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Short communication

An immunohistochemical study of the cytoskeletal proteins in the testis of the Sunda porcupine (*Hystrix javanica*)

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Abstract. In the testis of the Sunda porcupine (*Hystrix javanica*), the distribution pattern of cytoskeletal proteins was immunohistochemically examined to understand their functional roles in the testis. Immunoreactivity for α-smooth muscle actin (SMA) was found in the peritubular myoid cells of the seminiferous tubules and in the sub-tubular myoid cells of epididymal ducts. In intermediate filaments, desmin was exhibited in the sub-tubular myoid cells of the epididymal ducts, and vimentin immunoreactivity was detected in the Sertoli cells, Leydig cells, peritubular myoid cells, and sub-tubular myoid cells of the epididymal ducts. However, cytokeratin was not expressed in the testes or epididymides. Strong immunoreactivity for vimentin was demonstrated between the infranuclear part of the elevated nuclei and the basal membrane in the Sertoli cells. Furthermore, vimentin was extended from supranuclear to apical parts in some Sertoli cells. Immunoreactivity for α-tubulin was detected only in the Sertoli cells. It is concluded that, in the porcupine testes, desmin plays no role in the contractile function of peritubular myoid cells, vimentin may contribute to the anchoring and transition of the elevated nuclei in the Sertoli cells, and α-tubulin and vimentin in the Sertoli cells are likely involved in their structure and function as the main cytoskeleton.

Key words: cytoskeletal proteins, immunohistochemistry, porcupine, testis.

The Sunda porcupine (*Hystrix javanica*) belonging to the family Hystricidae (order Rodentia, suborder Hystricomorpha, infraorder Hystricognathi), is a small Indonesian endemic herbivore (Jori et al. 1998; Atkins 2004; Ozdemir et al. 2005) widely distributed in Java, Bali, Madura, Flores, Lombok, Tonahdjampea, Sulawesi, and the Sumbawa islands (Atkins 2004; Woods and Kilpatrick 2005). There is a few report about the reproductive biology of the male porcupines. In the testes of adult male Cape porcupines (*Hystrix africaeaustralis*), the spermiogenesis (completed spermatogenesis) was recognized throughout the year, and significant seasonal change of seminiferous tubule diameters was not observed (van Aarde and Skinner 1986). In male Sunda porcupines, however, the anatomical and histological characteristics of genital organs including testes have not been examined.

The cytoskeletal proteins of cells in general consist of three major components: microfilaments (actins), intermediate filaments (vimentin, desmin, cytokeratins, neurofilament proteins, and glial fibrillary acidic protein), and microtubules (tubulins). Their roles in cellular structure and function include maintaining cell shape and polarity, positioning of intracellular organelles, forming of cytoplasmic extensions, and anchoring of organelles to the plasma membrane.

In the testis, the localization of cytoskeletal proteins has been reported. In rat Sertoli cells, vimentin is expressed in all developmental stages from fetus to adult-

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hood, unlike cytokeratin and desmin (Paranko et al. 1986; Fröjdman et al. 1992; Rogatsch et al. 1996), and its formation was changed during the seminiferous cycle in adults (Zhu et al. 1997). In mature testes of humans, rats, Japanese black bears, and water buffalos, desmin was observed in the peritubular myoid cells of the seminiferous tubules (Virtanen et al. 1986; Komatsu et al. 1998; Cruzana et al. 2006), but in mature ovine and lesser mouse deer testes, desmin completely disappeared from the peritubular myoid cells (Steger and Wrobel 1994; Sasaki et al. 2010). The testicular distribution pattern of cytoskeletal proteins is different among species even if developmental stages are the same. In the present study, we examined the localization of the cytoskeletal proteins, α-smooth muscle actin (α-SMA), desmin, vimentin, cytokeratin, and α-tubulin in the Sunda porcupine testes to understand the functional role of these cytoskeletal proteins in the testis.

Materials and methods

Two testes with epididymides and two testes without epididymides were obtained from total four male Sunda porcupines in Karanganyar, central Java, Indonesia. Animals were sacrificed by an intraperitoneal overdose injection of ketamine hydrochloride and xylazine HCL. The testes with epididymides were immediately removed and fixed in Bouin's fixative for 24 h. Then, the samples were dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin, and cut serially in 4-μm thick slices. The sections were placed on aminopropyltriethoxy-silane-coated slides (S8226, Matsunami Glass Int. Inc., Osaka, Japan) and deparaffinized. The sections were stained with hematoxylin and eosin (HE), and immunohistochemically stained using the avidin-biotin peroxidase complex (ABC) method (Hsu et al. 1981).

The sections were treated by microwave in target retrieval solution (1:10, S1699; DakoCytomation, Inc., CA, USA) for 15 min, and then immersed in methanol containing 0.3% H_2O_2 for 10 min at room temperature (RT) to block the endogenous peroxidase activity. Afterwards, the sections were incubated with normal goat serum (1:50, S-1000, Vector Laboratories Inc., CA, USA) for 30 min at RT to prevent nonspecific staining and incubated overnight with each primary antibody at 4°C in a moisture chamber. For primary antibodies, mouse monoclonal anti-human α-SMA (1:1000, Clone 1A4, Sigma Chemical Co., St. Louis, MO, USA), mouse monoclonal anti-porcine eye lens vimentin (1:100, Clone V9, DAKO, Glostrup, Denmark), mouse monoclonal anti-porcine stomach desmin (1:50, code M724, DAKO, Glostrup, Denmark), mouse monoclonal anti-human lung tumor cell cytokeratin (1:100, Clone Lu-5, Biogenesis Ltd., Poole, UK), and mouse monoclonal anti-chick brain α-tubulin (1:200, Clone DM1A, Neomarkers, CA, USA) were used in this study. After being incubated with the primary antibodies, the sections were incubated with biotinylated goat anti rabbit IgG (1:200, BA-1000, Vector Laboratories Inc.) or biotinylated goat anti mouse IgG (1:200, BA-9200, Vector Laboratories Inc.) for 30 min, and then ABC reagent was applied for 30 min (1:2, PK-6100, Vectastain Elite ABC kit, Vector Laboratories). The binding sites were visualized with Tris-HCl buffer (pH 7.4) containing 0.02% 3,3'-diaminobenzidine hydrochloride (DAB) and 0.006% H_2O_2. After incubation, the sections were washed with 0.01 M phosphate buffered saline (PBS, pH 7.4), dehydrated in a graded series of ethanol, cleared in xylene, cover slipped, and observed with a conventional light microscope. The negative control sections omitted each primary antibody.

Results

On histological observation, the completed spermatogenesis was not observed in the testes of all Sunda porcupines (Fig. 1), although two spermatozoa which seem to be transferred from other parts of seminiferous tubules were noticed in one individual (Fig. 5). We were not able to observe the seminiferous tubules with completed spermatogenesis, although many sections were cut from a remaining sample paraffin block and observed. The spermatozoa were not detected in the epididymal ducts of observed all samples (Fig. 1B). However, the spermatogenic cells until round spermatids were differentiated in all animals (Fig. 1A). Thus, we think that these porcupines will achieve the spermiogenesis in all seminiferous parts before long, namely transitional period from immature to mature. In the Sertoli cells, moreover, the nuclei were arranged at varying heights (Figs. 1A, 4A).

On immunohistochemical observation, positive immunoreactivity for α-SMA was found in the peritubular myoid cells of the seminiferous tubules (Fig. 2A) and in the sub-tubular myoid cells of epididymal ducts (Fig. 2B). The walls of blood vessels also demonstrated immunoreactivity for α-SMA (Fig. 2B).

In intermediate filaments, desmin was detected in the sub-tubular myoid cells of epididymal ducts (Fig. 3A)

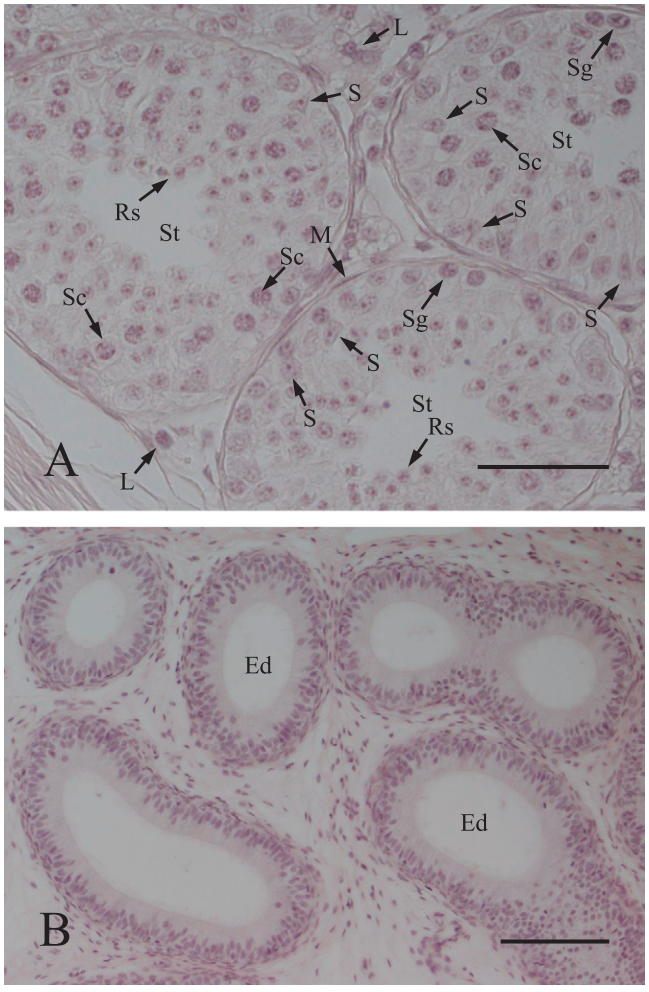

Fig. 1. Histological structure of the testis and epididymis of the Sunda porcupine. A, testis; B, epididymis. In testes, the spermatogenic cells until round spermatids were observed. The nuclei in the Sertoli cells were positioned at varying heights. Spermatozoa were not detected in the seminal tract. Ed, epididymal duct; L, Leydig cell; M, peritubular myoid cells; RS, round spermatid; S, Sertoli cell; Sc, spermatocyte; Sg, spermatogonia; St, seminiferous tubule. Bar: 50 μm (A), 100 μm (B). HE staining.

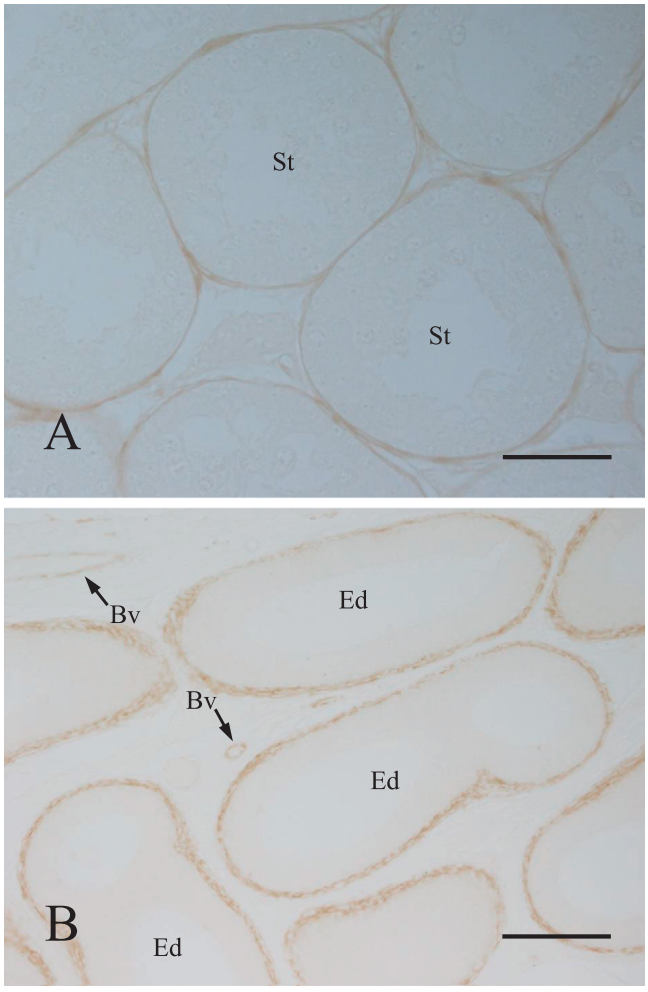

Fig. 2. Immunolocalization of α-smooth muscle actin (α-SMA) in the testis and epididymis. A, testis; B, epididymis. α-SMA was found in the peritubular myoid cells of the seminiferous tubules, the sub-tubular myoid cells of epididymal ducts, and the walls of blood vessels. Bv, blood vessel; Ed, epididymal duct; St, seminiferous tubule. Bar: 50 μm (A), 100 μm (B).

and the walls of blood vessels (Fig. 3B). Immunoreactivity for vimentin was observed in Sertoli cells, Leydig cells, and peritubular myoid cells (Fig. 4A). In Sertoli cells, strong immunoreactivity for vimentin was seen between the basal membrane and the nuclei, which were arranged above the spermatogonia (small arrowheads in Fig. 4A). Moreover, vimentin filaments protruding from the supranuclear part were observed in the Sertoli cells, regardless of their nuclear positions (large arrowheads in Fig. 4A). Vimentin immunoreactivity was also observed in the walls of blood vessels, and in the sub-tubular myoid cells of epididymal ducts and stromal cells of epididymides (Fig. 4B). In all samples, immunoreactivity for cytokeratin was not exhibited in the testes or epididymides.

Immunoreactivity for α-tubulin was detected only in Sertoli cells, extending from the basal area to the tip of the cytoplasm of each cell (Fig. 5).

Discussion

The present study clarified the presence and distribution patterns of α-SMA microfilament, desmin and vimentin intermediate filaments, and α-tubulin microtubules in the testes and epididymides of Sunda porcupines by immunohistochemical methods. However, localization of cytokeratin was not observed in the porcupine testes or epididymides.

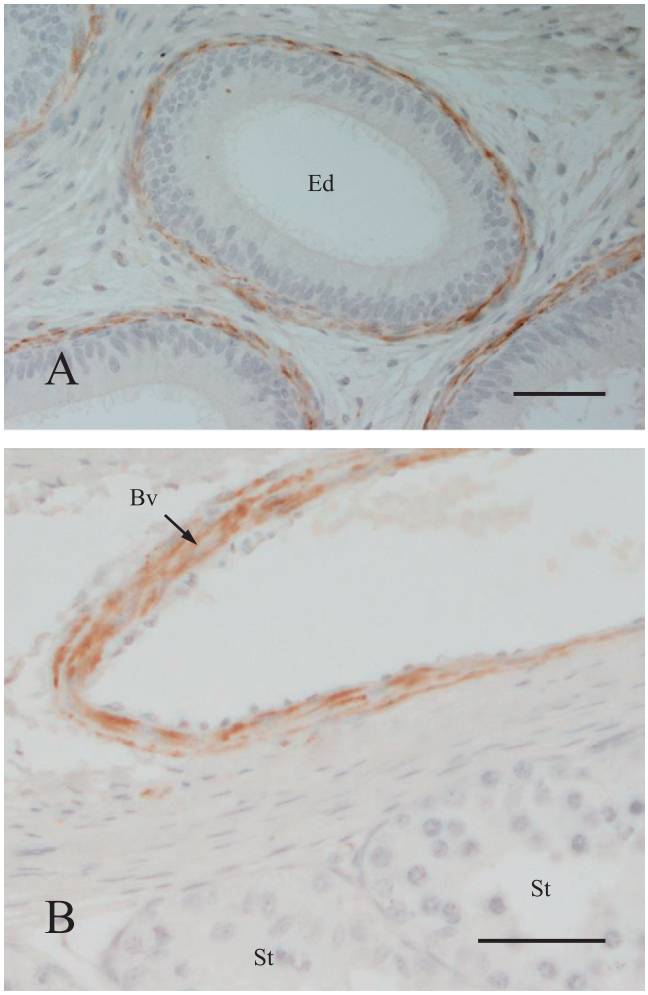

Fig. 3. Immunolocalization of desmin in the testis and epididymis. A, epididymis; B, blood vessel in the testis. Desmin was found in the sub-tubular myoid cells of epididymal ducts and the walls of blood vessels. Bv, blood vessel; Ed, epididymal duct; St, seminiferous tubule. Bar: 50 μm.

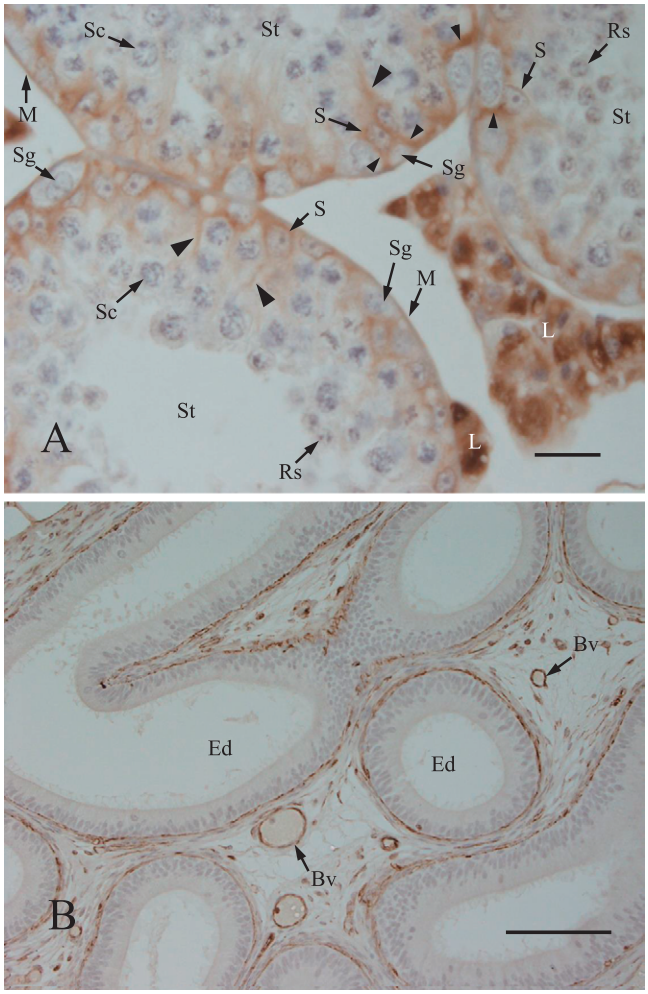

Fig. 4. Immunolocalization of vimentin in the testis and epididymis. A, testis; B, epididymis. In the testis, vimentin immunoreactivity was detected in the Sertoli cells, Leydig cells, and peritubular myoid cells. In the epididymis, vimentin was expressed in the sub-tubular myoid cells of epididymal ducts and the stromal cells. The walls of blood vessels were positive for vimentin. Small arrowheads, vimentin immunoreactivity in the infranuclear part; large arrowheads, vimentin immunoreactivity in the supranuclear part; Bv, blood vessel; Ed, epididymal duct; L, Leydig cell; M, peritubular myoid cells; RS, round spermatid; S, Sertoli cell; Sc, spermatocyte; Sg, spermatogonia; St, seminiferous tubule. Bar: 20 μm (A), 100 μm (B).

In the Sunda porcupine testis, α-SMA was detected in the peritubular myoid cells of seminiferous tubules and the sub-epithelial myoid cells of epididymal ducts in a completely circular formation. It has previously been reported in rats (Tung and Fritz 1990; Palombi et al. 1992) and cattle (Devkota et al. 2006) that incomplete circular formation of α-SMA is observed in the peritubular myoid cells of immature testes, and this formation is gradually completed with testicular development. In this study, α-SMA formation was already completed in the seminiferous tubules of Sunda porcupine testes that had developed until the round spermatid stage in spermatogenesis. It may be assumed that the complete formation of α-SMA is essential for completing spermiogenesis, i.e., spermatozoa production.

There are many reports stating that cytokeratin, desmin, and vimentin are related to testicular structural support and functions (Amlani and Vogl 1988; Fridmacher et al. 1992; Zhu et al. 1997; Sasaki et al. 1998, 2010; He et al. 2007). In the present study, cytokeratin was not expressed in the testicular cells such as Sertoli cells, spermatogenic cells, Leydig cells, or peritubular myoid cells. It has been reported that cytokeratin is present in the Sertoli cells of rats and humans in fetal and neonatal periods, and then completely disappears by adulthood (Paranko et al.

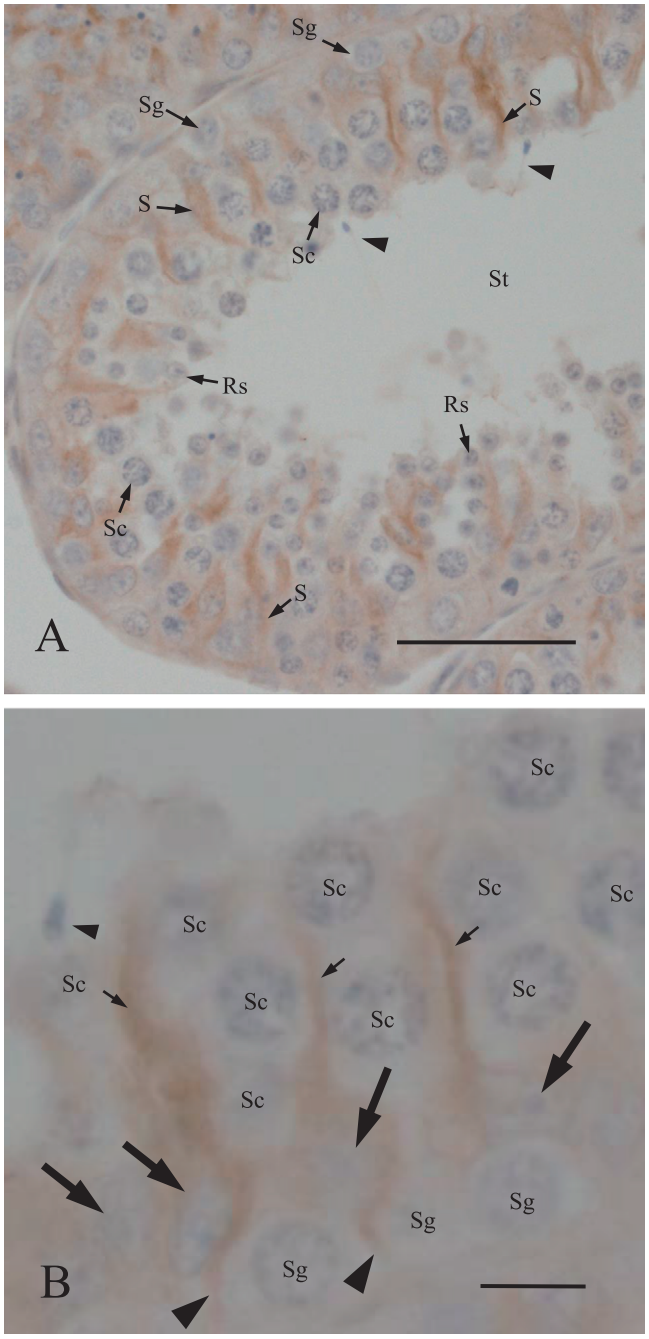

Fig. 5. Immunolocalization of α-tubulin in the testis. α-tubulin was found only in Sertoli cells. A, low magnification; B, high magnification. Small arrows, α-tubulin immunoreactivity in the supranuclear part; large arrows, nuclei of the Sertoli cells; small arrowheads, spermatozoa; large arrowheads, α-tubulin immunoreactivity in the infranuclear part; RS, round spermatid; S, Sertoli cell; Sc, spermatocyte; Sg, spermatogonia; St, seminiferous tubule. Bar: 50 μm (A), 10 μm (B).

1986; Fridmacher et al. 1992; Rogatsch et al. 1996; Sasaki et al. 1998). Therefore, in the Sertoli cells of the Sunda porcupine testes, cytokeratin may be expressed in earlier stages, such as fetal and/or neonatal, with transient functions, although cytokeratin was not detected in the testes just before complete spermatogenesis.

In the Sunda porcupine testes, desmin is expressed in the sub-epithelial myoid cells of epididymal ducts but not in the testicular cells, including the Sertoli cells, or peritubular myoid cells of seminiferous tubules. The present result is in agreement with previous findings in sub-adult and adult lesser mouse deer (Sasaki et al. 2010). On the other hand, in other mammalian species, desmin was found in the Sertoli cells of the fetal and early postnatal testes (Fröjdman et al. 1992; Cruzana et al. 2006), and widely in the peritubular myoid cells of seminiferous tubules from fetal until adult testes (Virtanen et al. 1986; Palombi and Di Carlo 1988; Fröjdman et al. 1992; Komatsu et al. 1998; Cruzana et al. 2006). It has been reported that desmin is expressed in ovine testes during the early prepubertal period, but disappears completely during puberty (Steger and Wrobel 1994). In the Sunda porcupine testes, it is possible that desmin from the Sertoli cells and/or peritubular myoid cells may vanish with postnatal testicular growth like as the transition observed in ovine testes. It is suggested that, in the developed testes of Sunda porcupines as well as mouse deer and sheep, desmin filaments cannot contribute to the contraction of peritubular myoid cells unlike α-SMA, and to the structural support of Sertoli cells unlike vimentin and α-tubulin.

In the Sunda porcupine, immunoreactivity for vimentin was detected in Sertoli cells, Leydig cells, peritubular myoid cells, and sub-tubular myoid cells of epididymal ducts. In the testis of Sunda porcupines, vimentin filament bundles, which connect the basal membrane and the nuclei of Sertoli cells, were observed (small arrowheads in Fig. 4A). In Sertoli cells, vimentin filaments play a role in anchoring the nucleus to the basal membrane, as well as in anchoring between Sertoli cells and spermatogenic cells (Amlani and Vogl 1988; Zhu et al. 1997; Vogl et al. 2000; Devkota et al. 2006; He et al. 2007). In immature bovine testes, temporal elevation of the nuclei with the extension of vimentin filaments has been reported (Devkota et al. 2006). In the Sunda porcupine testes, the nuclei of the Sertoli cells were positioned at different heights. It is thought that vimentin may function not only to anchor the basal membrane, but also to transit the nucleus toward the basal membrane when the nucleus resettles in the basal part. Furthermore, vimentin filaments extending toward the lumen of seminiferous tubules from the supranuclear area were observed in the porcupine testes (large arrowheads in Fig. 4A). These

vimentin filaments may be related with the anchoring of spermatogenic cells. Moreover, in Leydig cells, vimentin filaments were exhibited, although other cytoskeletal proteins examined in this study were not detected. Therefore, vimentin may be one of the most important cytoskeletal proteins controlling the structure and functions of Leydig cells.

In this study, α-tubulin was detected in the Sertoli cells, extending toward the apical part of the cytoplasm. In Sertoli cells, it is well known that microtubules have several functions such as spermatid migration, structural support for developing spermatogenic cells, shaping of the spermatid head and acrosome, and preservation of the cell structure (Vogl et al. 1983, 2000). In the porcupine Sertoli cells, microtubules are a main cytoskeletal component, along with vimentin, and may play important roles in supporting spermatogenic cells during their development as well as in preservation of cell shape.

In conclusion, the present study demonstrated the expression of α-SMA, desmin, and vimentin in the sub-tubular myoid cells of epididymal ducts, as well as immunoreactivity for vimentin and α-tubulin in the Sertoli cells, and vimentin in the Leydig cells in Sunda porcupine testes and epididymides just before complete spermatogenesis. For further understanding of the spermatogenic functions of cytoskeletal proteins, it will be necessary to examine porcupine testes that have completed spermiogenesis because vimentin and α-tubulin are involved in the anchoring and migration of elongated spermatids. This study may aid in developing the knowledge of testicular differentiation and development in order to investigate the dynamics of cytoskeletal proteins in the fetal and early postnatal porcupine testes.

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