The mitochondrial-dependent pathway is chronically affected in testicular germ cell death in adult rats exposed *in utero* to anti-androgens

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Abstract

In utero exposure to exogenous anti-androgenic compounds induces a wide range of abnormalities of the reproductive system, including hypospermatogenesis, cryptorchidism and hypospadias. By using rats exposed *in utero* to the anti-androgenic compound flutamide (0.4, 2 or 10 mg/kg per day), it has been shown that hypospermatogenesis in adult testes could be related to (i) a long-term apoptosis in germ cells but not in somatic Leydig and Sertoli cells as evidenced by the TUNEL approach and (ii) alterations in the mRNA and protein expression of pro- (Bax, Bak, Bid) and anti-apoptotic (Bcl-2, Bcl-w) members of the Bcl-2 family. Indeed, the number of apoptotic germ cells increased with the dose of flutamide administered and the apoptotic germ cells were mainly detected at androgen-dependent stages VII–VIII. Moreover, for the Bcl-2-related proteins that were expressed mainly in the germ cells, a decrease in the levels of anti-apoptotic peptides Bcl-w (60%, $P=0.003$) and Bcl-2 (90%, $P=0.0001$) was observed at 2 mg/kg per day

flutamide and an increase in levels of the pro-apoptotic Bax (2.3-fold, $P=0.0004$) was detected at 10 mg/kg per day. In contrast, the levels of pro-apoptotic peptide Bak that was mainly expressed in somatic cells decreased (70%, $P=0.0008$) at 10 mg/kg per day. Such alterations in Bcl-2-related peptides occurred mainly at the protein level except for Bcl-2 (72%, $P=0.0001$) and Bak (43%, $P=0.0002$) transcripts. Together, these results showed that the apoptosis observed in adult germ cells from rats exposed *in utero* to flutamide may result from a long-term alteration in the balance between pro- and anti-apoptotic Bcl-2-related molecules in favour of pro-apoptotic proteins. These data further supported the concept of an androgen-dependent fetal programming that is in relation with an alteration of the expression of Bcl-2-related genes/proteins promoting apoptosis in testicular germ cells of adult rats with fetal androgen disruption.

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Introduction

There is considerable support for the hypothesis that events occurring in fetal life could have life-long consequences on the health of the adult (Barker *et al.* 1989). Lucas (1991) defined programming as the physiological 'settings' by an early stimulus or insult occurring at a 'sensitive' period through, for example, interaction with the environment, resulting in long-term consequences on functions. The concept of fetal programming could be important for reproductive functioning, therefore underlining the importance of the neonatal period for reproductive development (for reviews see Davies & Norman 2002, Sharpe & Franks 2002). In this context, the recent apparent rise in testicular dysgenesis syndrome, which associates testicular cancer, undescended testes, hypo-

spadias and subfertility, potentially linked to fetal/neonatal exposure to environmental endocrine disruptors, illustrates such a concept. Indeed, *in utero* exposure to chemical compounds with anti-androgenic activity including, for example, pesticides (Gray *et al.* 1994, 1999, Kelce *et al.* 1998, Barlow & Foster 2003, Fisher 2004), phthalates (Mylchreest *et al.* 1998, Gray *et al.* 2000) and drugs such as flutamide (Imperato-McGinley *et al.* 1992, Kassim *et al.* 1997, McIntyre *et al.* 2001) can induce a wide range of abnormalities of the reproductive system, including small testes, cryptorchidism and hypospadias. However, although the effects of these anti-androgenic compounds on spermatogenesis have been established for many years, the cellular and molecular mechanisms underlying the loss of the mature germ cells still remain to be investigated. A possible explanation is that the hypospermatogenesis

is related to an apoptotic germ cell death process (Woolveridge *et al.* 1998, Omezzine *et al.* 2003).

In the present study, by using a model of adult rats exposed *in utero* to flutamide, we investigated whether the expression of the Bcl-2-related proteins, key factors in the mitochondrial-dependent pathway in the apoptotic cell death process, could be affected in this experimental model.

Materials and Methods

Experimental animals

Fifteen pregnant rats (Sprague–Dawley rats from Charles River Laboratories, St Aubin les Elbeuf, France) per group were administered vehicle (methylcellulose) or flutamide by gavage from day 10 of gestation (GD 10) up to the day before delivery (GD 21 or 22). This period includes the period of male internal and external sex differentiation. Because the expression of this compound was analyzed in all the testicular cell types including the germ cells, the doses of flutamide were chosen to avoid or minimize important germ cell loss (McIntyre *et al.* 2001). Indeed, alterations in testicular cellularity may confound the interpretation of the anti-androgen effects on gene expression in the different testicular cell types (for review see Ivell & Spiess 2002). Animals were given flutamide at doses of 0, 0.4, 2 or 10 mg/kg body weight per day adjusted daily based on body weight. Dams were weighed daily from GD 10 up to the day of delivery. After birth, the male rats were left with no flutamide treatment until postnatal day 90. At 90 days, male rats were killed by CO₂ inhalation. Each testis was weighed before being fixed or frozen. Only bilateral descended testes were studied in the present report. All studies on animals were conducted in accordance with current regulations and standards approved by the INSERM animal care committee.

Materials

TRIzol and dNTPs were obtained from Life Technologies (Eragny, France). Flutamide, obtained from Aldrich Chemical Co. (Mannheim, Germany), was dissolved in an aqueous solution of methylcellulose 400 (Fluka, Mulhouse, France) at 0.5% (w/v) and stored for a maximum of 1 week at approximately 5 °C (± 3 °C). Protease inhibitor cocktail was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Sigma (Meylan, France) was the source for random hexanucleotides, actin polyclonal antibody, Tween 20 and Biomax MR film. Taq polymerase was purchased from Promega Life Science (Madison, WI, USA). Horseradish peroxidase-labelled anti-rabbit IgG and the chemiluminescent kits were obtained from CovalAb (Lyon, France). Horseradish peroxidase-labelled anti-goat IgG (SC-2020) and the following primary

antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): rabbit polyclonal raised against human Bcl-2 (28 kDa, no. sc-492), rabbit polyclonal raised against mouse Bax (24 kDa, no. sc-526), rabbit polyclonal raised against human Bak (28 kDa, no. sc-832) and goat polyclonal raised against human Bid (23 kDa, no. sc-6538) or from StressGen Biotechnologies Corp. (Victoria, BC, Canada): rabbit polyclonal raised against human Bcl-w (21 kDa). Mayer's haematoxylin and the aqueous mounting medium (Faramount) were obtained from Dako (Trappes, France). The UltraVision Detection System for immunology was obtained from Lab Vision Corporation (Fremont, CA, USA). M-MLV and [α -³³P]dATP (1000–3000 Ci/mmol) were purchased from Amersham (Orsay, France). Primers were synthesized by PrologoFrance SAS (Paris, France).

TUNEL and immunohistochemistry

Testes were immediately fixed for 24 h in Bouin's fluid and stepwise dehydrated in graded ethanol baths and embedded in paraffin. Paraffin sections of Bouin-fixed testes were sectioned at 5 μ m. The sections were mounted on positively charged glass slides (Superfrostplus; Menzel-Glaser, Germany), deparaffinized, hydrated, treated for 20 min at 93–98 °C in citric buffer (0.01 M, pH 6), rinsed in osmosed water (2 $\times$ 5 min) and washed (2 $\times$ 5 min) in Tris-buffered saline, pH 7.8. Slides were then either used for TUNEL or immunohistochemistry. TUNEL reaction was performed as described earlier (Mauduit *et al.* 2001b), slides were counterstained for 2 min with Mayer's haematoxylin. In each rat testis, at least 100 random seminiferous tubules were counted, the results are expressed as the number of TUNEL-positive cells per 100 seminiferous tubules. For determination of the apoptotic index, counting of apoptotic germ cells and Sertoli nuclei was carried out at stages I–IV, V–VI, VII–VIII, IX–X and XII–XIV with a $\times 100$ oil immersion objective, and the results are expressed as the rate of apoptotic germ cells per Sertoli cells. For immunohistochemistry reaction, the Envision plus kit (Dako, Trappes, France) was used (as described by the manufacturer) with anti-Bid antibody. The UltraVision Detection System was used, as described by the manufacturer, with anti-Bax, anti-Bak, anti-Bcl-2 and anti-Bcl-w antibodies. Antibodies were diluted as follows: 1/50 anti-Bcl-2, 1/100 for anti-Bax, anti-Bak and anti-Bcl-w, and 1/500 for anti-Bid. As a negative control, the antibody diluent above replaced the primary antibody. No staining was observed in the negative control. The same antibody was used for immunohistochemistry and Western blot analyses.

Western blotting analysis

Testicular tissues were homogenized in 200 μ l ice-cold hypotonic buffer (25 mM Tris-HCl, pH 7.4; protease

Table 1 Primer sequences and PCR conditions

Primer name	Primer sequence	PCR product (bp)	Temperature (°C)
Bcl-2	5'-CTTTGTGGAAGTGTACGGCCCCAGCATGCG-3' 5'-ACAGCCTGCAGCTTTGTTTCATG-GTACATC-3'	232	67
Bcl-w	5'-AGACGAGTTTGAGACCC-3' 5'-CCATCCAATCCTGCAC-3'	231	63
Bax	5'-GGGAATTCTGGAGCTGCAGAGGATGATT-3' 5'-GCGGATCCAAGTTGC-CATCAGCAAACAT-3'	97	66
Bak	5'-TTTGGCTACCGTCTGGCC-3' 5'-GGCCCAACAGAACCACACC-3'	201	66
Bid	5'-CACGACCGTGAACTTTAT-3' 5'-GCTGTTCTCTGGGACC-3'	193	63
Cyclophilin A	5'-ACCCACCGTGTCTTCG-3' 5'-CATTTGCCATGGACAAGA-3'	300	
SCF	5'-TGGTGGCATCTGACACTAGTGA-3' 5'-CTTCCAGTATAAGGCTCCAAAAGC-3'	251 (soluble form) 167 (membrane-bound form)	58

inhibitor cocktail) and sonicated (10 s at 80 °C). Protein concentration was determined by the Bradford assay. Proteins (100 µg) were resolved on 10% SDS/polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes as described earlier (Mauduit *et al.* 2001a). Bax was diluted at 1/200, Bcl-2, Bak and Bcl-w were diluted at 1/100 and Bid was diluted at 1/250. The protein loading was checked by reprobing the blot with a rabbit IgG anti-actin antibody (1/500). The Biomax MR films were scanned on Gel doc 2000 (Bio-Rad) apparatus and quantitation was measured with Quantity one Software from Biorad (Marnes-la-Coquette, France).

PCR coamplification

Total RNAs were extracted from rat testis with TRIzol reagent. cDNAs were obtained as described earlier (Mauduit *et al.* 2001a). At the end of the experiment, the reverse transcription mix was diluted 1/2 with RNA-free water. For PCR, each target gene (Bcl-2, Bcl-Bax, Bak, Bid) was coamplified with a housekeeping gene (cyclophilin A), in the presence of 2 µl reverse transcription mix, 0.02U/µl Taq polymerase, 1 µM target primers, 0.5 M cyclophilin A primer, 100 µM dNTP and 0.075 µl [α -³²P]dATP. PCR amplification conditions were 95 °C for 5 min followed by 35 cycles for Bcl-2, Bax and Bid, 30 cycles for Bcl-w, 27 cycles for stem cell factor (SCF) and 24 cycles for Bak of 95 °C for 30 s, hybridization temperature (T_m) 30 s (see Table 1) 72 °C 30 s. PCRs ended with a step at 72 °C for 5 min. At the end of the experiment, coamplified target and housekeeping PCR products were resolved on an 8% polyacrylamide gel. Dried gels were exposed on a phosphor screen for 30–60 min. Quantitation of each PCR product was performed on a Cyclone phosphorimager with Optiquant software from Packard,

Meriden, CT, USA. The primers used are presented in Table 1. All PCR products were checked by direct sequencing. PCR analyses were carried out from the logarithmic phase of amplification. RT-PCR primers were designed inside separate exons to avoid any bias related to residual genomic contamination. Moreover, for all primers, no amplification was observed when PCR was performed on RNA preparations.

Data analysis

For TUNEL analyses and for protein and mRNA level quantitation, ten animals from different litters were used for each experimental condition. Three different independent experiments of *in utero* exposure to flutamide were carried out. The results from a representative experiment are shown here. For statistical analysis, ANOVA was performed to determine whether there were differences between groups ($P < 0.05$), and then the Bonferroni post-test was performed to determine the significance of the differences between the pairs of groups. A P value of 0.05 was considered significant. The statistical tests were performed on StatView software version 5.0 (SAS Institute Inc, Cary, NC, USA).

Results

Effects of *in utero* exposure to flutamide on apoptosis in adult rat testes

All adult animals exposed *in utero* to flutamide at doses of 0.4 or 2 mg/kg per day displayed bilateral descended testes (Table 2). In contrast, 33% of animals exposed to 10 mg/kg per day flutamide displayed cryptorchidism (Table 2). Among the cryptorchid animals, 40% displayed bilateral and 60% unilateral non-descended testes. In the

Table 2 Incidence of testicular weight and lesions in 90-day-old rats exposed to flutamide *in utero*. The testicular weights are means ± s.d.

	Flutamide (mg/kg per day)			
	0	0.4	2	10
No. of animals with descended/non-descended (cryptorchid) testes	34/0	31/0	25/0	10/5
No. of animals showing bilateral/unilateral non-descended testes	—	—	—	2/3
No. of animals (litters)	34 (5)	31 (5)	25 (5)	15 (3)
Testicular weight (g) of descended (D) testes and non-descended (ND) testes	1.79 ± 0.18	1.92 ± 0.15 NS	1.87 ± 0.22 NS	D: 1.78 ± 0.09 NS ND: 0.58 ± 0.1*

NS, not significantly different from control; **P*=0.0001 compared with control.

experiments presented here, all the animals displayed bilateral descended testes. The weight of descended testes from adult rats was not modified by *in utero* exposure to flutamide at the doses used (Table 2).

In utero exposure to flutamide induced an apoptotic cell death process. TUNEL-positive cells were exclusively observed in germ cells but not in somatic (Leydig and Sertoli) cells (Fig. 1*A* and *B*). A significant and flutamide dose-dependent increase in the number of apoptotic cells evaluated per 100 seminiferous tubules was observed at 0.4 (*P*=0.0001), 2 (*P*=0.0001) and 10 (*P*=0.0001) mg/kg per day as compared with untreated rats (Fig. 1*C*). Moreover, *in utero* exposure to flutamide induced apoptosis mainly in germ cells at stages VII–VIII of the seminiferous epithelium (Fig. 1*D*).

Immunolocalization of Bcl proteins in adult rat testes

In adult rat testis, Bcl-w was immunolocalized to somatic Leydig and Sertoli cells and mainly to pre-meiotic germ cells (Fig. 2*A* and Table 3). Bcl-2 was detected in Leydig cells and pachytene spermatocytes (Fig. 2*C*). The pro-apoptotic protein Bak was mainly localized to somatic Sertoli cells (Fig. 2*E*). Bax was expressed in Leydig cells and mainly in meiotic germ cells (Fig. 2*G*) and Bid was exclusively immunolocalized to round and elongated spermatids in adult rat testes (Fig. 2*I*). *In utero* exposure to flutamide did not affect the immunolocalization of the Bcl proteins to the different testicular cell types. However, Bcl-w (Fig. 2*B*) and Bcl-2 (Fig. 2*D*) immunostaining intensity appeared to be lower in germ cells from rats exposed *in utero* to flutamide compared with those of unexposed rats. Similarly, the anti-androgen seemed to lower Bak immunostaining intensity in Sertoli cells (Fig. 2*F*). While *in utero* exposure to flutamide did not affect Bid immunoreactivity (Fig. 2*J*), it seemed to induce a redistribution of Bax staining in the cytoplasm of germ cells in a punctate pattern (Fig. 2*H*).

Effects of in utero exposure to flutamide on Bcl-w and Bcl-2 mRNA and protein levels in adult rat testes

In utero exposure to flutamide did not modify Bcl-w mRNA levels in the adult rat testes (Fig. 3*A*) while it

induced a significant decrease in Bcl-w protein levels at 2 (60%, *P*=0.003) and 10 (80%, *P*=0.0004) mg/kg per day of the anti-androgen (Fig. 3*B*). *In utero* exposure to the anti-androgen induced a dose-dependent decrease in both Bcl-2 mRNA and protein levels (Fig. 4) in the adult rat testes. The largest decrease (72%, *P*=0.0001) in Bcl-2 mRNA levels was observed at 2 mg/kg per day (Fig. 4*A*). In parallel, Bcl-2 protein levels were also decreased after *in utero* exposure to the anti-androgen at 0.4 (72% decrease, *P*=0.0008), 2 (90% decrease, *P*=0.0001) and 10 (81% decrease, *P*=0.0002) mg/kg per day (Fig. 4*B*).

Effects of in utero exposure to flutamide on Bak, Bax and Bid mRNA and protein levels in adult rat testes

In these experiments, we examined whether *in utero* exposure to flutamide could induce changes in Bak, Bax and Bid mRNA and protein levels in the adult rat testes.

Bak mRNA levels were decreased at the different doses of flutamide with the highest reduction (65% decrease, *P*=0.0001) observed at 10 mg/kg per day (Fig. 5*A*). Similarly, Bak protein levels were also decreased in a flutamide dose-dependent manner with the highest reduction (70% of the control, *P*=0.0008) in Bak protein levels observed at 10 mg/kg per day flutamide (*P*=0.0008, Fig. 5*B*).

Since Bak was immunolocalized to Sertoli cells, we tested whether the decrease in Bak expression was specific. The decrease in Bak mRNA was specific in that such an alteration was not observed, for example, for another gene (stem cell factor, SCF) which was expressed exclusively in Sertoli cells (Rossi *et al.* 1991). *In utero* exposure to flutamide did not affect mRNA levels of either soluble or the membrane-bound form of SCF in the adult rat testis (Fig. 6).

In utero exposure to flutamide did not affect the transcriptional activity of Bax as no significant effect on Bax mRNA levels was observed in the adult rat testes (Fig. 7*A*) while it enhanced Bax protein levels. A significant (*P*=0.0004) increase (about 2.3-fold) in Bax protein levels was detected at 10 mg/kg per day of the anti-androgen (Fig. 7*B*).

Figure 1 Detection of apoptosis in the adult rat testis exposed *in utero* to flutamide. Testes were obtained from adult rats (A) unexposed or (B) exposed *in utero* to flutamide (10 mg/kg per day). Scale bar=50 μ m. (C) Number of TUNEL-positive germ cells per 100 seminiferous tubules; values are the means $\pm$ S.D. of ten rats per group. (D) Quantitative estimation of stage-specific changes in the incidence of germ cell apoptosis (expressed as numbers of TUNEL-positive germ cells per Sertoli cell) at designated stages of the seminiferous epithelium; values are the means $\pm$ S.D. of four rats per group.

In contrast to Bax and Bak, both Bid mRNA (Fig. 8A) and protein (Fig. 8B) levels were not affected in adult rat testes following *in utero* exposure to the anti-androgen.

Discussion

The data presented here indicated that *in utero* exposure to the anti-androgen flutamide induced, in adult rat testes, a chronic apoptotic cell death process in germ cells but not in somatic cells. Indeed, the *in situ* TUNEL approach revealed that germ cells represented the predominant apoptotic cell type in the testes of adult rat exposed *in utero* to flutamide. These alterations in the germ cell death process may represent early molecular events leading to the hypospmatogenesis observed after an *in utero*

exposure to higher doses of the anti-androgen (Imperato-McGinley *et al.* 1992, Kassim *et al.* 1997, McIntyre *et al.* 2001). Moreover, such an observation is compatible with a recent report from De Gendt *et al.* (2004) showing that a selective knock-out of the androgen receptor in Sertoli cells induced a dramatic increase in germ cell apoptosis in adult mice without affecting the Sertoli cell number. *In utero* exposure to flutamide induced apoptosis of germ cells mainly in the seminiferous tubules at androgen-dependent stages VI–VIII, whereas, germ cell apoptosis in physiological conditions occurred mainly at stages I–IV or XII–XIV, as testosterone plays a pivotal role in protecting germ cells at stages VII–VIII from programmed cell death (Lue *et al.* 1999), these observations suggested that *in utero* exposure to flutamide impaired, in the adult testes, the protecting action exerted by testosterone on germ cells.

Table 3 Immunolocalization of Bcl-2 family genes in the testicular cells. The results from Figure 2 are summarized here

	Leydig cells	Sertoli cells	Germ cells		
			Pre-meiotic	Meiotic	Post-meiotic
Anti-apoptotic					
Bcl-w	++	++	+++	+++	
Bcl-2	+		+++	+++	
Pro-apoptotic					
Bak	++	+++			
Bax	+++			+++	
Bid					+++

As the molecular mechanisms underlying the apoptotic process in germ cells in adult rat testes exposed *in utero* to anti-androgens remain unknown, the present study was aimed at identifying one of the signaling pathways potentially leading to the long-term apoptosis in germ cells in rat testes exposed *in utero* to flutamide. Our approach was focused on a possible alteration of the mitochondrial-dependent pathway in the germ cell death process. Indeed, it is accepted that the concerted action of Bcl-2 family proteins controls and directs apoptotic signals from either intrinsic or extrinsic stimuli towards the mitochondrial surface, and the Bcl-2 proteins act as ‘gatekeepers’ for the release of mitochondrial apoptogenic factors that activate proteolytic caspase cascade (Cain *et al.* 2002, Parone *et al.* 2002, Degli Esposti & Dive 2003). The Bcl-2 family of proteins consists of pro-apoptotic (Bax, Bak, Bid) and anti-apoptotic (Bcl-2, Bcl-w) proteins, which compete through dimerization to keep (anti-apoptotic) or alter (pro-apoptotic) the integrity of the mitochondrial membranes.

We first tried to answer the question as to whether *in utero* exposure to flutamide may affect the immunolocalization of the Bcl-2-related proteins in the testis. In the literature, immunolocalization in the testis of Bcl-2-related proteins showed that Bcl-2 was either expressed in germ cells and Leydig cells (Vilagrasa *et al.* 1997, Beumer *et al.* 2000, Oldereid *et al.* 2001, present study) or absent from mature testes (Meehan *et al.* 2001, Yan *et al.* 2000b). The reasons for the discrepancies observed in the localization of Bcl-2 in the testicular cell types between the different studies are not known. They could be accounted for by the use of (i) different technical approaches, i.e. Northern blotting, immunohistochemistry or *in situ* hybridization, (ii) different Bcl-2 antibodies directed against different epitopes or (iii) testicular tissues from different species (human, mouse or rat). Bcl-w was detected in Sertoli cells and mainly in germ cells (Print

et al. 1998, Ross *et al.* 1998, Yan *et al.* 2000b, Meehan *et al.* 2001, present study). *In utero* exposure to flutamide appeared to decrease Bcl-2 and Bcl-w immunostaining mainly at the level of germ cells (present study). The pro-apoptotic proteins were immunolocalized mainly to somatic Sertoli cells (Bak) and to Leydig and mainly germ cells (Bax) (Yan *et al.* 2000a, Meehan *et al.* 2001, present study). *In utero* exposure to the anti-androgen appeared to induce a decrease in Bak immunostaining intensity in Sertoli cells and to induce a redistribution of Bax immunoreactivity to a perinuclear compartment in germ cells (present study). A redistribution of Bax from a cytoplasmic to a perinuclear localization was yet reported in germ cells when apoptosis was triggered by mild testicular hyperthermia (Yamamoto *et al.* 2000) or observed at the beginning of the apoptotic process in a colorectal cell line (Mandal *et al.* 1998). Such a subcellular translocation of Bax (from cytosol to mitochondria) has been demonstrated to precede the apoptotic process (Zamzami *et al.* 1998). With regard to Bid, the protein was detected exclusively in germ (spermatids) cells (present report). *In utero* exposure to the anti-androgen did not affect the immunoeexpression of Bid in the different testicular cell types.

It was shown here that the apoptotic process observed in the adult rat testes exposed *in utero* to the anti-androgen may result from a long-term alteration of the ratio pro-/anti-apoptotic proteins in adult rat testes. Specifically, an increase in Bax and a decrease in Bcl-w protein levels (both expressed mainly in germ cells) were observed, leading to an increase in the Bax/Bcl-w ratio. It has been suggested that an elevated Bax/Bcl-w ratio might lead to an increase in the apoptotic process in germ cells (Knudson *et al.* 1995, Yan *et al.* 2000b). Furthermore, it is noteworthy that apoptosis occurred in germ cells but not in somatic cells (Sertoli, Leydig and peritubular myoid cells) from rats exposed *in utero* to an anti-androgen, although

Figure 2 Immunostaining of Bcl-2-related proteins in the adult rat testis exposed *in utero* to flutamide. Sections were treated with Bcl-w (A and B), Bcl-2 (C and D), Bak (E and F), Bax (G and H) or Bid (I and J) antibody. Testes were obtained from adult rats unexposed (A, B, C, D and E) or exposed *in utero* to 10 mg/kg per day flutamide (B, D, F, H and J). Scale bar=50 µm; inset scale bar=200 µm.

Figure 3 Effect of *in utero* exposure to flutamide on Bcl-w mRNA and protein levels in the adult rat testis. Bcl-w mRNA (A) and protein (B) levels were determined in adult rat testes not exposed (0) or exposed *in utero* to 0.4, 2 or 10 mg/kg per day flutamide. The Figure represents the values (solid symbols) and the mean (lines) determined from at least ten different animals from different litters. Representative autoradiograms are shown in the upper panels. Cyclo A, cyclophilin A. *Control versus flutamide (2 mg/kg per day), $P < 0.003$; **control versus flutamide (10 mg/kg per day), $P < 0.0004$.

these somatic cells are direct target cells of flutamide because they express the androgen receptor. In the fetal testes, androgen receptor is mainly expressed in peritubular myoid cells and not in Sertoli cells; therefore, the fetal androgen disruption occurring by *in utero* exposure to flutamide probably involves alterations in both peritubular myoid and Sertoli cells. The cellular and molecular mechanisms leading to the absence of the apoptotic cell death process (i.e. an anti-apoptotic process?) in testicular somatic cells remain unknown. However, based on our

Figure 4 Effect of *in utero* exposure to flutamide on Bcl-2 mRNA and protein levels in the adult rat testis. Bcl-2 mRNA (A) and protein (B) levels were determined in adult rat testes not exposed (0) or exposed *in utero* to 0.4, 2 or 10 mg/kg per day flutamide. The Figure represents the values (solid symbols) and the mean (lines) determined from at least ten different animals from different litters. Representative autoradiograms are shown in the upper panels. *Control versus flutamide (0.4 mg/kg per day), (A) $P < 0.0006$, (B) $P < 0.0008$; **control versus flutamide (2 mg/kg per day), (A and B) $P < 0.0001$; ***control versus flutamide (10 mg/kg per day), (A) $P < 0.0001$, (B) $P < 0.0002$.

present data, we suggest that the decrease in the expression of the pro-apoptotic Bak (in terms of mRNA and proteins) immunoexpressed in somatic cells (Yan *et al.* 2000b, present study) may represent the beginning of an explanation for the absence of apoptosis in somatic cells, particularly in Sertoli cells.

Although changes in the expression of some of the Bcl-2 family members in the testis have been reported in other

Figure 5 Effect of *in utero* exposure to flutamide on Bak mRNA and protein levels in the adult rat testis. Bak mRNA (A) and protein (B) levels were determined in adult rat testes not exposed (0) or exposed *in utero* to 0.4, 2 or 10 mg/kg per day flutamide. The Figure represents the values (solid symbols) and the mean (lines) determined from at least ten different animals from different litters. Representative autoradiograms are shown in the upper panels. *Control versus flutamide (2 mg/kg per day), $P < 0.0002$; **control versus flutamide (10 mg/kg per day), $P < 0.0001$; ***control versus flutamide (10 mg/kg per day), $P < 0.0008$.

studies following androgen disruption during adulthood (Woolveridge *et al.* 1999, 2001, Yan *et al.* 2000a, Jahnukainen *et al.* 2004), the novelty of our observations is that the changes in Bcl-2-related gene expression are chronic as they are observed in adult rat testes which were exposed to flutamide exclusively during fetal life but not during adulthood. The elevation of Bax protein levels and the increase in germ cell apoptotic number at stages

Figure 6 Effect of *in utero* exposure to flutamide on SCF mRNA levels in the adult rat testis. mRNA levels for the soluble (SCFs) and the membrane-bound (SCFm) forms of SCF were determined in adult rat testes not exposed (0) or exposed *in utero* to 0.4, 2 or 10 mg/kg per day flutamide. The Figure represents the values (solid symbols) and the mean (lines) determined from at least ten different animals from different litters. Representative autoradiograms are shown in the upper panels.

VII–VIII of the seminiferous epithelium (present study) were similarly observed in the testes of animals deprived of androgens during adulthood (Woolveridge *et al.* 1999, 2001, Yan *et al.* 2000a, Jahnukainen *et al.* 2003). In contrast, mild testicular hyperthermia induced apoptosis of germ cells at stages I–IV and XII–XIV, Bax protein levels remained unchanged while Bcl-2 protein levels increased significantly (Yamamoto *et al.* 2000). These observations therefore suggested that (i) the alterations in spermatogenesis observed in adult animals exposed *in utero* to flutamide appear to be related to abnormal androgen action and (ii) the germ cell apoptotic process exhibits specificities depending on the triggering mechanisms (androgenic deprivation versus heat). In this context, it is of interest to mention that, in our present study, only bilateral descended but not cryptorchid testes were used. The data in the present study suggested that the testicular expression of the Bcl-2 family genes is related to an

Figure 7 Effect of *in utero* exposure to flutamide on Bax mRNA and protein levels in the adult rat testis. Bax mRNA (A) and protein (B) levels were determined in adult rat testes not exposed (0) or exposed *in utero* to 0.4, 2 or 10 mg/kg per day flutamide. The Figure represents the values (solid symbols) and the mean (lines) determined from at least ten different animals from different litters. Representative autoradiograms are shown in the upper panels. *Control versus flutamide (10 mg/kg per day), $P < 0.0004$.

androgen-dependent fetal programming. The fetal androgen disruption appears to affect the Bcl-2 family gene expression in the testes at two different levels at least, i.e. (i) a transcriptional level leading to a decrease in the mRNA levels of anti- and/or pro-apoptotic factors (e.g. Bcl-2 and Bak) and (ii) a translational level leading to changes in protein levels of both anti- and/or pro-apoptotic factors. These changes could be associated (e.g. Bcl-2 and Bak) or not (Bcl-w and Bax) with changes in the corresponding mRNA levels. Finally, some members of the Bcl-2 family appeared not to be affected by the exposure to flutamide. Indeed, although Bid was immunoexpressed in a germ cell type, such as spermatids, known to be sensitive to androgen action, Bid mRNA and

Figure 8 Effect of *in utero* exposure to flutamide on Bid mRNA and protein levels in the adult rat testis. Bid mRNA (A) and protein (B) levels were determined in adult rat testes not exposed (0) or exposed *in utero* to 0.4, 2 or 10 mg/kg per day flutamide. The Figure represents the values (solid symbols) and the mean (lines) determined from at least ten different animals from different litters. Representative autoradiograms are shown in the upper panels.

protein levels were found not to be affected in adult rat testes exposed *in utero* to the different doses of flutamide used in the present study.

Although the regulatory mechanisms underlying the long-term changes in Bcl-2-related gene expression in the adult rat testes exposed *in utero* to flutamide remain to be investigated, they are probably different from those at the origin of the alterations in Bcl-2-related gene expression when androgen disruption occurs during adulthood (Woolveridge *et al.* 1999, 2001, Yan *et al.* 2000a). Indeed, when androgen disruption occurs during adulthood (Yan *et al.* 2000a), the elevated Bax/Bcl-w ratio is correlated with a decrease in plasma testosterone levels whereas, in our present study, the increase in Bax/Bcl-w ratio is probably not related to an androgen deficiency in the adult

rat for at least two reasons. First, circulating levels of testosterone in adult animals exposed *in utero* to anti-androgens such as flutamide (Goddard *et al.* 2003) and vinclozolin whose metabolites also prevent testosterone binding to its receptor (Wolf *et al.* 2000) were not significantly different from those found in untreated control animals. Similarly, *in utero* exposure to flutamide has also been reported not to affect intra-testicular testosterone levels in adult rat testes (Kassim *et al.* 1997). Secondly, in the seminiferous tubules, androgen receptors appear normally immunexpressed in Sertoli cells since its mRNA and protein levels were not affected (authors' unpublished data). Such observations would suggest that *in utero* exposure to flutamide would alter androgen action in the adult testes at a post-androgen receptor level and probably at the level of androgen targeted genes. While our present data suggested that *in utero* exposure to flutamide chronically affected the mitochondrial pathway, we cannot exclude the possibility that the death receptor pathway is also affected. Finally, in the context of fetal programming, the regulatory mechanisms underlying the chronic alterations in Bcl-2-related gene/protein expression in adult rat germ and somatic cells when the exposure to the anti-androgen specifically occurs during fetal life remain to be investigated. However, the possibility exists that the *in utero* exposure to the anti-androgen could alter the epigenetic control of some specific testicular genes expressed under androgen control. Such a possibility has been suggested for another endocrine disrupter (McLachlan 2001). Indeed, developmental exposure to the endocrine disruptor diethylstilboestrol elicits demethylation of the lactoferrin promoter in the mouse uterus. Those results suggested that oestrogens, either directly or through related signalling pathways, play a role in the imprinting of gene(s). Thus, when a gene programmed to respond to oestrogen at puberty is misprogrammed by developmental exposure to an endocrine disruptor, it will respond abnormally and the possibility exists that abnormalities in the functions of the organs occur (McLachlan 2001).

In summary, the present results have demonstrated long-term changes in the balance between pro- and anti-apoptotic molecules of the Bcl-2 family in terms of mRNA and protein levels in the adult rat testes exposed *in utero* to flutamide. Specifically, a decrease in anti-apoptotic factors such as Bcl-2 and Bcl-w levels associated with an increase in pro-apoptotic Bax levels were observed. The changes in the balance between pro- and anti-apoptotic molecules potentially induce caspase activation (Omezzine *et al.* 2003) which triggers the apoptotic cascade leading to a chronic germ cell death process observed in the adult rats when exposed *in utero* to flutamide.

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