Alternatively Spliced Glucocorticoid Receptor Messenger RNAs in Glucocorticoid-resistant Human Multiple Myeloma Cells

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Abstract

Glucocorticoids are highly effective chemotherapeutic agents used in the treatment of hematological malignancies including multiple myeloma. However, the clinical usefulness of this class of drugs is limited by the problem of resistance. In the following study, we have isolated two alternatively spliced transcripts of the glucocorticoid receptor from a complementary DNA library generated from the glucocorticoid-resistant myeloma cell line MM.1Re. In each of the clones, specific exons of the hormone binding domain are precisely deleted. Our data implicate alternate splicing as a mechanism by which a cell generates different receptor isoforms and as a consequence evades the effects of hormone.

Introduction

Glucocorticoids are among the most effective agents used in the treatment of hematological cancers including multiple myeloma (1-4). We and others have provided evidence that the efficacy of steroids is due to their growth-inhibitory and lymphocytolytic properties (5-7). However, not all patients initially respond to steroid therapy and those that do ultimately become resistant. Insight into the mechanisms of resistance is crucial for the timely and successful treatment of patients with this and other steroid-treated malignancies.

To address resistance to glucocorticoids in multiple myeloma, we have developed a cell line (MM.1) from a myeloma patient who had been treated intermittently with steroids and subsequently became resistant (5, 8). Upon establishing MM.1 in culture, a glucocorticoid resistant variant (MM.1Re) cell line was isolated from a dexamethasone-resistant subpopulation present in the parent line. For comparative purposes, a hormone-sensitive cell line (MM.1S) was also cloned. Here, we describe the cloning of two variant GR isoforms from MM.1Re with deletions in the hormone binding domain. Our data provide the first direct evidence that aberrant GR generated by alternative splicing events may contribute to resistance to glucocorticoids in multiple myeloma.

Materials and Methods

Cell Lines and Culture Conditions. MM.1 was isolated from the peripheral blood of a patient with multiple myeloma and sensitive and resistant cell lines obtained as described previously (8). Cells were grown in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml; Gibco), and Gultizone, 2.5 μg/ml (Irvine Scientific, Santa Ana, CA); and 5% carbon dioxide at 37°C.

Cloning of the cDNA for the Alternatively Spliced GR Transcripts. Highly pure polyadenylated RNA was isolated from MM.1Re and used to construct a randomly primed cDNA library. The cDNA was cloned into the EcoRI site of the Lambda ZAP II vector (Stratagene, La Jolla, CA). Approximately 5 × 10^6 pfu of the primary library (4.5 × 10^6 pfu/ml) were amplified to an estimated titer of 5 × 10^9 pfu/ml. Plaque DNA was isolated from 10 ml of the amplified stock. The DNA was amplified by PCR under standard conditions (35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3-10 min) using an upstream primer homologous to the 3' end of the DNA binding domain and vector sequences as the downstream primers. The PCR products were subcloned directly into the TA site of a pCR cloning vector (InVitrogen, San Diego, CA) and transformed into bacteria. Positive colonies were identified by hybridization with a GR probe and subsequently sequenced by Sanger dideoxy chain termination using [α-32P]dATP according to manufacturer’s instructions (United States Biochemical Corp., Cleveland, OH).

Results and Discussion

We have previously reported the characteristics of the glucocorticoid receptor in both the hormone-sensitive (MM.1S) and -resistant (MM.1Re) sublines of a human multiple myeloma cell line (5). MM.1S is sensitive to the cytolytic effects of glucocorticoid and synthesizes high levels of the wild type 7.1-kilobase GR mRNA. Hormone binding assays have indicated that the GR expressed by MM.1S bind [3H]dexamethasone with high affinity (1 × 10^-9 M). In contrast, MM.1Re is resistant to glucocorticoid-induced cytolyis, expresses an aberrant GR transcript of approximately 5.5 kilobases, and has little hormone-binding activity. We have mapped the 5.5-kilobase transcript using fragments of the GR cDNA as probes and found that it is shorter than wild type due to a deletion in its 3' end which includes the last 360 base pairs of the hormone-binding domain. To more precisely define the nature of the deletion, the cDNA for the GR expressed by MM.1Re was cloned. Initially we attempted to amplify the hormone binding domain of the GR in MM.1Re using reverse transcription PCR with oligonucleotide primers flanking that domain. However, when primers complementary to the 3' untranslated region were used, no amplification products were detected in MM.1Re. This indicated that the 3' untranslated region of the GR mRNA in MM.1Re is distinct from that of WT and, would not be obtained by this technique. As an alternative approach, we generated a randomly primed cDNA library from MM.1Re mRNA that had been shown by Northern analysis to contain high levels of the 5.5-kilobase GR transcript and low levels of the wild type 7.1-kilobase GR mRNA. Due to the long (2.3-kilobase) 3' untranslated region in the GR cDNA (9), random hexamers were used for priming the cDNA synthesis. As detailed in the experimental procedures, the PCR reaction was used to screen the library and PCR-amplified DNA fragments ranging in sizes from 300 base pairs to approximately 1.7 kilobases were subcloned and screened by hybridization with a 32P-labeled fragment comprising the full length hormone-binding domain. Inserts from positive colonies were then sequenced.

In addition to the wild type GR cDNA, two GR isoforms were isolated. The first, hGR-P, which was most highly represented in the screening process, directly correlates with our predictions based on the studies of the 5.5-kilobase transcript (Fig. 1A). Although the first portion of the hormone-binding domain of this clone is intact, the last...
Fig. 1. Cloning of the cDNA for alternatively spliced GR transcripts. The sequence of wild type GR is shown in comparison to clone hGR-P (A) where a failure to splice results in the retention of intron G adjacent to exon 7. When compared to wild type GR, the clone hGR-A (B) has a precise excision of exons 5, 6, and 7 results in the in frame juxtaposition of exons 4 and 8. A schematic representation of the alternatively spliced GR cDNAs is shown (C). Exons in the genomic sequence are shown as solid boxes and are numbered while introns appear as solid lines and are lettered. The two alternatively spliced cDNAs are presumed to be identical to wild type in all regions except the hormone-binding domain.

300 base pairs are missing. The cDNA diverges from wild type at the junction of exons 7 and 8 in which the splice donor site of exon 7 is present; however, the acceptor site of exon 8 is not (10). This failure to splice enables sequences from the intervening intron (intron G) to remain intact. Since intron G is approximately 13.5 kilobases in size (10) it is unlikely that the entire intron followed by exons 8 and 9 persists in the mature GR transcript. It is more likely that sequences within intron G signal polyadenylation and produce a truncated mRNA. Indeed, an in frame stop codon is present approximately 8 base pairs from the 5' end of intron G. Because it lacks exons 8 and 9, the hGR-P transcript would translate into a COOH-terminally truncated GR lacking a dimerization signal (11) and a transcriptional activation domain (12).

A second mutant, hGR-A, with an internal deletion in the first portion of the hormone-binding domain, was also cloned (Fig. 1B). hGR-A contains wild type sequences up to the border of exons 4 and 5. At this point, the splice donor site of the 3' end of exon 4 is juxtaposed in frame with the acceptor site at the 5' end of exon 8. Therefore, exons 5, 6, and 7 are precisely excised due to an alternate splicing event. The mRNA and protein encoded by this mutant are 555 base pairs and 185 amino acids, respectively, smaller than wild type. Thus, the predicted protein product of this transcript is missing a nuclear localization signal (13) and a transcriptional activation domain (14).

Previous in vitro studies of glucocorticoid resistance in leukemia and lymphoma cell lines have demonstrated that the majority of resistant variants (80–90%) fall in the category of receptorless (r−) based on their lack of hormone-binding activity in whole cell binding assays (15). This phenotype most often results from a repression of GR synthesis but also occurs when a cell expresses a GR with a defect in the hormone-binding domain. To date, all of the latter type of r− variants have been found to harbor point mutations in the hormone-binding domain (16). MM.1Re also falls into the r− category due to a lack of hormone-binding activity. However, the GR clones described here not only are the first mutant GR cDNA isolated from multiple myeloma but also comprise one of the first descriptions of r− variants derived from a hematological cancer in which specific exons of the hormone-binding domain are absent. The single precedent is the initial report on the cloning of the GR cDNA by Hollenberg et al. (9). In the latter, in addition to the wild type GR cDNA, an alternatively spliced GR cDNA is isolated from the lymphoblastoid cell line IM-9. In contrast to hGR-P and hGR-A, the clone (GR-β) diverges from wild type at the junction of exons 8 and 9.

Alternatively spliced transcripts coding for a steroid hormone receptor have also been detected in breast cancers. Estrogen receptor variants missing specific exons of the hormone-binding domain have been isolated from both human breast cancer cell lines and estrogen receptor-negative patient tumor samples (17–20). The studies described here raise the intriguing possibility that alternative splicing in the GR hormone-binding domain may be responsible for a change in the hormonal responsiveness of the myeloma cells MM.1Re. Clearly...
more studies are necessary to determine what role these GR isoforms may play in the loss of responsiveness to hormonal therapies in myeloma patients.

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References

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