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EXTRAADRENAL EXPRESSION OF STEROID 21-HYDROXYLASE AND 11β-HYDROXYLASE BY A BENIGN TESTICULAR LEYDIG CELL TUMOR

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Summary—We present an unusual case with bilateral testicular Leydig cell tumors displaying extraadrenal expression of steroid 21-hydroxylase and 11β-hydroxylase. Histological examination of a 38-yr-old man infertile due to azoospermia showed him to have bilateral testicular Leydig cell tumors. The *in vitro* steroidogenic potential of the tumors and their adjacent testicular tissue was evaluated using organ culture. Tumor tissue was found to secrete deoxycorticosterone (DOC), corticosterone (B) and cortisol, which are not produced in normal adult testis, into the medium, while testicular tissue adjacent to the tumors secreted a small amount of DOC and B. Northern blot analysis with cytochrome $P\text{-}450_{C21}$ complementary DNA (cDNA) and $P\text{-}450_{11\beta}$ cDNA as probes revealed that the tumor contained a considerable amount of mRNA for $P\text{-}450_{C21}$ and $P\text{-}450_{11\beta}$, while the mRNAs were not detected in the testicular tissues adjacent to the tumors. It is suggested that the high local levels of estrogen and/or progesterone within the Leydig cell tumors and their adjacent testicular tissues induced extraadrenal expression of steroid 21-hydroxylase and 11β-hydroxylase by the tumors and their adjacent testicular tissues.

INTRODUCTION

Extraadrenal steroid 21-hydroxylation, which converts progesterone (P) into deoxycorticosterone (DOC) or DOC sulfate, occurs in a variety of human tissues. Formerly, it was thought that a single specific form of cytochrome *P*-450, termed $P\text{-}450_{C21}$, mediated steroid 21-hydroxylation. However, recently Mellon and Miller [1] examined RNAs from a variety of human tissues by means of solution hybridization and nuclease protection, and demonstrated that one or more enzymes other than $P\text{-}450_{C21}$ might be responsible for human extraadrenal 21-hydroxylation. Therefore, the nature of the enzymes mediating extraadrenal 21-hydroxylation and the presence or absence of $P\text{-}450_{C21}$ mRNA in extraadrenal human tissues have become unclear.

Recently, we studied a case of bilateral testicular Leydig cell tumors that produced DOC, corticosterone (B) and cortisol (F) *in vitro*. In the present study, we show the presence of $P\text{-}450_{C21}$ and $P\text{-}450_{11\beta}$ in the Leydig cell tumors, as well as the presence of steroid 21-hydroxylase activity in the testicular tissues adjacent to the Leydig cell tumors.

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EXPERIMENTAL

Materials

Because a case report of this patient, describing mainly the clinical and pathological findings, has been already submitted to another journal [2], only a brief outline of the case will be given here. A 38-yr-old man with a complaint of infertility visited our clinic, where bilateral testicular tumors were found and subsequently resected. The histological diagnosis based upon microscopic and ultramicroscopic studies was benign Leydig cell tumors. Histological findings of testicular tissues adjacent to the tumors showed spermatogenic arrest. Endocrinological characteristics of both the Leydig cell tumors and their adjacent testicular tissues were examined by using a part of each tissue for organ culture and the rest for RNA extraction.

Specific chemicals

Human $P\text{-}450_{C21}$ complementary DNA (cDNA) [3] were kindly provided by Dr P. C.

White of Cornell University Medical Center, New York, U.S.A. Rat $P\text{-}450_{11\beta}$ cDNA [4] were a gift from Dr M. Okamoto of Osaka University Medical School, Osaka, Japan. [^{32}P]deoxyCTP (>6000 Ci/mol) were purchased from New England Nuclear Corp., Boston, Mass. U.S.A. Other chemicals were of the highest grade available.

Organ culture

Organ culture was performed according to the method previously described [5]. Briefly, a part of the tissues was washed with Ham's F-10 medium and then cut into small fragments (about 1 mm^3) which were arranged on a wire mesh grid covered with a Millipore filter (Millipore Corp., Bedford, Mass., U.S.A.) in a Falcon 3001 plastic dish (Becton Dickinson and Co., Oxnard, Calif., U.S.A.). The culture medium consisted of Ham's F-12 with 10% fetal calf serum. The tissue fragments were cultured at 33°C in a humidified atmosphere of 5% CO_2 and 95% air. 24 h after the culture, the media were collected and stored at −20°C until hormonal assays.

Hormonal assays

Assays were performed on the stored preoperative serum and cultured media. Testosterone (T), estradiol (E_2) and P were assayed with a double antibody radioimmunoassay kit (Shionogi Laboratory Inc., Osaka, Japan). 17α-Hydroxyprogesterone, androstenedione, DOC, B and F were also assayed with a double antibody radioimmunoassay kit (Shionogi Laboratory Inc.) following celite column chromatography. Cross-reactivity between each steroid was 1%.

RNA analysis

Parts of the Leydig cell tumors and their adjacent testicular tissues were frozen in liquid nitrogen after resection and stored at −80°C until the RNA preparation. RNA was prepared with the LiCl precipitation procedure [6]. RNA gel electrophoresis was then performed as previously described [7], with 50 μg of total RNA being applied to each lane. The RNAs were transferred to a Zetabind nylon membrane (Xydex Co., Bedford, Mass., U.S.A.) with the aid of VACUGENE (LKB, Uppsala, Sweden). RNA blots were serially probed with ^{32}P-labeled cDNA probes for human $P\text{-}450_{C21}$ and rat $P\text{-}450_{11\beta}$. Hybridization and washing were performed at high stringency condition. Before a blot was reused for another autoradiography, the radioactive probe was washed off until the blot showed no residual radioactivity.

RESULTS

Steroidogenesis in organ culture

The blank medium was negative for all steroids tested. Results of the steroid assays on the preoperative serum and the cultured media of the Leydig cell tumors and their adjacent testicular tissues are summarized in Table 1. As can be seen, high levels of serum P, 17α-hydroxyprogesterone, androstenedione and E_2 may be the results of production of those steroids by the Leydig cell tumors. Serum levels of androstenedione and E_2 returned to normal after the operation (postoperative serum levels of P and 17α-hydroxyprogesterone have not yet been measured, data not shown). The pattern of steroid release *in vitro* was different between the Leydig cell tumors and their adjacent testicular tissues, except for the E_2 level. Specifically, P, 17α-hydroxyprogesterone and androstenedione release from the Leydig cell tumors was greater than that from their adjacent testicular tissues. DOC, B and F were released from the Leydig cell tumors, but only DOC and B from their

Table 1. Hormonal concentrations in the preoperative serum and in the cultured media

	Preoperative serum		Cultured media	
			Leydig tumors	Adjacent testis
		(normal range)		
Progesterone (ng/ml)	9.1	0.16–0.42	13.0	3.8
17α-Hydroxyprogesterone (ng/ml)	140.0	0.05–3.70	55.0	4.2
Androstenedione (ng/ml)	15.0	0.6–2.5	17.5	1.0
Testosterone (ng/ml)	8.2	3.0–8.2	7.7	21.0
Estradiol (pg/ml)	110.0	10–60	110.0	160.0
Deoxycorticosterone (ng/ml)	0.3	<0.13	0.35	0.09
Corticosterone (ng/ml)	4.0	0.31–8.30	4.2	0.25
Cortisol (ng/ml)	NE	2.7–18.3	1.8	ND

NE: not examined; ND: not detected.
The blood sample was taken in the morning of the operation and the cultured media were collected 24 h after the organ culture. The blank medium was negative for all steroids tested.

adjacent testicular tissues. These steroids were not released from normal testicular tissues in another organ culture (data not shown). The slightly high level of serum DOC may be explained by its production by the Leydig cell tumors and their adjacent testicular tissues.

Steroidogenic enzymes

To confirm the unusual production of DOC, B and F by the Leydig cell tumors and their adjacent testicular tissues, we prepared total RNAs extracted from the Leydig cell tumors and their adjacent testicular tissues, from normal Leydig cell fractions obtained from a prostate cancer patient and from normal human adrenal obtained from a renal cell cancer patient. Northern blots of these RNAs were then probed with cloned cDNAs for $P\text{-}450_{C21}$ and $P\text{-}450_{11\beta}$. Figures 1 and 2 show a transfer blot of equal amounts of total cellular RNA from a normal adult Leydig cell, and from the Leydig cell tumors and their adjacent testicular tissues sequentially probed with cDNAs for human $P\text{-}450_{C21}$ and $P\text{-}450_{11\beta}$. When the blot was probed with human $P\text{-}450_{C21}$, small amounts of $P\text{-}450_{C21}$ mRNA were found in the Leydig cell tumors. No $P\text{-}450_{C21}$ mRNA was detected in the adjacent testicular tissues nor in the adult normal Leydig cells. When the blot was probed with rat $P\text{-}450_{11\beta}$, the amount of $P\text{-}450_{11\beta}$ mRNA in the Leydig cell tumors was roughly equivalent to that in the adrenal. No $P\text{-}450_{11\beta}$ mRNA was detected in the other tissues.

DISCUSSION

In the present study, we described an unusual case of Leydig cell tumors which displayed both $P\text{-}450_{C21}$ and $P\text{-}450_{11\beta}$ activities. At first, we examined steroids released from the Leydig cell tumors and their adjacent testicular tissues using the organ culture technique. Viability of the cultured tissues was confirmed by the responsiveness of the tissues to human chorionic gonadotropin stimulation using small amounts of the tissues (data not shown). We previously used this same method to study hormonal regulation of human testis [5]. DOC production from Leydig cell tumors has been reported in

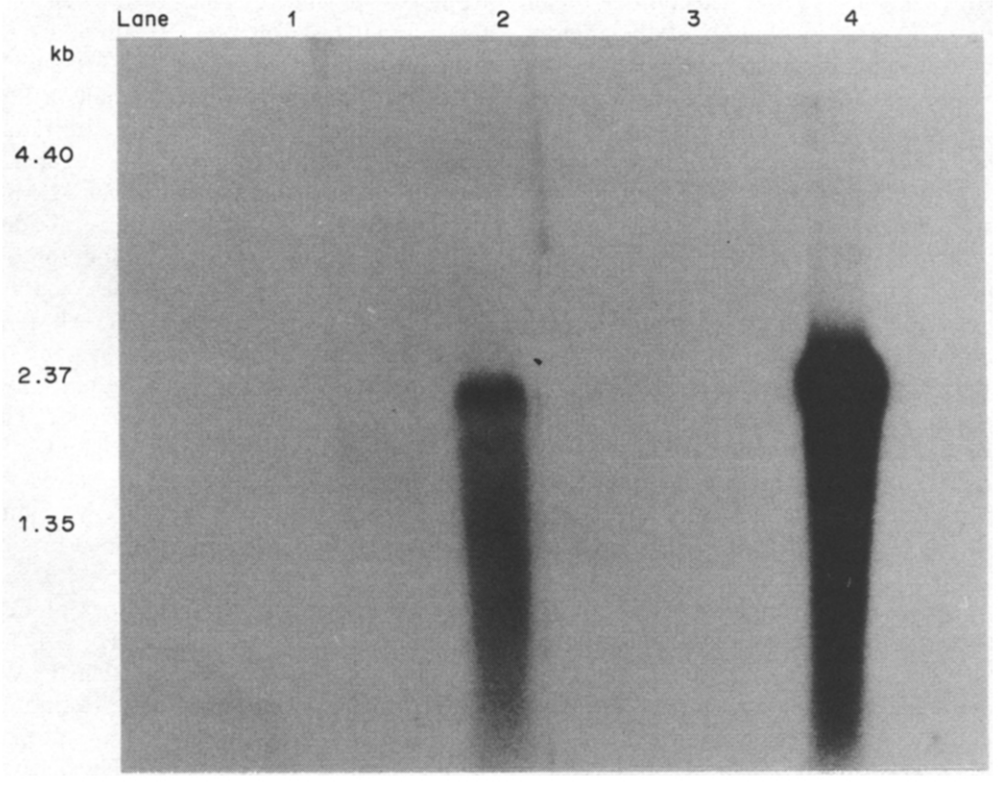

Fig. 1. Northern blot of equal amounts (50 μg) of total cellular RNA from purified normal adult Leydig cells (lane 1), from Leydig cell tumors (lane 2) and their adjacent testicular tissues (lane 3), and from a normal adult adrenal (lane 4), probed with a ^{32}P-labeled cDNA for human $P\text{-}450_{C21}$. A 2.2-kb hybridizing species was detected in RNAs from Leydig cell tumors as well as a normal adult adrenal, but not in RNAs from the other tissues.

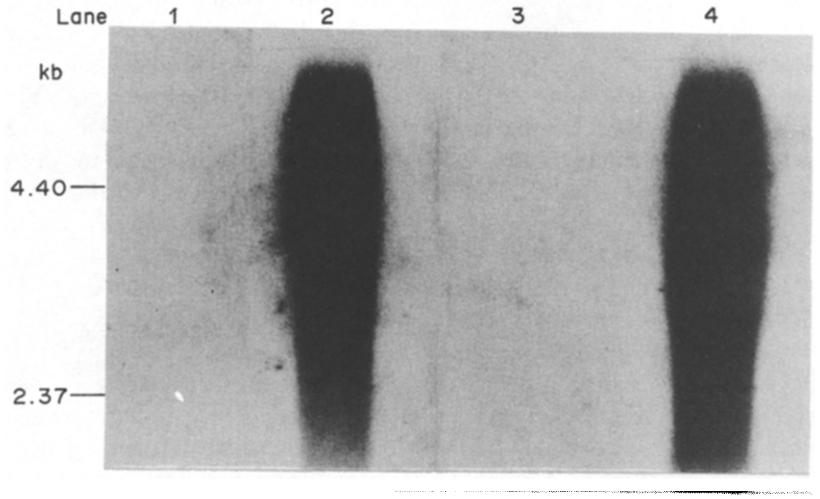

Fig. 2. Northern blot of equal amounts (50 μg) of total cellular RNA from purified normal adult Leydig cells (lane 1), from Leydig cell tumors (lane 2) and their adjacent testicular tissues (lane 3), and from a normal adult adrenal (lane 4), probed with a ^{32}P-labeled cDNA for rat P-450$_{11\beta}$. A positive band was detected in RNAs from Leydig cell tumors as well as a normal adult adrenal, but not in RNAs from the other tissues.

only one case [8], while there are two more reports which indirectly describe the presence of steroid 21-hydroxylase activity in Leydig cell neoplasms [9, 10]. The latter reports also indirectly demonstrated the presence of 11β-hydroxylase activity by the detection of small amounts of F after incubation of the tumors with radio-labeled precursor steroids. In our case, we directly showed, by using organ culture technique, that DOC, B and F were released from the Leydig cell tumors. We found that the adjacent testicular tissues of the tumors released both DOC and B. Histological changes in this part of the testis, which showed hypospermatic change, might have been induced by high concentrations of E_2 and P affected by the Leydig cell tumors. This hormonal environment may also induce the testis to produce DOC and B. Mellon and Miller [1] recently suspected the existence of enzymes other than P-450$_{C21}$ in fetal tissues having extraadrenal steroid 21-hydroxylase activity. In our case, the reason why P-450$_{C21}$ was not detected in the adjacent tissues of the Leydig cell tumors could not be clarified, because, unfortunately, we could not use such a highly sensitive method as Mellon and Miller [1] did. Of course, the tissues may have contained a small amount of P-450$_{C21}$ which could not be detected by Northern blot analysis. However, DOC, B and F produced by the Leydig cell tumors were clearly responsible for P-450$_{C21}$ and P-450$_{11\beta}$. This is the second report on Leydig cell tumors with P-450$_{C21}$ activity [11], while there has been no report on Leydig cell tumors with P-450$_{11\beta}$ activity. We should address the question of whether this tumor is truly a Leydig cell tumor or an adrenal rest tumor. Because if the tumor originates from an adrenal cell, then the endocrinological results are not surprising. In fact, Leydig cell tumors and adrenal rest cell tumors cannot be easily distinguished histologically. In this case, the tumor was confirmed to be originally a Leydig cell by microscopic and electron ultramicroscopic studies [2]. Moreover, the preoperative value for plasma ACTH was normal, 32.6 pg/ml (normal: < 60 pg/ml), and the bilateral adrenal glands detected by computed tomography were normal in size and shape. These data are not consistent with adrenal rest cell tumors which frequently occur in male patients with insufficiently treated congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Therefore, we judged that this tumor originated from a Leydig cell. Why this case displayed these activities, whether it was induced by abnormal hormonal environment or developed originally in the tumor, remained unclear. Most other reports on hormonal characteristics of Leydig cell tumors have shown high levels of E_2 [12–14], and have not mentioned steroid 21-hydroxylase or 11β-hydroxylase activities. Taking into consideration that the adjacent testicular tissues of the tumors displayed these activities, it may be reasonable to assume that the same activities shown in the Leydig cell tumors were also induced by the hormonal environment [15, 16].

Finally, it should be of interest that steroid 21-hydroxylase and 11β-hydroxylase activities could be induced in some hormonal environments, high in E_2 and/or P, in adult testicular tissue.

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