Leydig Cell Tumor of the Testis: Analysis of Testosterone Production and Secretion by Three-Dimensional Histoculture

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Abstract. We treated an 11-year-old boy with a testicular Leydig cell tumor. We analyzed the testosterone production of this tumor by immunolocalization of steroidogenic enzymes and in vitro three-dimensional histoculture. Spermatic venous blood from the tumor bearing testis had noticeably high concentrations of testosterone and androstenedione. The tumor had the characteristic ultrastructural features of steroid producing cells and was immunoreactive for P450scc (side chain cleavage), 3βHSD (hydroxysteroid dehydrogenase) and P450c17 (17α-hydroxylase). Three-dimensional collagen-gel-supported histoculture demonstrated that the tumor tissue in the culture maintained its histologic architecture, expression of steroidogenic enzymes, and secretion of testosterone into the medium for up to 7 days in culture. Histoculture preserved in vitro testosterone production in this case of testicular Leydig cell tumor.

Key words: Testis, Leydig cell tumor, Histoculture, Testosterone, Immunohistochemistry

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LEYDIG cell tumors of the testis are rare but the majority are associated with various steroid biosynthetic abnormalities including testosterone and estrogen overproduction [1-5]. Clinical hormonal analysis of testicular Leydig cell tumor has been extensively performed [1-5], but no detailed analysis of steroidogenesis of a Leydig cell tumor itself including in vitro studies has been reported.

We had the opportunity to examine an 11-year-old boy with a Leydig cell tumor of the testis with increased plasma androstenedione and testosterone concentrations and pseudoprecocious puberty. Pathological analysis of the resected specimens including electron microscopy and immunolocalization of steroidogenic enzymes, and in vitro studies of testosterone production via three-dimensional gel-supported primary culture of the tumor were used in order to investigate neoplastic steroid metabolism in this patient.

Case Report

The patient was an 11-year-old Japanese boy. His parents noticed linear and skeletal growth acceleration and virilization at the age of 9 years, but he did not receive medical attention at that time. He presented to the pediatrician with complaints of testicular enlargement and premature puberty at the age of 11 years and was referred to Tohoku University Hospital, Sendai, Japan for evaluation of a testicular mass.

His height was 160.05 cm and his weight was 58.0 kg on admission, both greater than the 95
percentile of Japanese boys of that age group. The patient had Tanner stage V pubic hair with increased penile length. His bone age was consistent with that of a 16-year-old male. No gynecomastia was observed. Physical examination revealed that the right testis was soft and 4 ml in volume, and the left testis was solid and 20 ml in volume. The prostate was palpable and enlarged, measuring 3.0 cm in its greatest dimension. Ultrasonography of the left testis demonstrated a well-circumscribed low echogenic mass measuring 3.3 × 2.3 × 2.1 cm. Laboratory studies included normal concentrations of hemoglobin, blood urea nitrogen, serum electrolytes, serum hCG, serum hCGβ, serum alpha fetoprotein, and serum alkaline phosphatase.

A left orchiectomy was performed. Prior to orchiectomy, blood was collected from the left spermatic vein. The resected specimen with spermatic cord weighed 37.5 g, and the testis measured 4.5 × 3.5 × 3.0 cm. A well-circumscribed and partly encapsulated tumor appearing brown on the cut surface measuring 3.5 × 2.9 × 2.5 cm was present. His postoperative course was unremarkable. Six months after the operation, he was 161.1 cm in height with the same bone age as prior to the operation.

Hormonal Studies

Results of the hormonal studies are summarized in Table 1. Steroids were assayed by Mitsubishi Chemical BCL Co. Ltd. (Tokyo, Japan). Samples for all steroid assays were extracted and purified prior to quantitation of steroids by RIA. Prior to surgery, serum concentrations of androstenedione, testosterone (T) and estradiol (E2) were increased (Table 1). FSH and LH concentrations were below the detection level, with no response to LH-RH hormone stimulation. (LH: 0, 0.4, 0.7, 0, 0 mIU/ml; FSH: 0, 0.7, 0.8, 0.6, 0.5 mIU/ml at 0, 15, 30, 60, and 120 min after the injection of 150 µg, i.v. respectively). The serum T was also determined in the basal state, 24, 48, and 72 h after hCG injection (5000 IU, i.m.). No significant increase in T was observed (0 h: 22.1, 24 h: 18.5, 48 h: 22.9, 72 h: 24.8 ng/ml). The concentrations of urinary 17-ketosteroids (17KS), free T, and total estrogen were increased prior to orchiectomy (Table 1). The concentration of urinary 17-hydroxycorticosteroids (17OHCs) was within normal limits. Serum concentrations of androstenedione, T and E2 and concentrations of urinary 17KS, free T, and total estrogen determined on the 7th day following surgery had returned to the normal range or a more appropriate prepubertal range (Table 1).

Concentrations of steroids in the blood from the left spermatic vein are summarized in Table 1. Progesterone, androstenedione, and testosterone concentrations were increased. The T: DHEAS ratio was 0.21 in the peripheral venous blood and 30.7 in the spermatic venous blood.

Material and Methods

Pathological and immunohistochemical studies

Tissue specimens were sectioned 4 mm thick immediately following excision, fixed in 8% paraformaldehyde buffered at pH 7.4, 10% formalin, or Bouin's fixative for 18 h at 4 °C and subsequently paraffin-embedded. A small portion of the tumor was fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and then transferred to 1% osmium tetroxide for postfixation. The immunostaining procedures and the primary antibodies used in this study have been described previously in detail [3, 6]. Immunohistochemistry for the following steroidogenic enzymes was performed: P450sc (cholesterol side chain cleavage), 3βHSD (3β-hydroxysteroid dehydrogenase), and P450c17 (17α-hydroxylase). The biotin-streptavidin (B-SA) amplified method with the Histofine immunostaining kit (Nichirei Co. Ltd., Tokyo, Japan) was used. Normal rabbit IgG or phosphate buffer at pH 7.4 was used instead of primary antibodies for negative controls, and no specific immunoreactivity was observed in these tissue sections.

Histoculture

Histoculture or a three-dimensional gel-supported native-state primary culture was performed based on the method previously reported by Hoffman et al. [7, 8], with some modifications. Briefly, the tumors were minced into 2 mm diameter pieces and placed on previously hydrated collagen gel matrices derived from pig skin (Upjohn Co. Ltd., Kalamazoo, MI). Collagen gel matrices were cut with scissors into 1 cm³ pieces, and one piece was
placed into each well of a 6-well plate. Six tissue pieces were placed on each piece of gel.

Eagle’s minimal essential medium (MEM) containing Earle’s salts (Sigma Co. Ltd., St. Louis, MO), nonessential aminoacids, gentamycin (0.1 mg/ml), and 10% fetal calf serum were added to the culture dishes in such a way that the upper part of the gel was not covered. A 100 μl aliquot of culture medium was removed from two wells 1, 3, 5 and 7 days after the start of the culture. Medium was concomitantly taken from control wells which contained collagen gels without tissues. The collagen gels were fixed in 10% formalin for 18 h at 4 °C at the same times that the culture media were collected. The immunohistochemical procedure performed on fixed collagen gels was the same as that described above.

Results

Pathology and immunohistochemistry

The tumor was composed of closely packed sheets or solid cords of relatively uniform, and polyhedral cells with clear or eosinophilic cytoplasm and round nuclei (Fig. 1a). Yellow-brown lipochrome pigment was observed in many tumor

Table 1. Summary of hormonal studies

<table>
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<tr>
<th></th>
<th>prog&lt;sup&gt;2&lt;/sup&gt; (ng/ml)</th>
<th>DHEA&lt;sup&gt;2&lt;/sup&gt; (ng/ml)</th>
<th>DHEA-S&lt;sup&gt;2&lt;/sup&gt; (mg/ml)</th>
<th>andro&lt;sup&gt;2&lt;/sup&gt; (ng/ml)</th>
<th>T&lt;sup&gt;2&lt;/sup&gt; (ng/ml)</th>
<th>E&lt;sup&gt;2&lt;/sup&gt; (pg/ml)</th>
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<td></td>
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<td>4.4</td>
<td>106</td>
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<td>65</td>
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<tr>
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<td>0.5</td>
<td>81</td>
<td>0.7</td>
<td>0.1</td>
<td>14</td>
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<td>0.8–7.0</td>
<td>40–350</td>
<td>0.6–5.0</td>
<td>3.8–9.9</td>
<td>20–60</td>
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<td>141</td>
<td>105</td>
<td>1400</td>
<td>3230</td>
<td>137</td>
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<tr>
<td>normal&lt;sup&gt;5&lt;/sup&gt; value</td>
<td>23.4 ± 4</td>
<td>NA&lt;sup&gt;6&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;6&lt;/sup&gt;</td>
<td>42.7 ± 6.3</td>
<td>688 ± 121</td>
<td>226 ± 38</td>
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(continued)

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<th></th>
<th>17OHCS (mg/day)</th>
<th>17KS (mg/day)</th>
<th>FT&lt;sup&gt;2&lt;/sup&gt; (mg/day)</th>
<th>TE&lt;sup&gt;2&lt;/sup&gt; (mg/day)</th>
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<td>13–160</td>
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<sup>1</sup> Hormonal values prior to orchietomy were measured on the day of the surgery and those following orchietomy were measured on postoperative day 7. <sup>2</sup> prog, progesterone; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; andro, androstenedione; T, testosterone; E2, estradiol; FT, free testosterone; TE, total estrogen. <sup>3</sup> Normal steroid concentrations and 24 h urine are those for the normal adult male (Mitsubishi Chemical BCL, Tokyo, Japan). <sup>4</sup> Hormonal values in spermatic venous blood represents those obtained immediately before orchietomy. <sup>5</sup> Normal steroids concentrations in the spermatic veins are values reported for normal adult male [2]. <sup>6</sup> NA: not available.
cells, but no Reinke's crystals were detected in any of the sections examined. The adjacent testis demonstrated compressed seminiferous tubules with fibrotic and thickened wall. The interstitium was edematous with scattered foci of Leydig cells. Electron microscopy demonstrated round nuclei, abundant vesicular smooth endoplasmic reticulum, and tubular mitochondria (Fig. 2). Tumor cells were immunohistochemically positive for P450scc, 3β-HSD (Fig. 1b) and P450c17 with some heterogeneity of staining.

Histoculture

The T concentrations in the culture medium are as follows: day 1: 23.6, 3: 52.3, 5: 61.4, and 7: 89.6 (ng/ml).

Testosterone concentrations gradually increased and peaked 7 days after the start of the culture. Foci of necrosis were observed, especially in the first 7 days of culture but the areas of necrosis were confined to less than 20% in all specimens. Areas of necrosis were similar at 1, 3 and 5 days of the histoculture. Tissue architecture including the stromal framework as well as the cytologic features of the Leydig cell tumor cells described above were present in all of the histoculture specimens (Fig. 3a). Tumor cells in the tissue culture specimens at 1, 3, 5 and 7 days were immunohistochemically positive for 3β-HSD (Fig. 3b) as well as for P450scc and P450c17 (data not shown) with an approximately equal frequency among viable tumor cells of these specimens. Testosterone levels in the medium of control cultures were below the detection levels.

Discussion

Leydig cell tumors of the testis have been demonstrated to produce and secrete various sex steroids [1-4]. In the present case, production and secretion of testosterone and androstenedione were confirmed by increased concentrations of these hormones both in peripheral and spermatic venous blood samples. It is, however, also important to investigate steroidogenesis in the resected specimen or in vitro in order to obtain an endocrine pathological correlation and examine the capability of the tumor tissues or cells to produce and

Fig. 1. Resected specimen of testicular Leydig cell tumor. a) light microscopic appearance (Hematoxylin and eosin stain). b) Immunohistochemistry of 3β-HSD (Magnification x 150).

Fig. 2. Ultrastructural features of the testicular Leydig cell tumor cells. N, nucleus; M, mitochondria (Magnification x 14000).
secretes steroid hormones. Light microscopic and ultrastructural examination can demonstrate whether the tumor cells are involved in steroidogenesis by identifying the histopathologic characteristics suggestive of steroid producing cells including eosinophilic or clear cytoplasm with polygonal round nuclei or ultrastructural features including well-developed smooth endoplasmic reticulum and mitochondria, as in this case. But electron microscopic examination cannot demonstrate which types of steroids are produced in the cells.

Analysis of steroidogenic enzymes in the resected specimens by immunohistochemistry or biochemical assays can provide important information as to the neoplastic steroidogenesis. Immunohistochemistry of steroidogenic enzymes can indicate the potential site(s) of specific steroid hormone(s) catalyzed by the expressed enzyme in the resected specimens as demonstrated in this case and previous reports [3]. But substrate availability or other intracellular conditions may not allow for production and/or secretion of steroids which could be generated by steroidogenic enzymes immunohistochemically identified within neoplastic cells. Biochemical assay of steroidogenic enzymes has similar problems. In addition, biochemical assay does not necessarily localize steroidogenesis within the resected specimens.

Primary culture of the resected tumors clinically associated with abnormal steroid metabolism can potentially provide important information about neoplastic steroidogenesis including testicular Leydig cell tumor [4]. Primary culture of human steroidogenic tissues or tumors has generally been performed by dispersing the cells and producing a monolayer of cells incubation [9] or short-term incubation of tissue slices [4]. These attempts have suffered from difficulty in supporting long term growth of human steroidogenic tumor cells and maintaining their in vivo structural and functional properties. In addition, cell types other than parenchymal or tumor cells, including interstitial cells, should be present in the culture system in order to approximate in vivo structural and functional relationships since interactions between various cell types can alter steroid metabolism [10]. A three-dimensional collagen gel-supported histoculture system allows tissues to be cultured with the preservation of native-state architecture and functions [7, 8, 11]. This system has been demonstrated to support the in vivo function and architecture of various human tumors and normal tissues for a relatively long period of time [11]. However, this histoculture system has not been applied to steroidogenic tumors or tissues in which complicated enzymatic steps are involved in the production of biologically active steroids.

Our present study demonstrates that a testicular Leydig cell tumor can maintain its histologic architecture, expression of steroidogenic enzymes, and secretion of testosterone into the medium for up to 7 days in culture. It is difficult to distinguish between the contributions of proceeding...
testosterone production from tumor cells and leakage from damaged tumor cells in analyzing the testosterone concentrations measured in the media at various times after culture. The largest changes in testosterone levels in media occurred between days 1 and 3, but there were no significant morphological changes of the tumor cells including necrosis observed in this period. Therefore, the increase in the testosterone levels in this period is considered to reflect the proceeding neoplastic testosterone production and the small change in the testosterone concentrations observed after this period may reflect decreased synthesis due to insufficient substrate availability or another cause. But the possibility that the leakage contributes to an increase in the testosterone concentration in the culture medium cannot be completely ruled out. Further studies are therefore still required to distinguish between active secretion and leakage of steroids when analyzing steroid secretion of neoplasmy histoculture.

Sponge-matrix-supported histoculture systems of human steroidogenic cells or tumors can provide important opportunities to study various aspects of steroidogenesis including possible effects of exogenous substances on neoplastic steroidogenesis through maintaining the histological architecture of the tumor.

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References