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 (by in situ hybridization) in steroid-sensitive and steroid-resistant subclones derived from the human CEM lymphoid cell line. In addition, GR was localized in lymphoid cells from patients with various hematological malignancies. In the absence of preincubation with the steroid, 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 the presence of a high receptor level, as measured by radioligand assays with the likelihood of response to glucocorticoids in patients with leukemia and lymphoma (10–12). Radioreceptor assays, however, failed to predict response in a number of cases (see Ref. 10), presumably due to the heterogeneity of the tumor tissues. In the case of breast cancers, this issue was addressed by measuring the level of estrogen receptor using immunocytochemistry (13–17) in order to predict response to endocrine therapy. We have now developed immunocytochemical and in situ hybridization assays to study the localization and regulation of GR and GR mRNA in human lymphoid cells.

Materials and Methods

Human Leukemic Cell Lines and Tissue Samples. The characterization of the IM9 and the CEM-C7 human cell line and the three types of glucocorticoid-lysis resistant clonal cell lines Cl(r”ly”), 4R4 and 3R43 (r”°ly”) and ICR27 (r°ly’) 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 x 10^6 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 cytospin centrifuge at 1100 rpm for 5 min (1 x 10^9 cells/slide). Following centrifuging, 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, cytospun without prefixation, were finally postfixed in either of these fixatives for 3 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.
REGULATION AND LOCALIZATION OF GLUCOCORTICOID RECEPTOR

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 individuals. 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 shown). 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 overlying 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 specific 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 individuals. 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 shown). At the electron microscope level, the GR was localized throughout the cytoplasm and to a weaker extent in the nucleus (Fig. 2).

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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, x 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-

volume. The number of silver grains is variable between individual cells. Following dexamethasone treatment, there was a significant \([P < 0.001]\) increase in the number of grains either at 4, 16, or 24 h [the only time points examined (Fig. 3)]. The labeling intensity was highest at 24 h. An absence of significant labeling was seen in control preparations in which the GR probe was substituted by pBR322 or in preparations previously digested by RNase I (Fig. 3).
strated 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.

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References


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