Leydig cell apoptosis in the rat testes after administration of the cytotoxin ethane dimethanesulphonate: role of the Bcl-2 family members

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Abstract

Ethane dimethanesulphonate (EDS) is cytotoxic to Leydig cells in the adult rat. To investigate the role and regulation of apoptosis in the Leydig cell, EDS (100 mg/kg i.p.) was administered to adult male rats and the testes examined 6, 12, 18, 24, 48 and 72 h later. Numbers of Leydig cells, identified by 3β-hydroxysteroid dehydrogenase immuno-histochemistry started to fall by 12 h after EDS injection and were almost undetectable by 72 h. Apoptotic cells in the interstitium, visualised by in situ end labelling of DNA, increased in number to reach a maximum 24 h after injection of EDS, and were undetectable by 72 h. In many tissues the apoptosis-related gene products act in cohort: Bcl-2 and Bcl-xl promoting survival of a cell, whilst Bax promotes cell death often positively regulated by the tumour-suppressor gene p53. Western blot analysis showed that: (1) Bcl-2 and p53 were absent from interstitial Leydig cells but were expressed in the seminiferous tubules. (2) Bax protein although expressed in the interstitium was not present in the Leydig cells. (3) Bcl-xl in Leydig cells was transiently increased after EDS.

In conclusion, EDS kills Leydig cells by apoptosis; however the control of Leydig cell death does not involve p53 or the Bcl-2 family members but may require other gene products yet to be identified.

Introduction

The methane sulphonic ester of ethylene glycol, ethane-1,2-dimethanesulphonate (EDS) is a unique testicular toxicant. Following a single dose the Leydig cell population is destroyed and subsequently regenerates, apparently from mesenchymal fibroblast-like precursors (Jackson et al. 1986a, Jackson et al. 1986b, Kerr et al. 1986, Morris et al. 1986, Morris 1996).

The in vivo treatment of rats with EDS leads to alterations in the cytoplasm and nuclei of the Leydig cells. The changes observed include vesiculation in the smooth endoplasmic reticulum, focal hypertrophy of the Golgi apparatus and clumping of the nuclear chromatin (Kerr et al. 1986, Morris et al. 1986). At later time points the Leydig cells exhibit further degenerate alterations leading to their disappearance through the phagocytic activity of macrophages (Kerr et al. 1985, 1986, Jackson et al. 1986b, Morris et al. 1986). These morphological changes are reminiscent of those described for apoptosis in other cell systems (Wyllie 1980, Earnshaw 1995, Kroemer et al. 1995), which suggests that Leydig cells are capable of engaging the programmed cell death pathway in response to certain stimuli. In support of this hypothesis, testes from rats treated in vivo with EDS show internucleosomal DNA cleavage 24 h after drug administration, a time at which only Leydig cell degeneration is thought to be occurring (Henriksen et al. 1995). Furthermore, crude interstitial cell preparations obtained from rats after hypophysectomy exhibit increased DNA fragmentation (Tapanainen 1993) suggesting that Leydig cell apoptosis may be a more general phenomenon than previously suspected.

Of the genes which have been shown to be involved in apoptosis, the Bcl-2 family members have been most widely studied. The Bcl-2 gene was initially identified as part of the most common translocation in human B cell follicular lymphoma (Bakhshi et al. 1985, Cleary et al. 1986) and was subsequently shown to prolong cell survival rather than increase cell proliferation (Vaux et al. 1988, Hockenbery et al. 1990). During embryogenesis Bcl-2 is expressed in a variety of tissues (Novak & Korsmeyer 1994) whereas in the adult it is mainly those cells which require long-term survival which exhibit Bcl-2 expression (Hockenbery et al. 1991). Knockout mice which lack the Bcl-2 protein are normal at birth but progress to exhibit a variety of complaints which are mainly confined to the

0022–0795/98/0157–0317 $08.00/0

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The Bcl-x gene has a high degree of homology to Bcl-2 and is also involved in the regulation of apoptosis (Boise et al. 1993); the gene product of the alternatively spliced long form (Bcl-xl) is functionally similar to Bcl-2 in inhibiting apoptosis. Prominent Bcl-x immunostaining has been shown in neurons, thymocytes, several cell types in the bone marrow, a variety of epithelial cells and reproductive tissues including spermatocytes and spermatids in the testes (Krajewski et al. 1994). Bcl-x knockout mice have an embryonic-lethal phenotype with the brain and spinal cord exhibiting massive apoptosis (Motoyama et al. 1995).

Another member of the Bcl-2 family, Bax, was the first protein to be shown to associate with Bcl-2 (Oltavi et al. 1993). Bax shows sequence homology to Bcl-2 and can block the ability of Bcl-2 to inhibit apoptosis (Oltavi et al. 1993) thereby suggesting that Bax may regulate apoptosis by functional antagonism through the formation of heterodimers. Bax knockout mice are normal at birth but develop lymphoid hyperplasia and male sterility (Knudson et al. 1995). The absence of mature spermatozoa and the presence of apoptotic germ cells suggest that Bax normally inhibits apoptosis in the spermatogenic cells. It would, therefore, appear that Bax expression can either promote or inhibit cell death depending on the cellular context. Alternatively Bax expression could be required during development to eliminate a specific cell type which, if retained, would produce inappropriate cell death during spermatogenesis.

Previous work has suggested that the tumour-suppressor gene p53 can promote apoptosis by the upregulation of Bax expression (Miyashita & Reed 1995). However, the complexity of the situation is such that p53-dependent cell death has been shown to occur in the presence of the transcriptional inhibitor actinomycin D or the translational inhibitor cyclohexamide (Caelves et al. 1994, Wagner et al. 1994) suggesting a transcription-independent mechanism. Furthermore, the adenovirus E1B 19 kDa protein and Bcl-2, both of which inhibit p53-dependent apoptosis, were shown to suppress transcriptional repression by p53 without exerting any effect on transcriptional activation (Shen & Shenk 1994, Sabbatini et al. 1995). Therefore, it is possible that p53 can influence the programme cell death machinery by either transcription activation, transcriptional repression, direct interaction with cellular proteins or even by its ability to reanneal single-stranded DNA.

We have previously shown that EDS induces Leydig cell apoptosis both in vitro and in vivo (Morris et al. 1997). In this study we have examined the time course of EDS-induced Leydig cell apoptosis by in situ end labelling of apoptotic DNA and 3β-hydroxysteroid dehydrogenase (3βHSD) immunohistochemistry. We have also determined the potential roles of p53, Bcl-2, Bcl-xl and Bax in Leydig cell apoptosis.

Materials and Methods

Reagents

All chemicals were of reagent grade. Penicillin, streptomycin and 10 × M199 media (with Hanks balanced salts) were obtained from Gibco Ltd (Paisley, Strathclyde, UK). Percoll was purchased from Pharmacia LKB (Uppsala, Sweden). Collagenase type I, DNase I, nitro-blue tetrazolium, NAD+, 5-androstane-3-ol-17-one, bovine serum albumin (BSA), Triton X-100, Tween 20 and Proteinase K were all obtained from Sigma Chemical Co. (St Louis, MO, USA). EDS was synthesised in our laboratory from ethylene glycol and methanesulphonyl chloride according to the method described by Jackson and Jackson (1984). The rabbit polyclonal anti-Bax antibody was prepared against a synthetic peptide corresponding to residues 44–59 of mouse Bax and immunoglobulin G (IgG) from the resulting serum was affinity purified on a Bax peptide column (Pullan et al. 1996). The rabbit polyclonal anti-Bcl-2 antibody was a gift from Dr G Evans (University of London, UK), the rabbit polyclonal anti-Bcl-xl antibody was obtained from Transduction Labs (Lexington, KY, USA), the rabbit polyclonal anti-3βHSD antibody was a gift from Professor I Mason (University of Edinburgh, UK), and the murine monoclonal anti-p53 antibody was purchased from Pharmingen (San Diego, CA, USA). The horseradish-peroxidase conjugated secondary antibodies (anti-rabbit IgG and anti-mouse IgG) and the PVDF membranes were obtained from Amersham Life Sciences (Little Chalfont, Bucks, UK) and the FITC-conjugated anti-rabbit IgG was from Sigma Chemicals. The ApopTag kit for in situ end labelling was purchased from Oncor Appligene (Chester-Le-Street, Co. Durham, UK). Protein standards and protein assay reagents were obtained from BioRad (Hemel Hempstead, Herts, UK).

Animals

Adult male Sprague-Dawley rats (250–300 g), purchased from Charles Rivers UK Ltd (Margate, Kent, UK), were housed four per cage in a light-controlled room (12 h light:12 h darkness; lights on at 0700 h). The animals were handled daily for at least 1 week prior to the beginning of experimentation; food and water were available ad libitum. Rats were randomly selected into eight groups of six and injected i.p. with either EDS (100 mg/kg body weight) in a vehicle of dimethyl sulphoxide:water (1:3 volume ratio) or with an equivalent volume of vehicle only (2 ml/kg body weight). The animals were killed by an overdose of anaesthetic at the time points indicated and the testes were removed and weighed. The left testis was processed for histology after fixing in Bouin’s solution, and the right testis was used to prepare an interstitial cell suspension and stored at −20 °C until processed for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).
Immunohistochemistry

3βHSD immunohistochemistry (IHC) was performed on paraffin-embedded testis sections (10 µm thick) which were deparaffinised and rehydrated in xylene and ethanol (100%, 95%, 70% and 40%). Slides were washed (2 × 10 min) in phosphate-buffered saline (PBS; 10 mM sodium phosphate dibasic, 1.8 mM potassium phosphate monobasic, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4), buffer A (PBS containing 0.1% Triton X-100; 2 × 5 min) and buffer B (PBS containing 0.1% Triton X-100, 2% BSA; 2 × 5 min). The testis sections were incubated overnight at 4 °C in buffer B containing anti-3βHSD antibody (1:200 dilution), washed in buffer B (2 × 15 min), and incubated for 2 h at room temperature in FITC-conjugated anti-rabbit IgG (1:250 dilution in buffer B). Following further washing in buffer B (2 × 5 min) and PBS (2 × 10 min), sections were dehydrated stepwise through ethanol (40%, 70%, 95% and 100%) and xylene, and mounted using DePeX.

In situ end labelling

In situ end labelling (ISEL) of apoptotic DNA was carried out on paraffin-embedded testis sections (10 µm) using the Oncor ApopTag kit. Briefly sections were deparaffinised and rehydrated in xylene and ethanol (100%, 95%, 70% and 40%). The testis sections were incubated with Proteinase K (20 µg/ml) for 15 min at room temperature, washed with Nanopure water (4 × 2 min) and incubated with equilibrium buffer for 5 min. Sections were incubated with terminal deoxynucleotidyl transferase reaction mixture for 1 h at 37 °C in a humidified atmosphere and the reaction stopped by immersion in buffer (10 min). The sections were washed in PBS (3 × 5 min) and incubated with peroxidase-conjugated anti-digoxigenin for 30 min at room temperature in a humid atmosphere. Following washing in PBS (4 × 5 min), testis sections were incubated with diaminobenzidine substrate for 10 min at room temperature, washed in Nanopure water (4 × 5 min), dehydrated stepwise in ethanol (40%, 70%, 95% and 100%) and xylene, and mounted with DePeX.

Cell counting

Two sections per testis, four testes per group, were assessed for both the ISEL and IHC procedures. Random interstitial spaces (25 per testis section) defined as the space enclosed by three or four circular tubules were counted for either the number of cells which contained a dark brown nucleus (ISEL) or a green fluorescent cytosol with an unstained nuclear profile (IHC). All counting was performed blind on a Nikon Optiphot-2 microscope using 400 × magnification. The testes weights did not significantly change over the period studied which allows for a direct comparison of cell numbers between treatments.

Leydig cell preparation

Leydig cells were prepared by a combination of collagenase digestion and Percoll gradient separation as described elsewhere (Lin et al. 1989, Moore & Morris 1993). Following killing by anaesthetic overdose the right testis was removed, decapsulated and digested with collagenase type I (0.10 mg/ml) in M199 media (1 × M199, pH 7.4, containing 4.2 mM sodium bicarbonate, 20 mM HEPES, 1 mg/ml BSA, 100 U/ml penicillin, 100 µg/ml streptomycin and 1000 Kunitz DNase I) for 10 min at 34 °C in a shaking water bath (200 cycles/min). An interstitial cell preparation was obtained by filtering the digest through 70 µm nylon mesh. The resultant cell suspension was centrifuged at 120 g for 20 min, the pellet washed twice in M199 and resuspended in 1 ml M199. The control seminiferous tubules, separated from the interstitial cells during the filtration procedure were collected and stored at −20 °C for use in Western blot analysis.

A Percoll-purified Leydig cell preparation was obtained by loading the cell suspension on the top of a discontinuous Percoll gradient. The gradients consisted of six density steps of 1.0900, 1.0625, 1.0450, 1.0350 and 1.0300 g/ml. The gradient was centrifuged at 800 g for 30 min, and cell fractions were collected from the bottom of the tube. The Leydig cell fraction was collected from the interface of densities 1.0900 and 1.0625. This fraction was diluted and centrifuged at 120 g for 20 min, resuspended and washed twice in M199. Leydig cell purity was assessed using the 3β-hydroxysteroid dehydrogenase staining method (Weibe 1976, Ziegler et al. 1983) with the formation of blue-purple formazan granules in the cytoplasm indicating the presence of Leydig cells.

Western blot analysis

Protein determinations were carried out on interstitial cell preparations from in vivo EDS-treated rats using the BioRad protein assay method (Bradford 1976). Samples (50 µg) were subjected to 10% SDS-PAGE at 20 mA (Laemmli 1970), followed by transfer onto a PVDF membrane at 35 V overnight. Membranes were blocked for non-specific binding using PBS containing 0.5% Tween 20 (PBST) and 5% non-fat milk and probed with the appropriate primary antibody for 2 h (Bcl-2, 1:10 000; Bcl-xl, 1:500; Bax, 1:1,000; 3βHSD, 1:5000; p53, 1:1000). The membranes were washed in PBST (6 × 10 min) and incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000). Following further washing (6 × 10 min) visualisation was achieved by chemiluminescence using Amersham enhanced chemiluminescence reagents. Quantification was carried out by densiometric scanning of the film and data were expressed as percentage of the control.
Statistical analysis

The Western blot analysis is expressed as the mean ± s.e.m. (n=6). Comparisons between the control group and the different times of EDS treatment were made by Kruskal–Wallis one-way analysis of variance and the Mann Whitney U-test.

Results

Histology

Typical examples of 3βHSD IHC fluorescence staining of interstitial spaces from control (A), 24 h post-EDS administration (B) and 72 h following EDS administration (C) are shown in Fig. 1. Cells staining positive for 3βHSD were clearly identified in both the control and 24 h EDS-treated testes, whereas only occasional staining was detected in the interstitial spaces from testes 72 h after EDS administration. Quantification of the observed reduction in the number of 3βHSD-positive cells is depicted in Fig. 1D, where the number of Leydig cells decreased starting at 12 h and falling to nearly zero by 72 h.

The ISEL of apoptotic DNA in the cells of the interstitium is shown in Fig. 2. The control (A) and 72 h post-EDS administration (C) showed no ISEL of DNA (although very occasional apoptotic cells were observed), whereas 24 h following EDS treatment (B) several apoptotic cells were clearly identified. Figure 2D shows the quantification of cells within the interstitial spaces which stained positive for apoptotic DNA.

Bcl-2 in tissues

Figure 3 shows the ability of this antibody to detect immunoreactive Bcl-2 protein in the thymus, spleen and testes from mouse and rat. For both the mouse and the rat there was significantly more Bcl-2 protein in the spleen and thymus compared with the testes. Within the rat testes immunoreactive Bcl-2 was detected within the seminiferous tubules only with the interstitial cell preparation (9% Leydig cells) and the Percoll-purified Leydig cell preparation (79% Leydig cells) showing no detectable Bcl-2 protein.

Comparisons of p53 and the Bcl-2 family members in the seminiferous epithelium, interstitial spaces and Leydig cells of the rat testes

Western blot analyses of Bcl-2 family members and p53 within the tubules, interstitium and Leydig cells of the testes are shown in Fig. 4. All family members examined (Bcl-xl and Bax) as well as p53 and 3βHSD were present within the rat testes to varying extents. The Bcl-xl protein could be detected within the seminiferous tubules, the interstitium and the Leydig cells. In contrast, the Bcl-2 (Fig. 3) and p53 proteins were only detected in the seminiferous tubules, and the 3βHSD protein (a Leydig cell-specific enzyme) was only found in the interstitium and Leydig cells. Bax protein was detected within the seminiferous epithelium and the interstitium, but no immunoreactive Bax was detected within the Leydig cells. The enrichment of the Leydig cells between the interstitial spaces...
cell (9%) and Leydig cell (79%) preparations showed a concomitant enhancement of immunoreactivity with both the 3βHSD and Bcl-xl proteins suggesting that these proteins are Leydig cell specific.

Western analysis of the degenerating interstitium

The levels of 3βHSD protein in the interstitial cell preparation from EDS-treated rats remained virtually unchanged until 48 h after EDS treatment; however by 72 h there was no detectable 3βHSD present (Fig. 5A and B). The Bax protein levels associated with interstitial cells following EDS treatment showed significant changes at 48 h following EDS which is probably due to the removal of the Leydig cells (Bax negative) from the interstitium (Fig. 6A and B). Bcl-xl protein levels, however, were significantly elevated at 6 and 12 h following EDS treatment returning to control levels thereafter (Fig. 7A and B).

Discussion

In the testes the enzyme 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴ isomerase (3βHSD) is localised exclusively within the Leydig cells (O'Shaughnessy & Murphy 1991, Dupont et al. 1993). The decrease in Leydig cell numbers following EDS, as determined by 3βHSD immunohistochemistry, is consistent with previous endocrinological and histological investigations where approximately 75% of Leydig cells were eliminated 24 h after administration (Kerr et al. 1987) and were completely absent by three days (Molenaar et al. 1985, Kerr et al. 1986, Morris et al. 1986). However, in the present study the Western blot analysis of immunoreactive 3βHSD in the interstitial cell preparations shows no difference in the levels of this protein until 48 h after EDS administration (Fig. 5). This apparent discrepancy can be explained by the differences in methodologies involved. Histologically only those cells with both cytoplasmic staining and a defined nucleus were counted as Leydig cells. However, the Western blot analysis detects all immunoreactive 3βHSD protein including that within apoptotic cells (where the nucleus has condensed or fragmented), apoptotic bodies (where no nucleus is present) and in neighbouring macrophages which have phagocytosed Leydig cell material.

Apoptosis is an active process in cells which is engaged in response to specific signals (Corcoran et al. 1994). One of the best characterised biochemical markers for apoptosis is the activation of endonucleases which cleave genomic DNA ultimately at the internucleosomal regions into fragments of 180 bp multimers (Cohen & Duke 1984, Arends et al. 1990, Brown et al. 1993). We have previously shown that internucleosomal DNA fragmentation can be

Figure 2 Apoptotic cells in the rat testicular interstitium identified by in situ end labelling of fragmented DNA. T=semiferous tubules, I=interstitial spaces. Magnification × 400. Arrows indicate ISEL-positive cells. (A) Testicular interstitium of control testis 6 h after i.p. injection of vehicle. (B) Testicular interstitium of EDS-treated testis 24 h after drug administration. (C) Testicular interstitium of EDS-treated testis 72 h after drug administration. (D) The number of cells per interstitial space staining positive for apoptotic DNA following EDS administration. Results are shown as means ± s.e.m. (n=4). The number of ISEL-positive cells ranged from 0 to 4.

Figure 3 Western blot analysis of Bcl-2 protein in various tissues (50 μg of each) from adult male mouse and rat.
induced in Leydig cells by \textit{in vitro} treatment with EDS (Morris \textit{et al.} 1997). The endonuclease digestion of genomic DNA produces 3'-hydroxyl groups which have been utilised for \textit{in situ} end labelling to identify apoptotic cells (Gavrieli \textit{et al.} 1992, Gorczyca \textit{et al.} 1993, Gold \textit{et al.} 1994). In the control sections only extremely rare ISEL-positive interstitial cells were detected which is consistent with the low turnover rate reported for Leydig cells (Teerds 1996). Following EDS treatment few apoptotic cells were seen at 6 h, but there were large increases at 12 h up to 24 h. This increase in apoptosis within the interstitium precedes the decrease in the 3\(^{-}\)\textit{HSD}-positive Leydig cell numbers by about 6 h. This pattern of staining suggests that it is the Leydig cells which are undergoing apoptosis with DNA fragmentation occurring a few hours prior to the removal of the apoptotic cells from the interstitium. These observations are in agreement with previous studies in which apoptotic changes in cell morphology were noted followed by the phagocytosis of the dying cell by neighbouring macrophages (Kerr \textit{et al.} 1986, Morris \textit{et al.} 1986).

The process of apoptosis in other cells has been shown to involve a number of different cellular proteins. One group of such proteins is the Bcl-2 family members which show sequence homology to one another and several have been shown to interact to modulate apoptosis. Bcl-2 has been shown to prolong cell survival rather than increase cell proliferation (Vaux \textit{et al.} 1988, Hockenbery \textit{et al.} 1990), although the exact mechanism by which Bcl-2 inhibits apoptosis and promotes cell survival remains elusive. The long half-life of the Leydig cell (Teerds 1996) indicates that this cell type is protected from programmed cell death, which suggests that it contains high levels of anti-apoptotic proteins such as Bcl-2. Immunoblotting shows that Bcl-2 protein was present within the rat testes, but the immunoreactivity was confined to the seminiferous epithelium with no detectable Bcl-2 protein within the interstitial or Leydig cell preparations. Studies in the human testes have shown that the Leydig cells exhibit little or no Bcl-2 immunostaining although they expressed high levels of the Mcl-1 protein (Krajewski \textit{et al.} 1995). The Mcl-1 protein exhibits a high degree of homology with Bcl-2 and can inhibit apoptosis (Reynolds \textit{et al.} 1994). The immunohistochemical localisation of Mcl-1 and Bcl-2 exhibit a reciprocal pattern of expression which suggests that the Mcl-1 protein has a unique role in the regulation of survival of terminally differentiated cells, like the Leydig cell (Krajewski \textit{et al.} 1995). A rat homologue for the \textit{Mcl-1} gene has yet to be identified.

\begin{figure}[h]
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\caption{Western blot analysis of 3\(^{-}\)\textit{HSD}, p53, Bcl-xl, and Bax in the seminiferous epithelium, the interstitium and Percoll-purified Leydig cells of rat testes. Fifty micrograms of protein were loaded in each lane. Lane T, seminiferous tubules; lane I, interstitial cell preparation (9\% Leydig cells); lane L, Percoll-purified Leydig cells (79\% purity).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Immunoreactive 3\(^{-}\)\textit{HSD} in interstitial cell preparations following EDS administration. (A) Western blot analysis of interstitial cell (50 \textmu g) preparations from control and EDS-treated rats from 6 to 72 h following administration. (B) Quantification of Western blot analyses. Densiometric scanning of Western blots was carried out using the BioRad molecular analyst software package. Results were plotted as a percentage of the 6-h control values and are represented as means \pm s.e.m. (\textit{n}=6). ***\emph{P}<0.005 compared with the 6-h control.}
\end{figure}
The Bcl-2-associated protein, Bax, homodimerises with Bcl-2 through two conserved domains which inhibit the ability of Bcl-2 to suppress apoptosis (Oltavi et al. 1993, Xiao-Ming et al. 1994). The Bax gene is upregulated in response to expression of the p53 protein (Miyashita & Reed 1995) and recently Bax has been demonstrated to act as a tumour suppressor required for a full p53-mediated apoptosis response (Yin et al. 1997). Several investigations in the testes have shown that p53 expression is confined to the primary spermatocytes within the seminiferous tubules (Almon et al. 1993, Schwartz et al. 1993, Sjoblom & Lahdetie 1996, Stephan et al. 1996). These studies agree with our data in which immunoreactive p53 was only detected within the seminiferous epithelium and not in the Leydig cells or other interstitial cells. However, Bax protein was detected in interstitial cells suggesting that Bax gene expression is not under the exclusive control of p53. The Leydig cells of the testicular interstitium are unusual since there is no detectable immunoreactive Bax protein present. This could explain the low turnover rate of the Leydig cell (Teerds 1996) or it might indicate that there are other apoptotic-promoting proteins present in these cells.

Another pro-apoptotic member of the Bcl-2 family of proteins which has been localised in high levels in the human testes is Bak (Krajewski et al. 1996). The tissue expression pattern of the Bak gene (Chittenden et al. 1995, Farrow et al. 1995, Kiefer et al. 1995) was shown to be widespread and included terminally differentiated cell types. In a yeast system, the Bak protein was demonstrated to bind to Bcl-xl (Farrow et al. 1995). Considering that Leydig cells contain little Bcl-2 protein in humans (Krajewski et al. 1995) and none in the rat (this study), together with the localisation of Bak in the Leydig cells of humans (Krajewski et al. 1996), might suggest that Bcl-xl and Bak are the main regulators of apoptosis in the Leydig cell. The Bcl-xl protein (Boise et al. 1993) inhibits apoptosis in a variety of cell systems. Within the human testes immunohistochemical analysis showed Bcl-x staining primarily in spermatocytes and spermatids (Krajewski et al. 1994) and we report its presence by immunostaining in interstitial and Leydig cells. Following EDS administration Bcl-xl levels are transiently elevated in interstitial cells. This increase in Bcl-xl occurred at 6 and 12 h which are time points immediately prior to and including the onset of apoptosis within the Leydig cells. A recent study investigating the effects of ionising radiation on the expression of Bcl-2 family members (Zhan et al. 1996) also showed a transient increase in Bcl-xl levels. This elevation of Bcl-xl was dependent on a wild-type p53 gene and it...
was speculated that the physiological function would be to limit the severity and duration of the Bax effect in these cells such that a proper threshold for apoptosis was achieved. The in vivo activation of apoptosis in the Leydig cell by EDS would appear not to involve the Bax protein, and no immunoreactive p53 was detected in these cells. Therefore, even though the Bcl-xl protein levels increase this is not a p53-mediated event, and Bax is unlikely to be involved in EDS-induced Leydig cell apoptosis. However, since Leydig cells are a terminally differentiated cell type the lack of p53 would seem understandable and cell cycle arrest unnecessary since the Leydig cells are in G0. Our data would suggest that the Leydig cell possesses another mechanism for the regulation of Bcl-xl expression which functions to maintain a proper threshold for apoptosis and to limit the severity and duration of another death promoter (possibly Bak). This hypothesis is supported by work on the activation of naive B cells by CD40L (Zhang et al. 1996) in which transient expression of Bcl-xl protected the cells from apoptosis by anti-Fas antibodies. Although it has been known for a number of years that EDS can induce Leydig cell degeneration (Jackson et al. 1986a, Jackson et al. 1986b, Kerr et al. 1986, Morris et al. 1986, Morris 1996) it is only recently that the mode of cell death has been established as apoptosis (Morris et al. 1997). The cellular events involved in EDS-induced Leydig cell apoptosis are at present unknown; however in vitro experiments suggest that the covalent binding of EDS to glutathione may be essential for cytotoxicity (Kelce & Zirkin 1993, Kelce 1994). In other cell systems the depletion of cellular glutathione is a trigger for apoptosis in response to cytotoxic treatments (Beaver & Waring 1995, Sugimoto et al. 1996).

In summary, we have shown that in vivo Leydig cells undergo programmed cell death in response to the cytotoxic EDS. The involvement of the Bcl-2 family members in Leydig cell apoptosis was unpredictable especially as we were unable to detect immunoreactive Bcl-2, Bax or p53 proteins in the Leydig cells, even when these cells were induced to undergo apoptosis. Bcl-xl protein levels transiently increased suggesting an attempt by the Leydig cells to protect themselves from the cytotoxic action of EDS. Other gene products yet to be identified may be required for engagement of apoptosis in the Leydig cell.

Acknowledgements

This work was supported by the MRC.

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Received 8 September 1997
Accepted 14 January 1998