Receptor and Centriole Pathways of Steroid Action in Normal and Neoplastic Cells

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Abstract

An experimental system designed for tracing the estradiol kinetics in target cells by specific antibodies has been applied to human breast cancer. Several major defects of the estradiol receptor mechanism have been demonstrated. The detected changes (lack of cytoplasmic receptors, impaired nuclear transfer of estradiol:receptor complexes, and abnormal nuclear retention of these complexes) have been demonstrated in most human breast cancers that appear to be composed of hormone-dependent and autonomous mixed-cell populations. These abnormalities could be the biological background for the overall or partial unresponsiveness of breast cancer to endocrine management.

The participation of steroids in the regulation of centriole activities is taken into account since immunoreactive steroids are traceable by UV and electron microscopy at the level of this cell organelle by steroid antibodies. Moreover, the presence of steroids in the pericentriolar material correlates well with the modulating appearance and activity of the centriole throughout the cell cycle. A new centriole pathway is suggested by which steroid hormones can regulate cell proliferation.

Introduction

Immunocytochemistry investigators recently succeeded in demonstrating sex steroid hormones in both source and target tissues (4, 30, 33, 34, 36, 39, 40, 52) (Figs. 1 to 4). This approach allowed the tracing of steroid hormones at different intracellular sites and provided some new insight into the biology and pathophysiology of steroid action. In regard to human breast cancer, the immunocytochemical approach led to the demonstration of some abnormal events in the steroid action mechanism that are relevant to the tumor hormone responsiveness (31, 32, 34, 36, 39, 40). The unpredictable response of human breast cancer to endocrine therapy is axiomatic, and the search for reliable markers of hormone responsiveness has been a major concern in recent years. The realization that most of the effects of steroid hormones are mediated by receptor proteins (2, 12, 22) has prompted attempts to evaluate the relationship between the presence of receptors in breast tumors and the response to endocrine therapy (15, 18, 24, 25, 27, 28). Thus, the best current index of the hormonal sensitivity of a breast cancer is the concentration of estrogen receptor proteins in the tumor tissue. Nevertheless, and contrary to expectation, the mere presence of estrogen receptors is not an absolute indicator of hormone dependency.

This report deals chiefly with the problem of cell heterogeneity in human breast cancer as far as hormone dependency is concerned, with special attention to some kinetic defects that can be detected in the steroid action mechanism by the fluorescent antibody technique.

Methodology

Three main cytochemical approaches can be envisaged for localizing steroid hormones in target tissues: (a) the use of labeled hormone, exploited already by Stumpf and Sar (46) and currently tried again as fluorescent hormone by Dandliker et al. (7); (b) the antibody to the purified receptor, obtained recently by Greene et al. (14) and expected to be a very efficient tool for precise localization of receptors; and (c) antibodies to steroid hormones, which we (33, 34, 36) and other groups (39, 40) presently use. This approach exploits the main property of the steroid receptor, i.e., high-affinity steroid binding, and appears to be a powerful technique for tracing the actual distribution of steroids in large populations of target cells. Moreover, not only the presence of the receptor, but also its working state can be appraised by this approach; this is an advantage especially remarkable with regard to human breast cancer.

The experimental system for monitoring the estradiol intracellular kinetics in human breast cancer cells has already been extensively described elsewhere (33, 34) and is only schematized in Table 1. It is important to note that, although the specificity of the fluorescent staining ultimately depends on the quality of the antibody, the reliability of the results depends heavily on the accuracy of control experiments.

Steroid Receptor Pathway

Cytoplasmic Receptor. The currently accepted model for the mechanism of action of steroid hormones includes interaction with cytoplasmic receptor proteins as an initial and essential intracellular event (2, 12, 22).

Under our experimental conditions the well-known cold immobilization of estradiol:receptor complexes in the cytoplasmic compartment allowed a selective fluorescent visualization of the estradiol bound at the cytoplasmic level (Figs. 5 and 6). Specificity for receptor binding was surmised from the results of the competitive inhibition by nonsteroid estrogenic (diethylstilbestrol) and antiestrogenic (nafonidine, tamoxifen) drugs. Marked differences in the cytoplasmic uptake were apparent among cells belonging to the same tumor (Fig. 7); it may be that this varying positivity can be related to some extent to the cell cycle (5).
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Table 1

<table>
<thead>
<tr>
<th>Experimental system</th>
<th>Control tests</th>
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<td>1. Isolated cell suspension is obtained by tissue mincing and squeezing.</td>
<td>Cell viability: dye exclusion</td>
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<td>2. Cells (3 x 10^6 to 5 x 10^6/ml) are incubated with 10 nm estradiol for 1 hr at 4°.</td>
<td>Binding specificity: competitive inhibition by diethylstilbestrol (10 μM), nafoxidine (0.1 nm), or tamoxifen (0.1 nm)</td>
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<td>3. Cells are carefully washed for 1 hr at 4°. Some aliquots of cells are then spread on slides by a cytocentrifuge and air dried at 4°.</td>
<td>Immune specificity: preimmune serum: estradiol-absorbed antiserum</td>
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<td>4. Other aliquots of cells are postincubated at 37° for 1 to 24 hr in TC199 plus 10% fetal calf serum. Aliquots are then treated as in Step 3.</td>
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<td>5. Indirect immunofluorescence is performed with rabbit anti-estradiol-6-BSA antiserum (final dilution 1:100) and goat fluorescein-conjugated anti-rabbit immunoglobulin antiserum (final dilution, 1:25). The temperature is kept at 4° throughout the procedure.</td>
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More frequently, 2 different cell populations were observed in the same tumor, one endowed with cytoplasmic estradiol receptor and the other devoid of binding capacity (Fig. 8). A quantitative evaluation could be made; yet a mere survey assessment supports the suggestion that almost all breast cancers are composed of hormone-dependent and autonomous cells as far as the cytoplasmic estradiol uptake is concerned.

Estrogen Receptor Movement to the Nucleus. After the binding of the hormone to its own receptor, the subsequent movement of the hormone-receptor complex from the cytoplasm to the nucleus is fundamental to the action of steroid hormones (2, 11, 12, 55). Previous studies from our laboratory monitored this temperature-dependent intracellular redistribution of estradiol following the initial uptake. A progressive increase in nuclear estradiol concomitant with the depletion of the cytoplasmic hormone was observed (Fig. 9), like that occurring in vivo (Fig. 15). Many tumors appeared to contain a varying number of cells with an evident cytoplasmic uptake but a failing nuclear transfer of the bound estradiol (Figs. 10 and 11). Two main aspects of the faulty movement to the nucleus have been observed: (a) failure of the bound hormone to move toward the nucleus, as if it were fixed at the cytoplasmic level; and (b) failure of the moving estradiol to penetrate the nucleus, around which the hormone appeared to concentrate in a perinuclear ring outside the nuclear membrane (Fig. 12). Data implicating a defective receptor, the receptor binding to the nuclear membrane, and/or a membrane-controlled access to the nuclear compartment have already been presented (16, 21, 29, 34, 42, 47, 49, 55). It may be that, in such neoplastic cells lacking the nuclear filling, a physiological nuclear membrane step no longer masked by the usual nuclear positivity can emerge.

Nuclear Binding and Retention. It is generally accepted (6, 12, 55), despite some conflicting opinion (51), that a key event in the regulation of gene expression by steroid hormones is the interaction of steroid:receptor complexes with a limited number of specific nuclear acceptor sites. Moreover, a prolonged nuclear retention, rather than just the nuclear binding, of complexes seems to be required to determine appropriate biological actions (6, 11, 12, 21). In this respect, some differential releasing rates of the estradiol accumulated in the nucleus were apparent during the following time course. Two main pools of nuclear-associated estradiol were identified: (a) a larger, fast-clearing one, which leaves the nucleus soon after; and (b) a finite number of long-lasting retention sites associated with the chromatin network (Fig. 13).

It seems noteworthy that in some tumors there were several cells that, although retaining the normal nuclear transfer, lacked the prolonged nuclear retention of the estradiol (Fig. 14); cell independence of endocrine control may be ascribed also to reduced or absent "true" nuclear acceptor sites. The observed defects (Fig. 16) in the nuclear translocation and binding times strengthen the previous assessment (19, 23) that nuclear-bound receptors may provide a better indicator of tumor responsiveness than does the assay of cytoplasmic receptors only.

Centriole Pathway

Steroid antibodies detected by immunocytochemical techniques strongly decorate the cell centrioles (Fig. 17) in steroid-target and nontarget cells (31, 35). The immunoperoxidase technique in electron microscopy has been instrumental in establishing the actual presence of immunoreactive steroids at the level of this cell organelle (Figs. 21 to 23). Moreover, after treatment with digitonin (a well-known reactant for 3β-hydroxysteroids), centrioles of cultured cells turn out highly birefringent by polarized-light microscopy (Fig. 20); 3β-hydroxysteroids are thus expected to be present in cell centrioles. In mitotic cells only one of the 2 centrioles present at each pole of the mitotic spindle is reactive to steroid antibodies (Figs. 18 and 19). This feature can be related to the known cycling activity of centrioles (10, 45); in fact the 2 parent centrioles normally separate at prophase, each accompanied by its still immature daughter, which will attain functional and morphological maturity only in the following interphase. Therefore, the presence of steroids at the centriole level seems to reflect the modulating activity of this organelle throughout the cell cycle.

The precise identity of the immunoreactive steroid(s) traced at this level is not yet established, and whatever relationship may exist between the centriolar and the receptor pathways is not clear. Only some suggestions encompassing steroid action and centriole function can be put forward at present. Centrioles are notoriously involved in the regulation of cell duplication and differentiation. The biology and biochemistry of centrioles are unexplored fields (10, 13, 43, 45); the centrioles are probably governed chiefly by local control, of which hardly anything is known. Nevertheless, it has already been suggested that steroid hormones can directly affect the structure and/or function of these organelles (3, 48) and influence cell mitotic behavior by interfering with the functional role of these microtubule-
organizing centers (37). It seems unlikely that tubulin, the major constituent of microtubule organelles and a well-known binder for many ligands (43, 44), is involved in this steroid localization. Instead of microtubules (20), steroid antibodies heavily stain the pericentriolar material, drawing a "negative" picture of the microtubule framework (Fig. 22). Centrioles themselves are not displayed by specific tubulin antibody by UV light and electron microscopy (8, 38); the lack of access of the tubulin antibody has been attributed to the presence of the pericentriolar material to which we trace immunoreactive steroids.

The pericentriolar amorphous cloud, which seems to be endowed with nucleic acids and specific centrosomeric properties (10, 13), fits well as the repository of the interplay between steroids and centriolar activity. In this respect it seems remarkable that, in mitotic cells, steroid antibodies do not detect the immature daughter centriole, which lacks only the pericentriolar amorphous material. The concomitant modulation of the centriole activity and its steroid supply strongly suggests a new pathway by which steroid hormones can affect the cell duplication, growth, and differentiation.

This observation represents a most important question to be answered in the future, although an arduous task to be accomplished, since it is directly related to the regulation of cell proliferation.

Conclusion

In light of present data, the biological background for cell control by steroid hormones does not look as simplistic as previously thought. With regard to the current receptor pathway, the labeled antibody approach indicates that several pathological variants of the tumor cell receptor pattern can influence endocrine responsiveness and the clinical course. Moreover, transcriptional or posttranscriptional defects downstream in the sequence of biochemical events may conceivably lead to unaffected tumor behavior, in spite of plentiful and working receptors. In fact, the investigation of some phenotypic effects (1) has been prompted (27, 28) in pursuit of a reliable postreceptor marker of hormone dependence. In this respect our present course concerns the evaluation of the predictive meaning of some steroid-induced enzyme activities in breast cancer cells, such as: (a) endogenous peroxidase, already proposed as a reliable marker for estrogen action (9, 17, 26, 31, 32); and (b) 17β-hydroxysteroid dehydrogenase, which seems to be regulated via progesterone receptors (41).

In this regard preliminary results have confirmed the assumption of the constant cellular heterogeneity of almost all breast cancers, while examining cell responsiveness to hormones. The existence of such cellular heterogeneity is a major concern in establishing the optimal therapeutic approach to breast cancer. We and others (15, 24, 32, 50) firmly believe that a correct approach of combined endocrine and cytotoxic treatments could be envisaged in a majority of cases. The definition of the optimal combination for customized therapy should take into account the chance offered by recruitment and synchronization of responsive cells (53, 54) attainable due to the regulation of growth rate parameters of breast tumors by estrogen.

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Fig. 1. Estradiol crystals treated by the labeled antibody technique brightly fluoresce under UV.

Fig. 2. Fluorescent antibodies display immunoreactive estradiol in follicles of a rat ovary. cl, corpora lutea.

Fig. 3. An evident nuclear localization of estradiol is traced by the fluorescent antibody technique applied to the myometrium obtained from a 20-day-old rat, 1 hr after the i.p. injection of estradiol (100 ng/g body weight).

Fig. 4. Endometrium from a rat treated as described in the legend to Fig. 3. Immunoperoxidase technique displays estradiol chiefly in nuclei of epithelial and stromal cells.
Figs. 5 to 8. Dispersed cells incubated with estradiol at 4° for 1 hr, washed, and stained with antiestradiol antibody.

Fig. 5. Hyperplastic breast cells. The hormone has been taken up at cytoplasmic level, where it is demonstrated by the fluorescent antibody.

Fig. 6. Human breast cancer cells exhibiting an homogeneous cytoplasmic uptake.

Fig. 7. Human breast cancer cells. The variable cytoplasmic staining seems related to cytological differences.

Fig. 8. Mixed populations of receptor-positive and receptor-negative cells obtained from the same breast cancer.

Figs. 9 to 12. Dispersed cells incubated with estradiol at 4° for 1 hr, washed, and postincubated for 1 hr at 37°. Immunofluorescence technique.

Fig. 9. A predominant nuclear localization of estradiol is clearly traced by fluorescent antibodies in these hyperplastic epithelial cells from human breast tissue.

Fig. 10. One of these clustered breast cancer cells fails to display any nuclear incorporation of estradiol.
Fig. 13. Hyperplastic cells from human breast tissue, postincubated at 37° for 5 hr. Long-lasting nuclear retention sites of estradiol appear as a fluorescent dotting of the chromatin network.

Fig. 14. Human breast cancer cells displaying scanty or no (O) retention of estradiol in the nucleus.

Fig. 15. Cryostatic section of an intraductal breast carcinoma. The predominant in vivo nuclear localization of the estradiol is clearly traced by the fluorescent antibody also, when applied on tissue sections.

Fig. 16. Cryostatic sections of 2 infiltrating breast cancers exhibiting a variable mixture of estradiol binding as displayed by the fluorescent antibody.

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Fig. 11. Breast cancer cells. Impaired nuclear transfer of the estradiol is evident in some cells, whereas in others the usual nuclear filling by translocated estradiol/receptor complexes is exhibited.

Fig. 12. Human breast cancer cells failing the nuclear transfer in spite of the cytoplasmic uptake. An evident perinuclear concentration of estradiol is shown by these cells; the nuclear transfer behaved properly only in the central cell of the cluster shown in a.
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Figs. 17 to 19. HEp-2 cells grown in monolayer and stained with antiestradiol antibody (see Ref. 35).

Fig. 17. Two prominent, brightly fluorescent centrioles stand out close to the nucleus. Immunofluorescence technique.

Fig. 18. In this mitotic cell, only 1 of the 2 centrioles present at each pole of the mitotic spindle is detected by the immunofluorescence technique.

Fig. 19. Mitotic cells at metaphase. a: horseradish peroxidase-labeled antibody decorates only the parent centrioles located at each side of the equatorial plate. b: the peroxidase reaction product draws an evident pericentriolar ring in the same cell by phase contrast.

Fig. 20. The treatment by digitonin brings out centrioles sharply defined when viewed by phase contrast (a) and highly birefringent by polarized light (b).

Fig. 21. Electron micrograph of a pair of interphase centrioles heavily stained by the immunoperoxidase reaction with antiestradiol antibody.

Fig. 22. The immunoperoxidase technique heavily stains the immunoreactive steroids of the pericentriolar material, drawing a negative picture of the centriole microtubule triplets.

Fig. 23. Architecture of the microtubule framework of the interphase diplosome of a cell exposed to preimmune rabbit serum.
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