Estrogen Receptor-β Expression in Human Testicular Germ Cell Tumors

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ABSTRACT

Purpose: Estrogen exposure has been linked to a risk for the development of testicular germ cell cancers. The effects of estrogen are now known to be mediated by estrogen receptor (ER)-α and -β receptor subtypes, but only ER-β has been found in human normal testis. The goal of the present study was to compare the localization and expression levels of these ER subtypes in testicular germ cell cancers (seminomas and nonseminomatous germ cell tumors) with normal testis. For completeness, expression of androgen and progesterone receptors was also investigated.

Experimental Design: Immunohistochemistry was used to localize the expression of steroid receptors in 39 archival testicular germ cell cancers and 5 morphologically normal testes. Expression of the steroid receptors at the transcript level was semiquantified by reverse transcription-PCR in 5 paired fresh-frozen specimens of normal and neoplastic testes.

Results: ER-α was not expressed in the human normal testis. It was also absent in all of the testicular germ cell cancers studied. In contrast, ER-β was strongly expressed in various germ cells of the normal testis. However, its expression was markedly diminished in seminomas, embryonal cell carcinomas, and in mixed germ cell tumors, at both transcriptional and translational levels. In contrast, ER-β remained highly expressed in endodermal sinus tumors and teratomas. Progesterone receptor, an estrogen-regulated gene, was localized to spermatagonia of the normal testis, but its expression dramatically reduced in seminomas. With the exception of spermatagonia, androgen receptor was found in all of the germ cells of the normal testis, but, aside from trace staining in 3 of 5 endodermal sinus tumor cells, it was not detected immunohistochemically in any other germ cell cancer.

Conclusions: We confirm expression of ER-β, but not ER-α, in normal testicular cells, suggesting that only the former ER subtype mediates the action of estrogen in the human male gonad. Our results provide the first evidence that only ER-β is expressed in testicular germ cell tumors. Its expression is down-regulated in seminomas and embryonal cell carcinomas but remains high in endodermal sinus tumors and in teratomas. The observed differences in ER-β expression levels among different testicular germ cell tumors may reflect divergent pathways of differentiation/dedifferentiation of these neoplasms from a common precursor. Collectively, these findings provide a possible mechanistic link between estrogen exposure and testicular cancer risk.

INTRODUCTION

Testicular germ cell tumors are the most common malignancy in males between 15 and 34 years of age, and represent a major cause of death attributable to cancer in this age group (1). Every year, ~7400 new cases of testicular tumors are diagnosed in the United States. The incidence of this type of cancer has increased progressively throughout the twentieth century (2). Germ cell tumors can be subdivided into seminoma and NSGCTs, which consist of embryonal cell carcinoma, choriocarcinoma, yolk sac tumor, and teratoma. Neoplasms that contain more than one tumor cell components, e.g. seminoma and embryonal cell carcinoma, are referred to as mixed germ cell tumors. Seminoma and NSGCTs not only present with distinctive clinical features, they also differ with respect to therapy and prognosis (3).

Whereas the etiology of testicular germ cell cancers remains undefined, exposure to certain hormones (in particular estrogen) at the time of testicular differentiation in utero has long been implicated as a risk factor for developing these neoplasms (4). An earlier case-control study (5) demonstrated first trimester exposure of the mother to exogenous estrogen produced a 8-fold rise in risk for testicular cancer in the son. More recently, other investigations (6, 7) confirmed increased

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4 The abbreviations used are: NSGCT, nonseminomatous germ cell tumor; ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGCNU, intratubular germ cell neoplasia of unclassified type.
incidences of testicular germ cell tumors in men with maternal exposure to diethylstilbestrol. Furthermore, a higher incidence of testicular cancer was observed among men exposed prenatally to excess maternal hormone as in the case of preterm birth, being a twin, and first births among young mothers (6, 8). In contrast, a protective effect was noted with in utero exposure to presumed low-estrogen states, such as those associated with heavy cigarette use and bleeding/threatened miscarriage (6). Adult exposure to excess estrogen as in conditions associated with development of gynecomastia (9), treatment of prostate cancer with estrogen (10), or occupational contact with estrogen mimics (e.g. organochlorines; Refs. 11, 12) have all been linked to a higher risk for testicular cancers. Experimentally, prenatal and postnatal administration of estrogens consistently causes testicular tumor development in some strains of rodents (13).

Despite the above-cited epidemiological reports linking estrogens to testicular cancer the mechanisms by which estrogens contribute to the pathogenesis of these neoplasms remains unclear. It has been reported that serum estrogen levels are elevated in patients with testicular germ-cell cancers (14, 15), a condition which likely resulted from local production by the tumor tissue, because aromatase activity has been demonstrated in Leydig cells, Sertoli cells, and various germ cells of the testis. Where aromatase activity has been reported to be strongly expressed in the efferent ductules, it has not been localized in testicular germ line or somatic cells of human testis (22). Whereas ER-α is expressed in target cells is mediated by its interaction and subsequent activation of ERs, which are members of the steroid and thyroid hormone receptor superfamily (19). After activation, these receptors, in association with a myriad of coactivators and repressors, act as nuclear transcription factors for targeted genes (20). From a teleological standpoint, the action of a hormone in a given tissue is substantiated by the identification of its specific receptor in that tissue.

Two ER subtypes, -α (the classical ER) and -β (a recently discovered subtype), have been identified and shown to exhibit functional differences as well as tissue-/cell type-specific expression (21). Recent investigations have demonstrated differential expression of ER-α and ER-β, and their variants in the human normal testis (22). Whereas ER-α has been reported to be strongly expressed in the efferent ductules, it has not been localized in testicular germ line or somatic cells of human testis (22). In contrast, ER-β immunostaining has been observed recently in spermatogonia, spermatocytes, and spermatids, as well as somatic cells, leading to the conclusion that the ER-β subtype is the principal mediator of estrogen action in promoting germ cell survival and development (17, 22–25).

Whereas the strong expression of ER-β in normal male germ cells is now established, analogous information is not available for testicular germ cell tumors. Earlier studies that used radioligand-binding methodologies for ER detection lacked the ability to discern which receptor subtype was expressed in testicular cancers (26–28). Similarly, immunohistochemical studies, conducted to localize ER in testicular tumors, used only antibodies directed against the α subtype (28).

In the current investigation we compared the expression ER-β, ER-α, AR and PR in 39 human testicular germ cell malignancies with that found in normal testicular specimens. ER-β expression was studied at the immunohistochemical level using a well-characterized, ER subtype-specific antibody, directed against the F domain of the human receptor (29). Furthermore, expression of these receptors was studied at the transcript level in 5 testicular germ cell cancers and results compared with expression levels in morphologically normal testicular tissues.

**MATERIALS AND METHODS**

**Testicular Tissues.** Thirty-nine archival cases from radical orchiectomies and 5 from simple orchiectomies (ages 21–57) were selected from the surgical pathology files of the University of Massachusetts Medical School. These specimens were collected during a 6-month period (at the end of 2000 and the beginning of 2001). The collection included 17 seminomas, and 22 NSGCTs consisting of 10 mixed germ cell tumors with embryonal cell components, 5 with endodermal sinus components, 3 cases of embryonal carcinoma, and 4 cases of mature teratomas. Serial sections, 5–6 μm, were cut from paraffin-embedded specimens, mounted on glass slides, and stored unbaked until immunohistochemistry was performed.

In addition, transcript expression was studied with mRNA extracted from 5 simple orchiectomy surgical specimens and compared those in extracts from morphologically “normal” testicular tissues from the ipsilateral testis. On the basis of gross appearance, tumor and “normal” tissues were separately dissected, and each piece was divided. One half of each sample was snap frozen in liquid nitrogen and stored at −80°C until ready for studies of receptor transcript expression. The other half was frozen in OCT embedding compound (Triangle Biomedical Sciences, Durham, NC) from which frozen sections were cut to confirm the diagnoses. Histological study revealed that 3 were pure seminomas, 1 was classified as a mixed germ cell tumor with embryonal cell components (embryonal + seminoma), and 1 was a mixed tumor with endodermal cell components (yolk sac + seminoma).

**Immunohistochemistry.** Primary antibodies used included GC17 polyclonal rabbit anti-ER-β (Biogenex, San Ramon, CA), mouse monoclonal anti-ER-α (Nova Castra, Newcastle-upon-Tyne, United Kingdom), rabbit polyclonal anti-AR (Upstate Biotechnologies, Lake Placid, NY), and rabbit polyclonal anti-PR (Santa Cruz Biotechnology, Santa Cruz, CA). The GC17 polyclonal rabbit anti-ERβ primary antibody has been fully characterized in our laboratory (29). The antibody recognizes the first 22 amino acid of the F-domain of the wild-type ER-β, exhibits high specific affinity for the wild-type receptor, and shows no cross-reactivity with ER-α.

The slide-mounted sections were baked for 1 h at 60°C, then deparaffinized with two xylene washes. They were rehydrated through a series of graded alcohol washes and finally rinsed in water. Heat-induced antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0). After the sections returned to room temperature, they were washed with water. Endogenous
peroxidase was blocked with 3% hydrogen peroxide in methanol for 30 min, and the slides were then rinsed and set in PBS. To reduce nonspecific antibody binding, the slides were incubated for 10 min in Power Block (Biogenex) at room temperature, then rinsed copiously in water and again set in PBS. Before application of the primary antibody, the sections were incubated in 10% normal goat serum for 15 min. The 10% goat serum was shaken off, and the sections were incubated overnight at 4°C with the primary antibody. Slide washing was performed by first briefly rinsing in PBS, then gently agitating for 20 s in Optimax detergent (Biogenex), and by finally thoroughly rinsing with PBS for 10 min on a rotator platform. The sections were incubated with the secondary antibody for 30 min at 37°C, and the wash sequence was repeated. The sections were incubated with avidin and biotinylated horseradish peroxidase from the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) for 1 h at room temperature, and the wash sequence was again performed. Staining was visualized using Sigmafast 3′, 3′-diaminobenzidine (Sigma, St. Louis, MO). The slides were then rinsed with water, counterstained with 10% hematoxylin, and progressively dehydrated through graded alcohols and xylene.

Human prostate cancer sections found previously to express ER-β and AR (29) were used as positive controls for the GC17 antibody. ER-α- and PR-positive human breast cancer specimens were used as positive controls for the ER-α and PR antibodies, respectively. All of the sections were independently reviewed by two pathologists (J. Z. and I. L.), and immunostaining was qualitatively graded as weak, moderate, or strong.

**RNA Isolation and RT-PCR.** Tumor and normal tissues from the 5 paired testicular specimens prospectively collected were homogenized and extracted for total tissue mRNA using the RNA Stat-60 reagent (Tel-Test Inc., Friendwood, TX) according to protocols provided by the manufacturer. The quality of each total RNA sample was checked and controlled by the following steps: (a) measurement of absorbance; (b) running of a denaturing RNA gel capable of detecting possible RNA degradation, as judged by the integrity and intensity of the 18S and the 28S rRNA signals; and (c) conducting a semiquantitative RT-PCR for the 18S rRNA at low cycle numbers. One μg of total cellular RNA was reverse-transcribed using the GeneAmp RNA PCR kit (Perkin-Elmer, CT), and 2 μl of the resulting cDNA was used in each PCR.

Intron-spanning primers were either obtained from published literature or designed using the Primer3 Output program. Primer sequences for ER-α, ER-β, PR, AR, and GAPDH are given in Table 1. PCR conditions were optimized for quantification of relative message contents under nonsaturating conditions. Preliminary experiments were conducted to ensure linearity for all of the semiquantitative procedures. Hot start PCR using AmpliTaq Gold DNA polymerase (Perkin Elmer) was used in all of the amplification reactions. The enzyme was activated by preheating the reaction mixtures at 95°C for 6 min before PCR. This protocol was chosen to minimize nonspecific product amplification. The routine PCR program was 30 cycles of 1 min at 94°C, 1 min at 60°C (annealing temperature), and 1 min at 72°C with the following modifications: (a) amplification for ER-β cDNA used an annealing temperature of 58°C; (b) amplifications of ER-α cDNA and AR cDNA were carried out at an annealing temperature of 55°C; (c) cycle number for ER-α cDNA amplification was 35; and (d) cycle-number for GAPDH was 28. Amplification of the correct sequence was verified by direct DNA sequencing of each PCR product from at least two different samples.

**Statistical Analyses.** One-way ANOVA was used to analyze whether there was a significant difference among the various group means. A multiple range test using the Tukey-B procedure was used to compare the individual group means. Significance was set at $P < 0.05$.

**RESULTS**

**Immunohistochemistry; ER-β**

**Normal Testes.** In the 5 specimens of normal testicular tissues and in the majority of morphologically normal tissues adjacent to cancers, intratubular germ cells demonstrated high expression of ER-β (Fig. 1A). Specifically, strong expression of the receptor was evident in spermatagonia, primary and secondary spermatocytes, but less so in elongate spermatids. ER-β immunostaining was in general absent in Sertoli cells (Fig. 1A).

**Seminoma.** In 14 cases, cancer cells were arranged in diffuse sheets separated by thin delicate septae visible at higher magnification. In 3 cases, foci of intratubular seminoma were also present. The overall intensity of ER-β expression was reduced or absent in the majority of tumor cells when compared with expression in germ cells of the normal testicular tissues in

<table>
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<td>528</td>
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<td>ER-α</td>
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<td>nt.41–60</td>
<td>650</td>
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<td></td>
<td>ER-α-2.5′ATC AAT GGT GCA CTG GTT GGG3′</td>
<td>nt.671–690</td>
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<td>PR</td>
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<tr>
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<td>PR-2.5′TGC TCG CCT AGT TGA TTG3′</td>
<td>nt.2330–2349</td>
<td>533</td>
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5 Internet address: http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi.
Fig. 1 A, a normal human seminiferous tubule immunostained for ER-β. Note the strong nuclear staining for the receptor in spermatogonia (double arrow) and in primary, secondary spermatocytes, and round spermatids. Staining is absent in Sertoli cells (single arrow), ×450. B, typical immunostaining of ER-β in a seminoma. Left, in this low-power field lightly stained cells are seen interspersed with negatively stained tumor cells, ×250. In a minority of seminomas receptor staining of tumor cells was more abundant and stronger than seen in this example. Right, the lightly stained tumor cells are better visualized in this representative higher magnification of the left panel. Staining appears to be localized in dispersed nuclear euchromatin of tumor cells. Compare this staining pattern and intensity with the strong even immunostained nuclei of normal testicular germ cells, ×425. C, ER-β immunostaining in an endodermal sinus component (yolk sac) of a mixed tumor. Note the strong nuclear staining in the tumor cells, ×250. D, representative section of an embryonal carcinoma immunostained for ER-β. Staining for the receptor is completely absent in embryonal cells forming a solid pattern. The same staining was found when embryonal components were present in mixed tumors, ×425. E, ER-β immunostaining in a mature teratoma. Note the strong nuclear uniform staining for the receptor in cells that have formed cartilage as well as in surrounding undifferentiated stromal cells. Strong receptor immunostaining was also observed in epithelial cells (not shown in this field), ×275. F, a normal human epididymal tubule for ER-α. Strong nuclear staining is evident in most cells lining the tubule. In contrast, staining for ER-α was absent in all cells of the normal testes and in all testicular germ cell cancers, ×300. Counterstained with hematoxylin.
12 of the 17 seminoma specimens (Fig. 1B). In the remaining 5 specimens only 25–50% of the tumor cells expressed the ER-β, whereas in most cells receptor staining was absent. When nuclear pleomorphism was present in a seminoma (anaplastic cells), a marked total absence of ER-β staining was strikingly evident in these atypical tumor cells. No differences in staining intensities were evident when solid tumors were compared with intratubular cancers. Variable receptor staining of lymphocytic infiltrates was found in the majority of cases.

Endodermal Sinus (Yolk Sac Tumors). Five mixed germ cell tumors had an endodermal sinus component. In all 5 of the cases, either a reticular or a glandular pattern predominated in the endodermal sinus component. Irrespective of the pattern in this component, there was strong expression of ER-β in neoplastic cells (Fig. 1C).

Embryonal Carcinoma. Both solid and glandular patterns were found in all 3 of the cases. In solid areas immunostaining for ER-β was either absent or very weakly positive in areas where tumor cells are arranged in a solid pattern (Fig. 1D), but was moderate in regions where glands were formed.

Mature Teratoma. Strong ER-β immunostaining was present in all 4 of the specimens, and was of equal intensity in stromal and epithelial components (Fig. 1E).

Mixed Germ Cell Tumors. Ten mixed cell tumors had both seminoma and embryonal cell components. In these cases, comparison with normal testicular germ cells showed that immunostaining for ER-β was weak in seminoma cells and greatly reduced or completely absent in embryonal cells.

Immunohistochemistry; ER-α, AR, and PR

Normal Testes. There was no expression of ER-α in any somatic or germ cell component in these specimens. However, strong nuclear staining for both ER subtypes was present in the nuclei of efferent tubular epithelial cells and in tubular principal cells of the epididymis (Fig. 1F). In 3 specimens there was sufficient tissue available to also study the localization of AR and PR. AR was highly expressed in 2 of 3 specimens where it was localized to the nuclei of primary and secondary spermatocytes, and mature spermatocytes, but not in the nuclei of spermatagonia. In addition, weak AR immunostaining was also present in Sertoli and Leydig cell nuclei. PR immunostaining was only detected in the nuclei of spermatagonia (Fig. 2A).

Seminoma. ER-α was not expressed in any seminoma cells. AR was also not visualized in any of the tumor cells. In 4 cases, a few immunopositive PR cells were found scattered among fields of negatively stained tumor cells (Fig. 2B).

Endodermal Sinus Tumors. Staining for ER-α or PR was not present in neoplastic cells. There was trace AR expression in cancer cells of 3 of 5 cases. There was no expression of PR seen in any of the tumors or in cells of adjacent normal testicular tissues.

Embryonal Carcinoma. Irrespective of the pattern formed by embryonal cells, ER-α, AR, or PR staining was not present.

Mature Teratoma. No ER-α, AR, or PR immunostaining was demonstrated in neoplastic cells.

Mixed Germ Cell Tumors. ERα and AR were also absent in these malignant germ cells. There was trace expression of PR in seminoma cells in 4 of 9 cases, but staining was absent in all of the embryonal cell components.

Receptor Transcript Levels Measured by Semiquantitative RT-PCR

Semiquantitative RT-PCR was used to assess the relative abundance (target mRNA signal over signal of GAPDH) of ER-β, PR, AR, and ER-α in total mRNA extracts of 3 pure seminomas and 2 mixed germ cell tumors (with significant seminoma cell component), and compared with relative receptor
transcript abundance in the ipsilateral normal testes in the same patients. ER-β mRNA was found to be expressed in all 5 of the normal testes, but expression was notably reduced (approximately 50%) in the 5 tumor specimens (Fig. 3A). Similarly, PR mRNA was present in normal testicular specimens but was barely detectable in all 5 of the tumors (Fig. 3B). In contrast, relative AR mRNA abundance was comparable between normal and cancerous testicular specimens (Fig. 3C).

**DISCUSSION**

Despite past epidemiological findings that linked estrogens to the development of testicular germ cell tumors (see “Introduction”) research in this area was hampered, in part, by the inability to localize ER in the testis or testicular neoplasms. This led many investigators, in the past, to conclude that ERs do not play a role in either normal germ cell function or malignant transformation (26, 27).

In 1996, a second ER subtype, termed ER-β, was discovered and found to be highly expressed in the testes and in tissues of the male reproductive tract (21). In the current study, we have confirmed recent findings that ER-β, but not ER-α, is expressed in normal human testis (22). Moreover, we find, in agreement with others (24), that ER-β is strongly expressed in spermatogonia, and primary and secondary spermatocytes. Because these cell types do not express ER-α, any direct effects of estrogen on them are likely mediated by ER-β. Intense attention has also been focused on the widespread exposure of gonadal cells to environmental “endocrine disruptors.” Such disruptors, including xenoestrogens and estrogen mimics, have been identified as possible culprits in male reproductive disorders, ranging from cryptorchidism and hypospadias to declining sperm counts (30). The localization of ER-β in normal testicular germ cells represents a potential mechanism through which these environmental estrogens affect male reproductive function at the testicular level.

Although in utero or adult exposure to estrogens has been implicated in male germ cell carcinogenesis, little is known of its mechanism of action. Expression of ER-β in spermatogonia, the purported precursor of germ cell cancers, is of particular interest, because these cells lie outside of the blood testis barrier maintained by the tight junctions of Sertoli cells (31). Without the protection afforded by this barrier, spermatogonia, unlike other germ cells within the seminiferous tubules, are subjected to influences of the systemic milieu. Significant levels of ER-β expression in spermatogonia may offer protection against circulating carcinogens, because this receptor subtype has been showed to regulate specific detoxification enzymes (32). Conversely, ER-β may mediate estrogen-induced carcinogenesis, because exposure of rat testis to estrogen was found to result in oxidative DNA damage, as evident from accumulation of 7,8-dihydro-8-oxo-2'-deoxyguanosine in testicular cell nuclei, via an ER-mediated process (18).

In the current study, we consistently detected decreased expression of both ER-β RNA and protein in seminomas, mixed cell tumors, and in embryonal carcinomas when compared with normal testes. Down-regulation of ER-β action is additionally suggested by decreased PR mRNA and protein expression seen in the same tumor-types, because the PR gene is known to be regulated by estrogen (33). In contrast, the immunohistochemical expression of ER-β remains strong in endodermal sinus
tumors and in mature teratomas. Conceptually, the differences in ER-\(\beta\) expression among seminomas, embryonal carcinomas, and other NSGCT tumors may be explained by the Tetrahedron model of histogenesis (3). This model proposes that all germ cell tumors arise from a common precursor called the IGCNU. IGCNU cells are considered to be transformed cells that arise directly from germinal cells. After transformation, the IGCNU cells give rise to seminomas, which are believed to be the precursors of all other germ cell cancers. Implied in this scheme is that cytological and immunohistochemical differences noted between seminomas and NSGCTs represents the acquisition and/or deletion of traits seen as this neoplasm evolves into other distinct tumor types. Viewed in this context, the down-regulation of ER-\(\beta\)-seen in seminomas, the complete loss of receptor expression in embryonal cell tumors, and the persistent strong expression in endodermal sinus tumors and teratomas may therefore reflect divergent pathways of differentiation/dedifferentiation as tumor cells progress in their development.

Whereas the functional consequences of differential ER-\(\beta\) expression among different germ cell tumors are not currently known, data from animal models and human cell culture studies suggest that ER-\(\beta\) may control and limit cell proliferation during breast, prostate, and colon cancer progression (34–36). Taken together, these considerations and our findings have led us to hypothesize that exposure to estrogens or estrogen-mimics, in some as of yet undefined manner, diminishes the ER-\(\beta\)-mediated growth restraint in spermatagonia, which favors unscheduled cell proliferation. The affected spermatogonia or their descendants may then be able to escape normal cell cycle regulation and be at a higher risk of undergoing malignant transformation. Furthermore, the diminution of ER-\(\beta\) expression in seminomas, the purported precursors of NSGCTs, its near total silencing in embryonal tumors, and the re-expression of the receptor in endodermal sinus tumors and teratomas may reflect the progressive evolution of these germ cell cancers into various types.

In conclusion, our findings provide a link by which estrogens and estrogen-like compounds may influence the genesis and progression of testicular germ cell cancers through their interaction with ER-\(\beta\).

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