Expression and Roles of Survivin in Experimental Rat Cryptorchidism

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ABSTRACT. Objectives: Cryptorchidism is one of the most common causes of infertility in men. In cryptorchidism, although it has been established that there is spermatogenetic disorder because of increasing apoptosis, the molecular mechanisms responsible have not yet been fully elucidated. The production of sperm is regulated by a balance between proliferation and apoptosis. Survivin is one of the inhibitors of the apoptosis protein (IAP) and its expression seems to correlate with not only apoptosis but also proliferation during spermatogenesis. We established experimental cryptorchid rats and investigated expression and the possible role of survivin in the cryptorchid testis.

Methods: Eight-week-old male Sprague-Dawley rats, weighing approximately 260-300g, were maintained, and experimental cryptorchid rats were established from among them. The animals were divided into four groups. One group was sacrificed to be evaluated without any surgical intervention (normal control, n=5). The other three groups were sacrificed following 3, 7 and 14 days of cryptorchidism (n=3 × 5). Johnsen's score for the rat and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay was used for evaluation of spermatogenesis and apoptosis, respectively. Survivin expression in experimental cryptorchidism was examined by the reverse transcription-polymerase chain reaction (RT-PCR), Western blot and immunohistochemistry.

Results: Apoptotic germ cells peaked early after cryptorchidism surgery (day 3) and testicular weight and Johnsen's score decreased progressively with longer periods of cryptorchidism. In the normal control and sham-operated testes, abundant expression of survivin protein was observed. On the other hand, the expression in cryptorchid testes decreased progressively with longer periods of cryptorchidism. As for immunohistochemistry, nuclear and cytoplasmic localization was observed in the normal control and sham-operated testes and the cytoplasmic stain did not decrease. In the cryptorchid testes, however, the nuclear stain became progressively stronger.

Conclusion: Survivin expression in testes could be used in the evaluation of spermatogenic disorders such as cryptorchidism.

Key words ① **survivin** ② **apoptosis** ③ **proliferation** ④ **spermatogenesis** ⑤ **cryptorchidism** ⑥ **testis** ⑦ **Sprague-Dawley rat**

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The production of sperm is regulated by a balance between proliferation and apoptosis[1]. It is estimated that 25-75 % of germ cells degenerate spontaneously during normal spermatogenesis[2,3]. Germ cell apoptosis is important in the regulation of the production of sperm[4]. Although apoptosis is a common phenomenon during spermatogenesis, its regulation has not been fully elucidated[5].

Survivin belongs to a group of inhibitors of apoptosis protein (IAP) that includes XIAP, c-IAP and c-IAP2. It contains a single baculovirus IAP repeat (BIR) domain and lacks a carboxyl-terminal RING finger. It is structurally distinct from most other IAP members[6) ~8]. Survivin expression is found during fetal development, embryogenesis and in the majority of cancers. Recently, its expression has also been found in growing tissues such as the testis, thymus and intestine[9) ~11].

Weiker *et al*[11]. reported finding survivin in normal human spermatogenesis, but also found a lack of survivin in infertile men with pre-meiotic maturation arrest and Sertoli-cell-only syndrome. Wang *et al*[12]. suggested that survivin expression in the rat testis is functionally linked to the dynamics of the germ cell population.

Cryptorchidism is one of the most common causes of infertility in men. It has been established that in cryptorchidism spermatogenetic disorder is caused by increasing apoptosis[12), 13]. Yin *et al*[14] reported that Fas-dependent and p53-dependent testicular germ cell apoptosis appeared in experimental cryptorchidism. The Bcl-2 family has also been shown to be involved in this apoptosis[15]. But it is still uncertain whether survivin is related to germ cell apoptosis in cryptorchidism.

In this study, against this background, we established experimental cryptorchid rats and investigated the expression and possible role of survivin in the cryptorchid testis using the reverse transcription-polymerase chain reaction (RT-PCR), Western blot and immunohistochemistry. We also evaluated the relationship between survivin expression and spermatogenesis.

MATERIALS AND METHODS

Animals and experimental procedure

Eight-week-old male Sprague-Dawley rats, weighing approximately 260-300g, were maintained under controlled temperature (22 ℃) and constant photoperiod conditions (12 hrs of light and 12 hrs darkness) with free access to water and rat chow. The animals were anesthetized with sodium pentobarbital and surgically induced with unilateral cryptorchidism. One testis was exposed through an incision above the inguinal canal. The epididymal fat pad was grasped with blunt forceps, with care being taken to avoid damage to the artery, and the testis was transferred through the inguinal canal into the abdominal cavity. Sham surgery was performed on the other testis, which was used as a control, by opening the inguinal canal and handling the epididymal fat. The animals were divided into four groups. One group was sacrificed to be evaluated without any surgical intervention (normal control, n=5). The other three groups were sacrificed following 3, 7 and 14 days of cryptorchidism (n=3 × 5). All animals were asphyxiated with CO2, and all testes were removed. These experiments were approved by the Animal Research Committee of Kawasaki Medical School and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

Evaluation of spermatogenesis

The testes samples were fixed in Bouin's solution for 4 hrs and embedded in paraffin. Sections (5 μm thick) stained with hematoxylin were observed under a light microscope. We evaluated the spermatogenesis of the seminiferous tubules using Johnsen' s score[17),18)]. This scoring system was advocated by Johnsen[19)] for evaluating the maturation of human seminiferous tubules. It is based on histologic findings of the testes and specifically focuses on the maturity of germ cells in the seminiferous tubules. Johnsen's score for the rat was devised by Lewis-Johnes and Kerrigan.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay and evaluation of apoptosis

The testes samples were fixed in 10% buffered formaldehyde and embedded in paraffin, and then were cut into 5 μm thick sections. The TUNEL assay was applied for histopathological apoptotic cell analysis using the Apoptosis Detection Kit (Chemicon, USA). The samples were incubated with a biotin conjugated monoclonal anti-digoxin (Sigma, Saint Louis, USA), counterstained with diaminobenzidine (DAB) and detected using a light microscope. Negative control sections were processed without TdT into the Apoptosis Detection Kit. TUNEL-positive nuclei were evaluated using an apoptosis index (AI), calculated by dividing the total number of labeled germ cells by total number of tubular cross-sections examined[20)]. A total of at least 50 cross-sections of circular seminiferous tubules per sample were counted.

RT-PCR analysis

Total RNA was extracted using the RNA isolation reagent (Tel-test, Texas, USA). The testes samples were homogenized in the RNA isolation reagent using Polytron homogenizer. cDNA was prepared from 1 μg total RNA with a first strand cDNA synthesis kit (Fermentas) and PCR reactions were performed with a Program Temperature Control System (ASTEC, Fukuoka, Japan). The primer sequences for survivin were 5'-CTGCACCCCAGAGCGGATGG-3' (forward primer) and 5'-CCCCACCCATAGATCCTGTCAGAGA-3' (reverse primer). Amplification of survivin cDNA was carried out for 30 cycles of denaturation at 94 ℃ for 30 sec, annealing at 65 ℃ for 30 sec, and elongation at 72 ℃ for 3 min. The primer sequences for β-actin were 5' -TGAGGGGGTCACCCACACTGTGCCCATCTA-3' (forward primer)and 5' -CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' (reverse primer). PCR products were visualized in 1.2 % agarose gel stained with ethidium bromide.

Western blot analysis

The testes samples were homogenized in ice-cold RIPA buffer containing 10μl/ml of a protease inhibitor cocktail (Sigma, Helsinki, Finland). The homogenized tissues were centrifuged at 13,000 × g for 20 min at 4 ℃ to extract whole testes protein. The total protein concentrations of supernatant extracts were measured using the Bio-Rad Protein Assay (Bio-Rad, USA). Samples containing 20 μg of total protein were loaded on 5-20 % stacking gel (Atto, Tokyo, Japan) and electrotransferred to a PVDF membrane. The membrane was blocked, at room temperature, in PBS-T (NaCl 137mM, KCl 2.7mM, $Na_2HPO_4 \cdot 12H_2O$ 8.1mM, KH_2PO_4 1.5mM, Tween-20 0.1%) containing 5 % non-fat skim milk powder, for 1 hr. The primary Ab, which is a mouse monoclonal anti-survivin Ab (Santa Cruz), was used at a dilatation of 1/300 for 16 hrs at 4 ℃. The secondary Ab, which is a goat anti-mouse Ab (Santa Cruz), was used at a dilatation of 1/1000 for 1hr at room

temperature. The membrane was revealed using a chemiluminescent reagent (Amersham Biosciences), and was stripped by incubation for 1 hr at 60 ℃ and reprobed, as above, using a monoclonal mouse anti-GAPDH primary Ab (Santa Cruz) at a dilatation of 1/300 and a goat anti-mouse secondary Ab.

Immunohistochemistry (IHC)

The testes samples were fixed in 10% buffered formaldehyde, embedded in paraffin, and cut into 5 μm thick sections. Immunohistochemical staining was performed using XT-Discovery (Ventana, Kanagawa, Japan) of the automatic IHC processing system with the indirect biotin-avidin method. Antigen retrieval was performed in citrate buffer with steam for 1 hr at 100 ℃. The rabbit monoclonal anti-survivin Ab (Cell Signaling) at a dilatation of 1/100 was used as the primary Ab. Biotinylated goat anti- rabbit secondary Ab at a dilatation of 1/200 was used. After developing color with DAB, the sections were counterstained with hematoxylin. In negative control sections, survivin blocking peptide (Cell Signaling) was added to the primary Ab.

RESULTS

Eight-week-old Sprague-Dawley rats were surgically induced with unilateral cryptorchidism, and sacrificed at 3, 7 and 14 days after surgery. Table 1 shows a comparison of testicular weight, Johnsen's score for rats and the AI between cryptorchid and control testes. Testicular weight and Johnsen's score decreased progressively with longer periods of cryptorchidism. The histological sections and TUNEL analysis showed dynamic changes in spermatogenesis and germ cell apoptosis, respectively (Fig. 1, Fig. 2). The change in impaired spermatogenesis was apparent progressively. Multinucleared giant cells were observed at days 3 and 7 of cryptorchidism (data not shown at day 3), and AI peaked at day 3 of cryptorchidism. Subsequently, AI

Table 1. Deceasing testicular weight and Johnsen's score for rats and an increasing apoptosis index in time-dependent manner. Values are presented as mean. († $p < 0.01$, vs. sham operated control, Mann-Whitney U-test)

	Day 0	Day 3	Day 7	Day14
Testicular weight (g)				
Normal control	1.67	—	—	—
Sham operated control	—	1.60	1.62	1.81
Cryptorchidism	—	1.55	0.99 †	0.74 †
Johnsen's score for rat				
Normal control	9.40	—	—	—
Sham operated control	—	9.20	9.20	9.60
Cryptorchidism	—	9.00	4.80 †	3.60 †
Apoptosis index (AI)				
Normal control	0.15	—	—	—
Sham operated control	—	0.42	0.45	0.34
Cryptorchidism	—	12.18 †	8.62 †	0.71

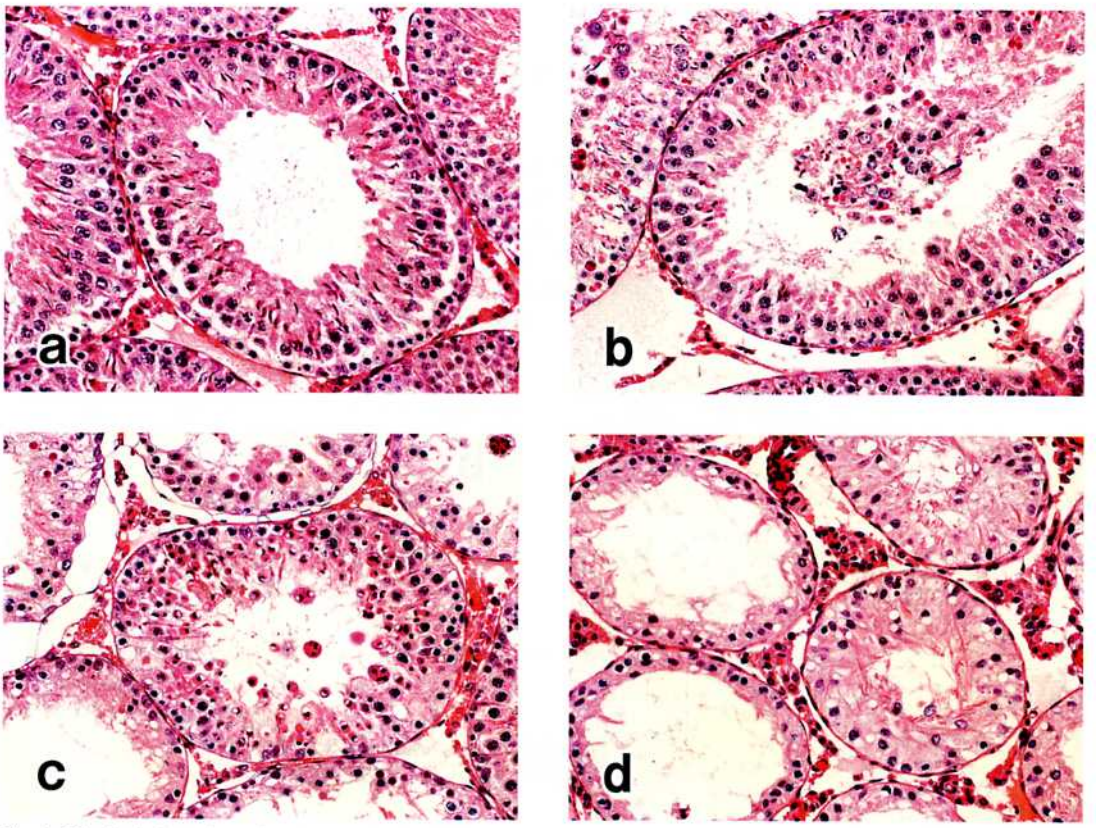

Fig. 1. Histological sections of rat testes stained with hematoxylin. (a) Normal control (day 0) testis: full spermatogenesis lying within the tubular lumen was observed (J-S 10). (b) Cryptorchid testis at day 3: almost full spermatogenesis was observed, but slough and obliteration of the lumen were also noted (J-S 9). (c) Cryptorchid testis at day 7: immature spermatids randomly arranged throughout the tubule were observed (J-S 5). Multinucleared giant cells were seen. (d) Cryptorchid testis at day 14: only a few spermatocytes were observed and no spermatid or spermatozoa were noted (J-S 4). J-S; Johnsen's score for rat

showed a decline at day 7 and reached a nadir at day 14.

Survivin mRNA expression between the cryptorchid and control testes was compared (Fig. 3). High survivin expression was exhibited in the normal control and sham-operated testes. There was no significant difference in the expression level between the two groups at day 3, but the survivin expression had decreased in the cryptorchid testes at days 7 and 14.

A major survivin protein product of about 16.5 kDa was revealed by Western blotting. Next, we performed Western blotting using a monoclonal anti-survivin Ab. Survivin protein expression between the cryptorchid and control testes was compared (Fig. 4). There was abundant expression of survivin protein in the normal control and sham-operated testes. In the cryptorchid testes, the expression began decrease at day 3 and decreased progressively with longer periods of cryptorchidism. A change in the expression of the cryptorchid testes began soon after surgery and was considerable. In that respect, reduction of survivin protein was occurred faster than diminished down-regulation of the mRNA.

Immunohistochemical survivin expression and localization between the cryptorchid and control testes were compared (Fig. 5). As with Western blotting, survivin protein expression was observed abundantly in

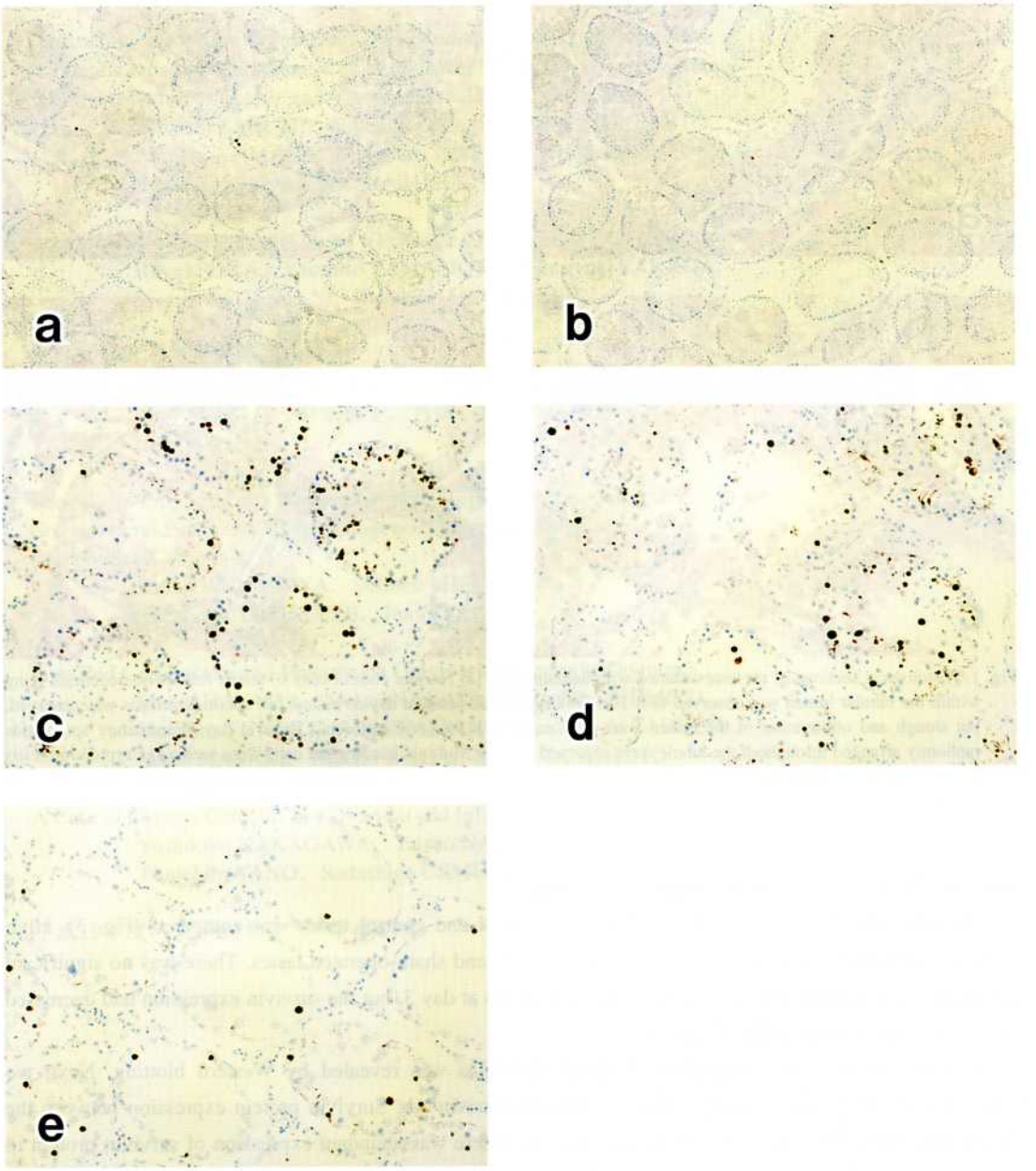

Fig. 2. TUNEL analysis of germ cells in cryptorchid testes. In the cryptorcild testis at day 3, apoptotic germ cells were observed. At day 14, only a few apoptotic germ cells were seen. normal control (a), sham-operated control at day 3 (b) , cryptorchid testes at day 3 (c), day 7 (d) and day 14 (e).

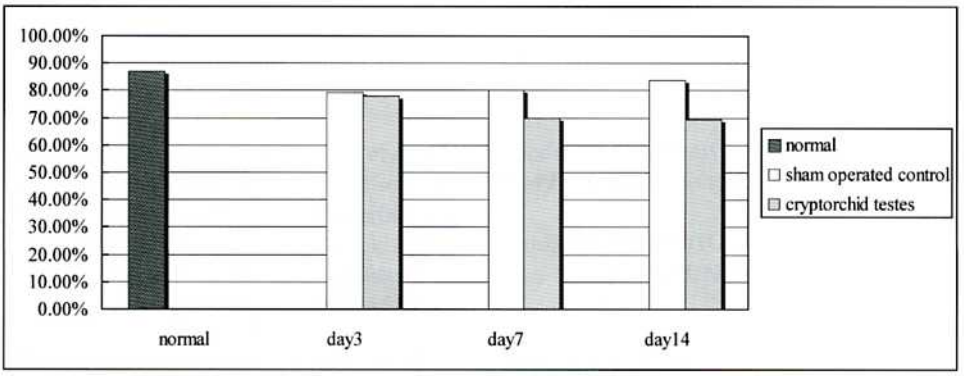

Fig. 3. Survivin mRNA expression of cryptorchid testes decreased progressively. Survivin expression was analyzed by the RT-PCR. The relative expression levels of survivin mRNA were normalized against that of β-actin. Densitometric analysis was performed using Dolphin 1D (Kurabo, Japan).

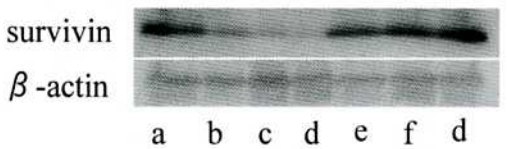

Fig. 4. Survivin protein expression decreased more rapidly than the mRNA expression. Western blot analysis of survivin expression in normal (a), cryptorchid testes at day 3 (b), day 7 (c), day 14 (d) and sham-operated at day 3 (e), day 7 (f), day 14 (g). A survivin protein product of about 16.5 kDa was observed. The blot was stripped and reprobed with a monoclonal anti-GAPDH Ab.

the normal control and sham-operated testes. Survivin protein was localized in both the nuclei and cytoplasm. The labeled germ cells were mostly spermatocytes, with a few spermatogonia and immature spermatids being noted. There was no signal in mature spermatids. Such a difference in the expression in the step of spermatogenesis was also seen in the cycle of the seminiferous epithelium. In the cryptorchid testes, survivin protein expression and the cytoplasmic stain did not decrease but the nuclear stain became progressively stronger with longer periods of cryptorchidism. In the cryptorchid testes at day 14 only a few labeled spermatocytes and spermatogonia per tubule were observed in particular and the expression was mostly localized in the nucleus.

DISCUSSION

The incidence of cryptorchid testes is 3.4% in newborns and 0.8% in adult men. The etiology of oligospermia and azospermia found in cryptorchid patients remains unclear[21),22)]. It seems that in experimental cryptorchid rats impaired spermatogenesis occurs as a result of increasing apoptotic germ cells and decreasing intratesticular testosterone levels[13),14)]. Shikone *et al*[22)]. reported that the increase in apoptosis in male germ cells after unilateral experimental cryptorchidism is regulated by local testicular factors. In the present study, we used unilateral experimental cryptorchidism of rats and found that apoptotic germ cells peaked soon after cryptorchidism surgery (day 3) and testicular weight and Johnsen's score

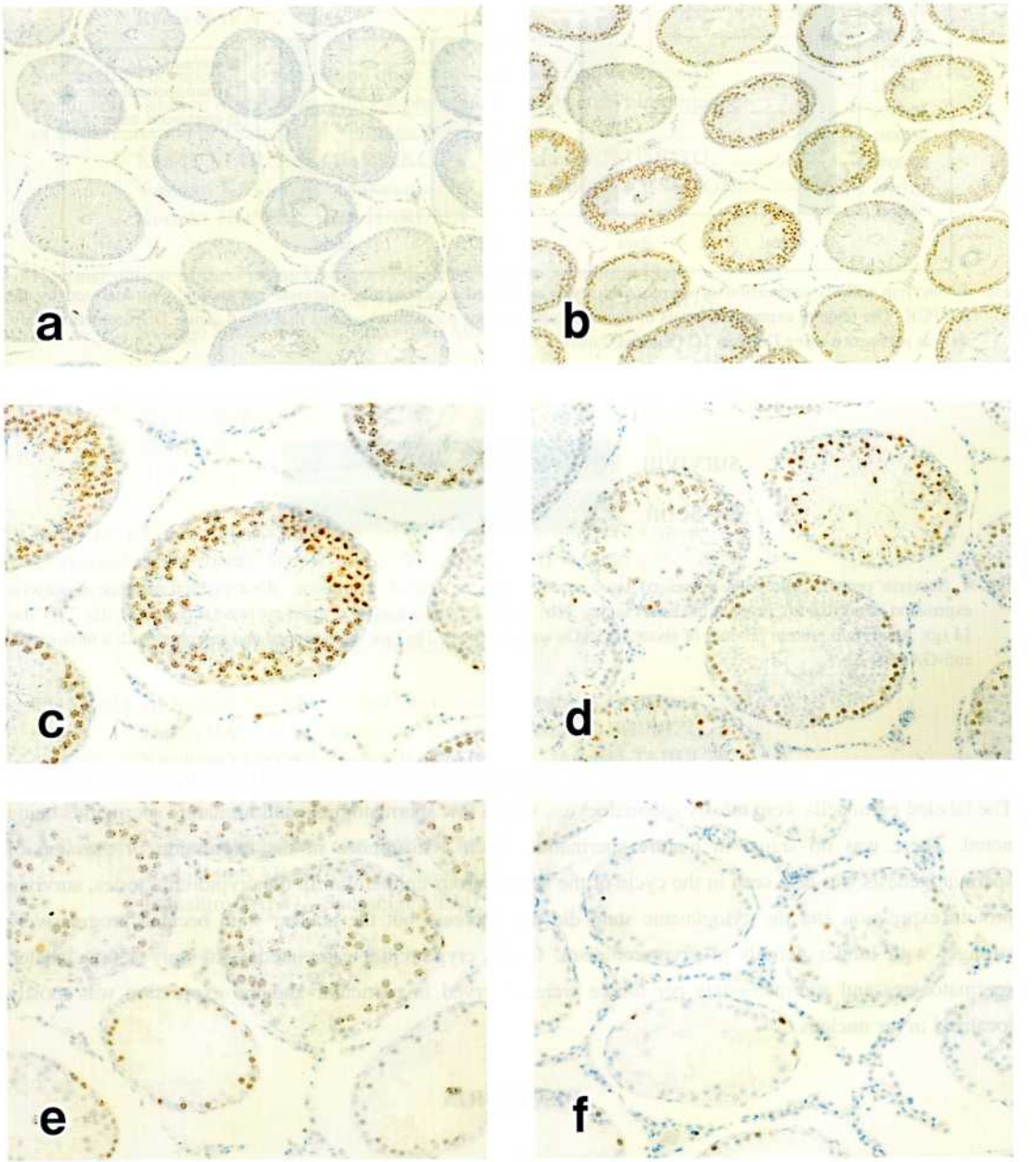

Fig. 5. Immunohistochemical analysis of survivin expression and localization. The sections of the normal control testes were used as a negative control. In the negative control sections, survivin blocking peptide was added to the primary Ab. In normal control sections, abundant expression was observed. The expression was localized in the nucleus and cytoplasm of germ cells. The labeled germ cells were mostly spermatocytes. The immunohistochemical expression of the cryptorchid testes and the cytoplasmic stain decreased and the nuclear stain became progressively stronger with longer periods of cryptorchidism. negative control (a), normal control (b, c), cryptorchid testes at day 3 (d), day 7 (e), day 14 (f).

decreased progressively with longer periods of cryptorchidism. The present findings were similar to some other investigations of experimental cryptorchidism[14), 16)]. Multinucleared giant cells were observed at days 3 and 7 of cryptorchidism. Chaki *et al*[13)]. reported that cell removal in cryptorchid seminiferous tubules was induced through giant cell formation.

Survivin regulates the G_2/M phase of the cell cycle in association with mitotic spindle microtubules, and it directly inhibits caspase-3 and caspase-7 activity[9)]. Expression of survivin protein was observed abundantly in the normal control and sham-operated testes. Although some survivin expression was found in spermatogonia and immature spermatids, the highest expression levels were in spermatocytes. Wang *et al*[12)]. reported the highest levels of survivin expression in rat seminiferous tubules occurred during the long first meiotic prophase of spermatocytes. Our result was consistent with their investigation. We consider that one of the survivin roles in testes is presumably meiotic and mitotic regulation of germ cells.

The present study is the first report on survivin expression in experimental cryptorchid rat testis. Impaired spermatogenesis occurs as a result of increasing apoptotic germ cells. Decreased expression of survivin was apparently exhibited progressively with longer periods of cryptorchidism by Western blotting and IHC. Weikert *et al*[11)] investigated survivin mRNA expression in azoospermic men with normal spermatogenesis and in men with specific spermatogenic disorders. In their investigation, survivin was found in normal spermatogenesis, but a lack of the expression was seen in some patients with pre-meiotic maturation arrest and in all patients with Sertoli-cell-only syndrome. They suggested the expression correlates with the stage of maturation arrest in patients presenting with spermatogenic disorders. We found that the difference in mRNA expression between the cryptorchid and control testes was smaller than that in the expression of protein. The results may be affected by acute damage for the experimental method. Survivin expression seems to be related more to spermatogenesis than to germ cell apoptosis. Therefore, our results suggested that survivin could presumably be a useful molecular marker of spermatogenesis.

Survivin protein levels have been shown to be upregulated in the rat seminiferous tubules in vivo by the stem-cell factor that regulates both the proliferation and apoptosis of germ cells[12)]. Cytoplasmic survivin immunoreactivity is lower in metaphase cells than in anaphase cells. During mitotic cell division, cytoplasmic survivin relocalizes to chromosomes between prometaphase and metaphase. At the beginning of anaphase, it is released from the chromosomes and diffuses back into the cytoplasm[23)]. Meiotic dividing germ cells in mammals are known to be prone to apoptosis and are especially vulnerable at metaphase[12),24)]. Wang *et al*[12)] observed a low cytoplasmic abundance of survivin in rat spermatocytes. It is conceivable that the capacity for survivin to directly interact with caspases is reduced when it localizes to chromosomes during metaphase[12)]. Thus, survivin is presumably a factor involved in the control of germ cell apoptosis and proliferation. In the present study, the immunohistochemical expression of the cryptorchid testes and the cytoplasmic stain did not decrease but the nuclear stain became progressively stronger with longer periods of cryptorchidism. In the cryptorchid testes at day 14, the expression was mostly localized in the nucleus of spermatocytes and spermatogonia. A redistribution of survivin from cytoplasmic to nuclear localization might be one of the regulating factors of apoptosis in cryptorchid rat testes.

In conclusion, this is the first report regarding survivin expression in experimental cryptorchid rat testes. Survivin expression in cryptorchid testes decreased in a time-dependent manner. It seems that survivin expression could be a useful molecular marker for evaluation of spermatogenic disorders such as cryptorchidism. One of the survivin roles in the testes is presumably meiotic and mitotic regulation of germ

cells. Survivin might play a key role in apoptosis in cryptorchid rat testes. It will be necessary to investigate the connection between survivin and a common part of the apoptosis pathway, such as caspases, in experimental cryptorchid rat testes.

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