[原著] Emergence of Spermatogenic Cells and Macrophages in Ductuli Efferentes Testis of Juvenile Rats

Emergence of Spermatogenic Cells and Macrophages in Ductuli Efferentes Testis of Juvenile Rats

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(Received on October 24, 2000, accepted on November 27, 2000)

ABSTRACT

In order to understand the postnatal developmental events of ductuli efferentes testis, the testis and ductus epididymidis of male Wistar rats in different developmental stages (1-9 weeks) were investigated by histological and immunohistological methods. A large number of cells appeared in the lumen of ductuli efferentes testis during the juvenile period (3-4 weeks). The majority of luminal cells had similar morphology to spermatids and spermatocytes. Some of the luminal cells showed morphological changes such as chromatin condensation and marginalization, and fragmented nucleus when they translocated to the ductuli. TUNEL staining for apoptosis was positive to some of the luminal cells. Small number of luminal cells in the ductuli were immunoreactive to anti-macrophages antibody suggests the appearance of macrophages in these cells. The timing of appearance of the luminal spermatogenic cells and macrophages in ductuli efferentes testis was in coincidence with the epithelial differentiation of DET. *Ryukyu Med. J., 20(2)53~59, 2001*

Key words: ductuli efferentes testis, spermatocyte, spermatid, macrophages, apoptosis

INTRODUCTION

The ductuli efferentes testis (DET), which originates from mesonephric tubules, are a series of tubules lined by ciliated epithelial cells[1]. A mature sperm travels through rete testis (RET) and DET to the ductus epididymidis (DE). Morphological and biochemical studies showed that DET are physiologically important, particularly in re-absorption of water, electrolytes and other substances. The maintenance of proper sperm concentration in the lumen of DE is also controlled by DET[2-8]. While recent studies have focused on the development, maturation and functions of DET, studies on the postnatal development to the puberty are very scanty. A androgen is an important hormone for males[9] in the initiation of the organogenesis of embryo, promotion of postnatal development and maintainance of sexual characteristics and functions. However great emphasis on the androgen has resulted in the neglect of other equally important synergic factors. An extensive exfoliation of developing germ cells in the testis of early puberty[10] is considered as an inefficient spermatogenesis due to androgen surge[10, 11]. Do the organisms really consume such a vast amount of energy just for an insignificant work? Or there is an unknown mechanism which modulates the biological phenomena for unknown reasons. We doubt the conclusion because in our present study, we found that a large number of germ cells and some macrophages appeared in the lumen of DET in juvenile rats and their emergence was coincidenced with the differential timing of DET and the reproductive tract as we investigated the postnatal development of DET in male Wistar rats by histological and immunohistological methods. The cell death of majority of these cells is not in seminiferous tubules but at the terminal of DET. These results do not lead to the conclusion that exfoliated cells are inefficient spermatogenesis. We discussed the biological significance of luminal cells to juvenile development of DET.

MATERIALS AND METHODS

Animals

Male Wistar rats (1 - 9 weeks) were used for the experiments. "Standards relating to the care and management of experimental animals" (Notification No.6, March 27,1980 from the Prime Minister's Office, Tokyo, Japan) was followed for the care and use of the animals. After diethyl ether anaesthesia, testis, DET and DE were dissected out. The adipose pad around the tissue was removed and the tissue was processed for histological, immunohistochemical studies and *in situ* cell death detection.

Histology

The testis, DET and DE were fixed in buffered 4 % paraformaldehyde, and dehydrated with graded ethanol. Specimens were then embedded in cold-polymerizing resin (Kuizer Histo −Technik, Technovit 7100, Germany) and sectioned serially in 4μm thickness with a glass knife using a

microtome (DuPont, JB-4A, USA). After hematoxylin and eosin staining, sections were observed under light microscope (Olympus, BX50, Japan). The number of luminal cells in DET was estimated by Abercrombie's method[12].

$$N = n \times 4 / (4+d)$$

N: corrected number of luminal cells;
n: the total number of those nuclei in DET counted in all sections;
d: average diameter in micrometers (μm) of five to ten of largest nuclei;
4: thickness of the section in micrometers (μm)

Immunohistochemistry

The testis and DET from 3-4 weeks rats were fixed in buffered 4% paraformaldehyde for 12 h, washed and treated with 0.5% glycine for 15min with 2 changes. Then the tissue was embedded in OCT compound (Sakura Fine chemicals, Tokyo) and rapidly immersed into liquid nitrogen to prepare frozen blocks. Sections of 5μm thickness were cut by cryostat microtome (Leica, CM1850, Germany) and mounted on APS-coated slides. Sections were rinsed in phosphate buffered saline (PBS) and treated with blocking reagent, protein block serum-free (DAKO A/S, Denmark), for 15 min to prevent nonspecific binding and then incubated with antibody against rat macrophage (IgG, mouse antibodies, KiM2R) obtained from Biomedicals AG, Switzerland at 4℃, overnight in a humid chamber. Sections that were not incubated with primary antibody were used as a negative control. After rinsing with PBS, sections were incubated with FITC-conjugated rabbit-anti-mouse immunoglobulin (DAKO A/S) at room temperature for 1h. Washed sections were sealed in aqueous gel/mount medium (Biomeda corp., USA) and observed by confocal laser scanning microscope (Olympus, FluoView, Japan).

In Situ Cell Death Detection

In situ cell death detection kit (fluorescein) was purchased from Boehringer Mannheim. The DET of rats were dissected and fixed in 10% formalin for 24 h and paraffin blocks were made. Sections in 4μm thickness were cut, mounted on APS-coated slides. Deparaffinised and rehydrated sections were treated with proteinase K (20μg/ml, 10mM Tris/HCl, pH7.4-8.0) in a humid chamber for 15 min. at 37℃. Sections were rinshed with PBS and then treated with TUNEL reaction solution in a humid chamber for 1 h at 37℃. Rinsed sections were sealed in aqueous gel/mount medium and then observed by a confocal laser scanning microscope. Sections incubated with corresponding buffer without TUNEL reaction solution served as negative control.

RESULTS

Histological characteristics of seminiferous tubules, rete testis, DET and epididymis

The DET and epididymis are identified in gross anatomy after the adipose tissue was removed. The testis was occupied by densely compacted seminiferous tubules (ST) (Fig. 1), which converges to rete testis (RET) (not shown in figure). Two or three slightly convoluted tubules arose from RET to form long proximal ductules of DET. These ductules became highly tortuous to form a bulbous coni vasculosi. Towards the epididymis, ductules anastomosed and formed a single, short, narrow and straight isthmic tubule (terminal tubule), which changed abruptly into the initial segment of DE (Fig. 1).

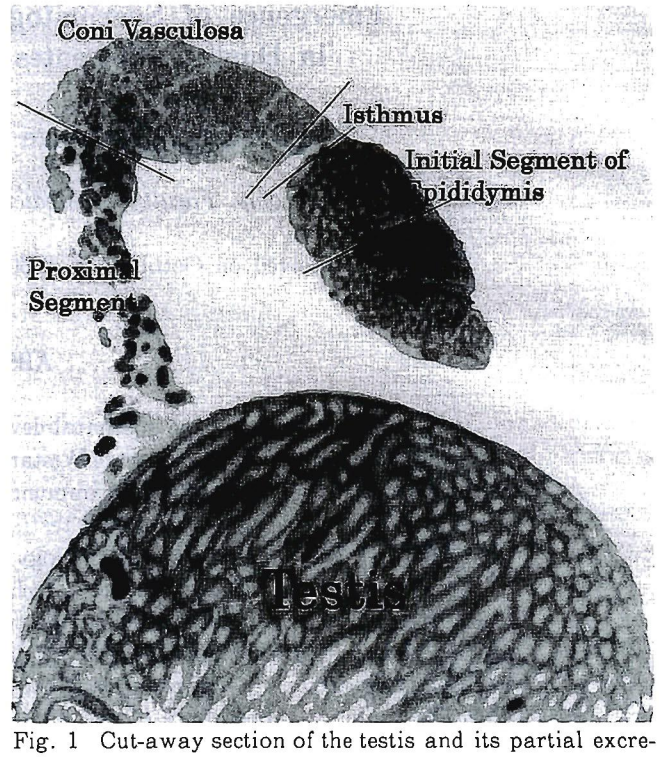

Fig. 1 Cut-away section of the testis and its partial excretory ducts. ×80.

One- to 2-week-old rats: Spermatogonia and Sertoli cells were closely arranged in the seminiferous tubules (ST). Developing spermatogenic cells such as primary spermatocytes were hardly observed (Fig. 2a). RET was covered by simple columnar epithelium. Partial epithelium and its attached basal membrane protruded into the lumen of RET (Fig. 2b). The DET and initial segment of DE were covered by simple cuboidal epithelium, and distinct differential configuration between them was not observed (Fig. 2c-d). The lumen of DET was narrow and round. Amorphous content was occasionally found in the lumen of DET and DE.

Three- to 4-week-old rats: The process of spermatogenesis advanced at this stage. Developing spermatogenic cells, especially spermatids in acrosome formation and primary spermatocytes in pachytene stage and in meiotic division were recognized. A large number of such cells were seperated from the wall of ST into the lumen (Fig. 3a). The higher columnar epithelium of RET in 1-2 week rats became simple cuboidal epithelium with the dilatation of lumen at this stage, and the lumen was rich of

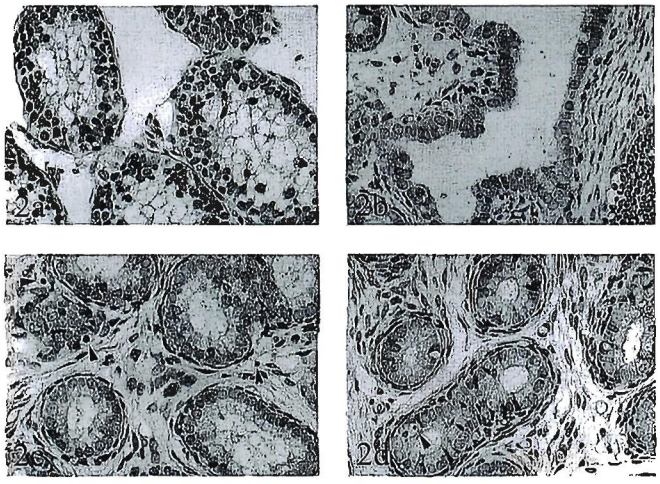

Fig. 2 Micrograph of ST, RET, DET and DE in 2 weeks rat. (a) ST, ×400; (b) RET, ×400; (c) DET. halo cell (arrow head), × 400; (d) initial segment of DE. halo cell (arrow head), ×400.

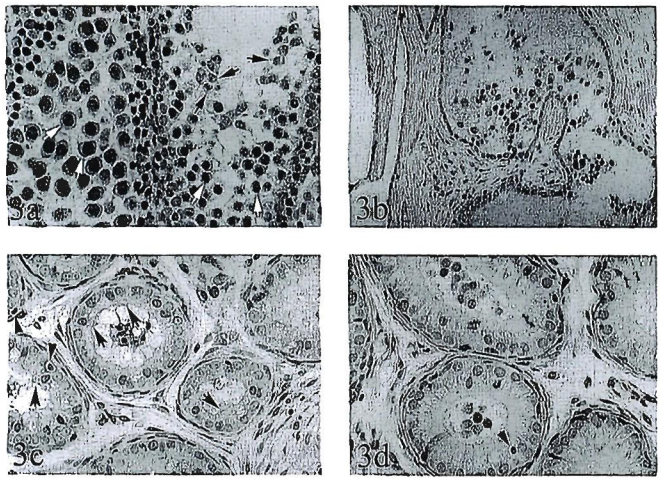

Fig. 3 Micrograph of ST, RET, DET and DE in 4 weeks rat. (a) ST. Except spermatogonium (type A and B), other types of germ cells are shown. pachytene spermatocytes (white arrows); spermatids in acrosome formation (black arrows), × 400; (b) RET, ×200; (c) DET. ciliated epithelial cells (arrows), halo cell (arrow head), × 400; (d) initial segment of DE. halo cell (arrow head), ×400.

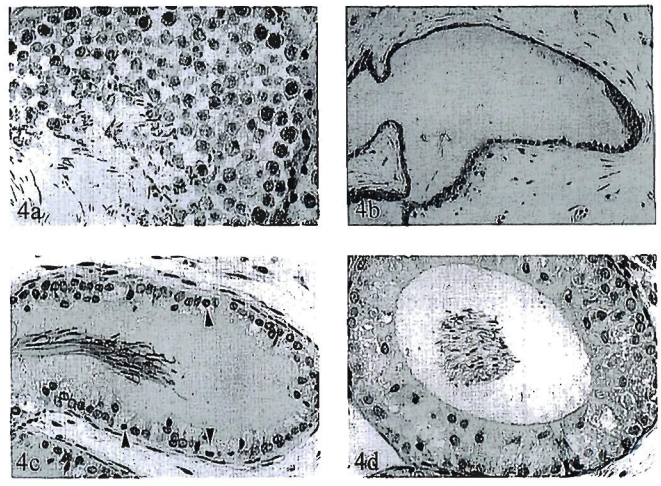

Fig. 4 Micrograph of ST, RET, DET and DE in 9 weeks rat. (a) ST, ×400; (b) border between RET and proximal segment of DET, ×200; (c) DET. halo cell (arrow head), ×400; (d) initial segment of DE, ×400.

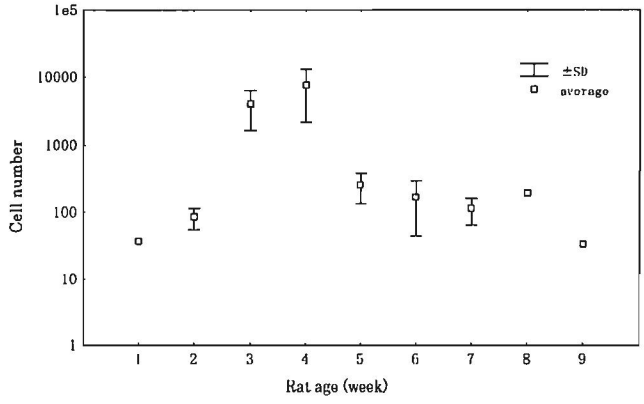

Fig. 5 The number of luminal cells in DET (postnatal 1 weeks to 9 weeks). The number of luminal cells is presented as the average(±standard deviation) of three rats at each stage.

luminal contents (Fig. 3b). The epithelia of DET and DE were differentiated into distinct configuration. The DET was lined with principal cells and relatively less ciliated cells (Fig. 3c) and its lumen was markedly dilated. The DE was lined with higher columnar principal cells with basal nucleus and basal cells and its lumen was even and round (Fig. 3d).

Five- to 9-week-old rats: ST, RET, DET and DE essentially have the same morphology as those in adult. Five to 7 rows of germ cells were sequentially and closely arranged in ST (Fig. 4a). The RT was covered with a simple squamous or cuboidal epithelium, of which its lumen appeared as wide and angular (Fig. 4b). Large number of mature sperms appeared in DET and DE from 8 weeks onwards (Fig. 4c-d).

Change in numbers and identification of non-sperm luminal cells in DET

Although the lumen of DET was almost empty, occasionally solitary luminal cell could be observed in 1-2 weeks rats. When postnatal development progressed, non-sperm cellular contents in DET markedly increased and its number reached the maximum at 4 weeks. After 5 weeks, these luminal cells sharply decreased. The changes in the number of non-sperm, luminal cells which appeared in DET from 1 to 9 weeks rats are shown in Figure 5. No sperm in DET were found in 1-4 weeks rats. Sperm can be seldom observed in the lumen of DET in 5 weeks however more number of sperms were observed after 8 weeks.

Luminal cells were scattered either in single or in clusters throughout the DET, however majority of them

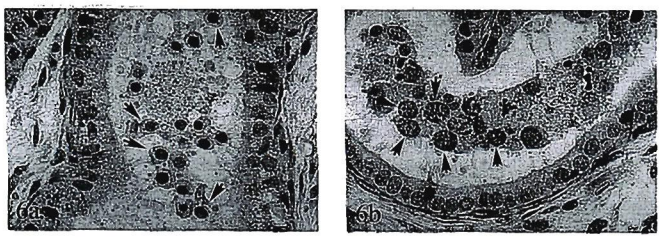

Fig. 6 Luminal cells in DET in 4 weeks rat. (a) round spermatid in acrosome formation (arrow), ×600; (b) patchytene spermatocytes (black arrow), ×600.

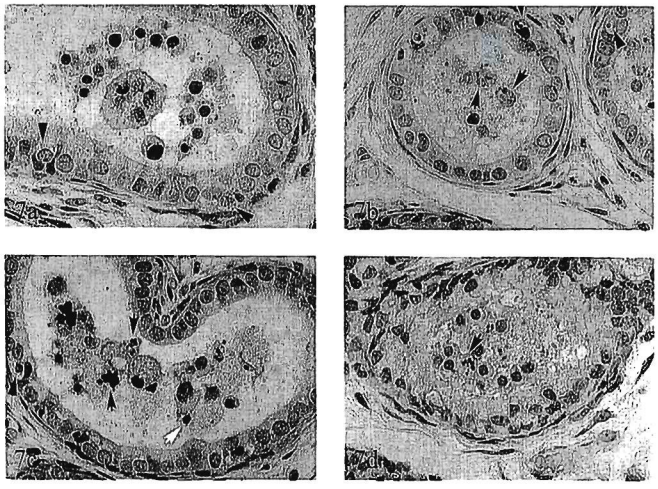

Fig. 7 Variation of luminal cells in DET. (a) multinucleated giant cells, halo cell (arrow head), ×600; (b) chromatin condensed (arrow), halo cell (arrow head), ×600; (c) chromatin condensation and marginalization, apoptotic body (black arrow), halo cell (arrow head), ×600; (d) apoptotic body (arrow), ×600.

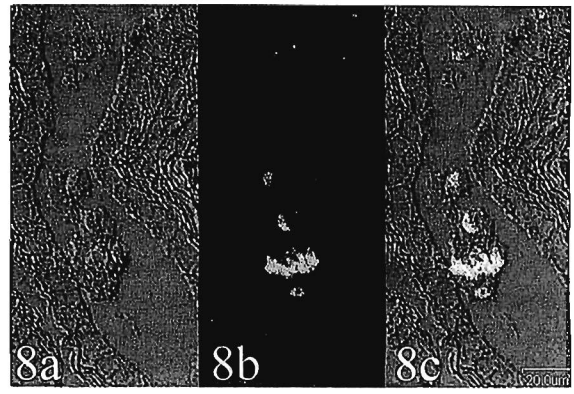

Fig. 8 Confocal micrograph for fluorescence TUNEL staining in 4 weeks rat. (a) interference channel; (b) fluorescence channel; (c) merged image of (a) and (b). Scale bar: 20 μm.

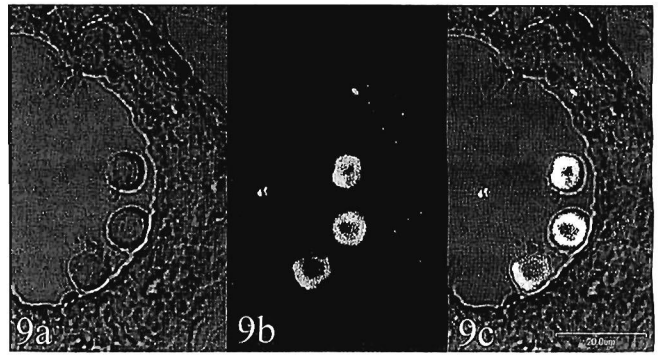

Fig. 9 Confocal micrograph for macrophages immunohistochemical staining in 4 weeks rat. (a) interference channel; (b) fluorescence channel; (c) merged image of (a) and (b). Scale bar: 20 μm.

were distributed in the coni vasculosa. Luminal cells were morphologically identical to round spermatids (Fig. 6a) and patchytene spermatocytes (Fig. 6b). These spermatids had acrosome vesicles at different stages in the perinuclear cytoplasm (Fig. 6a). The pachytene spermatocytes had thick and conspicuous chromosomes (Fig. 6b).

In the proximal coni vasculosa, the luminal cells were morphologically intact (Fig. 6a), while they showed marked morphological changes towards the terminals of coni vasculosa (Fig. 7). The luminal cells sometimes formed multinucleated giant cells (Fig. 7a) and showed chromatin condensation, marginalization and fragmentation (Fig. 7b-c). Typical apoptotic bodies, which appeared as small, round and basophilic bodies, were shown in the cytoplasm of some cells (Fig. 7d).

Halo cells

Halo cells with pale-stained or clear cytoplasm were found in the underlying lamina propia and in the base of the epithelium or near the lumen of DET and DE in every stage of rats (Fig. 2c-d, 3c-d, 4c, 7a-c).

In situ cell death detection of luminal cells in DET

FITC-labeled genomic DNA fragmentations (TUNEL staining) were detected in some of luminal cells in DET (Fig. 8). Fluorescence was shown either in single or in clusters in the nuclear region of the luminal cells. TUNEL-positive cells were principally situated in the terminal portion of the coni vasculosa.

Immunostaining of luminal cells in DET

Cells with positive cytoplasmic immuno-reactivity to anti-macrophages antibody were detected in the intertubular connective tissue of DET. A few number of luminal cells in DET were also immuno-reactive to the antibody (Fig. 9).

DISCUSSION

The laboratory rats grew into adults by 9 weeks and the week 3-5 was the juvenile period[13]. The present study demonstrated pronounced differential events in 3-4 weeks rats. The testis, DET and epididymis experienced rapid growth and notable epithelial changes during the period. The spermatogonia in seminiferous tubules developed into round spermatids. The epithelial cells of rete testis changed from columnar to simple squamous or cuboidal. Appearance of cilia in the epithelium of DET and difference in epithelium

between DET and epididymis were observed. These are in agreement with other previous reports[14-16].

At the juvenile period, developing spermatogenic cells such as spermatocytes in pachytene and in meiotic division and spermatids in acrosome formation were exfoliated into the lumen of seminiferous tubules. Our observations were similar to that reported by Alger, *et al.*[11] and Russell, et al.[10] Most interestingly, large numbers of luminal cells, which were identified as the exfoliated spermatogenic cells, appeared in the lumen of DET at the juvenile period, too. In our opinion, this is the first demonstration on the emergence of spermatogenic cells in excretory duct of reproductive system in juvenile rats.

Russell, et al.[10] studied an extensive exfoliation of developing germ cells such as spermatocytes and spermatids in seminiferous tubules to investigate the influence of androgen on the spermatogenesis at the pubertal stage. They considered that the exfoliation of germ cells at this stage was no more than the result of hormonal surge[10] and was due to inefficient spermatogenesis[10, 11]. But they did not discuss the significance of the exfoliation in seminiferous tubules and possible influence on the reproductive excretory tract. We demonstrated that luminal cells in DET come from exfoliated germ cells in seminiferous tubules. Cells with morphological changes of apoptosis were observed among luminal cells in DET and TUNEL reaction was detected in some of them, too. However most of them were located in terminal portion of coni vasculosa. The timing of appearance of luminal spermatogenic cells in DET coincided with the morphological differentiation of DET. These results did not suggest that DET was merely the drainage of those exfoliated germ cells but may play some specific roles in the postnatal development of excretory duct, especially in DET.

It is well known that androgen is an important factor for the development of reproductive system and maintenance of normal function of DET and caput epididymis[9, 17]. Hess, *et al.* reported that estrogen play a more important role in the development and maturation of DET[18]. In males, estrogen is present at low concentrations in blood, but can be extraordinary high in semen, and as high as 250pg ml^{-1} in rete testis fluids, which is higher than serum estrogen in the female[19-21]. In fact DET contains the highest concentration of estrogen receptors in rat[22, 23]. Janulis, *et al*[24] and Hess, *et al*[25] demonstrated that developing spermatocytes, spermatids and sperm possess an enzyme, cytochrome P450 aromatase, to convert androgen to estrogen. In male reproductive excretory ducts, the estrogen receptor first appears in the DET during development and only expressed in the DET in adults[24]. The main function of the DET is the re-absorption of fluid phase material with large molecular particles[2-8]. Hess, *et al.*[18] demonstrated that estrogen is the key factor that monitor or regulate the reabsorption in DET. The luminal cells we demonstrated are those that can transform androgen into estrogen. We assume that the luminal cells contribute to postnatal development of DET in the juvenile period as estrogen-producing cells, however, further evidences are required to prove this respect.

Apoptotic changes such as chromatin condensation and marginalization, and apoptotic bodies were observed among the luminal cells of DET and were further reinforced by TUNEL staining to indicate DNA fragmentation. Apoptosis is the final fate of luminal spermatogenic cells of DET.

We don't know yet how apoptotic luminal cells were eliminated. We observed multinucleated giant cells in HE-stained sections and macrophages demonstrated by immunohistochemistry. The antibody against macrophages employed in the present study is a kind of pan-macrophages marker but more sensitive to tissue and phagocytic macrophages[26-28]. The cells with positive immunoreactivity to the antibody were also detected in the intertubular interstitial matrix of DET. It hints that macrophages in the lumen of DET have the same phagocytic activity as histiocytes do and may share the same origin with interstitial histiocytes or directly originate from histiocytes. The histiocytes can migrate across the epithelia of DET because zonulate occludentes in DET, which constitutes the blood-epididymis barrier, are less developed and more discontinued, probably they belong to the assembly "leak type"[29-32]. It is well known that macrophages often appear where the apoptotic events occur. These evidences suggest that the clearance of apoptotic luminal spermatogenic cells may be associated with luminal macrophages in DET.

Halo cells were found in the underlying lamina propia and in the base of the epithelium or near the lumen of DET in our observation. The real nature of halo cells remains a controversy. While they have been referred to as lymphocytes, the possibility exists that they may be macrophages[33-38] if we compare the distribution of halo cells with that of macrophages as demonstrated by immunohistochemistry. Halo cells may provide an explanation regarding the source of macrophages when we consider them as macrophages.

In conclusion, a large number of spermatogenic cells such as pachytene spermatocytes, round spermatids appeared in the lumen of DET of juvenile rats; the timing of their emergence was coincidental with postnatal development and epithelial differenciation of DET. A apoptosis is the final fate of these luminal cells in DET while macrophages participate in the biological mechanics.

Acknowlegment

The authors thank Prof. Fumioki Yasuzumi for critical review of the manuscript. We are also grateful to Ms. Yukiji Sakiyama and Miss Makiko Moriyasu for their technical assistance.

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