Human immature germ cells and ejaculated spermatozoa contain aromatase and oestrogen receptors

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Abstract

It is now well established that oestrogens play a part in germ cell function. These hormones are synthesised by the cytochrome P450 aromatase (P450 arom) and act via two kinds of receptor (ERα and ERβ). Although the presence of aromatase and oestrogen receptors in mammalian testis is now well documented, the localisation of these proteins in human germ cells is not yet clear. The primary purpose of the current study was to look for the expression of aromatase and oestrogen receptors in human germ cells. Human immature germ cells were collected from semen samples with an excess of rounds cells (>20%) and purified spermatozoa were obtained after sedimentation on a discontinuous PureSperm gradient. Expression of aromatase and oestrogen receptors was determined by RT-PCR with specific primers, and by Western blot using monoclonal antibodies. RT-PCR products for aromatase, ERα and ERβ were amplified from total RNA isolated from human germ cells and spermatozoa. We identified an ERα isoform variant that lacks exon 4 in human germ cells and visualised P450 arom as a single band of 49 kDa in germ cells, as we have already reported for human ejaculated spermatozoa. By Western blot, we identified two proteins for ERβ at ~ 50 and ~ 60 kDa, which could correspond to the long and short forms of ERβ formed from the use of alternative start sites. In human ejaculated spermatozoa, ERβ protein was not detected, even though we could amplify mRNA. Using Western blot analysis and a monoclonal antibody specific for ERα, we detected two proteins in human immature germ cells: one of the expected size (66 kDa) and a second one of 46 kDa. In mature spermatozoa, only the 46 kDa band was observed and we speculate it may be related to the ERα isoform lacking exon I. In conclusion, we have identified P450 arom and ER proteins (full-length and variant) in human germ cells. Further studies are now required to elucidate the mechanism of action of oestrogens on human male germ cells, in terms of both genomic and 'non-genomic' pathways.

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Introduction

The irreversible conversion of androgens into oestrogens is catalysed by cytochrome P450 aromatase (P450 arom), a product of a unique gene called *Cyp19* (Simpson *et al.* 2002), which is located in the endoplasmic reticulum membrane. Although oestrogens have been considered to be female hormones, it is now well established that they have an important role in male reproduction. In adult males, Leydig cells have been considered for a long time as the major source of oestrogens, whereas Sertoli cells synthesise oestradiol in prepubertal males (Papadopoulos *et al.* 1986, Carreau *et al.* 1988). However, in the past decade there has been a growing body of evidence that germ cells could also synthesise oestrogens. In fact, the presence of aromatase has been demonstrated in germ cells of a great number of species (Nitta *et al.* 1993, Janulis *et al.* 1998, for review see Carreau *et al.* 1999).

In adult human testis, aromatase was previously immunolocalised to Leydig cells, but not Sertoli cells (Payne *et al.* 1976, Brodie *et al.* 2001). However, recently, aromatase has also been immunolocalised to the cytoplasm surrounding elongated spermatids (Turner *et al.* 2002), and we and others have reported the presence of aromatase in human ejaculated spermatozoa (Aquila *et al.* 2002, Lambard *et al.* 2003).

The effects of oestrogens on target tissues are mediated by specific receptors. Until now, two oestrogen receptors (ER) have been cloned: ERα (Green *et al.* 1986) and ERβ (Kuiper *et al.* 1996), both of which belong to the superfamily of nuclear hormone receptors. Numerous isoforms/variants have also been identified. Almost all these transcripts are generated by skipping of one or more exons, duplication or deletion of one or more exons, alternative usage of the 5′-untranslated exons or alternative usage of the coding exons (Hirata *et al.* 2003).

The presence of ER in the male reproductive tract of a large number of mammalian species is now well documented (O'Donnell *et al.* 2001). However, the distribution of the two receptor subtypes does not seem to be similar between species. In adult rodent testis, Leydig cells are immunopositive for ERα (Zhou *et al.* 2002), whereas caprine Leydig cells are immunonegative (Goyal *et al.* 1997). ERβ transcript was not found in the germ cells of adult rat testis (Mowa & Iwanaga 2001), but other studies have reported expression of ERβ (protein and mRNA) in the germ cells of rodent testis (Saunders *et al.* 1998, Van Pelt *et al.* 1999, Zhou *et al.* 2002).

In humans, data about the localisation of ER in seminiferous tubules are also controversial. Pelletier & El-Alfy (2000) did not observe any tubular labelling, for either ERβ or ERα. In contrast, Mäkinen *et al.* (2001) and Saunders *et al.* (2001) have detected ERβ immunoreactivity in spermatogonia, spermatocytes and early developing spermatids, but no staining in elongated spermatids and mature spermatozoa. No immunoexpression of ERα was detected in testes. Pentikaïnen *et al.* (2000) have demonstrated the presence of ERα in human germ cells and Durkee *et al.* (1998) and Luconi *et al.* (1999) have reported the existence of ERα in human ejaculated spermatozoa. To clarify these data further, the aim of this study was to determine if adult human testicular germ cells and ejaculated spermatozoa are potential sources, targets, or both, of oestrogens. For that purpose, we looked for the expression of aromatase, ERα and ERβ, as indicated by the presence of both transcripts and proteins, in human immature germ cells and ejaculated spermatozoa.

Materials and methods

Human semen sample preparation

Sperm samples ($n=18$) were obtained from healthy donors (mean age $32{\cdot}17 \pm 3{\cdot}03$ years) by masturbation after 3 days of sexual abstinence and allowed to liquefy for 30–60 min at room temperature before processing. Informed patient consent was obtained for the use of sperm samples in this study. The selected specimens had normal semen parameters according to the World Health Organisation guidelines (1999). Samples with more than $1{\cdot}10^6$ round cells/ml were excluded. A spermocytogram (analysis of sperm characteristics: volume, viscosity and pH of semen; number, mobility, vitality and morphology of spermatozoa) was performed in order to eliminate samples with cytoplasmic droplets. The liquefied semen samples were fractionated on discontinuous PureSperm gradient (JCD, Lyon, France) consisting of four successive layers with the following densities: 95, 76, 57 and 47·5%. A microscopic examination of the sperm-enriched fractions obtained (95% layer) was performed to control the quality of the preparations (motility, survival and morphology). The purity of this fraction (observed under microscope) was close to 100%, when compared with non-purified sperm (Fig. 1*a* and *b*).

Human leucocytes were obtained from healthy donors. Human granulosa cells (positive aromatase control) were collected from human follicular fluid from preovulatory follicles in the In Vitro Fertilisation Center (CHRU Clémençeau, Caen, France).

Isolation of human germ cells

Semen samples ($n=4$) with more than 20% of round cells (mainly germ cells) were selected (Fig. 1*c*). Although these samples showed a high number of round cells, the spermatic parameters were not altered. The liquefied semen samples were fractionated on discontinuous PureSperm gradient in order

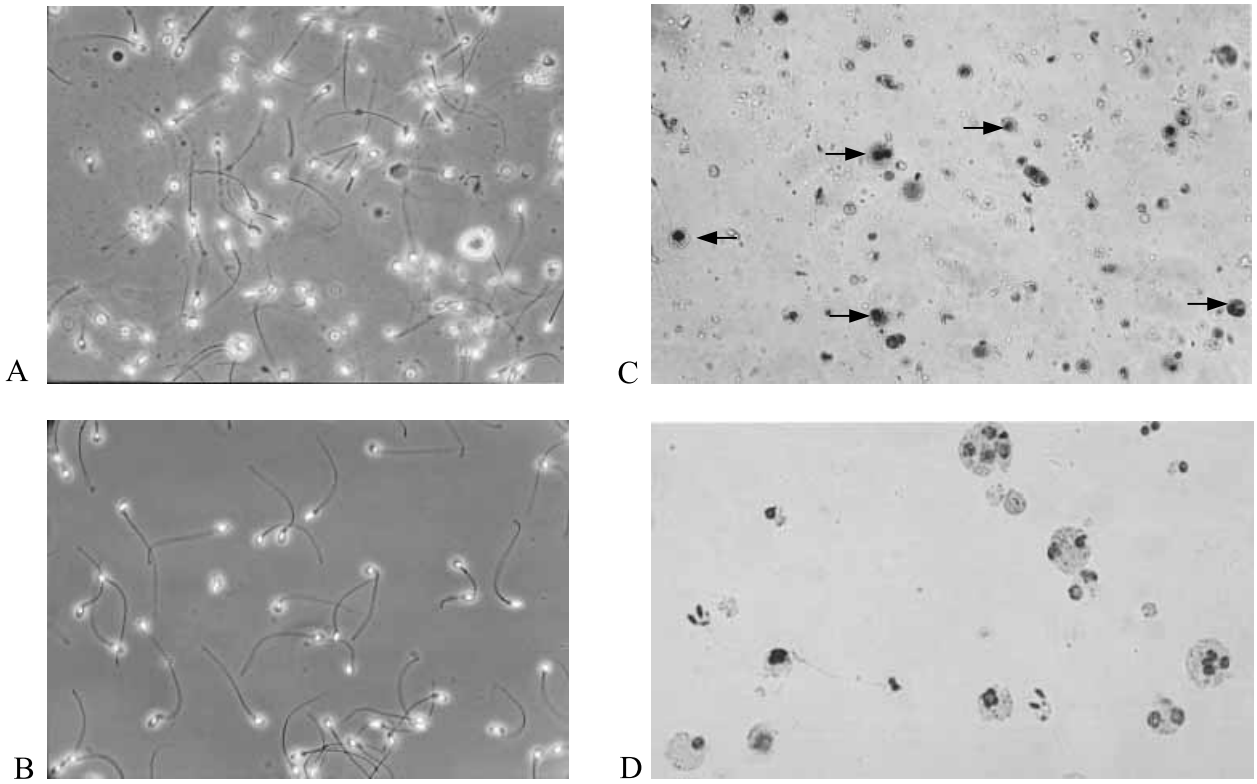

Figure 1 Microscopic examination of semen. (*a*) Human spermatozoa before purification on a discontinuous gradient (original magnification×400). (*b*) Human spermatozoa isolated from the 95% Puresperm fraction (original magnification×400). (*c*) Human semen with an excess of round cells (arrows) (original magnification×200). (*d*) Human immature germ cells isolated from the 47·5% Puresperm fraction (original magnification×200).

to eliminate the majority of spermatozoa. Round cells were collected from the 47·5% layer and washed twice with Earle's medium (Fig. 1*d*).

RNA extraction

Total RNAs from purified sperm fractions and granulosa cells were extracted using the guanidinium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987). Briefly, after centrifugation the cell pellets were homogenized on ice in a 1 M Tris buffer containing guanidinium thiocyanate (4 M). The RNA was then isolated with a phenol–chloroform–isoamylic acid solution. It was precipitated twice from the aqueous phase with isopropanol, washed with 75% ethanol, dried on speed-vac and dissolved in diethyl pyrocarbonate-treated water and then stored at −80 °C. The purity of RNA samples was checked spectroscopically by measuring the optic density at 260 and 280 nm and by evaluating the ratio 260/280 nm.

RT-PCR assay

Four hundred nanograms total RNA were reverse-transcribed to first-strand cDNA as follows: 1 h at 37 °C with 200 IU M-MLV-RT (Promega, Charbonnières, France), 500 μM dNTP, 0·2 μg oligo-dT (12–18 mers) and 24 IU RNasin in a final volume of 10 μl, then 5 min at 94 °C.

The cDNAs were further amplified by PCR using selected oligonucleotides. PCR was performed in the presence of 1·5 mM $MgCl_2$, 200 μM dNTP, 1·5 IU Taq polymerase and 50 pmol forward and reverse primers (Life Technology) in a final volume of 50 μl. The applied PCR primers and the expected lengths of the resulting PCR products are shown in Table 1. All primers were chosen in different exons in order to eliminate any potential contamination by genomic DNA; the cycle profiles used are described in Table 2.

Contamination by leucocytes and Sertoli cells in our germ cell preparations was assessed by

Table 1 Oligonucleotide sequences used for RT-PCR

Gene	Sequence (5′-3′)	Size of PCR product (bp)	Reference
Aromatase	5′-TGAATATTGGAAGGATGCACAGAC-3′ 5′-TGGAATCGTCTCAGAAGTGTAACGAG-3′	189	Lambard *et al*. 2003
CD45	5′-TGCAGATGCCTACCTTAATGC-3′ 5′-CACATTGCAGCACTTCCATT-3′	844	Ralph *et al*. 1987 GenBank accession No. Y00062.1
c-kit	5′-AGTACATGGACATGAAACCTGG-3′ 5′-GATTCTGCTCAGACATCGTCG-3′	780	de los Santos *et al*. 1998
ERα	5′-GACTATGCTTACGGCTACCATT-3′ 5′-TGGTTCCTGTCCAAGAGCAAGTTA-3′	674	Hillier *et al*. 1998
ERα exon-1-deleted	5′-CCGGTTTCTGAGCCTTCTG-3′ 5′-TCCTTGGCAGATTCCATAGC-3′	264	Green *et al*. 1986 GenBank accession No. X03635.1
ERβ	5′-TAGTGGTCCATCGCCAGTTATCAC-3′ 5′-GCACTTCTCTGTCTCCGCACAA-3′	438	Hillier *et al*. 1998
GAPDH	5′-TGAACGGGAAGCTCACTGGCATGGCCTT-3′ 5′-GTGTGGTGGGGGACTGAGTGTGGCAGGGAC-3′	431	Lambard *et al*. 2003
Protamines-2	5′-GGATCCACAGGCGGCAGCATCGCT-3′ 5′-GCATGTTCTCTTCCTGGTTCTGCA-3′	103	Siffroi and Dadoune 2001
SCF	5′-AACCCTCAAATATGTCCCCG-3′ 5′-CTGCCCTTGTAAGACTTGGC-3′	584 or 500	Teyssier-Le Discorde *et al*. 1999

amplifying CD45 and SCF transcripts respectively; c-kit (a germ cell marker) was used to verify the presence of germ cells.

For all PCR amplifications, negative (water only) and positive controls were included. All cDNA fragments were run on a 1·5% agarose gel stained with ethidium bromide and visualised under UV transillumination.

Table 2 Cycling conditions for the different sets of pairs

Gene	Cycle profile
Aromatase	95°C/1 min, 60°C/1 min, 72°C/1·5 min
CD45	95°C/45 s, 60°C/45 s, 72°C/1 min
c-kit	95°C/1 min, 52°C/1 min, 72°C/1 min
ERα	95°C/45 s, 60°C/45 s, 72°C/2 min
ERα exon 1	95°C/1 min, 58°C/1 min, 72°C/1·5 min
ERβ	95°C/45 s, 60°C/45 s, 72°C/2 min
GAPDH	95°C/1 min, 60°C/1 min, 72°C/1 min
Protamines-2	95°C/45 s, 56°C/45 s, 72°C/45 s
SCF	95°C/1 min, 56°C/1 min, 72°C/1 min

DNA sequence analysis

The RT-PCR products were extracted from the agarose gel by using a gel band purification kit (Amersham Biosciences). The purified DNAs were amplified and then sequenced using a DNA sequencing kit (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction) and procedure for fluorescence-based DNA sequencing with Taq polymerase (Applied Biosystems).

Protein extraction

The spermatozoa isolated from the 95% PureSperm layer and the mixture of germ cells were washed twice with Earle's medium. The pellets were resuspended in lysis buffer [100 mM Tris-HCl pH 7·4, 20% glycerol, 150 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, containing one protease inhibitor tablet (Roche)/10 ml buffer]. Samples were homogenised and sonicated and protein concentrations determined using Bradford's assay (1976).

Western blot

Five microlitres denaturing loading buffer (20% sucrose, 5% SDS, 5% β-mercaptoethanol, 0·075% bromophenol blue) were added to aliquots of protein and boiled for 10 min. The samples were electrophoresed at 30 mA for 2 h on 10% SDS-polyacrylamide gel using a running buffer consisting of 25 mM Tris, 192 mM glycine pH 8·3, 0·1% SDS and the proteins were then transferred onto nitrocellulose enhanced chemiluminescence (ECL) membranes (Amersham) in a transfer buffer (Tris 20 mM, glycine 150 mM, 20% methanol) at 200 V for 45 min. The membranes were blocked for 2 h at room temperature in 4% non-fat dried milk in 0·1% Tween 20, 10 mM Tris and 15 mM NaCl pH 7·4 (TTBS), incubated overnight with the primary antibody [anti-aromatase 1:500 (Code MCA 2077 Serotec UK), anti-ERα 1:100 (Santa-Cruz, France) or anti-ERβ 1:250 (Code EMR02, Novocastra, Newcastle, UK)], all of which were diluted in TTBS containing 2% non-fat dried milk. The antigen–antibody complexes were detected by incubation of the membranes for 90 min with appropriate secondary antibodies (sheep anti-mouse; Amersham) and developed using the ECL Plus Western blotting detection system (Amersham).

Proteins extracted from human granulosa cells were used as positive control.

Results

Characterisation of germ cells

In order to eliminate any contamination of samples by leucocytes, we looked for the white blood cell specific CD45 transcript in the preparations of round cells; samples containing detectable levels of CD45 transcripts were excluded. We were also unable to amplify Sertoli cell factor (SCF) mRNA (specific for Sertoli cells) in our germ cell preparations. Conversely, all isolated round cells contained transcripts for c-kit, which is a positive control for testicular germ cells (Fig. 2*a*). In fact, it has been demonstrated that c-kit is expressed in premeiotic and meiotic germ cells of the mouse (Albanesi *et al.* 1996), but this result does not exclude the presence of post-meiotic germ cells in our human samples.

No detectable levels of mRNA coding either CD45 or c-kit were found in purified semen samples (Fig. 2*b*).

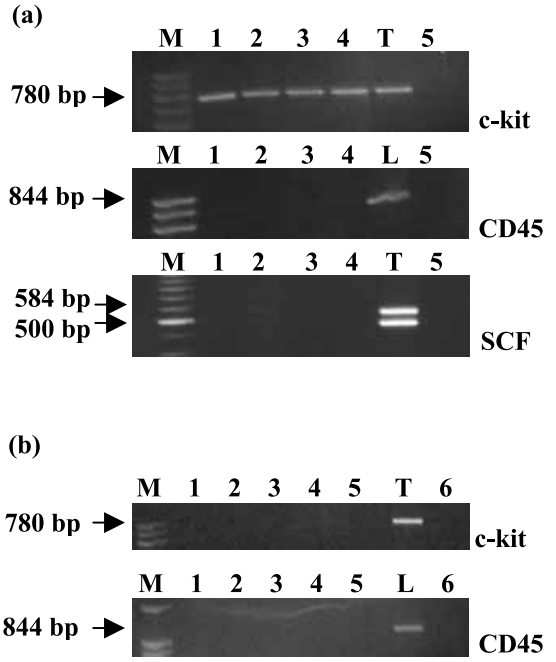

Figure 2 Agarose gel showing the PCR products of c-kit, CD45 and SCF. (*a*) RNA extracted from human germ cells (lanes 1–4), human testis (T) and human leucocytes (L). RNA was omitted in lane 5. M, DNA ladder (100 bp). Two bands were observed in the RT-PCR product for SCF, one at 584 bp and the other at 500 bp, assigned respectively to transcripts encoding the soluble and membrane forms of SCF. (*b*) RNA extracted from human spermatozoa isolated from the 95% PureSperm fraction (lanes 1–5), human testis (T) and human leucocytes (L). RNA was omitted in lane 6. M, molecular mass standard (100 bp).

Detection of aromatase and oestrogen receptor transcripts

We looked for the expression of aromatase and oestrogen receptor transcripts in human germ cells. The PCR amplification was carried using 30 cycles for all transcripts. Aromatase, oestrogen receptors (both types) and GAPDH mRNA were present in four different preparations of human germ cells (Fig. 3). There was no detectable signal in samples processed without reverse transcriptase (data not shown). We observed two bands for ERα transcripts, one at the expected size and another at a smaller size. The PCR product corresponding to the smaller band was sequenced and found to be an ERα isoform that lacks exon IV (Fig. 4).

After 35 cycles of amplification, we detected both types of oestrogen receptor mRNA in purified semen samples ($n=9$) (Fig. 5), in addition to the

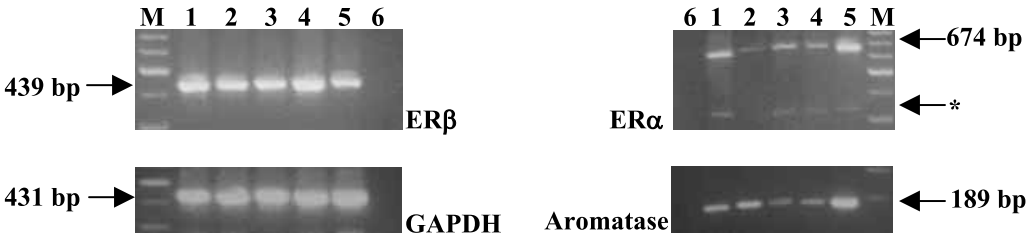

Figure 3 Analyses of ERβ, ERα, cytochrome P450 aromatase and GAPDH RT-PCR products in human germ cells after separation on 1·5% agarose gels. Lanes 1–4: RNA extracted from human germ cells; lane 5: RNA extracted from granulosa cells; lane 6: water instead of RNA. Arrows indicate the PCR products and the expected size of amplified fragments. *An unexpected PCR product for ERα. M, DNA ladder (100 bp).

aromatase transcripts. A strong signal was obtained when protamine-2 mRNA was amplified. For each sample, when a control was added without the reverse transcription step, no signal was detected.

Presence of aromatase and oestrogen receptor proteins

Western blot using an antibody against human P450 arom revealed a positive signal having a molecular mass of 49 kDa in protein extracted from 'mixed' germ cells (Fig. 6). This band migrated a little behind the 53 kDa aromatase protein detected in human granulosa cells, as we have already described in human ejaculated spermatozoa (Lambard *et al.* 2003). Western analysis of human isolated germ cells showed a signal for both ERβ and ERα (Fig. 6). Using an antibody specific to the C-terminal of full-length ERβ, we detected two bands for ERβ in isolated human germ cells, one at ~ 60 kDa and a weaker one at ~ 50 kDa (Fig. 6). In spite of the presence of ERβ mRNA in human spermatozoa, we were unable to find the protein (data not shown). Incubation of granulosa cell samples with our ERβ antibody that had been preabsorbed with the recombinant protein did not result in detection of any protein (data not shown).

Using an antibody directed against the C-terminal region of ERα, we also detected two bands in human germ cells, one at 66 kDa and the other at 46 kDa (Fig. 6). In human spermatozoa, we only detected a protein at 46 kDa (Fig. 7). The 66 kDa band corresponds to the classic reported

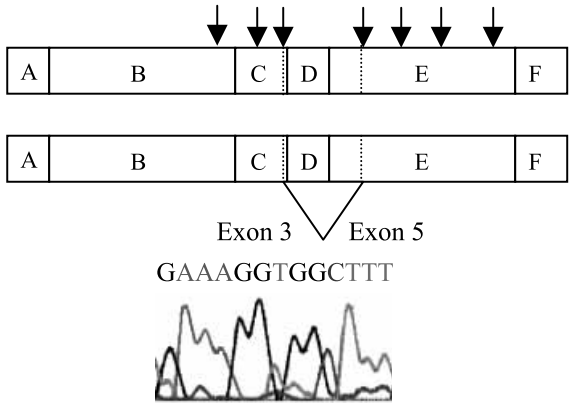

Figure 4 Schematic representation of the exon-4-deleted ERα isoform aligned to the wild-type ERα. Arrows indicate exon boundaries. Wild-type ERα contains eight different exons coding for a protein divided into structural domains (A–F). The DNA-binding domain is located in region C, whereas the ligand-binding hormone is located in region E. The domain D, which is deleted in this ERα variant, represents the hinge region.

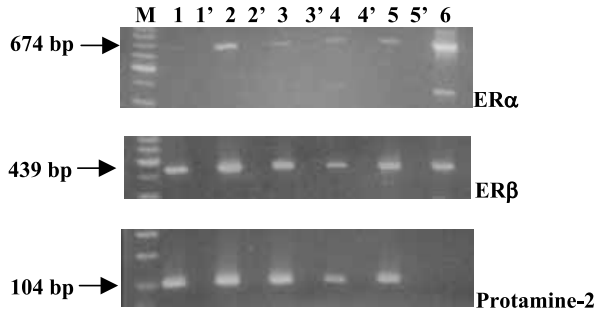

Figure 5 Analyses of ERα, ERβ and protamine-2 RT-PCR products in human purified spermatozoa after separation on 1·5% agarose gels. Lanes 1–5: RNA extracted from human spermatozoa isolated from the 95% PureSperm fraction; lane 6: RNA extracted from granulosa cells. M, DNA ladder (100 bp ladder). Arrows indicate the PCR products and the expected size of amplified fragments. No reverse transcriptase was added in RT-PCR reactions for products loaded in lanes 1' to 5'.

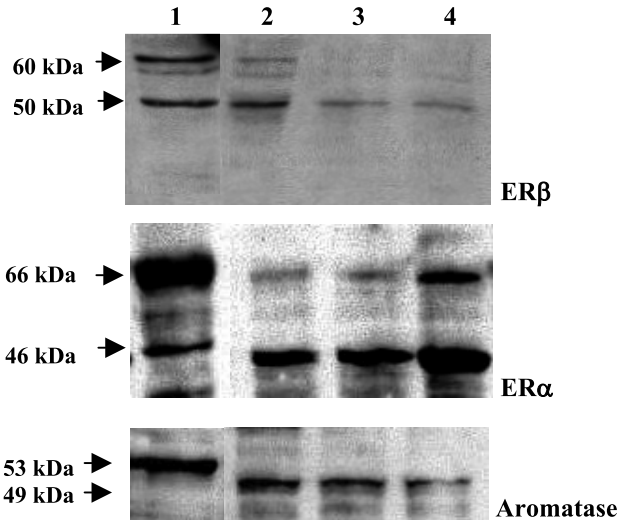

Figure 6 Western blot analysis of ERβ, ERα and cytochrome P450 aromatase proteins in human isolated germ cells. Total cellular proteins were extracted from human granulosa (lane 1, used as a positive control) and human germ cells (lanes 2–4); 30 μg of protein were loaded in each lane. Molecular masses (kDa) are indicated on the left of the blot.

molecular mass of human ERα, whereas the 46 kDa band could correspond to the isoform that lacks exon I (Flouriot *et al.* 2000).

As our PCR primers for ERα were located in exon 2 and in exon 6, we were able to amplify the wild-type and distinguish this form from the exon-4-deleted variant of ERα. However, using these primers we can not distinguish between the wild-type and exon-1-deleted variant. Therefore, we also performed RT-PCR using primers flanking exon 1. It has been reported that this truncated transcript results from splicing of the 5′UTR variant exon E or F directly to exon 2 (Flouriot *et al.* 2000), so we chose a primer located in exon 2 and another primer that recognised both exon E and exon F, to look for the expression of this exon-1-truncated transcript. In human germ cells, two bands were obtained (Fig. 8). The 620 bp band corresponded to the wild-type, whereas the lower band (264 bp) was related to the ERα exon-1-deleted variant mRNA (Green *et al.* 1986, GenBank accession No. X03635·1). In human spermatozoa, we amplified only the 264 bp product (Fig. 8). These results were consistent with detection of the 46 kDa protein.

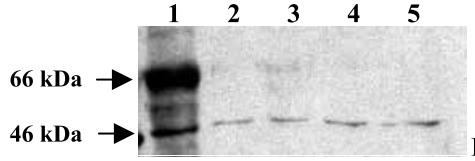

Figure 7 Western blot analysis of ERα protein in human spermatozoa. Total cellular proteins were extracted from human granulosa (lane 1, used as a positive control) and human spermatozoa isolated from the 95% PureSperm fraction (lanes 2–5); 30 μg of protein were loaded in each lane. Molecular masses (kDa) are indicated on the left of the blot.

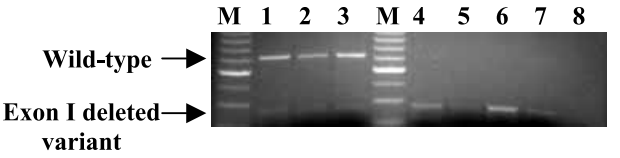

Figure 8 Detection of ERα exon-1-deleted variant mRNA by RT-PCR in human germ cells and human purified spermatozoa. Lanes 1–3: RNA extracted from human germ cells; lanes 4–7: RNA extracted from human spermatozoa isolated from the 95% PureSperm fraction; lane 8: RNA replaced by distilled water. M, DNA ladder (100 bp ladder). Arrows indicate the PCR products and the expected size of amplified fragments.

Discussion

The existence of ERα, ERβ and aromatase in the adult human testis is the subject of numerous studies, but the expression and cellular localisation of ER subtypes is not yet clear. The primary purpose of our study was to look for the expression of aromatase and oestrogen receptors in human germ cells in order to add an alternative view to the conflicting data.

In this study, we detected the expression of aromatase and ER in human germ cells, in terms of both mRNA and protein. In ejaculated spermatozoa, we demonstrated the presence of both mRNA and protein ERα, whereas in the case of ERβ we detected the presence of only mRNA.

In mammals, it is now well established that germ cells represent a new source of oestrogens (Nitta *et al.* 1993, Tsubota *et al.* 1993, Levallet *et al.* 1998, Carreau 2001). In adult human testis, aromatase has been immunolocalised to the cytoplasm surrounding elongated spermatids (Turner *et al.* 2002) and in the cytoplasmic droplet of ejaculated spermatozoa (Rago *et al.* 2003), but was absent from other germ cells (Turner *et al.* 2002).

The presence of ER in germ cells is also well documented, and most of the studies favoured ERβ as the predominant form in mammalian germ cells (Saunders *et al.* 2002, Zhou *et al.* 2002). In human germ cells, the presence of both types of ER is controversial. Although Pelletier & El-Alfy (2000) have reported no tubular staining, other authors (Enmark *et al.* 1997, Mäkinen *et al.* 2001, Saunders *et al.* 2002) have immunolocalised ERβ to human germ cells. Using fixed human testes and Western blot analysis, Saunders *et al.* (2001) and Mäkinen *et al.* (2001) were unable to demonstrate the presence of ERα in testis, whereas Pentikaïnen *et al.* (2000), who performed immunohistochemistry on squash preparations of segments of human seminiferous tubules, demonstrated the existence of ERα protein. The discrepancies between these studies could be explained by the different methodologies used.

The human germ cells used were obtained from semen samples with excessive shedding of immature germ cells (>20%). It has been shown that germ cells constitute the main component of non-sperm cells in semen from men without infections (Auroux *et al.* 1985, Jassim & Festenstein 1987, Smith *et al.* 1989). Moreover, it has also been reported that purified immature germ cells isolated from semen samples could be useful for diagnostic and research purposes (Gandini *et al.* 1999). However, these cells have not been isolated from testis and could reflect a defect of spermatogenesis.

We visualised the aromatase as a single band of 49 kDa, as we have already reported for human ejaculated spermatozoa (Lambard *et al.* 2003). The small variation in aromatase molecular mass observed between granulosa cells and human germ cells could be due to the level of glycosylation; however, as already described (Sethumadhavan *et al.* 1991, Moslemi *et al.* 1997), glycosylation does not seem to have an impact on the enzymatic activity.

Using Western blot, we detected two bands for ERβ, which could correspond to use of alternative start sites in the mRNA. In human ejaculated spermatozoa, we detected only the ERβ mRNA and not the protein. This result is in agreement with the findings of Saunders *et al.* (2001, 2002). This transcript may represent some remnants of spermatogenesis or spermiogenesis. The putative existence of translationally repressed mRNAs in spermatozoa has been reported, and they could also represent a paternal contribution for the initiation of the first zygote division (Siffroi & Dadoune 2001).

Using a monoclonal antibody specific to the C-terminus of full-length of ERα, we detected two bands in human germ cells: one at the expected size (66 kDa) and a weaker one at 46 kDa. This latest form has also been identified in several cellular types such as endothelial cells (Figtree *et al.* 2003) or osteoblasts (Denger *et al.* 2001), and could correspond to an isoform of ERα that lacks exon 1. That isoform is a powerful inhibitor of the 66 kDa ERα when they are coexpressed in the same cell (Flouriot *et al.* 2000), but the same variant has also been implicated in the rapid oestrogen signalling pathway (Figtree *et al.* 2003), particularly by mediating acute activation of endothelial nitric oxide synthase in response to oestrogen stimulation (Li *et al.* 2003). The 46 kDa protein is the only form identified in spermatozoa. This result is consistent with those of several studies showing the presence of specific binding sites for 17β-oestradiol on the human sperm membrane (Cheng *et al.* 1981, Durkee *et al.* 1998). It has been demonstrated previously that oestradiol increased motility, oxidative metabolism, longevity of spermatozoa and oocyte penetration (Idaomar *et al.* 1989). Recently, it has been shown that oestradiol and phytoestrogens improve mouse sperm capacitation (Adeoya-Osiguwa *et al.* 2003). Thus oestrogens may act via a membrane oestrogen-binding protein, to exert a rapid effect. However, this putative receptor is not yet well identified and some additional studies are necessary to clarify the protein and the pathways involved.

Our data showed the existence of another alternatively spliced isoform of ERα in human germ cells; this isoform lacks a region that exactly corresponds to exon 4. This variant has been identified in human breast cancer cell lines and in brain (Pfeffer *et al.* 1993, Skipper *et al.* 1993); it does not possess the hinge domain and lacks a part of the ligand-binding domain. The role of the putative protein is unknown, but some data suggest that this isoform either could have a cellular distribution and oestrogen-binding affinity different from the normal receptor (Pfeffer *et al.* 1993) or could act as a ligand-independent transcription factor (Skipper *et al.* 1993). Indeed, by Western blot analysis we did not find any size variant corresponding to the 54 kDa protein in our cell preparations.

The role of oestrogens in male reproduction is now better understood and is supported by many studies. Firstly, the administration of an aromatase inhibitor in rat (Tsutsumi *et al.* 1987) and monkey (Shetty *et al.* 1998) leads to a reduction in numbers of round and elongated spermatids. Secondly, aromatase-deficient (ArKO) mice became infertile as a result of an impairment of spermiogenesis associated with a decrease in sperm motility and an inability to fertilise oocytes (Robertson *et al.* 1999, 2001). Six cases of oestrogen deficiency caused by an inactivating mutation of the *Cyp19* gene have been described (Morishima *et al.* 1995, Carani *et al.* 1997, Deladoey *et al.* 1999, Murata *et al.* 2001, Herrman *et al.* 2002, Kottler *et al.* 2002); analysis of spermatic parameters in three patients revealed a decreased motility (Carani *et al.* 1997, Herrman *et al.* 2002, Kottler *et al.* 2002). Thirdly, αERKO mice are infertile because of an alteration in fluid reabsorption in the proximal parts of the epididymis (Hess *et al.* 1997). An inactivating mutation of the ERα gene in man has been reported by Smith *et al.* (1994); the number of spermatozoa was in the normal range, although their viability was diminished. In contrast, βERKO mice do not have altered spermatogenesis (Krege *et al.* 1998).

Oestrogens could influence the development of germ cells at several levels: stem cell number and spermatid maturation. Indeed, the proliferation of rat gonocytes is induced by 17β-oestradiol (Li *et al.* 1997). Moreover, male rats treated perinatally with an aromatase inhibitor showed a decrease in the number of spermatozoa in the testis (Gerardin *et al.* 2002). Hypogonadal mice, in which germ cell development never progresses beyond the pachytene stage, developed elongated spermatids after treatment with oestradiol (Ebling *et al.* 2000). In addition, in bank voles treated with oestradiol during the resting season, a recrudescence of spermatogenesis has been demonstrated (Bilinska *et al.* 2002). The same observations were recorded in hamsters kept under short-day photoperiod and treated with oestradiol (Pak *et al.* 2002). Indeed, it has been shown that the recrudescence of spermatogenesis in rodents and some steps in spermiogenesis are under the control of oestrogen (for reviews see O'Donnell *et al.* 2001, Carreau *et al.* 2002). Oestradiol has also been proposed as a germ cell survival factor in the human testis (Pentikaïnen *et al.* 2000).

In conclusion, this study was undertaken to look for the expression of aromatase and oestrogen receptors in human immature germ cells and ejaculated spermatozoa. We demonstrated the existence of aromatase and several ERβ and ERα isoforms, as both mRNA and proteins, in human germ cells. However, the roles of oestrogens in human testis are not clearly defined, and further studies are required to elucidate the mechanism of action of oestrogen, in terms of both genomic and non-genomic pathways.

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