Environmental toxicant-induced germ cell apoptosis in the human fetal testis

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BACKGROUND: Disorders of the male reproductive system are increasing in prevalence. The term testicular dysgenesis syndrome emphasizes the importance of developmental influences on the aetiology of conditions including cryptorchidism, testicular germ cell cancer and reduced spermatogenesis. Men whose mothers smoked during pregnancy have lower sperm production. Cigarette smoke contains agents acting on the aryl hydrocarbon receptor (AHR). We have investigated the presence of AHR in the developing human testis and the effects of functional activation.

METHODS AND RESULTS: Immunohistochemistry determined AHR to be expressed by germ cells in the human testis between 7 and 19 week gestation, but not by other cells. Treatment of cultured fetal testis with an AHR ligand present in tobacco smoke increased markers of cell apoptosis, and this was prevented by an AHR receptor antagonist. Immunohistochemistry indicated that apoptosis was restricted to germ cells. CONCLUSIONS: Germ cells in the developing human testis are a target for regulation by AHR ligands. Activation of AHR by environmental toxicants and AHR-induced apoptotic pathways may be the mechanism of action underlying the epidemiological findings of reduced spermatogenesis in men exposed to cigarette smoke before birth, and may also be of importance in other conditions comprising the testicular dysgenesis syndrome.

Keywords: testis; environmental toxin; spermatogenesis; development; smoking

Introduction

Adult male reproductive function is considerably influenced by events in the intrauterine and immediate post-natal periods. The testicular dysgenesis syndrome encompassing abnormalities including cryptorchidism, testicular germ cell cancer and reduced spermatogenesis. Men whose mothers smoked during pregnancy have lower sperm production. Cigarette smoke contains agents acting on the aryl hydrocarbon receptor (AHR).

Testicular development follows migration of primordial germ cells (PGCs) from the extraembryonic mesoderm of the yolk sac to the indifferent gonad. Sexual differentiation occurs at ~8 weeks gestation (i.e. 6 weeks postconception) in the human, when PGCs become surrounded by Sertoli cells, forming testicular cords, and continue to proliferate throughout fetal life (Hilscher, 1991). This increase in number is rapid, with a 10-fold increase in germ cell number in the 3 weeks following sexual differentiation. Sertoli cells proliferate in parallel, maintaining a constant ratio between germ cell and Sertoli cell numbers (Bendsen et al., 2003).

This period is therefore critical for the establishment of normal testicular structure and function, and is a period of potential vulnerability of the testes to external influences. One potential external influence is cigarette smoke. It is well established that smoking is associated with a significant reduction in sperm concentration and quality (Vine et al., 1994; Kunzle et al., 2003). There is also evidence for adverse effects on the developing testis, as epidemiological studies have shown that sons exposed to maternal smoking in utero have a reduction in sperm concentration of between 20 and 48% in comparison to unexposed men (Storgaard et al., 2003; Jensen et al., 2004). Tobacco smoke contains polycyclic aromatic hydrocarbons (PAHs), environmental toxicants produced primarily as a by-product of fossil fuel combustion. PAHs bind to and activate the aryl hydrocarbon receptor (AHR) (Hankinson, 1995), which is a member of the per-Arnt-Sim family of transcriptional factors. The AHR is involved in toxin metabolism and also has physiological roles in the development of several organ systems (Pocar et al., 2005). An endogenous ligand has not been identified.
Treatment of fetal mice in utero with the AHR ligand benzo(a)pyrene reduced the fertility of both male and females in adulthood (Mackenzie and Angevine, 1981). Maternal exposure to dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD), also an AHR ligand, has also been demonstrated to have irreversible effects on the developing reproductive tract in rats including reduced sperm production (Mably et al., 1992a; Gray et al., 1995, 1997; Sommer et al., 1996; Wilker et al., 1996; Faqi et al., 1998), and reduced the size of the reproductive organs (Mably et al., 1992b; Bjerke and Peterson, 1994; Roman et al., 1995, 1998; Gray et al., 1997; Roman and Peterson, 1998). Both in vitro and in vivo exposure of mouse fetal ovaries to PAHs results in an increase in germ cell apoptosis, subsequently demonstrated to be mediated through an increase in Bax expression (Matikainen et al., 2001, 2002).

The aim of this study was therefore to explore the expression, distribution and effect of functional activation of the AHR in the human fetal testis to investigate mechanisms underlying the effects of environmental AHR agents on testicular development. This study investigated expression of Bax, cleaved caspase 3 and cleaved PARP, which are established markers of the regulation, execution and a target of the apoptotic pathway, respectively, (Gross et al., 1999); n the case of Bax, a link with AHR activation in the developing gonad has been previously demonstrated (Matikainen et al., 2001).

Materials and Methods

Tissue
Human fetal testes were obtained following medical termination of pregnancy during both the first and second trimesters (64 days to 20 week gestational age). Women gave consent according to national guidelines and the study was approved by the Lothian Research Ethics Subcommittee (Polkinghorne, 1989). Termination of pregnancy was induced by treatment with mifepristone (200 mg orally) followed 48 h later by misoprostal (800 mcg) three hourly per vaginum. None of the terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination before termination and confirmed by subsequent direct measurement of foot length.

Testes were dissected and either snap frozen and stored at −70°C, fixed in Bouin’s for 2 h, followed by processing for immunohistochemistry or further dissected for in vitro culture experiments.

Immunohistochemistry
Paraffin-embedded testes were cut into 5-μm sections and mounted onto electrostatically charged microscope slides (VWR, Poole, UK), dried overnight and then dewaxed and rehydrated using conventional methods. Endogenous peroxidases were quenched in 3% hydrogen peroxide in methanol for 30 min at room temperature. After a wash in water, slides were transferred into Tris-buffered saline (PBS) for 5 min and blocked for 30 min in normal serum (Diagnostics Scotland) diluted 1:4 in PBS containing 5% BSA. Sections were blocked with avidin (0.001 M; 15 min) and then biotin (0.001 M; 15 min; both from Vector Laboratories) with washes in PBS in between. AHR antibody (Affinity BioReagents, Cambridge, UK) was diluted 1:150 and applied to sections at 4°C overnight in a humidified chamber. AHR was visualized by tyramide-enhanced fluorescein via a peroxidase conjugated goat anti-mouse secondary antibody diluted 1:200. Sections were counterstained with propidium iodide 1:1000. Fluorescent images were captured using a LSM510 confocal microscope. Negative controls were incubated with mouse IgG, omitting primary antiserum.

Tissue culture
Fetal testes were dissected and cultured in minimum essential medium α with phenol red (GibcoBRL, Life Technologies, Paisley, UK) supplemented with 2 mM pyruvate, 2 mM glutamine, 1 x ITS supplement (Sigma) and 3 mg/ml BSA and with penicillin, streptomycin and amphotericin as previously described (Robinson et al., 2003). Fetal testes (14–19 weeks) were dissected in prewarmed and gassed medium to yield tissue fragments ~1 mm². Between six and eight fragments were transferred in 500 μl medium to wells containing medium to yield tissue fragments ~1 mm². Between six and eight fragments were transferred in 500 μl medium to wells containing medium to yield tissue fragments ~1 mm². Between six and eight fragments were transferred in 500 μl medium to wells containing medium to yield tissue fragments ~1 mm². Between six and eight fragments were transferred in 500 μl medium to wells containing medium to yield tissue fragments ~1 mm². Between six and eight fragments were transferred in 500 μl medium to wells containing medium to yield tissue fragments ~1 mm². Between six and eight fragments were transferred in 500 μl medium to wells containing medium to yield tissue fragments ~1 mm². Between six and eight fragments were transferred in 500 μl medium to wells containing medium to yield tissue fragments ~1 mm².
Fetal testes were homogenized in lysis buffer containing 80 mM Tris (pH 6.8), 1% (v/v) glycerol, 1% (w/v) sodium dodecyl sulphate (SDS) and a Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Total protein quantification was performed using the BioRad protein assay kit.

Proteins were boiled in a 1:3 volume of 4× reduced sample buffer [20% 250 mM Tris (pH 6.8), 4% SDS, 10% β-mercaptoethanol and 0.1% bromophenol blue]. Samples of 10 μg total protein was loaded onto 15% SDS–polyacrylamide gel electrophoresis (PAGE) gels with high-molecular weight Rainbow protein markers run in parallel (Amersham Biosciences, Buckinghamshire, UK). Gels were blotted onto polyvinyl difluoride membrane (Amersham Biosciences) and then blocked in TBS and Tween [10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20] containing 5% powdered milk before overnight incubation at 4°C. Bound antibody was detected using HRP-linked secondary antibodies to Bax (Santa Cruz), cleaved PARP (Cell Signaling Technology, Danvers, USA) and β-actin (Santa Cruz) were diluted 1:1000, 1:1000 and 1:100 in TBS and Tween 20 containing 5% powdered milk. Bound antibody was detected using HRP-linked secondary antibodies diluted 1:10 000 (Cell Signaling Technology), and proteins were revealed and quantified by phosphoimager analysis using the Typhoon 9400 system (Amersham Biosciences) and normalized for protein loading using β-actin.

AHR protein in the human fetal testis
Expression of AHR was detected by immunofluorescence in all specimens of human fetal testis examined, between the gestational ages of 64 days and 20 weeks (Fig. 1A and B). AHR was exclusively localized to the cytoplasm of germ cells, without expression by other cell types in either the testis cords or interstitium at any gestation examined. In the first trimester, AHR was expressed by all germ cells, however, by the second trimester, AHR was only expressed by a subpopulation of germ cells. Immunoblotting confirmed the expression of AHR protein in the human fetal testis, with a single band detected of molecular weight 95 kDa (Fig. 1C).

Bax and cleaved caspase 3 expression in fetal testis
Apoptotic cells in the testis were identified by immunolocalization of Bax and cleaved caspase 3. Bax was expressed in all specimens of human fetal testis examined, being located in both the Sertoli and germ cells, with no expression in any cells of the interstitium (Fig. 2C). An apparent increase in Bax expression was observed in germ cells in human fetal testis cultured with DMBA-DHD (Fig. 2G). Cleaved caspase 3 is another marker of apoptosis. Immunohistochemical examination of uncultured human fetal testes and human fetal testis cultured with the vehicle, revealed no cleaved caspase 3 expression (Fig. 2D and F). Testis cultured with DMBA-DHD however showed expression of cleaved caspase 3 in a small number of cells in the testicular cords (Fig. 2H). Morphologically, these cells appeared to be germ cells, with no other cell types in either the testis cords or interstitium expressing cleaved caspase 3.

PAHs increase apoptosis in fetal testis
The effect of PAHs on markers of apoptosis in the fetal testis was investigated using an in vitro culture system and immunoblot analysis. Preliminary experiments demonstrated that DMBA-DHD treatment increased Bax expression by ~2-fold in a concentration dependent manner (P = 0.01, Fig. 3A). Further experiments again resulted in a 2-fold increase in Bax expression from 6.9 ± 1.9 to 14 ± 1.7 relative to β-actin (P < 0.001, Fig. 3B). Treatment of fetal testis with 1 μM αNF had no effect alone but completely reversed this proapoptotic effect of DMBA-DHD in germ cells (Fig. 3B and C). 8.3 ± 3.0 αNF alone, 8.3 ± 1.0 αNF with DMBA-DHD, P = 0.02 versus DMBA-DHD alone, ns versus control, n = 5).

Expression of a further marker of apoptosis, the caspase product cleaved PARP, showed a 3-fold increase from 1.08 ± 0.38 to 3.12 ± 0.5 relative to β-actin (P = 0.02) (Fig. 4). Treatment of fetal testis with 1 μM αNF had no effect alone but completely reversed the proapoptotic effect of DMBA-DHD in germ cells (Fig. 4) (1.8 ± 0.5 αNF alone,
1.54 ± 0.5 αNF with DMBA-DHD, $P = 0.03$ versus DMBA-DHD alone, ns versus control, $n = 5$).

**Discussion**

This study provides direct evidence that exposure to toxins found in tobacco smoke results in germ cell apoptosis in the developing human testis. Epidemiological studies have demonstrated that sperm production is dose-dependently reduced by 20–48% in the sons of mothers who smoked during pregnancy, and testicular volume is also reduced (Storgaard et al., 2003; Jensen et al., 2004). Fecundability was also reduced in men exposed to cigarette smoke *in utero* in a study of 430 Danish couples (Jensen et al., 1998), although a previous study found no such effect (Baird and Wilcox, 1986).

The results from these experiments demonstrate expression of AHR in germ cells but not in other cell types in first and second trimester human fetal testis. AHR has been previously demonstrated in adult human testis (Schultz et al., 2003), with widespread expression in almost all interstitial and tubular cells. Expression fell with increasing gestation, with the AHR expressed in all germ cells in the first trimester but only in a subpopulation in the second trimester. This may indicate that the AHR is only expressed at certain developmental stages as PGCs mature to gonocytes/prespermatogonia (Gaskell et al., 2004). The AHR is an orphan receptor member of the per-Arnt-Sim family of transcriptional factors. It is involved in toxin metabolism, but there is also a substantial body of evidence regarding its role in embryological development of different organ systems, which may reflect a physiological function (Pocar et al., 2005). AHR has also been implicated as playing a role in normal vascular biology and AHR activation modulates transcription of genes involved in regulating the cell cycle, apoptosis and oxidative stress (Denison and Heath-Pagliuso, 1998). In males, knockout of the AHR resulted in reduced weight of the testes and other organs, although adult sperm production was normal (Lin et al., 2003). In female mice, knockout of the AHR resulted in an increase in primordial follicles between Days 2–4, suggesting a potential physiological role of the AHR in prenatal germ cell apoptosis (Benedict et al., 2000). The data shown here demonstrate highly selective expression of AHR in only the germ cells of the developing human testis during both the first and second trimesters, giving a stage of development during which any physiological role of AHR may be important. However, it also provides a basis for potentially adverse effects by environmental toxicants that act as AHR ligands. These include dioxin and PAHs produced in cigarette smoke.

Human fetal testis was cultured in the presence of DMBA-DHD, the active metabolite of the PAH. DMBA that is produced primarily in cigarette smoke was used to investigate whether AHR activation resulted in apoptosis. Three markers of apoptosis were utilized: Bax, a proapoptotic member of the Bcl-2 family of cell death regulators, cleaved caspase 3 and the caspase target PARP. DMBA-DHD resulted in an increase in expression of Bax and cleaved PARP, which was prevented by the use of the AHR antagonist αNF (Merchant et al., 1990), demonstrating mediation of this effect via the AHR.

**Figure 2:** Effect of AHR activation on Bax and cleaved caspase 3 expression in fetal testis

H&E stain of uncultured 16 weeks human fetal testis (A); H&E stain of cultured 16 week human fetal testis (B). Bax localization in uncultured 16 weeks human fetal testis (C); 16 weeks testis control culture (E); +1 μM DMBA-DHD (G); +1 μM αNF (I) and +1 μM DMBA-DHD plus 1 μM αNF (K). The arrowhead indicates Bax expression by germ cells; the arrows indicate germ cells not expressing Bax. Cleaved caspase 3 localization in uncultured 16 weeks human fetal testis (D); 16 weeks testis control culture (F); +1 μM DMBA-DHD (H); +1 μM αNF (J) and +1 μM DMBA-DHD plus 1 μM αNF (L). The arrowhead demonstrates cleaved caspase 3 expression by germ cells; the arrows indicate germ cells not expressing cleaved caspase 3. Scale bars 50 μm in B, 30 μm in A, D and F and 20 μm in C, E and G–L.
We were unable to demonstrate expression of cleaved caspase 3 by immunohistochemistry in uncultured tissue or by immunoblotting, which may reflect very low expression, but clear staining in a small number of cells was detected after culture with DMBA-DHD. Morphologically, the cells affected by apoptosis appeared to be germ cells. These data therefore indicate that PAHs acting through the AHR induce apoptosis of germ cells in the developing human testis.

A PAH induced increase in germ cell apoptosis via an increase in Bax expression has been demonstrated in the mouse fetal ovary in vitro, and in utero exposure of female mice also resulted in marked loss of germ cells (Matikainen et al., 2001, 2002). This has not been previously demonstrated in the male, either rodent or human, although there is considerable evidence as to the adverse effects of in utero or lactational exposure to AHR ligands. In rats, maternal exposure to TCDD causes reduced sperm production (Mably et al., 1992a; Gray et al., 1995, 1997; Sommer et al., 1996; Wilker et al., 1996; Faqi et al., 1998), and reduced volume of the testes and accessory organs (Mably et al., 1992b; Bjerke and Peterson, 1994; Roman et al., 1995, 1998; Gray et al., 1997). Treatment of adult rodent testis with the AHR ligands benzo(a)pyrene and TCDD resulted in increased apoptosis in seminiferous tubules (Merchant et al., 1990; Denison and Heath-Pagliuso, 1998; Benedict et al., 2000; Revel et al., 2001; Lin et al., 2003; Schultz et al., 2003; Gaskell et al., 2004).

Germ cell tumours are believed to arise from fetal germ cells that have not progressed through the normal stages of development, and continue to express markers such as OCT4 (Jones et al., 2004). We have previously demonstrated OCT4 expression by germ cells at similar gestations to those expressing AHR (Gaskell et al., 2004). The prevalence of testicular germ cell tumours is rising in most developed countries. This has been proposed to be part of the testicular dysgenesis syndrome, also comprising testicular maldescent, hypospadias and disorders of spermatogenesis manifesting as infertility in adult life, all of which are thought to be manifestations of perturbed prenatal development and associated with exposure to environmental toxicants (Skakkebak et al., 2001; Sharpe, 2006). The environmental ‘hormone disruptor’ 4-octylphenol (4-OPL) has been demonstrated to markedly reduce germ cell number in the fetal human testis in vitro (Bendsen et al., 2001). 4-OPL has estrogenic activity and estrogen receptors are expressed by germ cells in the human fetal testis (Gaskell et al., 2003). Interactions between AHR and estrogen receptor activation and degradation are increasingly recognized (Ohtake et al., 2003; Hockings et al., 2006; Ohtake et al., 2007) and may provide a common pathway between these data.

In conclusion, the present data demonstrate that germ cells in the developing human testis are a target for regulation by AHR ligands. In addition to unknown physiological processes, activation of AHR by environmental toxicants and AHR-induced apoptotic pathways may be the mechanism of action underlying the epidemiological findings of reduced spermatogenesis in men exposed to cigarette smoke before birth.

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