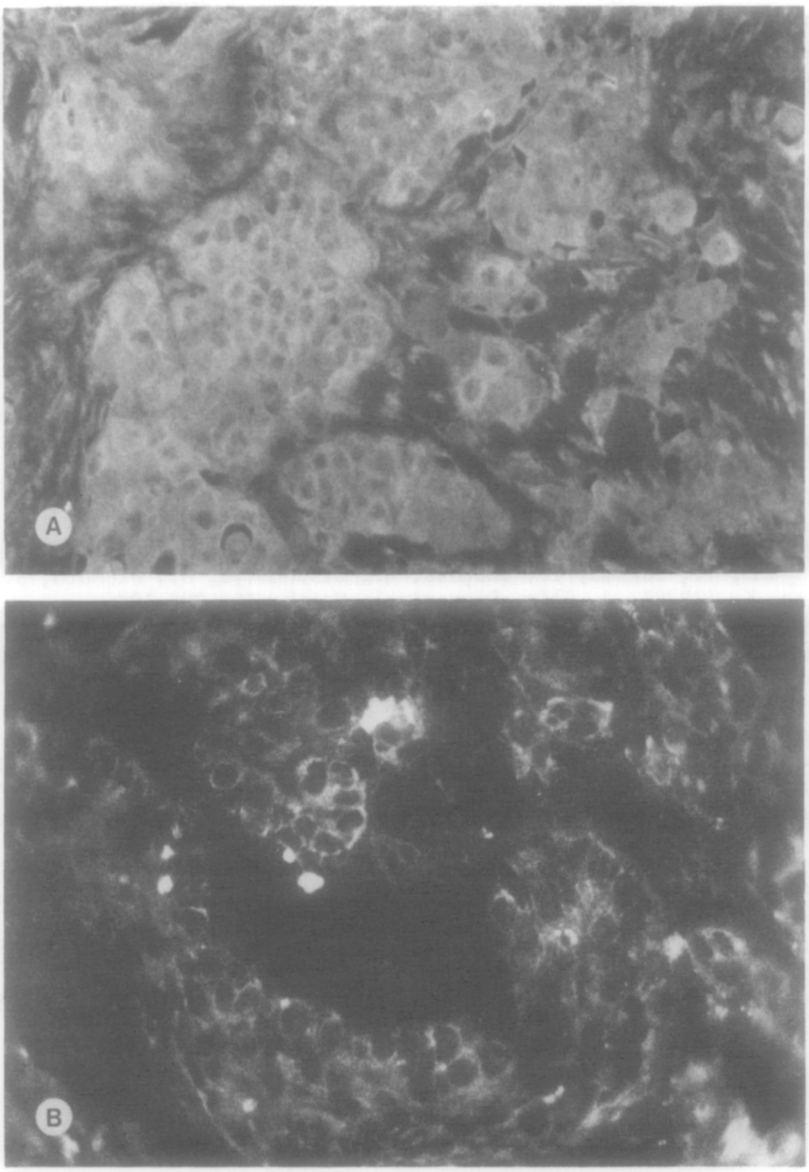

Fig. 3 Immunofluorescent localization of SBP in breast adenocarcinomas cells. A : aprocrine type, B : common type. Sections show positive cytoplasmic fluorescence surrounding the negatively stained nuclei (A) or sharp membrane fluorescence (B). Note the negative staining of the stroma.

REFERENCES

1. Sandberg, A.A., Rosenthal, H., Schneider, S.G. and Slaunwhite, W.R. (1966). In : Steroid Dynamics, 59, (Pincus, G., Nakao, T. and Tait, J.F. editors) Academic Press, New York.
2. Anderson, D.C. (1974). Clin. Endocrinol. 3, 69-96.
3. Petra, P.H., Stanczyk, F.Z., Senear, D.F., Namkung, P.C., Novy, M.J., Ross, J.B.A., Turner, E. and Brown, J.A. (1983). J. Steroid Biochem. 19, 669-706.
4. Pearlman, W.H., Crépy, O. and Murphy, M. (1967). J. Clin. Endocrinol. 27, 1012-1018.

5. Wenn, R.V., Kamberi, I.A., Vossough, P., Kariminejad, M.H., Torabee, E., Ayoughi, F., Keyvanjah, M. and Sarberi, N. (1977). Acta Endocrinol. 84, 850-859.
6. Vermeulen, A., Stoïca, T. and Verdonck, L. (1971). J. Clin. Endocrinol. Metab. 33, 759.
7. Tulchinsky, D. and Chopra, J.J. (1974). J. Clin. Endocrinol. Metab. 39, 164-169.
8. Habrioux, G., Egloff, M., Boukari, M. and Degrelle, H. (1979). Ann. Endocrinol. (Paris) 40, 155-158.
9. Vermeulen, A. and Andó, S. (1979). J. Clin. Endocrinol. Metab. 48, 320-326.
10. Egloff, M., Savouré, N., Tardivel-Lacombe, J., Massart, C., Nicol, M. and Degrelle, H. (1981). Acta Endocrinol. 96, 136-140.
11. Burke, C.W. and Anderson, D.C. (1972). Nature 240, 38-40.
12. Pardridge, W.M. (1981). Endocrine Reviews 2, 103-123.
13. Bordin, S. and Petra, P.H. (1980). Proc. Natl. Acad. Sci. USA 10, 5678-5682.
14. Egloff, M., Vendrely, E., Tardivel-Lacombe, J., Dadoune, J.P. and Degrelle, H. (1982). C.R. Acad. Sci. Paris 295, 107-112.
15. Egloff, M., Vendrely, E., Tardivel-Lacombe, J., Dadoune, J.P. and Degrelle, H. (1982). J. Steroid Biochem. 17, XLVIII, Abstract n° 143.
16. Murayama, Y., Sakuma, T., Udagawa, H., Utsunomiya, J., Okamoto, S. and Asano, K. (1978). J. Clin. Endocrinol. Metab. 46, 998-1006.
17. Harris, A.L., Smith, I.E., Dowsett, M., Jeffcoate, S.L., Coombes, R.C., Powles, T.J. and Neville, A.M. (1981). The Lancet, January 31, 279.
18. Berta, L., Gaidano, G.P., Navello, C., Bonatesta, C., Valenzano, C., Mossetti, C. and Priolo, E. (1982). Int. J. Breast Mammary Pathol. - Senologia 1, 125-129.
19. Namkung, P.C. and Petra, P.H. (1982). J. Steroid Biochem. 17,ci Abstract n° 301.
20. Egloff, M., Vranckx, R., Tardivel-Lacombe, J. and Degrelle, H. (1981). Steroids 4, 455-462.
21. Sternberger, L.A. (1979). In : Immunocytochemistry (Ed. by Wiley, J. and Sons Inc., New York) pp 24-58.
22. Krøll, J. (1973). Scand. J. of Immunol. 2, suppl. 1, 83-87.
23. Mickelson, K.E., Teller, D.C. and Petra, P.H. (1978). Biochemistry 17, 1409-1415.
24. Petra, P.H. and Lewis, J. (1980). Analytical Biochem. 105, 165-169.