Castration Induces Apoptosis in the Mouse Epididymis during Postnatal Development

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Abstract. The effect of castration on apoptosis in the mouse epididymis during postnatal development was examined. The weight of the epididymis slowly increased from day 0 (day of birth) to day 20 after birth, followed by a rapid increase thereafter. Castration on days 0, 5, 10, 20, 30, 40 and 60 increased apoptotic indices (percentages of apoptotic cells) of epithelia of the caput (head), corpus (body), and cauda (tail) epididymis, their apoptotic indices reaching maximal levels on day 2 after castration with the exception of a maximal apoptotic index on day 4 in the tail after castration on day 60. The maximal levels of apoptotic indices of the head, body and tail after castration on days 0, 5, 10 and 20 were significantly lower than those after castration on days 40 and 60. DNAs extracted from the epididymides 2 days after castration on days 0, 5, 10 and 60 showed a ladder pattern on agarose gel electrophoresis, which is a characteristic of apoptosis. When testosterone propionate (10 μg/g body weight) was injected twice a day into mice which had been castrated on day 10, 30 or 60, the increases in apoptotic indices of the head, body and tail of the epididymis were completely inhibited. The weights of the paired epididymides 6 days after castration on days 0, 5, 10, 20, 30, 40 and 60 were significantly lower than those of sham-operated mice, indicating the secretion of androgen by the testes from birth to adulthood. The present results indicated that androgen deprivation caused by castration induces apoptosis in the epithelium of the epididymis of mice from birth to adulthood, and suggested that a proportion of epithelial cells, the survival of which is dependent on the testes, is smaller in the epididymides during a slow growth stage than in the epididymides after this stage.

Key words: Castration, Apoptosis, Epididymis, Mouse, Development

THE epididymis is a single long coiled tubule that connects the efferent ducts to the vas deferens, and is subdivided into four regions: initial segment, caput (head), corpus (body), and cauda (tail) epididymis [1]. The function of the epididymis is not confined to the transport of spermatozoa, and the epididymis plays an important role in maturation of spermatozoa; the intraluminal environment of the epididymis induces spermatozoa to acquire the capacity to ferti-

lize eggs [1–3]. Furthermore, the tail of the epididymis functions as a repository of matured spermatozoa in a metabolically quiescent state [1–3].

The maintenance of the structure and function of the epididymis of mature rodents depends on androgen secreted by the testes; deprivation of androgen by castration induces not only atrophy but also cell death of epithelial cells in the epididymis [2, 4–7]. Fan and Robaire [6] have shown that the castration-induced cell death in the epithelium of the epididymis occurs as apoptosis as in the epithelia of other accessory sex organs such as the ventral prostate and seminal vesicles [8–12]. Although deprivation of androgen induces epithelial apoptosis in the epididymis of mature rodents, not all epithelial cells die and

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a part of the epithelial cells survives even under deprivation of androgen as shown in the rat ventral prostate [13]. This means that there are two populations of epithelial cells in the epididymis of mature rodents in terms of androgen dependency for their survival.

The rodent epididymis grows not only after puberty but also before puberty [5, 14], and its growth is dependent on androgen secreted by the testes [15, 16]. Consistent with androgen dependency of the growth of the rodent epididymis before puberty, it is reported that the rodent testes before puberty secrete androgen as those after puberty [14, 17–19], and that androgen concentrations in the epididymides of prepubertal mice are comparable to those in the epididymides of postpubertal mice [14]. Although androgen-dependent growth of the rodent epididymis before and after puberty has been shown [15, 16], the role of androgen secreted by the testes in the survival of epithelial cells of the rodent epididymis during postnatal development has not yet been examined. Therefore, we investigated whether the survival of epithelial cells in the epididymis during postnatal development is dependent on androgen secreted by the testes. For this purpose we examined the effects of castration at various ages on apoptosis of epithelial cells of the mouse epididymis.

Materials and Methods

Mice

BALB/c mice 2-3 months old were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Female and male mice were kept together in cages, and when mice became pregnant, they were put into individual cages, and examined for birth of offsprings at 12 a.m. every day. All male newborn mice were collected and 6 mice were nursed by each mother in a cage. The day of birth was designated as day 0 of age. Mice were kept at 25°C under controlled light conditions (12 h light/12 h darkness), and allowed free access to water and a pellet diet. Castration of mice, which had been anesthetized by cooling on ice, was carried out on days 0 and 5 via the abdominal route using a dissection microscope according to the procedure described by Pfeiffer [20], and castration of mice from day 10 on was carried out via the scrotal route under pentobarbital anesthesia. The sham-operation was carried out by pulling out the testes with the epididymides and putting them back where they were without removing the epididymides. All experiments with mice were approved by the Animal Care Committee of Hyogo College of Medicine, and all mice were treated with humane care.

Injection of testosterone propionate (TP)

TP was suspended at a concentration of 10 µg/0.01 ml of vehicle (0.9% (w/v) NaCl, 0.4% (v/v) Tween 80, 0.5% (w/v) carboxymethylcellulose, and 0.9% (v/v) benzylalcohol), and TP at a dose of 10 µg/g body weight was injected subcutaneously into the interscapular space twice a day.

Growth of the epididymis

Mice castrated on day 0 or non-castrated mice were killed by decapitation on days 0, 10, 20, 30, 40, 60 and 65, and a pair of epididymides of each mouse was removed and weighed.

Effect of castration at various ages on apoptotic index of epithelium

Mice were castrated on days 0, 5, 10, 20, 30, 40 and 60 and were killed on day 0 (day of castration) and on days 2, 4 and 6 after castration by decapitation. A pair of epididymides of each mouse was then promptly removed and weighed. The epididymides were fixed in phosphate (0.15 M) buffered 10% formalin (pH 7.4). After fixation, the epididymis was cut into three regions, i.e., head, body and tail, and each region was embedded in paraffin separately. Transverse sections (5 µm thick) of each region were prepared and stained with hematoxylin and eosin. About 1,000 epithelial cells in tissue sections were examined, and the percentage of apoptotic cells was defined as the apoptotic index. Apoptotic cells were identified by well-established criteria [21, 22]. Cells with the following characteristics were regarded as apoptotic cells: shrunken cells with fragments of condensed nuclei and eosinophilic-condensed cytoplasm, and cells with cytoplasmic fragments containing condensed chromatin.
Effect of TP injections on apoptotic index

Mice on day 10, 30 or 60 were castrated at 9 a.m. Castrated mice were divided into two groups: one group was injected with TP (10 μg/g body weight) twice a day (at 9 a.m. and 5 p.m.), and the other group was given no injection. These mice were killed at 10 a.m. on days 2, 4 and 6 after castration (day 0: the day of castration), and the apoptotic index of each portion of the epididymis was determined as described above.

Weight of paired epididymides after castration and sham-operation

Mice were castrated or sham-operated on days 0, 5, 10, 20, 30, 40 and 60 and killed 6 days after the operation by decapitation, and a pair of epididymides was weighed.

Analysis of DNA fragmentation by agarose gel electrophoresis

Mice were castrated on days 0, 5, 10 and 60 and killed 2 days after castration by decapitation. The epididymides were then promptly removed and weighed. DNA was extracted using Isotissue DNA extraction kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer’s instructions. About 50 mg of the epididymides was used for DNA extraction. Five micrograms of the extracted DNA was labeled with 0.5 μCi of α-32P-deoxyctydine triphosphate (3000 Ci/mm; Amersham, Little Chalfont, Buckinghamshire, England) using a Tacs apoptotic DNA laddering kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer’s instructions. After precipitation of the labeled DNAs in the presence of glycogen and transfer RNA, the labeled DNAs were electrophoresed in 3% (w/v) agarose gel at 100 V. After electrophoresis, the gel was dried without heating in a slab-gel dryer and exposed to a Kodak X-Omat film at −80°C as described by Tilly and Hsueh [23].

Statistical analysis

Data are presented as mean ±SE. Data of 3 and more than 3 groups were analyzed with Dunnet’s t-test for multiple comparison. Data of two groups were analyzed with Student's t-test. A P value less than 0.05 was considered significant.

Results

Growth of the epididymis

Fig. 1 shows the weights of the paired epididymides of intact mice and mice castrated on day 0, from birth to day 65. The weights of intact mice increased slowly from day 0 to day 20, and rapidly from day 20 on, reaching a maximal level on day 60. When the histology of the epididymis was examined, spermatozoa were found in the epididymis from day 40 on. By contrast, the weights of the paired epididymides of castrated mice were below 2 mg from birth to day 65, and were significantly lower than those of intact mice.

Effect of castration on apoptosis in epithelium of epididymis

To examine dependence of epithelial survival on testicular androgen at various developmental stages of mice, mice were castrated on days 0, 5, 10, 20, 30, 40 and 60, and the apoptotic index (percentage of

![Graph](image-url)

**Fig. 1.** Weights of the paired epididymides of intact mice and mice castrated on day 0 after birth. Weights of the paired epididymides of intact mice (○—○) or mice castrated on day 0 after birth (●—●) are shown. Each point represents a mean ±SE of 3–10 mice.

*P<0.05, Significant difference from the values of castrated mice by Student’s t-test.
apoptotic cells) was determined in epithelia of the head, body and tail of the epididymis on day 0 (day of castration) and on days 2, 4 and 6 after castration.

Tables 1, 2 and 3 show apoptotic indices of epithelia in the head, body and tail of the epididymis, respectively. Castration at any age increased their apoptotic indices on day 2 after castration. Apoptotic indices of the epithelia of the head, body and tail reached maximal levels on day 2 after castration except for the apoptotic index of the tail after castration on day 60. Average apoptotic indices after castration were higher on day 4 than on day 2 in the head after castration on days 5 and 10, and in the body after castration on days 5 and 60. However, there was no significant difference between apoptotic indices on days 2 and 4 ($P > 0.05$, by Dunnet’s $t$-test). The apoptotic index of the tail after castration on day 60 became maximal only on day 4 ($P > 0.05$, by Dunnet’s $t$-test).

Fig. 2 shows an apoptotic cell in the epithelium of

<table>
<thead>
<tr>
<th>Table 1. Effect of castration at various ages on apoptotic index of the head of the epididymis.</th>
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<tbody>
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<td><strong>Age of castration</strong></td>
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<td>Day 0</td>
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<td>Day 5</td>
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<td>Day 40</td>
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<td>Day 60</td>
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</table>

Male mice were castrated on day of birth (day 0) and on days 5, 10, 20, 30, 40 and 60 after birth, and the apoptotic index of the epithelium in the head of the epididymis was determined on day of castration (day 0) and on days 2, 4 and 6 after castration. Numbers in parentheses indicate numbers of mice.

$^a$ mean ± SE.

$^b$ $P < 0.05$, Significant increase from the values on day 0 (day of castration) by Dunnet’s $t$-test.

<table>
<thead>
<tr>
<th>Table 2. Effect of castration at various ages on apoptotic index of the body of the epididymis.</th>
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<td><strong>Age of castration</strong></td>
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<td>Day 0</td>
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<td>Day 40</td>
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<td>Day 60</td>
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</tbody>
</table>

Male mice were castrated on day of birth (day 0) and on days 5, 10, 20, 30, 40 and 60 after birth, and the apoptotic index of the epithelium in the body of the epididymis was determined on day of castration (day 0) and on days 2, 4 and 6 after castration. Numbers in parentheses indicate numbers of mice.

$^a$ mean ± SE.

$^b$ $P < 0.05$, Significant increase from the values on day 0 (day of castration) by Dunnet’s $t$-test.
Table 3. Effect of castration at various ages on apoptotic index of the tail of the epididymis.

<table>
<thead>
<tr>
<th>Age of Castration</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>0.3±0.2 (6)</td>
<td>2.7±0.3 (12)(^a)</td>
<td>1.3±0.2 (9)(^b)</td>
<td>1.3±0.2 (14)(^b)</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.9±0.1 (8)</td>
<td>2.6±0.6 (7)(^b)</td>
<td>2.0±0.4 (8)</td>
<td>2.0±0.5 (7)</td>
</tr>
<tr>
<td>Day 10</td>
<td>0.7±0.1 (6)</td>
<td>2.7±0.3 (10)(^b)</td>
<td>1.7±0.3 (6)(^b)</td>
<td>1.5±0.3 (9)</td>
</tr>
<tr>
<td>Day 20</td>
<td>0.7±0.2 (8)</td>
<td>3.6±0.5 (5)(^b)</td>
<td>1.7±0.3 (5)</td>
<td>1.2±0.4 (4)</td>
</tr>
<tr>
<td>Day 30</td>
<td>1.2±0.2 (7)</td>
<td>5.0±0.3 (9)(^b)</td>
<td>2.6±0.3 (5)(^b)</td>
<td>1.0±0.1 (7)</td>
</tr>
<tr>
<td>Day 40</td>
<td>0.2±0.02 (6)</td>
<td>10.3±0.7 (5)(^b)</td>
<td>2.9±0.6 (6)(^b)</td>
<td>2.2±0.3 (7)(^b)</td>
</tr>
<tr>
<td>Day 60</td>
<td>0.4±0.1 (12)</td>
<td>4.6±0.6 (12)(^b)</td>
<td>9.9±1.0 (12)(^b)</td>
<td>1.9±0.5 (13)</td>
</tr>
</tbody>
</table>

Male mice were castrated on day of birth (day 0) and on days 5, 10, 20, 30, 40 and 60 after birth, and the apoptotic index of the epithelium in the tail of the epididymis was determined on day of castration (day 0) and on days 2, 4 and 6 after castration. Numbers in parentheses indicate numbers of mice.

\(^a\) mean±SE.

\(^b\) \(P<0.05\), Significant increase from the values on day 0 (day of castration) by Dunnet’s t-test.

Fig. 2. Apoptotic cell in the epithelium in the tail of the epididymis 2 days after castration on day 60. Arrow indicates an apoptotic cell. Hematoxylin & eosin, ×912.

The epididymis 2 days after castration on day 60. It showed characteristics of apoptotic cells, i.e., shrunk cells with fragments of condensed nuclei and eosinophilic-condensed cytoplasm. To confirm occurrence of apoptosis after castration, DNAs were extracted from the epididymides of mice castrated on days 0, 5, 10 and 60 after birth, on day 2 after castration, and DNA fragmentation was examined by agarose gel-electrophoresis (Fig. 3). An agarose gel electrophoresis of DNAs from these mice showed a ladder pattern which is characteristic of apoptosis [21, 22, 24].

To examine whether castration-induced apoptosis in the epididymis is due to androgen deprivation, TP

Fig. 3. Agarose gel electrophoresis pattern of DNAs extracted from the epididymis. Mice were castrated on day 0, 5, 10 and 60 after birth, and the epididymides were removed 2 days later. DNAs (5 μg) extracted from the epididymides were labeled with \(^32\)P-deoxyctydine triphosphate, and electrophoresed in 3% (w/v) agarose gel.
at a dose of 10 μg/g body weight was injected twice a day into mice which had been castrated on day 10, 30 or 60 (Fig. 4). TP injection completely inhibited increases in apoptotic indices of the head, body and tail of the epididymis.

Apoptotic indices reached maximal levels on day 2 after castration except for the apoptotic index of the tail after castration on day 60 (Tables 1–3). To compare the extent of apoptosis after castration at various ages, the apoptotic indices on day 2 were illustrated (Fig. 5). The apoptotic indices of the head 2 days after castration on days 40 and 60 were similarly high, and were significantly higher than those after castration on days 0, 5, 10, 20 and 30. The apoptotic index of the tail 2 days after castration on day 40 was highest, and was significantly higher than those after castration on days 0, 5, 10, 20, and 30. The apoptotic index of the tail 2 days after castration on day 60 was significantly higher than those after castration on days 0, 5, and 10, but not higher than those after castration on days 20 and 30. However, when the maximal apoptotic index of the tail after castration on day 60, i.e., the apoptotic index 4 days after castration, was compared to apoptotic indices 2 days after castration at various ages (Table 3), it was significantly higher than apoptotic indices 2 days after castration on days 0, 5, 10, 20, and 30 (P<0.05, by Dunnet’s t-test).

![Graph showing apoptotic index of head, body, and tail after castration on day 10, 30, and 60.](image)

**Fig. 4.** Effect of androgen replenishment on castration-induced apoptosis in the epididymis. Mice on day 10, 30 or 60 were castrated at 9 a.m., and these mice were divided into 2 groups. One group (●—●) was injected with TP (10 μg/g body weight) twice a day (at 9 a.m. and 5 p.m.), and the other group (○—○) was not. These mice were killed at 10 a.m. on days 2, 4 and 6 after castration (day 0: day of castration), and an apoptotic index of each portion of the epididymis was determined. Each point represents a mean±SE of 5–13 mice.

* a P<0.05, Significant difference from the values on day 0 by Dunnet’s t-test.

* b P<0.05, Significant difference from the values of mice that received TP injections by Student’s t-test.
Fig. 5. Apoptotic indices of epithelia in the head, body and tail of the epididymis on day 2 after castration. Apoptotic indices on day 2 after castration shown in Tables 1, 2 and 3 are illustrated. Open (□), hatched (□) and black (■) bars indicate the apoptotic indices of the head, body and tail of the epididymis, respectively. Each bar indicates a mean ± SE.

\* P<0.05, Significant difference from the values for day 40 by Dunnet’s t-test.

\* P<0.05, Significant differences from the values for day 60 by Dunnet’s t-test.

Table 4. Effects of castration at various ages on the weights of the paired epididymides on day 6 after castration.

<table>
<thead>
<tr>
<th>Age of the operation</th>
<th>Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Castrated mice</td>
</tr>
<tr>
<td>Day 0</td>
<td>1.5 ± 0.1 (4)</td>
</tr>
<tr>
<td>Day 5</td>
<td>3.0 ± 0.2 (6)</td>
</tr>
<tr>
<td>Day 10</td>
<td>3.4 ± 0.1 (5)</td>
</tr>
<tr>
<td>Day 20</td>
<td>6.1 ± 0.4 (4)</td>
</tr>
<tr>
<td>Day 30</td>
<td>11.2 ± 1.5 (6)</td>
</tr>
<tr>
<td>Day 40</td>
<td>13.8 ± 1.3 (4)</td>
</tr>
<tr>
<td>Day 60</td>
<td>25.1 ± 0.6 (6)</td>
</tr>
</tbody>
</table>

Male mice were castrated or sham-operated at various ages, and the paired epididymides of these mice were weighed on day 6 after the operation. Numbers in parentheses indicate numbers of mice.

\* mean ± SE.

\* P<0.01, Significant difference from the values of castrated mice by Student’s t-test.

Effect of testes on weight of paired epididymides

To examine the androgen secretion by the testes of mice from birth to adulthood, the weights of the paired epididymides of mice castrated and sham-operated on days 0, 5, 10, 20, 30, 40 and 60 were compared 6 days after the operation (Table 4). The weights of the epididymides of castrated mice were significantly lower than those of sham-operated mice regardless of ages of castration.

Discussion

Apoptotic cells were morphologically identified on tissue sections stained with hematoxylin and eosin according to well-established criteria, i.e., shrunken cells with fragments of condensed nuclei and eosinophilic-condensed cytoplasm, and cells with cytoplasmic fragments containing condensed chromatin [21, 22]. Epithelial cells in the head, body and tail of the epididymis contain several types of cells: principal, basal, clear, apical and halo cells [1, 25, 26]. Halo cells are small round cells with dense nuclei and pale cytoplasm [1, 26], and are considered to be lymphocytes or monocytes [26]. However, apoptotic cells can be distinguished from halo cells, and cannot be mistaken for them. The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) method has been widely used as another method of identifying apoptotic cells [27]. This method is assumed to be more sensitive for detecting apoptotic cells than morphological identification because it can also detect apoptotic cells with DNA fragmentation which do not show the morphological characteristics. However, our experiences show that treatment with proteinase K often affects the number of positive cells, and that apoptotic cells with morphological characteristics are rather hard to stain with TUNEL method. Furthermore, false positive cells have been reported in other
tissues [28, 29]. Therefore, for the quantitative estimation of apoptosis in many tissue sections, apoptotic cells were identified morphologically on tissue sections stained with hematoxylin and eosin.

Castration on days 0–60 increased apoptotic indices of epithelia in the head, body and tail of the mouse epididymis. Furthermore, agarose gel electrophoresis of DNAs extracted from the epididymides of mice castrated on days 0, 5, 10 and 60 showed a ladder pattern which is characteristic of apoptosis [21, 22, 24]. Therefore, these results indicate that castration induces apoptosis in the epithelium of the mouse epididymis during postnatal development as well as in adulthood.

Fan and Robaire [6] reported that castration of mature rats caused apoptosis in the epithelium of the epididymis, and that the time course and the extent of apoptosis differed among the regions of the epididymis. In mature rats, apoptosis occurred first in the head, subsequently in the body and finally in the tail, and the extent of apoptosis was much greater in the head than those in the body and tail. In the present study with mice, the peak apoptotic index was delayed in the tail, but there was no apparent regional difference in the extent of apoptosis. Fan and Robaire [6] determined the number of apoptotic cells per 100 tubules or the percentage of apoptosis-positive tubules for quantitative estimation of apoptosis, while we determined the percentage of apoptotic cells in the epithelium. Although there is a difference in the method for quantitative estimation of apoptosis, the different results may be ascribed largely to species difference.

Expression of androgen receptors has been shown in both the epithelium and stroma of the epididymides of mice at birth [30]. Furthermore, it has been reported that mouse testes secrete androgen during postnatal development [14, 19], and that the androgen concentrations (testosterone plus dihydrotestosterone) in the mouse epididymides during postnatal development are comparable to those in the mouse epididymides in adulthood [14]. These studies suggest that androgen secreted by the testes works on the epididymis during postnatal development. In fact, there was a significant difference in the weight of the paired epididymides between castrated and sham-operated mice on day 6 after the operation even if castration was performed during postnatal development, indicating the action of androgen secreted by the testes on the mouse epididymis during postnatal development. In addition, injections of testosterone propionate into mice castrated on day 10, 30 or 60 completely inhibited an increase in the apoptotic index. Therefore, it is assumed that castration-induced apoptosis during postnatal development is due to androgen deprivation. The epididymis receives androgen by two routes: via the bloodstream and via testicular fluid [31]. However, Jean Faucher et al. [32] have shown that in mature mice about 85% of the androgen reached the epididymis by way of the bloodstream. Therefore, androgen reaching via the bloodstream seems to be the most important factor for epithelial survival of the mouse epididymis.

The extent of apoptosis induced by castration before day 30 was apparently less than that by castration after day 30, when the extent of apoptosis was estimated by comparing the maximal apoptotic indices. As reported in the ventral prostate [13], the epithelial cells of the epididymis contain androgen-dependent and androgen-independent cells in terms of their survival. Therefore, this result indicates that the epithelium of the mouse epididymis before day 30 contains a larger population of androgen-independent cells in terms of their survival than that after day 30.

The maximal apoptotic indices in the body and tail after castration on days 40 and 60 were significantly higher than those after castration on day 30, but the maximal apoptotic indices in the head were not significantly higher than that after castration on day 30. This implies that the epithelium of the head matures earlier than the body and tail in terms of androgen-dependent survival of epithelial cells.

Various gene products, such as c-fos, transforming growth factor-β, p53, Bax, Bcl-2, and Fas have been reported to be involved in castration-induced apoptosis in the rodent male accessory sex organs [10, 11, 33, 34]. In addition, the role of vascular regression after castration has been emphasized in the initiation of apoptosis [35]. The study of the roles of these gene products and vascular regression in castration-induced apoptosis of the epididymis may provide some insights into differences in apoptosis during postnatal development and in adulthood.
Acknowledgment

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