In-vitro effects of FSH and testosterone withdrawal on caspase activation and DNA fragmentation in different cell types of human seminiferous epithelium

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BACKGROUND: Caspases are downstream elements of apoptosis-mediated pathways initiated by the Fas ligand/Fas receptor system which is supposed to play a central role in the regulation of apoptosis in the human seminiferous epithelium. However, caspase activity in different cell types of this epithelium has never been addressed. METHODS AND RESULTS: We evaluated caspase activity and DNA integrity in Sertoli and germ cells within in-vitro cultured segments of human seminiferous tubules after induction of apoptosis by FSH or testosterone withdrawal. FSH withdrawal increased the incidence of DNA fragmentation in meiotic (primary spermatocytes) and post-meiotic (spermatids) germ cells without producing any detectable effect on caspase activity in these cells and without affecting DNA integrity or caspase activity in Sertoli cells. Testosterone withdrawal stimulated caspase activity and produced DNA fragmentation in Sertoli cells, but showed only a weak effect on DNA fragmentation in germ cells and did not alter germ cell caspase activity. CONCLUSIONS: These findings confirm the central role of caspases in apoptosis of Sertoli cells. However, they also suggest that acute apoptosis of germ cells in the adult human testis occurs in a caspase-independent way and is controlled by Sertoli cells via an as yet undetermined mechanism.

Key words: caspase activity/DNA fragmentation/FSH/seminiferous epithelium/testosterone

Introduction

Programmed cell death (apoptosis) is required for normal spermatogenesis in mammals (Rodriguez et al., 1997; Tres and Kierszenbaum, 1999) and is believed to ensure cellular homeostasis and the fine balance between germ cells and Sertoli cells (Kierszenbaum, 2001). Also in the normal human testis, spontaneous apoptosis occurs in all germ cell compartments (Oldereid et al., 2001). However, various testicular injuries, including heat (Lue et al., 1999) and toxicant (Ku et al., 1995; Li et al., 1996) exposure, radiation (Meistrich, 1993), freezing and thawing (Tesarik et al., 2000a) or withdrawal of hormonal support (Sinha Hikim et al., 1995, 1997; Tesarik et al., 1998a; Woolveridge et al., 1999) have been shown to enhance the apoptotic process as compared with the physiological condition. In the human testis, apoptosis appears to be the final result of various testicular and systemic pathologies (Gandini et al., 2000).

Massive germ cell apoptosis is associated with maturation arrest (Tesarik et al., 1998b; Jurisicova et al., 1999). However, high frequencies of mature sperm showing different types of apoptosis-related cell damage were also observed in men with complete, though mostly disturbed, spermatogenesis (Gorczyca et al., 1993; Aravindan et al., 1997; Sakkas et al., 1999; Barroso et al., 2000; Gandini et al., 2000), and this condition was shown to be associated with lower sperm concentration (Oosterhuis et al., 2000), production of reactive oxygen species (Barroso et al., 2000) and impaired fertilizing ability (Sun et al., 1997; Host et al., 2000).

The mechanisms controlling the process of apoptosis occurring during normal spermatogenesis and in response to different pathological situations may not be the same and are still largely unknown (Kierszenbaum, 2001). Most of the earlier studies dealt with different aspects of DNA damage in germ cells. Little attention has been paid to Sertoli cells and to cell signalling pathways upstream of DNA damage.

This study uses the previously characterized in-vitro system in which apoptosis of germ and Sertoli cells is induced by the withdrawal of FSH and testosterone respectively (Tesarik et al., 1998a). This system is used to analyse the relationship between caspase activation and DNA damage in both germ and Sertoli cells maintained in vitro within explanted segments of the human seminiferous tubules.

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Materials and methods

Source and preparation of the seminiferous epithelium

Pieces of testicular tissue were obtained by open testicular biopsy from 12 patients with obstructive azoospermia and apparently normal spermatogenesis. The biopsies were clinically indicated to recover sperm for assisted reproduction attempts. The patients had given their informed consent with the use of part of the tissues obtained by testicular biopsy for this study.

The tissue pieces allocated to this study were partly disintegrated by stretching between two sterile microscope slides. Seminiferous tubules released by this treatment were cut transversally into short segments that were separated from the rest of the tissue with a finely drawn Pasteur pipette. All these manipulations were carried out in Gamete medium (Scandinavian IVF Science, Gothenborg, Sweden) at laboratory temperature.

In-vitro incubation

Segments of seminiferous tubules were incubated in Gamete medium at 30°C as described previously (Tesarik et al., 1998a). Samples obtained from different sites of both testes of individual patients were mixed together and then divided into four aliquots which were incubated either in control medium, medium without FSH supplementation, medium without testosterone supplementation, or medium without both FSH and testosterone supplementation. The control medium was Gamete medium supplemented with 50 IU/l of human recombinant FSH (Puregon; Organon, Oss, The Netherlands) and 1 µmol/l testosterone (water soluble, cat. T-5035; Sigma, St Louis, Missouri, USA). These concentrations of FSH and testosterone had previously been shown to ensure the optimal survival of germ and Sertoli cells in samples from men with normal spermatogenesis during 48 h of in-vitro culture (Tesarik et al., 1998a).

In-situ labelling of active caspases

Freshly prepared or in-vitro cultured seminiferous tubule segments were further mechanically disintegrated to a suspension of single cells and Sertoli–germ cell clusters by repeated vigorous aspiration into a tuberculin syringe. This suspension was centrifuged at 500 g for 10 min and resuspended in 2 ml of Gamete medium to which CaspACE™ FITC-VAD-FMK in-situ marker (Promega, Madison, WI, USA) was added. FITC-VAD-FMK is a fluorescent analogue of the cell permeable pan-caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyel-aspartyl-($\mu$-methyl)-fluoromethylketone) in which the fluorescein isothiocyanate (FITC) group has been substituted for the carboxybenzoyl (Z) group to create the fluorescent marker which penetrates into intact living cells where it irreversibly binds to activated caspases. The final concentration of FITC-VAD-FMK in the incubation medium was 10 µmol/l. The choice of this concentration was based on preliminary experiments in which it gave the optimal signal-to-noise ratio. The incubation was carried out at 30°C for 20 min. After the incubation, the samples were centrifuged at 500 g for 10 min, resuspended in 10 ml of phosphate-buffered saline (PBS; Sigma, St Louis, MO, USA), centrifuged under the same conditions and resuspended in 0.2 ml of PBS. One part of this cell suspension was examined in the native state in a fluorescence microscope (Nikon, Tokyo, Japan). The other part was used for preparation of smears on microscope slides. After air-drying, the smears were fixed with 5% glutaraldehyde in 0.05 mol/l cacodylate buffer (pH 7.4) and processed for the simultaneous detection of DNA fragmentation (see below). Beginning with the incubation with FITC-VAD-FMK, all steps of the procedure were carried out in the dark to prevent photobleaching.

To assess the specificity of FITC-VAD-FMK labelling, samples were pre-incubated (30°C, 30 min) with 50 µmol/l Z-VAD-FMK (Promega) in Gamete medium before the final incubation with FITC-VAD-FMK. The fluorescent analogue was added to the samples pre-incubated with the non-fluorescent inhibitor at the end of the pre-incubation time without any intermediate washing step. These samples were subsequently treated in the same way as described above.

Evaluation of DNA fragmentation

Aliquots of fresh and in-vitro incubated samples were treated with 1000 U/ml collagenase IV (Sigma, C-5138) as described (Crabbé et al., 1997) to achieve complete disintegration of cell clusters into single cells. The cell suspensions were then smeared on microscope slides, fixed with 5% glutaraldehyde in 0.05 mol/l cacodylate buffer (pH 7.4) and processed for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) using Cell Death Detection Kit (Boehringer, Mannheim, Germany) containing FITC-labelled dUTP. This method adds the FITC-labelled nucleotides to the exposed ends of multiple DNA fragments resulting from the apoptosis-induced internucleosomal DNA breakage; this generates an in-situ fluorescent signal in the respective nuclei. For each sample at least two slides were treated by TUNEL using the standard procedure according to the manufacturer’s instructions, whereas at least two other slides were processed by a modified procedure adapted to human testicular germ cells which combines TUNEL with simultaneous immunocytochemical visualization of a germline marker (a monoclonal antibody against proacrosin) (Tesarik et al., 1998b). The standard procedure enabled the distinction between apoptotic and necrotic patterns of DNA fragmentation by the inclusion of a live-dead stain (propidium iodide) and was particularly useful for the evaluation of apoptosis in elongated spermatids and spermatozoa where the distinction from non-germline cells was easy on a morphological basis and no specific germline staining was thus needed. Propidium iodide was part of the commercial TUNEL kit (Boehringer), and it was used according to the manufacturer’s instructions. The modified procedure, including the germline marker, was useful for the distinction of pre-meiotic and meiotic germ cells from non-germ cells, but it precluded the inclusion of the live-dead stain. Nevertheless, most human germ cells staining positive by TUNEL after glutaraldehyde fixation are apoptotic and not necrotic as demonstrated previously (Sinha Hikim et al., 1998; Tesarik et al., 1998b). The smear preparations of cells previously processed for in-situ labelling of active caspases (see above) were treated by using the standard TUNEL procedure recommended by the manufacturer.

A positive control was performed by incubating segments of seminiferous tubules at 37°C instead of 30°C for 24 h before processing for TUNEL. This incubation was carried out in Gamete medium without hormone supplementation. More than 30% of germ cells consistently became TUNEL positive under these conditions.

Image generation

Microscopic preparations were evaluated with a Nikon Eclipse 6600 microscope (Nikon, Tokyo, Japan), and pictures were taken with the use of a JVC TK-1280E colour video camera (JVC, Tokyo, Japan). Images were captured with the use of Arkon FISH software, version 5.12 (purchased from Nikon Instruments, Florence, Italy).

Quantitative evaluations

Two hundred cells of each type (Sertoli cells, primary spermatocytes, and elongating/elongated spermatids) were evaluated for each patient and each experimental condition (time of incubation and medium supplementation with FSH and testosterone). The evaluation of
Caspase activity in Sertoli cells was carried out in native preparations (cell suspensions) because of the frequent loss of Sertoli cell cytoplasm during smear preparation. The evaluations of DNA fragmentation in Sertoli and germ cells and of caspase activity in germ cells were carried out in fixed smear preparations.

Cells to be evaluated were determined by viewing randomly chosen microscope fields (at least 10). All cells of a given type that were found in each field and that did not show detectable signs of degeneration (paucity or total lack of cytoplasm in Sertoli cells, and positive nuclear staining with propidium iodide in the case of germ cells) were included in the evaluation until the number of 200 cells was reached. Caspase activity in cytoplasmic fragments lacking a nucleus was not taken into account.

Statistical analysis
Testicular cells recovered from one patient represented one replicate. Percentages of cells showing caspase activity and DNA fragmentation were calculated for each cell type for which quantitative evaluation was performed (see above). Quantitative data (mean ± SEM) were analysed by $\chi^2$ and Kruskal–Wallis tests.
Results

**Cellular localization of active caspases and fragmented DNA in freshly explanted seminiferous epithelium**

In freshly explanted seminiferous tubules, the only seminiferous epithelium cells with active caspases were Sertoli cells and spermatids. In Sertoli cells, the caspase activity was always detected in the cytoplasm only (Figure 1), never in the nucleus. Caspase activity was detected both in Sertoli cells forming clusters with each other and with germ cells (Figure 1) and in isolated intact Sertoli cells (Figure 2). No distinction between these two situations was made in quantitative evaluations because preliminary data (not shown) indicated that the occurrence of caspase activity did not differ between the clustered and the isolated Sertoli cells.

In spermatids, the caspase activity was also strictly extranuclear and became topographically restricted to a caudal cytoplasmic compartment during the process of spermatid elongation (Figure 3). Caspase activity was not detected in meiotic or pre-meiotic germ cells (Figure 1). When samples were pre-incubated with Z-VAD-FMK before the incubation with FITC-VAD-FMK (negative control), no fluorescence was observed in any cell type (Figure 4).

Fragmented DNA was detected by TUNEL in nuclei of a few Sertoli and germ cells showing the same staining patterns as described previously (Tesarik et al., 1998a,b). However, when the TUNEL reaction was performed with cells previously treated with the pan-caspase marker FITC-VAD-FMK, the two markers were never simultaneously positive in the same cell. Even though both of these markers were visualized with the use of the same fluorochrome (FITC), this conclusion was possible because, in single-labelled preparations, the FITC-VAD-FMK never marked the cell nucleus, whereas the TUNEL labelling was always strictly intranuclear.

Quantitative data on the representation of cells containing active caspases and of those with damaged DNA are given below.

**Changes in the quantitative representation of cells containing active caspases and those with fragmented DNA after incubation in the presence or absence of FSH and testosterone**

In aliquots of seminiferous tubule preparations that were incubated in control medium (supplemented with 50 IU/l FSH...
Caspase activity in seminiferous epithelium

Selective withdrawal of testosterone from culture medium resulted in a superimposition of the changes observed after isolated withdrawal of each of these hormones (Table III).

The withdrawal of both FSH and testosterone from culture medium resulted in a superimposition of the changes observed after isolated withdrawal of each of these hormones (Table III).

Discussion

Human male germ cells carry Fas receptor while Sertoli cells carry both Fas receptor and Fas ligand (Francavilla et al., 2000), suggesting that, as in other mammals, Fas ligand released from Sertoli cells by proteolytic processing might induce apoptosis by an autocrine (directed against Sertoli cells) or paracrine (directed against germ cells) mechanism of action (Kierszenbaum, 2001). Signalling through Fas receptor may proceed via two alternative pathways. One of these pathways involves recruitment of the adaptor molecule Fas-associated death domain protein (FADD) and activation of procaspase-8 which in turn cleaves and activates downstream caspases such as procaspase-3, -6 and -7, whereas the other proceeds through release of cytochrome c from mitochondria and assembly of the apoptosome complex that leads to the activation of procaspase-9 and subsequent activation of the downstream caspases (Scaffidi et al., 1998). In both of these alternative pathways, sequential activation of different types of caspases plays a central role. In agreement with this theoretical background, in-vitro apoptosis of human male germ cells can be blocked by both immunoneutralization of Fas ligand and caspase inhibition (Pentikainen et al., 1999). However, the present study is the first to address the dynamics of caspase activation in different cell types of human seminiferous epithelium in response to apoptosis-inducing conditions.

The data obtained in this study have shown that caspase activation, occurring in Sertoli cells in response to testosterone withdrawal, coincided with a progressive increase in the proportion of Sertoli cells showing DNA fragmentation. Even though this study did not address the implication of the Fas system in these phenomena, it may be hypothesized that the caspase activation resulted from the autocrine action of Fas ligand. Contrary to testosterone, withdrawal of FSH did not produce any measurable effect either on caspase activation or DNA integrity in Sertoli cells. The insensitivity of Sertoli cell DNA to acute FSH withdrawal is in agreement with our previous observations (Tesarik et al., 1998a).

In germ cells, caspase activity was consistently found only

Sertoli cells containing active caspases as well as of those with fragmented DNA as early as 24 h after the beginning of incubation. Similar to the freshly explanted seminiferous tubules, the caspase labelling was always strictly cytoplasmic, and it never coincided with nuclear labelling for damaged DNA in the same cell.

In contrast to Sertoli cells, no changes in the incidence of caspase activation or DNA fragmentation were detected in the primary spermatocyte and elongating/elongated spermatid populations after 24 h of incubation in testosterone-depleted medium, although a slight but significant ($P < 0.01$) increase in the proportion of cells with damaged DNA was detected in the two cell populations after 48 h of incubation. This increase was not accompanied by any changes in the proportion of cells containing active caspases (Table II).

The withdrawal of both FSH and testosterone from culture medium resulted in a superimposition of the changes observed after isolated withdrawal of each of these hormones (Table III).
at post-meiotic stages of development, particularly at the elongating and elongated spermatid stage. At these latter two stages, caspase activity was restricted to a post-nuclear cytoplasmic compartment which is shed away from maturing spermatids as a residual body during the process of spermiogenesis. A similar topographically restricted localization of caspase-1, c-jun, p53 and p21, coinciding with apoptosis-related externalization of phosphatidylserine, has been described in rat spermatids (Blanco-Rodriguez and Martinez-Garcia, 1999), suggesting a role for apoptosis in residual body extrusion. This topographically restricted apoptosis occurred in the presence of a healthy nucleus (Blanco-Rodriguez and Martinez-Garcia, 1999). Similarly, in the present study caspase activity and apoptosis-related DNA fragmentation were never observed to occur simultaneously in the same spermatids, suggesting that this localized apoptotic process was physiological and possibly dependent on the presence of a healthy nucleus. Moreover, FSH withdrawal did not produce an increase in the percentage of spermatids showing caspase activity, although it increased the incidence of DNA fragmentation in spermatid nuclei.

The above data suggest that, unlike the physiological apoptosis during spermiogenesis, the early apoptotic changes occurring in germ cells in response to FSH withdrawal are caspase-independent. Even though CED-3 protease activation is an obligatory step in apoptosis in Caenorhabditis elegans, recent studies have shown that apoptosis may be caspase independent in some mammalian cells under certain conditions (Qi and Sit, 2000). For instance, caspase-independent apoptosis can occur in in-vitro cultured retinal cells exposed to oxidative stress (Carmody and Cotter, 2000), in serum-starved cortical neurons (Hamabe et al., 2000), in MCF-7 breast carcinoma cells exposed to hydrogen peroxide (Kim et al., 2000), in

### Table I. Effects of FSH withdrawal from culture medium on FITC-VAD-FMK binding and TUNEL labelling of Sertoli and germ cells

<table>
<thead>
<tr>
<th>Time of incubation and FSH inclusion</th>
<th>% labelled cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sertoli cells</th>
<th>Spermatocytes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Spermatids</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>VAD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>TUNEL</td>
<td>VAD&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>0 h</td>
<td>7 ± 1</td>
<td>8 ± 2</td>
<td>–</td>
<td>13 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 h</td>
<td>6 ± 1</td>
<td>9 ± 2</td>
<td>–</td>
<td>14 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
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<td>9 ± 3</td>
<td>–</td>
<td>26 ± 4&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
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<td>FSH–</td>
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<td>8 ± 2</td>
<td>–</td>
<td>16 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>48 h</td>
<td>8 ± 1</td>
<td>10 ± 3</td>
<td>–</td>
<td>39 ± 4&lt;sup&gt;f&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>Data are mean ± SEM of eight replicates performed with samples from eight different patients. Two hundred cells were evaluated for each cell type in each replicate.

<sup>b</sup>Abbreviated form of FITC-VAD-FMK, a fluorescent analogue of the cell permeable pan-caspase inhibitor carbobenzoxy-valyl-analyl-aspartyl-(O-ethyl)-fluoromethylketone.

<sup>c</sup>Values with different superscript within columns are different (P < 0.01).

TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

### Table II. Effects of testosterone (T) withdrawal from culture medium on FITC-VAD-FMK binding and TUNEL labelling of Sertoli and germ cells

<table>
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<tr>
<th>Time of incubation and FSH inclusion</th>
<th>% labelled cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sertoli cells</th>
<th>Spermatocytes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Spermatids</th>
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<tr>
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<td>TUNEL</td>
<td>VAD&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>0 h</td>
<td>7 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8 ± 2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
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<td>24 h</td>
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</tr>
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<td>T–</td>
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<td>–</td>
<td>16 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>–</td>
<td>24 ± 3&lt;sup&gt;e&lt;/sup&gt;</td>
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</table>

<sup>a</sup>Data are mean ± SEM of eight replicates performed with samples from eight different patients. Two hundred cells were evaluated for each cell type in each replicate.

<sup>b</sup>Abbreviated form of FITC-VAD-FMK (see Table I).

<sup>c</sup>Values with different superscript within columns are different (P < 0.01).

TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.
Caspase activity in seminiferous epithelium

Table III. Effects of simultaneous FSH and testosterone (T) withdrawal from culture medium on FITC-VAD-FMK binding and TUNEL labelling of Sertoli and germ cells

<table>
<thead>
<tr>
<th>Time of incubation and FSH and testosterone inclusion</th>
<th>% labelled cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sertoli cells</th>
<th>Spermatocytes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Spermatids</th>
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<td>TUNEL&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>8 ± 2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
<td>13 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>24 h</td>
<td>6 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9 ± 2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
<td>14 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>FSH+, T+</td>
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<td>FSH−, T−</td>
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<td>–</td>
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<td>FSH+, T+</td>
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<td>39 ± 5&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
<td>56 ± 6&lt;sup&gt;f&lt;/sup&gt;</td>
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<sup>a</sup>Data are mean ± SEM of eight replicates performed with samples from eight different patients. Two hundred cells were evaluated for each cell type in each replicate.

<sup>b</sup>Only primary spermatocytes are included in this category.

<sup>c</sup>Abbreviated form of FITC-VAD-FMK (see Table I).

<sup>d</sup><sup>e</sup><sup>f</sup>Values with different superscript within columns are different (P < 0.01).

TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

thapsigargin-treated AT3 and TSU-Pr1 prostate cancer cells (Denmeade et al., 1999), or in tert-butyl hydroperoxide-treated SH-SY5Y neuroblastoma cells (Krishnamurthy et al., 2000).

Interestingly, in many systems in which partly or totally caspase-independent apoptosis was observed, the cell death was induced by oxidative stress (Carmody and Cotter, 2000; Kim et al., 2000; Krishnamurthy et al., 2000). Similarly, oxidative stress plays an important role in apoptosis of human germ cells in which the apoptotic process induced by withdrawal of survival factors (serum or hormones) is attenuated by lowering partial oxygen pressure from 21% to <10% (Erkkila et al., 1999). The recent discovery of a caspase-independent nuclease (endonuclease G) which releases from mitochondria and translocates to the nucleus during apoptosis (Li et al., 2001) provides a plausible explanation for these phenomena. The implication of endonuclease G would also explain the observations on apoptosis-related phenomena in late spermatids and sperm that lack caspase activity but are rich in mitochondria. The expression and activity of endonuclease G in germ cells remains to be evaluated.

With regard to the way processes of caspase activation and apoptosis are supposed to act in the seminiferous epithelium under in vivo conditions, the present data concerning the effects of testosterone withdrawal on Sertoli cells are surprising. To our knowledge, out of numerous studies in the literature that have looked at the effects of in-vivo testosterone withdrawal in a range of rodents and non-human primates in search for an efficient and reversible male contraceptive regimen (Anderson, 2000), none has ever reported any effect on Sertoli cell apoptosis. In contrast, artificial separation from basement membrane does induce apoptosis in Sertoli cells, and this effect can be partly prevented by a mix of survival factors including FSH and testosterone (Dirami et al., 1995). Does this mean that Sertoli cell apoptosis is a mere artefact of the in-vitro conditions? It is probable that the immediate cause of Sertoli cell apoptosis in this particular explant culture system is the loss of the original cell–basement membrane associations.

However, this study also shows clearly that testosterone efficiently prevents caspase activation in Sertoli cells exposed to this apoptosis-inducing condition, which is an entirely new observation. Disturbances of the association between Sertoli cells and basement membrane, leading to Sertoli cell desquamation, are known to occur as non-specific phenomena in different testicular pathologies. Hence, Sertoli cells in a diseased testis can be expected to be more vulnerable to testosterone depletion as compared with a healthy testis. Interestingly, a recent study has demonstrated the occurrence of varying degrees of Sertoli cell cytoplasmic degradation and the development of inappropriate cell associations in chimpanzee seminiferous tubules after only 21 days of in-vivo androgen deprivation (Smithwick and Young, 2001).

On the other hand, all available evidence from clinical trials of steroid-based male contraception, which was supposed to imply a deep suppression of intratesticular testosterone levels, shows that the men participating in these trials recover their testes to normal size and with normal sperm counts after cessation of the contraceptive regimen. However, a recent study in the cynomolgus monkey model (Weinbauer et al., 2001) has shown that, unexpectedly, suppression of intratesticular testosterone did not occur in animals exposed to a long-acting testosterone ester, although serum LH was completely abolished and FSH was suppressed in a dose-dependent manner. Profound suppression of FSH by externally administered steroids may thus be the main factor of steroid-based male contraception rather than a deep suppression of intratesticular testosterone. Moreover, men participating in clinical trials of male contraception were healthy volunteers with normal testicular function. In view of the present data, caution is needed to ensure the normal testicular function of men exposed to steroid-based contraceptive regimens and to other treatments (such as GnRH analogues) that may decrease intratesticular testosterone levels.

The present data indicate a central role of Sertoli cells in the regulation of apoptosis in the adult human testis. Apoptosis
of germ cells, at least that induced in vitro by testosterone withdrawal, occurs secondarily, after a primary alteration has occurred in Sertoli cells. The implication of Sertoli cells in apoptosis of germ cells in response to FSH withdrawal in vitro is also very probable because germ cells are believed to lack a functional FSH receptor (Böckers et al., 1994). The nature of the Sertoli cell-derived prosurvival mediator(s) involved in these FSH and testosterone effects remains to be determined. If these hypothetical prosurvival factors are depleted, germ cells may become increasingly sensitive to oxidative stress which may be the immediate death-inducing factor.

It also remains to be determined to what extent these in-vitro phenomena reflect those produced in vivo by testosterone and FSH withdrawal. For ethical reasons, this question is difficult to address directly in humans, and experimental studies in an appropriate animal model are needed. It is possible that the in-vitro effects of testosterone withdrawal observed in this study merely represent an acute response which may be followed by a more generalized caspase activation involving different stages of germ cells. In fact, caspase-3 activation has been shown to occur in rat germ cells by 1 week after in-vivo reduction of intratesticular testosterone, and internucleosomal DNA cleavage in germ cells continued increasing through the subsequent 3 weeks (Kim et al., 2001).

If germ cell apoptosis occurring as an acute response to hormone withdrawal is not mediated by caspases, a question arises as to the biological significance of the up-regulation of Fas receptor in rat (Lee et al., 1999) and human (Pentikainen et al., 1999) germ cells occurring after in-vitro induction of massive cell death, which is also found in spermatids from patients with post-meiotic maturation arrest (Francavilla et al., 2000). The latter observation may simply be related to the arrest of spermiogenesis leading to retention of the residual body, which is rich in apoptosis-related proteins (see above), rather than a true activation of the Fas signalling pathway. Hence, unlike the autocrine action of the Fas system in Sertoli cells, this study does not provide any element in support of a paracrine action of this system in adult human germ cells.

The present data may have implications in the recent efforts aimed at restoring pathologically disturbed spermatogenesis by in-vitro germ cell maturation (Tesarik et al., 1999, 2000b,c). It appears that in-vitro maturation block may be overcome in vitro in those cases in which the disturbed function of Sertoli cells can be improved by exposing them to conditions that would hardly be possible to reproduce in vivo, such as high hormone concentrations (Tesarik et al., 1998a), the presence of drugs augmenting intracellular concentration of second messengers (Tesarik et al., 2000d) or low temperature (Tesarik et al., 1998a). On the other hand, the efficiency of germ cell in-vitro survival and differentiation is dubious when a great majority of Sertoli cells are apoptotic or when the cause of spermatogonial arrest is inherent in germ cells. Combined evaluation of DNA integrity and caspase activity in different cell types of the human seminiferous epithelium may help, distinguish between these different situations.

References


Caspase activity in seminiferous epithelium


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