

Ricardo D. Moreno · Carlos Lizama · Natalia Urzúa ·
Solange P. Vergara · Juan G. Reyes

Caspase activation throughout the first wave of spermatogenesis in the rat

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Abstract Early in postnatal life, the first wave of spermatogenesis is accompanied by an initial wave of germ cell apoptosis. This may reflect an adjustment in the number of germ cells that can be adequately maintained by Sertoli cells. Two major pathways (intrinsic and extrinsic) are involved in the process of caspase activation and apoptosis in mammalian cells. The extrinsic pathway is characterized by the oligomerization of death receptors such as FAS or tumor necrosis factor, followed by the activation of caspase-8 and caspase-3. The intrinsic pathway involves the activation of procaspase-9, which in turn activates caspase-3. Extensive information is available concerning apoptotic inducers and their possible mechanisms in the adult rat. However, no data exist regarding the molecular and cellular mechanisms governing physiological cell death during puberty in the male rat. We have studied caspase activation throughout the first wave of spermatogenesis in the rat under physiological conditions, by combining the TUNEL procedure with the localization of active caspases in germ cells. We observed TUNEL-positive germ cells in rats of 5–40 days of age, the highest number being found in 25-day-old rats. TUNEL-positive and caspase-3-positive germ cells appeared as long chains of interconnected germ cells in 25-day-old rats. Caspase activation was assayed by either immunohistochemistry with antibodies against active caspase-3, -8, and -9, or by determining enzymatic activity in seminiferous

tubules extracts. Both techniques showed activation of caspase-3, -8, and -9 in 25-day-old rats and low enzymatic activity at other ages. Confocal scanning laser microscopy indicated that active caspase-3, -8, and -9 co-localized with TUNEL-positive cells. Thus, caspase-3, -8, and -9 are active in apoptotic germ cells during the first wave of rat spermatogenesis. The extrinsic pathway of apoptosis may therefore play an important role in germ cell apoptosis during puberty in the rat.

Keywords Spermatogonia · TUNEL · Apoptosis · Testis · Caspase · Rat (Sprague Dawley)

Introduction

During the first wave of spermatogenesis in the rat, a massive wave of apoptosis occurs to maintain an optimal Sertoli to germ cell ratio (Boulogne et al. 1999; Jahnukainen et al. 2004; Rodriguez et al. 1997). In adult rat testes, apoptosis occurs primarily at stages XII–XIV and I of spermatogenesis during which differentiating type A spermatogonia, zygotene, both early and late pachytene spermatocytes and meiotically dividing cells are eliminated (Blanco-Rodriguez 1998; Blanco-Rodriguez and Martinez-Garcia 1997; Boulogne et al. 1999; Huckins and Oakberg 1978; Jahnukainen et al. 2004; Rodriguez et al. 1997).

At the cellular level, apoptosis is characterized by cytoplasmic and nuclear condensation, membrane blebbing, and cell shrinkage (Leist and Jaattela 2001; Wyllie et al. 1980). At the molecular level, apoptosis involves the activation of the pro-apoptotic members of the Bcl-2 family of proteins (bad, bax, bak, bok, bid, Bmf, or Puma) and activation of a specific family of cysteine proteases named caspases (Antonsson and Martinou 2000; Cory and Adams 2002). Two major pathways (intrinsic and extrinsic) are involved in the process of caspase activation and apoptosis in mammalian cells. The intrinsic pathway for apoptosis involves the release of cytochrome c from the mitochondria into the cytosol where it binds to apoptotic protease-activating factor 1 (Apaf-1). This results in the activation of

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R. D. Moreno (✉) · C. Lizama · N. Urzúa · S. P. Vergara
Departamento de Ciencias Fisiológicas, Facultad de Ciencias
Biológicas, Pontificia Universidad Católica de Chile and
Millennium Nucleus for Developmental Biology,
Alameda, 340 Santiago, Chile
e-mail: rmoreno@bio.puc.cl
Tel.: +56-2686-2885
Fax: +56-2222-5515

J. G. Reyes
Instituto de Química,
Pontificia Universidad Católica de Valparaíso,
Av. Brasil 2695, Valparaíso, Chile

the initiator caspase-9 and the subsequent proteolytic activity of executioner caspase-3, -6, and -7 (Ceconi 1999; Hengartner 2000; Newmeyer and Ferguson-Miller 2003). The extrinsic pathway for apoptosis involves the binding of a death receptor, such as Fas, to its ligand, Fas L (Algeciras-Schimmich et al. 2002; Krammer 2000). The binding of Fas L to Fas induces the trimerization of Fas receptors, which recruit the adaptor protein FADD (Fas-associated death domain), through homotypic interactions with shared death domains. FADD also has a death effector domain or DED, in its N-terminal region (Algeciras-Schimmich et al. 2002; Krammer 2000). The Fas/FADD complex recruits the initiator caspase-8 or -10 via interactions between the DED of the FADD with the DED contained in the caspase molecules. Caspase-8 or -10 then activates the effector or executioner caspase -3, -6, and -7 resulting in cellular disassembly (Algeciras-Schimmich et al. 2002; Hengartner 2000; Krammer 2000). Both pathways converge on effector caspase-3, -7, and -6, which drive the terminal events of programmed cell death.

In many cell types (such as type I), caspase-8 directly activates the effector caspases, whereas in other cell types (such as type II), Fas triggering induces the mitochondrial apoptotic machinery (i.e., the intrinsic pathway), which in turn activates the effector caspases (Scaffidi et al. 1999). This crosstalk between the intrinsic and extrinsic pathways depends on caspase-8-mediated cleavage of the proapoptotic Bcl-2 member Bid and the subsequent release of cytochrome c from the mitochondria, a process that results in caspase-9 activation via apoptosome formation (Newmeyer and Ferguson-Miller 2003; Said et al. 2004; Scaffidi et al. 1999; Waterhouse et al. 2005).

Exposure of adult rats to local testicular heating, testosterone treatment, or estrogen induces apoptosis via the intrinsic pathway (i.e., activation of procaspase-9 followed by procaspase-3 activation; Kim et al. 2001; Vera et al. 2004, 2005). Similarly, exposure of Sertoli cells to toxicants or estrogen-like chemicals induces an increase in Fas signaling and activation of procaspase-8 (Boekelheide et al. 1998; Choi et al. 2004). Moreover, diethylstilbestrol, an estrogen-like chemical, induces Fas overexpression and activation of procaspase-8, -9, and -3 (Mishra and Shaha 2005; Nair and Shaha 2003). Thus, spermatogenic cells seem to have the ability selectively to activate either the intrinsic or the extrinsic pathways of apoptosis depending on external stimuli.

Our present aim has been to study the presence and activation of caspases throughout the first wave of spermatogenesis in the rat and to determine which pathway (extrinsic or intrinsic) is activated by germ cells during apoptosis. Our results show a coordinated activation of caspase -3, -8, and -9 during the first wave of spermatogenesis, with maximum activity in 25-day-old rats; this coincides with the highest rate of TUNEL-positive cells. Additionally, we demonstrate that the final outcome after completion of the apoptotic process is the development of interconnected germ cells.

Materials and methods

Animals

Male rats (Sprague Dawley) between 5 to 60 days of age were acquired from the Animal Facility of our Faculty. The rats were housed under a 12L:12D cycle, with water and rat chow being provided *ad libitum*. They were killed by cervical dislocation after exposure to CO₂ for 30 s. Investigations were conducted in accordance with the rules laid down by the Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and by the National Research Council.

TUNEL analysis

Apoptotic fragmentation of DNA in histological sections of rat testes was evaluated by TUNEL analysis (Dead End System; Promega, Madison, Wis.). Standard protocols for paraffin sections were followed (Grataroli et al. 2002). Samples were observed under phase contrast and fluorescence microscopy (Optiphot-2, Nikon, Japan) by using filters for wavelengths at 460–500 nm (excitation) and 510–560 nm (barrier). Micrographs were taken with a digital camera (CoolPix 4500, Nikon, Japan). TUNEL-positive germ cells were quantified in each tissue section by counting the number of TUNEL-positive cells in each round seminiferous tubule. The apoptotic index was calculated as the average number of TUNEL-positive cells per seminiferous tubule. Three testicular histological sections were taken per rat, with a minimum of 100 randomly selected tubules in each tissue section. The data are presented as the mean (\pm SD) from three rats for each specified age.

Caspase activity measurements

Isolated seminiferous tubules were washed by sedimentation with ERK medium (3 \times) and homogenized in a buffer containing 1 M NaCl, 1 mM EDTA, 10 μ g/ml phenylmethane sulfonyl-fluoride, 1% Triton X-100, 20 mM TRIS-HCl pH 7.4. Colorimetric substrates for caspase-3 (Ac-DEVD-pNA), caspase-8 (Ac-IETD-pNA), and caspase-9 (Ac-LEHD-pNA) were purchased from Calbiochem (Darmstadt, Germany) and were labeled with chromophore *p*-nitroaniline (pNA). pNA is released upon caspase cleavage. Free pNA produces a yellow color that is monitored by a spectrophotometer at 405 nm. The amount of yellow color produced upon cleavage is proportional to the amount of caspase activity present in the sample. To minimize cross reactivity, inhibitors of caspase-3 (DEVD-fmk), caspase-8 (IETD-fmk), or caspase-9 (LEHD-fmk) were included at a concentration of 1.5 M when the activity of caspase-8 and caspase-3, respectively, were measured. To normalize caspase activity the results were expressed as units of enzyme activity per gram of protein (U/g tissue).

One international unit of caspase was defined as the amount of caspase hydrolyzing 1 μmol substrate/min at 25°C (Ding et al. 2000; Faraco et al. 1999). Protein concentration was measured by using the BCA method (Pierce, Rockford, Ill.).

Immunofluorescence

Rat testes were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5–7 μm thick) were cut and re-hydrated. Nonspecific binding sites were blocked by incubating the sections in phosphate-buffered saline (PBS) plus 2% bovine serum albumin (PBS-BSA) for 1 h. Tissue

sections were then incubated (overnight at 4°C in a humidified chamber) in a rabbit polyclonal antibody against cleaved (active) caspase -3, -8, or -9 at a concentration of 0.01 mg/ml (Cell Signaling, Beverly, Mass.; Sanchez-Gomez et al. 2003), washed in PBS, incubated with Alexa 488 conjugated to goat anti-rabbit IgG (Molecular Probes, Eugene, Ore.) for 1 h at room temperature, washed, mounted in a fluorescence protector medium (VectaShield, Burlingame, Calif.), and observed under phase-contrast and fluorescence microscopy (Optiphot-2, Nikon, Japan) with filters for wavelengths at 460–500 nm (excitation) and 510–560 nm (barrier). Micrographs were taken with a digital camera (CoolPix 4500, Nikon, Japan). To establish the co-localization of

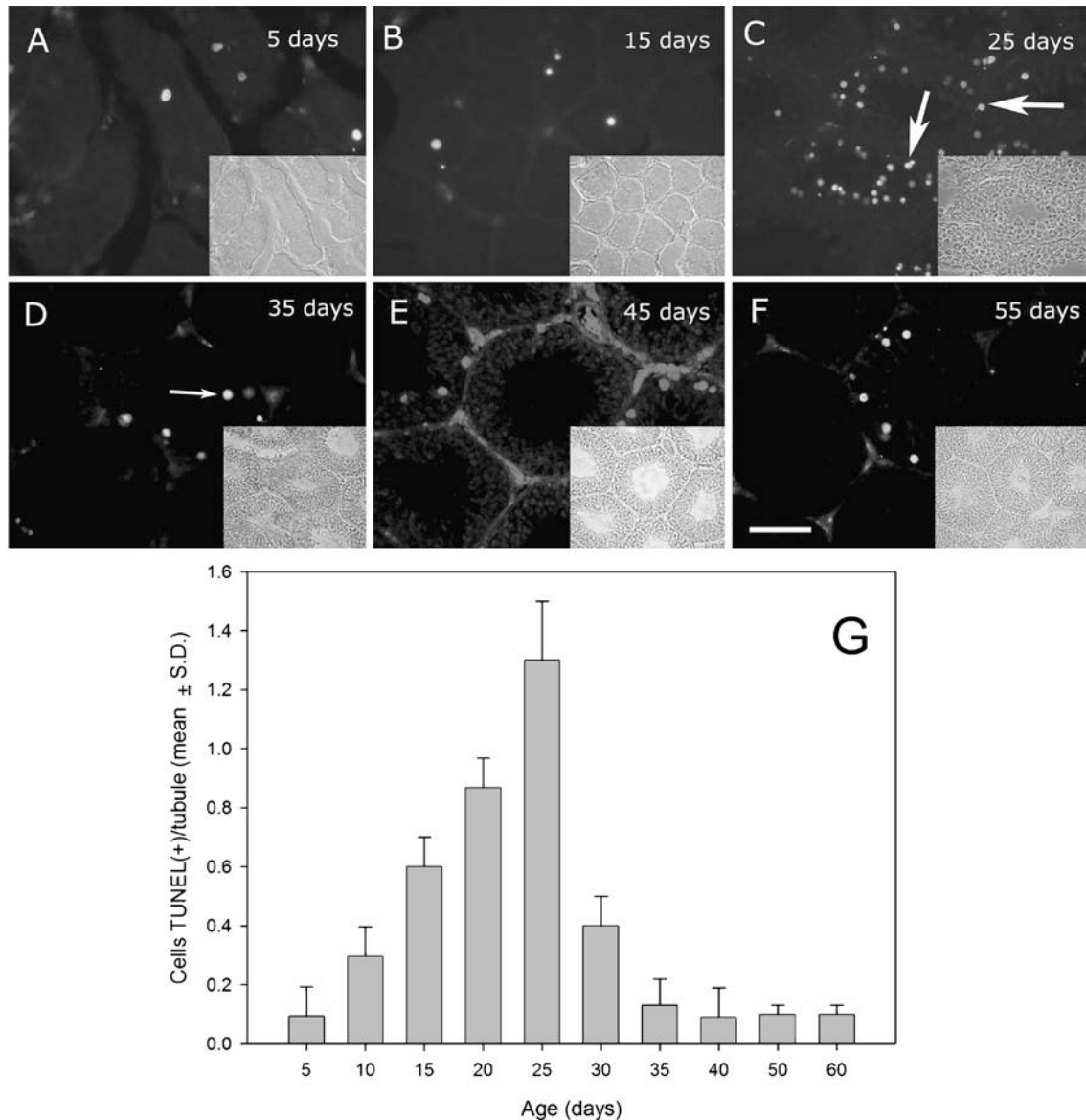


Fig. 1 TUNEL assay during the first wave of spermatogenesis. **a–f** Images of TUNEL-stained (Dead End) sections of rat testis (ages: 5–55 days). Note that the number of TUNEL-positive cells (*bright spots*) increases dramatically from 20 to 25 days (peak value). Most of the apoptotic cells are found near the lumen of each

tubule (*arrows*). *Inserts*: Respective phase-contrast images. *Bar* 100 μm . **g** Apoptotic index. TUNEL-positive germ cells were counted in at least 100 randomly chosen seminiferous tubules at specified ages. Values represent the mean (\pm SD) of three rats in triplicate

caspsases and TUNEL-positive cells, samples were observed by laser scanning confocal microscopy (Pascal, Zeiss, Germany).

Statistics

Statistical analyses were performed with SPSS 11.5 software, by using analysis of variance and the Bonferroni test (Sokal 1995).

Results

TUNEL assays showed few apoptotic cells in testicular sections of rats aged 5, 10, and 15 days (Fig. 1a,b). We observed a dramatic increase in the number of apoptotic cells in 20-day-old and then again in 25-day-old rats (Fig. 1d). The number of TUNEL-positive cells sharply decreased from day 30 and then remained low throughout adulthood (Fig. 1e–g). These results suggest a high rate of apoptosis during the first wave of spermatogenesis in the rat.

TUNEL assays of histological sections of testis showed several positive cells grouped in clusters of two or more cells (Fig. 1). Closer inspection of the apoptotic germ cells in the testis of 25-day-old rats showed groups of two, four, eight, 16, and more TUNEL-positive cells that possibly represented clones of interconnected germ cells (Fig. 2a,b). Most of these clones were located toward the lumen of the seminiferous tubules (Fig. 2a,a'). Indirect immunofluorescence against active caspase-3 showed isolated germ cells and clusters of four or more interconnected germ cells in

testis cross sections of 25-day-old rats (Fig. 2c,d). To characterize the cell types undergoing apoptosis in 25-day-old rats, either TUNEL or caspase-3-labeled testis sections were counterstained with periodic acid-Schiff (PAS) and hematoxylin. Our results showed that TUNEL-positive (Fig. 3a) and active caspase-3 (Fig. 3c) signals were well preserved after PAS-hematoxylin staining. Counterstaining clearly indicated that cells positive for both TUNEL and activated caspase-3 were spermatocytes according to their morphology and their position in the seminiferous tubules (Fig. 3b,d). Of interest, all observed germ cells positive for TUNEL and caspase-3 in this study were found predominantly in the second or third row of the epithelium (Figs. 2c,d, 3).

These results suggested that, in the rat, the highest rate of apoptosis during the first wave of spermatogenesis occurred at 25 days of age and that most apoptotic germ cells were clones of pachytene spermatocytes probably interconnected by cytoplasmic bridges.

Caspase activation in germ cells during first wave of spermatogenesis

To evaluate the pathways involved in the apoptosis that occurred during the first wave of spermatogenesis, testis sections were incubated with antibodies directed against active caspase-3, -8, and -9. Immunofluorescent staining of different tissue sections for active caspase-3, -8, or -9, followed by TUNEL assays, showed a clear co-localization of both labels (Fig. 4). Although TUNEL-positive cells were also positive for the three active caspases, some caspase-positive cells were not TUNEL-positive, possibly

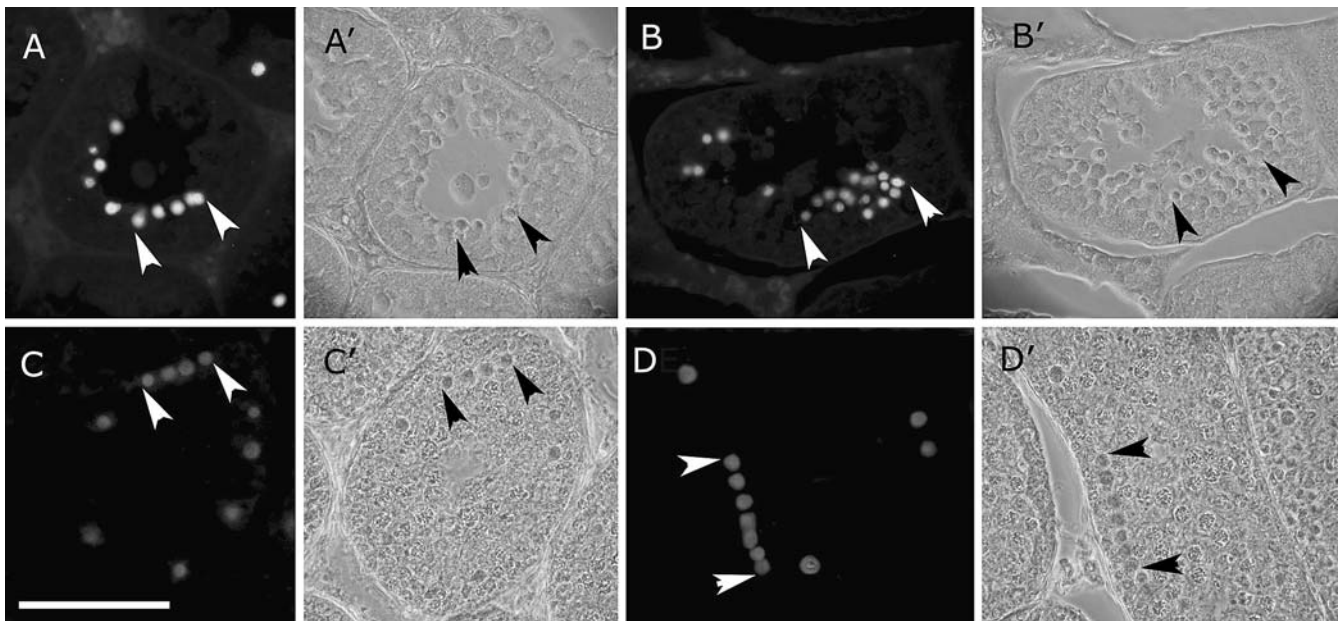
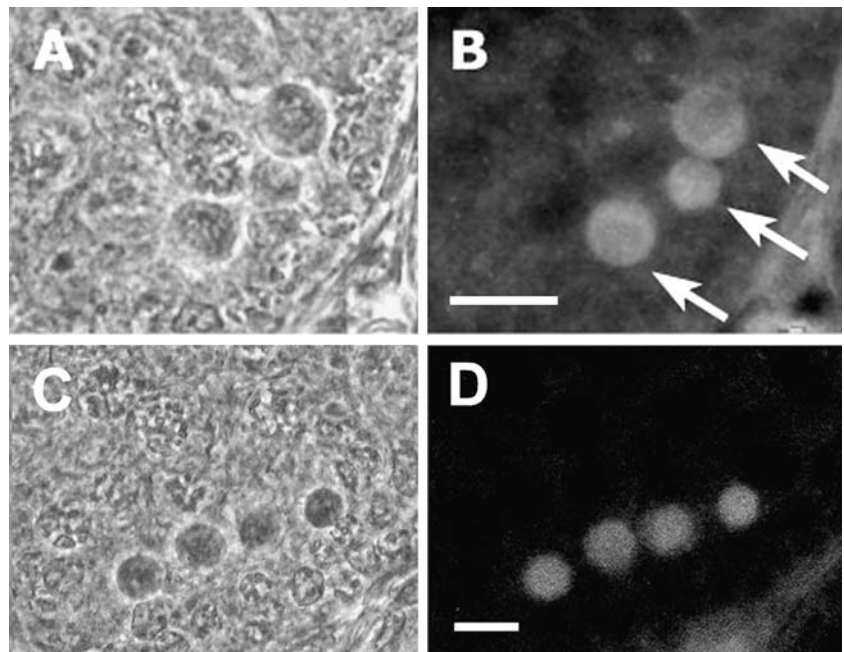


Fig. 2 Groups of apoptotic germ cells in 25-day-old rats (*arrow-heads* physical limits of each cell cluster). **a, b** Groups of 5–16 TUNEL-positive germ cells are found in clusters near the lumen. Some cells appear to be in a different focal plane. **a', b'** Phase-

contrast images. **c, d** Groups of four or eight germ cells are labeled with anti-active caspase-3 antibody. **c', d'** Phase-contrast images. Note that almost all the labeled germ cells are located in the second and third rows from the basement membrane. *Bar* 100 μ m

Fig. 3 Apoptosis in pachytene spermatocytes of 25-day-old rats testis. PAS-hematoxylin staining (a) and indirect immunofluorescence against active caspase-3 (b) in the same cross section of a 25-day-old rat testis. PAS-hematoxylin staining (c) and TUNEL image (d) of the same cross section of a 25-day-old rat testis. Caspase-3 immunoreactions and TUNEL-positive spermatocytes appear to be joined by intercellular bridges (arrows cf. PAS-hematoxylin-stained image). The identification of pachytene spermatocytes was based on their localization in the seminiferous epithelium and nuclear chromatin pattern. Bar 20 μm



because the cells were in the process of cell death prior to DNA fragmentation (Fig. 4a,e, arrows). Quantification of

the number of caspase-positive cells throughout the first wave of spermatogenesis indicated that the highest number

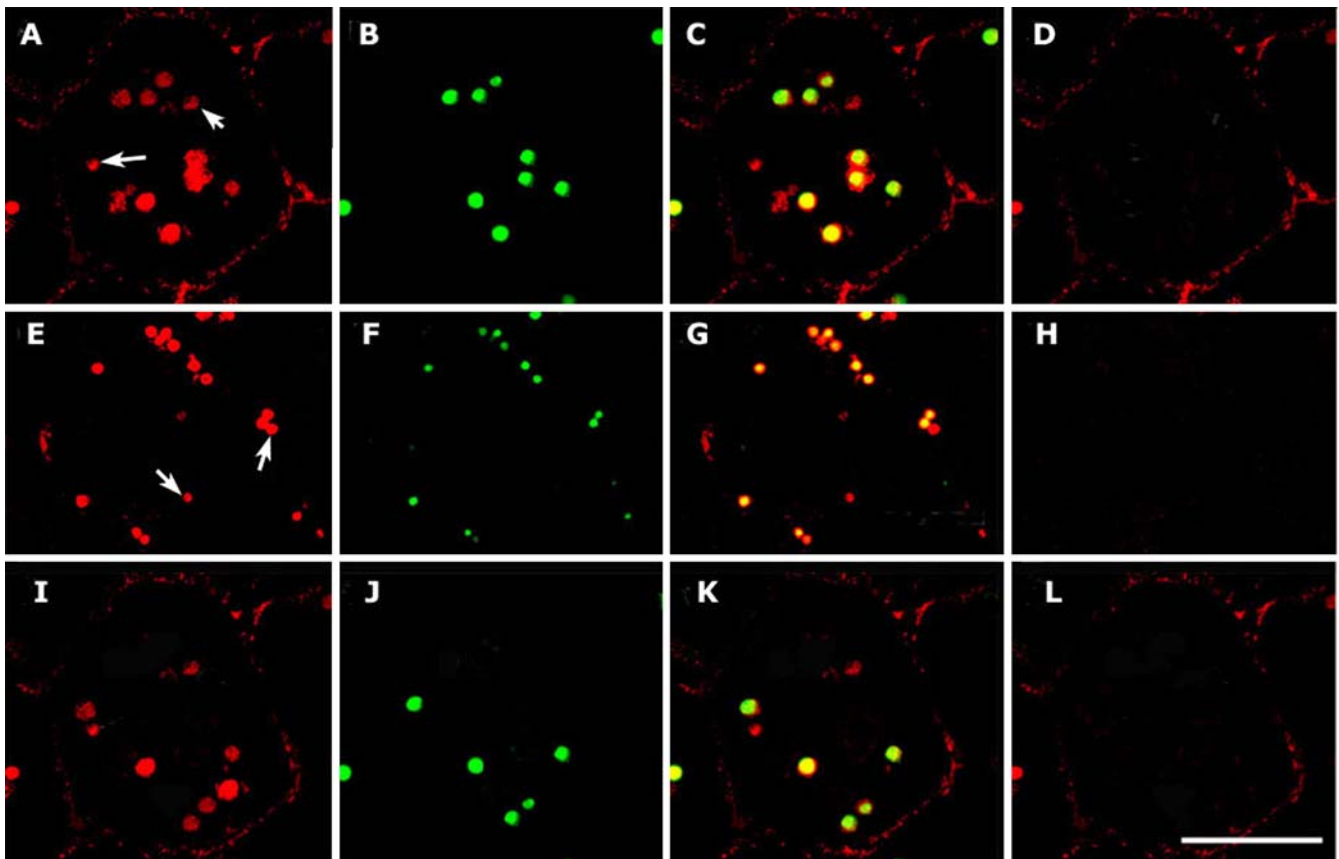


Fig. 4 Co-localization of TUNEL and active caspases in testes of 25-day-old rats. Cross sections stained with an antibody against active caspase-3 (a), caspase-9 (e), or caspase-8 (i). The same section stained with TUNEL (b, f, j) is also presented. Merging of

both images shows co-localization of active caspases and TUNEL-positive cells (c, g, k). Control sections for immunofluorescence were stained without primary antibody and for TUNEL without terminal deoxynucleotidyl transferase (d, h, l). Bar 100 μm

of positive cells for active caspase-3, -8, and -9 were found in the testis of 25-day-old rats (Fig. 5a–d). Interestingly, we did not find any caspase-9-labeled cells in testis sections of rats between 5 and 15 days old or older than 30 days (Fig. 5b). As controls, no signal was detected when the primary antibody was replaced with pre-immune serum or when the enzyme terminal polymerase was omitted in the TUNEL reaction (Fig. 4d,h,l).

To reinforce our immunohistochemical data, we determined the enzymatic activity of caspase-3, -8, and -9 in homogenates from isolated seminiferous tubules. Caspase-9 activity was found only in testis from rats aged 20 and 25 days old, confirming our previous results obtained with immunohistochemistry, which gave the highest activity in 25-day-old rats (Fig. 6a). Caspase-3 activity was low at 5–15 days of age but increased reaching maximum activity at day 25. This activity then declined and remained

constant in adult rats (Fig. 6b). The activity for caspase-8 followed a similar pattern to that describe for caspase-3, with the highest activity being found at 25 days of age (Fig. 6c). Therefore, caspase-3, -8, and -9 were present and specifically activated in germ cells during the first wave of spermatogenesis.

Discussion

We show here that three major caspases are activated in the testis of 25-day-old rats, and that this activation coincides with the highest proportion of TUNEL-positive germ cells. On the other hand, only caspase-8 and -3, but not caspase-9, are active in rats 5–10 days old. Activation of procaspase-9 has been shown in several models of hormone deprivation or testicular intoxication (Choi et al.

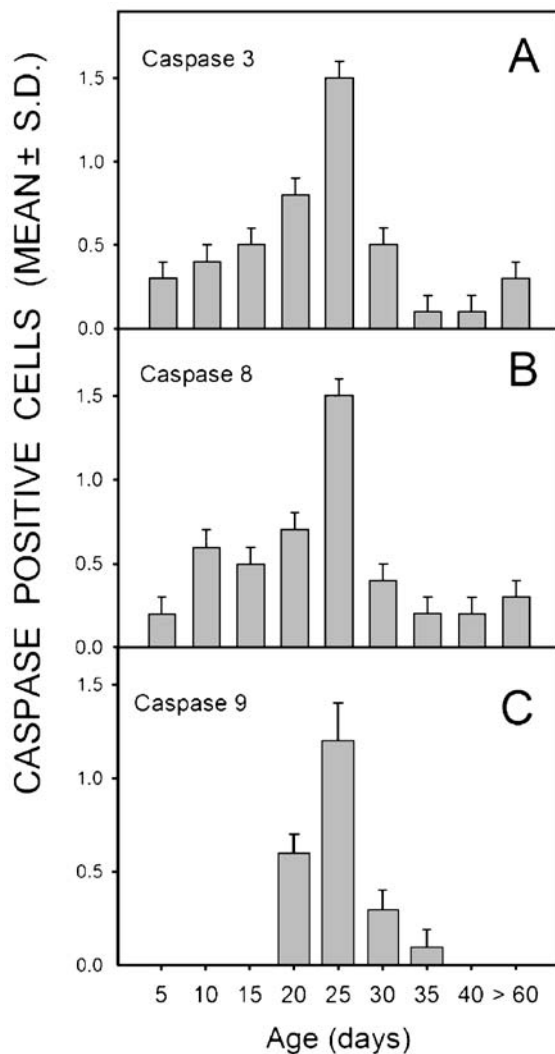


Fig. 5 Germ cells displaying active caspases during the first wave of spermatogenesis in the rat. Quantification of germ cells with active caspase-3 (a), caspase-8 (b), and caspase-9 (c) in rats aged 5 and 35 days. Positive germ cells were counted in at least 100 randomly chosen seminiferous tubules at the specified ages. Values represent the mean (\pm SS) of three rats in triplicate

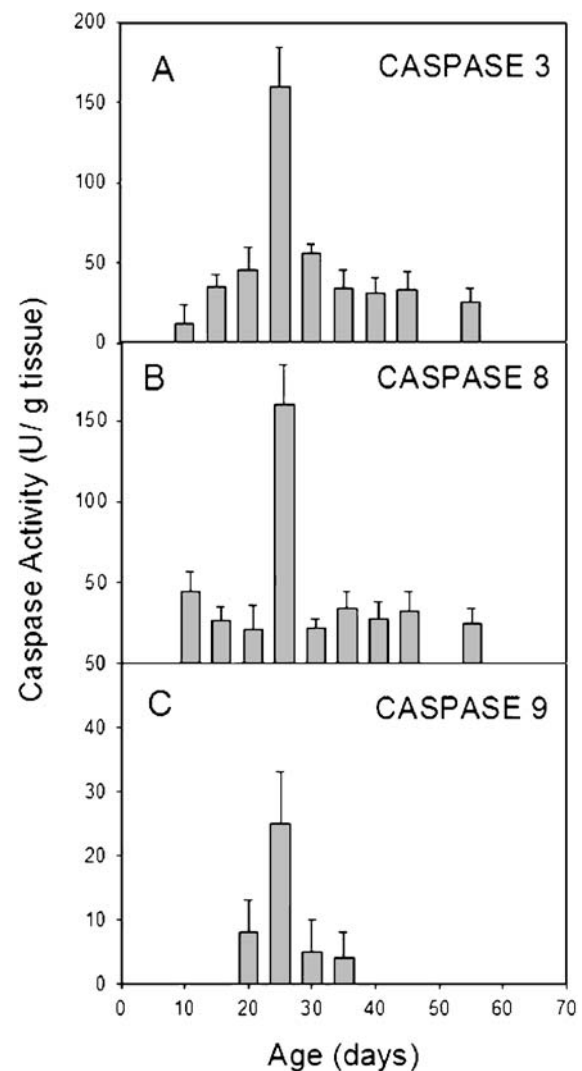


Fig. 6 Caspase activation during the first wave of spermatogenesis. Enzyme activity (in caspase units/g tissue) of caspase-3 (a), caspase-8 (b), and caspase-9 (c) in homogenates of isolated seminiferous tubules at the specified ages. Each determination was from three animals in triplicate ($n=3$)

2004; Mishra and Shaha 2005; Miura et al. 2002; Nair and Shaha 2003; Vera et al. 2005). Active caspase-9 is only present in germ cells of rats aged 20, 25, and 30 days. This sharply contrasts with the activation pattern of caspase-8 and -3 that have a sustained activity throughout all the studied ages. These results suggest that caspase-8 (and probably the extrinsic pathway of apoptosis) can be activated at various stages of germ cell development. On the other hand, caspase-9 is activated more stage-specifically, triggering the intrinsic apoptotic pathway in primary spermatocytes. In this context, the proapoptotic proteins BAX and BAD show a peak in expression levels in testis of rats aged 18–26 days (Jahnukainen et al. 2004; Yan et al. 2000), consistent with the idea that the intrinsic pathway is involved in triggering physiological apoptosis in primary pachytene spermatocytes during the first wave of spermatogenesis. Because caspase-8 is also transiently active around 25 days of age in the rat, we propose that pachytene spermatocytes are type II cells using both the intrinsic and extrinsic pathways. An intriguing result is that the enzymatic activity of caspase-9 is lower in comparison with that of caspase-3 and/or caspase-8. Pachytene spermatocytes may not have efficient machinery to activate the intrinsic pathway completely and may have to trigger the extrinsic apoptotic pathway to induce full caspase activation. Additionally, these results suggest that physiological apoptosis and toxicant-induced apoptosis follow different pathways (Castanares et al. 2005; Vera et al. 2004, 2005). Spermatogonia are also present in 25-day-old rats and might also be part of the apoptotic germ cell population. Therefore, we cannot exclude the possibility that spermatogonia are involved in the apoptosis observed at day 25 (Wang et al. 1998). Spermatogonia may account for a low proportion of the TUNEL-positive cells in 25-day-old rats and, thus, may have been underrepresented in our results. However, after a close examination of both TUNEL-positive and caspase-3-positive cells in 25-day-old rat testis, we have found that apoptotic cells were predominantly found in the center of the tubules, and never adjacent to the wall of seminiferous tubules, suggesting that, if any spermatogonia are present in the apoptotic cell population, they should have detached from the basal membrane of the seminiferous tubules. If this is the case for spermatogonia, the apoptotic mechanism elicited in spermatogonia might be related to a particular mode of cell death named anoikis (Frisch and Screaton 2001). This type of cell death has been observed in adherent cells and is triggered following detachment from the extracellular matrix. Anoikis involves Fas-induced apoptosis and subsequent caspase-8 activation (Aoudjit and Vuori 2001; Rytomaa et al. 1999). A similar mechanism might thus be involved in germ cell demise in 25-day-old rats.

Our TUNEL assays have revealed isolated germ cells or germ cells grouped in clusters of up to 16 cells in the testis of 25-day-old rats. According to our results, these cells are spermatocytes undergoing apoptosis as they are positive for both TUNEL and caspase-3. Previous investigations with preparations of whole seminiferous tubules have shown apoptotic type A spermatogonia in long chains of up

to 28 cells (Huckins and Oakberg 1978). In vitro studies indicate that apoptosis can initially affect a single member of a spermatogonial cell cohort, and that single non-viable spermatogonia cells remain joined to viable spermatogonial cells (Hamer et al. 2003; Tres et al. 2004). We demonstrate here that spermatocytes also enter into apoptosis as long chains of up to 16 interconnected cells. Intercellular bridges have long been assumed necessary for the maintenance of the synchronous development of germ cells (Ren and Russell 1991; Russell et al. 1987, 1990). Intercellular bridges allow the sharing of molecular signals between rat spermatids and may function in a similar way in spermatocytes and in spermatogonia (Ventela et al. 2003). Therefore, the synchronous development of germ cell progenies interconnected by intercellular bridges might account for the distribution of anti-apoptotic and proapoptotic signals in spermatogonia and pachytene spermatocytes during the first wave of spermatogenesis.

In conclusion, we have demonstrated the coordinated activation of caspase-3, -8, and -9 in rat pachytene spermatocytes during the first wave of spermatogenesis. In this context, some of the death receptors present in germ cells, such as Fas or DR5, might be actively involved in triggering caspase activation during this wave of spermatogenesis in the rat.

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