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Rapid Development of Leydig Cell Tumors in a Wistar Rat Substrain

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ABSTRACT: In 78% of the Wistar rats (substrain U) studied, spontaneous Leydig cell tumors developed between the ages of 12 and 30 months. The first signs of tumor development, in the form of nodules of Leydig cells, were already apparent in 1-month-old U-rats. These nodules of Leydig cells were found in all animals studied. In no other strain of rats has this type of abnormality been observed at such an early age. The first Leydig cell tumors were noticed between the ages of 12 and 14 months. The tumor tissue appeared to have developed from a rapid, focal outgrowth of a nodule. The tumor Leydig cells were found to be sensitive to the cytotoxic action of the specific Leydig cell toxicant ethane dimethane sulphonate (EDS), although not all tumor cells were killed. Inhibin-like immunoreactivity could be detected in both normal and tumor Leydig cells, and plasma levels varied considerably within the different groups of rats. Moreover, no significant changes in plasma levels of inhibin-like immunoreactivity were found during the aging period when Leydig cell tumors were formed or after EDS administration when nearly all Leydig cells were killed. Therefore, the possible contribution of Leydig cells and tumor cells to the total content of inhibin-like immunoreactivity in the testis and plasma may be of less importance than expected. Some significant fluctuations in plasma testosterone concentrations were found during aging; however, there appeared to be no correlation between plasma testosterone levels and the appearance of large Leydig cell tumors. This indicates that testosterone production by tumor cells is limited. Plasma luteinizing hormone (LH) levels did not vary significantly up to the age of 12 months and were undetectable in rats in which tumors had developed. This indicates that the tumor cells probably produce an LH-suppressing product. This product is not likely to be estradiol because estradiol levels do not change significantly during tumor development. When isolated tumor cells were implanted intratesticularly, tumor development took place in U-rats but not in another Wistar substrain. Since the abnormalities preceding tumor formation are already apparent at the age of 1 month in U-rats, this Wistar substrain may provide a good model to study Leydig cell tumor development.

Key words: Leydig cell tumors, Wistar rat substrain U, aging, inhibin, estradiol.

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Spontaneous Leydig cell tumors are rare in humans and in most strains of rats; malignancy of these tumors occurs even less frequently. Fischer 344 rats are an exception: Coleman et al (1977) reported nodular interstitial cell hyperplasia, preceding interstitial cell neoplasia, in male Fischer rats by 10 to 12 months of age. Around the age of 24 months, approximately 95% of the Fischer rats exhibit some degree of interstitial neoplasia (Thompson et al, 1961; Jacobs and Huseby, 1967; Coleman et al, 1977; Walsh, 1979). It has been suggested that Leydig cell neoplasm is the result of excessive, long-term stimulation by luteinizing hormone (Huseby, 1981). Other authors have stated that the development of Leydig cell tumors may be correlated with elevated levels of prolactin and estradiol (Turek and Desjardins, 1979). However, Sweeney et al (1983) and Amador et al (1985) have shown that hyperprolactinemia can inhibit Leydig cell tumor development. Thus, the exact etiology of tumor formation in Fischer rats is not known. The slow development and often low frequency of neoplastic Leydig cells in different strains of rats have hampered the elucidation of the primary causes of tumor formation. Recently, we found a substrain of Wistar rats with a high incidence of abnormalities in their interstitial tissue that is detectable at the age of 6 months. At 14 months, Leydig cell tumors were present. The developmental, and some endocrinologic, aspects of the tumor formation were studied using morphologic, immunohistochemical, and biochemical techniques.

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Materials and Methods

Materials

Ethane dimethane sulphonate (EDS) is not commercially available and was prepared as described by Jackson and Jackson (1984). Bovine serum albumin (BSA), fraction V, was obtained from

Fluka (Hicol BV, Oud Beyerland, The Netherlands). A polyclonal antiserum against 58 kd inhibin, purified from bovine follicular fluid (de Jong et al, 1987), was raised in a rabbit. Peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody was obtained from Sanbio BV (Uden, The Netherlands).

Treatment of Animals

Male Wistar rats of the U-substrain (henceforth called U-rats) were obtained from the Netherlands Cancer Institute, Amsterdam. The U-rat is a Wistar inbred strain that was developed in 1958 at the Veterinary School in Utrecht. The strain has since been maintained at the Netherlands Cancer Institute where the rats have been used for perfusion studies. Until now, nothing was known about tumor development in these rats.

Groups of at least three to five U-rats were asphyxiated with carbon dioxide between the ages of 1 and 30 months. One hundred and ten rats were used for this investigation. Ethane dimethane sulphonate (30 mg/ml in a mixture of dimethyl sulfoxide and water; 1:3, v/v) was administered in a single intraperitoneal injection (75 mg/kg body weight) to groups of U-rats aged 6, 10, and 24 months. Rats were asphyxiated 2, 4, 19, or 60 days later.

In another series of experiments, tumor Leydig cells were isolated from the testes of 24-month-old U-rats in which nearly the complete testicular space was filled with tumor cells by collagenase dispersion. In these testes, the amount of Leydig cells outside the tumor tissue was negligible compared with the large number of tumor cells. Cells were further purified by centrifugation in a Ficoll solution (Rommerts et al, 1985). After isolation, approximately 1.0×10^6 cells were injected intratesticularly into 6-month-old U-rats or in 4-month-old Wistar R-substrain rats (henceforth called R-rats). The U-rats were asphyxiated 12 months later; R-rats were asphyxiated 6 months after implantation of the tumor cells.

Histologic and Immunohistochemical Procedures

Testes were fixed in Bouin's solution. After dehydration, the material was embedded in Technovit 7100 plastic, a glycol methacrylate (Kulzer & Co, GmbH, Wehrheim, West Germany), or in paraffin for immunohistochemical purposes. Five-micrometer thick sections were cut. Plastic-embedded sections were stained by the periodic acid Schiff (PAS) technique and Gill's hematoxylin (Polysciences Inc., Warrington, PA). One testis from a U-rat into which tumor cells had been implanted at the age of 6 months was cut into small pieces, fixed with a combination of 1% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.2), and postfixed with 1% buffered osmium tetroxide. After dehydration, this material was embedded in Epon 814, cut into 1-μm thick sections, and stained with toluidine blue.

Testis tissue of U-rats was frozen in liquid nitrogen and stored at −20°C. 3β-Hydroxysteroid dehydrogenase (3β-HSD) histochemistry was performed on 10-μm thick frozen sections according to Loyda et al (1979). Control sections incubated in the absence of substrate or co-factor showed no staining.

For the immunohistochemical localization of inhibin-like material, 5-μm thick paraffin-embedded sections were used. Sections were deparaffinized, and endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 30 minutes. Slides were washed in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4), followed by preincubation with 10% normal goat serum in PBS for 30 minutes. Slides were then incubated for 60 to 120 minutes at room temperature with either the inhibin antibody diluted 1:200 in PBS with 0.2% Tween 20, with 1% normal rabbit serum, or with 1% bovine serum in PBS with 0.2% Tween 20. Following incubation, slides were rinsed with PBS and then incubated for 45 minutes with peroxidase-conjugated goat anti-rabbit immunoglobulin G in PBS with 0.2% Tween 20. Slides were again washed in PBS, and bound antibody was visualized with a 0.5 mg/ml solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.05 mol/L Tris-HCl (pH 7.6) and 0.1% hydrogen peroxide for 4 minutes. Nonspecific staining with rabbit or bovine serum could not be detected, indicating that the inhibin antibody bound specifically. Specificity of the second antibody (goat anti-rabbit immunoglobulin G) was checked by omitting the first antibody incubation step. Nonspecific staining of the second antibody was not found. Slides were counterstained with hematoxylin according to the method of Mayer (Romeis, 1968)

Cell Counts

Identification of different types of interstitial cells was based on their nuclear morphology and localization in the interstitial tissue according to de Kretser and Kerr (1988) and Hardy et al (1989), and the staining characteristics of the cytoplasm. Leydig cells were identified by their oval- to round-shaped nucleus and characteristic distribution of heterochromatin, in combination with the specific blue-purple staining of their cytoplasm. Nuclei of macrophages were somewhat smaller and more irregularly shaped than those of Leydig cells. The cytoplasm of the macrophages stained pink (PAS-positive). Pericytes had fusiform nuclei and were located in direct apposition to the vascular endothelium. Nuclei of myoid cells were directly apposed to the basal lamina of the seminiferous tubules and were part of the boundary tissue surrounding tubules, together with a peripheral layer of lymphatic endothelial cells. Other interstitial cells (eg, mesenchymal cells), which were located in the central regions of the interstitial space, had fusiform nuclei. These cells were counted together. Nuclei of Sertoli cells were only counted when the nucleolus was present in the nuclear cross section.

Cross sections of the whole testis were made in areas chosen at random. Nuclei of cells in these areas were counted using a square lattice grid inserted in the eyepiece of the microscope. At least five different sections (100 to 200 μm apart) were studied, and cells were counted until 1,000 Sertoli cells were scored. The number of nuclei counted per cell type (henceforth called number of cells) was expressed per thousand Sertoli cell nuclei according to the method of Heller et al (1971).

Other Measurements

Blood was collected via heart puncture, and plasma was stored at −20°C. Concentrations of luteinizing hormone (LH) and estradiol in plasma were measured by radioimmunoassay (RIA) as described by Welschen et al (1975). For LH, results are expressed in terms of National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) rat LH-RP1; intra- and interassay coefficients of variation were 9.1% and 15.6%, respectively. Testosterone concentrations in plasma were assayed by RIA as described by Verjans et al (1973); intra- and interassay coefficients of variation

were 8.1% and 11.8%, respectively. Inhibin-like immunoreactivity in plasma and testicular homogenates was measured by RIA using antiserum and labeled material purchased from Dr. D. M. Robertson (Department of Anatomy, Monash University, Clayton, Victoria, Australia). This RIA showed minimal cross-reactivity with activin and isolated α- and β-subunits following reduction and alkylation of inhibin, but considerable cross-reaction with the pro-α_c dimer of inhibin. Results are expressed relative to the potency of a standard pool of bovine follicular fluid, with an arbitrary potency of 1 U/μg protein (Grootenhuis et al, 1989). Student's t-test was performed for statistical analysis. Differences were considered significant when $P < 0.01$.

Results

Development of Leydig Cell Tumors

In all testes of 1-month-old U-rats (the youngest age group studied), small clusters (nodules) of tightly packed interstitial cells were found (Fig 1A). The nuclear and cytoplasmic features of these cells were reminiscent of Leydig cells. The cells were medium sized with hexagonal cross sections and distinct cell boundaries, and they possessed a regular round or oval nucleus, often with a nucleolus, which is characteristic of active Leydig cells. Blood capillaries were sometimes present in these nodules, but macrophages and other types of interstitial cells were absent. The nodules of Leydig cells were sometimes partly surrounded by cells with elongated nuclei and thin cytoplasmic extensions, possibly endothelial or fibroblast-like cells. In another substrain of Wistar rats, substrain R, these nodules were not found at any age.

With age, the nodules increased in cell number and size. In most rats between the ages of 4 and 8 months, the average diameter of the nodules was 162 ± 20 μm (mean ± SD; Fig 1B), while at 10 months the size of the nodules had increased to 230 ± 48 μm. In one 10-month-old rat, a considerably larger nodule was found with a diameter of approximately 620 μm (Fig 1C). The number of Leydig cells in those parts of the interstitium that were devoid of nodules did not undergo any significant changes between the ages of 4 and 10 months. Relative cell numbers were 1,084 ± 47 at 4 months and 1,356 ± 197 at 10 months of age (mean cell numbers ± SD, expressed per thousand Sertoli cells). In 12-month-old U-rats, the number of Leydig cells in areas devoid of nodules had increased to 1,500 ± 113 ($P < 0.05$). No signs of Leydig cell hyperplasia were found up to the age of 12 months. Hyperplasia of Leydig cells is characterized by a nonfocal, diffuse increase in the number of Leydig cells, and often seminiferous tubules are entrapped within these large fields of Leydig cells (Mostofi and Bresler, 1976).

The first Leydig cell tumors were detected around the age of 14 months. The tumor tissue comprised approximately 20% to 50% of the surface area of midsagittal sections of the testis. The histology of the cells present in these tumors was similar to that of Leydig cells in the nodules. Occasionally, binuclear cells were found, but abnormal mitotic figures were not seen (Fig 1D). The number of mitotic figures in the tumor tissue was very low, indicating that the tumor cells do undergo proliferation, although this probably occurs at a very low rate. The proliferation of tumor cells may have caused the increase in the size of the tumors during aging. No seminiferous tubules entrapped by tumor tissue were found, but compression of testicular tissue at the periphery of the tumor occurred. Within the tumor tissue, many blood vessels and fibroblast-like cells were found. Large vacuoles and some hemorrhagic areas were present. The walls of the blood vessels were thicker than normal (Fig 1F). In total, testicular tissue of 46 animals between the ages of 1 and 14 months was studied. In the testes of 44 animals, nodules or tumor tissue could be detected. In two 14-month-old rats, no nodules were found, even after sectioning of the whole testis. In addition, the number of Leydig cells was considerably lower (531 and 304 per thousand Sertoli cells) in these rats compared with the others, and mast cells were frequently found. The mast cells were not found in the testes of U-rats with nodules or tumor tissue, or in the testes of R-rats. This indicates that the interstitial composition of these two rats is, for unknown reasons, completely different from what is normally found in U-rats and in nontumor-bearing R-rats.

In 78% of the testes from U-rats older than 14 months, large areas of tumor tissue, comprising 20% to 90% of the surface area of midsagittal testicular sections, were found. Within the tumor tissue of some animals, another smaller type of cells was found (Fig 1E). This cell type was characterized by a small, elongated nucleus with a considerable amount of heterochromatin that stained intensely, scanty cytoplasm, and a less prominent cell boundary. This is characteristic for Leydig cells in an unstimulated state (Morii et al, 1988). Intermediate cell types, mostly located in between two areas of large tumor cells and having the morphologic characteristics of active cells, and small Leydig tumor cells were present as well. In these areas, necrosis sometimes occurred and macrophages were frequently present (Fig 1E). Vacuoles and hemorrhagic areas were found mainly within the fields that consisted of the larger Leydig tumor cells. The tumor tissue was nearly always surrounded by one or more layers of fibroblast-like cells.

In 22% of the U-rats aged 16 months or older, only nodules of Leydig cells were found. Most of these nodules consisted of the large Leydig cell type, but some nodules consisting of small tumor cells were also found (Fig 1F). Degeneration of the seminiferous epithelium was observed near the nodules, but spermatogenesis was normal in other parts of the testis.

No changes in the numbers of other types of interstitial cells (eg, peritubular-myoid cells, or mesenchymal cells)

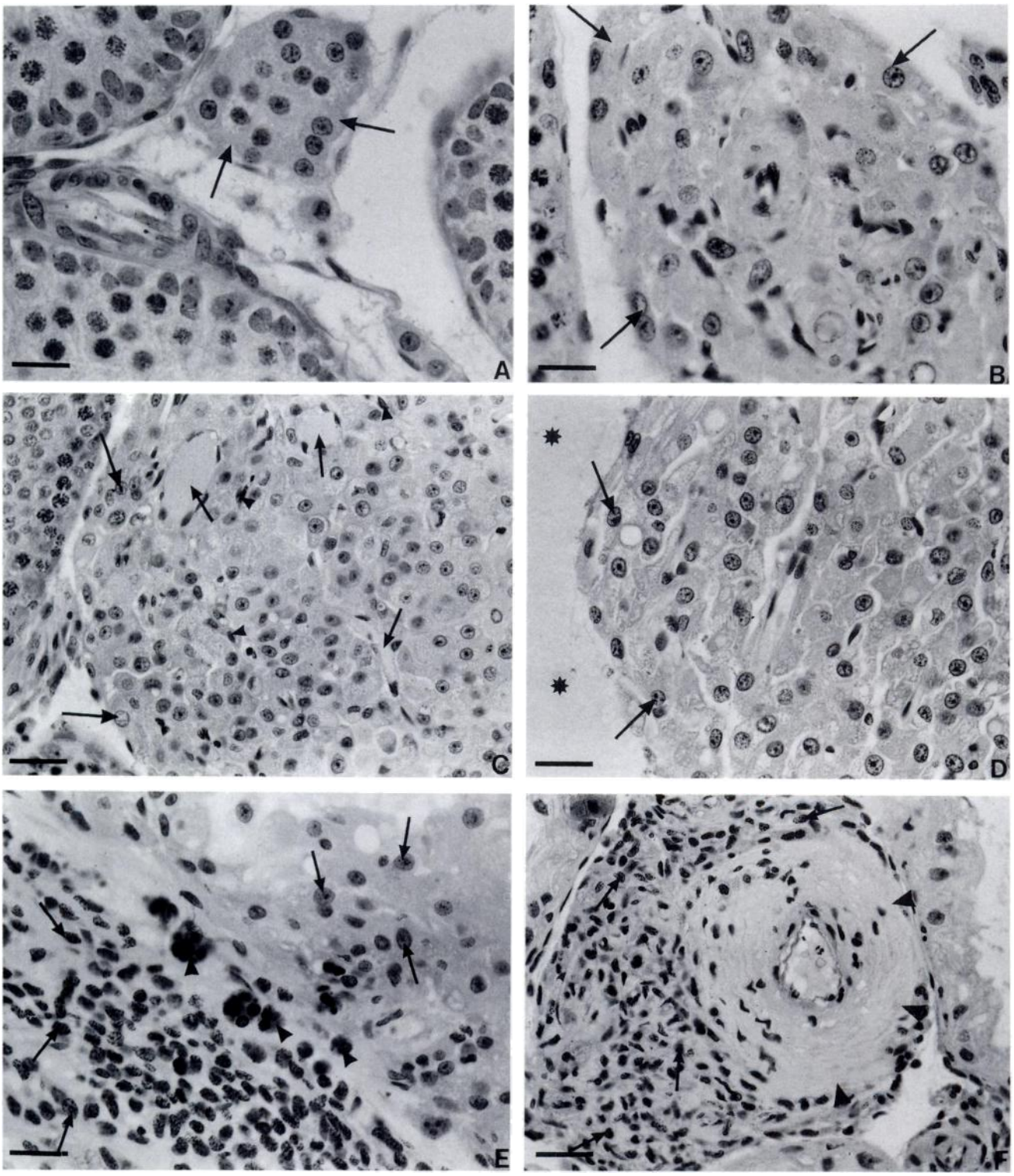

FIG. 1. Representative midsagittal sections of testes from 1- to 24-month-old U-rats. (A) Interstitial area of a 1-month-old rat in which a small cluster of Leydig cells is located (arrows; magnification = 582×, scale bar = 17 μm). (B) Nodule of Leydig cells (arrows) near a seminiferous tubule of an 8-month-old rat (magnification = 450×, scale bar = 22 μm). (C) Part of a large nodule of Leydig cells (arrows) found in a 10-month-old rat; fibroblast-like cells (arrowheads) and blood vessels (small arrows) are also visible (magnification = 288×, scale bar = 34 μm). (D) Tumor tissue of a 14-month-old rat. Large Leydig tumor cells (arrows) and part of a large vacuole (asterisks), which is located between the tumor cells, are indicated (magnificantion = 324×, scale bar = 31 μm). (E) Tumor tissue of a 24-month-old rat. Both large and small Leydig tumor cells (arrows) were found; macrophages (arrowheads) are also present (magnification = 288×, scale bar = 34 μm). (F) Nodule of small Leydig tumor cells (arrows) surrounding a blood vessel in a 24-month-old U-rat. The walls of the blood vessels are thickened (arrowheads, magnification = 288×, scale bar = 34 μm).

were found in areas that were devoid of nodules and tumor tissue during aging. Gross morphologic examination in some rats around the age of 24 months did not reveal metastases of the Leydig cell tumors or other types of tumors in the adrenals, liver, lymph nodules, and lung tissue.

Leydig Cell Tumors and Hormone Production

In U-rats between 4 and 24 months of age, some significant fluctuations in plasma testosterone levels were found. There appeared to be no correlation, however, between plasma testosterone levels and the appearance of large Leydig cell tumors (Table 1). Plasma LH levels did not undergo any significant changes up to the age of 12 months (Table 1). In rats aged 14 months and older possessing significant amounts of tumor tissue, LH levels were below the detection limit of the assay (Table 1). This may indicate that tumor cells secrete products that suppress LH secretion. Plasma estradiol levels did not undergo significant changes between 4 and 24 months of age, even in those animals that possessed large Leydig cell tumors (Table 1).

Histochemical staining of frozen sections for the presence of 3β-HSD revealed strong staining of nodules and Leydig cell tumors in 24-month-old U-rats (Fig 2A). The 3β-HSD staining of the normal Leydig cells located outside the nodules and tumors was less intense than in the nodule/tumor containing areas (Fig 2A).

Immunohistochemical staining of paraffin-embedded sections of 6-month-old U-rats with a polyclonal antibody against 58 kd bovine inhibin revealed that all Leydig cells (normal cells and cells located in nodules) showed inhibin-like immunoreactivity (Fig 2B). Faint positive staining was visible in the seminiferous tubules. The same distribution of inhibin-like immunoreactivity was found in testis sections of adult R-rats that did not possess Leydig cell tumors or nodules. When Leydig cells were destroyed by administration of the specific Leydig cell toxicant EDS (Kerr et al, 1985; Molenaar et al, 1985; Bartlett et al, 1986), staining for inhibin-like immunoreactivity was not found in the interstitial tissue of U-rats, although faint positive staining was still present in the seminiferous tubules (Fig 2C). In 21-day-old R-rats, inhibin-like immunoreactivity was found in both the interstitium and seminiferous tubules, as reported by Rivier et al (1988). The plasma levels of inhibin-like immunoreactivity varied considerably within the same groups of animals, ranging from 0.05 to 2.8 U/mg protein. No correlation was found between the development of Leydig cell tumors during aging and plasma levels of inhibin-like immunoreactivity. Moreover, no effect on the levels of inhibin-like immunoreactivity in plasma and testicular homogenates could be found in 6- and 10-month-old U-rats after EDS administration and the consequent destruction of the Leydig cell population. These remained within the same range as in untreated rats.

TABLE 1. *Levels of hormones in plasma of Wistar rats (substrain U) measured at various ages**

Age (months)	Plasma levels of		
	Testosterone (mmol/L)	LH (ng/ml)	Estradiol (pmol/L)
4	9.3 ± 1.2	80.9 ± 55.5	23.3 ± 7.2
6	4.0 ± 1.0†	116.3 ± 67.8	23.3 ± 3.5
8	3.3 ± 0.6†	56.0 ± 13.5	24.3 ± 3.2
10	3.0 ± 0†	119.0 ± 13.9	20.7 ± 0.6
12	7.5 ± 1.3	123.0 ± 31.2	21.3 ± 7.5
14	3.0/3.0	<20/180	29/23
16	2.7 ± 0.6†	<20	28.0 ± 7.0
24	4.5 ± 3.8	143/<20/<20	49.7 ± 19.3
18 (Leydig cell implant)	>50/>50	<20/<20	168/76

Values are presented as the means ± SD of three animals unless otherwise indicated. The detection limit of the LH assay was 20 ng/ml.

* In some rats, Leydig tumor cells were implanted intratesticularly at the age of 6 months; these animals were killed at the age of 18 months.

† Significantly different from values measured in 4-month-old U-rats ($P < 0.005$) by Student's t-test modified according to Bonferroni.

Implantation Experiments

Leydig tumor cells were isolated from the testes of 24-month-old U-rats and injected unilaterally into the testes of adult, 6-month-old U-rats or into 4-month-old R-rats. The R-rats were asphyxiated after 6 months. Tumor cells transplanted into the testes of R-rats had not developed into nodules or tumor tissue 6 months after the implantation. There were some indications from palpation that the testes of U-rats had increased in size and that tumors were developing 6 months after implantation. The U-rats therefore were not killed at this stage but were asphyxiated 12 months after implantation. At the time of asphyxiation, both the implanted testes and the contralateral testes were in the scrotal position. The weights of the implanted testes were between 6.0 and 7.2 g, while the weights of the contralateral testes were between 1.3 and 1.4 g (Fig 3A). Histologic examination revealed that the injected testes of U-rats were completely filled with tumor tissue (Fig 3B); both large and small tumor cells were present, and remnants of seminiferous tubules were found within the tumor tissue (Fig 3C). Necrosis was rare in the tumors that developed spontaneously, but necrotic areas containing considerable numbers of macrophages and hemorrhagic areas were present in the implanted tumors. Although measured in only two animals, it appeared that plasma testosterone and estradiol levels were considerably higher in rats with implanted tumor tissue than in 4-month-old U-rats (Table 1). Plasma LH levels were always below the detection level of the assay (Table 1).

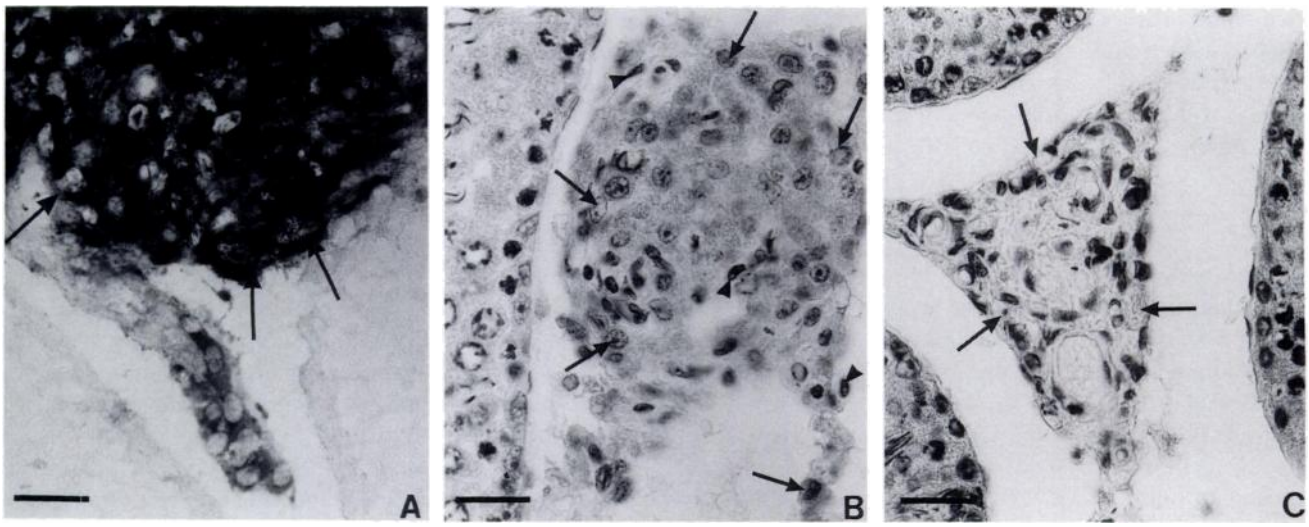

FIG. 2. Enzyme-immunohistochemical localization of 3β-HSD and inhibin-like immunoreactivity in the testes of U-rats. In (B) and (C), cell nuclei are stained with hematoxylin. (A) Frozen section of testis tissue from a 24-month-old rat stained for 3β-HSD. Tumor Leydig cells are indicated by arrows (magnification = 288×, bar = 34 μm). (B) Testis tissue of a 6-month-old rat. Inhibin staining is visible in the Leydig cells located in nodules and in areas devoid of nodules (arrows), whereas other interstitial cells show no staining (arrowheads). Faint staining is found in the seminiferous tubules (magnification = 359×, scale bar = 28 μm). (C) No inhibin-like immunoreactivity can be detected in the remnants of a nodule in testis tissue from a 10-month-old rat (arrows). Faint staining was found in the seminiferous tubules. The absence of Leydig cells is a result of EDS administration (magnification = 378×, scale bar = 26 μm).

Cytotoxic Effects of EDS on Leydig Tumor Cells

Ethane dimethane sulphonate, which destroys Leydig cells in adult rats, was given to three groups of 24-month-old U-rats, and the testis tissue was examined histologically at different times after administration. Two days after EDS administration, the tumor cells in the testes of these rats showed severe signs of degeneration, and many pycnotic nuclei of large and small Leydig tumor cells were found (Fig 4A). However, normal Leydig cell nuclei were occasionally found between the degenerating cells. Nineteen days after administration, a rapid and nearly complete regeneration of Leydig tumor cells was evident. Many mitotically active Leydig cells were observed (Fig 4B), but vacuoles and hemorrhagic and necrotic areas were noticed more frequently than in untreated rats of the same age. Sixty days after administration, the histology of the tumor tissue was no different from that of tumors found in untreated, 24-month-old U-rats: both types of Leydig tumor cells were present.

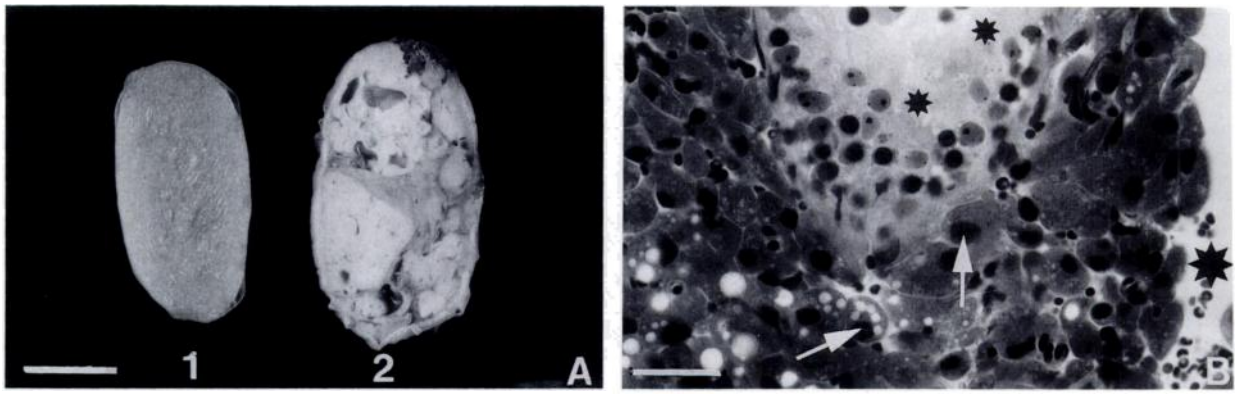

FIG. 3. Leydig tumor cells derived from 24-month-old U-rats were transplanted into the testes of 6-month-old U-rats. Rats were killed at the age of 18 months. (A) Longitudinal sections of contralateral testis (1) and testis into which tumor cells were transplanted (2; magnification = 2×, scale bar = 5 mm). (B) Testis tissue fixed in glutaraldehyde and paraformaldehyde and post-fixed with osmium tetroxide. Leydig tumor cells are indicated by arrows. Remnants of an entrapped seminiferous tubule (small asterisks) and hemorrhagic areas (large asterisk) are visible (magnification = 306×, scale bar = 33 μm).

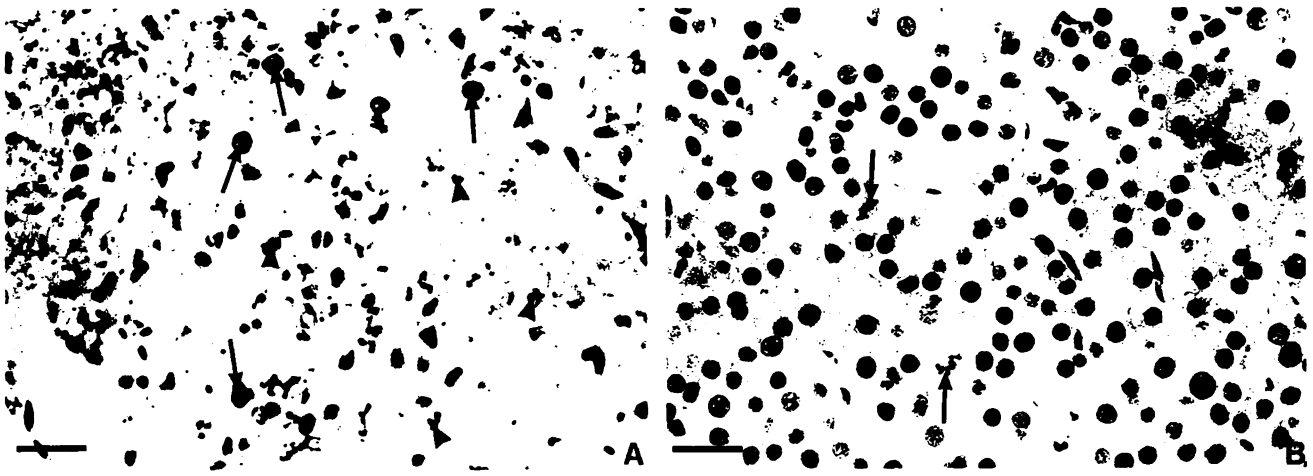

FIG. 4. Twenty-four-month-old U-rats received a single injection of EDS. (A) Two days after EDS administration, massive degeneration of tumor tissue is found (arrowheads). Some large Leydig tumor cells seem to be unaffected (arrows). (B) Nineteen days after EDS administration, the tumor has largely regenerated, and mitosis of Leydig tumor cells frequently occurs (arrows; magnification = 358×, scale bar = 28 μm).

Discussion

The development of Leydig cell tumors occurs at a high incidence and early age in Wistar rats of the U substrain. In these U-rats, small clusters of tightly packed Leydig cells are present in the interstitial tissue at the age of 1 month. We have never observed this type of clustering of Leydig cells in the interstitial tissue of the R substrain of Wistar rats nor have we found tumors in these R-rats. Fetal Leydig cells are often located in clusters and have been reported to persist in normal rats during the first 7 days after birth (Kerr and Knell, 1988; Kuopio et al, 1989). Nevertheless, it is not likely that the clusters or nodules of Leydig cells present in 1-month-old U-rats are fetal Leydig cells since it has been shown recently that these clusters of fetal Leydig cells disintegrate between 7 and 14 days after birth (Kuopio et al, 1989). Our results suggest that the first signs of abnormal Leydig cell development could be the presence of nodules in 1-month-old rats. Moreover, these nodules may be the origin of the larger nodules and tumors. Which factors stimulate the formation of nodules, from which type of interstitial cell the Leydig cell nodules develop, and why the distribution of the nodules is patchy are unknown. The fact that these nodules are already present in prepubertal rats may indicate that the genetic background is important for the formation of the nodules. In contrast to these findings, the first Leydig cell nodules are found around the age of 10 months in Fischer 344 rats in which a high incidence of spontaneous Leydig cell tumor development also occurs (Coleman et al, 1977).

The Leydig cell nodules persisted in older U-rats and gradually increased in size, but in general, the growth rate of the nodules was very slow and mitotic figures of Leydig cells were scarce. Between 12 and 14 months of age, the first Leydig cell tumors were noticed. The morphologic data suggest that the tumor tissue developed from a rapid, focal outgrowth of a Leydig cell nodule and not as a result of a gradual and diffuse increase in the number of Leydig cells. This is supported by the fact that compression of seminiferous tubules was found at the periphery of the tumor, while no remnants of entrapped seminiferous tubules could be detected within the tumor tissue. The latter pattern is characteristic of diffuse hyperplasia (Mostofi and Bresler, 1976). It is not clear what stimulus is causing the rapid outgrowth of nodules into Leydig cell tumors and why this occurred in only 78% of the animals. It is unlikely that an increase in plasma LH levels is responsible for the induction of tumor formation since no rise in LH was found during aging in the U-rats. On the other hand, Huseby (1981) has suggested that the response of Leydig cells to LH stimulation may become abnormal in aging Fischer rats, resulting in the formation of tumors. In this case, a change in LH levels would not be necessary to induce tumor development. Clearly, the role of LH in nodule and tumor development needs to be investigated further. In 22% of the U-rats, nodules persisted up to 30 months without developing into tumors, in contrast to the Leydig cell tumor incidence in Fischer 344 rats that is approximately 100% around the age of 30 months (Coleman et al, 1977).

Two types of Leydig cells were often found within the tumor tissue: large cells that are histologically identical to the nodular Leydig cells and small cells with a more elongated nucleus and scanty cytoplasm. The presence of these two types of Leydig cells has also been reported in the tumor tissue of Fischer 344 rats (Mostofi and Bresler, 1976; Turek and Desjardins, 1979; Morii et al, 1988). Both types

of tumor cells are sensitive to the cytotoxic action of EDS as has also been reported for tumor-bearing Fischer 344 rats (Morii et al, 1988). However, in contrast to Leydig cells in R-rats (Kerr et al, 1985; Molenaar et al, 1985; Bartlett et al, 1986), not all tumor cells in U-rats are killed by EDS. The presence of these EDS-insensitive Leydig tumor cells might explain why the regeneration of the tumor tissue occurred so rapidly (within approximately 19 days). Since a considerable amount of mitotic tumor cells was found 19 days after EDS administration, it is likely that the regeneration process is mainly the result of proliferative activity of the surviving tumor cells. In R-rats, the first new normal Leydig cells develop from precursor cells. This process takes 14 to 21 days (Molenaar et al, 1986; Teerds et al, 1990). Although it is not clear why some Leydig tumor cells are insensitive to the cytotoxic action of EDS, it has been shown that immature Leydig cells are insensitive to this toxicant (Edwards et al, 1988; Rommerts et al, 1988; Risbridger et al, 1989). Hence, it may be possible that some tumor cells are in a less differentiated stage than others and are therefore insensitive to EDS.

The tumor cells have characteristics in common with normal Leydig cells. Inhibin-like immunoreactivity could be detected in normal and in nodular Leydig cells. Similarly, de Jong et al (1990) showed the presence of inhibin-like immunoreactivity in human tumor Leydig cells. However, changes in plasma and testicular levels of inhibin-like immunoreactivity could not be detected during aging when Leydig cell numbers increased in U-rats or when EDS was administered and all Leydig cells were killed. Hence, the possible contribution of Leydig cells and tumor cells to the total content of inhibin-like immunoreactivity in testis and in plasma may be of less importance than expected. In contrast to these findings, other authors have found changes in plasma levels of inhibin in the presence of endocrine tumors, eg, granulosa cell tumors, and have suggested that inhibin is a marker for the presence of endocrine tumors (Lappohn et al, 1989). In the current study, no correlation was found between plasma levels of inhibin-like immunoreactivity and the presence of an endocrine (Leydig cell) tumor, suggesting that more care must be taken in using this parameter as a marker for the presence of an endocrine tumor in men.

Another characteristic that is shared by tumor cells and normal Leydig cells is the presence of the steroidogenic enzyme 3β-HSD, which is essential for androgen synthesis. Although some significant changes in testosterone concentrations were detected in the plasma of U-rats aged from 4 to 24 months, no correlation between plasma testosterone levels and the appearance of large Leydig cell tumors was found. This suggests that testosterone production by nodular and tumor Leydig cells is limited. The tumor cells probably produce an LH-suppressing product that is not likely to be estradiol, since plasma estradiol levels do not undergo any significant changes during tumor development. From studies of Fischer 344 rats, it can be suspected that tumor cells produce significant amounts of progesterone or another steroid precursor of testosterone (Bartke et al, 1985).

Plasma levels of testosterone and estradiol increased significantly only in the U-rats implanted with tumor cells. This increase in plasma testosterone levels may be explained by the fact that when tumors become very large, the amount of testosterone produced by the tumors also increases, giving rise to higher plasma levels than in the other U-rats. Thus, these very large tumors either secrete estradiol in considerable amounts, as has been shown in Fischer rats (Turek and Desjardins, 1979), or the increased amount of testosterone is metabolized to estradiol peripherally.

A surprising finding was that intratesticularly implanted Leydig tumor cells only multiplied in U-rats, indicating that the intratesticular environment in U-rats is different from that in R-rats. It is not likely that the different times of asphyxiation after implantation (12 and 6 months for U-rats and R-rats, respectively) affected the results, since we could not detect any signs of nodule or tumor development in R-rats 6 months after implantation, while palpation indicated that something was developing in the U-rat testes.

In conclusion, a high incidence of Leydig cell tumor development occurs during aging in the Wistar rat U substrain. Abnormalities preceding tumor formation are already apparent at the age of 1 month. This strain of rats may therefore provide a good model to study Leydig cell tumor development and the factors that may influence this process.

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