Morphometrical Evaluation of Germ Cell Apoptosis in Infertile Men

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Abstract. Apoptosis associated with programmed cell death plays an essential role in the control of germ cell number in the testes. Although male germ cell apoptosis has been well characterized in different animal models, only a few studies of apoptosis in human testes are presently available. In 43 infertile men with azoospermia of varying aetiology, testicular tissue was obtained by testicular biopsy. Apoptosis of testicular germ cells was determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling method in situ. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling-positive cells were found in the testicular tissue of all patients with azoospermia, except in Sertoli cell-only syndrome. The apoptotic index was higher in germ cell hypoplasia and in normal spermatogenesis in comparison with germ cell arrest. This study was performed to confirm the presence and determine the frequency of apoptosis in infertile men.

Introduction

Apoptosis is a process of natural death that can occur in tissue under both normal and pathological conditions. This physiological or programmed cell death is an active, triggered process occurring under genetic control (Kerr, 1994). Morphologically, apoptosis is characterized by chromatin aggregation, nuclear and cytoplasmic condensation, and fragmentation of the dying cell into a cluster of membrane-bound segments (apoptotic bodies), rapidly recognized, phagocytized and digested by either macrophages or adjacent epithelial cells (Verhaegen, 1998).

Apoptosis provides a way to remove redundant cells at the end of their lifespan and thus acts as a homeostatic mechanism, maintaining the correct number of cells in the body by balancing their production and death. In the testis, this process seems to play a pivotal role in spermatogenesis and in maintaining the proper functioning of the testis (Porcelli et al., 2006). Spontaneous death of certain classes of germ cells has been shown to be a constant feature of normal spermatogenesis in a variety of mammalian species, including human. Recent studies on various animal models have demonstrated that apoptosis is the underlying mechanism of germ cell death during normal spermatogenesis. Male germ cell apoptosis occurs through two major pathways, involving either mitochondria (intrinsic) or cell surface death receptors (extrinsic) (Shaha, 2007). The stimuli for germ cell apoptosis are internal cues that control proper homeostasis of the testicular tissue, or external agents including testicular toxins, heat stress and chemotherapeutic agents. In addition, an imbalance of hormones can lead to the apoptosis of germ cells (Ruwandipura et al., 2008). During spermatogenesis, more than half of the differentiating spermatogenic cells undergo apoptosis before they mature into spermatozoa (Wang et al., 2006). Up to 75% of the spermatagonia die in the process of programmed cell death before reaching maturity (Dunkel et al., 1997).

Sertoli cells and germ cells, the only cell types within the seminiferous epithelium, are in close contact. Sertoli cells, spanning the thickness of the seminiferous epithelium, supervise spermatogenesis by providing structural and nutritional support to germ cells. The seminiferous epithelium of the testis is a rapidly proliferating tissue in which germ cells degenerate spontaneously. It is generally accepted that Sertoli cells control the germ cell population through one of the best-known apoptotic pathways, the Fas/FasL paracrine signal transduction system, in which the Fas ligand (FasL) expressed by Sertoli cells induces apoptosis when it binds with its receptor, Fas, expressed by the germ cells (Pen-
tikainen et al., 1999; Celik-Ozenci et al., 2006; Porcelli et al., 2006).

**Material and Methods**

Testicular biopsy was performed in 43 infertile patients of varying aetiology with average age of 32 ± 2 years (from 22 to 45 years). Twenty-eight patients were affected by non-obstructiveazoospermia, four patients by obstructiveazoospermia, five patients by anejaculation and six patients by severe oligo-, astheno- and teratozoospermia. Patients were infertile for 6 ± 5 years (from 0.5 to 20 years).

**Testicular tissue sampling**

Testicular tissue was obtained using an open testicular biopsy technique in the conventional testicular sperm extraction (TESE) method. Each testicular specimen was analysed for the presence of spermatozoa (successful or unsuccessful TESE), and for the number and motility of spermatozoa. The samples were immediately fixed in Bouin’s solution for standard histological interpretation of spermatogenesis and in neutral-buffered formalin for detection of apoptotic cells.

**Histological analysis**

On the basis of standard qualitative histological interpretations of haematoxylin-eosin (HE)-stained sections, the biopsy specimens were classified as Sertoli cell-only syndrome (SCOS), germ cell arrest (GA, a total arrest at a particular stage), germ cell hypoplasia (hypospermatogenesis, GH, all stages of spermatogenesis are present but reduced to a varying degree) (McLachlan et al., 2007) or normal spermatogenesis (N).

**Testicular tissue preparation**

Biopsy samples of testes were fixed in 10% neutral buffered formalin for 12 h at 4 °C. Longer fixation has been reported to reduce the ability to produce terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining (Davison et al., 1995). Specimens were dehydrated with ethanol and embedded in paraffin. Sections 5 µm thick were cut and mounted on silanized slides. After the deparaffinization, the sections were washed in PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl). One 5-µm thick section from each block was stained with Harris HE for conventional morphological examination.

**TUNEL assay for apoptosis**

Apoptosis of testicular germ cells was determined by the TUNEL method in situ using an ApopTag Plus In Situ Apoptosis Detection Kit (Oncor, Gaithersburg, MD). The recommended staining procedure was followed in principle: digoxigenin-UTP-labelled DNA was detected with anti-digoxigenin-peroxidase antibody followed by peroxidase detection with 0.05% diaminobenzidine (DAB) and 0.02% H2O2. However, the time for treating slides with proteinase K (Oncor) was reduced from 15 min to 5 min to avoid excessive destruction of seminiferous tissue, and Mayer’s haematoxylin was used to counterstain the nuclei lightly. The positive control was Oncors control slide, containing unstained rat mammary gland obtained at the fourth day after weaning. For a negative control we mixed water instead of TdT enzyme into the reaction buffer.

**Morphological analysis**

Morphological analysis was made by light microscopy on HE-stained slides.

**Morphometrical analysis**

Morphometrical analysis was performed on systematically sampled TUNEL-positive testicular germ cells. An x10 ocular fitted with M-42 test system and an x100 oil immersion objective (total magnification x1000) were employed using a digital cell counter. The apoptotic index as a percentage of TUNEL-positive cells, assessed by morphological analysis, was estimated. The frequency of apoptotic cells was expressed as the apoptotic index, i.e. the number of apoptotic cells (or apoptotic bodies) per 1000 germ cells.

**Statistical analysis**

The germ cell apoptotic index in all testicular specimens was correlated in conventional histological diagnosis of spermatogenesis (N, GH, GA and SCOS). Statistical significance was set after Student’s t-test at P < 0.05.

**Results**

TUNEL-positive germ cells were found in early germ cell arrest, germ cell hypoplasia and in normal spermatogenesis (Fig. 1).

The apoptotic index (Fig. 2) was highest in germ cell hypoplasia (2.0 ± 2.7) and was statistically significant (P < 0.025) in comparison with germ cell arrest (0.7 ± 0.4). The apoptotic index of normal spermatogenesis (1.0 ± 0.4) was higher than in germ cell arrest, but the difference was not statistically significant. As expected, no apoptosis was observed in the tissue samples of SCOS (Fig. 1).

**Discussion**

Spontaneous death of certain classes of germ cells has been shown to be a constant feature of normal spermatogenesis in a variety of mammalian species, including human. Studies on various animal models have demonstrated that apoptosis is the underlying mechanism of germ cell death during normal spermatogenesis (Hikim et al., 1998; Inaba et al., 1998; Kimura et al., 2003; Chaki et al., 2006; Francis et al., 2006; Hong et al., 2007; Bozec et al., 2008).

Variations in apoptosis frequency are associated with differential cAMP-responsive element (CRE) modulator expression in male infertility states. Lin et al. (1998)
suggest that CRE modulator may be important for spermatid development and a stage-specific regulator of human spermatogenesis. Factors involved in the regulation of germ cell death are being actively investigated. Testicular cell death was effectively regulated by lactate, which may be regarded as a potential compound for optimizing in vitro methods involving male germ cells for assisted reproduction (Erkkilä et al., 2002). In most of the testicular cells, mitochondrial respiration appears to play a crucial role in controlling primary cell death cascades. On the basis of their investigation, Erkkilä et al. (2006) concluded that the mitochondrial adenosine triphosphate (ATP) production machinery plays an important role in regulating in vitro induced primary pathways of human male germ cell apoptosis, and that there seem to be secondary pathways of human testicular cell apoptosis that do not require mitochondrial ATP production.

In the present study, a significant increase of germ cell apoptosis has been detected in the histopathological state of hypospermatogenesis in comparison with apoptosis found in germ cell arrest. Our results are in accordance with the results of Lin et al. (1997a) and Kimura et al. (2003), who found a significantly increased ratio of apoptotic bodies per total cell, and increased apoptotic bodies per Sertoli cell were observed in GA and hypospermatogenesis states in comparison to SCOS and normal spermatogenesis. Hassan et al. (2009) reported significantly higher apoptotic indices in fertile and infertile...
men with varicocele in comparison with obstructive azoospermmia as a control group. On the other hand, Fujisawa et al. (1999) reported decreased apoptosis in the tests of infertile men with varicocele as compared with normal men.

With the in situ 3’end-labelling technique, the apoptotic cells in the seminiferous tubules of our biopsy specimens were identified as being mostly spermatogonia according to the position of apoptotic cells or apoptotic bodies in the testicular tubule. Using an in situ apoptosis detection method, Heiskanen et al. (1996) were able to demonstrate that both interstitial cells and germ cells were affected and that the specific germ cells undergoing apoptosis were exclusively spermatogonia. Fujisawa et al. (1999) found out that spontaneous apoptosis in normal human testes occurred primarily in germ cells near the basement membrane, spermatogonia and spermatocytes. Other authors (Dunkel et al., 1997; Lin et al., 1997b) reported that apoptosis was most frequently identified in spermatocytes, while occasionally some spermatids also showed signs of apoptosis.

We found germ cell apoptosis in 72% of testicular specimens of 43 infertile men. The germ cell apoptotic index was relatively low. One of the reasons for this could be the very short time course of the apoptotic process, lasting 30 min or less from shrinkage of the cell to phagocytosis (Verhaegen, 1998).

Schmelz et al. (2005) reported a strong relationship between the apoptotic index and the age of the patient, showing a significant apoptotic index dependence on age. We have not found any correlation between the male age and the germ cell apoptotic index. This might be due to the fact that the men in our study were aged from 22 to 45 years and were much younger than the elderly men (above 60 years) showing increased germ cell apoptosis.

The results of the study of Helal et al. (2002) suggest that gonadal apoptosis occurs in germ cells, Sertoli cells and Leydig cells at all gestational ages. According to the examined human foetal testicular tissue, apoptotic death was highest in the Leydig cells, followed by germ cells and Sertoli cells. There was a significant positive correlation between the apoptosis of germ cells and Sertoli cells and a negative correlation between healthy germ cells and Sertoli cells. Decreasing total Sertoli cell count with ageing was found by Petersen et al. (2000) using one of the newer stereological methods, the optical fractionator.

Like some other authors (Brinkworth et al., 1997; Dunkel et al., 1997; Lin et al., 1997a; Tay et al., 2007, Hassan et al., 2009) we did not find TUNEL-positive cells in the eight patients with SCOS, thus indicating that the Sertoli cells, as post-mitotic terminally differentiated cells, are in some way protected against programmed cell death (Johnson et al., 2007). The results of in vitro experiments suggest that the basement membrane plays a crucial role in Sertoli cell survival when it is used as a solid substratum for culture; in the absence of basement membrane, follicle-stimulating hormone (FSH) and other regulators of Sertoli cell function cannot prevent Sertoli cell apoptosis (Richardson et al., 1998). Using hypogonadal hgn/hgn rats as a model for the study of Sertoli cell deficiency, Yagi et al. (2006) reported early postnatal apoptosis of Sertoli cells. Despite these results, Weikert et al. (2004) didn’t find surviving mRNA expression in SCOS specimens.

Based on this finding, we conclude that testicular apoptosis is increased in germ cell hypoplasia and in normal spermatogenesis as compared with germ cell arrest. Research must be expanded to the molecular level to explain the increased testicular apoptosis in hypospermatogenesis compared to germ cell arrest. We expect that further research will also show whether there is some correlation between the proportion of apoptotic testicular germ cells and the clinical picture of azoospermic patients.

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References


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