Demonstration of the Intracellular Localization and Up-Regulation of Glucocorticoid Receptor by *in Situ* Hybridization and Immunocytochemistry

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Abstract

Glucocorticoid response in target cells closely correlates with glucocorticoid receptor (GR) level. We have compared the localization of GR (by immunocytochemistry) and GR mRNA levels (*in situ* hybridization) in steroid-sensitive and steroid-resistant subclones derived from the human CEM lymphoid cell line. In addition, GR was localized at the light and electron microscopic level predominantly in the cytoplasm and to a weaker extent in the nucleus. In the presence of steroid, the GR was shown to translocate to the nucleus in the steroid-sensitive but not in the steroid-resistant cell lines. Furthermore, GR and GR mRNA levels were increased following glucocorticoid treatment of the sensitive cell line but not the resistant one. These data support a role for receptor regulation and translocation in the overall mechanism of glucocorticoid hormone action.

Introduction

Due to their lympholytic actions, glucocorticoids are often incorporated in multidrug regimens for treating lymphomas and leukemias. The exact molecular mechanisms that result in cell lysis are unknown, but evidence supporting various mechanisms has been presented (1-4). Glucocorticoid action is mediated by a cellular receptor which behaves as a *trans*-acting factor and binds specific DNA sequences in target cells (5, 6). Absence or greatly decreased number of glucocorticoid receptors in normal rat tissues is associated with resistance to steroid (7, 8) and the magnitude of response to steroid correlates with GR3 level (9). This is in agreement with the clinical data which showed or greatly decreased number of glucocorticoid receptors in normal rat tissues is associated with resistance to steroid (7, 8) and the magnitude of response to steroid correlates with GR3 level (9). This is in agreement with the clinical data which showed

Materials and Methods

Human Leukemic Cell Lines and Tissue Samples. The characterization of the IM9 line and the CEM-C7 human cell line and the three


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The abbreviations used are: GR, glucocorticoid receptor; PBS, phosphate-buffered saline; cDNA, complementary DNA.

types of glucocorticoid-llysistant clonal cell lines Cl(r'ly'), 4R4 and 3R43 (r'3R43) and ICR27 (r1ly') derived from or related to it have been described previously (10, 18). Cell lines were cultured as suspensions in RPMI 1640 (GIBCO, Grand Island, NY) containing 5% fetal calf serum. Sera were previously treated with charcoal-dextran (Fisher Scientific Products and Pharmacia, respectively) to remove endogenous steroids. Cells were maintained in a tissue culture incubator at 37°C with a humidified atmosphere of 95% air and 5% CO2 at a density of 0.5-2×106 cells/ml.

Peripheral blood, bone marrow, or lymph node biopsy samples were obtained from patients with hematological malignancies. Single cell suspensions were prepared from the lymph nodes by mincing and filtration through a wire mesh; the mononuclear cell layer was subsequently recovered. Heparinized blood or bone marrow aspirate were layered on Ficoll-Hypaque (Pharmacia) and centrifuged, and mononuclear cells were recovered at the interface. Following washing in PBS containing 1% fetal calf serum, the cells were incubated with or without dexamethasone before processing for fixation and immunocytochemistry.

Preincubation with Dexamethasone. In order to study the effect of incubation with glucocorticoid on the glucocorticoid receptor level and distribution, cells were first collected by low speed centrifugation and washed twice with and resuspended in RPMI medium with or without serum. Subsequently, cells were incubated in presence of 10-4 M dexamethasone or the vehicle used to dissolve the steroid (ethanol, final concentration 0.1%) for either 30 or 60 min (short term) or 2, 4, 8, 16, and 24 h (long term) at 37°C. Subsequently the cells were collected by centrifugation as above and processed for immunocytochemistry or *in situ* hybridization.

Immunocytochemistry of the GR. In a previous study we have compared a number of fixation and preparative protocols, thus optimizing an immunocytochemical procedure for GR. This procedure was further adapted to localize the GR in human leukemic cells (19, 20) and summarized below. The cells prefixed in Bouin's solution were resuspended in 4% bovine serum albumin (Sigma) and were subsequently adhered onto gelatin-coated glass slides using a Shandon-Elliott cyt centrifuge at 1100 rpm for 5 min (1×106 cells/slide). Following cytopinning, the cells were air dried and then stored at -20°C until use for up to 3 months. In the case of 100% methanol or 95% ethanol-5% acetic acid only, the cells, cytopsin without prefixation, were finally postfixed in either of these fixatives for 30 min. In all cases cytopsins were air dried and either frozen at 20°C or processed directly for immunocytochemistry.

The tissue slides were processed for immunocytochemistry as described (7) with either of the glucocorticoid receptor antibodies listed below. Briefly, the slides were washed in PBS after the primary antibody reaction, and the reacted antibodies were revealed using biotin-labeled anti-mouse or anti-rabbit IgG and the avidin-biotin complex (Vector Laboratories, Burlingame, CA) between each step; four washes were done. The peroxidase was revealed by the diaminobenzidine (Sigma) cytochemical reaction. In this report, "staining" and "stain" refer to the dark brown reaction resulting from the peroxidative polymerization of diaminobenzidine. In order to identify cell morphology, slides were weakly counterstained with 0.2% methylene blue.

Glucocorticoid Receptor Antibodies. In order to demonstrate the validity of the immunocytochemical reaction three independently developed antibodies were used: (a) three polyclonal antibodies to the purified human glucocorticoid receptor purified from IM9 cells (21) were used (Nos. 882, 884, and 202); (b) a monoclonal antibody (No.
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In Situ Hybridization of Glucocorticoid Receptor mRNAs. CEM cells were cultured as above, fixed in 4% paraformaldehyde for 15 min at 4°C, and cytopsins onto chromosulfuric acid-cleaned and gelatin-coated glass microscope slides as described for immunocytochemistry in suspension. The cell preparations were stored at −20°C until hybridization and subsequently properly handled to avoid RNA degradation. Prehybridization and hybridization were carried out as described (24). Briefly, the cells were incubated in the hybridization buffer [8× standard saline-citrate, 100 μl; formamide, 100 μl; Sarkosyl (IBI) 20×, 10 μl; 1.2 M phosphate, 20 μl; Denhardt’s 100× (0.02 g/ml bovine serum albumin; 0.02 g/ml polyvinylpyrrolidone, Sigma; 0.02 g/ml Ficoll, Sigma), 2 μl containing an optimal concentration of 2.5 × 10⁶ cpm/100 μl/slide of an sp6-derived, 35S-labeled glucocorticoid receptor complementary RNA (25). Following hybridization for 18 h at 42°C slides were washed in 2× standard saline-citrate for 30 min with one change at room temperature and 30 min with two changes at 55°C. After a brief rinse in distilled water to remove the buffer salts, slides were dehydrated in ethanol and processed for light microscopic autoradiography. The specific labeling was assessed by comparing the number of grains overlaying cells hybridized with either the pBR322 probe from the value obtained for the glucocorticoid receptor complementary RNA probe. Additional control incubations were done to demonstrate the specificity of the in situ labeling. One control consisted of using an equal amount of 35S-labeled PBR complementary DNA probe. In other controls, the cell preparation was treated by RNase (10 μg/ml) or DNase (10 μg/ml). Only the former treatment resulted in an almost complete absence of label. The number of grains was counted either visually with the help of a hemocytometer or with an automated computer-assisted image analysis system developed in our laboratory.

Results

Immunocytochemical Localization of GR in CEM Cells. In the absence of glucocorticoid, the GR was localized predominantly in the cell cytoplasm of all cell lines studied (Fig. 1). Some cells showed mixed nuclear and cytoplasmic labeling, but no exclusive nuclear staining was observed, in agreement with earlier studies (7). These observations were reproducible with each of the GR antibodies mentioned above. Within any given cell preparation, the intensity of staining was variable among individual cells. In order to address the issue of heterogeneity, the wild-type C7 cells were subcloned using the soft agar technique (26). Individual subclones were processed for GR localization; in each case the staining intensity among cells was as heterogeneous as the wild-type C7 cells (data not shown5). At the electron microscope level, the GR was localized throughout the cytoplasm and to a weaker extent in the nucleus (Fig. 2).

Nuclear Translocation and Up-Regulation of the Glucocorticoid Receptor by Glucocorticoids. Our earlier studies (20, 27) have shown a significant increase in nuclear GR staining of CEM C7 cells following dexamethasone treatment for 30 or 60 min. Moreover, a slight but detectable increase in immunoreactive cytoplasmic GR was observed following those short-term incubations. In this study, increasing duration of glucocorticoid treatment (2–24 h) resulted in a gradual and overall increase in staining intensity in the cytoplasm and, more particularly, the nucleus of most C7 cells (Fig. 1). Unlike the C7 cells (steroid sensitive), dexamethasone had no detectable effect either on the translocation or up-regulation of immunostained GR in any of the steroid-resistant cell lines (Fig. 1, C and D).

Localization of GR mRNA Sequences by in Situ Hybridization. Fig. 3 shows specific hybridization of cRNA probe to GR mRNA. The radioactive grains overlaying the cells are mostly found on the cell periphery which corresponds to the cytoplasm; the nucleus of these lymphoid cells is occupying most of its

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Fig. 1. Immunocytochemical localization of the GR in various lymphoid cells. Except where indicated, cell preparations were reacted with an antibody to a synthetic GR peptide. (A) 4R4 cells without preincubation with dexamethasone (DEX); (B) C7 cells incubated in presence of dexamethasone for 24 h (note increased immunoreactivity (brown color) in both nucleus and cytoplasm); (C) 4R4 cells without preincubation with dexamethasone; (D) 4R4 preincubated with dexamethasone for 1 h, no difference in staining between C and D is observed; (E) immunocytochemical control in which the GR antibody was replaced by preimmune serum; (F) preparation of human bone marrow cells from an IgGu monoclonal gammapathy. All preparations were counterstained in methylene blue. × 1000.

11) to the glucocorticoid receptor purified from human HeLa cells (22) and used in our earlier studies (8); (c) an antibody recently developed using a synthetic 14-amino acid peptide of the NH2 terminus region of the human glucocorticoid receptor (23).4 This antibody recognizes nuclear and cytosolic receptor and was fully characterized by four independent criteria: immunocytochemistry; radioimmunooassay; immunoprecipitation; and “Western” blotting. To confirm the specificity of the immunocytochemical staining, the reaction was shown to be eliminated by absorbing the antibody with partially purified receptor (7) as indicated in Fig. 1B or by substituting the receptor antibodies by preimmune serum or nonspecific IgG.

Electron Microscopy. Cells were fixed in 4% paraformaldehyde for 30 min and immunocytochemically stained while in suspension using a protocol similar to that indicated above and were washed 4 times in PBS before being postfixed in 1% osmium tetroxide, in 0.1 M phosphate buffer, pH 7.2, in order to render the diaminobenzidine reaction products electron dense. Subsequently, the cell pellet was conventionally dehydrated and embedded in Epon. These sections were cut and observed in a Philips 400 electron microscope at 80 kV without counterstain.

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Fig. 2. Electron microscope localization of GR in C7 cells. The cells were stained and processed as described in the text using the polyclonal antibody to the human GR (20). Note the electron-dense staining in the center cell (C). Another cell is shown which is very weakly reactive. Less staining is observed in the nucleus. × 18,000.

Discussion

In this paper, we have described the application of immunocytochemistry and in situ hybridization for the detection of GR in cells from human lymphoid cells. Two issues have been addressed using these techniques; the cellular localization and the autoregulation of GR.

For the past few years, the classical “two-step” mechanism (5) in which the cytosolic steroid receptor translocates to the nucleus following steroid binding has been repeatedly challenged. Immunocytochemical and enucleation studies on the estrogen and progesterone receptors have demonstrated exclusively nuclear localization of these receptors even in the absence of steroid (16, 17, 28). Thus, studies on the estrogen and progesterone receptor are at variance with rapidly accumulating evidence from various laboratories that the glucocorticoid receptor is largely cytoplasmic and translocates to the nucleus following steroid binding (29, 32). In several of these GR studies, particularly those using monoclonal antibodies, the epitopes detected are close to the DNA or to the steroid binding domain of the receptor molecule (33). In this study, use of a novel polyclonal antibody generated to the amino terminus domain demonstrated similar localization of the GR. These results herein were further substantiated using two independently prepared antibodies (a polyclonal and monoclonal). It is therefore evident that the subcellular localization of GR is different from that of estrogen and progesterone receptors. The nuclear translocation of GR may have physiological relevance and may be clinically useful in predicting glucocorticoid response. In fact, nuclear translocation of the GR occurs only in the steroid-sensitive CEM-C7 human leukemic cell line but not in the steroid-resistant subclone 4R4 or other resistant cell lines derived from the wild-type C7 cells. Biochemically, this seems to be due to a defect whereby the receptor loses its steroid-binding capability upon activation (34). All these data support a role for the steroid in the mechanism of steroid hormone action.

The second issue addressed in this paper concerns the autoregulation of GR. This study stems from our experiments in which incubation of pituitary cells and lymphoid cells in culture even for short term (1 h) resulted in a significant increase in GR immunoreactivity (20). In an earlier study, we demon-
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It was demonstrated that glucocorticoid up-regulates GR and GR mRNA levels in lympholysis-sensitive cells (35). Eisen et al. (36) similarly observed that glucocorticoid induces its mRNA and protein level in the CEM-C7 cells. The rapid effect of GR induction (<1 h) suggests that in addition to increasing GR mRNA level, the steroid may act at a posttranscriptional level such as stabilizing and/or increasing translational efficiency of GR mRNA sequences. The present findings are unexpected in the light of earlier studies demonstrating that glucocorticoid induces a down-regulation of GR binding sites in pituitary AtT-20 pituitary tumor cells (37), human HeLa cell line (38), human and mouse lymphocytes (39-41), and GR mRNA level in rat hepatoma cells (42), normal rat tissues (43), and IM9 cell line (44). This discrepancy between our data and those reports may be explained on the basis of a tissue-specific regulation of GR gene expression. In fact, we have previously reported that the regulation of GR expression by dopamine in the anterior versus intermediate pituitary lobe is tissue specific (8). Most hormone receptors are down-regulated by homologous ligands (45), whereas a few examples are known in which the opposite phenomenon occurs. Prolactin is one example in which the hormone has been shown to induce its own receptor in vivo (46, 47). In addition, interleukin 2 and epidermal growth factor were shown to up-regulate the synthesis of their own receptor (48, 49). The phenomenon of GR up-regulation in leukemic cells may be physiologically relevant in potentiating the steroid-sensitive cell to maximize the lytic response to glucocorticoid.

Acknowledgments

We thank Drs. Jeff Harmon and John Cidlowski for their generous supply of GR antibodies and Dr. K. R. Yamamoto for the GR cDNA probe.

References


Fig. 3. In situ hybridization of GR mRNA using a 32P-labeled GR complementary RNA probe as described in the text. (A) C7 cells without dexamethasone. (B) C7 cells incubated with dexamethasone for 18 h; note a marked increase in the number of silver grains as compared to A; note also not all cells are labeled with the same intensity. (C) control preparation in which the cell preparation was pretreated with RNase inasmuch as before hybridization, no labeling is observed. Cells were counterstained by hematoxylin and eosin. × 1000.
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