A Variant Glucocorticoid Receptor Messenger RNA Is Expressed in Multiple Myeloma Patients

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Abstract

In multiple myeloma cells resistant to glucocorticoids, we have previously identified a variant glucocorticoid receptor (GR) transcript (P. A. Moalli et al., Cancer Res., 53: 3877–3879, 1993). Here, we report a reverse transcription-PCR assay to assess whether this aberrant GR transcript is present in myeloma patients. We detected both the wild-type and variant GR transcripts in the patient isolate that was the source of our myeloma cell lines, in patients refractory to steroid treatment, and in healthy control subjects. Simultaneous amplification of wild-type and variant GR mRNAs indicates that the variant GR is more highly expressed in cells that are resistant to glucocorticoids. We hypothesize that the variant GR is a normal mRNA transcript that acts to modulate glucocorticoid responsiveness, and increased expression contributes to a resistant phenotype.

Introduction

Multiple myeloma is a disease caused by neoplastic plasma cells that synthesize abnormal amounts of immunoglobulin fragments (1). It represents approximately 10% of hematological cancers and is associated with a median survival of 2.5–3 years. Glucocorticoids are among the most active agents for the treatment of this malignancy. Unfortunately, a small percentage of patients have de novo clinical resistance to this therapy, and responding patients ultimately become refractory to steroids. Insight into the mechanisms of resistance is essential if therapeutic advances with this and other steroid-treated tumors are to be made. We have previously described a variant glucocorticoid receptor transcript (hGR-P) in a multiple myeloma cell line that is resistant to the killing effects of glucocorticoid (2, 3). hGR-P diverges from the wild-type GR at the junctions of exons 7 and 8; a failure to splice at the exon 7/8 junction results in the retention of a portion of the intervening sequence of intron G (Fig. 1). To determine if the hGR-P transcript is clinically significant, we have developed a RT-PCR assay to detect the presence of this transcript in bone marrow aspirates from myeloma patients. Our data suggest that hGR-P is normally expressed at low levels and that increased expression of this alternatively spliced GR is associated with steroid resistance.

Materials and Methods

Cell Lines and Culture Conditions. MM.1 cells were isolated from the peripheral blood of a patient with multiple myeloma who was refractory to glucocorticoid treatment (4). Sensitive and resistant cell lines were established from the original patient isolate as described previously (3). Cells were grown in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/ml Fungizone in 5% carbon dioxide at 37°C.

RNA Isolation. The WBC component of bone marrow or peripheral blood was enriched by separation on a Ficoll gradient. A minimum of 10^7 cells was disrupted with 4 M guanidine isothiocyanate, and total RNA was isolated by centrifugation through a CsCl cushion (5).

RT-PCR Assay. One μg of total RNA is reverse transcribed using 100 μM random hexamers (Pharmedia, Piscataway, NJ), 50 units of Superscript reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), 20 units Rnasin (Promega, Madison, WI), 1 mM deoxynucleotide triphosphates, and 6 mM MgCl2 in 1X PCR buffer (PerkinElmer/Cetus). After a 90-min incubation at 42°C, the reaction is heated to 95°C for 15 min to inactivate the enzyme and to denature the DNA-RNA heteroduplex. The PCR reaction is assembled in the same reaction tube. The MgCl2 concentration is adjusted to 2 mM, and the deoxyribose triphosphates are re-added to compensate for any hydrolysis during the 95°C incubation step. A trace amount (2 μCi) of 32P-labeled nucleotide is included in the reaction mix. Taq polymerase (2.5 units; Perkin/Elmer/Cetus) is added to each reaction. The oligonucleotide primers for the PCR reactions were synthesized in the biotechnology facility at Northwestern University and are diagrammed in Fig. 1. Their sequences are listed below:

- Primer #1, 5' - TAGGCCFFGATA1TFCATGC-3'; primer #2, 5' - CAAGTATGCTAGAATCCAAGAG-3'; and primer #3, 5' - GTTCCTAGGCTCATCTTTCATGC-3'. The upstream primer (#1) is common to both the wild-type and the variant GR transcripts and falls within exon 5 in the hormon-binding domain of the receptor. The downstream primer for the hGR-P variant (#3) is within the sequences for intron G, which is uniquely included in the hGR-P transcript. The downstream primer for the wild-type receptor (#2) is within exon 8, which is not present in the hGR-P transcript. The thermocycles consisted of 94°C for 1.5 min, 58°C for 1 min, and 72°C for 2 min for 35 cycles, followed by a 10-min extension at 72°C. The PCR reaction products were fractionated on a 6% nondenaturing acrylamide gel, and the gel was dried and exposed to X-ray film (Hyperfilm; Amersham, Arlington Heights, IL) for signal detection. In some instances, the density of the autoradiographic signal was quantified using an LKB Ultrascan XL laser densitometer.

PCR Sequencing. PCR products were gel purified and directly sequenced using 32P-labeled oligonucleotide primers. The double-stranded DNA cycle sequencing system from BRL (Gaithersburg, MD) was used according to the instructions of the manufacturer.

Results and Discussion

To define the assay parameters, we used total RNA from glucocorticoid-sensitive and -resistant multiple myeloma cell lines. Glucocorticoid-resistant myeloma cells are defined as having unimpaired growth in the presence of 1 × 10^-6 M dexamethasone (3).4 DNA segments of the correct sizes were generated by both the wild-type receptor primers and the hGR-P primers (Fig. 2). These segments were further characterized by direct PCR sequencing and confirmed to be the correct GR sequences. No GR-specific DNA segments were amplified in reactions using tRNA as the starting material, indicating

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that there is no contamination of the assay reagents with the GR cDNAs from the laboratory (Fig. 2, Lane 6). RT-PCR analysis of peripheral WBC from a healthy control subject indicates the presence of both wild-type and the hGR-P transcripts (Fig. 2, Lane 8). The low levels of hGR-P expression suggest that the variant OR may be a normally occurring transcript. In addition, a minus reverse transcriptase reaction on the same normal control RNA did not express any GR products (Fig. 2, Lane 9), indicating that there is no cDNA contamination in the total RNA isolation procedure. Similar results were obtained from the analysis of peripheral blood from seven additional healthy control subjects (data not shown).

To compare the levels of the GR transcripts within a sample, both the hGR-P and wild-type receptor primers were included in the same reaction. This was done to control for the amount of variability between reactions (Fig. 2) and allows for a semiquantitative comparison of wild-type receptor expression to that of hGR-P. The density of the autoradiographic signal was determined using a scanning densitometer, and these values are expressed as a percentage of the total amount of glucocorticoid receptor signal (total glucocorticoid receptor signal = WT-GR + hGR-P). These results indicate that glucocorticoid-resistant cells (Fig. 2, Lanes 2, 3, and 4) express relatively more of the hGR-P variant than the glucocorticoid-sensitive cells (Fig. 2, Lanes 1, 5, and 7). In contrast, the glucocorticoid-sensitive cells express more of the wild-type receptor mRNA.

In separate amplification reactions, RT-PCR analysis of the original patient isolate used to establish the MM.1 myeloma cell line demonstrates that the hGR-P variant transcript is present in the patient and is, therefore, not an artifact of tissue culture (Fig. 3, Lane 4). Analysis of additional multiple myeloma patients indicates that the hGR-P variant is also present in patients who have failed glucocorticoid therapy (Fig. 3, Lanes 5 and 6) as well as in a myeloma patient at presentation (Fig. 3, Lane 7). Since these analyses are from separate amplification reactions, direct quantitative comparisons of RT-PCR results are not possible without an internal standardization control.

Based on these results, we hypothesized that the variant receptor, hGR-P, may be involved in regulating the glucocorticoid responsiveness of a cell, and when expressed in excess, hGR-P may contribute to the hormone resistance of the cell. The phenomenon of alternative splicing of a hormone receptor that may contribute to a resistant phenotype has been described for the estrogen (6) and thyroid hormone receptors (7, 8) as well as for the GR. Recently, Strasser-Wozak et al. (9) noted that a unique GR splice variant involving a point mutation in the 3'-splice junction of intron G is associated with steroid hormone resistance. The clinical significance of the alternatively spliced steroid receptors has been explored by Murphy et al. (10).
They have analyzed breast tumors for the presence of a variant estrogen receptor mRNA and found higher expression of the variant receptor relative to wild-type receptor in tumors with markers of poor prognosis compared to those with good prognosis. At the present time, we are evaluating additional patient samples to determine if the amount of hGR-P expression in myeloma patients has prognostic and therapeutic significance. In addition, we are pursuing the identification and characterization of the variant protein product.

References

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