Expression in Hematological Malignancies of a Glucocorticoid Receptor Splice Variant That Augments Glucocorticoid Receptor-mediated Effects in Transfected Cells

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ABSTRACT

Glucocorticoids play an important role in the treatment of a number of hematological malignancies, such as multiple myeloma. The effects of glucocorticoids are mediated through the glucocorticoid receptor $\alpha$, the abundance of which can be modulated by alternative splicing of the glucocorticoid receptor mRNA. Two splice variants of the glucocorticoid receptor mRNA have been described: glucocorticoid receptor $\beta$, which reportedly has a dominant negative effect on the actions of the glucocorticoid receptor $\alpha$, and glucocorticoid receptor P, of which the effects are unknown. In this study, we have investigated the expression levels of these two splice variants at the mRNA level in multiple myeloma cells and in a number of other hematological tumors. Although the glucocorticoid receptor $\beta$ mRNA was, if at all, expressed at very low levels, considerable amounts (up to 50% of the total glucocorticoid receptor mRNA) glucocorticoid receptor P mRNA was present in most hematological malignancies.

INTRODUCTION

GCs$^4$ constitute an active component of chemotherapeutic regimens for various B-cell lymphoid malignancies, such as Hodgkin’s disease, indolent and aggressive NHL, MM, ALL, and CLL (1–3). In contrast, AML or chronic myeloid leukemia and preleukemic states such as the myelodysplastic syndromes usually do not respond favorably to GC treatment (4). In lymphoid malignancies, dexamethasone induces a rapid apoptotic response in vitro, which may be antagonized by interleukin 6 and other cytokines (5). However, the beneficial effects of GC therapy are limited because of the occurrence of resistant tumor cell clones, which evolve during GC treatment. During treatment, the malignant cells may develop resistance to the GC-mediated cytolysis (1).

Genetic abnormalities of the GR are not a common cause of GC resistance in hematological malignancies. Small deletions and point mutations have been found in a highly resistant MM cell line (6) and in other leukemic cell lines (7, 8). However, analysis of fresh leukaemia cell samples obtained from GC-resistant patients with CLL has not revealed any GR point mutations (3).

Prolonged exposure of resistant cells to GCs has been reported to up-regulate GR expression (9). However, in these MM cell lines, the GC-induced response is not correlated with the number of GRs (9). In even highly refractory MM patients resistant to high doses of GCs, the number of GRs/tumor cell is comparable with that in sensitive tumor cells (10). Consequently, it seems likely that resistance to GCs is mediated either by postreceptor mechanisms or that it is associated with the presence of nonfunctional GR isoforms (9). Hence, the question arises as to which mechanisms are required for GCs to induce apoptosis. It is important to know whether, and to what extent, abnormal GRs or GR isoforms are involved in the multistep process leading to treatment-refractory disease. Several years ago, a variant GR mRNA, designated GR-P, was observed in tumor cells obtained from a GC-refractory myeloma patient (11). The GR-P protein is a truncated GR protein of 676 amino acid residues (Ref. 11; Fig. 1). GR-P differs from GR-$\alpha$, which is the active form of the GR, and the well-known natural splice variant GR- $\beta$ (12), because the exons 2–7 and a part of intron 7 encode it and the exons 8 and 9 are missing. GR-$\alpha$ uses exons 2–8 and part of 9 as a coding region, whereas GR-$\beta$ uses the exons 2–8 and part of 9 as a coding region, as is indicated in Fig. 1. In this study, we demonstrate that GR-P mRNA is present in fresh tumor cells obtained at diagnosis from patients with MM, ALL, or NHL who were sensitive to treatment with GCs. In contrast to this, the expression of the GR- $\beta$ splice variant was very low or undetectable in these samples. Transfection studies in several different cell lines demonstrated that GR-P increases the activity of GR-$\alpha$ in COS cells and in HeLa cells but not in CHO cells. This suggests that cell-specific factors play a role in this process and that the ratio of GR-P:GR-$\alpha$ may reflect different grades of GC responsiveness, with a higher ratio corresponding to a higher sensitivity.

MATERIALS AND METHODS

Tumors and Tumor Cell Lines. After having obtained approval from the local Medical Ethics Committee and informed consent from the patients, fresh bone marrow cells were collected at diagnosis from the posterior iliac crest of 16 previously untreated patients with MM. These patients were included in a clinical study protocol of the Dutch Hemato-Oncology Group (HOVON). After marrow collection, these MM patients were treated with 3 cycles of i.v. vincristine, Adriamycin, and dexamethasone. Thereafter, the clinical response was assessed according to the criteria of the Southwest Oncology Group. For the present analysis, patients were classified as responders (those who had achieved a complete response or a partial response) and nonresponders. To determine the pattern of GR expression in MM patients as compared with other hematological malignancies, we also studied tumor cells obtained from the bone marrow of untreated patients with ALL (n = 5) and NHL (n = 5), who subsequently
showed a clinical response to GC treatment and from patients with AML (n = 5), a disease that is unresponsive to GC treatment. Bone marrow aspirates were collected in Hanks’ HEPES medium and purified using buffy coat or a Ficoll-Hypaque gradient and adherence depletion. Samples with <80% tumor cells as determined by microscopy were discarded. Normal lymphocytes were obtained from healthy volunteers and purified by Ficoll-Hypaque gradient centrifugation.

**RNA Isolation.** Total RNA was isolated using the Trizol standard protocol (Life Technologies, Inc., Gaithersburg, MD). Dr. Gert-Jan van Steenbrugge (Department of Urology, Erasmus University) kindly provided total RNA from control tumor cell lines and prostate carcinoma xenografts.

**RT-PCR Assays.** One μg of total RNA was reverse transcribed using 1 pmol of oligo-dT primers (Pharmacia Biotech, Roosendaal, the Netherlands), 2.0 units Superscript reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 0.5 unit RNasin (Promega Benelux, Leiden, the Netherlands), and 1 mM deoxynucleotide triphosphates in reverse transcriptase buffer (Life Technologies, Inc.). The total volume was adjusted with distilled H2O to 20 μl. One-tenth of the reverse transcriptase reaction mixture was used directly for the PCR reaction in a total volume of 20 μl, with 1.0 unit of SuperTaq polymerase (Sphaero Q; Leiden, the Netherlands), 300 nM of the relevant primer and 5 mM MgCl2. The downstream primers for GR-α and GR-β differ from GR-α by usage of exon 9β as a coding region, and GR-P is an mRNA encompassing part of intron 7 but lacking exons 8 and 9. N-TERM: NL-α-terminal (variable) domain; DBD, DNA binding domain; HBD, hormone binding domain. The location of the PCR primers used and the lengths of the amplified fragments are indicated under the mRNA structure. Primer pairs 1–2 versus 1–3 were used to distinguish GR-α + GR-β from GR-P. Primer pairs 4–5 versus 4–6 were used to distinguish GR-α from GR-β.

**Cell Culture and Transfections.** Monkey kidney (COS-1), CHO, and human cervical epithelial carcinoma cells (HeLa) were maintained in DMEM-Ham’s F-12 tissue culture medium (Life Technologies, Inc.) supplemented with 5% charcoal dextran-treated FCS (Life Technologies, Inc.). The RPMI 8226 parent cell line was kindly provided by Dr. W. S. Dalton (Lee Moffitt Cancer Center, Tampa, FL). The UM3 cell line was derived and provided by Dr. H. M. Lokhorst (Department of Hematology, University Hospital Utrecht, the Netherlands). These cells were grown in suspension culture in RPMI 1640 with 10% DCC-FCS and 0.1% gentamicin. The UM3 cell line was derived and provided by Dr. H. M. Lokhorst (Department of Hematology, University Hospital Utrecht, the Netherlands). These cells were grown in suspension culture in RPMI 1640 with 10% DCC-FCS and 0.1% gentamicin. For transcription regulation studies, cells were plated at 6 x 105 cells/well (3.5 cm2; COS-1 and HeLa cells) or at 3 x 105 cells/well (CHO cells). They were grown for 24 h and transfected overnight by calcium phosphate precipitation, as described previously (13, 14). For LUC assays, cells were transfected with 10 ng/well of pRSV-hGRα and either 0, 1, 5, 10, 50, or 100 ng/well pcDNA3hGRβ plasmid, containing the hGR-β receptor, driven by the CMV promoter. All transfections were supplemented to a total of 100 ng/well of CMV promoter-containing plasmid with the pcDNA3.1 plasmid. In addition to this, 100 ng/well of MMTV-LUC plasmid were added. pTZ carrier DNA was added to bring the total amount of DNA to 2 μg/well. After an incubation period of 24 h, dexamethasone was added, and after another 24 h, the cells were harvested for the LUC assay, as described previously (13, 14). RPMI 8226 and UM3 MM cells at 105 cells/well were transfected in suspension using the FuGENE transfection reagent (Roche Diagnostics Nederland B.V., Almere, the Netherlands) according to the supplier’s protocol with a FuGENE: DNA ratio of 6:1. For LUC assays, cells were transfected with 24 ng/well of pRSV-hGRα and either 0, 5, 10, 20, 40 ng/well pcDNA3hGRβ plasmid, containing the hGR-β receptor, driven by the CMV promoter. All transfections were supplemented to a total of 240 ng/well of CMV promoter-containing plasmid with the pcDNA3.1 plasmid. In addition to this, 100 ng/well of MMTV-LUC plasmid were added. pTZ carrier DNA was added to bring the total amount of DNA to 2 μg/well. After an incubation period of 24 h, dexamethasone (100 nmol/l) was added, and after another 24 h, the cells were harvested for the LUC assay, as described previously (13, 14).

**Signal Quantification.** X-ray films were scanned using a Hewlett Packard scanner (Hewlett Packard Company, Wilmington, DE). The data resulting from the transfection studies were analyzed by ANOVA, and when significant differences between groups were present, multiple comparisons were carried out using the Student-Newman-Keuls test.
RESULTS

Expression of GR-P in Multiple Myeloma. We have screened bone marrow samples, obtained before the start of treatment from 16 patients with MM for the presence of mRNAs encoding the GR variants. Of these patients, 8 (Fig. 2, Lanes 10, 11, 13, 14, and 16–19) had shown a rapid response to vincristine/adriamycin/dexamethasone combination therapy, whereas 8 patients responded only slowly or were refractory (Fig. 2, Lanes 1, 4–9, and 15). The expression of GR-P varied drastically among the patients, and in some cases, GR-P mRNA levels exceeded those of GR-α + GR-β, e.g., patient 17, albeit that in this patient the total signal was rather low. Quantification of GR-α + GR-β mRNA ratios did not show a clear difference between sensitive or less sensitive MM patients. Separate analysis of the levels of GR-α and GR-β mRNAs showed that GR-β was expressed in all samples at 1–5% of the total GR message (not shown). In five samples of normal lymphocytes, GR-P constituted <20% of the total GR message (GR-α + GR-β + GR-P). An example is shown in Fig. 2, Lane CON.

Expression of GR-P in Other Hematological Malignancies. Expression levels of the GR-P variant were also determined in purified tumor cells obtained from the bone marrow samples of patients with ALL, AML, and NHL. Substantial levels of GR-P expression were found in many of the samples (Fig. 2). However, as was found in the MM samples, there was a considerable variation in GR-P expression. In ALL patients, the relative level of GR-P ranged from 23% of the total GR message (patient 21) to 54% (patient 23). NHL patients all expressed GR-P at very high levels, as can be observed in patients 32, 33, and 34 (44, 41, and 43% of the total GR message, respectively). In samples from patients with AML, a hybrid expression pattern of GC variants was observed. Although only four of five samples could be analyzed successfully, apparently 2 patients exclusively expressed either GR-α only (patient 28) or GR-P only (patient 26), whereas in two other samples, both GR-α and GR-P were detected (patients 29 and 30). In all of these samples, GR-β expression was undetectable under these conditions.

Expression of GR-P in Other Tumors. In samples from paragangliomas, consisting of very slowly dividing cells as opposed to the other tumors, which consist of more rapidly dividing cells, no expression of GR-P was detected (Fig. 2). In ovarian carcinoma and mammary carcinoma cell lines, GR-P was expressed at constant levels, at ~27% of the total GR mRNA, which is slightly higher than that found in normal lymphocytes (the highest level found was 20%). In the liver cell line HepG2 and in the androgen-sensitive prostate carcinoma cell-line LNCaP-FGC, GR-P was expressed at low levels, comparable with those seen in normal lymphocytes. In prostate carcinoma xenografts, GR-P expression varied from 12 to 30% of the total message (Fig. 2). GR-β was not expressed in these tumors.

Effect of GR-P on Cell Type-specific Activity of GR. To study the effect of GR-P on GR-α activity, transient transfection assays were performed using several cell lines of different origins. COS-1 cells were cotransfected with 10 ng of hGR-α expression plasmid and increasing concentrations (0–100 ng) of GR-P expression plasmid as indicated in Fig. 3A. In the absence of GR-P, dexamethasone stimulated GR-α-mediated luciferase expression ~100-fold. When GR-P was cotransfected with GR-α, it induced a significant concentration-dependent increase in the dexamethasone-induced luciferase activity, resulting in a maximal stimulation of 2.5-fold over the stimulation by dexamethasone in the absence of GR-P (P < 0.05). In HeLa cells, the stimulatory effect of GR-P was also observed when GR-α was co-transfected (P < 0.01; Fig. 3B). In the absence of exogenous GR-α in HeLa cells, which have a functional endogenous GR-α, a similar trend was observed (P < 0.05; Fig. 3D). Induction levels in HeLa cells in the absence of exogenous GR-α were one third of those observed when GR-α expression plasmid was present (results not shown). In contrast to the effects of GR-P in COS-1 and HeLa-cells, GR-P had an inhibitory effect in CHO cells (Fig. 3C). When CHO cells were cotransfected with GR-P and GR-α, a reduction of the maximal stimulation by 50% was observed, relative to the dexamethasone-induced stimulation in the absence of GR-P (P < 0.01; Fig. 3C). However, in the absence of cotransfected GR-α, CHO cells showed no response to dexamethasone (Fig. 3E), despite the presence of endogenous GR-α in these cells. Most importantly, cotransfection of GR-P also potentiated the GR-α-mediated dexamethasone effect significantly (P < 0.01; Fig. 4) in two MM cell lines, RPMI 8226-S (Fig. 4A) and UM3 (Fig. 4B).

DISCUSSION

The cause of GC resistance in hematological malignancies is unclear. Direct involvement of the GR may include genetic alterations in the GR gene or altered expression of the GR or its splice variants. Indeed, genetic alterations in the GR have been reported to occur in a MM cell line (6); a deletion of 8 bp in the 3′ untranslated region (exon 9α) of the GR gene was found in the MM cell line U266. This 8-bp sequence contained an estrogen response element half site (5′-TGACCT-3′), which may serve to interact with regulatory factors, including the estrogen receptor (6). Analysis of a number of GC-sensitive and -resistant cell lines derived from the clonal leukemic cell line CEM-C7 suggests that mutations in the GR gene frequently cause glucocorticoid resistance in vitro (7, 8). The lack of apoptotic responses to GC in GC resistant cells is activator protein-1 independent but may involve the less effective induction of a labile nuclear factor-kB inhibitory factor (8). We have shown recently that mutations in the GR gene of patients with generalized GC resistance may achieve a differential interaction with some of these factors (13). One report has addressed the question as to whether mutations in the human GR gene can also account for the development of refractoriness to GC treatment in CLL patients (3). However, none of the resistant patients studied revealed any additional mutations in the GR gene in their tumor cells. The results suggested that mechanisms other than altered ligand or DNA binding of the receptor should be respon-
Gr-P encoding plasmid pCDNA3hGR

levels (45–55% of the total GR mRNA) in lymphocytes isolated from cells. Moreover, GR-P expression was also found at considerable MM cells, as has been suggested (11), but also in relatively sensitive and to a lesser extent in AML. These levels of expression of the GR-P myeloma plasma cells. GR-P mRNA was also present in ALL, NHL, mRNA), we observed a consistently high expression of this isoform in untreated hematological malignancies. Relative to its expression in MM patients (11), and we have investigated expression of its mRNA reversal of multidrug resistance (15).

GCs. GC treatment may thus be an alternative mechanism for the inhibition of cell growth in the OPM-1 and RPMI 8226 cell lines may thus be a postreceptor mechanism. Multidrug-resistant MM cell lines, GR mRNA levels were up-regulated upon stimulation with doxorubicin (15). In the relatively doxorubicin-sensitive cell line R10, this reduction was not counteracted by GC treatment. However, in the highly doxorubicin-resistant R40 and R60 cell lines, GR mRNA levels were up-regulated upon stimulation with GCs. GC treatment may thus be an alternative mechanism for the reversal of multidrug resistance (15).

A splice variant of the hGR termed GR-P has been identified in MM patients (11), and we have investigated expression of its mRNA in untreated hematological malignancies. Relative to its expression in normal peripheral blood lymphocytes (10–20% of the total GR mRNA), we observed a consistently high expression of this isoform in myeloma plasma cells. GR-P mRNA was also present in ALL, NHL, and to a lesser extent in AML. These levels of expression of the GR-P suggest a potential role for the GR-P, not only in highly refractory MM cells, as has been suggested (11), but also in relatively sensitive cells. Moreover, GR-P expression was also found at considerable levels (45–55% of the total GR mRNA) in lymphocytes isolated from normal bone marrow aspirates (not shown). These data indicate that the GR-P form is uniformly present in malignant hematological cells at diagnosis. Thus far, its role in these cells is difficult to assess. In our small series of MM patients, refractoriness to GC therapy did not seem to be associated with grossly altered ratios of the GR-P:GR-α + GR-β mRNA (Fig. 2). Possibly, GC treatment may induce some changes in the level of GR-P expression later during the course of the disease. The present data indicate that GR-P expression is not a phenomenon observed exclusively in highly refractory cases of myeloma, as was assumed in earlier studies (11). Although we have focused on MM in this study, other GC-responsive diseases such as ALL and NHL were also included, and essentially the same pattern was observed. In contrast, AML patients showed a more variable pattern, which seems in line with the clinical observation of lower or absent response of these tumors to GC treatment.

GR-P was also found at a variable degree of expression in a panel of other tumors, in xenografts from prostate carcinomas, and in tumor cell lines from ovarian carcinoma, mammary carcinoma, HepG2 cells, and LNCap-FGC-cells. These data indicate that high levels of GR-P expression are not restricted to hematological malignancies and may be involved in the GC response of tumors originating from different tissues. Interestingly, the GR-P variant was absent in benign neuroendocrine tumor cells (paragangliomas).

Because it was observed that GR-β was expressed at very low levels in MM and could not be detected at all in other hematological malignancies or in solid tumors, it can be concluded that the GR-P splice variant is the only one that is predominantly generated in (hematological) malignancies.

We and others (14, 16, 17) have shown that GR-β does not inhibit GR effects in COS-1 and in other cells, whereas other groups have shown that the GR-β acts as a dominant inhibitor of GR action (12, 18). The observation that the GR-P splice variant investigated in this study stimulates GR activity in COS-1, HeLa, and MM cells while it
inhibits GR effects in CHO cells is new. Our experiments in the RPMI 8226-S and UM3 myeloma cell lines (Fig. 4) demonstrate that a specific dexamethasone response is mediated through GR-α, and that GR-P may up-regulate activation of the dexamethasone effect. This finding may be of important clinical relevance because of the observed wide variation of GR-P expression in naive myeloma cells obtained from patients.

The effects of GR-P observed in our transfection experiments are relatively small, 2.5-fold increases only at relatively high concentrations of added GR-P expression plasmid. However, it is possible that in this system the amount of cotransfected GR-α is so high that relatively much GR-P is necessary to make its effects visible. Moreover, the effects observed in CHO cells occur already when GR-α and GR-P are transfected in equal quantities (10 ng each; see Fig. 3C). Such equal amounts of both splice variant mRNAs have also been observed in several tumor samples (e.g., Fig. 2, Lanes 17, 23, and 34). In contrast to the situation in these transfection experiments, where a constant amount of GR-α was used in the presence of increasing concentrations of GR-P, the situation in vivo is different. There the generation of each copy of GR-P mRNA proceeds at the expense of a copy of GR-α mRNA. Reductions in the number of GR-α molecules/cell have been shown previously to reduce glucocorticoid sensitivity (19).

The mechanism by which the effects of GR-P occur are unknown. However, the differences observed between the cell types suggest that the specific cellular environment plays a role in determining the nature of these effects. GR-P may have a conformation that favors dimerization to ligand-bound GR-α, depending upon the relative abundance of the GR-α and GR-P proteins, upon which the dimer translocates to the nucleus to stimulate transcription of target genes in a more efficient manner compared with regular GR-α homodimers. Alternatively, the balance between GR-α and GR-P formation in the in vivo context may modulate the effects of glucocorticoids. In this way, decreased expression of GR-P in chemotherapy-resistant hematopoietic tumor cells could explain enhanced resistance to glucocorticoid therapy. Clinically, such mechanisms are of great importance, because the development of resistance to GCs during treatment contributes to treatment failure. Also, here we have only investigated one mode of glucocorticoid action with respect to the effects of GR-P, stimulating effects of the GR operating via direct interaction of the GR dimer with discrete sequences in the promoter region of the target gene (the MMTV glucocorticoid-responsive element). It will be interesting to investigate the role of GR-P in systems designed to dissect other modes of GR action, such as in activator protein-1- and nuclear factor-κB-mediated glucocorticoid signal transduction. We have shown previously (13) that several mutations in the GR gene, responsible for generalized GC resistance, exhibit different response profiles in such systems. Recently, it was shown that GR-P does bind to the promoter region of the proopiomelanocortin gene (20) but apparently does not repress gene expression in that context. We are currently also investigating which external signals influence the relative abundance of the GR splice variants in peripheral mononuclear leukocytes and if changes in this distribution affect the regulation of endogenous glucocorticoid-regulated genes, such as several cytokine genes.

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REFERENCES