Estrogen-induced growth inhibition of human seminoma cells expressing estrogen receptor β and aromatase

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Abstract

It is now well established that estrogens participate in the control of normal spermatogenesis and endogenous or environmental estrogens are involved in pathological germ cell proliferation including testicular germ cell tumors. Studying a human testicular seminoma cell line, JKT-1, we show here that 17β-estradiol (10−12 to 10−6 M) induced in vitro a significant dose-dependent decrease of cell growth. This antiproliferative effect was maximum after 4 days of exposure at a physiologically intratesticular concentration of 10−9 M, close to the Kd of ER, and reversed by ICI 182780, an ER antagonist, suggesting an ER-mediated pathway. By RT-PCR and Western blot we were able to confirm that JKT-1, like tumor seminoma cells and normal human testicular basal germ cells, expresses estrogen receptor β (ERβ), including ERβ1 and ERβ2, a dominant negative variant, but not ERα. Using immunofluorescence and confocal microscopy, ERβ1 was observed as perinuclear intracytoplasmic spots in JKT-1 and tumoral seminoma cells without significant translocation of ERβ into the nucleus, under 17β-estradiol exposure. Double staining observed by confocal microscopy revealed that ERβ colocalized in JKT-1 cells with cytochrome C, a mitochondrial marker. We report for the first time the expression of a functional aromatase complex in seminoma cells as assessed by RT-PCR, Western blot and enzymatic assay. Seminoma cells are able to respond to estrogens through a possible autocrine or paracrine loop. These preliminary results support estrogen-dependency of human testicular seminoma, the most frequent tumor of young men, and suggest potential pharmacological use. Whether this estrogen control, however, involves an ERβ-mediated stimulation of cell apoptosis and/or an ERβ-mediated inhibition of cell proliferation, remains to be further determined.

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Introduction

Estrogens are important regulators of cell proliferation in many reproductive and extra-reproductive tissues in both sexes. This control is cell-specific, depending on the expression of two related estrogen receptors (ERs), ERα and ERβ, which are transcriptional factors (see review in Nilsson et al. 2001). There is compelling evidence that ERα mediates the proliferative effects of estrogens in several so-called estrogen-dependent cancers, such as breast, uterus and ovary. Of particular interest, ERβ, when coexpressed with ERα, reduces tumor cell proliferation (Speirs 2002), and its loss is associated with advanced tumoral stage in several cancers such as breast (Lazennec et al. 2001), ovary (Rutherford et al. 2000), prostate (Weihua et al. 2001) and colon (Konstantinopoulos et al. 2003).

Recently it has been suggested that estrogens, the archetype female hormones, could play a role in the control of male germ cell proliferation (see review in Jones & Simpson 2000). Indeed, estrogen is found at a higher level in mature testis than in circulating plasma in relation to its production through testosterone conversion by aromatase complex (Carreau et al. 2002). Of particular interest, knockout (KO) mice deficient in aromatase display infertility with aging (Fisher et al. 1998), and men with aromatase mutation present abnormal spermograms (Simpson 1998). In human testis, gonocytes (fetal germ cells which differentiate into spermatogonia after birth) (Gaskell et al. 2001) and most adult germ cells (Mäkinen et al. 2001) express ERβ. Estrogens have been shown in vitro to control gonocyte proliferation in the rat (Li et al. 1997) and recent analysis of neonatal testes from ERβKO mice has confirmed the link between endogenous estrogens, ERβ and gonocyte survival (Delbes et al. 2004). However, the precise role of estrogens and their receptors (Dupont et al. 2000) and the underlying mechanism(s) in the control of normal and malignant germ cell proliferation remain to be determined.

This issue is of particular concern since environmental estrogens have been blamed for the increasing incidence
of infertility and testicular cancer, the most frequent cancer of the young man (Skakkebaek et al. 1998). Indeed, deleterious effects of adult male reproductive function have been observed after fetal or prenatal exposure to diethylstilbestrol, a potent synthetic estrogen (Walker 1989, Giusti et al. 1995). However, little is known of the possible estrogen-dependency of seminoma, the most frequent malignant testicular germ cell proliferation, partly due to the lack of available in vitro models. We therefore took advantage of a pure human testicular seminoma cell line, JKT-1 (Kinugawa et al. 1998, Roger et al. 2004), to assess the in vitro effects of estrogens on seminoma cell proliferation, to characterize ER subtypes and to test for a functional aromatase complex.

Materials and methods

Tissue preparation and cell culture

Normal and seminoma testicular tissues obtained according to the French ethical guidelines were immediately snap-frozen or fixed and embedded in paraffin wax. The three testicular tumors were classified as pure seminoma by histological analysis and by positive staining for placental alkaline phosphatase (PlAP), a specific seminoma marker (Giwercman et al. 1991). The JKT-1 cells, described as a pure human seminoma cell line (Kinugawa et al. 1998, Roger et al. 2004), were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco BRL). For estrogen stimulation, JKT-1 cells were plated in the above complete medium, estrogen-starved for 24 h in fresh phenol red-free DMEM supplemented with 10% charcoal-stripped serum (3 g charcoal-coated dextran/50 ml FBS), before adding every culture day 17β-estradiol (Sigma), ICI 182780 (Falsodex; Astra-Zeneca, Birmingham, UK) or ethanol as vehicle control. Human granulosa–lutein cells were obtained from the in vitro fertilization center of CHRU of Caen. MCF-7 cells (LGC-Promochem, Molsheim, France), a human breast cancer cell line, were cultured in DMEM 10% serum.

Cell proliferation study

JKT-1 cells were treated with steroids diluted in ethanol and counted for 1–6 days and compared with controls (cells cultured in steroid-free medium with ethanol). Results were expressed as percentages of variation as compared with the control. A non-parametric Mann–Whitney test was used for statistical analysis.

RT-PCR analysis

Total RNAs were prepared from normal human testis, seminoma tumors, JKT-1, MCF-7 and granulosa cells. RT-PCR analysis was performed as described previously (Roger et al. 2004). ER primers (Eurogentec, Seraing, Belgium) were designed from published sequences (Otsuki et al. 2000, Mäkinen et al. 2001, Lambard et al. 2004) (see Table 1). Cycling parameters were as follows: denaturation at 94 °C for 1 min; annealing for ERβ, ERα and aromatase respectively at 58, 59 and 60 °C, for 1 min, and extension at 72 °C for 1 min; 35 cycles were performed then followed by a final extension step of 10 min.

Western blot analysis

Sections of normal testis and seminoma tumors as well as cultured JKT-1, MCF-7 and granulosa cells were directly lysed in 600 µl Brij/NP40 lysis buffer (50 mM Tris–HCl, pH 7-5, 1% NP40, 1% Brij 96, 1 mM Na3VO4, 10 mM sodium fluoride, 10 mM β-glycerophosphate, 2 mM EDTA, 1 mM aprotinin, 25 mM leupeptin, 1 mM pepstatin, 2 mM phenylmethyloxysulfonfluoride). Lysates were sonicated 10 s on ice twice, then centrifuged for 20 min at 22,000 g. Lysates were analyzed by Western blotting as described previously (Defamie et al. 2001) with a rabbit anti-ERα

Table 1 Sequences of the human ER and aromatase primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer</th>
</tr>
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<tbody>
<tr>
<td>hERα F</td>
<td>5′-AGA-CAT-GAG-AGC-TGC-CAA-CC-3’</td>
</tr>
<tr>
<td>hERα R</td>
<td>5′-GCC-AGG-CAC-ATT-CTA-GAA-GG-3’</td>
</tr>
<tr>
<td>hERβ F</td>
<td>5′-TCA-CAT-CTG-TAT-GCG-GAA-CC-3’</td>
</tr>
<tr>
<td>hERβ R</td>
<td>5′-CTG-ACC-ACT-TCC-GAA-GTC-GG-3’</td>
</tr>
<tr>
<td>hERβ2 F</td>
<td>5′-CGA-TGC-TTT-GGT-TTG-GTG-AT-3’</td>
</tr>
<tr>
<td>hERβ2 R</td>
<td>5′-CTT-TAG-GCC-ACC-GAG-TTG-ATT-3’</td>
</tr>
<tr>
<td>Aromatase F</td>
<td>5′-AAA-GGA-AAT-CCA-TGT-TAT-TGG-TGG-3’</td>
</tr>
<tr>
<td>Aromatase R</td>
<td>5′-GTA-TCT-TCT-GTA-GTC-TCT-CAAC-ACCT-GTG-3’</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
antibody (sc-543; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a goat polyclonal anti-ERβ antibody raised against a peptide mapping near the carboxy-terminus of ERβ which recognizes both ERβ1 and ERβ2 and which does not cross-react with ERα (sc-6822; Santa Cruz), a rabbit polyclonal anti-ERα antibody raised against the amino-terminus of ERα (Upstate Biotechnology, Lake Placid, NY, USA) or a mouse monoclonal anti-aromatase antibody (1:500, kindly provided by Dr Saunders, Serotec, UK) which recognizes mammalian aromatase (Turner et al., 2002), in 0.1% Tween 20, 10 mM Tris, 15 mM NaCl, pH 7.4, containing 1% non-fat dried milk.

Immunocytochemical procedures

JKT-1 and MCF-7 cells were prepared for immunofluorescence analysis as described previously (Defamie et al., 2001, Roger et al., 2004). Slides of frozen testicular tissue and tumors were fixed in methanol at −20°C for 7 min, washed twice in PBS and then saturated in PBS/saponine 0.5%/SVF or fetal calf serum 5% for 30 min. ERα and ERβ were detected using a rabbit anti-ERα (Santa Cruz; 200 mg/ml) and a goat anti-ERβ (Santa Cruz; 200 mg/ml). After three washes in PBS/saponine 0.5%, the antibodies were detected using an FITC-labeled anti-rabbit (Dako, 1/50) and an FITC-labeled anti-goat (Dako, 1/50 in PBS with 5% goat serum). Nuclei were stained by propidium iodide (30 µg/ml). Colocalization of ERβ was performed by using a mouse anti-cytochrome C antibody (Becton Dickinson) revealed by an anti-mouse-Texas Red sheep antibody (Amersham). Sections were examined with a confocal laser scanning microscope (Leica TCS SP).

Aromatase activity

Microsomal fractions from JKT-1 and granulosa cells (used as a positive control) were prepared as previously described (Levallet et al., 1998). The aromatase activity was assessed by measurement of 3H2O released from [1β-3H]androst-4-ene-3,17-dione (New England Nuclear, Les Ulis, France) as described before (Lephart & Simpson, 1991). Blank values were obtained from identical incubations in the absence of microsomes. The activity was expressed in pmol 3H2O produced per milligram of protein per hour.

Results

Effects of estrogens on seminoma cell proliferation

We examined first the effects of 17β-estradiol and ICI 182780, an ER antagonist, on seminoma cell proliferation. As shown on Fig. 1A, treatment of JKT-1 cells with estradiol (10−12 to 10−6 M) for 24 h induced a

![Figure 1](https://example.com/figure1.png)

**Figure 1** Effect of 17β-estradiol on JKT-1 cell proliferation. (A) Dose–response study. JKT-1 cells were incubated for 48 h with steroid-free medium or medium containing 10−12 to 10−6 M 17β-estradiol. Competition between ICI 182780 and 17β-estradiol at the final concentration of 10−6 M is also represented. The results are given as means±S.E.M. of three independent experiments, and show the percentage of cell numbers compared with control (steroid-free medium with ethanol). *P<0.05. (B) Kinetic study. Cell proliferation was evaluated during 6 days of culture with or without 17β-estradiol at low concentrations of 10−12 and 10−9 M. The results are given as means±S.E.M. of three separate experiments, with two replicates per experiment considering the percentage of variation of cell number compared with control (steroid-free medium with ethanol). *P<0.05.
dose-dependent inhibition of cell growth, reaching a 30% decrease at $10^{-6}$ M estradiol as compared with control cells cultured without estradiol. Co-treatment with ICI 182780 ($10^{-6}$ M), was able to reverse significantly ($P<0.05$) the inhibition induced by $10^{-6}$ M estradiol alone (Fig. 1B). JKT-1 cells were then cultured for 6 days without reaching sub-confluency with and without low concentrations of estradiol ($10^{-9}$ and $10^{-12}$ M). Relative inhibition of cell proliferation increased from the first day to the sixth day, reaching 30% on the fourth day at the physiological concentration of $10^{-9}$ M.

**ER expression in seminoma cells**

Considering ERα, neither the transcript nor the protein could be identified in the pure seminoma cell line, JKT-1 (Fig. 2A and B) compared with MCF-7, a human breast cancer cell line used as positive control and normal testicular tissue, which also expressed a transcript at the expected size. Consistently, indirect immunofluorescence analysis confirmed the lack of expression of ERα in JKT-1 cells (Fig. 2C) compared with a strong nuclear staining in MCF-7 cells. Considering ERβ, RT-PCR analysis identified the ERβ1 transcript at the expected size of 346 bp in JKT-1 cells (Fig. 3A). In addition to ERβ1, RT-PCR detected also the transcript of ERβ2 (also referred as ERβCx), a dominant negative splice variant (Saunders et al. 2002) (Fig. 3A). Both transcripts were also identified in three seminoma tumoral fragments and in normal testicular tissue (Fig. 3A). Using an anti-ERβ antibody raised against the carboxy-terminal part of ERβ and which recognizes both ERβ1 and ERβ2 isoforms, an intense band was detected by Western blotting at 59 kDa (Fig. 3B) corresponding to the molecular mass of ERβ1.

**Figure 2 ERα expression.** RT-PCR analysis (A), Western blot analysis (B) and immunofluorescence analysis by confocal microscopy (C) in JKT-1 ('J') cells, MCF-7 ('M') cells, and normal testicular tissue ('T'). JKT-1 cells, shown in phase contrast (left) are negative for ERα. MCF-7 cells presenting an intense nuclear staining are shown as a positive control.
long form. This band was associated with a weaker 53 kDa band, which may represent ERβ isoform (Fig. 3B). The same pattern was obtained with a second polyclonal antibody (Upstate Biotechnology) recognizing the amino-terminal part of ERβ (data not shown). Two bands were detected with a similar pattern in tumoral seminoma cells and normal testicular tissue (Fig. 3B). Indirect immunofluorescence analysis with nuclear propidium iodide counterstaining observed by confocal microscopy revealed that ERβ was expressed in normal testicular tissue, in myoepithelial cells surrounding the seminiferous tubules and in intratubular basal germ cells which showed a punctuate cytoplasmic localization (Fig. 3Cb), as observed in tumoral seminoma cells (Fig. 3Cd and e). In JKT-1 cells, ERβ was identified as intense perinuclear intracytoplasmic spots associated with a weak nuclear staining (Fig. 3Cg and h). Further, no ERβ signal was seen in the cells in which the primary antibody was omitted (Fig. 3Cc, f and i). Moreover, no evident nuclear translocation could be observed under a 24 h estrogen exposure (Fig. 4A) and differed from what has been reported usually for steroid receptors (Picard et al. 1990). Double-staining experiments, using immunofluorescence and confocal microscopy, allowed partial colocalization of ERβ, identified by an FITC-antibody, with cytochrome C, a mitochondrial marker, revealed by a Texas Red antibody (Fig. 4B).

Aromatase expression

P450 aromatase mRNAs were detected by RT-PCR at the expected size of 424 bp, in JKT-1 cells, as in granulosa cells used as a positive control (Fig. 5A). Such an expression was also identified in normal human testicular tissue and in three seminoma tumors, although with a weaker intensity (Fig. 5B). Western blot analysis of microsomes from JKT-1 cells revealed the expression of the aromatase protein as a 55/49 kDa doublet including one band at the expected mass of 55 kDa, as obtained with granulosa cells used as the positive control (Fig. 5B). The second band of 49 kDa may correspond to a less-glycosylated isoform already described in germ cells (Lambard et al. 2004). The tritiated-water release assay demonstrated that this aromatase complex was functional and allowed the measurement of a basal activity of

Figure 3 ERβ expression. RT-PCR analysis with primers for ERβ1 and ERβ2 (A); Western blot analysis using the Santa Cruz anti-ERβ antibody which recognizes both ERβ1 and ERβ2 (B); immunofluorescence analysis by confocal microscopy (C) of normal testicular tissue (‘T’) (a–c), tumoral seminoma cells (‘S’) (d–f) and JKT-1 (‘J’) (g–i) with nuclei counterstained with propidium iodide (b, c, e, f, h, i). First antibody has been omitted as an immunofluorescent negative control in c, f and i. Note that on testicular sections the peritubular myoepithelial cells and the intratubular basal germ cells are positive for ERβ (white arrow). Tumoral seminoma cells (d, e), as JKT-1 cells (g, h), show intense perinuclear intracytoplasmic staining with very little intranuclear staining. The same staining was found in three different seminoma tumors examined (data not shown).
96.0 ± 26.4 fmol/h per mg protein (mean ± s.d. of three activities measured in duplicate). This activity was compared with the much higher one (4.71 pmol/h per mg protein) observed with granulosa cells.

**Discussion**

Our study provides several lines of evidence suggesting that testicular seminoma might be an estrogen-dependent cancer. First, we reported a previously unrecognized inhibitory effect of estrogen on seminoma cell proliferation. Indeed, addition of exogenous 17β-estradiol inhibited JKT-1 cell growth at the physiological concentration of 10^{-9} M, supporting the in vivo relevance of this result. Seminosas, like most testicular tumors, are believed to arise from malignant fetal gonocytes (Giwercman et al. 1991). Seminoma cells share with gonocytes several specific markers such as PIAP that we identified in JKT-1 cells (Roger et al. 2004). Li et al. (1997) have reported that estradiol was able to stimulate rat gonocyte proliferation in vitro, but this mitogenic effect was comparable with that triggered by platelet-derived growth factor and supposed to be mediated through a rapid, membranous, receptor kinase-dependent pathway. Such a rapid and membranous estrogen induction often associated with
activation of MAP kinases, has now been described in many models (Kelly & Levin 2001). However, it did not seem to be involved in our model, since 17β-estradiol linked to BSA did not reproduce any growth inhibition (data not shown). More recently, analysis of neonatal testes of ERβKO transgenic mice and organotypic testis culture under estrogen exposure, have allowed Delbes et al. (2004) to propose that estrogens may physiologically control perinatal gonocyte proliferation through both an ERβ-mediated induction of apoptosis and an ERβ-mediated inhibition of proliferation (Delbes et al. 2004). Moreover, in several estrogen-dependent cancers such as breast (Lazennec et al. 2001), prostate (Weihua et al. 2001), ovarian (Rutherford et al. 2000) and colon cancers (Fiorelli et al. 1999; Konstantinopoulos et al. 2003), estrogen has been shown to decrease, via ERβ, tumor cell proliferation. This suppressive effect of ERβ may occur via a negative control of cell cycle gene transcription, as shown for cyclin D1 whose promoting sequence contains AP-1 sites (Liu et al. 2002). It has also been suggested in several cell models that estrogens could stimulate apoptosis (Jenkins et al. 2001, Song & Santen 2003, Moore et al. 2005), possibly through an ERβ-mediated process (Qiu et al. 2002).

Expression of ERβ in testicular tumors, including seminoma, has been identified (Pais et al. 2003) and found by real-time PCR to be down-regulated when compared with normal testicular tissue (Hirvonen-Santti et al. 2003). By studying JKT-1 cells and three tumors, we confirmed that seminoma cells express ERβ but not ERα. In addition to ERβ1, we detected by RT-PCR, for the first time, the expression of ERβ2 (also referred to as ERβC), a splice variant of ERβ1 (Saunders et al. 2002), as reported in normal germ cells (Mäkinen et al. 2001). ERβ1 was readily detected by Western blotting at the expected mass of 59 kDa, using two different antibodies raised against the carboxy- or amino-terminal part of the protein. The associated 53 kDa band observed is likely to represent ERβ2 or alternatively another isoform corresponding to one of the splice variants described for ERβ (Lehman & Wilson 1998), especially in testicular cells (Aschim et al. 2004). An ERβ-mediated inhibitory control of JKT-1 cells under estrogen exposure is supported by the following observations: (i) ERβ but not ERα was expressed in JKT-1 cells; (ii) the antiproliferative effect induced by estradiol occurred at a concentration close to the Ki of ER (1–4 × 10−10 M); (iii) this inhibition was reversed by the classic ER antagonist ICI 182780; and (iv) ERβ has now been shown to act as a suppressive agent in several estrogen-dependent cancers (Speirs 2002). Very recently ERβ has been localized in the mitochondria in rat primary neurons, human cardiomyocytes and human lens cells (Cammarata et al. 2004, Yang et al. 2004); it is suggested to participate to the regulation of several estrogen-dependent mitochondrial functions (Yang et al. 2004), such as apoptosis (Moore et al. 2005). The cytoplasmic perinuclear localization for ERβ that we found in JKT-1 cells without any nuclear translocation under estrogen exposure, its colocalization with cytochrome C, and the estradiol-induced cell growth inhibition observed, may suggest such a mechanism.

The molecular mechanism(s) responsible for this estrogen-dependent seminoma cell growth inhibition is now under investigation in our laboratory. Using neutralization of ERβ expression by siRNA, cell cycle gene expression and apoptosis analysis we should be able to demonstrate whether an ERβ-mediated inhibition of cell proliferation and/or an ERβ-mediated stimulation of apoptosis are involved in this process.

So far, aromatase has been reported in mature testis in both somatic and germ cells (Levallet et al. 1998; Lambard et al. 2004), and was suggested to be responsible for gynecomastia in one patient bearing a seminoma (Duparc et al. 2003). We report here for the
first time the characterization of a functional aromatase complex (transcript, protein and enzymatic activity) in a pure human seminoma cell line, underlining the ability of intratesticular seminoma cells to convert testosterone into estradiol and to respond to it through an autocrine and/or a paracrine loop.

Altogether, these results suggest estrogen-dependency for testicular seminoma and support a possible participation of endogenous or environmental estrogens in testicular carcinogenesis. Whether the expression level of ERβ in seminoma could represent a prognostic marker related to the degree of malignancy as described for other estrogen-dependent cancers (Speirs 2002), and whether its apparent suppressive role could be used for a therapeutic goal, require further investigation.

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