Biosynthesis and metabolism of steroid hormones by human adrenal carcinomas

J.W. Brown and L.M. Fishman

Adrenal Research Laboratory, Medical Research Service, VA Medical Center and Division of Endocrinology, Department of Medicine, University of Miami School of Medicine, Miami, FL, USA

Abstract

Over a 15-year period, our university-based laboratory obtained 125 adrenal tumors, of which 15 (12%) were adrenal cortical carcinomas. Of these, 6 (40% of the carcinomas) occurred in patients with clear clinical manifestations of steroid hormone excess. Adrenal cortical carcinoma cells derived from the surgically resected tumors in 4 of these patients were isolated and established in primary culture. Radio tracer steroid interconversion studies were carried out with these cultures and also on mitochondria isolated from homogenized tissues. Large tumors had the lowest steroidogenic activities per weight, whereas small tumors had more moderately depressed enzyme activities relative to cells from normal glands. In incubations with pregnenolone as substrate, 1 mM metyrapone blocked the synthesis of corticosterone and cortisol and also the formation of aldosterone. Metyrapone inhibition was associated with a concomitant increase in the formation of androgens (androstenedione and testosterone) from pregnenolone. Administration of metyrapone in vivo before surgery in one patient resulted in a similar increase in plasma androstenedione, though plasma testosterone levels were not significantly affected. In cultures of two of four tumors examined, dibutyryl cAMP stimulated 11ß-hydroxylase activity modestly; ACTH also had a significant stimulatory effect in one of these tumors. Unlike results obtained with normal or adenomatous adrenal cortical tissues, mitochondria from carcinomatous cells showed a lack of support of either cholesterol side-chain cleavage enzyme complex or steroid 11ß-hydroxylase activity by Krebs cycle intermediates (10 mM isocitrate, succinate or malate). This finding is consistent with the concept that these carcinomas may tend to function predominantly in an anaerobic manner, rather than through the oxidation of Krebs cycle intermediates.

Introduction

Adrenal cortical carcinoma is a rare disease whose course is often fatal within a short time of recognized onset. This malignancy represents approximately 0.02% of all cancers (1,2) and within the general population occurs with a frequency of about two cases per million individuals per year (3). Nearly two-thirds of diagnosed patients are women. There are numerous reports in the literature describing animal models of adre-
nal carcinoma (4-9). Comparatively few biochemical studies, however, have been reported with primary human tumors (10-14), although a substantial literature now exists concerning the clinical presentation, diagnosis and treatment of this disorder (15-19).

Functional tumors often present with manifestations of prolonged glucocorticoid excess (e.g., central obesity, glucose intolerance, abdominal striae, protein wasting and psychiatric disturbance). Occasionally, the malignant nature of this disorder may result in the masking of some symptoms, such as the emaciating effects of a rapidly growing tumor nullifying the tendency to weight gain otherwise seen with glucocorticoid excess. Adrenal carcinoma can also result in presentation with clinical and laboratory evidence of androgen excess, including hirsutism and virilization in women and increased levels of testosterone and related androgens. More rarely, mineralocorticoid excess occurs (2,20), with high aldosterone and hypokalemia (i.e., Conn’s syndrome); two malignancies of this type are included in the current study. Most of the other tumors reported here were nonfunctional (and thus similar to those reported by Shons and Gamble (21) and Terzolo et al. (22)). Two glucocorticoid-producing neoplasms and two androgen-producing adrenal carcinomas are also described in the present study.

Adrenal carcinomas, like adenomas, usually produce glucocorticoid hormones independently of pituitary-adrenal axis ACTH control. This phenomenon results in an absent or blunted ACTH response, although ACTH-responsive tumors have been reported in some animal models (23). To date, there is no definitive biochemical information to localize the site of the defect in ACTH responsiveness; potential mechanisms might include defective ACTH receptors (24), altered ACTH receptor enzyme coupling or adenylate cyclase activity (25,26), or defects in the cAMP cascade (27). Various combinations of these factors could, of course, be involved and might vary in different tumors.

We present in this communication the results of studies of the biosynthesis of steroid hormones in cell cultures from human adrenal carcinomas and of the in vitro modulation of steroidogenesis in these tumor cells by physiologic and pharmacologic agents.

Material and Methods

Isolation and culture of cells

Adrenal carcinomas were obtained from individuals undergoing surgery for adrenal tumors. Subsequent pathologic examination confirmed the diagnosis of malignancy.

Excised tissue was immersed in sterile Dulbecco’s phosphate-buffered saline (PBS), pH 7.4, at 5°C, and then transported on ice to the laboratory, where it was dissected under sterile conditions and sectioned into fragments measuring approximately 0.5 x 0.3 x 0.3 cm. Forty ml of PBS containing 0.3% w/v type IV collagenase was added to these fragments. Tissue was then digested for approximately 20 min using magnetic stirring bars (140-200 rpm) at room temperature (24°C). Free cells and cellular debris were aspirated and transferred with the supernatant to an equal volume of Medium 199 supplemented with 15% v/v dialyzed fetal calf serum (FCS). Fresh collagenase-supplemented PBS (40 ml) was added to tissue residue and reincubated for 20 min under the conditions indicated previously. Dislodged cells were aspirated and added to the supernatant to an equal volume of Medium 199 supplemented with 15% v/v dialyzed fetal calf serum (FCS). Fresh collagenase-supplemented PBS (40 ml) was added to tissue residue and reincubated for 20 min under the conditions indicated previously. Dislodged cells were aspirated and added to the earlier fraction. Cells were then centrifuged in plastic tubes for 60 s at 2,000 rpm in a Damon IEC HN-S centrifuge. Cell pellets from 4 to 6 tubes were pooled and washed with 40 ml of Medium 199 supplemented with 15% FCS. Fresh collagenase-supplemented PBS (40 ml) was added to tissue residue and reincubated for 20 min under the conditions indicated previously. Dislodged cells were aspirated and added to the earlier fraction. Cells were then centrifuged in plastic tubes for 60 s at 2,000 rpm in a Damon IEC HN-S centrifuge. Cell pellets from 4 to 6 tubes were pooled and washed with 40 ml of Medium 199 supplemented with 15% FCS. Cells were re-washed with 40 ml of this solution before final centrifugation and suspension in Medium 199 (supplemented with 15% FCS and 1X Gibco Antibiotic-Antimycotic Mix) to cell densities of 0.2 to 1.0 x 10^6 cells per ml of medium. Three ml of this
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Studies of steroidogenesis in culture

Cells were allowed to equilibrate by incubation for 2 days before assays were initiated. The conversion of tritium-labelled substrates into steroid hormones and intermediates was studied. Tritium-labelled substrates (11-deoxycortisol (DOC) or pregnenolone) were added to 1.0 ml of Medium 199 supplemented with 15% FCS and antibiotics, yielding a solution containing 1.0 µCi per ml ([3H]-DOC specific activity = 41.8 Ci per mmol; [3H]-pregnenolone specific activity = 19.3 Ci per mmol). DOC was used as substrate for 11β-hydroxylase assays rather than 11-deoxycortisol because our studies have shown that the latter can be channeled to a quantitatively significant extent into non-11β-hydroxylase pathways (e.g., androgen synthesis). Furthermore, by using DOC as substrate we were able also to estimate the biosynthetic potential for the formation of aldosterone in these cultures without compromising the analysis of 11β-hydroxylase activity.

Dibutyryl cAMP (1 mM), ACTH (10 nM), and NADPH-generating system (consisting of glucose-6P, glucose-6P-dehydrogenase and NADPH) and metyrapone (1 mM) were each examined for their ability to alter the rate and nature of steroid product formation. Incubations of cells with labelled substrates were terminated after 24-h periods by aspiration of medium and steroid extraction with methylene chloride. Culture dishes were washed once with 1.0 ml of PBS prior to this separation. The supernatant fractions (medium + PBS wash) were vortexed with 10 ml of redistilled methylene chloride and further extracted 3 times with this solvent. Methylene chloride fractions were then pooled and dried under nitrogen. Both radiolabelled substrates and products were found almost exclusively in the aspirated medium; cell monolayers contained negligible quantities of radioactive materials.

Mitochondrial studies of steroidogenesis

Subcellular fractionation techniques to obtain purified mitochondria were carried out on tumor tissue after homogenization. Tissue was coarsely minced and homogenized in a solution containing 0.3 M sucrose using an all-glass homogenizer. This homogenate was centrifuged at 380 g for 20 min at 4°C to precipitate nuclei and residual large cell fragments. Mitochondria were then obtained by centrifugation at 10,000 g for 20 min at 4°C. The resultant pellet was suspended in incubation buffer and assayed for cholesterol side-chain cleavage enzyme complex activity ([14C]-cholesterol conversion into pregnenolone and progesterone; cholesterol specific activity = 59.4 mCi/mm) and steroid 11β-hydroxylase activity ([14C]-DOC conversion into corticosterone; DOC specific activity = 58.5 mCi/mm). Assay incubations were carried out for 60 min at 37°C before termination.

The effects of succinate, isocitrate and malate (each, 10 mM) and of an NADPH-generating system were studied for support of both mitochondrial cholesterol side-chain cleavage enzyme complex and steroid 11β-hydroxylase activities. The NADPH-generating system contained the following agents at indicated concentrations: 3.0 mM glucose-6P, 0.6 mM NADP, and 5.0 units per ml glucose-6P-dehydrogenase.

Purification of steroids

Steroids were purified on silica gel thin layer chromatography plates (Kieselgel 60 F-254) in different combinations of the selected solvent systems described below. Rf values were compared with known standards for product identification. In mitochondrial
studies, [3H]-labelled substrates and products were added and copurified with the fractions being characterized. Recoveries were estimated for each sample (mitochondrial fractionation studies) or average recoveries were determined (cell culture steroid biosynthesis studies) and used in the calculation of product formation. Samples were run 2-3 times in combinations of the following solvent systems: methylene chloride, methanol, water (225:15:1.5, v/v/v); petroleum ether, ethyl ether, acetic acid (48:50:2, v/v/v); benzene, acetone (4:1, v/v), or methylene chloride, ethyl ether (50:20, v/v). These chromatography procedures and the re-crystallization studies of the products isolated are described in detail elsewhere (28-31).

Steroid hormones were eluted from chromatograms by three consecutive extractions with a solution containing methylene chloride and methanol (9:1, v/v). Radioactivity was then quantitated in Liquifluor-toluene scintillation fluid using a Packard Tri-Carb counter. Where appropriate, dual-labelling experiments were employed to determine steroid recovery by analysis of both [3H] and [14C] isotopes.

**Results and Discussion**

**Clinical experience with adrenal tumors**

The data in Table 1 summarize the various types of adrenal tumors studied in our laboratory over a 15-year period. Adrenal cortical tumors (46%) were slightly more common than adrenal medullary tumors (38%). Other types of identified adrenal neoplasms (neurofibrosarcomas, myelolipomas, neurofibroma, neuroblastoma, and cystic lipoma) together accounted for 6%. An additional 10% of adrenal neoplasms were not identified pathologically. Fifteen (26%) of the 58 adrenal cortical tumors were carcinomas; of these 15, 40% (6 tumors) were non-functional, 20% (3 tumors) were unclassified or unknown with regard to function, 13% (2 tumors) caused Cushing’s syndrome,
13% (2 tumors) were associated with Conn’s syndrome, and 13% (2 tumors) caused virilization (Table 2).

Steroid enzyme activity

Table 3 shows a comparison of 11ß-hydroxylase activities measured in cell cultures from four adrenal carcinomas relative to cell cultures from two normal human adrenal glands. Data on the conversion of [3H]-labelled DOC into corticosterone and aldosterone are presented. Rates of aldosterone formation in tumor cells were found to be low. Corticosterone formation by tumor cells was also markedly lower than in normal cells and cells from progressively larger neoplasms yielded consistently lower steroid interconversion. The largest tumor we studied (in excess of 500 g), in fact, had no detectable 11ß-hydroxylase activity by our analyses; using DOC as substrate, we observed neither corticosterone nor aldosterone formation by these cells. The correlation of size and enzyme activity is depicted graphically in Figure 1.

In one carcinoma (tumor weight = 401 g), very low steroid 11ß-hydroxylase activity was demonstrated, as measured by rates for the conversion of DOC into corticosterone, but there was a relatively normal rate of aldosterone biosynthesis on a per cell basis. The finding that aldosterone could be produced from DOC at normal rates in vitro in the presence of an 11ß-hydroxylase defect is not surprising since this enzyme is not typically rate-limiting for aldosterone formation.

Another patient (tumor weight = 144 g) showed clear evidence of mineralocorticoid excess, with elevated plasma aldosterone and 18 OH-DO (Table 4). Cortisol, however, was not elevated and, in studies described below, was found to be normally inhibited by metyrapone. In vitro (Table 3), cells from this tumor produced somewhat decreased amounts of aldosterone on a per cell basis and also showed a depressed biosynthetic capacity for glucocorticoids. In this tumor, 11ß-hydroxylase activity was greatly depressed as judged by conversion of labelled DOC to corticosterone, thus perhaps accounting for the absence of cortisol overproduction even though plasma levels of aldosterone were relatively high. Again, this finding may be accounted for by the fact that 11ß-hydroxylase is not the rate-limiting enzyme for aldosterone formation but is for cortisol biosynthesis.

Plasma DHEA and androstenedione were within normal limits in this individual, but plasma testosterone was markedly elevated. It is probable that this finding could also have been related to the 11ß-hydroxylase defect. The observation of elevated plasma...
11-deoxycortisol levels (shown in Table 4) supports this concept, as do the in vitro cell culture studies with metyrapone described below.

**In vivo metyrapone responses**

One might anticipate that a deficiency in 11ß-hydroxylase activity, either inherent in the neoplastic tissue or induced by pharmacologic inhibition of this enzyme by metyrapone, could result in increased androgen formation since substrate availability for androgen synthesis would be increased as a result of decreased conversion of 11-deoxycortisol into cortisol. When metyrapone was administered to the patient with the aldosterone-producing adrenal carcinoma, serum cortisol and aldosterone levels both declined by 70% or more (Table 4). There was a concurrent 9-fold increase in plasma 11-deoxycortisol and >2-fold increase in plasma androstenedione levels. As described subsequently, in vitro cell culture studies from another tumor showed a similar pattern of enhanced androgen pathway activity during 11ß-hydroxylase inhibition with metyrapone.

**In vitro metyrapone responses**

Figure 2 illustrates changes in the corticosterone-aldosterone biosynthetic pathway after in vitro exposure of adrenal carcinoma cells to 1 mM metyrapone. The rate of DOC accumulation increased markedly, while corticosterone formation from pregnenolone was almost completely suppressed. Of note is the fact that 11ß-hydroxylase inhibition was evidently severe enough in this instance to result in a decrease in the formation of aldosterone. Small increases were observed in progesterone biosynthesis. These findings are all consistent with the well-known inhibitory effects of metyrapone on 11ß-hydroxylase activity and on the utilization of intermediates dependent on this enzyme for further conversion.

The cortisol pathway (Figure 3) showed similar effects of metyrapone, with an inhibition of cortisol biosynthesis and an increase in 17 OH-progesterone accumulation. An unexpected result in this experiment was the finding of a slight (yet statistically not significant) decrease in the rate of 11-deoxycortisol accumulation in the presence of metyrapone. This observation might be explained in part by the rechanneling of steroid synthesis intermediates into the androgen pathway, as previously suggested. As might be expected from this formulation, androstenedione and testosterone biosynthesis...
sis were both increased in the presence of metyrapone (Figure 4).

**In vitro responses to ACTH and cAMP**

The effects of ACTH and dibutyryl cAMP on cell cultures from different tumors were studied to explore the well-documented phenomenon of unresponsiveness of adrenal carcinomas *in vivo* to stimulation by this trophic hormone. The efficacy of an NADPH-generating system was also assessed in order to determine whether steroidogenesis could be stimulated by these co-factors in the absence of responses to ACTH or dibutyryl cAMP. Since tumor cells may have inadequate NADH, and therefore NADPH generation, because of an anaerobic shift away from Krebs cycle metabolism, exogenously generated NADPH was provided to study its effect on the depressed 11ß-hydroxylase activity described earlier. As indicated in Table 5, exogenous NADPH only very modestly enhanced (~2 times) 11ß-hydroxylase activity in two of three tumors examined, so it is unlikely that lack of endogenous NADH/NADPH is the major factor causing decreased 11ß-hydroxylase activity in these tumors.

Three tumors were examined for response to ACTH stimulation, with one demonstrating a modest steroidogenic response (Table 5). Dibutyryl cAMP also stimulated steroid interconversions in cells from this tumor, as well as in cells from one of three other carcinomas tested. Two of the four tumors examined showed no response whatever to either ACTH or dibutyryl cAMP. It is interesting to note that the two unresponsive tumors were the two largest tumors studied (401 and >500 g).

These preliminary observations using primary cell cultures from human adrenal carcinomas suggest that defects occur in both ACTH receptor and/or post-receptor response mechanisms. It is likely that the specific biochemical nature of these defects varies in different tumors.

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**Figure 2** - Conversion of [3H]-labelled pregnenolone (Preg) to progesterone (Prog), 11-deoxycorticosterone (DOC), corticosterone (B) and aldosterone (Aldo) in primary adrenal carcinoma cultures in the absence (C) and presence of 1 mM metyrapone (M). Data are reported as the mean ± SD of 4-6 determinations. Conditions of incubation and assay are described in Material and Methods.

**Figure 3** - Conversion of [3H]-labelled pregnenolone (Preg) to progesterone (Prog), 17-hydroxyprogesterone (17 OH-Prog), 11-deoxycortisol (S) and cortisol (F) in primary adrenal carcinoma cultures in the absence (C) and presence of 1 mM metyrapone (M). Data are reported as the mean ± SD of 4-6 determinations. Conditions of incubation and assay are described in Material and Methods.
In vitro effects of Krebs cycle intermediates on steroidogenesis

Many types of tissue are known to show an increase in anaerobic metabolism after malignant transformation. Therefore, we investigated the effect of Krebs cycle intermediates (i.e., substrates involved in aerobic NADH and NADPH generation) in support of mitochondrial 11ß-hydroxylase activity. Unlike results obtained with mitochondria from normal adrenal cortical tissues, adrenal carcinoma mitochondrial 11ß-hydroxylase and cholesterol side-chain cleavage enzymes both showed a lack of effectiveness of isocitrate, succinate, or malate but were readily stimulated by NADPH (Figure 5). These results are consistent with the hypothesis that these tumors, in common with other malignancies, may shift toward partial anaerobic function and may be deficient in a number of Krebs cycle enzymes found in normal tissues.

Conclusions

Using cell culture and subcellular fractionation techniques, we have examined the steroid biosynthetic activities and related biochemical characteristics of human adrenal cortical carcinomas. Our studies of these primary malignant adrenal neoplasms indicate the following: 1) Not surprisingly, tumors vary greatly in their steroidogenic activity. 2) There is a clear trend for small tumors to have higher 11ß-hydroxylase activities per cell than large tumors. 3) In vitro responses to ACTH and cAMP are uniformly low and usually absent in large tumors. 4) Metyrapone inhibits 11ß-hydroxylase activity in vitro in adrenal carcinomas, as it does in normal adrenal tissue; a concomitant modest increase in androgen formation often occurs. 5) Steroidogenesis in adrenal carcinoma tissue is not readily supported by Krebs cycle intermediates, which typically are associated with enhancement of this activity in
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Preliminary data suggest that a minor role in the deficiency in 11ß-hydroxylase activity may be played by defective NADPH generation. It is more likely, however, that the process of neoplastic dedifferentiation results in deficient transcription of steroidogenic genes and in decreases in levels of steroidogenic enzymes (e.g., 11ß-hydroxylase). Immunocytochemical and cDNA hybridization studies may shed further light on this matter. Further studies are needed to elucidate more specific mechanisms for the deficiency in 11ß-hydroxylase activity observed and for the loss of ACTH responsiveness.

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References


