Molecular Human Reproduction Vol.7, No.5 pp. 403–408, 2001

Expression of Bcl-2 family proteins and spontaneous apoptosis in normal human testis*

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We investigated the frequency of spontaneous apoptosis and expression of the Bcl-2 family of proteins during normal spermatogenesis in man. Testicular tissue with both normal morphology and DNA content was obtained from necro-donors and fixed in Bouin's solution. A TdT-mediated dUTP end-labelling method (TUNEL) was used for the detection of apoptotic cells. Expression of apoptosis regulatory Bcl-2 family proteins and of p53 and p21(Waf1) was assessed by immunohistochemistry. Germ cell apoptosis was detected in all testes and was mainly seen in primary spermatocytes and spermatids and in a few spermatogonia. Bcl-2 and Bak were preferentially expressed in the compartments of spermatocytes and differentiating spermatids, while Bcl-x was preferentially expressed in spermatogonia. Bax showed a preferential expression in nuclei of round spermatids, whereas Bad was only seen in the acrosome region of various stages of spermatids. Mcl-1 staining was weak without a particular pattern, whereas expression of Bcl-w, p53 and p21(Waf1) proteins was not detected by immunohistochemistry. The results show that spontaneous apoptosis occurs in all male germ cell compartments in humans. Bcl-2 family proteins are distributed preferentially within distinct germ cell compartments suggesting a specific role for these proteins in the processes of differentiation and maturation during human spermatogenesis.

Key words: apoptosis/Bcl-2 family proteins/immunohistochemistry/spermatogenesis/testis

Introduction

A fine balance between cell proliferation and cell loss is necessary for normal tissue homeostasis. Apoptosis constitutes a strictly regulated process that is important for the removal of surplus, aged or damaged cells, as well as unnecessary cells in the course of normal animal development, and its deregulation is associated with the pathogenesis of a wide range of diseases (Hetts, 1998). Apoptosis is morphologically characterized by nuclear fragmentation, formation of membrane-encased apoptotic bodies containing organelles, and cell shrinkage (Kerr *et al*., 1994), and is distinctly different from necrosis.

Spermatogenesis is a complex process where spermatogonia (stem cells) divide and differentiate into mature spermatozoa. The relative inefficiency of human spermatogenesis has been well documented, with the human testis producing comparatively low numbers of sperm cells per unit weight of testis compared to various animal species (Brinkworth *et al*., 1997). The reason for this is not known. From animal studies, germ cell deletion during normal spermatogenesis has been estimated to result in the loss of up to 75% of the potential numbers of mature sperm cells in the adult testis (Dunkel *et al*., 1997). Apoptosis is probably the major mechanism responsible for this cell loss. Despite its putative implications for improved fertility control and clinical management of infertility in men, the mechanisms of germ cell death in men are poorly understood (Sinha Hikim *et al*., 1998).

The members of the Bcl-2 family of proteins have been reported to be involved in the regulation of apoptosis in various cell types, and may either inhibit (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w) or promote apoptosis (Bax, Bcl-x_S, Bak, Bad) (Sinha Hikim and Swerdloff, 1999). The significance of the Bcl-2 family of proteins has not been established in the human testis. Furthermore, the associations of these proteins with other proteins that influence apoptosis in the human testis, e.g. the tumour suppressor proteins p53 and p21(Waf1), are also unknown.

In the present study, spontaneous apoptosis was assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) of DNA fragments to detect apoptotic cells in paraffin-embedded testis sections. The expression of the apoptosis regulatory proteins, Bcl-2, Bcl-x, Bax, Mcl-1, Bad, Bcl-w and Bak as well as of p53 and p21(Waf1) was determined using immunohistochemical methods.

*Presented in part at the 16th ESHRE Annual Meeting, Bologna, 2000.

Materials and methods

Testicular tissue was obtained from necro-donors in connection with organ transplantation ($n = 15$). The causes of death were intracranial bleeding or head injury. Clinical information other than that pertinent to organ transplantation was not available. The obtained information did not indicate any abuse of drugs or alcohol.

Sample preparation

Biopsies were placed in Roswell Park Memorial Institute cell culture medium, supplemented with fetal calf serum (10%), penicillin/streptomycin (1%) and pyruvate (110 mg/l), immediately after removal, for a median of 8 h, range 2–13 h. Testicular samples were fixed in freshly prepared Bouin's solution for 1–2 days and then embedded in paraffin.

Specimens for histopathology and immunohistochemical staining were cut successively as 5 μm thick sections and mounted on gelatin-coated slides. One section was stained with haematoxylin and eosin for the evaluation of spermatogenesis in each testis. Only samples which revealed morphologically normal spermatogenesis with normal tubular cellularity and late spermatids in all tubular cross-sections, and that showed normal DNA content distributions (Thorud *et al.*, 1980), were included in the final study. Suspensions of testicular cells from fresh tissue were obtained after collagenase treatment, and stained with Hoechst 33258 (Calbiochem-Boehringer, La Jolla, CA, USA) (Bjørge *et al.*, 1996), and analysed with an Argus 100 Flow cytometer (Skatron, Lier, Norway). The percentages of haploid, diploid and tetraploid cells were calculated from two-parameter cytograms based on fluorescence and forward light scatter.

Immunohistochemical analysis

In-situ detection of apoptosis

The Apop Tag Plus In Situ Apoptosis Detection Kit (peroxidase) (Oncor, Gaithersburg, MD, USA) was used to detect apoptotic cells. This method (TUNEL) uses terminal deoxynucleotidyl transferase (TdT) to end label the DNA fragments resulting from apoptosis with deoxyuridine triphosphate (dUTP). Both morphologically identifiable apoptotic nuclei and apoptotic bodies as well as pre-apoptotic cells with morphologically intact nuclei can be identified. In brief, deparaffinized sections were treated with proteinase K (20 μg/ml) for 15 min at room temperature prior to using TdT to label the 3′-OH ends of DNA with digoxigenin-labelled nucleotides (1 h incubation at 37°C). The sections were then treated with antidigoxigenin antibody-peroxidase conjugate for 30 min at room temperature, stained with diaminylbenzidine (DAB) for 3–6 min to produce the characteristic brown colour of positive cells, counterstained with haematoxylin, mounted and examined microscopically. For each batch, a positive control section from the mammary gland of weaning mice was included. Apoptotic germ cells were counted in 50 tubular cross-sections from each of the testes that showed spermatogenesis within normal range. The apoptotic cells were classified as being spermatogonia, primary spermatocytes, spermatids or Sertoli cells.

Expression of Bcl-2 proteins (Bax, Bcl-2, Bad, Bak, Bcl-w, Mcl-1 and Bcl-x)

The immunohistochemical method used to detect these proteins has been described previously (De Angelis *et al.*, 1998). The specificities of the antibodies (Santa Cruz Biotechnology, CA, USA) used to detect these proteins were determined by immunoblotting of different normal and tumour tissues and cell lines (De Angelis *et al.*, 1998). When choosing these antibodies, they were the only ones reproducibly staining tissues known to express the respective antigens, and even then staining was only observed after the sections were subjected to pressure cooking. Briefly, tissue sections were deparaffinized by two washes in xylene for 5 min each and then dehydrated in absolute ethanol. To block endogenous peroxidase, the sections were incubated in 3% (vol/vol) H_2O_2 in methanol (45 s), dehydrated with ethanol and washed in phosphate-buffered saline (PBS) for 5 min. They were then heated in a pressure cooker for 5 min in 10 mmol/l citric acid buffer (pH 6.0) followed by rinsing in lukewarm tap water. The sections were placed in Tris-buffered saline (TBS), pH 7.8, for 5 min, and then blocked in TNK buffer (100 mmol/l Tris 7.6-7.8, 550 mmol/l NaCl, 10 mmol/l KCl) which contained 2% (wt/vol) bovine serum albumin (BSA), 0.1% Triton X-100, and 1% normal goat or donkey serum. Rabbit anti-human bax, Bcl-2, Bak, Mcl-1 and Bcl-x polyclonal antibodies and goat anti-human bad and Bcl-w polyclonal antibodies (Santa Cruz Biotechnology, CA, USA; 1:10 and/or 1:20 dilutions of 100 μg/ml stock in TNK buffer) were added and the sections were incubated overnight in a humidified chamber placed at 4°C. The sections were then washed once with PBS and incubated for 1 h at room temperature in a humidified chamber with biotinylated goat anti-rabbit antibody (1:500) (Vector Labs, Burlingame, CA, USA) or biotinylated donkey anti-goat antibody (1:100) (Santa Cruz Biotechnology, CA, USA) in TNK buffer, followed by washing with PBS, incubation for 30 min at room temperature with streptavidin-horseradish peroxidase (1:20) in TNK buffer, then placement in development solution containing 0.06% DAB and 0.1% (v/v) H_2O_2 in TNK buffer (without serum, BSA and Triton X-100). The sections were finally counterstained with haematoxylin, mounted and examined microscopically. Positive cells stained brown. Positive and negative (without the relevant primary antibody) control sections from a normal colorectal mucosal tissue and colorectal tumours were included in each batch.

Expression of p53 and p21(Waf1) proteins

After dewaxing in xylene, the sections were dehydrated in ethanol and rinsed in distilled water. For antigen retrieval, the slides were incubated in 10 mmol/l citric acid buffer (pH 6.3) in a microwave oven (750 W) for 5 min, five times. After cooling for 30 min at room temperature, the sections were washed in Tris buffer for 5 min and incubated at room temperature with the DO1 mouse monoclonal antibody to p53 (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) at 1:100 dilution of stock. The p21(Waf1) antibody (Oncogene Research Products, MA, USA) was used diluted 1:20. For each batch of immunostained sections, one positive and one negative control (a tumour section known to be positive for p53 and p21, with and without the primary antibody) were included. Staining was performed with labelled streptavidin-biotin methos (LSAB) using DAB chromogen. To test the possible influence of Bouin's fixative on p53 and p21(waf1) immunostaining, some samples were also fixed in buffered formalin.

Results

The median age of the subjects in which the testis revealed spermatogenesis within normal range was 44 years (range 27–69 years, $n = 11$). Thus four testes were excluded due to impaired spermatogenesis. The DNA content distributions were measured by flow cytometry. The mean (± SD) for the haploid population (1C) was 55.1% ± 11.5, 26.1% ± 8.5 for the diploid (2C) and 18.9% ± 6.5 for the tetraploid population (4C). This is well within the normal range as reported previously (Thorud *et al.*, 1980).

Assessment of apoptotic fraction by the TUNEL method

Table I shows the distribution of apoptotic cells within the various germ cell classes, of the 11 testes with spermatogenesis

Table I. The frequency of apoptotic germ cells in normal human testis (n = 11)

Cell types	Apoptotic cells per 50 tubular cross sections	
	Median (range)	Mean (SD)
Spermatogonia	1 (0–19)	4.6 (6.9)
Primary spermatocytes	13 (3–71)	26.2 (25.1)
Spermatids	10 (1–58)	17.0 (18.3)
Total apoptotic germ cells	31 (6–133)	47.8 (47.3)

judged to be normal. Figure 1 shows a testicular section stained with the TUNEL method where the nuclei of the apoptotic cells have a brown colour. Germ cell apoptosis was observed in all testes with a frequency varying considerably between individual samples. Apoptotic cells were observed mainly as isolated single cells, and there were more apoptotic primary spermatocytes than spermatids (Table I). Apoptotic cells within the two latter cell types were seen only in all testes, whereas apoptotic spermatogonia were seen in the majority of testes. Occasional Sertoli cells with positively stained nuclei were seen in some testes. In general, nuclear morphology was sufficient to differentiate between the various types of apoptotic germ cells, but in some instances the location of the apoptotic cell and neighbouring cells within the tubule was used for classification.

Expression analysis by immunohistochemistry

Bax, Bcl-2, Bcl-x, bak, mcl-1 and bad were all expressed in germ cells whereas expression of Bcl-w was not detected. Positive staining for Bcl-x was preferentially localized to the cytoplasm of spermatogonia (Figure 2). It should be noted that the Bcl-x antibody used in this study detects both the short (Bcl-x_S) pro-apoptotic form as well as the long (Bcl-x_L) anti-apoptotic form. Bcl-2 was expressed in most tubular cells with preferential expression in the cytoplasm of the differentiating spermatids close to the luminal surface (Figure 3). Bax was strongly positive in the nuclei of round spermatids and of a few primary spermatocytes, but also stained weakly in the cytoplasm of other tubular cells (Figure 4). Bak was preferentially expressed in the primary spermatocytes and spermatids close to the luminal surface, and the cell membrane seemed to be more strongly stained than the cytoplasm. Some positivity for Bak was also seen in spermatogonia (Figure 5). Bad was expressed in round and elongating spermatids and in spermatozoa and was localized to the acrosomal region (Figure 6a,b). Mcl-1 was weakly expressed in the cells in the basal parts of the seminiferous epithelium, whereas Bcl-w expression was not detected in germ cells of any testes, although positivity was seen in interstitial cells (data not shown). Expression of p53 and p21(Waf1) proteins were not detected by immunohistochemistry in germ cells in any of the testes with either formalin or Bouin's fixation. To examine if there was any co-localization of apoptotic cells and expression of Bcl-2 family proteins, sections were stained with the TUNEL method, and parallel sections were incubated with antibodies against each of the proteins. As indicated from the Table, these numbers are total numbers within 50 tubular cross sections. No correlations were found between apoptotic cells and protein expression, and neither did the frequency of apoptosis within the testes influence the pattern of protein expression.

Discussion

Mammalian spermatogenesis requires intricate regulation of cell proliferation and cell death, and apoptosis appears to be the underlying major mechanism of germ cell death during normal spermatogenesis in various mammals including rats, hamsters, mice and humans (Sinha Hikim and Swerdloff, 1999). Although male germ cell apoptosis has been well characterized in various animal models, few studies regarding germ cell apoptosis and the expression of different apoptotic markers in the human testis are presently available (Dunkel *et al*., 1997). The physiological significance of germ cell apoptosis is not yet clear, although it appears that apoptosis serves as a mechanism for maintaining an appropriate number of germ cells that can be adequately supported and matured by the Sertoli cells (Dunkel *et al*., 1997). Previously, testicular tissue from patients undergoing operations for malignant disease (Brinkworth *et al*., 1997; Erkkilä *et al*., 1997) and from autopsy material (Sinha Hikim *et al*., 1998) has been used to study spontaneous apoptosis in humans. The chances of studying perturbed spermatogenesis in testes from organ donors, where spermatogenesis is judged to be normal by histomorphology as well as by DNA content distributions as in the present study, are assumed to be very small. We therefore consider our material to be representative of normal human testes.

The in-situ end-labelling technique used in this study relies on the presence of the characteristic DNA strand breaks caused by the activation of endogenous nucleases during apoptosis, and has been routinely used to detect apoptotic cells in various tissues (De Angelis *et al*., 1998; Sinha Hikim *et al*., 1998). This method seems to be rather specific for apoptotic cell death in testicular tissue, as necrotic germ cells in cadmium-treated rats were clearly devoid of any specific labelling for DNA fragmentation (Sinha Hikim *et al*., 1997). Germ cell apoptosis was observed in all testes in the present study, and we found that the distribution of apoptosis in human testes included all three classes of germ cells, confirming the observation of Sinha Hikim *et al*. (1998). Consistent with results from the later report, the majority of apoptotic events were seen within spermatocytes and spermatids, with less apoptosis in spermatogonia. Contrary to that report, however, in our study a higher apoptotic frequency was found in spermatocytes compared to spermatids, which is also consistent with results from an in-vitro study of human testis (Pentikäinen *et al*., 1999). Assuming an average of 20–25 Sertoli cells per cross-section, the average of about 50 apoptotic cells per 50 tubular cross sections in our study is close to the results of Sinha Hikim *et al*. (1998), who found 5–10 apoptotic cells per 100 Sertoli cells. Fixation in Bouin's solution renders good morphology for allocating apoptotic cells with retained nuclear morphology to their respective germ cell classes. We therefore consider our apoptotic germ cell evaluation to be reliable, and

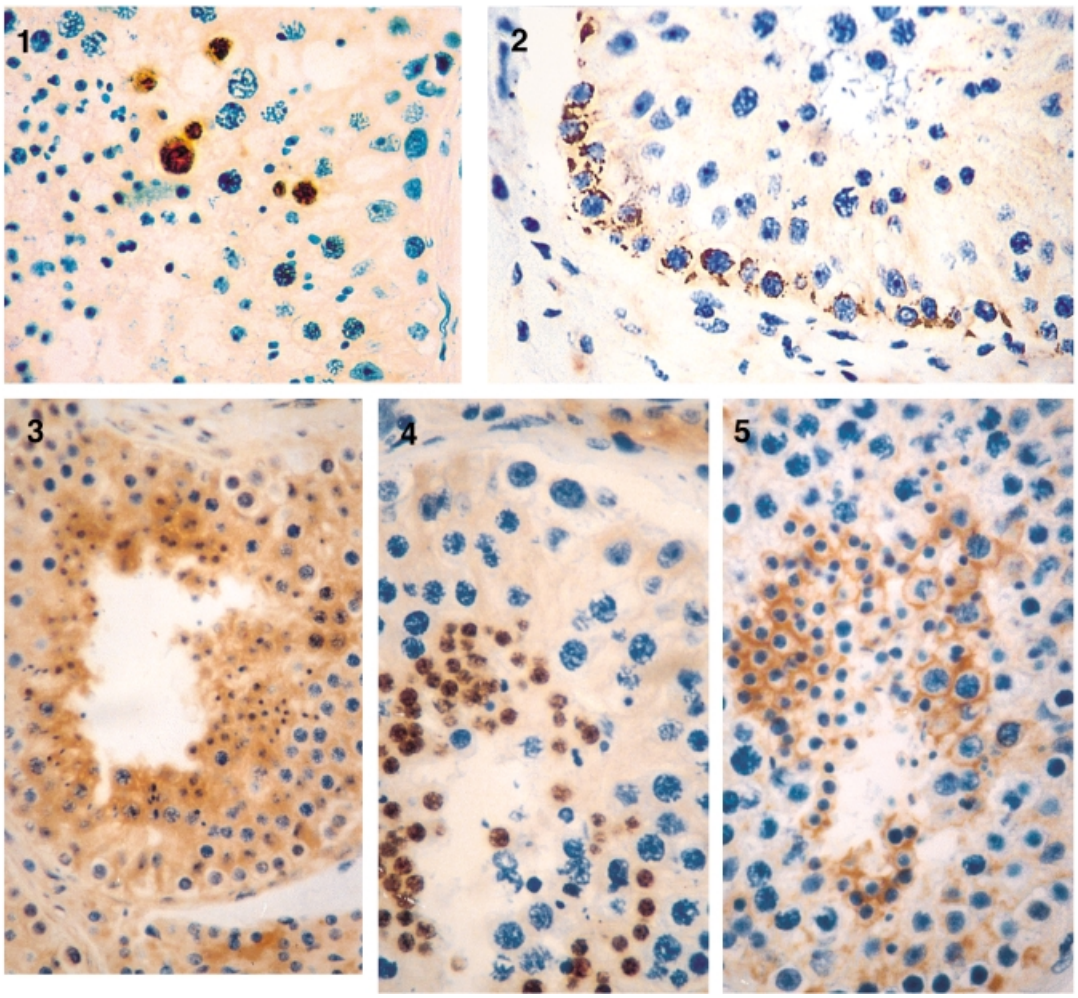

Figure 1. Spontaneous apoptosis in normal human testis visualized by terminal deoxynucleotidyl transferase nick end labelling. Apoptotic spermatids and spermatocytes can be seen. Original magnification ×400.

Figure 2. Immunohistochemical detection of Bcl-x proteins (long and short forms) showing weak staining in most tubular cells with preferential expression in the cytoplasm of spermatogonia in normal human testis. Original magnification ×400.

Figure 3. Immunohistochemical detection of Bcl-2 protein showing cytoplasmic expression in most tubular cells with preferential staining close to the luminal surface in normal human testis. Original magnification ×200.

Figure 4. Immunohistochemical detection of Bax protein showing preferential expression in nuclei of round spermatids in normal human testis. Original magnification ×400.

Figure 5. Immunohistochemical detection of Bak protein showing preferential expression on the cell surface of spermatocytes and spermatids close to luminal surface in normal human testis. Original magnification ×400.

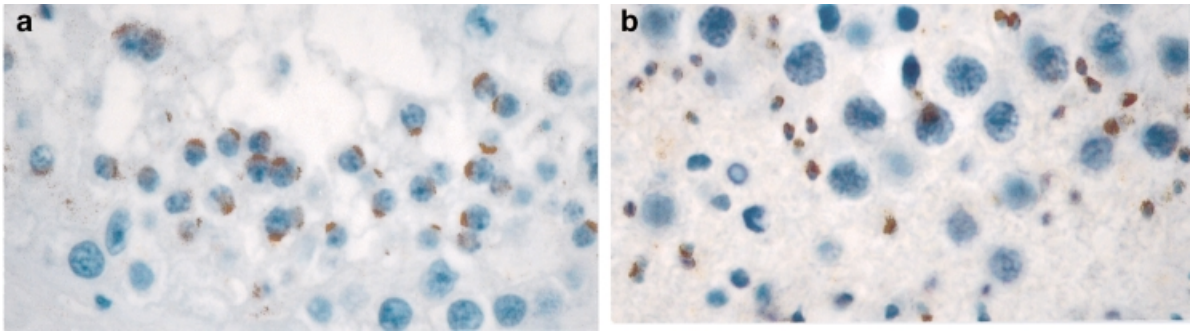

Figure 6. Immunohistochemical detection of Bad protein showing expression in the acrosomal regions of round spermatids (**a**) and elongating spermatids and spermatozoa (**b**) in normal human testis. Original magnification ×600.

it is consistent with the hypothesis that apoptosis is the major mechanism of germ cell death during normal human spermatogenesis. Available evidence from animal studies suggests that germ cell apoptosis is a hormonally regulated process (Sinha Hikim *et al*., 1995; Erkkilä *et al*., 1997), but the triggering factors for spontaneous germ cell apoptosis during normal spermatogenesis are not known. There was a rather large variation in total numbers of apoptotic cells between testes, despite the fact that spermatogenesis was judged to be normal in all testis evaluated. Such variations may be found among men with normal spermatogenesis and thus constitute perturbations that healthy males undergo during normal daily life. Spontaneous germ cell apoptosis may also be considered in the context of a reported reduction of sperm quality in the Western world during the last 50 years (Carlsen *et al*., 1992). A high frequency of spontaneous apoptosis may thus be the consequence of increased exposure to toxic agents with subsequent low sperm counts. The observation that some Sertoli cells were positive by the TUNEL assay may be explained by the fact that these cells phagocytose nuclear fragments of neighbouring apoptotic germ cells, consistent with another study (Pentikäinen *et al*., 1999). However, the possibility that Sertoli cells themselves may undergo apoptosis cannot be excluded.

The Bcl-2 family of proteins play an important role in the control of apoptosis acting at the effector stage (Kroemer, 1997). However, the significance or function of the proteins in this family has not yet been established in the testis (Dunkel *et al*., 1997). A significant and remarkable finding in the present study is the preferential Bax staining of nuclei in round spermatids and in a few primary spermatocytes. There is also a weak cytoplasmic Bax staining of tubular cells in general. Since Bax is an apoptotic promoter, this is consistent with the idea that round spermatids may be particularly prone to apoptosis when DNA is damaged. We found numerous apoptotic cells among round spermatids in normal human testis, and the preferential expression of Bax in these cells may thus be related to induction of apoptosis. This would, however, not explain the majority of apoptotic events among spermatocytes. Another explanation may therefore be that Bax is related to the differentiation of round spermatids into more differentiated stages. The observation that some primary spermatocyte nuclei are also Bax-positive is consistent with the observation that genetically manipulated mice lacking Bax expression showed disturbed spermatogenesis characterized by the lack of development of haploid cells (Knudson *et al*., 1995). The distribution of Bax expression shown in this study suggests that Bax is important for germ cell differentiation and maturation in humans.

Although expressed weakly in all types of tubular cells, Bcl-2 and Bak showed a very similar and preferential expression in cells close to the tubular lumen, suggesting that these proteins may be involved in the interaction between the luminal environment and spermatocytes and spermatids. These are the cells among which most spontaneous apoptotic events are found in the human testis. Since Bcl-2 is anti-apoptotic and Bak is pro-apoptotic, their co-localization may indicate an important function in regulation of human testicular apoptosis. Such a distribution, however, is also consistent with these proteins having a crucial role during differentiation and maturation of human germ cells. The weak, but preferential staining of Bcl-x in spermatogonia may be related to regulation of apoptosis in this germ cell compartment. Under staining conditions giving a strong positive reaction in testicular interstitial cells, Mcl-1 stained all tubular cells very weakly, whereas no detectable expression of Bcl-w was seen under the same conditions. These results, however, do not exclude a particular function of these proteins during testicular development or germ cell apoptosis. Another significant observation was the preferential expression of Bad in the acrosomal region of the spermatids. This protein may thus be involved in the maturation and differentiation of the sperm acrosome, the function of which is essential for fertilization. That Bad may also be involved in regulation of apoptosis in germ cells cannot be excluded, but is not supported by our results. No co-localization was found between apoptotic cells and Bcl-2 family protein expression, and the protein staining pattern was not related to the frequency of apoptotic cells per testes. While this does not support the involvement of the proteins in regulating spontaneous germ cell apoptosis, it does not exclude such a possibility.

In the present study of testicular samples from men with morphologically normal testis and with considerable apoptotic activity, p53 protein was not detected immunohistochemically. Neither could we detect the expression of p21(Waf1) protein which is the effector of p53 when arresting cells in the G_1 phase of the cell cycle to allow for DNA repair after damage. The lack of immunostaining of these proteins with the present method is generally consistent with normal protein concentrations. p53 concentrations usually increase and are detectable by immunohistochemistry following DNA damage. This indicates that spontaneous apoptosis in male germ cells in man may be p53 independent. Thus Fas ligand (Apo-1, CD95) and Fas, another system involved in regulation of apoptosis, may be candidates for inducing spontaneous apoptosis in human testis. This is consistent with Fas-Fas ligand being expressed in Sertoli cells (Bellgrau *et al*., 1995). It will be of interest to study the role of apoptosis and apoptosis regulatory proteins in male infertility, where these parameters might help to characterize the nature of perturbed spermatogenesis, and possibly be of prognostic value.

In conclusion, the results from the present study demonstrate that apoptosis is detected in the normal human testis involving all classes of germ cells. The preferential expression of Bax, Bcl-x, Bcl-2, Bad and Bak in the various germ cell compartments strongly suggests that these apoptotic proteins may be involved in differentiation and maturation through the various stages of human spermatogenesis. Lack of detection of p53 and p21(Waf1) proteins by immunohistochemistry may suggest that these proteins are not involved in the induction of spontaneous apoptosis in morphologically normal human testes.

Acknowledgements

We acknowledge the excellent cooperation of the Transplant Coordinators at the Surgical Department, National Hospital of Norway, and

Medinnova SF. We also thank Grethe Guldbrandsen for skilful technical assistance.

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Received on October 4, 2000; accepted on February 7, 2001