Heterogeneity of Induced Testicular Interstitial Cell Tumors of Mice as Evidenced by Steroid Biosynthetic Enzyme Activities

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SUMMARY

Quantitative enzyme studies (17α-hydroxylase, 17,20-desmolase, 21-hydroxylase, and 20α-hydroxydehydrogenase), using as a source of enzyme small fragments (25-48 mg) of estrogen-induced testicular interstitial cell (Leydig cell) tumors of mice and their first generation transplants in castrate male isogeneic hosts, show rather conclusively that such tumors are composed of heterogeneous populations of hormone-producing cells. Our data supported the original concept that, during tumor induction or shortly thereafter, several enzyme systems involved in steroid biosynthesis in Leydig cells decrease in an apparently random fashion. However, it appeared evident that at least in certain neoplastic interstitial cells the activity of 20α-hydroxydehydrogenase, an enzyme whose functional significance has not yet been clearly established, increased significantly.

INTRODUCTION

Earlier studies (11) quantitating several steroid biosynthetic enzyme systems in transplantable testicular interstitial cell (Leydig cell) tumors of mice revealed quantitative differences of considerable magnitude in the various tumor lines investigated. In one instance, 2 sublines that had originated from the same primary tumor were very different. In general, the enzyme content of the tumors paralleled rather well their hormone production in vivo as judged by biologic effects upon the host animals. Since all of these tumors had been carried through several serial passages prior to enzyme quantitation, the question arose: Did these differences result entirely from further genetic change during serial transplantation or are primary Leydig cell tumors made up of heterogeneous populations of hormone-producing cells? The present studies were designed to test the latter possibility.

If a large tumor was derived from a heterogeneous population of cells, the cells in a localized area most likely would have been derived from a common ancestry. A method was developed, therefore, by which steroid biosynthetic enzyme activities could be quantitated in small fragments obtained from large tumors. The results demonstrate clearly that primary Leydig cell tumors induced in mice by the chronic administration of diethylstilbestrol, as well as their 1st generation transplants, are composed of heterogeneous populations of neoplastic interstitial cells.

MATERIALS AND METHODS

Tissues. Three primary testicular interstitial cell tumors induced in (BALB/c x A/Bi)F1 hybrid mice by the subcutaneous implantation of a fused 10% stilbestrol-cholesterol pellet were studied. Two or 4 fragments (25-48 mg) from each of these tumors were homogenized separately; in each instance, an aliquot equivalent to 10 mg of tissue was incubated using progesterone-4-14C as a substrate, and a 2nd 10-mg equivalent was incubated with 17α-hydroxyprogesterone-4-14C. At the time of sacrifice other fragments of each tumor were implanted subcutaneously into castrate male isogeneic hosts that were then placed on a diet containing 0.2 μg stilbestrol per gm (each animal consumed approximately 2.5 gm of diet per day). When these tumors grew to an appropriate size, the animals were sacrificed and 2 or 4 small fragments (25-38 mg) were removed from different areas of each tumor and handled in the same manner as the fragments from the primary tumors. The total weight of each tumor was determined, and the weights of the seminal vesicles obtained as an indicator of androgen production by the tumor (11).

Testes from normal (BALB/c x A/Bi)F1 males 2 months of age were used as control tissue. In 1 experiment whole testes were homogenized while, in a 2nd, small fragments approximately the same size as the fragments from the tumors were employed.

Since the tumor fragments from the 1st generation transplants may have contained some subcutaneous connective tissue, normal nonfatty subcutaneous connective tissue from hybrid male mice was collected and handled in a manner similar to the tumor fragments.

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Incubation Procedure. In all experiments the tissues employed were homogenized in a Ten Broeck glass tissue homogenizer employing 0.15 M phosphate buffer (pH 7.4), and containing 40 mM nicotinamide. The volume was adjusted so that 0.5 ml of homogenate was equivalent to 10 mg of tissue. Prior to the incubations, the isotopically labeled substrates were taken to dryness in the incubation container using a stream of nitrogen. They were collected in the bottom of the vessels by washing repeatedly with ethyl alcohol and drying alternately. Just prior to the incubation, the substrates were dissolved in small amounts of absolute ethyl alcohol (2 drops in the case of the micro incubations and 0.1 ml in the case of macro incubations). Media containing the cofactors were then added as was the desired amount of homogenate: the final media consisted of 0.15 M phosphate buffer (pH 7.4) containing 40 mM nicotinamide, 0.4 mM ATP, 0.4 mM diphosphopyridine nucleotide, 0.1 mM reduced triphosphopyridine nucleotide, and 1 mM fumaric acid.

Under these conditions of incubation it has been demonstrated that 17α-hydroxylase and 17,20-desmolase activities can be quantitated in normal mouse testes (19). The products formed increase linearly over a 1-hr period and are proportional to the amount of tissue added when 100 mg of tissue or less are incubated in 10 ml of media containing 100 μg/μmoles of progesterone or 200 μg/μmoles of 17α-hydroxyprogesterone, provided that at least half of the added substrate remains unchanged at the conclusion of the incubation. Therefore, in order to quantitate these enzyme systems in small fragments of tissue, 10 μg/μmoles of progesterone-4-14C or 20 μg/μmoles of 17α-hydroxyprogesterone-4-14C3 were incubated with 10 mg equivalents of tissue homogenate in a total volume of 1 ml. All incubations were carried out at 37.5°C for 1 hr in a Dubnoff shaking incubator with air as the gas phase. Unstopped 50-ml Erlenmeyer flasks were used for the macroincubations while, for the microincubations, 50-ml round-bottom, ground glass-stoppered centrifuge tubes were employed in order to reduce evaporation.

At the conclusion of the incubation period, enzymic reactions were stopped by the addition of 0.5 ml of 1 N HCl; this was added directly to the media in the macro incubation while 9 ml of cold water were added to the micro incubates prior to adding the acid. The radioactive steroids were extracted from the aqueous incubate with ether:chloroform (4:1, v:v) 5 successive times, using 20 ml of solvent for the 1st extraction and 10 ml of solvent for each successive extraction. The pooled extracts were dried in a flash evaporator at 55-60°C, and the residues were quantitatively transferred to graduated conical centrifuge tubes employing chloroform:methanol (1:1, v:v). Aliquots were counted to determine total recovery which ranged from 94 to 102%, and the samples were dried under a nitrogen stream.

Paper Chromatography. The dried extracts were dissolved in chloroform:methanol (1:1, v:v) and chromatographed on 40-cm wide strips of Whatman No. 1 filter paper in Zaffaroni type solvent systems (4) after the addition of 100 μg each of 17α-hydroxyprogesterone, 4-androstenedione, and progesterone as internal carriers. The initial chromatography was carried out in the hexane:benzene (1:1)/formamide system until the solvent front reached a point approximately 2 cm from the tip of the paper. The chromatograms were dried overnight, and the areas of the added carriers were located over an ultraviolet scanning lamp (9). The strips were then scanned on a 4-Pi Actigraph (Nuclear-Chicago) to locate the position of the radioactive compounds (1).

Regularly, when progesterone was used as a substrate, 4 major peaks were detected on these initial chromatograms and, for the sake of convenience, were designated A, B, C, and D. These areas of radioactivity were cut from the paper strip and eluted with 10 ml of methanol while the fragment of the chromatogram was hung from a bent 25-gauge hypodermic needle attached to a 10-ml syringe containing the solvent. The eluate, collected in a graduated conical centrifuge tube, was made homogeneous, and aliquots were taken to quantitate the radioactivity present using the ambient temperature Nuclear-Chicago liquid scintillation spectrophotometer (5).

Upon rechromatography, Area A, which was at the origin of the initial chromatogram, was shown to contain undetectable amounts of material with a polarity of 17α-hydroxyprogesterone or of lesser polarity. The amounts of radioactivity in this area were generally very small.

After the addition of 100 μg each of deoxy corticosterone and testosterone, the eluates of Area B, already containing carrier 17α-hydroxyprogesterone, were acetylated with nonradioactive acetic anhydride employing the conditions described previously (7). The acetylation media were extracted and the mixtures of steroids rechromatographed in hexane:benzene (2:1, v:v)/formamide. This accomplished a clear separation of 17α-hydroxyprogesterone, 11-deoxycorticosterone acetate, and testosterone acetate. The amount of radioactivity associated with each of these compounds was determined, as described above for the initial chromatogram.

To the eluates from Area C containing the carrier 4-androstenedione, 100 μg 4-pregnen-20a-ol-3-one were added; these mixtures were then rechromatographed in a hexane/proplylene glycol system, impregnating the filter paper with methanol: propylene glycol (1:2, v:v) to locate the position of the radioactive compounds isolated, all were further purified by additional paper chromatographies in different solvent systems and prepared for crystallization. Known amounts (10-20 mg depending upon the
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Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate used</th>
<th>17α-Hydroxylase</th>
<th>17α-Hydroxyprogesterone-4,14C</th>
<th>17α-Hydroxyprogesterone-4,14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of incubation</td>
<td>Micro</td>
<td>Macro</td>
<td>Micro</td>
<td>Macro</td>
</tr>
<tr>
<td>Amount of substrate (mMoles)</td>
<td>10</td>
<td>100</td>
<td>20</td>
<td>200</td>
</tr>
<tr>
<td>Tissue (mg)</td>
<td>10</td>
<td>100</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Vol of media (ml)</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>% of product formed</td>
<td>43.6a</td>
<td>45.9a</td>
<td>32.9b</td>
<td>33.8b</td>
</tr>
<tr>
<td>mMoles of product formed</td>
<td>4.4a</td>
<td>45.9a</td>
<td>6.6b</td>
<td>67.6b</td>
</tr>
<tr>
<td>per hr per gm of tissue</td>
<td>440a</td>
<td>459a</td>
<td>660b</td>
<td>676b</td>
</tr>
</tbody>
</table>

Comparative results of macro- and microincubations carried out with normal mouse testes.

- 17α-Hydroxyprogesterone plus 4-androstenedione plus testosterone.
- 4-Androstenedione plus testosterone.
- 4-Pregnene-20α-ol-3-one formed from progesterone. The quantitation of these products was calculated from the radioactivity in the appropriate areas of the 2nd chromatograms, correcting for the small losses that occurred during elution and rechromatography.

**RESULTS**

**Normal Testes.** In order to be certain that the small volumes used in the micro incubations did not significantly alter the results obtained, an initial experiment was carried out in which aliquots of a single homogenate of whole normal testes were incubated under the 2 conditions. For each type of incubation and both substrates used, duplicate flasks were prepared, and the figures averaged. As seen in Table 1, the results obtained with the micro procedure were essentially identical to those with the well-established macro procedure. Small amounts of deoxycorticosterone were also formed during these incubations, but the actual amounts formed in the micro incubations were too small for sufficiently accurate quantitation to make a comparison with the macro method meaningful. As is usually the case when using normal testicular tissue and isotopically labeled progesterone of relatively low specific activity, the 20α-hydroxydehydrogenase activity was too low to be quantitated.

In a 2nd experiment, 6 fragments of about 25 mg each were removed from 2 different normal testes, and each was carried through the micro incubation procedure. The results are presented graphically in Chart 1. Although there were minimal differences in the absolute amounts of enzyme activities from fragment to fragment, probably due to slight differences present in the ratios of germinal to interstitial tissue, the ratio of 17,20-desmolase activity to 17α-hydroxylase activity was remarkably constant. With all fragments, 21-hydroxylation occurred with a range of 0.10 to 0.15 μMoles of 11-deoxycorticosterone formed from progesterone, but 20α-hydroxydehydrogenase activity was not detected.

**Testicular Tumors.** The results obtained with tumor 164L are summarized in Table 2 and depicted graphically in Chart 2. It is obvious that the 2 fragments from the primary tumor differed markedly with respect to 20α-hydroxydehydrogenase and 21-hydroxylation activities while 17α-hydroxylase and 17,20-desmolase activities were similar. The fragments from the 1st generation transplants varied significantly not only between different tumors, but also between fragments from the same tumor. Since the transplanted tumors, in particular, may have contained some areas of necrosis and thus the fragments may have been composed partially of inactive cell debris, the ratios of the various enzyme activities were calculated. These ratios were found to vary as widely as did the actual values for the enzyme activities, indicating that the tumors were composed of different populations of hormone-producing cells.

Tumor 164R developed in the other testis of the same animal, and the results obtained with this tumor are depicted in Chart 3. It is evident that the 2 fragments from this primary tumor differed in their enzyme content from those taken from the tumor on the opposite side, particularly with regard to 20α-hydroxydehydrogenase and 21-hydroxylation activities. 17α-Hydroxylase and 17,20-desmolase activities were considerably...
Table 2

<table>
<thead>
<tr>
<th>Tissue Weight of seminal vesicles (mg)</th>
<th>Primary tumor</th>
<th>First transplant generation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Enzymes measured:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 17α-hydroxylase a</td>
<td>0.29</td>
<td>0.27</td>
</tr>
<tr>
<td>2. 21-hydroxylase b</td>
<td>0.09</td>
<td>1.48</td>
</tr>
<tr>
<td>3. 20α-hydroxy-dehydrogenase c</td>
<td>2.54</td>
<td>0.88</td>
</tr>
<tr>
<td>4. 17α,20-desmolase d</td>
<td>1.39</td>
<td>1.15</td>
</tr>
</tbody>
</table>

Ratios of enzymes measured:

- 4/1: 4.6 | 4.3 | 26.5 | 3.5 | d | 6.2 | 1.7 | 4.1 | 2.6 | 6.3
- 2/1: 0.31 | 5.48 | e | c | e | e | e | e | e | 1.48 | e
- 3/1: 8.76 | 2.51 | 10.25 | 2.89 | e | 0.22 | 8.06 | 0.17 | e | 4.23

Product formed (m moles) by various enzymes present in 10 mg fragments obtained from either primary tumor (164L) or various first transplant generation tumors A, B, C, and D.

- Enzymes were measured as m moles of product formed by each enzyme from 10 m moles of progesterone-4-14C in 1 hr.
- Enzyme was measured as m moles of product formed by the enzyme from 20 m moles of 17α-hydroxyprogesterone-4-14C in 1 hr.
- Undetectable.
- Ratios were not calculated because 17α-hydroxylase activity, taken as unit, was present in amounts lower than the limits of detection.

Chart 2. Comparison of enzyme activities in two small fragments of primary testicular tumor 164L and in fragments from four first generation transplants. Enzyme activities are calculated as described in footnote of Table 2. A small bar extending below the 0 line indicates enzyme activity below the limit of detection. 20α-OH-de(H)ase, 20α-hydroxydehydrogenase; 21-OH-ase, 21-hydroxylase; 17α-OH-ase, 17α-hydroxylase; T. Wt., tumor weight; S. V., weight of seminal vesicle-coagulating gland complex.

higher in the fragments from the 1st generation transplants than in those from the primary tumor, and this is due, at least in part, to the suppressant effect of the higher level of estrogenization to which the primary tumor had been subjected (13). As a matter of fact, in the incubations of 5 of these 8 fragments, more than half of the 17α-hydroxyprogesterone substrate had been converted to 4-androstenedione and testosterone at the conclusion of the incubation period so that, in these instances, the 17α-hydroxylase activity has undoubtedly been underestimated.

Tumor 254 was a larger tumor so that 4 fragments could be obtained for incubation. As illustrated in Chart 4, the 17α-hydroxylase and 17α,20-desmolase activities were not strikingly different in the 4 fragments, with the 17α,20-desmolase activity being uniformly low. No 21-hydroxylase activity was detected in any of the fragments, though 3 showed some 20α-hydroxydehydrogenase activity. Again, both 17α-hydroxylase and 17α,20-desmolase activities were increased in the fragments from the 1st generation transplants and varied considerably from fragment to fragment. Except for 1 fragment, 20α-hydroxydehydrogenase activity was low, but 7 of the 8 fragments possessed 21-hydroxylase activity.

Although neither 17α-hydroxylase nor 17α,20-desmolase is rate-limiting for the formation of testosterone by the normal mouse testis, in a previous study (11) a rough correlation was found to exist between these activities and the production of androgens when the enzymes were quantitated, employing homogenates of the entire tumor transplant. Charts 2, 3, and 4 illustrate rather strikingly that such is not the case when only small portions of large tumors are assayed. For instance, fragments from tumor 254, transplant D, had relatively little biosynthetic enzyme activity, yet the animal's seminal vesicles weighed 300 mg and fragment Ab, derived from the same primary tumor, contained higher levels of enzyme activity. Nevertheless, the host's seminal vesicles were only about half as large.

**Connective Tissue.** Normal mouse testes possess very little 20α-hydroxydehydrogenase activity, yet this enzyme was present in significant amounts in many of the tumor fragments incubated. Since connective tissue has been reported to contain this enzyme (2), and certainly fragments from the transplanted...
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Chart 3. Comparison of enzyme activities in two small fragments of primary testicular tumor 164R and fragments from three first generation transplants. Abbreviations are the same as in Chart 2.

Chart 4. Comparison of enzyme activities in four small fragments of primary testicular tumor 254, and in fragments from four first generation transplants. Abbreviations are the same as in Chart 2.

DISCUSSION

The enzyme studies presented here, as well as the biologic data relative to androgen production as reflected in the seminal vesicular weights of castrate male hosts bearing 1st generation transplants, demonstrate clearly that estrogen-induced testicular interstitial cell tumors in mice are composed of a heterogeneous population of hormone-producing cells. Whether this heterogeneity results from a multifocal tumor formation in the testis or from different alterations occurring in a unifocally arising tumor cannot be decided on the basis of these experiments. Histologic examination of testes containing small tumors certainly indicates that multifocal tumor formation does occur, though this does not rule out the occurrence of the second phenomenon. It also should be pointed out that, on morphologic and biologic grounds, evidence has been obtained in this laboratory suggesting that, with certain tumors, some degree of heterogeneity may persist for as many as 20 serial passages.

The data presented here also do not help in determining the mechanisms by which functional Leydig cell tumors frequently lose function upon serial transplantation (10). It could be either that a nonfunctional population of cells in time outgrows the functional ones, or that functional cell populations, through further change, gradually lose their ability to produce hormones.

Although it is evident in this study that one cannot judge the function of a large Leydig cell tumor in the mouse by in vitro studies employing only a small fraction of the entire tumor, it would seem that the original conclusion drawn is still valid (11): that is, that during or immediately following the neoplastic transformation of Leydig cells, steroid biosynthetic enzymes are diminished in activity or lost completely in an apparently random fashion. Since interstitial cells comprise only about 5% of the cells in a normal testis, while the tumors are composed primarily of neoplastic Leydig cells, the concentration of 17a-hydroxylase and 17,20-desmolase per functional cell was lower in all of the fragments studied. The same seems to be true of 21-hydroxylase also, for, with the macro method where this activity can be quantitated accurately in normal testes, 3–5 mμmoles of 11-deoxycorticosterone generally is formed per 100 mg of tissue or roughly 5 mg of interstitial tissue. However, 20α-hydroxydehydrogenase seems to be the exception. Generally, its presence is not detected using the macro method employed here (limits of detection = 0.05 mμmoles of 4-pregnen 20α-ol-3-one) though, by incubating progesterone of very high specific activity (O. V. Dominguez, unpublished data) or by employing greater concentrations of progesterone (3, 8), it has been demonstrated in normal murine testes so that one does not have to assume that it arose de novo during neoplastic transformation. Yet, in 1 fragment of primary Tumor 164L, 25% of the progesterone substrate was converted to 4-pregnen-20α-ol-3-one and, in 2 fragments of 1st generation transplants, more than 15% of the substrate was converted to this compound. The biologic significance of this enzyme has not yet been firmly established, but it does not seem to be involved in androgen biosynthesis by the normal mouse testis (6). Nominal increases in 20α-hydroxydehydrogenase activity have also been described in irradiated murine testes (3, 8), though it does not seem to be increased by estrogen stimulation per se in the mouse (L. T. Samuels and R. A. Huseby, unpublished data).
REFERENCES


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