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·*Original Article*·

Apoptotic Sertoli cell death in hypogonadic (*hgn/hgn*) rat testes during early postnatal development

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Abstract

Aim: To determine the involvement of apoptotic cell death in postnatal pathogenesis in mutant strain of hypogonadic (*hgn/hgn*) rats testes. We evaluated the numbers and types of cells undergoing apoptotic cell death. **Methods:** Tissue sections were stained by the TUNEL method for *in situ* detection of apoptotic cells, with specific antibodies used as markers of testicular somatic and germ cells. **Results:** We found that apoptosis in the *hgn/hgn* testes during the early postnatal period occurred primarily in Sertoli cells, which should actively proliferate during this stage of differentiation. These findings strongly suggest that the normal allele of *hgn* is involved in the direct or indirect control of differentiation and proliferation of Sertoli cells. **Conclusion:** To our knowledge, this is the first report demonstrating early postnatal apoptosis of Sertoli cells, suggesting that the *hgn/hgn* rat is a unique model for the study of Sertoli cell deficiency. *(Asian J Androl 2006 Sep; 8: 535–541)*

Keywords: apoptosis; *hgn/hgn*; male hypogonadism; Sertili cell; testis; TUNEL; vimentin

1 Introduction

Sertoli cells are important for both the development and function of the testes. In embryonic gonads, precursor Sertoli cells express the sex-determining gene *Sry*, which induces formation of testes cords by activating expression of male specific genes. In adult testes, Sertoli cells support germ cells at various stages of their differentiation and carry out spermatogenesis in cooperation with other testicular cell types and under hormonal control. Therefore, defects in the proliferation and differentiation of Sertoli cells result in testicular dysfunction [1], and the establishment of a normal number of Sertoli cells is critical for a normal level of sperm production during adulthood [2]. Postnatal Sertoli cell proliferation, however, occurs only during a short postnatal period, which, in rats, occurs only 2 weeks after birth [3].

To study testicular development, we have used a mutant strain of hypogonadic (*hgn/hgn*) rats. Male *hgn/hgn* rats are sterile because of dysplastic testes development [4], female *hgn/hgn* rats show reduced fertility as a result of ovarian hypoplasia [5], and rats of both sexes show progressive renal failure from renal hypoplasia [6]. All of these defects are controlled by a single recessive allele (*hgn*) located on chromosome 10 [7]. Sertoli cells in *hgn/hgn* testes have low proliferative activity during

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the early postnatal period, and there are too few Sertoli cells to normally distribute along the basement membrane of the seminiferous tubules. Furthermore, a considerable number of apoptotic cells have been detected in postnatal *hgn/hgn* testes, and, based on histological criteria, these apoptotic cells are likely Sertoli cells [8].

Physiological apoptotic cell death is observed during both testicular development and spermatogenesis, and it has been reported that these apoptotic cells are germ cells but not somatic cells [9, 10]. Germ cell apoptosis, which occurs directly or is secondarily induced by the dysfunction of somatic cells, has been well documented in testicular pathology [11]. To our knowledge, however, in both physiological and pathological conditions, there have been no other reports describing Sertoli cell apoptosis during the early postnatal period. To characterize the testicular phenotype of *hgn/hgn* rats, it is important to evaluate apoptotic cell death during postnatal testicular development. Therefore, we assayed the distribution of Sertoli cells and the number of apoptotic cells in the postnatal testes of *hgn/hgn* and normal rats by immunostaining for the markers of testicular somatic and germ cells and by the TUNEL method for *in situ* detection of apoptotic cells. The markers used in the immunostaining were vimentin (VM) [12] for somatic cells, Mullerian inhibiting substance (MIS) [13] for Sertoli cells, and Vasa homologue (VSH) [14] for germ cells.

2 Materials and methods

2.1 Preparation of paraffin sections

The experimental procedure and care of animals were in accordance with the guidelines of the Animal Care and Use Committee of Nippon Veterinary and Life Science University [6, 8]. We used male rats from the *hgn* inbred strain maintained in our department [4, 5, 7, 8]. Sister-brother mating of the strain has continued for more than 50 generations. They were killed at postnatal days (PD) 0, 1, 3, 7 and 12 by overdose of ether, and their testes were removed. Male *hgn/hgn* rats were distinguished from phenotypically normal (*+/hgn* or +/+) littermates by weighing their testes using an electric balance [4, 7, 8]. Their testes were fixed in Bouin's solution for 2–5 h. The fixative was replaced with alcohol, and the testes were embedded in paraffin and sectioned into 3 μm thick slices [6, 8].

2.2 Double staining

TUNEL staining was performed as previously reported using an *in situ* apoptosis detection kit (TaKaRa Bio, Shiga, Japan) [8]. After deparaffinization, the tissue sections were processed in a microwave oven (3 × 1 min) in apoptosis-preparation solution (Nara Pathological Institute, Nara, Japan) and then immersed in 0.01 mol/L Tris-buffered saline (TBS; pH 7.5). Fluorescein isothiocyanate (FITC)-conjugated dCTPs were incorporated into nick DNA for 90 min at 37ºC in the presence of terminal deoxynucleotidyl transferase (TdT). The sections were rinsed in TBS and incubated with Horseradish peroxides (HRP)-conjugated anti-FITC antibody for 30 min at 37ºC. The slides were incubated with 3,3´-diaminobenzidine tetrahydrochloride (DAB) for 10 min, and the reactions were stopped by immersion in tap water. The sections were subsequently processed for anti-VM or anti-VSH immunostaining. After immersion in 0.1 mol/L glycine-HCl buffer (pH 2.0) for 15 min, the slides were washed in PBS (0.1 mol/L, 1× 5 min [anti-VM]; 0.01 mol/L, 1 × 5 min [anti-VSH]) and incubated for 10 min in PBS containing 10% normal rabbit or goat serum to block nonspecific antigen-antibody reactions. The sections were incubated with anti-VM antibody (clone V9, 1:40 dilution, LabVision Co., Fremont, CA, USA) [11] for 48 h at 4ºC or anti-mouse VSH antibody (rabbit polyclonal, 1:1000 dilution; the kind gift of Dr Toshiaki Noce, Mitsubishi Kagaku Institute of Life Sciences, Minami-Ooya, Machida, Tokyo, Japan) [14] for 24 h at 4ºC, and washed in PBS (3 × 5 min). The primary antibodies were detected with streptavidin and biotin complex methods (Histofine SAB-AP kit; Nichirei Co., Tokyo, Japan) [8]. A Fast Blue substrate kit (Nichirei Co., Tokyo, Japan) was used to detect the activity of alkaline phosphatase conjugated with streptavidin. After stopping the reaction by immersion in tap water, the stained sections were mounted in soluble mounting medium and examined under a light microscope (BX50, Olympus Co., Tokyo, Japan), with images obtained by a Penguin 600CL digital camera system (Pixcera Co., Oosaka, Japan) attached to the microscope [6, 8].

2.3 Apoptotic cell counts

This experiment utilized testes from three normal and three *hgn/hgn* male rats at each time point. More than ten areas, each of which was defined by a square field (SF, 76738.16 μm^2) in the finder of CCD camera of the digital camera system (Pixcera Co., Oosaka, Japan), were randamoly selected from the histological sections of each

testis, and the numbers of TUNEL-positive cells were counted. The values were averaged and compared between the normal and *hgn/hgn* male rats using Mann-Whitney *U*-test.

2.4 *Immunostaining of MIS*

Tissue sections were processed in a microwave (5 × 3 min) in 0.01 mol/L citric acid buffer (pH 6.0) to reactivate their antigenicity, soaked in water, immersed in 2 mol/L HCl for 20 min, and soaked in PBS. The sections were immersed in methanol containing 3% periodic acid to inactivate internal peroxidases and incubated in PBS containing 10% normal rabbit serum for 60 min to block nonspecific antigen-antibody reactions. Sections were incubated overnight at 4ºC with antibodies against MIS (goat polyclonal, 1:500 dilution; Santa Cruz Biotechnology, CA, USA). The sections were rinsed in PBS and incubated with Histofine SAB-PO (G) kit (Nichirei, Tokyo, Japan) for goat polyclonal primary antibodies. The slides were incubated with 3,3′-DAB and counterstained with hematoxylin.

3 Results

TUNEL and VM double staining revealed temporal and special distributions of VM-positive Sertoli cells and TUNEL-positive apoptotic cells in the seminiferous tubules during the early postnatal period. Figure 1 shows photographs of doubly stained testes on PD 1–12. Although VM was detected in several types of testicular somatic cells, in seminiferous tubules, VM was detected in the cytoplasm under the nucleus of each Sertoli cell. In normal testes, therefore, intense immunostaining associated with Sertoli cells formed an alignment along the basement membrane on PD 0–3 (Figure 1A, C, E), and, on PD 7–12, the VM filament of each Sertoli cell extended towards the tubular lumen (Figure 1G, I and Figure 2C). In *hgn/hgn* testes, there were fewer VM-positive Sertoli cells than in normal testes, and we often found the spot-like distribution of the VM associated with Sertoli cells in the tubular lumen (Figure 1B, D, F and Figure 2B). On PD 7–12, the Sertoli cells in *hgn/hgn* testes failed to form an axial extension of VM filaments (Figure 1H, J and Figure 2D). In *hgn/hgn* testes on PD 3–7, interstitial fibrous tissue, which was positive for VM, increased (Figure 1F, H).

There were few TUNEL-positive cells in the seminiferous tubules of normal testes on PD 0–7, whereas many TUNEL-positive cells were observed in the tubules on PD 12 (Figure 1A, C, E, G, I and Figure 3). In *hgn/hgn* testes, however, a considerable number of TUNEL-posi-

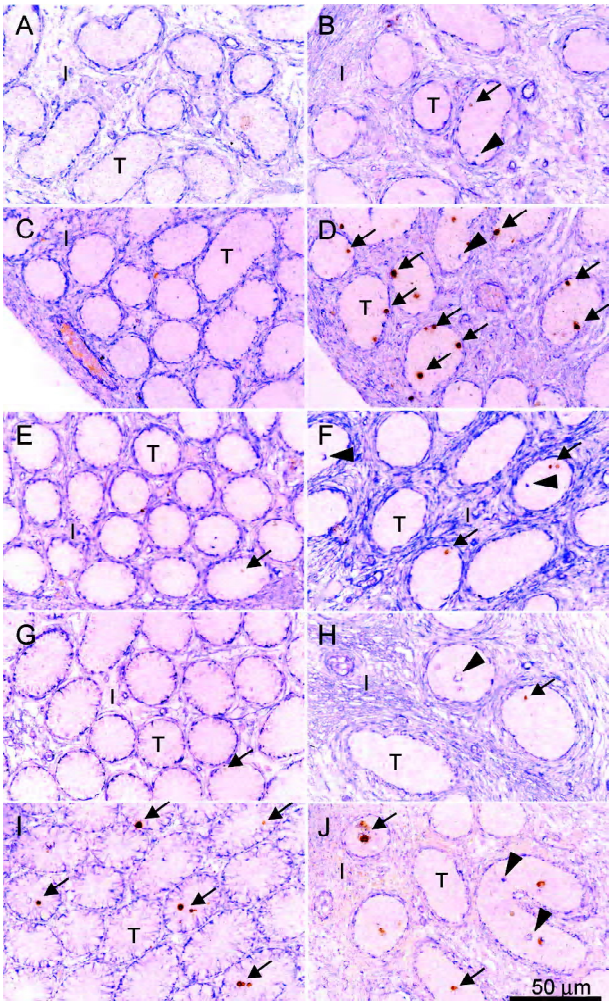

Figure 1. TUNEL and vimentin (VM) staining in normal (A, C, E, G, I) and *hgn/hgn* (B, D, F, H, J) testes on PD 0 (A, B), 1 (C, D), 3 (E, F), 7 (G, H), and 12 (I, J). The nuclei of apoptotic cells were stained brown (arrow), and the VM filaments in the cytoplasm were stained blue. In normal testes, TUNEL-positive apoptotic cells were rarely detected on PD 1–7 (A, C, E, G), whereas many of these cells were observed on PD 12 (I). In *hgn/hgn* testes, TUNEL-positive cells were detected on all days examined, and many TUNEL-positive cells, located near the basement membrane, were found on PD 1 (B). The palisade-like distributions of Sertoli cells showing the intense immunostainings of VM under their nuclei were distributed along the basement membrane of seminiferous tubules in normal testes (A, C, E, G, H). In *hgn/hgn* testes, some spot-like distributions of VM were found near the center portion of the tubular lumen (arrowhead). In contrast to normal testes on PD 7 and 12 (G, I), Sertoli cells failed to extend their VM filaments towards the tubular lumen in *hgn/hgn* testes (H, J). T, seminiferous tubules; I, interstitial tissue.

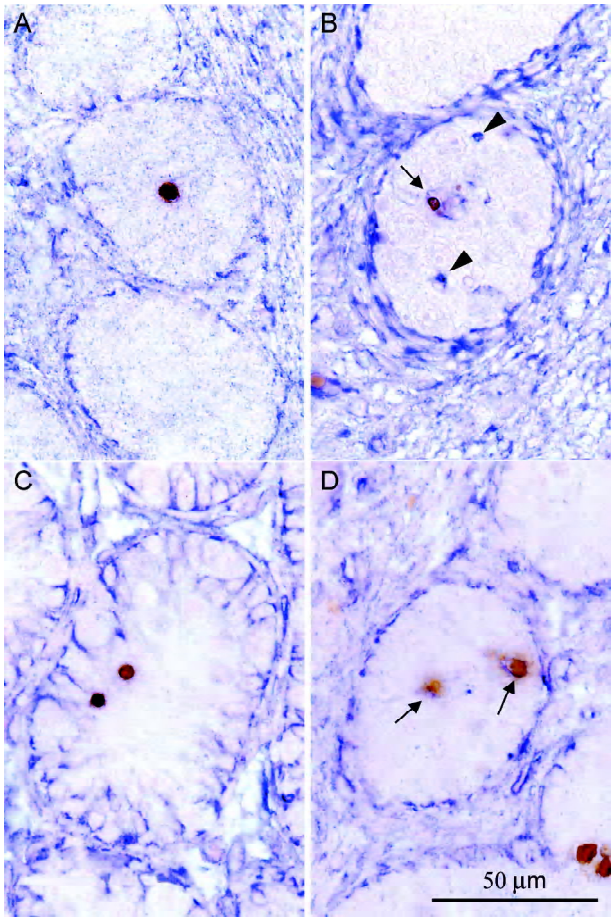

Figure 2. Photographs of TUNEL and vimentin (VM) staining in normal (A, C) and *hgn/hgn* (B, D) testes on PD 1 (A, B) and PD 12 (C, D). In normal testes at both time points, TUNEL-positive cells were located near the center of the tubules and were not stained with anti-VM antibody (A, C). In *hgn/hgn* testes on PD 1, some VM-positive Sertoli cells were detached from basement membrane (arrowhead), and the TUNEL-positive nucleus was associated with the abnormal distribution of VM filaments (B). In *hgn/hgn* testes on PD 12, TUNEL positive nuclei were often associated with a few dot-like immunostainings of VM (arrow) (D).

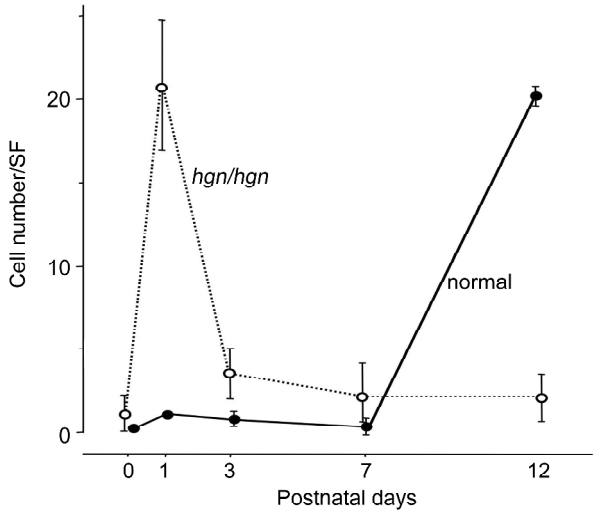

Figure 3. TUNEL-positive cells were counted in the square field (SF, 76738.16 μm^2) defined by the finder of CCD camera. Black and dotted lines represent the cell number in normal and *hgn/hgn* testes, respectively, on PD 1–12. Each point and vertical bar represents mean ± SME (n = 3).

tive cells were observed on all postnatal days examined, with the highest number of apoptotic cells detected on PD 1 (Figure 1B, D, F, H, J and Figure 3). In normal testes, TUNEL-positive cells were located in the central portion of the tubular lumen, and these cells did not stained with anti-VM antibody (Figure 1I and Figure 2A, C). In *hgn/hgn* testes on PD 1, most of apoptotic cells containing TUNEL-positive nuclei were observed near the basement membrane of the seminiferous tubules. TUNEL-positive cells were also found in the central portion of the tubules in *hgn/hgn* testes on PD 1, and immunostaining with antibody to VM was observed around the TUNEL-positive nuclei (Figure 2B). In *hgn/hgn* testes on PD 12, TUNEL-positive cells were located in the center of the tubules, and these cells were positive or negative for VM (Figure 2D).

Because VSH is located in the cytoplasm of germ cells [14], the immunostaining with antibody to VSH formed circles around the large nuclei of gonocytes. In TUNEL and VSH double staining on PD 1, the TUNEL-positive nucleus was surrounded by the immunostaining of VSH in normal testes (Figure 4A). In *hgn/hgn* testes, however, TUNEL-positive small nuclei were present outside the circles of VSH immunostaining in the cytoplasm of gonocytes (Figure 4B). MIS has been detected in the cytoplasm of Sertoli cells [13]. Because the number of Sertoli cells was apparently smaller in *hgn/hgn* testes than that in normal testes, the total area containing MIS in the seminiferous tubules was smaller in *hgn/hgn* testes, compared to that in normal testes (Figure 4C, D). In normal testis on PD 1, the nuclei of MIS-positive cells were uniform in shape and formed palisade-like distributions along the basement membrane of the tubules (Figure 4C). In *hgn/hgn* testis, the nuclei of MIS-positive Sertoli cells were irregular and small, and Sertoli cells often showed nuclear condensations (Figure 4D).

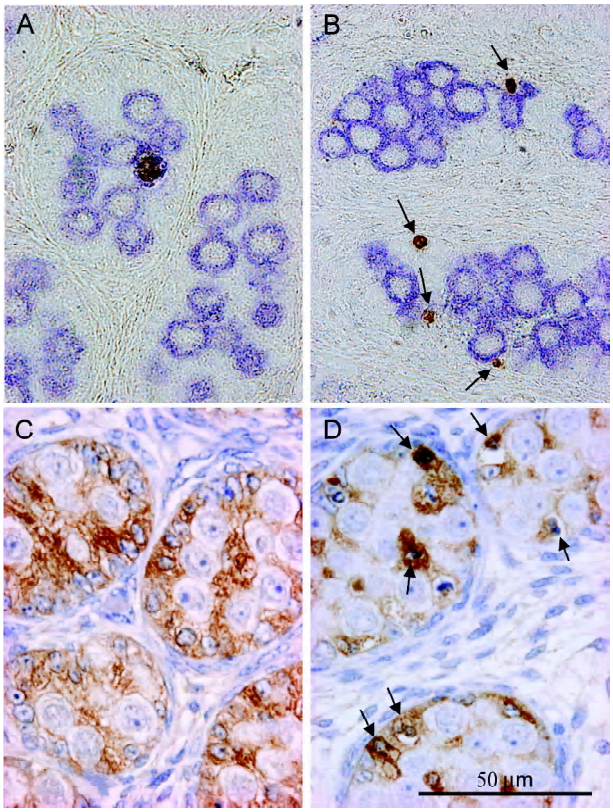

Figure 4. Photographs of TUNEL and Vasa homologue (VSH) double staining (A, B) and Mullerian inhibiting substance (MIS) immunostaining (C, D) in normal (A, C) and *hgn/hgn* (B, D) testes on PD 1. In double staining (A, B), the nuclei of apoptotic cells were stained brown, and the VSH in the cytoplasm of gonocytes were stained blue. In normal testes, the TUNEL-positive nucleus was surrounded by the immunostaining of VSH located in the cytoplasm of genocytes. In *hgn/hgn* testis, TUNEL-positive small nuclei were often observed, and they were present outside of the circles formed by the immunostaining of VSH (A, B). MIS was located in cytoplasm of Sertoli cells (C, D). In *hgn/hgn* testis, condensed nuclei were often observed, and their cytoplasm was positive for MIS (arrow, D).

4 Discussion

We previously reported that the postnatal pathogenesis of *hgn/hgn* testes included apoptotic cell death in the seminiferous tubules. Because we also observed a decreased number of cytokeratin-positive cells and a disappearance of follicle-stimulating hormone (FSH) mRNA expression during early postnatal development, we predicted that the apoptotic cells might be Sertoli cells [8]. Although Sertoli cell dysfunction is a major cause of male reproductive problems [1], there have been no other animal models with defective development of functional Sertoli cells during postnatal testicular development. In addition, to our knowledge, there have been no other reports describing apoptotic Sertili cell death during early postnatal development, the period during which Sertoli cells actively proliferate in normal testes.

The *hpg* mice have a decreased number of Leydig, Sertoli and germ cells [15]. By contrast to *hgn/hgn* rats, during the postnatal developmental stage, no apoptotic cell death has been reported in the Sertoli cells of *hpg* mice. The pathological difference between *hpg* mice and *hgn/hgn* rats might be a result of the difference between their primary causes. The *hpg* mice, which lack GnRH, show hypogonadotropic hypogonadism [15]. The primary cause of hypogonadism in *hgn/hgn* rats is present in the testes, because they have high levels of gonadotropins and low levels of testosterone in plasma [16].

We could not find any reports describing *in situ* detection of apoptotic Sertoli cells with double staining of TUNEL and immunostaining. In the present study, using TUNEL and VM double staining, we showed postnatal alteration in the number of TUNEL-positive apoptotic cells and identified VM-positive apoptotic cells in the seminiferous tubules of *hgn/hgn* testes. VM is an intermediate filament protein, which is expressed in somatic cells derived from mesenchymal cells. In the seminiferous tubules, VM is expressed by Sertoli cells, not by germ cells. In contrast to cytokeratin and MIS [12, 13], whose expressions are reduced with Sertoli cell differentiation, VM is expressed in Sertoli cells through their differentiation and maturation. To confirm the apoptotic cells as being Sertoli cells in *hgn/hgn* testes on PD 1, TUNEL and VSH double staining, as well as immunostaining of MIS, were also performed.

In the immunostaining of VM, we observed defective distributions of VM filaments in the Sertoli cells of *hgn/hgn* testes. Abnormal spot-like distributions of VM filaments in the tubular lumen of *hgn/hgn* testes indicate the detachment of apoptotic Sertoli cells from the tubular basement membrane [8]. During the postnatal development of normal testes, VM filaments extend towards the tubular lumen in coordination with the cytological differentiation of Sertoli cells [12]. Because abnormal distribution of VM filaments is related to defective differentiation of Sertoli cells, the absence of axially oriented VM filaments in early postnatal *hgn/hgn* testes strongly suggest that the normal allele of the *hgn* locus

directly or indirectly controls the differentiation of Sertoli cells.

In normal testes development during the early postnatal period, Sertoli cells proliferate rapidly, and spermatogonia differentiate from gonocytes and resume mitosis [1–3]. During this time, the cells undergoing physiological apoptosis are germ cells, increasing gradually up to 3 weeks after birth. This is regarded as the establishment of the proper ratio of germ cells to functional Sertoli cells [9, 10]. We found few TUNEL-positive apoptotic cells in normal testes on PD 0–7, whereas a considerable number of apoptotic cells, which were positive for TUNEL but negative for VM, were detected on PD 12. Therefore, it is likely that most of the TUNEL-positive cells in normal testes are germ cells.

In *hgn/hgn* testes on PD 1, most of the TUNEL-positive cells were located near the basement membrane. Some of the TUNEL-positive cells on PD 1 and most on PD 12 were located in the center of the tubules, and these cells were often stained with anti-VM antibody. However, the cellular distribution of VM is apparently abnormal in the TUNEL-positive apoptotic cells. It has been reported that the progression of apoptosis involves the disruption of cytoskeletal network [17]. This process might be facilitated by the cleavage of VM filaments [17]. Although, at present, the biochemical condition of VM filaments in apoptotic Sertoli cells is unclear, the presence of VM immunostaining associated with TUNEL-positive neculei indicates that *hgn/hgn* testes include apoptotic Sertoli cells during their postnatal development.

This observation is also supported by the result of TUNEL and VSH double staining. Genes homologous to Vasa, which encodes Asp-Glu-Ala-Asp (DEAD)-family protein of ATP-dependent RNA helicase in Drosophila, have been cloned in many animal species, and the expression of VSH is highly specific for gem cells [14]. The immunostaining with antibody to VSH formed circles around the large nuclei of gonocytes. In *hgn/hgn* testis on PD 1, TUNEL-positive nuclei were certainly present outside of the circular staining of VSH associated gonocytes.

MIS, a specific secretory product of Sertoli cells, causes regression of the mullerian ducts in embryonic development, and it has been reported that MIS is expressed in postnatal Sertoli cells [13]. Although we could not establish TUNEL and MIS double staining for technical reasons, immunostaining of MIS revealed that MIS-positive Sertoli cells had condensed small nuclei characteristic of apoptotic cells. These results indicate that premature Sertoli cells are the major cell type showing TUNEL-positive apoptosis in *hgn/hgn* testes during the early postnatal period. In addition, the absence of functional Sertoli cells from *hgn/hgn* testes indicates that the *hgn/hgn* rat is a unique model for studying Sertoli cell dysfunction.

Recently, we localized the *hgn* locus to an 840 kb region on rat chromosome 10 [18]. In this region, we detected the insertion of a 25 bp duplication into an exon of the *Spag5/astrin/MAP126* gene, which codes for a protein associated with the spindle microtubule (H. Suzuki, personal communication). Although *Spag5* knockout mice have normal fertility [19], the depletion of *Spag5* by RNA interference in HeLa cells causes mitotic arrest, with dispersed distribution of chromosomes and consequent apoptotic cell death [20]. Abnormal mitotic cells with dispersed chromosomes have been observed in *hgn/hgn* testes during the early postnatal period [8]. Immunological staining with antibodies to GATA-4, which is a marker for Sertoli cells, indicates that the cells showing abnormal mitosis in *hgn/hgn* testes are Sertoli cells (Dr H. Suzuki, personal observation). Therefore, apoptotic cell death of Sertoli cells would result from the disorganization of the spindle apparatus caused by defective *Spag5*. Confirmation of this hypothesis, however, requires further investigation.

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