A Patient with Adrenocortical Carcinoma: Characterization of Its Biological Activity and Drug Resistance Profile

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ABSTRACT

We describe a patient with a metastasized adrenocortical cancer who exhibited excessive production of both glucocorticoids and mineralocorticoids combined with suppressed androgen production. Unusual steroid metabolites found in the patient’s urine have not been described previously in association with this tumor type. Investigation of the multidrug resistance phenotype in single-cell suspensions of the tumor revealed low expression of multidrug resistance protein but high expression of P-glycoprotein (Pgp) and lung resistance-related protein. Functional Pgp in these tumor cells was shown by the modulatory effect of PSC833 on daunorubicin accumulation. Mitotane, at a concentration achieved in this patient’s plasma, completely reversed the Pgp-related resistance both in the Pgp-overexpressing KB8–5 cell line and in the patient’s tumor cells. On the basis of these in vitro results, the patient was treated with a combination of multidrug resistance drugs (doxorubicin, vincristine, and etoposide) plus mitotane as a Pgp modulator. This treatment was ineffective, however. A chemosensitivity assay demonstrated that the tumor cells were highly resistant to the drugs used. The adrenocortical cancer cells expressed mutant p53, and no evidence for induction of apoptosis by these drugs was found.

INTRODUCTION

ACC is a rare neoplasm with an annual incidence of 1.5 to 2 per million people (1–3). About half of adrenal carcinomas are associated with hypercortisolism (Cushing’s syndrome) with or without hyperandrogenism. Hyperaldosteronism is extremely rare (4, 5). Because the majority of these tumors are deficient in one or more enzymes involved in normal steroidogenesis, aberrant production of various precursor steroids is typical. Analysis of the steroid profile can be helpful in elucidating the pathophysiology of these tumors as well as in explaining the patient’s symptoms.

For ACC, the overall mortality rate after 5 years is 75–90%, and the mean survival duration is 14.5 months. The only potentially curative treatment is complete surgical excision, but most patients present with metastatic disease, which is associated with a very poor prognosis. The rarity of the tumor makes it difficult to perform controlled studies to assess the impact of any therapy on survival. Mitotane [1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2-dichloroethanone] may be successful in reducing hypercortisolism but has limited antitumor efficacy, yielding mostly partial and short-lived responses in 30–60% of patients (6–8). Chemotherapy for metastatic ACC is likewise ineffective (9, 10), possibly because of MDR (11).

MDR, either intrinsic or acquired, has been defined as cross-resistance to structurally and functionally unrelated natural drugs. One type of MDR is caused by the overexpression of plasma membrane drug transporters, resulting in lower intracellular drug concentrations (12, 13). To date, two different plasma membrane drug transporters have been cloned, namely the MDRI-encoded Pgp (14) and the MRP (15). Recently, a novel MDR-associated protein, the LRP, has been characterized as a major vault protein that is localized in the cytoplasm and is overexpressed in MDR cell lines of different origins (16).

Another type of resistance is caused by the inability of cells to undergo apoptotic death. Certain genes involved in oncogenic transformation, such as p53 and bcl-2, appear to modulate apoptosis. p53 is a tumor-suppressor gene, and loss of p53 function is associated with carcinogenesis and drug resistance (17). Functional inactivation may be due to mutations of p53, as is the case in many human malignancies. Overexpression of bcl-2 may prevent drug-induced cell death and thereby cause a broad spectrum of drug resistance. The survival-promoting activity of bcl-2 is opposed by bax, a protein that forms heterodimers with bcl-2 and accelerates cell death. The ratio of bcl-2:bax is an important determinant of cell survival or death following an apoptotic stimulus (18).

In this report, we describe a patient with a metastasized ACC. The urinary steroid profile was analyzed by gas chromatography-mass spectrometry. In addition, the MDR phenotype of the tumor cells was characterized in an attempt to optimize the treatment of this patient. On the basis of these studies, we treated the patient with a combination of various MDR drugs together with mitotane. Finally, the expression of p53, bcl-2 and bax, all involved in apoptosis, were studied in ACC cells.
MATERIALS AND METHODS

Case Report

A 42-year-old man was referred to our hospital because of hypertension, hypokalemia, and hyperglycemia. His blood pressure was 180/120 mm Hg, and he exhibited a classic cushingoid appearance. Serum sodium was normal, but the serum potassium was 1.8 mm, and urinary potassium was 150-200 mm, indicating renal potassium loss. The patient had metabolic alkalosis (pH 7.53; HCO3- 39.9 mm) with a urinary pH of 5.0. Serum cortisol was persistently elevated (±800 nm) with no diurnal pattern. Free cortisol in the urine ranged from 1500 to 5300 nmol/24 h (normal, <270). Serum adrenocorticotropic was below the detection limit. Serum aldosterone was 3.4 nm (normal, <0.35) with a renin activity of 670 fmol/liter/s (normal, 300-1200). Serum testosterone was 5.7 nm (normal, >8.0), with normal prolactin, luteinizing hormone, and follicle-stimulating hormone levels. Serum DHEAS was <0.01 nm (normal, 5.2-8.7). Serum glucose varied from 8 to 14 mm.

Abdominal computed tomographic scanning showed a 12-cm tumor in the left adrenal region with multiple liver metastases and destruction of the first two lumbar vertebral bodies. The findings of left ventricular hypertrophy, grade III retinopathy, and proteinuria (3-5 g/day) suggested malignant hypertension of long duration. Hypertension was treated with high doses of nifedipine, labetolol, and spironolactone but failed to normalize blood pressure. The patient needed up to 120 mmol of oral potassium daily to maintain serum potassium in the normal range. A liver biopsy confirmed the diagnosis of a metastasized ACC.

Subsequently, the primary tumor was removed and part of the tumor was dissociated enzymatically according to a standard procedure (19). The patient was then treated with mitotane in increasing dosages up to 16 g/day. Serum mitotane concentrations of 20-33 mg/liter, considered to be therapeutically adequate (20), were achieved. This therapy normalized the urinary cortisol concentration and allowed withdrawal of potassium supplementation but failed to induce tumor regression. In view of the high expression of Pgp in the tumor cells and the ability of mitotane to inhibit Pgp and increase accumulation of MDR drugs (21), therapy was subsequently continued with the MDR drugs DOX (10 mg/m²/day), VCR (0.4 mg/m²/day), and VP-16 (75 mg/m²/day) given as a 96-h continuous infusion every 3 weeks in combination with mitotane. This therapy resulted in stable disease for several months. After 6 cycles, treatment was discontinued because of progressive disease. The patient died a few months later.

Urinary Steroid Analysis

Urinary steroid profiling was performed as described previously (22). Aldosterone and tetrahydroaldosterone were measured by gas chromatography-mass spectrometry using a Hewlett-Packard 5890 gas chromatography apparatus equipped with a CP Sil 5 CB capillary column (25 cm × 0.25 mm) coupled to a VG 70-250 S mass spectrometer (VG Instruments, Manchester, England).

Cells

A single-cell suspension of the ACC was made by dissociation with 0.14% collagenase and 0.02% DNase (19). After centrifugation on Ficoll-Hypaque, 70% of the cells were viable. Cells were maintained in HITES (RPMI 1640 supplemented with hydrocortisone, insulin, transferrin, 17β-estradiol and sodium selenite) + 10% FCS (23) but failed to proliferate in vitro, and no cell line could be derived from these cells. For functional assays, cells were recovered for 2 days, because the modulatory effects on drug accumulation are greater after recovery of these dissociated tumor cells.

The human epidermoid carcinoma Pgp-overexpressing MDR subline KB8-5 [obtained from Dr. I. Roninson (Department of Genetics, University of Illinois)] with its parental cell line KB3-1 (obtained from American Type Culture Collection, Rockville, MD) and the human small cell lung cancer cell line GLC4 with the MRP- and LRP-overexpressing cell line GLC4/ADR (24) were used as controls.

Drug Resistance Proteins and Functional MDR Assays

The proteins Pgp and MRP were detected by fluorescence-activated cell sorting analysis, and LRP was stained by immunocytochemistry as described previously (25, 26).

Cellular DNR accumulation for measuring Pgp function and calcein efflux for measuring MRP function were performed as described (19, 27).

Chemosensitivity Assays

Chemosensitivity experiments were performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (28). Fifty thousand cells/well were exposed to DOX, VCR, VP-16, and mitotane or to the combination of these drugs. The effects of 2 μM PSC833 on DOX, VCR, and VP-16 chemosensitivity were measured as well. Results were expressed as a percentage of control cells.

Apoptosis

Western Blot of bcl-2, bax, and p53. A mouse antihuman monoclonal antibody (ITK Diagnostics) was used for detection of bcl-2, a rabbit antihuman polyclonal antibody (Santa Cruz Biotechnology) for bax, and a mouse antihuman monoclonal antibody (Oncogene Science) for p53. A mouse antihuman monoclonal antibody against actin (Boehringer-Mannheim) was used as a control for protein loading. Expression of bcl-2, bax, and p53 was measured after culturing ACC cells for 72 h without drugs or in the presence of combinations of DOX, VCR, VP-16, and mitotane, respectively, at two concentrations: (a) 50 nm, 5 nm, 500 nm, and 10 mg/liter or (b) 5 μM, 0.5 μM, 50 μM, and 10 mg/liter.

TUNEL Assay. After a 72-h exposure of the tumor cells to LC50 concentrations of DOX, VCR, VP-16, or mitotane alone or in combination, the presence of apoptotic bodies was investigated on cytospin preparations using the fluorescent in situ DNA end-labeling TUNEL assay.

Effect of Pgp Modulators on Cortisol Excretion by ACC Cells in Vitro

The release of cortisol was determined by plating 10⁵ cells in 200 μl of HITES (without steroids) with 10% FCS at 37°C in...
round-bottomed wells. Cells were incubated with either 2 μM PSC833, 4 μM cyclosporin A, 8 μM bepridil, 8 μM verapamil, or control medium.

RESULTS

Urinary Steroid Profile
The urinary steroid excretion pattern was highly abnormal, both qualitatively and quantitatively (Table 1). A number of steroids were present that are undetectable in normal urine. These included metabolites of 11β-hydroxyprogesterone; metabolites containing a βa-hydroxyl group, which normally appear in the urine of newborns (29); a metabolite of 21-deoxycortisol, which is found in cases of 21-hydroxylase deficiency (30); and unusual metabolites of corticosterone. These metabolites can be found only when the corticosterone production is excessive, as in case of 17α-hydroxylase deficiency. The tumor cortisol, which is found in cases of 21-hydroxylase deficiency, was present in these tumor cells.

PSC833. The accumulation of DNR could be increased by blocking Pgp with 2 μM PSC833, a concentration that with acceptable toxicity can be achieved in patients (Table 3).

Modulation of Pgp Function by Mitotane. As shown in Table 3, 30 mg/liter mitotane, a concentration reached in the patient’s plasma, completely inhibited Pgp-mediated DNR transport both in the ACC cells and in Pgp-overexpressing MDR KB8-5 cells. The absence of any effect on DNR accumulation in the chemosensitive cell line KB3-1 indicated that mitotane is a direct modulator of Pgp-mediated transport. Mitotane seemed to be an even more effective Pgp modulator than PSC833 in KB8-5 cells as well as in ACC cells.

MRP Function. MRP-specific efflux of calcein was studied with and without 100 μM VCR as a modulator (27). The efflux of calcein by ACC cells was not inhibited by VCR (data not shown), indicating that no or little functional MRP was present in these tumor cells.

Cortisol Production and Pgp Modulators in Vitro
After incubation of the ACC cells for 2 days, 1.7 nmol cortisol/10⁶ cells was released into the medium, which was not affected significantly by Pgp modulators (data not shown).

Chemosensitivity Assays
The ACC cells were highly drug resistant (Fig. 1). The concentration at which 50% of the cells died (LC₅₀) was 150 μM for DOX, 350 μM for VCR, 200 μM for VP-16, and 15 mg/liter for mitotane. By comparison, in childhood acute lymphoblastic leukemia, which is highly responsive to chemotherapy, the LC₅₀ values of DOX (0.6 μM), VCR (0.8 μM), and VP-16 (3.7 μM; Ref. 31) are all much lower than the corresponding LC₅₀ values in this patient’s ACC cells. The combination of 50 nm DOX, 5 nm VCR, 500 nm VP-16, and 10 mg/liter mitotane resulted in 25% cell death, whereas the use of 5 μM DOX, 0.5 μM VCR, 50 μM VP-16, and 10 mg/liter mitotane induced 60% cell death. These concentrations are much higher than those achieved by continuous infusion of 10 mg/m²/day DOX, 2 mg/day VCR, and 75 mg/m²/day VP-16, which correspond to steady-state plasma levels of 20 nm DOX (32), 1–5 nm VCR (33), and 3–8 μM VP-16 (34). ACC cells, in contrast to Pgp-expressing KB8-5 cells, did not become more sensitive after coincubation of these drugs with 2 μM PSC833.
Table 3 Effect of PSC833 and mitotane on DNR accumulation

<table>
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<th>KB3-1 Percentage</th>
<th>KB8-5 Percentage</th>
<th>ACC Percentage</th>
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<td>60 mg/liter mitotane</td>
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<td>218</td>
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*Data represent means of the experiments performed in triplicate on fresh ACC cells. Cells were incubated for 60 min at 37°C with 0.5 μM [3H]DNR with and without a modulator. The effect of modulator is expressed as the ratio of [3H]DNR accumulation with a modulator: accumulation of [3H]DNR in control cells.*

Markers of Apoptosis

Western Blot. Fig. 2 shows the presence of p53 protein in tumor cells, indicative of mutant p53. Expression of bcl-2 was low compared to that in the breast cancer cell line MCF-7. bax levels were high and comparable to those seen in MCF-7 cells. After a 72-h exposure to low and high concentrations of the combination of DOX, VCR, VP-16, and mitotane, the expression of both bcl-2 and bax declined, resulting in an unchanged bcl-2:bax ratio.

TUNEL Assay. No apoptotic bodies could be detected using the TUNEL assay on cytospins of ACC cells treated with LCso concentrations of the above-mentioned drugs. At the LCso of VP-16, all cells showed DNA damage, detected by staining of the nucleus, although no signs of apoptosis were observed.

DISCUSSION

This patient produced glucocorticoids and mineralocorticoids in large excess. Some urinary steroid metabolites have never been described previously in association with an ACC. Their presence reflected both excessive corticosterone production and abnormal enzymatic pathways in tumor tissue. Although plasma total cortisol levels were only moderately elevated, the urinary free cortisol concentration was very high, which is explained by the limited capacity of cortisol-binding globulin to bind cortisol in the circulation. Adrenal tumors often secrete large amounts of androgens, but in this patient the excretion of DHEAS, androstenedione, and etiocholanolone was decreased. Clearly, the production of androgens by the normal adrenal was low because of the complete suppression of adrenocorticotropic production by tumor products. Primary hyperaldosteronism is often caused by adrenal adenomas or bilateral adrenal hyperplasia and is rarely observed in cases of adrenal carcinoma (4, 5). The combination of hypercortisolism and hyperaldosteronism due to an adrenal carcinoma is unique and created major problems for the patient, notably hypertension and renal potassium loss. Multiple mechanisms were certainly responsible for this patient's hypertension (35, 36), which could neither be controlled by high doses of antihypertensive agents with different targets nor ameliorated by removal of the primary tumor. Competition at the aldosterone receptor by tumor products accounted for the inability of high-dose spironolactone to reduce renal potassium loss.

The ACC cells produced cortisol in vitro, and because cortisol is transported by Pgp (37), the effect of Pgp modulators on the release of cortisol was investigated. The absence of an effect of Pgp modulators in our experimental setup might be attributable to the rapid diffusion of this lipophilic compound across the cell membrane. Another possibility is that the inhibitory effect of Pgp modulation was masked by excessive tumor production of cortisol.

Initially, we treated the patient with mitotane, which was effective in normalizing cortisol production but failed to induce tumor regression. To come to a more effective treatment, we characterized the MDR phenotype of the dissociated ACC cells. The cells expressed low levels of MRP but high levels of Pgp and LRP. This is in contrast to normal adrenal cells, which show high expression of all three MDR associated proteins (26, 38, 39). Our studies of functional drug transport showed that Pgp-mediated DNR transport was inhibited by PSC833 and even more effectively by mitotane. These findings pointed to mitotane as a good candidate for MDR reversal in vivo.

On the basis of these experiments, we decided to treat the patient with the MDR drugs DOX, VCR, and VP-16 together...
with mitotane, according to a study by Dr. Fojo3 at the National Cancer Institute. Unfortunately, after a few months of stable disease, the tumor became progressive after six cycles. At a later moment, we investigated the toxicity of these drugs toward ACC cells in vitro. The tumor cells were highly resistant to these drugs, even at much higher concentrations than reached in the patient. The addition of PSC833 had no effect on LC50. A possible explanation is that the PSC833 concentration might have been too low to compete with the LC50 drug concentrations for Pgp-binding sites. However, the LC50 concentration of mitotane (15 mg/liter) was achieved in the patient's plasma. These in vitro results suggest that mechanisms other than Pgp-mediated transport might have been responsible for the resistance of the ACC cells to chemotherapy.

Given the high expression of LRP in the tumor cells, it is tempting to speculate that LRP may have been implicated in the resistance of these cells to chemotherapy. Although the role of LRP in drug resistance is not yet clear, one study of patients with ovarian cancer (40) and another study of patients with leukemia (41) demonstrated correlations between LRP expression and the response to treatment and survival. The current unavailability of a functional assay for LRP prevented us from investigating the function of this protein in the single tumor cell suspension.

Most chemotherapeutic agents kill cells by inducing programmed cell death, but we found no apoptotic bodies after treating the ACC cells with LC50 concentrations of DOX, VCR, VP-16, and mitotane alone or in combination. The ACC cells showed mutated p53, which has been associated with drug resistance. Notably, the bcl-2:bax ratio was low as compared to the ratio in the chemosensitive leukemic cell line HL60 and that in breast cancer MCF-7 cells. A low bcl-2:bax ratio is favorable for apoptotic cell death. However, after drug exposure, no increase of bax was observed. Both bcl-2 and bax declined, resulting in an unchanged ratio. There is a reciprocity of bcl-2 and p53 expression in human colorectal adenomas and carcinomas (42), because wild-type p53 and some p53 mutants down-regulate bcl-2 by binding to the bcl-2 promoter (43). In light of these reports, it is noteworthy that this tumor showed clear expression of mutated p53 and a low level of bcl-2. The role of these genes in drug resistance remains to be elucidated.

In conclusion, this patient with ACC exhibited not only a unique pattern of abnormal steroid metabolities but also an over-production of glucocorticoids and mineralocorticoids, which accounted for his cushingoid appearance, hypertension, and disturbed potassium metabolism. Despite promising in vitro results suggesting that mitotane could reverse Pgp-related drug resistance in this patient's tumor cells, combination MDR chemotherapy plus mitotane yielded no significant antitumor effect in vivo. This finding, coupled with the presence of mutant p53 and the failure of high concentrations of MDR drugs to induce apoptosis in vitro, points to the probability of non-Pgp-mediated mechanisms underlying the resistance of the patient's tumor to therapy.

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REFERENCES


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Footnote:
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