Ultrastructural Localization of the Progesterone Receptor by an Immunogold Method: Effect of Hormone Administration

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Abstract. The progesterone receptor has been localized in the rabbit uterus by immunocytochemistry at the electron microscopic level, using monoclonal antibodies and the protein A-gold technique. The progesterone receptor in uterine stromal cells was mainly localized in the nucleus; however, a small fraction of antigen was present in the cytoplasm, where it was associated with the rough endoplasmic reticulum and with free ribosomes. The plasma membrane was not labeled. In the nucleus, the receptor was always associated with condensed chromatin or areas surrounding condensed chromatin, whereas the nucleolus was not labeled. In the chromatin, receptor distribution varied according to the hormonal state: in the absence of progesterone, the receptor was randomly scattered over the clumps of condensed chromatin; after administration of the progestin R5020, it was mainly detected in the border regions between condensed chromatin and nucleoplasm and, to a lesser extent, over dispersed chromatin in the nucleoplasm. These areas have been shown to be the most active sites of gene transcription.

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In target cells, steroid hormones form complexes with specific receptors which in turn modulate gene expression (for review, see reference 10). Data have recently accumulated on the binding of steroid-receptor complexes to regulatory regions of cloned genes (34). However, little is known about the topology of these regulations in the intact cell. The recent availability of monoclonal antibodies has allowed the study of the estrogen receptor by immunocytochemical methods at the light microscopic level (19, 29). Surprisingly, and in contrast to results of cell fractionation experiments, the receptor was found only in the nucleus, both in presence and in absence of hormone. Using monoclonal antibodies against the rabbit progesterone receptor (PR) and immunocytochemistry we have found a similar localization (35). These parallel findings suggest that all (or most) of the steroid receptors can be found in the nuclei of their target cells, even in the absence of their ligand.

However, the exclusively intranuclear distribution of steroid receptor antigen found at the light microscopic level could not exclude the presence of small amounts of receptor in the cytoplasm, undetected at this level of resolution. Moreover, the intranuclear distribution of steroid receptors and their association with different nuclear structures (in the presence or in the absence of hormone) remained unknown.

The aim of the present study was to investigate the above-mentioned problems and thus, for the first time, show the ultrastructural localization of a steroid hormone receptor. Using monoclonal antireceptor antibodies and the protein A-gold technique (39), we observed that, in uterine stromal cells, PR was located mainly in the nucleus, whereas only a small fraction was present at specific sites in the cytoplasm. In addition, immunoelectron microscopy of nontreated and progestin-treated rabbits revealed differences in the ultrastructural distribution of immunoreactive PR within the nucleus. The observed redistribution was compatible with a role for hormone-activated PR in the modulation of gene expression.

Materials and Methods

Animals and Preparation of Tissues

Immature female rabbits (New Zealand, 1 kg) were primed with estrogen as described (26). 30 min before being killed some rabbits received a subcutaneous injection of either the synthetic progestin R5020 (17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione; Roussel-Uclaf, Romainville, France) (10 mg in 0.5 ml sesame oil), or of vehicle alone. The characteristics of these hormonal treatments were selected to match previous biochemical (24-27) and immunocytochemical studies (35). Western blot experiments have shown that 30 min after such a progestin injection only 10-20% of receptor molecules remain in the cytosol (23). The ultrastructural distribution of progesterone receptor was compared in these two situations, which differ by the fact that receptor is either unliganded or bound to the hormone.

Rabbit uteri were removed immediately after cervical dislocation. For control in light microscopy, a slice (2 mm long) was taken in the middle part of the uterus, fixed in 1% glutaraldehyde, and embedded in paraffin (35). For electron microscopy, fragments (≤1 mm³) from endometrium or myometrium were fixed with 1% glutaraldehyde in Sorensen buffer, pH 7.4, for 6 h at 4°C. Occasionally, free aldehyde groups were blocked by subsequent incubation of fragments in 0.5 M NH₄Cl in phosphate buffer for 1 h (38). Postfixation with Oso₄ was omitted. Dehydration through an ethanol series at 4°C, infiltration with propyleneoxide, and embedding in Epon 812 were performed according to the usual procedure. Semithin sections, 0.5 μm thick, were obtained from these blocks and mounted on glass slides. Thin sections were cut with a diamond
level, the progesterone receptor is present in the nuclei of the

Results

Antisera

Monoclonal mouse IgG against PR from rabbit uterus (M60-10 alone or a mixture of M60-10, Mi11-5, M15-31, Mi12-2) was used for immunolabeling in electron microscopy, as it had been for previous light microscopic studies (35).

The specificity of these studies of these antibodies have been described (24, 25, 27).

They included density-gradient experiments (25), precipitation of PR-progesterin complexes with a second antibody or protein A (25), immunoelectron analysis of crude uterine cellular extracts (27), and immunofluorescence chromatography experiments (24).

Mouse monoclonal antibodies (IDA and AIDA, a gift from Dr. P. Legrain, Institut Pasteur, Paris), unrelated to the receptor under study, served as controls.

They were antidiotype antibodies raised against mouse myeloma anti-antireceptor antibodies (21). In all cases, the control antibody was of the same class as the antireceptor antibody (for instance, IgG2a class in the case of the Mi60-10 antibody).

Immunocytochemical Labeling

For light microscopy, paraffin sections (4 μm thick) or semithin sections of Epon-embedded uterus were stained by an indirect immunoperoxidase method or by the peroxidase-antiperoxidase method, as previously described (35).

Before the immunolabeling procedure, semithin sections were deplastified with sodium ethoxide (20).

For electron microscopy, PR was localized on thin sections using the protein-A gold technique developed by Roth (39), but the etching step with H2O2 was replaced by a treatment with sodium metaperiodate (4). All solutions were filtered on 0.45-μm Millipore filters (Millipore Corp., Bedford, MA) immediately before use. Background labeling was reduced by incubating sections with 1% ovalbumin for 5 min before labeling and by using 0.09% Tween in all incubations and washes. In brief, grids were floated on a drop of diluted mouse monoclonal antibodies (M60-10, 1.5-3 μg/ml) in PBS containing 1% ovalbumin, for 3 h at room temperature in a humid chamber. Then the grids were washed with PBS and incubated for 1 h with protein A conjugated to colloidal gold particles 15 nm in diameter (or 10 nm where indicated) (diluted 1:40 in PBS containing 1% ovalbumin) (Janssen Pharmaceutica, Beersel, Belgium).

After successive washings in PBS and distilled water, the grids were air-dried. They were post-stained with uranyl acetate (4% in distilled water) for 5 min and viewed in a Siemens electron microscope type CT 350 at 80 kV. Some sections were stained by the uranyl-EDTA lead method of Bernhard (8) after immunogold labeling; in this case, sections were stained (2 min) with uranyl acetate, layered on a 0.1 M EDTA solution for 20 min, and final post-stained (1 min) with lead citrate.

Controls were prepared as follows. Several dilutions of M60-10 antibodies (IgG2a class) were initially studied (1.5-20 μg/ml), and the dilutions used (1.5-20 μg/ml) were finally selected because they gave an optimal staining of progesterone receptor-containing nuclei and a low background over mitochondria and extracytoplasmic matrix. Control tests, which all gave negative results, involved the substitution of monoclonal M60-10 antibodies either by a non-progesterone receptor-related monoclonal antibody of the same class (IDA, IgG2a used at the same concentration, or by monoclonal anti-progesterone receptor antibodies that had been adsorbed by the pure receptor (24) before the immunolabeling (35).

For each hormonal situation, three rabbits, two randomly chosen blocks per uterus, and 4-10 grids per block were studied. A mean of 30 grids per situation and washes. In brief, grids were floated on a drop of diluted mouse monoclonal antibodies (M60-10, 1.5-3 μg/ml) in PBS containing 1% ovalbumin, for 3 h at room temperature in a humid chamber. Then the grids were washed with PBS and incubated for 1 h with protein A conjugated to colloidal gold particles 15 nm in diameter (or 10 nm where indicated) (diluted 1:40 in PBS containing 1% ovalbumin) (Janssen Pharmaceutica, Beersel, Belgium).

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For the analysis of nuclear localization, four nuclear compartments were defined: the nucleoplasm, the nucleolus, and the extranucleoplasmic matrix (35).

The specificity of the immunogold staining was shown by controls made by (a) replacing antiprogestosterone receptor antibodies by nonrelated antibodies of the same class, at the same concentration (Fig. 3c), (b) incubating the sections with the protein A-gold complex in absence of antibodies, or (c) preadsorbing the antibodies with pure progesterone receptor (Fig. 3b). In all cases only a low background was observed, randomly distributed on all cellular structures and over the extracellular space.

Furthermore, application of the EDTA regressive stain (8) results in a preferential staining of ribonucleoprotein structures and thus allows us to distinguish them from chromatin.
Figure 1. Preliminary experiments at the light microscope level. (a and b) Effect of hormone administration on progesterone receptor localization. (c) Effect of tissue-embedding in Epon. Uteri from progestin-treated (b) and nontreated (a and c) rabbits were embedded in paraffin (a and b) or in Epon (c). For the detection of the receptor, sections were treated either by the indirect peroxidase-antiperoxidase method (a and b) or by the indirect peroxidase method using peroxidase-labeled rabbit IgG (c). Under all conditions, typical immunostaining is seen in cell nuclei of luminal and glandular epithelium (E) and stroma (S). Note the stronger staining in stromal cells as compared with epithelial cells. There was no counterstaining. Bar, 25 μm.

When this method was applied to thin sections after the immunogold staining, it clearly showed that the receptor was associated with chromatin and not with ribonucleoproteins (Fig. 4).

**Nuclear Localization of PR After Administration of Progestin**

The intranuclear concentration and distribution of the receptor were changed in the uterine stromal cells of rabbits treated by progestin (R5020) (Fig. 5). The overall labeling was decreased by hormone treatment (compare Figs. 3 and 5a). Such changes of receptor concentration have previously been observed in biochemical studies (18, 30) and have sometimes been called “receptor processing” (31) or “down regulation.” Moreover, the general organization of the chromatin changed in these cells after the progestin was administered. The clumps of condensed chromatin became smaller and were spread out within the nucleus (see in Fig. 5a). PR immunoreactivity was also associated with chromatin, as confirmed by the regressive EDTA technique (8). However, in contrast to what was seen in rabbits not treated by the progestin, the immunogold stain was spread out as was condensed chromatin. The decrease in
receptor labeling and its dispersion inside the nucleus rendered the interpretation of its distribution more difficult. However, the examination of many different samples (30 grids) enabled us to define clearly its localization: gold particles were observed mainly at the border between the small clumps of condensed chromatin and the nucleoplasm (Fig. 5, a and d). Some labeling was also present over dispersed chromatin in the nucleoplasm (Fig. 5c). In contrast, the clusters of interchromatinic granules were not labeled above background. These granules were clearly visible after a standard uranyl acetate staining. Occasionally, a few gold particles were still associated with the remaining thin perinuclear (Fig. 5a) and perinucleolar (Fig. 5e) condensed chromatin. The specificity of the labeling was again established by the controls described above in the case of non-progestin-treated animals: nonspecific labeling with receptor unrelated monoclonal antibodies was very low (Fig. 5b), and competitive inhibition of the staining by pure receptor was observed (not shown).

**Presence of a Small Amount of PR in the Cytoplasm of Target Cells**

At the light microscope level, immunocytochemical studies have not shown any estrogen (19, 29) or progesterone (35) receptors in the cytoplasm. We took advantage of the high...
chromatin (CC) appears bleached by the EDTA treatment, whereas 0.5 μm. was followed by the EDTA regressive technique (8). The condensed chromatin clumps. of interchromatinic granules this question. Using the immunogold method, we have con-

Figure 4. Nuclear localization of progesterone receptor in uterine stromal cells in the absence of hormone. Immunogold labeling of PR was followed by the EDTA regressive technique (8). The condensed chromatin (CC) appears bleached by the EDTA treatment, whereas ribonucleoproteic structures in the nucleoplasm, among them clusters of interchromatinic granules (IG), contrast well. Most progesterone receptor immunoreactivity is associated with bleached condensed chromatin clumps. NM, nuclear membrane. M, mitochondria. Bar, 0.5 μm.

resolution offered by the electron microscope to reexamine this question. Using the immunogold method, we have con-

Discussion

By light microscope immunocytochemistry using monoclonal antibodies, estrogen and progesterone receptors have been observed to reside in the nuclei of their target cells (19, 29, 35). In contrast, different localizations of steroid hormone receptors (only cytoplasmic [16, 33, 37], only nuclear [15], both cytoplasmic and nuclear [1, 7, 14, 17], translocation from the cytoplasm to the nucleus after hormonal treatment [7, 16, 33, 37]) have been reported in other light immunocytochemical studies using polyclonal antibodies. Some of these discrepancies, especially when extranuclear localization was observed, might have been due to the fact that the polyclonal antibodies recognized not only the receptor but also other antigens.

The present study represents the first visualization at the electron microscope level of a steroid receptor in a target cell using specific monoclonal antibodies.

Various methodological difficulties had to be resolved to observe the in situ localization of PR. Previous immunocytochemical studies at the light microscopic level had already been in part focused on the determination of fixation conditions that thereafter could be used for ultrastructural immunochemistry. In this respect, the possibility of detecting immunoreactive receptor after glutaraldehyde fixation proved to be important, since this fixative gives a good preservation of cellular ultrastructure. This, added to the use of the protein A-gold technique, has allowed a fine identification of the labeled nuclear and cytoplasmic structures. Due to their high electron density, gold particles are easily detected in the electron microscope and, in contrast to the peroxidase-antiperoxidase technique, do not obscure the ultrastructural details of the labeled structures. The fixation and embedding conditions used probably did not lead to an artifactual redistribution of the antigen for the following reasons: glutaraldehyde rapidly penetrates cells and cross-links proteins; and consistent and unequivocal staining of PR with little or no background was constantly observed in the numerous experiments (30 different grids; see Materials and Methods). We are now examining the possibility of using other fixatives, but this necessitates long preliminary studies. In each case, it is necessary to define the precise experimental conditions that give a good preservation of ultrastructural details without impeding the immunological recognition of the receptor.

The specificity of the antireceptor antibodies has previously been thoroughly discussed (24, 25, 27). The same localization of PR immunoreactivity was observed using either M160-10 monoclonal antibodies or a mixture of four other monoclonal antibodies (35). In addition, a comparable result was obtained using a goat polyclonal monospecific antibody prepared against the purified (chromatographed on immunoaffinity column [24], electrophoresed, and electroeluted) 110 kD form of PR (Logeat, F., unpublished observations). The absence of staining by gold-protein A alone, or by receptor unrelated monoclonal antibodies or presaturated PR antibodies also indicates the specificity of the immunocytochemical staining.

Most of the PR molecules were intranuclear in uterine stromal cells, as well as in epithelial, glandular, and smooth muscle cells (Perrot-Applanat, M., unpublished observations). In stroma cells and in the absence of hormone, they were localized mainly in the condensed chromatin which is known to be for the most part transcriptionally inactive (12). Since receptors are easily extracted when the tissue is homogenized, they are probably loosely bound to some component of condensed chromatin. By contrast, after the administration of hormone, the relative labeling was increased at the border
Figure 5. Nuclear localization of progesterone receptor in uterine stromal cells after administration of the progestin R5020. Note the more euchromatic appearance of these nuclei (N). Progesterone receptor immunoreactivity is observed over small clumps of condensed chromatin (CC), mainly at their periphery (a and d). (c) In nuclei exhibiting highly dispersed chromatin, labeling is also observed over dispersed chromatin (DC). (e) The nucleolus (Nu) is not significantly labeled; Pnc, perinucleolar chromatins. (b) Control incubation with non-receptor-related antibody. Note the complete absence of immunocytochemical staining. IG, interchromatinic granules. Bar, 0.5 μm.

Biochemical studies have indicated that various steroid hormone receptors may be associated with the nuclear matrix (2).

This description of receptor distribution is based on the analysis of a total of 12 different blocks, obtained from three progestin-treated and three nontreated rabbits, and the examination of >200 electron micrographs. It is also supported by preliminary manual counts of the immunogold labeling (unpublished observations). Of course, these qualitative observations should be completed by a quantitative evaluation of dense chromatin and nucleoplasm. Some labeling corresponded to dispersed chromatin in the nucleoplasm. These localizations correspond to the regions known to be most active in extranucleolar gene transcription (11, 12). Nash et al. (32) have previously shown that the increase of RNA synthesis in rat liver nuclei provoked by cortisol was correlated with the increase of the amount of perichromatin fibrils localized at the periphery of condensed chromatin. In addition, it has been shown that the newly transcribed RNA seems to be closely associated with the nuclear matrix (5).
of the distribution of the labeling; we are now reexamining our micrographs with an automated image analyzer.

The partial redistribution of the PR within the nucleus of stromal cells upon administration of hormone may correspond to a translocation of the receptor from one site to another. Alternatively, it may be due to the fact that the receptor simply accompanies the change in localization of at least some specific genes. It is noticeable that, in stromal cells, the administration of the progestin markedly decreases the proportion of condensed chromatin present in the form of large clumps. Thus, specific genes initially present in the condensed regions are probably shifted towards the euchromatin or to the periphery of condensed chromatin. If it interacts with these genes, the receptor may perhaps passively change its localization. Similar changes in the ultrastructure of target cell nuclei upon administration of steroid hormones have previously been described (6, 40, 42). Thus, estradiol transformed the condensed chromatin into dispersed chromatin in uterine epithelial cells (6, 42). Moreover, as described in this study, dispersion of chromatin has also been observed in rat stromal cells under progesterone treatment (40). The mechanism of this modification of chromatin structure is unknown. To determine whether the receptor translocates from one site to another or only changes its localization in a passive way, it would be necessary to localize in intact interphase nuclei specific hormonally regulated genes before and after hormone administration.

In various uterine cell types, there seems to be a relationship between the importance of the change in the organization of the chromatin provoked by the steroid and the importance of receptor redistribution in the nucleus. We have recently examined the distribution of PR in myometrial cells. As observed for stromal cells, PR immunoreactivity was associated with condensed chromatin. However, progestin injection does not, in myometrial cells (in contrast to in stromal cells), provoke a major change in chromatin structure (Perrot-Applanat, M., unpublished observations). Similarly, the redistribution of receptor in the nucleus, if it follows the general pattern described above (receptor scattered over condensed chromatin in the absence of hormone, enhanced labeling at the border of condensed chromatin and nucleoplasm in the presence of hormone), appears to be less pronounced in the myometrial cells. Now that the methodology is available, ultrastructural localization studies with other receptors and in other target cells should reveal how general the present observations are.

Progesterone (and/or estradiol) have been shown to cause various effects in stromal cells: stimulation of cell division (28), an increase in rough endoplasmic reticulum (9). These cells, under proper stimuli and in the presence of progesterone, develop into decidual tissue (13).

Various other questions are raised by this study: What is (are) the component(s) of condensed chromatin to which receptors are bound in the absence of hormone? Do hormone-receptor complexes interact only with accessible DNA, or do other features of the chromatin (spatial organization, specific proteins, etc.) also play a role? The localization of accessible DNA in the nucleus was studied by Bendayan (3) using
incubations of thin sections with DNase-gold complexes in conditions under which the enzyme bound to DNA without digesting it. In Epon-embedded tissue, most gold particles were associated with dispersed chromatin. A comparison between both studies suggests that the distribution of steroid-receptor complexes cannot be simply explained by that of accessible DNA.

The presence of low, but clearly above background, labeling in the cytoplasm provides evidence of the existence of a small concentration of the receptor in this compartment, which was probably under the limit of detectability of the light microscope. This receptor is apparently associated with membrane-bound or free ribosomes. It may represent newly synthesized protein or, alternatively, it may be exerting a biological function on the translational machinery of the cell. Biochemical evidence in favor of such a localization and such a role has been published (22, 41).

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