Animal Model

Overexpression of Aromatase Leads to Development of Testicular Leydig Cell Tumors

An in Vivo Model for Hormone-Mediated Testicular Cancer

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Despite recent advances in diagnosis and treatment of testicular cancer, its causes remain unknown. The most common conditions known to be associated with testicular cancer are cryptorchidism, infertility, and overexposure to pesticides or radiation. Recent studies also indicate hormones may play a crucial role in testicular tumorigenesis. Our studies show that about half of the male transgenic mice overexpressing aromatase in testis were infertile and/or had larger than normal testicles. Gross pathology and histological analysis showed the mice to have Leydig cell tumors, unilaterally or bilaterally. Serum estradiol levels for transgenic mice were at least twice as high as those for nontransgenic mice. Expression of aromatase and estrogen receptor were also very high in testicular tissue of transgenic mice compared to nontransgenic mice. Consistent with increased estrogenic activity in the testicular tissue, we also saw an increase in the levels of genes involved in cell cycle that are regulated by the estrogen. To obtain a better understanding of the biological significance of testicular tumorigenesis, a reliable animal model is necessary to clarify the mechanisms and correlations associated with human cancers. Here we describe such a model, which shows that overexpression of aromatase results in increased estrogen production and a changed hormone milieu, leading to the induction of testicular cancer (Leydig cell tumors). This predictable and useful model is a potential tool for the study of testicular tumorigenesis, hormonal carcinogenesis, synergistic action of other carcinogens on hormone-induced tumors, and tumor dependency on endocrine factors. (*Am J Pathol* 2000, 156:347–353)

Testicular tumors are the leading cancer in men between 20 and 39 years of age, accounting for approximately 20% of neoplasms in this age group. The etiology and pathogenesis of human testicular tumors are poorly defined. Ninety-five percent of all testicular neoplasms arise from germinal cells and are termed testicular germinal cell tumors. Non-germinal cell (ie, Sertoli and Leydig cell) neoplasms account for the remaining 5% of testicular tumors. Leydig cell tumors are the most common tumors of the gonadal stroma.¹ In rodents, reproductive system tumors in general are uncommon, with the few exceptions of Leydig cell and ventral prostatic neoplasms in some rat strains.² Rare spontaneously developing Leydig cell tumors have been reported in non-inbred mice.^{3–8} Testicular tumors were also induced in rodents by chronic administration of estrogens. However, there is no *in vivo* model on which to test the importance of tissue estrogen in testicular tumorigenesis. Our recent studies have demonstrated that estrogen produced locally in the breast tissue is sufficient to initiate preneoplastic changes associated with breast cancer.⁹ In addition, aromatization or *in situ* estrogen production by aromatase has been considered to play an important role in the development of human breast cancer.^{10,11} However, the role of *in situ* estrogen production as a result of aromatase expression in testicular cell tumorigenesis is not clearly defined. Few consistent factors from animal studies have been described to substantiate Leydig cell tumorigenesis. Cryptorchidism has been described as a consistent risk factor for testicular cancer in men. In mice, estrogen or surgically induced cryptorchidism is associated with Leydig cell tumorigenesis.¹² The presence of a functioning pituitary is also necessary for estrogen induction of Leydig cell tumors in the adult mouse.¹²

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Previous reports of Leydig cell tumors in rodents include either spontaneously occurring tumors or induction with chronic estrogen supplementation.¹³ Leydig cell tumors can be induced by pre- and postnatal estrogen treatment in mice, depending on the strain used, and in adult hamsters, but not rats.¹⁴ Each of the following hormonal exposures has been shown to cause testicular tumor formation in rodents: chronic exposure to estrogenic compounds of adult mice (inbred strains A and BALB/c) and hamsters; prenatal exposure to estrogenic compounds of mice and humans; and any treatment or condition that induces cryptorchidism in mice and humans.¹⁴ To obtain a better understanding of the biological significance of testicular tumorigenesis, specifically hormone-induced tumorigenesis, a reliable *in vivo* model is necessary to clarify mechanisms and correlations associated with human cancers. Here we describe such a model, which shows that overexpression of aromatase results in increased estrogen production and a changed hormone milieu, leading to the induction of testicular cancer (Leydig cell tumors).

Materials and Methods

Experimental Animals

The generation of aromatase transgenic mice (previously referred as MMTV-int-5/aromatase transgenic mice) and their characterization have been described previously.⁹ Briefly, aromatase cDNA was expressed under the control of mouse mammary tumor virus promoter (MMTV-LTR), which is active in male reproductive organs as well as in mammary tissues. Male transgenic mice overexpressing aromatase were maintained in a standard colony and were used as breeders. Mice were housed in a centralized animal facility accredited by the American Association for Accreditation of Laboratory Animal Care and the United States Department of Agriculture and maintained according to the recommendations established in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Serum Sample Collection

Blood was collected from mice via retro-orbital sinus every other week or from the cardiac puncture at the time of sacrifice. To collect blood from the retro-orbital sinus, mice were sedated with 100 μ l of 0.1 ml/10 grams of a mixture of fentanyl (0.011 ng/ml), midazolam (0.1 mg/ml), and droperidol (0.5 mg/ml) by intraperitoneal injection. A drop of piperocaine ophthalmic solution was instilled in the eye using a heparinized microhematocrit capillary tube to provide local anesthesia. Pooled serum samples from retro-orbital collection or samples collected using cardiac puncture were used to measure the serum estradiol (E_2) levels.

Serum Estradiol Levels

Serum concentrations of estradiol were measured by double antibody radioimmunoassay using commercially available reagents (Diagnostic Products Corp., Los Angeles, CA). Using an equivalent of 200 μ l of serum in duplicate, the assay had a sensitivity of 2.5 pg/ml and an upper limit of 500 pg/ml. Assaying increasing volumes of serum from 50 to 200 μ l produced a displacement line parallel to the standard curve. Intra- and interassay coefficients of variation averaged <10.% and 6.7%, respectively. Differences in mean estradiol levels were compared, and significance was determined using paired Student's *t*-test.

Histology and Immunohistochemistry

For routine histology, tissues were rapidly removed and fixed in 10% formalin overnight, and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin and eosin (H&E). For immunostaining of testicular tissue, unstained tissue sections were quenched in 3% hydrogen peroxide in phosphate buffered saline (PBS). After washing with PBS the sections were incubated in PBS containing 1% goat serum at room temperature followed by incubation with primary antibody (1:200 dilution) overnight at 4°C. Aromatase polyclonal antibody¹⁵ was a gift from Dr. Evan Simpson of the University of Texas South-west Medical Center (Dallas, TX). Secondary antibodies (goat anti-rabbit secondary antibody and biotinylated goat anti-rabbit secondary antibody) were obtained from Vector Laboratories (Burlingame, CA) and staining with diaminobenzidine tetrahydrochloride was carried out per the manufacturer's instructions. Sections were rinsed with deionized water, counterstained in hematoxylin, and mounted with coverslips for evaluation by light microscopy.

Biochemical Analysis of Testicular Tissue

Total RNA from testicular tissues of aromatase transgenic mice and control nontransgenic litter mates was isolated as described before.¹⁶ Equal amounts of total RNA (exact amounts of total RNA used are indicated in figure legends) from both aromatase transgenic animals and nontransgenic animals were analyzed for both aromatase mRNA and estrogen receptor ($ER\alpha$) mRNA levels by RT-PCR as described before.^{9,17} Initially, the quality of the total RNA from each sample was confirmed using agarose gels and ethidium bromide staining for 28 and 18S RNA. To further demonstrate that an equal amount of total RNA was used from each sample to determine aromatase and $ER\alpha$ expression, we examined the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, as an invariant control by RT-PCR. After initial equalization of the each sample based on GAPDH concentration, a fraction (1 μ l) of the reverse-transcribed mixture, the first step in reverse-transcriptase-polymerase chain reaction (RT-PCR), was used for PCR amplification of GAPDH; the remaining mixture (19 μ l) was used for the amplification of either aromatase or

Figure 1. Morphological and histological appearance of testis from nontransgenic mice and testicular tumors from aromatase transgenic mice. Male reproductive organs from both nontransgenic mice (**A**) and aromatase mice suspected of testicular tumor (**B**) were removed and after initial gross examination tissues were fixed and sections were stained with H&E. Histological comparison of testis from nontransgenic mice and Leydig cell testicular tumor from aromatase transgenic mice (**C** and **D**). Testicular tumors from aromatase transgenic mice with varying levels of tumor cells filling entire testis. Leydig cell tumor filling entire testis (**E** and **F**). Presence of few germinal cells from disorganized seminiferous tubule in the middle of Leydig cell tumor (**G**). Germinal cells are indicated with an **arrowhead**. Presence of disorganized seminiferous tubule in the middle of Leydig cell tumor (**H**). Disorganized seminiferous tubule is indicated with an **arrowhead**. Overgrown necrotic Leydig cell tumor from aromatase transgenic mice (**I**).

ER α PCR product in the same cycle of amplification. The densitometric data from ethidium bromide staining of RT-PCR products on agarose gels were used for calculating the differences in the expression of various mRNA levels in different tissue samples. Data were equalized to GAPDH if required when calculating the differences in the expression of either aromatase or ER α between the samples. All of the analyses were carried out using at least three separate samples. The representative data are presented.

Testicular tissues from both aromatase and nontransgenic control animals were also used to determine the ER α protein levels using Western blot analysis. Briefly, testicular tissue was homogenized in lysis buffer, 60 μ g total protein from each sample was separated on polyacrylamide gels and transferred to nylon membrane. Nonspecific binding of antibodies were blocked by incubation for at least 4 hours at room temperature with Tris-buffered saline (TBS) containing 0.05% Triton X-100 (TBST) and 5% nonfat dry milk. Filters were incubated with respective primary antibodies in TBST-milk overnight at 4°C, and specific binding was visualized by using anti-mouse IgG followed by enhanced chemiluminescent detection (ECL kit; Amersham). The densitometric data from Western blots (X-ray image of chemiluminescent

proteins) were used for calculating the differences in the expression of receptor protein levels in various samples.

Results

Clinical History and Pathology

Originally, select male breeders from this colony were suspected of being infertile because of poor reproductive performance. The ages of these mice ranged from 6 to 12 months. About half of the aromatase male breeders ($n = 30$) were determined to be infertile. On close examination some of these mice were found to contain larger than normal testicles (Figure 1, A and B). Grossly, the testicular tumors from aromatase transgenic mice ranged in size from 1 to 3 cm in diameter. Some tumors were bilateral and well circumscribed, having a soft to firm consistency. The cut surface of the tumor was homogeneous and yellow-tan (data not shown). Only the larger tumors were associated with necrosis. Histologically, these tumors were made up of large interstitial cells that were polygonal in shape, with round nuclei and abundant clear to granular eosinophilic cytoplasm. The supporting stroma was highly vascular with multiple cystic areas

Figure 2. Expression of aromatase mRNA in nontransgenic and aromatase transgenic testicular tissue. Total RNA (100 ng) from testicular tissue of nontransgenic mice (NT) and aromatase transgenic mice (T) were analyzed for the expression of aromatase mRNA using RT-PCR analysis as described in materials and methods. RT-PCR amplification of GAPDH as an invariant control, shown to demonstrate the equal amount of RNA in each sample (inset). Densitometric data from ethidium bromide-stained gels are used for graphical representation.

containing numerous red blood cells (Figure 1, D-I). We have observed tumors in various human Leydig cell tumors; 30 to 40% of cases show intracytoplasmic, eosinophilic rod-shaped crystals of Reinke.¹⁸ However, crystals of Reinke were not observed to be present in any of the mouse tissues we examined. No cellular atypia or mitoses were observed. Gross and histological evaluation of other tissues were normal. These results clearly suggest that the pathological changes were restricted to testicular tissue.

Aromatase Is Overexpressed in Testicular Tissue

Our previous studies, as expected, showed the overexpression of aromatase in transgenic mammary glands, so we examined whether transgene is similarly overexpressed in testicular tissue. Our results using RT-PCR analysis (Figure 2) show that, compared to aromatase mRNA levels in testicular tissue of control animals, the levels of aromatase mRNA in testicular tissue of aromatase animals are almost fourfold higher. These results suggest that increased aromatase expression may play a role in change in the hormonal milieu of testicular tissue and may also affect circulating hormonal levels.

Serum Estradiol Levels Are Higher in Transgenic Males

To determine whether the overexpression of aromatase in testicular tissue changes the hormonal milieu in these

Figure 3. Immunohistochemical localization of aromatase in Leydig cell tumor. Sections from normal testis (A) and testicular tumors from aromatase transgenic mice (B and C) were reacted without and with aromatase primary antibody. No reaction was observed with secondary antibody alone without the primary antibody in either normal or aromatase testis (A and B). Immunoreactive aromatase was present within interstitial Leydig cells and absent from other cells (C).

animals, serum estradiol levels were estimated. Results clearly indicate that estradiol levels are significantly higher for transgenic mice in comparison to nontransgenic mice. The serum estradiol levels for transgenic mice were at least twice as high as those for nontransgenic mice. Mean estradiol levels for transgenic and nontransgenic mice were 5.71 and 2.55 pg/ml, respectively. Differences in mean estradiol levels were compared, and significance was determined by paired Student's *t*-test ($P < 0.001$).

Leydig Cells in Tumor Express Aromatase

To characterize the histological features of Leydig cell tumors and to verify the cellular localization of aromatase overexpression, we carried out immunohistochemical staining for the aromatase protein expression. In general, the testes from transgenic mice were found to contain immunoreactive aromatase within the cytoplasm of interstitial Leydig cells (Figure 3C). The pattern of staining within the testicular tissue was not uniform and ranged from focal to locally extensive in distribution. Immunoreactive aromatase was present within the interstitial Leydig cells and absent from other interstitial cells such as Sertoli cells, fibroblasts, and cells within the seminiferous tubules. Areas of intense staining were noted in the pe-

Figure 4. Expression of estrogen receptor α protein and mRNA and in nontransgenic and aromatase transgenic testicular tissue. Equal amount of protein (60 μ g) from nontransgenic (NT) and aromatase transgenic (T) testicular tissue were analyzed by Western blot analysis using specific primary antibodies (**top panel**). Total RNA (200 ng) from testicular tissue of nontransgenic mice (NT) and aromatase transgenic mice (T) was analyzed for the expression of ER α mRNA using RT-PCR analysis as described in materials and methods (**inset, bar graph**). RT-PCR amplification of GAPDH as an invariant control is shown to demonstrate the equal amount of RNA in each sample. Densitometric data from ethidium bromide stained-gels are used for graphical representation (**bottom panel**).

riphery of the tumor tissue, whereas areas of light to no staining were observed in the more central areas. In addition, the degree of immunostaining appeared to be strongest in testes with more advanced stages of neoplasia. Nontransgenic tissue showed very weakly positive reactivity with aromatase antibody, indicating very low expression of aromatase (data not shown). The negative controls (without primary antibody) of testicular sections from nontransgenic, nonsyngeneic mice were negative for aromatase (Figure 3, A and B).

ER α Overexpressed in Transgenic Testicular Tissue

Our previous studies have shown that overexpression of aromatase in transgenic mammary glands leads to up-regulation of ER α as well as induction of various histopathological changes mammary epithelial cells.^{9,19} Therefore, we have examined whether overexpression of aromatase in testicular tissue that results in increased circulating estrogen levels also leads to up-regulation of ER α . Our results (Figure 4, top panel) show that, compared to ER α protein levels in testicular tissue of control animals that are too low to be detected, the levels of ER α protein in testicular tissue of aromatase transgenic ani-

Figure 5. Expression of cyclin D1 and PCNA protein in nontransgenic and aromatase transgenic testicular tissue. Equal amounts of protein (60 μ g) from nontransgenic and aromatase transgenic testicular tissue were analyzed by Western blot analysis (**inset, bar graph**). Densitometric data from Western blot X-ray films are used for graphical representation.

mals is very high. Similarly, the expression of ER α mRNA is fourfold higher in aromatase transgenic mice testicular tissue compared to nontransgenic testicular tissue. These results suggest that increased biosynthesis of estrogen as a result of aromatase overexpression leads to up-regulation of ER α in testicular tissue.

Up-Regulation of Cell Cycle Genes

To determine whether overexpression of aromatase in testicular tissue leads to changes in estrogen-dependent cell cycle proteins, we estimated the levels of cyclin D1 in testicular tissues from transgenic and control nontransgenic mice. The data presented in Figure 5 clearly show that the testicular tissue cyclin D1 protein level in transgenic mice is about threefold higher than that in control animals. No difference was observed in the levels of PCNA between nontransgenic and transgenic tissue. These results suggest that estrogen-mediated epigenetic changes may play a significant role in testicular tumorigenesis.

Discussion

The results of this study clearly demonstrate that the overexpression of aromatase plays a significant role in the formation of Leydig cell tumors and that mouse Leydig cells are a target for estrogenic action and involved in estrogen-mediated tumorigenesis. Our studies also show overexpression of aromatase in Leydig cells. Previous studies have suggested that the pathophysiology of Leydig cell tumors in man is based on a primary Leydig cell abnormality.²⁰ The presence of ER α in human testicular Leydig cell tumors is consistent with the Leydig cells being the source of estrogen in both rodent and human

testis.^{21,22} Recent studies by Berensztein et al²³ and Valenski et al²⁴ with human tissue samples have shown that aromatase activity and estrogen synthesis is higher in Leydig cell tumor fraction than in normal tissue surrounding the tumor of the same patient. Studies by Kermal et al²⁵ have shown further that estradiol-producing tumors in men responded well to human chorionic gonadotropin (hCG) administration in regulating aromatase activity and estradiol production. Our studies clearly show the level of expression of ER α and aromatase are higher in the testicular tissue of these mice. In contrast, the expression of both aromatase and ER α was lower in testicular tissue of nontransgenic mice (Figures 2–4). This study and a number of previous animal and human studies clearly suggest that aromatase may play a critical role in Leydig cell tumorigenesis.

Estrogen plays a critical role in regulating the G1 cyclins in a number of target tissues. Our previous studies with mammary and testicular tissue and other studies with breast cancer cells^{26,27} have shown that estrogen also plays direct role in activation of genes involved in the G1 phase of the cell cycle. These studies have shown that estrogen induces cyclin D1 levels significantly in mammary tissues, whereas the levels of cyclin E, cdk2, and the p21 and p27 cdk inhibitors are relatively constant. However, the p21 cdk inhibitor shifts from its association with cyclin E-cdk2 to cyclin D1-cdk4, thus suggesting that cyclin D1 has an important role in steroid-dependent cell proliferation and that estrogen, by regulating the activities of G1 cyclin-dependent kinases, can control the proliferation of breast cancer and other hormone-dependent cells.^{26,27} Cyclin D1 is a very important cell cycle protein that has been demonstrated to play a significant role in mammary gland development and human mammary carcinomas. Recent work has postulated that cyclin D1 binds directly to ER α s and potentiates transcription of ER α -regulated genes.²⁷ Our results not only confirm increased ER α expression in testicular tissue, but also show that serum estradiol levels are significantly higher in transgenic mice than in nontransgenic litter mates. This finding indicates that enhanced circulating estrogen production does occur. The increased estradiol levels may be due to the increased aromatization and direct secretion from the Leydig cells. Our expression studies directly support this notion. Based on our observations we suggest that an enhanced ER α phenotype of Leydig cells and increased peripheral aromatization may contribute to the Leydig cell tumorigenesis.

One advantage of using this transgenic mouse model, in contrast to previous animal models, is that this is a non-invasive model that does not use exogenous estrogen administration. We believe the spontaneous development of testicular tumors in these transgenic mice better characterizes the natural physiological and molecular progression of events that occur in humans who develop testicular tumors, specifically Leydig cell tumors. In conclusion, the results from this study provide the first qualitative and quantitative relationship between testicular aromatase, ER α expression, serum estradiol levels, and Leydig cell tumor development in a transgenic animal model. We have demonstrated that the aromatase

transgenic mouse model mimics the human Leydig cell tumor and should provide future insight into the molecular pathogenesis of Leydig cell tumors and possibly other testicular tumors.

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