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Abstract

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KEYWORDS: dynamin 2, endocytosis, spermatogenesis

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Original Article

Localization of Dynamin 2 in Rat Seminiferous Tubules during the Spermatogenic Cycle

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Dynamin is a protein essential to endocytosis. Dynamin 2, a dynamin isoform, is expressed most intensely in testicular tissue; however, precise localization has never been studied. Therefore, we investigated the expression of dynamin 2 in rat testicular tissue using immunohistochemical methods, and discuss here the physiological function of this protein. Testicular tissues were obtained from Wistar rats at 10, 21 and 63 days of age. Immunohistochemistrical examination and Western blot analysis were conducted using dynamin 2 specific antibody. Western blot analysis showed that expression in 21- and 63-day-old rats was more intense than that in 10-day-old rats. Dynamin 2 expression was observed using immunohistochemical method in the seminiferous tubules of all rats. In the 63-day-old rats, the expression was intense, especially in spermatids in the earlier maturation stages and in spermatocytes, and was observed in Sertoli cells. However, in spermatids, the expression gradually declined as spermatids matured to spermatozoa. In the 21-day-old rats, the expression was evident in spermatocytes and Sertoli cells, but that in the 10-day-old rats was weak. Intense expression of dynamin 2 during spermatogenesis suggests that this protein plays an important role in this process.

Key words: dynamin 2, endocytosis, spermatogenesis

Dynamin is a 100-kDa GTPase which plays an important role in endocytotic processes [1]. In recent years, it has been demonstrated that the function of dynamin is essential for clathrin-mediated endocytosis of synaptic vesicles in nerve terminals [2-5]. Furthermore, experimental data involving cultured cells has revealed that dynamin regulates the entry of molecules into the cell via endocytosis [6, 7]. During endocytosis, dynamin assembles to form a polymer at the cell surrounding the neck of clathrin-coated pits and pinches off the vesicles from the cell membrane [8, 9].

There are several isoforms found in dynamin: dynamin 1 is expressed exclusively in the neurons, dynamin 2 is ubiquitously expressed, including in the testicular tissues, and dynamin 3 is expressed in the neurons, lungs and testicular tissues [3]. Although the function of dynamin 3 is not evident, that of dynamin 1 and dynamin 2 has been investigated intensively, and it has been determined that these isoforms participate in endocytosis. Dynamin 2 is expressed intensely in rat testicular tissues [6, 10]; however, its precise localization has not yet been studied.

On the other hand, spermatids have been studied in detail and their development can be categorized into 19

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steps depending on maturation stage in mature rats, with higher step numbers indicating greater maturation. During Steps 1 to 8, spermatids are round spermatid, while during Steps 9 to 19, they are elongating spermatids [11]. Each seminiferous tubule can be classified into one of 14 stages according to its composition of germ cells [11].

In this study, we investigated the localization of dynamin 2 in rat testicular tissues using these classifications. In addition, the physiological function of dynamin 2 is discussed.

Materials and Methods

Western Blot Analysis. Western blot analysis was performed as previously described [12]. Testicular tissue obtained from Wistar rats, aged 10, 21 and 63 days, was lysed with lysis buffer (6.25 mM Tris-HCl, pH 6.8, 0.25 M sucrose, 2% SDS, 1.25% 2-mercaptoethanol), and samples were subsequently prepared by sonication. Approximately 30 μg protein were subjected to 6% SDS-PAGE. The samples were then electrotransferred to nitrocellulose membranes, followed by incubation with blocking buffer [10% equine serum, 5% skim milk and TBS (10 mM Tris-HCl, pH 7.6, 150 mM NaCl)] for 2 h. Membranes were subsequently incubated with primary antibody (anti-dynamin 2 goat polyclonal antibody, purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted by 1% gelatin and TTBS (20 mM Tris-HCl, pH 7.6, 500 mM NaCl, 0.05% Tween-20) solution for 2 h at 37 °C. Membranes were then incubated with HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) diluted with the identical solution utilized for the primary antibody for 1 h at 25 °C. Target protein was visualized with 0.05% 4-methoxy-1-naphthal (Aldich Chemical Company, Inc., Milwaukee, WI, USA).

Immunohistochemistry. Immunohistochemical analysis was conducted following the techniques previously described [13]. Wistar rats, aged 10, 21 and 63 days, were deeply anesthetized with sodium pentobarbital. Testicular tissues were removed and subsequently fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Samples were cryo-protected with 10% sucrose in 0.1 M phosphate buffer (pH 7.4) for 2 h, frozen embedded with Tissue-Tek (O C T compound). The blocks of rat testis tissue were cut into 10 μl sections using a cryostat. The sections were then thaw-mounted on glass slides and were then incubated with blocking buffer containing 10% rabbit serum, 10 mM sodium phosphate (pH 7.4) and 150 mM NaCl for 30 min, followed by incubation with the primary antibody 1:200 dilution overnight at 25 °C. The sections were incubated with rabbit secondary biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) and with ABC reagent (Vecstatin ABC Kit, Vector Laboratories), and subsequently stained with DAB-H_2O_2 and counterstained for cell nuclei with hematoxylin. Glycerin-embedded sections were observed with a microscope. Negative control sections were stained using goat serum in lieu of the primary antibody.

In the 63-day-old rats, each spermatid was categorized into one of 19 steps, and each seminiferous tubule was classified into 14 stages according to the criteria presented by Perry [11] in a previous study.

Results

Dynamin 2 Expression in Rat Testicular Tissues. Western blot analysis revealed the presence of 100-kDa dynamin 2-specific bands in all rat testicular tissues. Dynamin 2 expression was approximately identical in 21- and 63-day-old rats. However, dynamin 2-specific bands in the testicular tissue of 10-day-old rats were siginificantly less intense than those in the tissue of older rats (Fig. 1).

Dynamin 2 Localization in Rat Testicular Tissues. Intense dynamin 2 signals were detected in the seminiferous tubules of the 63-day-old rats (Fig. 2). Each seminiferous tubule was examined using an enlarged view (Fig. 3A–E). At all stages, the most intense signal was observed in spermatocytes, both primary and secondary. Sertoli cells and spermatogonia also exhibited a positive signal. Signals were also observed in interstitial cells, although only weakly.

In Stage III, Step 3 round spermatids and Step 16 elongating spermatids were detected (Fig. 3A); moreover, the cytoplasm of Step 3 round spermatids displayed an intense signal comparable to that of spermatocytes. On the other hand, the cytoplasm of Step 16 elongating spermatids, which was located in the inner portion, demonstrated a less intense signal relative to that of both spermatocytes and Step 3 round spermatids (Fig. 3A).

Step 6 round spermatids and Step 18 elongating spermatids in Stage VI (Fig. 3B), and Step 7 round spermatids and Step 19 elongating spermatids in Stage

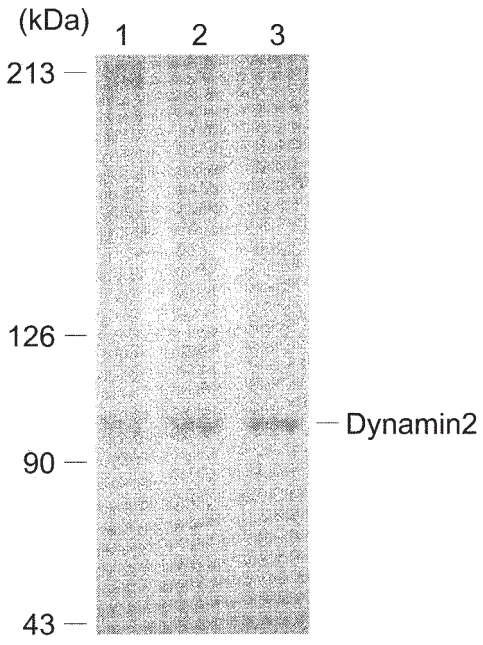

Fig. 1 Dynamin 2 expression in rat testicular tissues by western blot analysis. Lane 1, 10-day-old rat testis; Lane 2, 21-day-old rat testis, Lane 3, 63-day-old rat testis. A 100-kDa protein was detected in all rats, however, expression in 10-day-old rats was less than that in older rats.

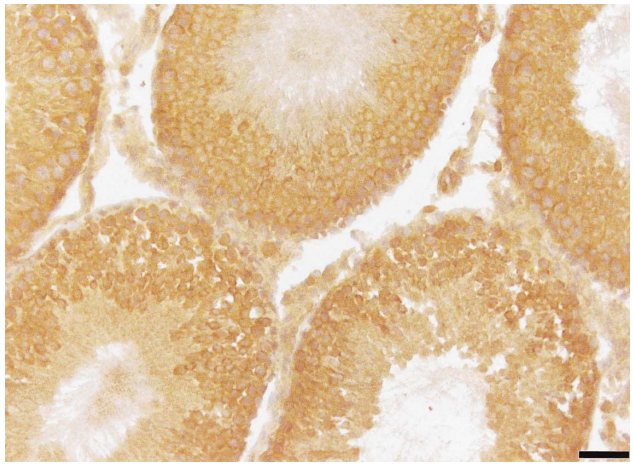

Fig. 2 Dynamin 2 localization in rat testicular tissues (ABC-DAB staining, hematoxylin staining with anti-dynamin 2 antibody, Bar = 50 μm) Dynamin 2 signals were detected in seminiferous tubules. Roman numerals indicate each seminiferous tubule stage.

VII (Fig. 3C) were detected. However, the signals of round spermatids of Step 6 (Fig. 3B) and 7 round spermatid (Fig. 3C) were weaker than that of Step 3 round spermatids (Fig. 3A). The signals of elongating spermatids of Steps 18 (Fig. 3B) and 19 (Fig. 3C) were also weaker than that of Step 16 spermatids (Fig. 3A). Step 9 elongating spermatids observed in Stage IX (Fig. 3D), and Step 12 elongating spermatids observed in Stage XII (Fig. 3E) expressed more intense signals than Step 16 spermatids (Fig. 3A). No specific signal was evident in either of the seminiferous tubules in the negative control samples (Fig. 3F).

Intense dynamin 2 signal was also present in the spermatocytes in 21-day-old rat testicular tissue (Fig. 3G). No immune-specific signal was detected in the seminiferous tubules in negative control samples (Fig. 3H).

In 10-day-old rat testicular tissue, dynamin 2 expression was weak in the spermatocytes (Fig. 3I). No immune-specific signal was detected in the seminiferous tubules in negative control samples (Fig. 3J).

In our observations, the most intense dynamin 2 expression was apparent in spermatocytes, round spermatids and Sertoli cells. In 63-day-old rats, round spermatids in the earlier developmental stages showed intense signals in comparison with those of spermatocytes, however signal intensity declined as the spermatids matured.

Discussion

The present investigation demonstrates that 100k-Da dynamin 2 is expressed in 10-, 21- and 63-day-old rats. Dynamin 2 expression in 21- and 63-day-old rats is more intense than that in 10-day-old rats. Immunohistochemically, dynamin 2 expression in 10-day-old rats was significantly weaker than in the older animals. It is known that spermatogenesis begins at around 15 days after birth. Less intense expression of dynamin 2 before the onset of spermatogenesis suggests that there exists a strong relationship between this protein and spermatogenesis. Since dynamin 2 expression seems to be identical in 21- and 63-day-old rats, it is possibile that the physiological role of dynamin 2 has already been established by 21 days of age.

Dynamin 2 signal was expressed in germ cells and Sertoli cells. Our study demonstrates a wide range of localization of dynamin 2 signal in the cytoplasm. The

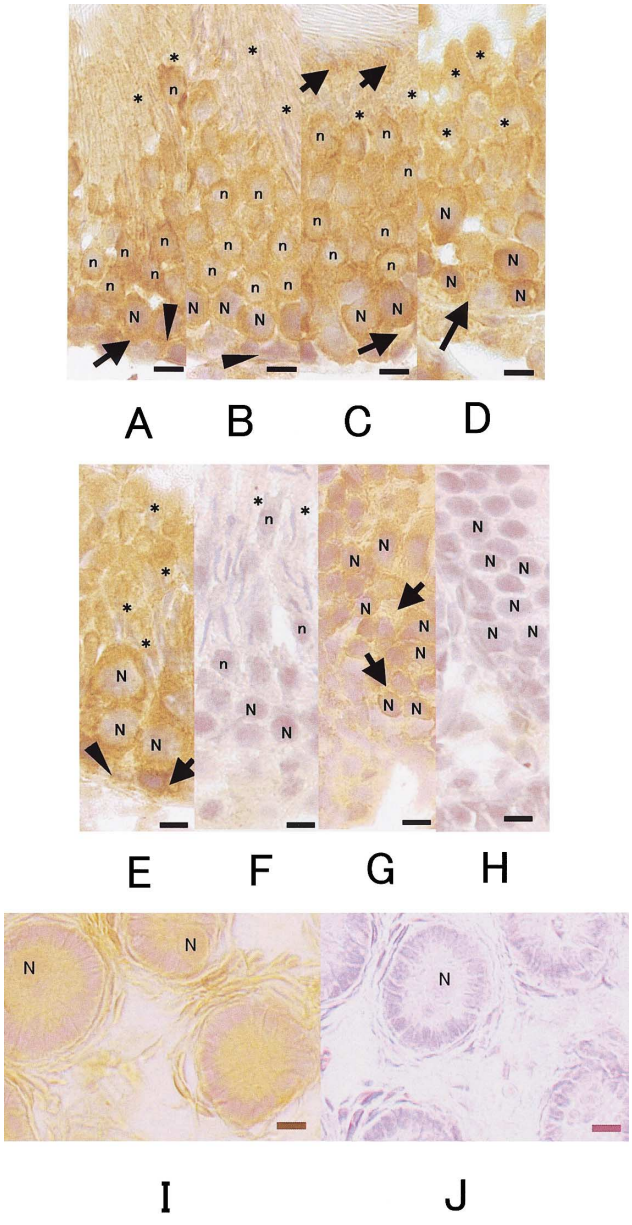

Fig. 3 Dynamin 2 localization in rat testicular tissues (ABC-DAB staining, hematoxylin staining, Bar = 10 μm). **A**, 63-day-old rat (Stage III): Step 3 and Step 16 spermatids were found in the seminiferous tubules. The intensity of signals in Step 3 spermatids was identical to that in spermatocytes, while that in Step 16 spermatids was weak; **B**, 63-day-old rat (Stage VI) and **C**, 63-day-old rat (Stage VII): In these figures, spermatids of Steps 6 and 18 (Fig. 3B) and Steps 7 and 19 (Fig. 3C) spermatids are detected. The signals in spermatids of Steps 6 and 7 was less intense than those in Step 3 spermatids, and that in spermatids of Steps 18 and 19 was less intense than that in Step 16 spermatids; **D**, 63-day-old rat (Stage IX) and **E**, 63-day-old rat (Stage XII): Step 9 (Fig. 3D) and Step 12 (Fig. 3E) spermatids were observed. Expression in spermatids of both Steps 9 and 12 was more intense than that in Step 16 spermatids; **F**, 63-day-old rat (negative control): No specific signal was detected in the seminiferous tubules; **G**, 21-day-old rat: Signals were detected most intensely in spermatocytes, and were also observed in Sertoli cells and spermatogonia; **H**, 21-day-old rat (negative control): No specific signal was detected in the seminiferous tubules; **I**, 10-day-old rat: Both spermatocytes and Sertoli cells were found in the seminiferous tubules. Signals in both types of cell were weak; **J**, 10-day-old rat (negative control): No specific signal was detected in the seminiferous tubules. N, spermatocyte (nucleus); n, round spermatid (nucleus); *, elongating spermatid; arrow, Sertoli cell (cytoplasm); arrowhead, spermatogonia.

most intense dynamin 2 signal was apparent in the spermatocytes in 21-and 63-day-old rat testicular tissues. In 63-day-old rats, round spermatids in earlier stage of maturation showed an intense signal in comparison with that detected in spermatocytes. The intensity of the signal declined as the spermatids matured. Dynamin 2 expression in spermatogonia was weak. Differences in signal intensities were not detected in Sertoli cells in any of the stages.

Sertoli cells are considered to directly influence spermatogenesis by providing nutrients and secreting various substances for germ cells. Adjacent Sertoli cells form tight junctions to create the blood-testis barrier at an age of between 16 and 18 days in rats [14]. Seminiferous tubules are partitioned into the basal and adluminal compartments via the blood-testis barrier, with the latter containing germ cells after the leptotene stage, such as spermatocytes, spermatids and mature spermatozoa [15]. The development of germ cells in adluminal compartments is sustained exclusively by Sertoli cells [16], which must obtain various substances from outside of the seminiferous tubules. Endocytosis of several proteins, such as transferrin, is reported to be active in the base of Sertoli cells [17, 18] and dynamin 2 is thought to play an essential role in this process.

Electron Microscopy studies have shown that androgen-binding protein is transported from Sertoli cells to germ cells by clathrin-mediated endocytosis [19]. It is recognized that spermatogenesis is dependant upon testosterone [20]. In fact, based on the results of an experiment using transferrin, it was reported that the transportation of transferrin via endocytosis in spermatocytes and round spermatids is active, while that in elongating spermatids is inactive [21]. In addition, immunocytochemical localization of clathrin places its localization at the surface of primary spermatocytes and spermatids

[22]. In the same experiment, intense dynamin 2 expressions were detected in both spermatocytes and round spermatids, and this expression declined as the spermatids matured. These findings are thought to be consistent with our current understanding of spermatogenesis and transportation of neutrients. Spermatocytes and round spermatids undergo meiotic stages, hence, these cells are thought to need to import more nutrients. This suggests a relationship between nutrient transportation in spermatogenesis and dynamin 2 expression. Recent reports have proposed that dynamin, in addition to its role in pinching off clathrin-coated pits, also serves as a regulatory factor for the coated vesicles [23].

The function of endocytosis-related proteinssuch as amphiphysin I and synaptojanin in testicular tissues has been analyzed in several recent studies [24, 25]. Specifically, intense expression of amphiphysin I, which binds to dynamin, has been shown in Sertoli cells [25]. It has been claimed that, based on the increase of expression levels of amphiphysin I, endocytosis in Sertoli cells may become significant, coinciding with the onset of spermatogenesis [25]. As a result, a functional relevance between dynamin 2 and amphiphysin I is suggested.

Based on the observation that dynamin 1 is intensely expressed in neurons but shows nearly no expression in testicular tissues [3], dynamin 2 could be proven to be essential for transportation processes in testicular tissues. In this study, we suggest that dynamin 2 plays an important role in spermatogenesis.

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