Postnatal Developmental Changes in Immunohistochemical Localization of α-Smooth Muscle Actin (SMA) and Vimentin in Bovine Testes

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Abstract. The present study demonstrates the postnatal developmental changes in immunohistochemical localization of α-smooth muscle actin (SMA) and vimentin in the bovine testis. In the peritubular myoid cells of seminiferous tubules and the sub-epithelial and stromal cells of straight tubules and the rete testis, α-SMA starts appearing at around 4 months of age. Peritubular α-SMA attains the continuous mature pattern at around 5 months of age whereas sub-epithelial and stromal α-SMA increases with advancing age. Vimentin is localized in the perinuclear zone of Sertoli cells, peritubular and vascular wall cells, a few interstitial cells, and in the basal part of the epithelia of straight and rete tubules. Developmental changes are only evident in the Sertoli cell vimentin, which is basal and weak at birth and increases moderately until 4 months of age. From around 5 to 8 months of age when the Sertoli cells are under morphological transformation, vimentin intensity is considerably increased and the characteristic vimentin extensions connect the Sertoli nuclei to the basal membrane. These extensions get shorter at around 9 month of age as the Sertoli nuclei are positioned basally. The mature Sertoli cell perinuclear vimentin is strong and stable without infranuclear extension. In conclusion, the age of appearance of α-SMA coincides with the onset of postnatal division of spermatogonia, and vimentin may play a key role in stabilizing Sertoli cell nuclei during their transformation in bovine.

Key words: Actin, Bovine testis, Sertoli cell, Vimentin

The cytoskeleton is an active cytosolic protein framework comprised of microfilaments (actins; diameter: 6–7 nm), intermediate filaments (cytokeratins, desmin, and vimentin; diameter: 8–10 nm), and microtubules (tubulins; diameter: 25 nm). 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 [1]. As a specific marker of smooth muscle differentiation [2], seminiferous peritubular α-smooth muscle actin (SMA) has been detected in the first few postnatal days in the rat testis [3, 4] and in the early postnatal ovine testis [5]. Increasing patterns of α-SMA in the ovine testis [5] and changes in the actin filament arrangement in the rat testis [6] during postnatal development have suggested a relationship for SMA with testicular development and functions. However, no postnatal localization patterns or possible functional relationships for SMA in the bovine testis have been reported so far.

In the testis, Sertoli cells support germ cells and also constitute the seminiferous tubules. Sertoli cells possess a highly organized cytoskeleton, and vimentin, the most common intermediate filament [7, 8], is located around the nucleus and provides it with structural support [5, 9–11]. During pubertal processes, mammalian Sertoli cells undergo transformation [12, 13] that is likely related to cytoskeletal changes [11, 14]. Increases in Sertoli cell vimentin in the prepubertal ovine testis [5] and changes of its distribution area during postnatal development in rats [11] have indicated vimentin activity during postnatal development. Although perinuclear localization of vimentin has been demonstrated in the pre-Sertoli and adult Sertoli cells of the bovine testis [10, 15], its distribution and role during pubertal development are not yet understood. The objectives of the present study were to investigate the postnatal developmental changes in localization of α-SMA and vimentin in the bovine testis and to discuss their functional relationships.

Materials and Methods

The present investigation was carried out on 55 testes (0 months to 7 years old) collected from Holstein bulls (n=33) reared in Hokkaido and Iwate prefectures in Japan. The normally growing scrotal testes (n=8) from unilateral cryptorchid bulls were also included in the study. The testes were collected immediately after slaughter or surgical castration and fixed in Bouin’s solution. Tissue samples were taken from the parenchyma and mediastinum. After dehydration in ascending series of ethanol and paraffin embedding, 5 µm sections were made and mounted on to glass slides. Some sections were stained with hematoxylin-eosin (HE) for observation of general histology.

Immunohistochemical staining of α-SMA and vimentin was carried out using the avidin-biotin peroxidase complex (ABC) method [16]. After deparaffinization, sections were microwaved (500 KW) for 20 min while being dipped into distilled water, and then treated in 0.3% H2O2 in methanol to block the endogenous peroxidase activity. Mouse monoclonal anti-α-smooth muscle actin (1:5000; Clone 1A4, Sigma Chemical Co., St. Louis, USA) and polyclonal anti-calf lens vimentin (1:150; MEDAC, Hamburg, Germany) were used as primary antibodies. The following incubations were carried out in moist chambers after a barrier was created around the sections using a DakoCytomation Pen (DakoCytomation, Kyoto, Japan): a) incubation with normal horse serum (1:50, for monoclonal antibody) or with normal goat serum (1:50, for polyclonal antibody), b) incubation with primary antibodies, c) incubation with secondary antibodies (for monoclonal antibody: biotinylated antibodies to mouse IgG raised in horse, 1:200; for polyclonal antibody: biotinylated antibodies to rabbit IgG raised in goat, 1:200; BA-2000, Vector Laboratories, Inc., Burlingame, CA, USA), and d) incubation with ABC (1:2, PK-6100, Vectastain® Elite ABC Kit, Vector Laboratories, Inc.) followed by time controlled coloring with 0.2% 3,3’-diaminobenzidine tetrahydrochloride (DAB) and 0.005% H2O2 in Tris-HCl buffer (pH 7.4). All incubations were carried out for 30 min at room temperature, except for incubation with primary antibodies, which was conducted overnight at 4 °C. Finally, the sections were counterstained with Mayer’s hematoxylin solution. The control sections were treated with normal horse and normal goat serum instead of α-SMA and vimentin primary antisera, respectively. Similar procedures were followed for all samples.

Results

Summaries of the immunohistochemical localization patterns of α-SMA and vimentin in the bovine testes during postnatal development are shown in Tables 1 and 2. The spermatogenic cells,
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Sertoli cells, and Leydig cells showed negative immunoreactions to α-SMA at all ages. Actin filaments of the walls of blood vessels were always positive (Fig. 1A-H), and were considered as the positive control. Bovine testicular cells that showed developmental changes in their α-SMA immunoreactivities were the seminiferous peritubular myoid cells, sub-epithelial myoid cells of the straight tubules and rete testis, and stromal myoid cells in the rete testis. The immunoreactivities of the peritubular cells were negative from birth (Fig. 1A) until 3 months of age (Fig. 1B). Similar pattern was observed in the straight tubules and rete testis during this period (Fig. 1C). In the peritubular cells of 4-month-old testes, however, an intermittent positive reaction was observed (Fig. 1D). At this age, immunoreactions were weak in sub-epithelia and very weak in stroma of the straight and rete tubules (Fig. 1E). This indicates that α-SMA appears at around 4 months of age in the bovine testis. All testes from 5 months old (Fig. 1F) or older bulls (Fig. 1G) demonstrated regular positive α-SMA around the seminiferous tubules, termed as the mature pattern. However in the straight tubules and rete-testis, the intensity increased gradually until 12 months of age and assumed the mature pattern (Table 1, Fig. 1H).

With regard to vimentin immunoreactions, spermatogenic cells were negative and a few interstitial cells, peritubular myofibrils, and blood vessels were positive with an unchanged pattern throughout postnatal development (Fig. 2A-G). Similarly, the positive immunostaining pattern in the basal part of the epithelia of the straight tubules and rete testis also did not change (Fig. 1H, I).

<table>
<thead>
<tr>
<th>Table 1. Summary of the immunohistochemical localization and intensity of α-smooth muscle actin (SMA) in bovine testes during postnatal development</th>
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<td>Age (month)</td>
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<td>Testes (n=)</td>
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<td>Animals (n=)</td>
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<tr>
<td>Spermatogenic cells</td>
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<td>Sertoli cells</td>
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<td>Interstitial cells</td>
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<td>Peritubular myoid cells*</td>
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<td>Sub-epithelial myoid cells of the straight tubules and rete testis</td>
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<td>Stromal myoid cells of the rete testis</td>
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<td>In blood vessels</td>
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–: Negative; ±: very weak, +: weak, ++: moderate and +++: intensely positive.
*: Intermittent at 4 months of age and regular at 5 months of age, and onward. ND: Not determined.

<table>
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<tr>
<th>Table 2. Summary of immunohistochemical localization and intensity of vimentin in bovine testes during postnatal development</th>
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<tr>
<td>Spermatogenic cells</td>
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<tr>
<td>Sertoli cells (Perinuclear)*</td>
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<tr>
<td>Interstitial cells (a few)</td>
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<tr>
<td>Peritubular myofibrils</td>
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<td>Basal part of the epithelia of the straight tubules and rete testis</td>
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–: Negative; +: weak, ++: moderate and +++: intensely positive.
*: Characteristic infranuclear vimentin extensions from Sertoli cell perinuclei to basal membrane at 5, 7, and 8 months of age. ND: Not determined.
Furthermore, bovine Sertoli cells were the only cells to show remarkable changing patterns during postnatal development. Testes from 0-day-old bulls (at birth) demonstrated weak positive immunoreactivities in the narrow basal perinuclear zone of the Sertoli cells (Fig. 2A) that increased moderately until 4 months of age (Fig. 2B). A remarkable increase in immunostaining intensity and changes in the pattern was observed in 5- to 8-month-old testes. This was characterized by bundles of prominently stained vimentin extending from Sertoli cell nuclei to the basal membrane from 5 months of age (C) until 8 months of age (D) and also at 10 months of age (F), which showed relatively immature seminiferous tubules as compared to 9-month-old testis (E). The perinuclear vimentin was intense, but the infranuclear extensions shortened as the Sertoli cell nuclei were basal at 9 months of age (E) that almost resembled a 14-month-old mature pattern (G). The moderate positive patterns in the basal epithelial part of the straight tubules (arrow heads) and rete testis (big arrows) were similar at 1 month (H) and 12 months of age (I). A few interstitial cells, blood vessel walls, and peritubular cells were weak positive at all ages (A–G). Bars: A and B: 25 µm; C–I: 50 µm.
nuclei were at different distances from the basal membrane. Infranuclear vimentin filaments were observed supporting the Sertoli cell nuclei and connecting them to the basal membrane. A somewhat similar pattern was also observed in the testes of one 10-month-old bull that showed delayed maturity (Fig. 2F). Shortening of vimentin extension was noticed in 8-month and clearly observed in the 9-month-old testis (Fig. 2E) as the mature Sertoli cells appeared with nuclei positioned close to the basal membrane. This was followed by the appearance of the mature pattern of a strong and stable perinuclear Sertoli cell vimentin without any infranuclear extensions, but having a few nuclei bearing a short supranuclear flame (Fig. 2G).

Discussion

The histological observations in our study were generally consistent with previous reports for the bovine testis during postnatal development [17], except for one 10-month-old bull that showed delayed pubertal development. Application of microwave moist heat prior to immunohistochemical procedures probably enhanced the immunoreactive sensitivity in our experiment. Although only qualitative, our results clearly demonstrate the typical postnatal pattern of α-SMA and vimentin distribution in the bovine testis.

As shown in previous reports in the rat [4], monkey [18], human [19], domestic fowl [20], Japanese black bear [21], sheep [5], and bull [10] testis, we demonstrated α-SMA localization in the seminiferous peritubular myoid cells and vascular smooth muscle cells. Moreover, we also demonstrated α-SMA immunoreactions in the straight tubules and rete testis as well. The age of appearance of α-SMA in the bovine testis coincided with the age of initiation of postnatal division of spermatogonia, which starts around 4 months of age [17]. This is in contrast with the postnatal detection of α-SMA in rats [3, 4] and sheep [5], which was observed during the first postnatal week and from early postnatal age, respectively. However, changes in the structural arrangement of peritubular actin filaments from circular to longitudinal have been reported in the rat testis in relation to the advancement of initial spermatogenesis [6]. It has also been reported that structural actin (F-actin) in the bovine testis appears at around 4 weeks of age in peritubular cells [15]. We speculate that the α-SMA isomer appears relatively late. It has been suggested that actin filaments in peritubular myoid cells serve in a contractile capacity aiding the transport of spermatozoa in the tubular lumen [22]. Appearance of α-SMA at around 4 months of age in bovine peritubular myoid cells and immediate attainment of the mature pattern may help to increase contractility of seminiferous tubules and may also secrete some paracrine factors required for regulation of Sertoli cell functions [23] that are vital for spermatogenic process. Moreover, its relative delay in assuming the mature pattern in the rete testis may indicate that spermatozoa enter into the rete testis only during the later stages of puberty. It has previously been reported that appearance of α-SMA in the peritubular cells of monkey testes was induced by androgen [18]. We speculate that androgen, which begins increasing at around 4 months of age in bulls [17, 24], may initiate the appearance of α-SMA and onset of postnatal division of spermatogonia in the bovine testis.

Our finding of Sertoli cell perinuclear localization of the vimentin is consistent with previous reports in the rhesus monkey [25], rat [9, 11, 26], human [11, 27, 28], South American camelids [29], sheep [5], and bull [15]. Our observations of vimentin distribution from day-0 to 5 months of age and in mature bulls are in agreement with the previous studies in the bovine testis [10, 15]. Similarly, as reported in rhesus monkeys [25] and in Japanese black bears [23], we have localized vimentin in a few interstitial cells and vascular walls of the bovine testes. Contrary to the observations in the Sertoli cells of the ground squirrel [30] and rat [11] and in agreement with previous reports in the ovine [5] and bovine [10], we were not able to observe any changes in the distribution of vimentin during the seminiferous cycle. The most striking findings of our study is the demonstration of a considerable increase in the vimentin immunostaining intensity and of changes in its pattern from 5 to 8 months of age when Sertoli cells were undergoing active morphological transformation [12, 17]. The characteristic infranuclear vimentin bundles extending from Sertoli cell perinuclei to the basal membrane during
this stage probably help in stabilizing the differentiating Sertoli cell nuclei, thus preventing dislocation of the nuclei and finally pulling them back close to the basal membrane at around 9 month of age. This is in contrast with a previous study in the bovine testis [15] that reported negative vimentin immunoreactions at 30 and 40 weeks of age. This kind of discrepancy may be due to different embedding media being used in different experiments [15]. Methodological protocols such as the application of microwave heat might have increased the sensitivity of our experiment. Although an increase in vimentin has been reported in the Sertoli cells of prepubertal sheep [5], our results are more consistent with the findings for the postnatal day-14 rat, which had prominently stained vimentin bundles connecting the Sertoli cell nuclei to the basal membrane [11]. However, we were not able to observe supranuclear vimentin, as observed in developing rats [11]. Although we demonstrated a few mature Sertoli cell nuclei with a short supranuclear flame, as reported in mature sheep [5], they were not uniformly distributed and their role was not clear. Our results here demonstrate a more specific role of vimentin in the positioning of bovine Sertoli cell nuclei during postnatal transformation and maturation. Furthermore, the vimentin localization pattern may indicate the maturation status of bovine Sertoli cells, as one 10-month-old bull showing relatively immature seminiferous tubules also demonstrated Sertoli cell infranuclear vimentin extensions similar to transforming Sertoli cells around 5 to 8 month of age. Further study with a larger number of animals around this age may be required to clarify this. The characteristic vimentin localization pattern during pubertal development that was observed for the first time in this study might shed further light on the developmental study of postnatal bovine Sertoli cells.

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References


