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 K_d 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 tumoral seminoma cells and normal human testicular basal germ cells, expresses estrogen receptor β (ER β), including ER β 1 and ER β 2, a dominant negative variant, but not ER α . Using immunofluorescence and confocal microscopy, ER β was observed as perinuclear intracytoplasmic spots in JKT-1 and tumoral seminoma cells without significant translocation of ER β into the nucleus, under 17β -estradiol exposure. Double staining observed by confocal microscopy revealed that ER β 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 ER β -mediated stimulation of cell apoptosis and/or an ER β -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), ER α and ER β , which are transcriptional factors (see review in Nilsson *et al.* 2001). There is compelling evidence that ER α mediates the proliferative effects of estrogens in several so-called estrogen-dependent cancers, such as breast, uterus and ovary. Of particular interest, ER β , when coexpressed with ER α , 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.* 2003) and most adult germ cells (Mäkinen *et al.* 2001) express ER β . Estrogens have been shown *in vitro* to control gonocyte proliferation in the rat (Li *et al.* 1997) and recent analysis of neonatal testes from ER β KO mice has confirmed the link between endogenous estrogens, ER β 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

Table 1 Sequences of the human ER and aromatase primers

Primer	
Name	
hERα F	5'-AGA-CAT-GAG-AGC-TGC-CAA-CC-3'
hERα R	5'-GCC-AGG-CAC-ATT-CTA-GAA-GG-3'
hERβ F	5'-TCA-CAT-CTG-TAT-GCG-GAA-CC-3'
hERβ R	5'-CGT-ACC-ACT-TCC-GAA-GTC-GG-3'
hERβ2 F	5'-CGA-TGC-TTT-GGT-TTG-GTG-AT-3'
hERβ2 R	5'-CTT-TAG-GCC-ACC-GAG-TTG-ATT-3'
Aromatase F	5'-AAA-GGA-AAT-CCA-TGT-TAT-TGG-TGG-3'
Aromatase R	5'-GTA-TCT-TCT-GTA-TGC-TCT-CAA-CAC-ACT-GTG-3'

F, forward; R, reverse.

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 (PIAP), a specific seminoma marker (Giwercman *et al.* 1991). The JKT-1 cells, described as a pure human seminoma cell line (Kinugawa *et al.* 1998), also expressing PIAP (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 phenylmethylsulfonyl fluoride). 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

antibody (sc-543; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a goat polyclonal anti-ER β antibody raised against a peptide mapping near the carboxy-terminus of ER β which recognizes both ER β 1 and ER β 2 and which does not cross-react with ER α (sc-6822; Santa Cruz), a rabbit polyclonal anti-ER β antibody raised against the amino-terminus of ER β (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. ER α and ER β were detected using a rabbit anti-ER α (Santa Cruz; 200 mg/ml) and a goat anti-ER β (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 $\mu\text{g/ml}$). Colocalization of ER β 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 $^3\text{H}_2\text{O}$ released from $[1\beta\text{-}^3\text{H}]\text{androst-4-ene-3,17-dione}$ (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 $^3\text{H}_2\text{O}$ 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 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 $\pm$ 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 $\pm$ 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). $*P < 0.05$.

Figure 2 ER α 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 ER α . MCF-7 cells presenting an intense nuclear staining are shown as a positive control.

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 ER α , 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 ER α in JKT-1 cells (Fig. 2C) compared with a strong nuclear staining in MCF-7 cells. Considering ER β , RT-PCR analysis identified the ER β 1 transcript at the expected size of 346 bp in JKT-1 cells (Fig. 3A). In addition to ER β 1, RT-PCR detected also the transcript of ER β 2 (also referred as ER β Cx), 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-ER β antibody raised against the carboxy-terminal part of ER β and which recognizes both ER β 1 and ER β 2 isoforms, an intense band was detected by Western blotting at 59 kDa (Fig. 3B) corresponding to the molecular mass of ER β 1

Figure 3 ER β expression. RT-PCR analysis with primers for ER β 1 and ER β 2 (A); Western blot analysis using the Santa Cruz anti-ER β antibody which recognizes both ER β 1 and ER β 2 (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 ER β (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).

long form. This band was associated with a weaker 53 kDa band, which may represent ER β 2 or another ER β isoform (Fig. 3B). The same pattern was obtained with a second polyclonal antibody (Upstate Biotechnology) recognizing the amino-terminal part of ER β (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 ER β was expressed in normal testicular tissue, in myoepithelial cells surrounding the seminiferous tubules and in intratubular basal round germ cells which showed a punctuate cytoplasmic localization (Fig. 3Cb), as observed in tumoral seminoma cells (Fig. 3Cd and e). In JKT-1 cells, ER β was identified as intense perinuclear intracytoplasmic spots associated with a weak nuclear staining (Fig. 3Cg and h). Further, no ER β 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 ER β , 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 4 ER β expression in JKT-1 cells analyzed by immunofluorescence and confocal microscopy. (A) Estrogen exposure (10^{-6} M) for 4 and 24 h. Lack of significant nuclear translocation of ER β . (B) ER β revealed by FITC and cytochrome C revealed by Texas Red; colocalization in yellow (merged).

96.0 ± 26.4 fmol/h per mg protein (mean $\pm$ 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 17 β -estradiol inhib-

ited JKT-1 cell growth at the physiological concentration of 10^{-9} M, supporting the *in vivo* relevance of this result. Seminomas, like most testicular tumors, are believed to arise from malignant fetal gonocytes (Giwerzman *et al.* 1991). Seminoma cells share with gonocytes several specific markers such as PLAP 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

Figure 5 P450 aromatase expression. Analysis of P450 aromatase mRNAs and protein expression by RT-PCR (A) and Western blot (B) in JKT-1 cells ('J'), human granulosa cells ('G'), human normal testis ('T'), and one out of three seminomas tested ('S'). RT-, negative control.

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 17 β -estradiol linked to BSA did not reproduce any growth inhibition (data not shown). More recently, analysis of neonatal testes of ER β KO 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 ER β -mediated induction of apoptosis and an ER β -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 ER β , tumor cell proliferation. This suppressive effect of ER β 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 ER β -mediated process (Qiu *et al.* 2002).

Expression of ER β 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 ER β but not ER α . In addition to ER β_1 , we detected by RT-PCR, for the first time, the expression of ER β_2 (also referred to as ER β Cx), a splice variant of ER β_1 (Saunders *et al.* 2002), as reported in normal germ cells (Mäkinen *et al.* 2001). ER β_1 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 ER β_2 or alternatively another isoform corresponding to one of the splice variants described for ER β (Lehman & Wilson 1998), especially in testicular cells (Aschim *et al.* 2004). An ER β -mediated inhibitory control of JKT-1 cells under estrogen exposure is supported by the following observations: (i) ER β but not ER α was expressed in JKT-1 cells; (ii) the anti-proliferative effect induced by estradiol occurred at a concentration close to the K_d of ER ($1-4 \times 10^{-10}$ M); (iii) this inhibition was reversed by the classic ER antagonist ICI 182780; and (iv) ER β has now been shown to act as a suppressive agent in several estrogen-dependent cancers (Speirs 2002). Very recently ER β 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 ER β 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 ER β expression by siRNA, cell cycle gene expression and apoptosis analysis we should be able to demonstrate whether an ER β -mediated inhibition of cell proliferation and/or an ER β -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 ER β 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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