Glucocorticoid Receptor Structure and Function in Glucocorticoid-resistant Small Cell Lung Carcinoma Cells

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ABSTRACT

Human small cell lung carcinomas (SCLCs) frequently express the adrenocorticotropic hormone precursor gene proopiomelanocortin. Glucocorticoids usually fail to inhibit this ectopic adrenocorticotropic hormone production, in contrast to their effects in the pituitary. We have shown three human SCLC cell lines to be globally resistant to glucocorticoid action; in two of these lines this occurs despite the presence of glucocorticoid receptors (GR+). Accordingly, we have cloned and sequenced the GR coding region from one of these two GR+, SCLC cell lines, COR L24, and identified compound heterozygous mutations. One allele had a single nucleotide substitution of A to G in the NH2-terminal domain, which altered Asp to Ser at amino acid 363. The other allele contained a trunculeotide insertion at the 5’ boundary of exon 4, which introduced an additional amino acid, Arg462, between the two zinc fingers of the DNA binding domain. In cotransfection studies using the glucocorticoid responsive mouse mammary tumor virus-luciferase Ser336 did not alter receptor function. In contrast, Arg462 encoded a GR with 48% Vmax activity compared to wild-type receptor (P < 0.001), with an unchanged EC50. Thus, GR mutations may contribute to the glucocorticoid-resistant phenotype of GR+ COR L24 cells, which could confer survival advantage to this highly malignant neuroendocrine tumor.

INTRODUCTION

Human SCLCs2 are the commonest cause of the ectopic ACTH syndrome. These tumors often express the ACTH precursor gene POMC, although secretion of bioactive peptide products is usually restricted to those tumors with significant neuroendocrine differentiation (1). Up to 50% of patients with SCLC have biochemical features suggestive of excess ACTH-like bioactivity (1), although their clinical presentation is usually dominated by the underlying cancer rather than endocrine manifestations (2). ACTH-related peptide production by these extrapituitary tumors is characteristically unresponsive to glucocorticoid inhibition, in contrast to the anterior pituitary expression (3).

We have established an in vitro model of ectopic ACTH production, using a panel of human SCLC cell lines that express the POMC gene and secrete POMC-derived peptides (4). One cell line, COR L24, expresses GR, with normal affinity for dexamethasone (Kd, 5.7 nM) and at adequate concentration for signaling (Bmax, 11 fmol/million cells). Despite this, the cells fail to suppress expression of POMC in response to even high concentrations of dexamethasone, up to 2000 nM (5).

In addition to the glucocorticoid resistance of the endogenous POMC gene, both positive and negative regulated reporter genes transfected into the cells exhibited severe resistance (6), thereby excluding a defect within the POMC gene itself. Furthermore, expres-

MATERIALS AND METHODS

Cell Culture. The COR L24 cells were derived from a patient with histologically confirmed bronchogenic SCLC and were generously donated by Dr. P. Twentyman (MRC Clinical Oncology and Radiotherapeutics Unit, Cambridge, UK). The cells were cultured as described (9). The COS 7 cells were obtained from the European Collection of Animal Cell Culture (Porton Down, Wilts, UK). They were cultured in DMEM supplemented with 10% FCS, 4 mM L-glutamine, and 1 mM sodium pyruvate. Dexamethasone was purchased from Sigma Chemical Co. (Poole, Dorset, UK).

Plasmids. pMMTV-luc was constructed by excising the MMTV long terminal repeat from pMSG-CAT (Pharmacia, Upplands, Sweden) at HindIII and SmaI sites and ligating it into the pX-P-2 (10) poly linker using T4 ligase (Boehringer-Mannheim). The resulting plasmid has the MMTV promoter driving a luciferase reporter gene. Wild-type GR expression vector (pRS-HGR) was the kind gift of Dr. R. Evans (Salk Institute for Biological Studies, La Jolla, CA; Ref. 11). The expression vector for SER336 (pRS-GR-SER), in the same vector as pRS-hGR, was the kind gift of Drs. M. Karl and G. P. Chrousos (Developmental Endocrinology Branch, National Institute of Child Health and Human Development, NIH, Bethesda, MD; Ref. 12). pRSV-CAT has been described before (13). The expression vector for GR-Ag453 was constructed in the same vector as pRS-hGR using standard recombinant DNA techniques.

Transfection. Cells were washed twice in serum-free medium and then resuspended with DNA in a 4-mm pathlength Bio-Rad electroporation cuvette (Bio-Rad, Hemel Hempstead, UK). All cells were electroporated with 200V at 1500 μF capacitance (Electrophore 2000; SEDD, Liege, Belgium). The transfected pools of cells were divided into treatment and control groups to normalize for transfection efficiency between groups (6). Cells were harvested after 48 h and were lysed in 25 mM Tris phosphate, pH 7.8, 10 mM magnesium chloride, 0.1 mg/ml BSA, 15% glycerol, 1% Triton X-100, and 1 mM EDTA. Luciferase activity was measured in a Berthold Lumat LB9501 luminometer in the presence of 0.8 mM ATP and 0.3 mM n-luciferin. Cells were cotransfected with pRSV-CAT to control for transfection efficiency, and results are expressed as fold-induction of luciferase activity (in light units) per 100 pg CAT protein. In some experiments, absolute luciferase activity is presented: in these cases, the CAT protein level between groups varied less than 15%. Two hundred μl of cell lysate were used to measure CAT using the CAT ELISA (Boehringer-Mannheim), as described by the manufacturer.
Extraction of RNA, Synthesis of cDNA, and Amplification by PCR. 
Poly(A)⁺ RNA was isolated using MicroFast Track kit (Invitrogen, Ltd., Abingdon, UK). This was reverse transcribed using an oligo(dT) primer and AMV reverse transcriptase for 1 h at 42°C. The reaction products were heated to 99°C for 5 min and then stored at −20°C until use. Typically, 1 μl was used in each PCR reaction, with 2.5 mM magnesium chloride, 50 pmol of each primer, and 2 units Taq polymerase in 1× PCR buffer (Boehringer-Mannheim). Reaction conditions were: 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s. Thirty cycles were performed. PCR primers were synthesized by Severn Biotech, Ltd. (Kidderminster, UK). Sense primers were located at cDNA positions 50 and 1101 and antisense primers at 1254, 1924, and 2502. The reactions gave rise to three fragments of 1204, 823, and 1401 bp. The sequences were (5' to 3'): 50, AGC TAA GTT GTT TAT CTC GG; 1101, TGT GAG TCT TGC AGG AC; 1254, GTT GTC ATC TCC AGA TCC TTT; 1924, ACT GCA GTA GGG TCA TTT GG; and 2502, CGA TCT TCT TTA AGG CAA CC.

Cloning and Sequencing GR from COR L24 Cells. The PCR products were resolved on 1% agarose gels run in 0.04 M Tris acetate, 0.002 M EDTA buffer. The expected size bands were seen and were excised from the gel; then the DNA was extracted using the USBiolith kit (USB, Cleveland, OH). The DNA was ligated into the vector pCR II (Invitrogen). Double-stranded DNA was sequenced using Sequenase (USB, Cleveland, OH).

RESULTS

Identification of GR SER³⁶³ Mutation in COR L24 Cells. Using an reverse transcription-PCR approach, the coding region of the GR was amplified in three overlapping segments and subsequently cloned. Sequence analysis of 10 clones revealed one GR allele with a single nucleotide substitution, A to G, at nucleotide 1220 (Fig. 1). This changed the predicted codon from asparagine to serine at amino acid 363, which lies between the tau 1 transcriptional domain and the first zinc finger of the DNA binding domain (Fig. 2).

Identification of GR ARG⁴⁵³ Mutation in COR L24 Cells. We identified the other allele, without mutation at 1220, and this allele was found to harbor a trinucleotide insertion, TAG, at position 1485 (Fig. 3). This insertion results in the downstream introduction of an additional arginine at amino acid 453, at the base of the second zinc finger of the DNA-binding domain (Figs. 2 and 3). This allele was also found to have a point mutation at 1938 T to C, which did not alter the predicted amino acid from phenylalanine 602 or create novel splice sequences.

Expression of GR SER³⁶³ Mutant in COS 7 Cells. The SER³⁶³ mutant GR has been described before in familial glucocorticoid resistance and has been shown to retain transcriptional activity in response to dexamethasone (12). However, as with experimentally introduced mutations involving the NH₂-terminal domain, the mutant GR may have reduced transcriptional potency compared to wild-type GR. To measure the transcriptional effectiveness of this mutant, we expressed the mutant in COS 7 cells with the pMMTV-luc reporter gene in the presence of 100 nM dexamethasone. Because we were interested to determine potency, we used a range of receptor expression vector concentrations. It was found that 1 ng of wild-type GR expression vector was sufficient for dexamethasone to induce reporter gene expression by 300 ± 30%. Over a range of expression vector concentrations up to 100 ng/ml, there was no significant difference between the wild-type receptor and the SER³⁶³ mutant GR (Fig. 4a).

Expression of GR SER³⁶³ in COR L24 Cells. Both wild-type and SER³⁶³ GR expression vectors were transfected into COR L24 cells again with the MMTV-luc reporter gene. No significant difference in the DNA dose-response curve was noted (Fig. 4b). Thus, the SER³⁶³ GR has equivalent activity to wild-type GR in both COS 7 cells and in the resistant COR L24 cells. There was no difference in the dexamethasone dose-response curve between the wild-type GR and SER³⁶³ (data not shown). However, there is clearly a difference in the responsiveness of the COR L24 cell line compared to the COS 7 cell at a given concentration of plasmid. COR L24 cells require nearly 1000-fold more GR expression vector to cause equivalent dexamethasone induction of the reporter gene (Fig. 4). This is unlikely to reflect differences in transfection efficiency because results are corrected for CAT expression from a cotransfected RSV-CAT construct.

Expression of GR ARG⁴⁵³ Mutant in COS 7 Cells. An expression vector for the GR ARG⁴⁵³ mutation was constructed, and the function of this mutant was compared to that of wild-type GR in COS 7 cells. The luciferase activity obtained at a dexamethasone concentration of 100 nM using the GR-ARG⁴⁵³ expression vector was 8663 ± 486 (mean ± SE) light units, compared to 17986 ± 970 light units with the wild-type GR expression vector (n = 6; P < 0.001). This represented a reduction of transcriptional activity of 48% of wild-type. A dexamethasone dose-response curve confirmed that ARG⁴⁵³ had impaired function compared to wild-type GR and showed that the dose-response curve was “flattened” over the range of concentrations used, with a reduction in V₅₀ but no change in the EC₅₀ (Fig. 5).

To model the situation found in vivo in COR L24 cells, both GR-ARG⁴⁵³ and GR-SER³⁶³ were expressed simultaneously in COS 7 cells. There was no evidence of dominant negative activity of the GR-ARG⁴⁵³ on the function of the GR-SER³⁶³ mutant molecule (data not shown). Equally, there was no evidence that the GR-ARG⁴⁵³ had a dominant negative action on the wild-type GR in the same assay (data not shown). Expression of GR-Arg⁴⁵³ in COR L24 cells yielded similar results to those observed in COS 7 cells, excluding a cell type-specific effect of this mutant (data not shown).

DISCUSSION

We have previously described the presence of GRs in COR L24 SCLC cells capable of binding to ligand and found the concentration to be similar to that described in other cell systems responsive to...
dexamethasone. In contrast, another cell line, DMS 79, appears to express negligible ligand-binding receptors, probably because of glucocorticoid resistance (6, 14). Despite expressing receptors, COR L24 cells do not respond to dexamethasone when endogenous or transfected positive- or negative-regulated reporter genes are measured (5, 6). Expression of wild-type GR restored signaling, suggesting functional failure of the endogenous GR, possibly as a result of mutation in the coding region (6). In the present studies, we have examined the sequence and function of the endogenous GR from COR L24 cells.

We have shown that both GR alleles are expressed in COR L24 cells, and that both alleles show variations from the normal GR sequence. The first identified variant is a single adenine-to-guanine base substitution in the second position of codon 363, changing an asparagine to a serine residue. This allele has been identified previously in a kindred with familial glucocorticoid resistance. However, the proband described has compound heterozygous mutations within the GR gene. The GR-Ser\textsuperscript{363} mutation did not segregate with the phenotype in this family, and glucocorticoid resistance was attributed to instability of the RNA transcript arising from the other allele, as a result of a disrupted splice donor site at the 3′ boundary of exon 6 (12).

Previous expression studies using the SER\textsuperscript{363} mutant have focused on ligand dose-response in the presence of maximal concentrations of mutant receptor rather than on the transcriptional potency of the mutant at maximal ligand concentrations. Therefore, it was possible that subtle differences in receptor transcriptional potency may exist. To examine this, the transcriptional effects of different amounts of the two expression vectors on reporter gene activity were measured. Initially, we used the COS 7 cell line, which does not express endogenous glucocorticoid receptors, and found that over a wide range of receptor expression vector concentrations, the SER\textsuperscript{363} mutant and the wild-type GR expression vector showed equal transactivation potential in response to 100 nm dexamethasone at a given concentration of receptor. We also wished to compare the function of the SER\textsuperscript{363} with wild-type in the COR L24 cells, in case differences in receptor function were dependent on cell type. Again, no differences were seen. Although these data suggest that the SER\textsuperscript{363} mutant does not contribute to the glucocorticoid-resistant phenotype observed in COR L24 cells, it is relevant that SER\textsuperscript{363} has been described in another glucocorticoid-resistant cell line (14) and in a kindred with familial glucocorticoid resistance (12). It is possible that SER\textsuperscript{363} does affect sensitivity to glucocorticoid when expressed at physiological concentrations within the cell.
The second mutation identified in the SCLC cells was the introduction of three nucleotides at position 1485 of the GR. This is a most unusual finding, the more extraordinary as the same nucleotides are found in the GR from the glucocorticoid-resistant primate, the Cotton Top marmoset (15). The marmoset GR also has a number of other amino acid differences compared to the human, and the relative importance of the DNA-binding domain remains undetermined. The addition of a basic amino acid, Arg\textsuperscript{453}, at this position might be expected to alter the conformation of the two zinc fingers and so reduce DNA affinity. Because the marmoset can overcome glucocorticoid resistance at the expense of high circulating glucocorticoid concentrations, it is likely that the ARG\textsuperscript{453} molecule would retain at least partial function. We have shown that the ARG\textsuperscript{453} retains transactivation potential, but that its maximal effect is less than 50% of that seen with the wild-type GR. The GR comprises three main functional domains, NH\textsubscript{2}-terminal, DNA-binding, and ligand-binding, which are capable of independent function (7, 8). Therefore, it is not surprising that the GR-Arg\textsuperscript{453} retains normal ligand binding activity and, therefore, normal dexamethasone dose-response effects on a reporter gene. The presumed defect in DNA binding appears to reduce the maximal transcriptional activity of the receptor, causing a diminished $V_{\text{max}}$. We cannot exclude differences in protein translation between GR-Arg\textsuperscript{453} and wild-type as contributing to the functional differences observed.

Despite our demonstration of compound heterozygous mutations in the GR from SCLC cells, it seems unlikely that they account for the degree of glucocorticoid resistance observed in the cell line with a point substitution encodes a GR protein, which we have shown to be fully functional in a cotransfection assay; the other allele contains a trinucleotide insertion but retains $\sim$50% of wild-type activity, with no evidence of dominant negative activity. Mutations in the coding region of the GR have been linked to some cases of familial glucocorticoid resistance (16, 17). However, in one kindred, reduced receptor expression as a result of splice site disruption was implicated (12), suggesting that expression of one functional allele is not sufficient for normal signaling. A recent report has shown that the reason for loss of glucocorticoid receptor expression in another human SCLC cell line, DMS 79, stems from aberrant splicing of the primary transcript with retained intron sequence (GR-P). This disrupts the ligand binding domain (14). This transcript has also been reported to arise in glucocorticoid-resistant myeloma (18). Such aberrant splicing cannot underlie resistance in COR L24 cells, because these cells retain ligand binding activity (6) and the GR-P fails to exhibit dominant-negative activity (14).

However, it is possible that the receptor mutations identified here in SCLC do not completely explain the observed glucocorticoid resistance. It seems likely that the multiple factors that determine cell sensitivity to glucocorticoid, including level of receptor expression and other factors such as AP-1 (19–21) and calreticulin (22, 23), contribute with expression of only one fully functional GR allele to cause the severe glucocorticoid-resistant phenotype of COR L24 cells. The relative contributions of each of these factors is difficult to judge, although it is striking that a novel GR mutant with impaired function is found in these resistant cells. It will be important to determine if similar mutations occur in SCLC in vivo.

Glucocorticoids are important for many aspects of tissue develop-
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ment, cellular differentiation, and metabolism. Glucocorticoid receptors are expressed in most solid tumors and in all lymphoid malignancies (24). Disruption of glucocorticoid-induced apoptosis confers clear survival advantage on leukemic cells, and this glucocorticoid resistance is acquired after transformation as a separate “hit,” usually with mutation of the GR (25). Very little is known about the effects of glucocorticoid receptors on solid tumors, but there may be a more subtle effect on cancer cell proliferation, as in the case of alveolar cell carcinoma of the lung (26). It may be that the mutation found in the SCLC cell line COR L24 confers a survival advantage on the cells by making them resistant to the effects of glucocorticoids. This has implications for human SCLC tumorigenesis.

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