Leydig cell re-generation and expression of cell signaling molecules in the germ cell-free testis

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Abstract

Leydig cells in the rat testis can be specifically ablated with ethane dimethane sulfonate (EDS) and will subsequently re-generate. In this study, we have characterized Leydig cell re-generation and expression of selected cell-signaling molecules in a germ cell-free model of EDS action. This model offers the advantage that re-generation occurs on a stable background without confounding changes from the regressing and repopulating germ cell population. Adult rats were treated with busulfan to remove the germ cell population and Leydig cells were then ablated with EDS. Testicular testosterone levels declined markedly within 24 h of EDS treatment and started to recover after 8 days. After EDS treatment there were marked declines in levels of Leydig cell-specific mRNA transcripts coding for steroidogenic enzymes cytochrome P450 11a1 (Cyp11a1), cytochrome P450 17a1 (Cyp17a1), 3β-hydroxysteroid dehydrogenase type 1 (Hsd3b1), 17β-hydroxysteroid dehydrogenase type 3 (Hsd17b3) and the LH receptor. Levels of all transcripts recovered within 20 days of EDS treatment apart from Hsd17b3, which remained undetectable up to 20 days. Immunohistochemical localization of CYP11A1 during the phase of early Leydig cell re-generation showed that the Leydig cell precursors are spindle-shaped peritubular cells. Studies on factors which may be involved in Leydig cell re-generation showed there were significant but transient increases in platelet-derived growth factor A (Pdgfa), leukemia inhibitory factor (Lif), and neurofilament heavy polypeptide (Nefh) after EDS, while desert hedgehog (Dhh) levels declined sharply but recovered by 3 days. This study shows that the Leydig cell precursors are peritubular cells and that expression of Pdgfa and Lif is increased at the start of the re-generation process when precursor proliferation is likely to be taking place.

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Introduction

During normal male fetal development the Leydig cells actively secrete androgen to ensure normal masculinization. Androgen secretion then declines before a second generation of Leydig cells establishes the normal adult pattern of secretion during puberty (Klonisch et al. 2004). In the mouse and rat, the two Leydig cell populations appear to represent distinct cell lineages and show clear differences in their regulation and pattern of development. The fetal Leydig cells arise soon after testis differentiation around 14.5 days of intrauterine development (Majdic et al. 1998) while the adult population starts to develop just before puberty, around postnatal day 12 in the rat (Ariyaratne et al. 2000a). In the mouse, a number of factors have been identified which are involved in fetal Leydig cell differentiation. These include desert hedgehog (DHH), platelet-derived growth factor A (PDGFA), and the Arx homeobox (ARX) and, in each case, gene knockout studies have shown that these factors are required for normal fetal Leydig cell development (Kitamura et al. 2002, Yao et al. 2002, Brennan et al. 2003). In contrast, the factors initiating and regulating development of the adult population of cells remain unclear. A recent study has reported the isolation of stem cells from the pre-pubertal testis, which give rise to Leydig cells in vivo following transplantation (Ge et al. 2006). These stem cells express receptors for PDGFA, leukemia inhibitory factor (LIF), and kit ligand (KITL) and proliferate in the presence of these mitogens (Ge et al. 2006). This would appear to suggest a central role for these growth factors, and in particular PDGF, in the development of the adult Leydig cell cohort.

Despite isolation of the Leydig cell stem cells, the identity of the precursor cells that give rise to the adult Leydig cells remains uncertain and controversial. A number of studies have indicated that Leydig cell precursor cells are predominantly peritubular, based on morphological and immunohistochemical evidence (Haider et al. 1995, Russell et al. 1995, Haider & Sevros 1998, Ariyaratne et al. 2000a, 2000b). A recent study, however, has reported that the progenitor cells are vascular smooth muscle cells and pericytes, based on the apparent neuronal and glial properties of these cells (Davidoff et al. 2004). Before an understanding of adult Leydig cell differentiation and
development can be achieved, the origin of the cells needs to be established and further studies are needed.

A convenient model for the investigation of adult Leydig cell development is the ethane dimethane sulfonate (EDS)-treated rat. In the adult animal, a single injection of EDS leads to rapid ablation of the existing adult Leydig cells followed by Leydig cell repopulation in a manner that appears to recapitulate the normal developmental process (Teerds 1996, Ariyaratne et al. 2003). This model, therefore, offers the possibility for studying Leydig cell differentiation in a relatively stable environment without the other ongoing background developmental processes that occur naturally as the animal grows through puberty. A potential problem of the EDS model, however, is that loss of Leydig cells and, therefore, androgen secretion is rapidly followed by atrophy of the androgen-dependent spermatogenic epithelium, which repopulates as the Leydig cells re-appear (Sharpe et al. 1990). This, of itself, is likely to have a marked effect on sertoli cell activity, which will confound studies on Leydig cell re-generation. In an attempt to circumvent this problem, we have adapted a model of EDS-induced Leydig cell depletion which used germ cell-free animals (Molenaar et al. 1986). Clearly, this will avoid problems of germ cell depletion and re-generation following EDS. In addition, Leydig cell repopulation occurs more rapidly in rats in which the spermatogenic epithelium is absent either experimentally (Molenaar et al. 1986) or developmentally (Edwards et al. 1988a). This shortening of the re-generation process is likely to increase the chances that transient changes in gene expression will be detected. We report here on the characteristics of Leydig cell depletion and re-generation and the expression of cell signaling molecules following EDS treatment in germ cell-depleted testes.

Results

Effects of busulfan and EDS on testis morphology and androgen levels

The busulfan regime used in this study caused a decline in testis weight from 1.96 ± 0.09 to 0.77 ± 0.02 g 6 weeks after the second treatment. This was associated with depletion of germ cells so that tubules were largely devoid of germ cells at the start of EDS treatment (Fig. 1A and B). The intertubular nuclei remaining after busulfan treatment had the characteristics of Sertoli cell nuclei (Fig. 1C). The Leydig cells appeared densely packed in the interstitial space (Fig. 1C).

Treatment with EDS caused a further decline in testis weight over the next 8 days followed by a recovery up to 20 days (Fig. 2A). Testosterone levels dropped markedly over the first 24 h following EDS and reached a nadir at 3–5 days before starting to recover at 8 days. By 20 days the levels of testosterone were about 25% of controls (Fig. 2B). Leydig cell numbers were depleted 24 h after EDS, and by 3 days post-treatment were almost completely ablated with only a very occasional, morphologically distinct, Leydig cell apparent in the interstitial space (Fig. 1C).

Immunohistochemical expression of CYP11A1

In untreated rats and in busulfan-treated rats prior to EDS, there was strong expression of cytochrome P450 11a1 (CYP11A1, a marker of steroidogenic cells) in the interstitial Leydig cells. This staining was no longer present by 3 days after EDS (Fig. 1A–C). By 8 days after EDS treatment staining for CYP11A1 had started to re-appear in some peritubular cells and remained exclusively associated with the peritubular cells up to 20 days (Fig. 1D–F). At 8 days there were no CYP11A1-positive cells developing next to blood vessels, which were not also lying in a peritubular position. At day 20 the immunostained cells mainly formed clumps closely opposed to the external surface of the tubule although some cells appeared within the interstitial space (Fig. 1F).

Testicular mRNA expression levels following EDS

Leydig cell-specific genes

Levels of mRNA encoding the three steroidogenic enzymes CYP11A1, cytochrome P450 17α1 (CYP17A1) and 3β-hydroxysteroid dehydrogenase type 1 (HSD3B1), and the luteinizing hormone receptor (LHR) showed an expression profile after EDS which closely resembled changes in intratesticular testosterone (Fig. 3). There was a rapid fall in expression 24 h after treatment reaching a nadir around 3–5 days with recovery starting on day 8. The notable difference to testosterone levels is that expression of these genes returned to normal by 20 days whereas testosterone was still reduced by 75% at 20 days. The other major steroidogenic enzyme specific to the Leydig cells is 17β-hydroxysteroid dehydrogenase (HSD17B3), and levels of Hsd17b3 mRNA declined rapidly after EDS to become undetectable by 3 days. Unlike the other steroidogenic enzymes, however, levels of Hsd17b3 showed no recovery of expression up to day 20. The levels of Pdgfra mRNA declined about 75% after EDS and remained low up to 20 days.

Cell-signaling molecules

Within 24 h of EDS treatment there was a marked, sixfold increase in Pdgfra mRNA transcript levels (Fig. 4). This change in expression was transient and levels returned to normal by 3 days. There was a similar, though smaller, increase in levels of Lif after EDS. In contrast, levels of Dhh mRNA transcripts declined by about 80% within the first 24 h of EDS treatment then returned to normal by 3 days, while insulin-like growth factor 1 (Igf1) levels declined to 30% of normal at 24 h and remained low up to 5 days. Other signaling molecules measured
Kitl and wingless-related MMTV integration site 5A (Wnt5a) showed no significant change in mRNA levels following EDS treatment.

Other genes

Levels of nuclear receptor subfamily 5a1 (Nr5a1, also known as Sf1) transcripts declined by about 50% immediately after EDS treatment but did not differ from control thereafter until 20 days when transcript levels were significantly increased (Fig. 5). Similarly, the levels of Arx transcripts declined immediately after EDS but were not significantly different to control thereafter (Fig. 5). Levels of neurofilament heavy polypeptide (Nefh) increased significantly 24 h after EDS treatment and then returned to normal whilst there was no significant change in levels of nestin (Nes) transcripts.

Discussion

In the normal rat, the adult Leydig cell population begins to develop around day 12 after birth and reaches adult numbers by about day 40 (Ariyaratne & Chamindrani Mendis-Handagama 2000, Ariyaratne et al. 2000a). The underlying mechanism that initiates this process is unknown but recent studies have shown that the testicular interstitium of neonatal rats contains stem cells that can differentiate into steroidogenic cells in vitro or follow transplantation in vivo (Ge et al. 2006). Current
evidence suggests that Leydig cell re-population of the adult testis following EDS treatment is very similar to the normal developmental process (Teerds 1996, Ariyaratne et al. 2003) and it is likely that the same stem cells act as precursors of re-populating Leydig cells. The EDS model offers, therefore, a convenient method for the study of Leydig cell development while use of germ cell-free animals, as described here, allows this to be done on a relatively stable background with no other on-going, age-related, developmental events and without the massive degeneration and re-population of the germ cells which normally occurs following EDS.

Following EDS treatment, changes in the levels of mRNA species known to be specific to the Leydig cells followed, in the most part, changes in testosterone levels. The clear exception was Hsd17b3 mRNA which declined at the same rate as other steroidogenic enzymes but did not show any recovery and remained un-detectable up to 20 days. Failure of Hsd17b3 expression to recover at the same rate as other enzymes may explain why testosterone levels remained relatively low at day 20. In an earlier study we showed that 17-ketoreductase activity (enzyme activity catalyzed by HSD17B3) declined more slowly after EDS than other steroidogenic enzyme activities and remained detectable at all times (O’Shaughnessy & Murphy 1991). This apparent discrepancy is probably due to the presence of other members of the 17β-hydroxysteroid dehydrogenase family in the testis (Sha et al. 1996).

In the first study of EDS action on the germ cell-free testis it was reported that Leydig cell repopulation after EDS was faster than in normal rats (Molenaar et al. 1986). This is confirmed by our current study which shows that levels of testosterone and most Leydig cell mRNA markers began to recover by 8 days after EDS treatment. This contrasts with normal rats in which testosterone levels begin to recover between 14 and 21 days after EDS (Molenaar et al. 1986, Edwards et al. 1988b, Sharpe et al. 1990, Ariyaratne et al. 2003). It is known that germ cell depletion affects Sertoli cell function (McKinnell & Sharpe 1997, Guitton et al. 2000, O’Shaughnessy et al. 2008) and so the accelerated re-generation of active Leydig cells in germ cell-depleted testes may be due to altered Sertoli cell activity. Alternatively, LH is required for re-generation of Leydig cells after EDS treatment (Molenaar et al. 1986, Teerds et al. 1989) and levels of LH are higher in germ cell-free animals treated with EDS compared with normal EDS-treated animals (Morris & Jackson 1978, Molenaar et al. 1986). The more rapid Leydig cell re-generation in germ cell-free animals may, therefore, reflect increased tropic stimulation of the developing precursor cells.

Previous studies of adult Leydig cell differentiation, either during normal development or following EDS...
in the adult, have identified peritubular, spindle-shaped/mesenchymal cells as the immediate precursor cells (Haider et al. 1995, Russell et al. 1995, Ariyaratne et al. 2000a, 2000b). In addition, spindle-shaped peritubular cells have been described recently as Leydig cell stem cells (Ge et al. 2006). In contrast, it has recently been proposed that adult Leydig cells differentiate from perivascular smooth muscle cells and pericytes (Davidoff et al. 2004). This is based on the hypothesis that Leydig cells show neural characteristics and that cells expressing Nes, which is a stem cell marker in the nervous system, appear to differentiate from the perivascular region of the testis after EDS and may then go on to form adult Leydig cells (Davidoff et al. 2004). In addition, expression of Neff, an intermediate filament protein expressed in the neuronal/glial lineage subsequent to Nes, also increases transiently in perivascular cells. Our studies agree that there is an increase in testicular Neff expression following EDS although no significant change in Nes was seen. More crucially, however, we saw no evidence of perivascular development of Leydig cells. All cells expressing CYP11A1 during the initial phases of differentiation (around 8 days) were peritubular. From this evidence we cannot rule out a perivascular origin for Leydig cells, and there are other studies suggesting a minor contribution from this source (Haider et al. 1995, Haider & Servos 1998), but it is unlikely to be the major site of Leydig cell precursor differentiation.

PDGF is a major mitogen for mesenchymal cells and there is a growing body of evidence that links PDGF signaling with Leydig cell differentiation and development. In Pdgfa-null mice, for example, adult Leydig cells fail to develop, possibly because of a failure of precursor proliferation (Gnessi et al. 2000). Consistent with this hypothesis, the putative Leydig cell stem cells express Pdgfra and PDGFA stimulates stem cell proliferation (Ge et al. 2006). The early, transient rise in Pdgfa mRNA levels after EDS treatment is, therefore, of considerable interest and would be consistent with a role for PDGFA in Leydig cell precursor proliferation and differentiation. In addition to PDGFA, the myoid cell factor LIF also stimulates Leydig cell stem cell proliferation (Ge et al. 2006). Levels of Lif mRNA increased in the testis 24 h after EDS treatment and then returned to basal levels within 3–5 days. In embryonic stem cells LIF withdrawal is a stimulus for differentiation (Ward et al. 2004) and it is possible that the transient change in levels of Lif after EDS acts both to enhance stem cell proliferation and then to permit Leydig cell differentiation.

DHH is a cell signaling molecule produced by the sertoli cells and essential for normal differentiation of the fetal and adult Leydig cell populations (Clark et al. 2000, Yao et al. 2002). In DHH-null mice, there is failure of adult Leydig cell differentiation although this may be through failure of precursor proliferation or development (Clark et al. 2000). Following EDS treatment levels of mRNA encoding, DHH declined transiently but...
significantly at 24 h. It is not clear whether this may be related to subsequent Leydig cell re-generation or is simply a response to declining testosterone levels. Levels of \( \text{Igf1} \) mRNA also declined after EDS although, unlike \( \text{Dhh} \), levels remained significantly reduced up to 8 days. The pattern of \( \text{Igf1} \) expression after EDS is consistent with partial expression in the Leydig cells as reported previously (Moore et al. 1993).

This study characterizes a model system with which to study Leydig cell degeneration and re-generation without marked changes in the germ cell population. Leydig cell re-generation is also more rapid in this system, which may make it simpler to identify changes in gene expression associated with these changes. Our results also provide further support for a peritubular origin of adult Leydig cells and suggest that increasing levels of PDGFA and LIF following Leydig cell ablation may contribute to precursor proliferation.

**Materials and Methods**

**Animal treatments**

Adult Sprague–Dawley rats (350–400 g) were purchased from Harlan UK (Bicester, Oxfordshire, UK). To remove the germ cell population in these animals they were treated with the cytotoxic drug busulfan that selectively ablates spermatagonia (Jackson et al. 1962). Rats are sensitive to the toxic effects of busulfan, and initial studies were designed to identify a treatment regime that was effective in ablating the germ cell population without clear systemic toxicity. The most effective regime identified was a modification of one described earlier (Jiang 1998) and required two injections (i.p.) of busulfan (10 mg/kg) spaced 4 weeks apart. Under this regime, daily observations of the animals indicated that there was no gross toxicity. Six weeks after the second injection of busulfan the animals were injected (i.p.) with EDS (100 mg/kg) or vehicle. Animals were killed by CO\(_2\) inhalation at different times after EDS treatment. One testis was processed immediately for RNA extraction while the other was weighed, cut into two halves and one half fixed in Bouin’s medium while the other half was frozen rapidly in liquid N\(_2\) and stored at \(-20^\circ\text{C}\).

**Gene expression studies**

Total RNA was extracted from one testis of each animal by homogenization in TRizol (Invitrogen Ltd). At the start of the extraction process, luciferase mRNA (5 ng) was added to each sample to act as an external standard for real-time PCR (Baker & O’Shaughnessy 2001). Extracted RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript II, Invitrogen Ltd) as described previously (O’Shaughnessy & Murphy 1993, O’Shaughnessy et al. 1994). Levels of mRNA were measured

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abbreviation</th>
<th>Primers</th>
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<tbody>
<tr>
<td>Cytochrome P450 side chain cleavage</td>
<td>Cyp11a1</td>
<td>GACGCGACTCTCTTCTCCTGCG</td>
</tr>
<tr>
<td>3β-hydroxysteroid dehydrogenase(^a)</td>
<td>Hsd3b1</td>
<td>CCCATCTGGGTGGTTTGAACATTT</td>
</tr>
<tr>
<td>Luteinising hormone receptor</td>
<td>Lhr</td>
<td>GACGAGAACAGGAGGTTTGCTGG</td>
</tr>
<tr>
<td>Cytochrome P450 17α-hydroxylase</td>
<td>Cyp17a1</td>
<td>CTCCTCTAACATGTCACCTGGTGG</td>
</tr>
<tr>
<td>17β-hydroxysteroid dehydrogenase type 3</td>
<td>Hsd17b3</td>
<td>CTGCGGCTCTGGAATTGCC</td>
</tr>
<tr>
<td>Desert hedgehog</td>
<td>Dhh</td>
<td>CTGCGGCTCTGGAATTGCC</td>
</tr>
<tr>
<td>Wingless-related MMTV integration site 5A</td>
<td>Wnt5a</td>
<td>CTGCGGCTCTGGAATTGCC</td>
</tr>
<tr>
<td>Platelet-derived growth factor (z)</td>
<td>Pdgfl</td>
<td>CTGCGGCTCTGGAATTGCC</td>
</tr>
<tr>
<td>Pdgf-receptor (z)</td>
<td>Pdgfrz</td>
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</tr>
<tr>
<td>Kit ligand</td>
<td>Kitl</td>
<td>CTGCGGCTCTGGAATTGCC</td>
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<td>Igf1</td>
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<td>Leukemia inhibitory factor</td>
<td>Lif</td>
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<td>Neurofilament heavy polypeptide</td>
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<td>Nestin</td>
<td>Nes</td>
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<td>Arx homeobox</td>
<td>Arx</td>
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<tr>
<td>Nuclear receptor 5a1</td>
<td>Nr5a1</td>
<td>CTGCGGCTCTGGAATTGCC</td>
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\(^a\)Amplifies both type1 and type2 isoforms.
by real-time PCR using the SYBR green method with a Stratagene MX3000 cycler. Reactions contained 5 μl 2× SYBR mastermix (Stratagene, Amsterdam), primers (100 nM), and template in a total volume of 10 μl. The thermal profile used for amplification was 95 °C for 8 min followed by 40 cycles of 95 °C for 25 s, 63 °C for 25 s, and 72 °C for 30 s. At the end of the amplification phase a melting curve analysis was carried out on the products formed. All primers were designed by Primer Express 2.0 (Applied Biosystems, Warrington, UK) using parameters previously described (Czechowski et al. 2004). The primers used are described in Table 1.

**Immunohistochemistry**

Testes were fixed overnight in Bouin’s medium and stored in 70% ethanol. Wax sections (5 μm) were incubated with primary antibody overnight at 4 °C and endogenous biotin was blocked using an avidin/biotin blocking kit (R&D systems Europe Ltd, Abingden, UK). The antibody used was rabbit antibody CYP11A1 (gift from A H Payne). Sections were washed and incubated for 30 min with biotinylated secondary antibody (R&D Systems Europe Ltd, Abingden, UK). Bound anti-body was visualized using 3,3-diaminobenzidine tetrahydrochloride (R&D Systems Europe Ltd). Negative controls without the primary antibody were included in each experiment.

**RIA**

To measure total intratesticular testosterone, steroids were extracted from the testes with ethanol and measured by RIA as previously described (Sheffield & O’Shaughnessy 1989).

**Statistical analysis**

Data were analyzed by single-factor ANOVA followed by Fisher’s multiple comparison test (Minitab, Minitab Ltd, Coventry, UK). Where necessary, data were log-transformed before analysis to avoid heterogeneity of variance.

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