Glucocorticoid Receptor Expression during Differentiation of Human Promyeloic Leukemia Cells

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ABSTRACT

The human promyeloic leukemia cell line HL-60 can be triggered in culture to differentiate into several cell types of the myeloid lineage in response to a variety of chemical stimuli. We used this cell system in order to investigate the changes in glucocorticoid receptors which occur concomitantly with such cellular differentiations. Neutrophilic granulocytes obtained by the addition of dimethyl sulfoxide or retinoic acid to the culture medium showed only slight changes in cellular glucocorticoid receptor levels and receptor-specific mRNA as compared to undifferentiated control cells. Monocytic cells induced by incubation with dihydroxyvitamin D₃ had a moderate increase in receptor hormone-binding activity. However, differentiation toward macrophages by exposure to phorbol ester resulted in a 5- to 6-fold increase in both cellular hormone-binding capacity and immunochemically cross-reacting receptor protein. An even greater increase in glucocorticoid receptor-specific mRNA was observed. These data suggest that the receptor is regulated at the mRNA level and that de novo receptor synthesis occurs during macrophage differentiation, thus making these cells potentially more susceptible to glucocorticoid-induced effects.

INTRODUCTION

Glucocorticoids are hormones used widely in medicine because of their many actions on a variety of mammalian target cells, including those of the hematopoietic system. Thus, the immunosuppressive and anti-inflammatory effects of corticosteroids are of great interest. Pharmacological doses are known to trigger apoptosis in certain lymphoid cells (1–3), a response which is exploited in the treatment of lymphoid malignancies. However, the major immunosuppressive and anti-inflammatory effects of these steroids appear to be mediated by macrophages and other members of the myeloid cell family (4). Indeed, various regulatory or modulator effects of glucocorticoids have been described in myeloid cells and are either negative or positive in nature. These include the inhibition of production of thromboxane A₂, tumor necrosis factor, interleukin-β, the granulocyte/macrophage colony-stimulating factor, and plasminogen activator and decreased activities of cyclooxygenase and phospholipase A₂ (5–10). On the other hand, the expression of the receptors for interleukin 1α and 1β are stimulated by glucocorticoids (11). These responses are typical for fully differentiated, rather than immature, myeloid cells.

Specific intracellular receptors for glucocorticoids are known to play a pivotal role in mediating the effects in target cells (for reviews, see Refs. 12–15). Thus, changes in glucocorticoid receptor expression may accompany the differentiation of myeloid precursors to mature cells which respond in the above-mentioned ways. Alternatively, the activity of cellular receptors may change during differentiation. In an attempt to study these questions, we used HL-60 cells, a permanent line of human promyeloic leukemia cells which have the capability to develop in vitro into different cell types, depending on the culture conditions (16). We stimulated these cells to differentiate toward macrophages, monocytes, or neutrophilic granulocytes and measured glucocorticoid-binding activity, the levels of immunoreactive receptor protein, and the relative cellular contents of glucocorticoid receptor mRNA. We observed a significant increase in these receptor parameters during macrophage differentiation but not when cells were differentiated into neutrophils.

MATERIALS AND METHODS

Reagents. [1,2.4.5′-H]Triamcinolone acetonide (1 TBq/mmol), [α-32P]UTP (110 TBq/mmol), and [α-32P]dCTP (220 TBq/mmol) were obtained from Amersham (Amersham, England), and [6,7,8,9]dexamethasone mesylate (1.6 TBq/mmol) was from Dupont/New England Nuclear (Boston, MA). TPA and oligodeoxymyridylate-cellulose were obtained from Sigma Chemical Co. (St. Louis, MO), all-trans-retinoic acid was from Serva (Heidelberg, Germany), and 1,25-dihydroxyvitamin D₃ was from Duphar B.V. (Weesp, Netherlands). Laboratory chemicals were of reagent grade.

Cell Culture. HL-60 cells (16) were maintained in continuous suspension culture in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) at 37°C under 5% CO₂. Viable cell counts were performed using trypan blue dye exclusion. Cells were routinely supplied with fresh complete medium 48 h before harvesting or using for hormone-binding experiments. This turned out to be essential for obtaining reproducible results because preliminary experiments showed a general decrease in hormone-binding ability after several days without medium change. The addition of insulin and transferrin (each 5 μg/ml) could partially substitute for this complete medium change.

For inducing cell differentiation, DMSO (180 μM), retinoic acid (1 μM), dihydroxyvitamin D₃ (10 nM), or TPA (50 nM) were added to the culture medium, and cultures were thereafter maintained for up to 5 days. Differentiation experiments were routinely set up at cell densities of 2.5 × 10⁷/ml, except for TPA treatment which was started at 10⁻⁷ cells/ml.

Hormone-binding to Intact Cells. Specific binding of [3H]triamcinolone acetonide to intact cells was determined as before (17). Binding capacities and dissociation constants were obtained from Scatchard analysis.

Cell Extracts. Cells were harvested by centrifugation at 1000 × g, washed with phosphate-buffered NaCl solution, and frozen in liquid nitrogen. Extracts were prepared from frozen cell pellets (18).

Polyacrylamide Gel Electrophoresis and Immunoblotting. Equal amounts of proteins, as determined by the biuret method, were separated on standard SDS-polyacrylamide gels (10%) with rabbit muscle phosphorylase b (97.400), bovine serum albumin (66.000), bovine liver catalase (60.000), and rabbit muscle aldolase (39.000) as molecular weight markers. Protein transfer onto Immobilon-P (polyvinylidene difluoride) membranes (Millipore, Bedford, MA) was as described previously (19). Detection of the receptor polypeptide was either by antiserum H.H. diluted 1:250 or antiserum aPl (20) diluted 1:2000. The rabbit antiserum H.H. against the human glucocorticoid receptor peptide Ser₉₀-Thr₇₄₆ was kindly provided by Dr. M. N. Alexis. Incubation with horseradish peroxidase-conjugated second antibodies (Sigma) at a dilution of 1:1000 and development with 3,3′-diaminobenzidine/NiCl₂ were carried out as described before (19). For quantitation, we scanned the photographs on translucent film with an Ultrascan XL, laser densitometer (Pharmacia Biotech, Uppsala, Sweden). In control experiments, extracts of undifferentiated cells were reacted with 100 nM [3H]dexamethasone mesylate (21) at 0°C for 3 h, and the receptor complexes were partially purified by chromatography on DNA-cellulose (21) prior to SDS-gel electrophoresis and immunoblotting. The

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2 The abbreviations used are: triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxyprogesterone-1,4-diene-3,20-dione cyclic 16,17-ketal with acetone; dexamethasone mesylate, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-progesterone-1,4-diene-3,20-dione-21-mesylate; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; TPA, 12-O-tetradecanoylphorbol-13-acetate; cDNA, complementary DNA.

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membranes were sliced and counted for radioactivity. The immunostained material at Mr 100,000 was found to be the only labeled protein band, thus proving identity with the glucocorticoid receptor.

**Analysis of mRNA Levels.** RNA was isolated (22), enriched for polyadenylated mRNAs (23), separated on 1% agarose/formaldehyde gels with rRNAs as markers, and blotted onto nitrocellulose filters (Hybond-C extra; Amersham) in citrate-buffered NaCl solution (23). Prehybridization was for 3 h at 42°C in 50% formamide under standard conditions (23), followed by hybridization with a receptor cDNA fragment (1260 base pairs) radiolabeled with [32P]dCTP by the random priming method (24) using a kit (Boehringer Mannheim, Mannheim, Germany). The plasmid GR(AB)-Gal containing the receptor cDNA fragment (EcoRI-KpnI) was kindly provided by Dr. P. Chambón. Filters were washed with 0.1% SDS containing buffers first at room temperature and then at 42°C and finally exposed to X-ray film. Autoradiograms were quantified by densitometric scanning with the UltroScan XL laser densitometer. To make sure that equal amounts of RNA were loaded onto gels, nitrocellulose filters were stained with methylene blue after autoradiography (23).

**Nuclear Transcription Assay.** Nuclear run-off assays were performed as described previously (25). Briefly, differentiated and undifferentiated cells were washed with isotonic phosphate-buffered NaCl solution, preswollen on ice in hypotonic buffer and broken by 20 to 40 strokes in a glass Dounce homogenizer. Nuclei (109) were incubated with [32P]UTP and unlabeled ATP, CTP, and GTP. The labeled RNA was isolated and hybridized to excess denatured plasmid GR(AB)-Gal immobilized on nitrocellulose filters, and the filters were exposed to X-ray film. For quantitation, spots were excised and counted for radioactivity.

**RESULTS**

**Glucocorticoid-binding Activity during Differentiation of HL-60 Cells.** Promyeloic HL-60 cells were treated with the phorbol ester TPA for differentiation toward macrophages. This led to significant morphological changes (16, 26) concomitant with the formation of cell clusters adhering to the cell culture vessel. Maximum differentiation was attained after 3 days, at which time cell proliferation had ceased. In other experiments, cells were exposed to DMSO or retinoic acid for differentiation into neutrophilic granulocytes. These cells were somewhat smaller and more spherical than control cells.

Fig. 1 shows the specific binding of radiolabeled steroid to cells pretreated for 3 days with TPA (triangles) or for 5 days with DMSO (squares). DMSO-treated cells had receptor levels very similar to those of undifferentiated control cells (not shown), while differentiation induced by TPA resulted in greatly increased hormone-binding ability. We obtained 110,000 ± 3,000 steroid-binding sites per cell (mean and range of four experiments), and there was no change during our 3- to 5-day experiments. Scatchard analysis of the data (Fig. 1B) revealed the presence of a single class of binding sites for the steroid in all experiments. The dissociation constant Kd of the receptor-hormone complexes was 13 ± 4 nM (mean ± SD; total number of experiments was 23).

The time course of receptor induction during TPA differentiation is depicted in Fig. 2 (solid triangles). Hormone binding was found to increase significantly by 24 h of incubation and reached a maximum at 72 h (5- to 6-fold). Interestingly, there was no significant change in receptor level by 5 h of TPA treatment. This suggests that the increase in hormone-binding ability is not due to a direct effect of the phorbol ester via protein kinase C stimulation but, rather, depends on cellular differentiation.

Fig. 2 also shows that exposure of HL-60 cells to DMSO or retinoic acid (solid and open squares, respectively) resulted in only a small and transient increase in glucocorticoid-binding activity. We similarly treated HL-60 cells with 1,25-dihydroxyvitamin D3 in order to stimulate monocytic differentiation (16). This resulted in an approximately 2-fold increase in cellular hormone binding after 4 days of incubation (Fig. 2, open triangle).

**Levels of Receptor Protein during Differentiation of HL-60 Cells.** We next asked whether the above observed changes in steroid binding reflect actual increases in receptor protein levels or whether TPA induction of macrophage differentiation affects hormone-binding activity of cellular receptors, i.e., their biological availability. We prepared extracts of cells pretreated in various ways and analyzed them by SDS-polyacrylamide gel electrophoresis and immunoblotting with antisera directed against the human glucocorticoid receptor polypeptide, which has an Mr of approximately 100,000.

As shown in Fig. 3, the receptor antisera H.H. detects a protein band of M, 100,000, the intensity of which varied between samples from differently treated cells. Some additional bands were also stained with this antibody but were found to be unrelated to the receptor because they did not show up by affinity labeling with [3H]dexamethasone mesylate (cf. “Materials and Methods”). Fig. 3 shows that the level of immunoreactive receptor material increased significantly by 24 h of TPA treatment (lane 2). After 3 days of differentiation toward macrophages (lane 3), the receptor signal was about 5-fold above that of undifferentiated cells (lane 1), as revealed by scanning immunoblots (Fig. 3, bottom). By contrast, HL-60 cells differentiated into
granulocytes by DMSO treatment (lane 4) expressed the receptor polypeptide at levels indistinguishable from control cells. These results demonstrate that changes in cellular hormone-binding activity induced by differentiation are caused by changes in receptor protein levels.

In additional experiments we used antiserum aP1 directed against the hormone-binding domain of the human glucocorticoid receptor polypeptide (20). This antibody also cross-reacted with several other proteins, but the band at Mr 100,000 was likewise identified by labeling with dexamethasone mesylate as the bona fide receptor. Again, this signal increased 5- to 6-fold when the cells were exposed to TPA but remained at control levels after DMSO treatment (data not shown).

Receptor-specific mRNA during Differentiation of HL-60 Cells.

For measuring the levels of receptor mRNA upon cellular differentiation, we used a cDNA fragment as a hybridization probe which encodes amino acids 1-420 of the human glucocorticoid receptor. This was chosen in order to minimize cross-hybridization with mRNAs for other members of the steroid and thyroid receptor family (27). In all our Northern blots (cf. Fig. 4), we observed two signals at 7.2 and 5.2 kilobases, respectively, corresponding to two related transcripts as previously described (20, 28-31).

Fig. 4 shows blots with RNA from differently treated cells. We observed a dramatic increase in receptor mRNA upon cellular differentiation (lanes 2 and 3) but no significant changes upon granulocyte differentiation (lane 4) as compared to the control (lane 1). Scanning of autoradiographs (Fig. 4, bottom) revealed 9- and 13-fold increases in specific mRNAs after 1 and 3 days of exposure to the phorbol ester, respectively. While the experiments of Fig. 4 were performed with RNA previously chromatographed over oligo-deoxythymidylate-cellulose, we obtained similar results with total cellular RNA (data not shown). Taken together, these observations clearly support the view that the increased levels of glucocorticoid receptor protein and hormone-binding activity detected upon differentiation of HL-60 cells toward macrophages are due to greatly increased cellular amounts of receptor mRNA.

In order to determine whether the observed increase in receptor mRNA level is due to enhanced transcription, we carried out nuclear run-off experiments (25). As shown in Fig. 5, we obtained receptor-specific mRNA signals of about equal intensities with nuclei from undifferentiated HL-60 cells and from cells treated either with TPA for 1 or 3 days, respectively, or with DMSO for 5 days. Similarly, scintillation counting did not show any significant differences (Fig. 5). These data suggest that the rate of transcription of the receptor gene does not change upon differentiation of HL-60 cells; increased mRNA levels in TPA-differentiated cells may, rather, be due to increased message stability.

DISCUSSION

The glucocorticoid receptor plays a pivotal role in mediating the actions of glucocorticoids in target cells. Thus, regulation of receptor activity and/or level may influence the hormonal response. In various target cells, glucocorticoids have been shown to regulate their own receptors at the mRNA level (14, 20, 30-33). In previous studies we observed that different lines of mouse lymphoma cells respond to very...
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Previously, Hirai et al. (35) noted a 2- to 4-fold increase in glucocorticoid binding per cell upon TPA treatment of several myeloid leukemia cell lines, including HL-60, and considered this change an effect of macrophage differentiation. Increased hormone binding was also described in a somewhat related human leukemic cell line, U937, when exposed to TPA (5, 10). Using of the HL-60 cell system, we prove in the present study that increased steroid-binding capacity is caused by proportionately higher levels of immunoreactive receptor protein rather than enhanced glucocorticoid receptor activity. Moreover, we show that this augmentation in receptor content is a consequence of significantly increased receptor mRNA levels in cells stimulated to macrophage differentiation. Hence, de novo receptor synthesis is involved.

The increased level of receptor mRNA in TPA-differentiated cells could be due to either elevated rates of transcription from the glucocorticoid receptor gene during differentiation or greater stability of the message. In an attempt to distinguish between these alternatives, we used differentiated and control cells for nuclear run-off experiments. In these studies, we did not obtain any evidence for increased numbers of preinitiated receptor mRNA molecules upon differentiation. Therefore, it appears likely that enhanced glucocorticoid receptor mRNA and protein levels in macrophage-like HL-60 cells result from increased message stability. Clearly, more detailed experiments will be required in order to unequivocally establish this point. In fact, regulation of mRNA stability is one of the posttranscriptional control mechanisms for gene expression in both prokaryotic and eukaryotic cells. For example, one of the mechanisms controlling the expression of the heat shock protein hsp70 during both heat shock and subsequent recovery is the regulation of mRNA degradation (36). Also, the turnover of the messages for c-fos and c-myc plays a crucial role in the expression of the respective proteins (37), and steroid hormone treatment of target cells may affect specific mRNA stability (7, 38, 39).

Glucocorticoid receptors are known to exist in at least two molecular forms: the unactivated receptor is able to bind the hormonal ligand but does not interact with DNA, while the activated receptor form has the ability to bind to DNA (40). Activation to the DNA-binding state involves subunit dissociation of the heteromeric receptor structure (18) and is a prerequisite for interaction with control sequences in the genome. The unactivated receptor of lymphoid cells is a heterotetramer composed of one receptor polypeptide in close association with two molecules of the heat shock protein hsp90 and one p59 protein subunit (41). This structure has recently also been detected in intact cells in the absence of steroid (42) and, therefore, is the original receptor form ready for accepting the hormonal signal. It is reasonable to assume that glucocorticoid receptors of other cell types, e.g., myeloid cells, have a similar subunit structure. The components hsp90 and p59 are known to be present in target cells in a large excess over the receptor polypeptide itself and even form complexes with each other and with other cellular components (43, 44). Therefore, up-regulation of the receptor polypeptide by de novo synthesis as observed here during in vitro macrophage differentiation will directly result in the formation of the multimeric receptor structure. No additional regulatory events appear to be required which would affect the receptor-associated components hsp90 and p59.

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