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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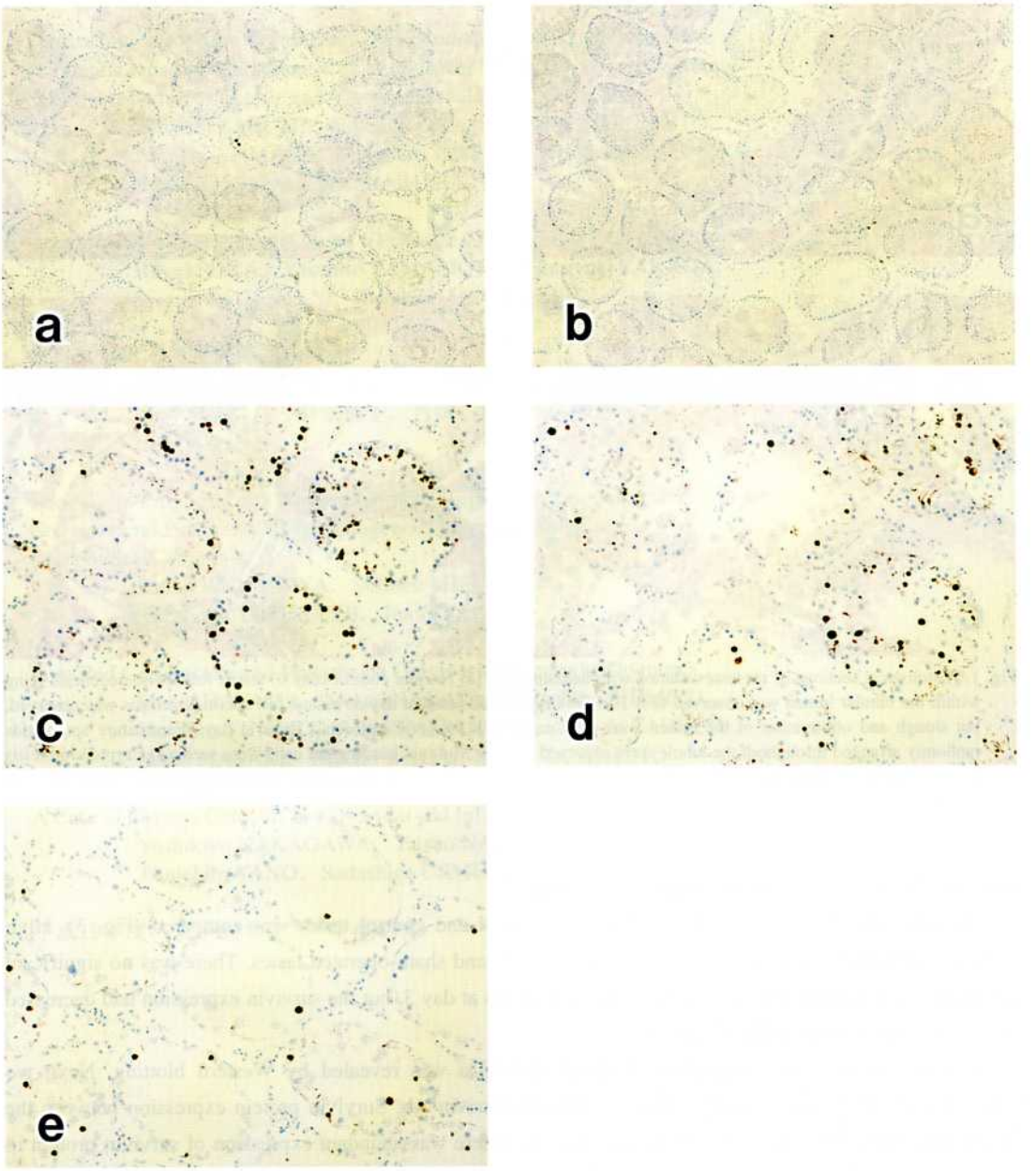

Fig. 2. TUNEL analysis of germ cells in cryptorchid testes. In the cryptorcild testis at day 3, apoptotic germ cells were observed. At day 14, only a few apoptotic germ cells were seen. normal control (a), sham-operated control at day 3 (b) , cryptorchid testes at day 3 (c), day 7 (d) and day 14 (e).

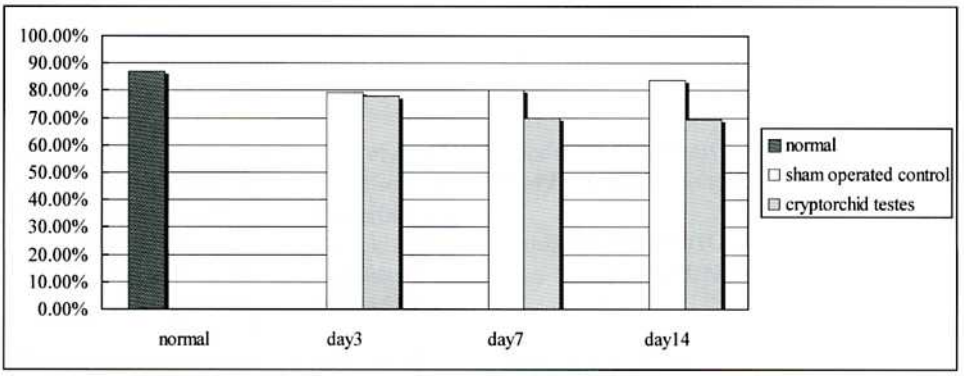

Fig. 3. Survivin mRNA expression of cryptorchid testes decreased progressively. Survivin expression was analyzed by the RT-PCR. The relative expression levels of survivin mRNA were normalized against that of β-actin. Densitometric analysis was performed using Dolphin 1D (Kurabo, Japan).

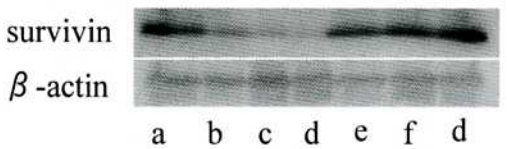

Fig. 4. Survivin protein expression decreased more rapidly than the mRNA expression. Western blot analysis of survivin expression in normal (a), cryptorchid testes at day 3 (b), day 7 (c), day 14 (d) and sham-operated at day 3 (e), day 7 (f), day 14 (g). A survivin protein product of about 16.5 kDa was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

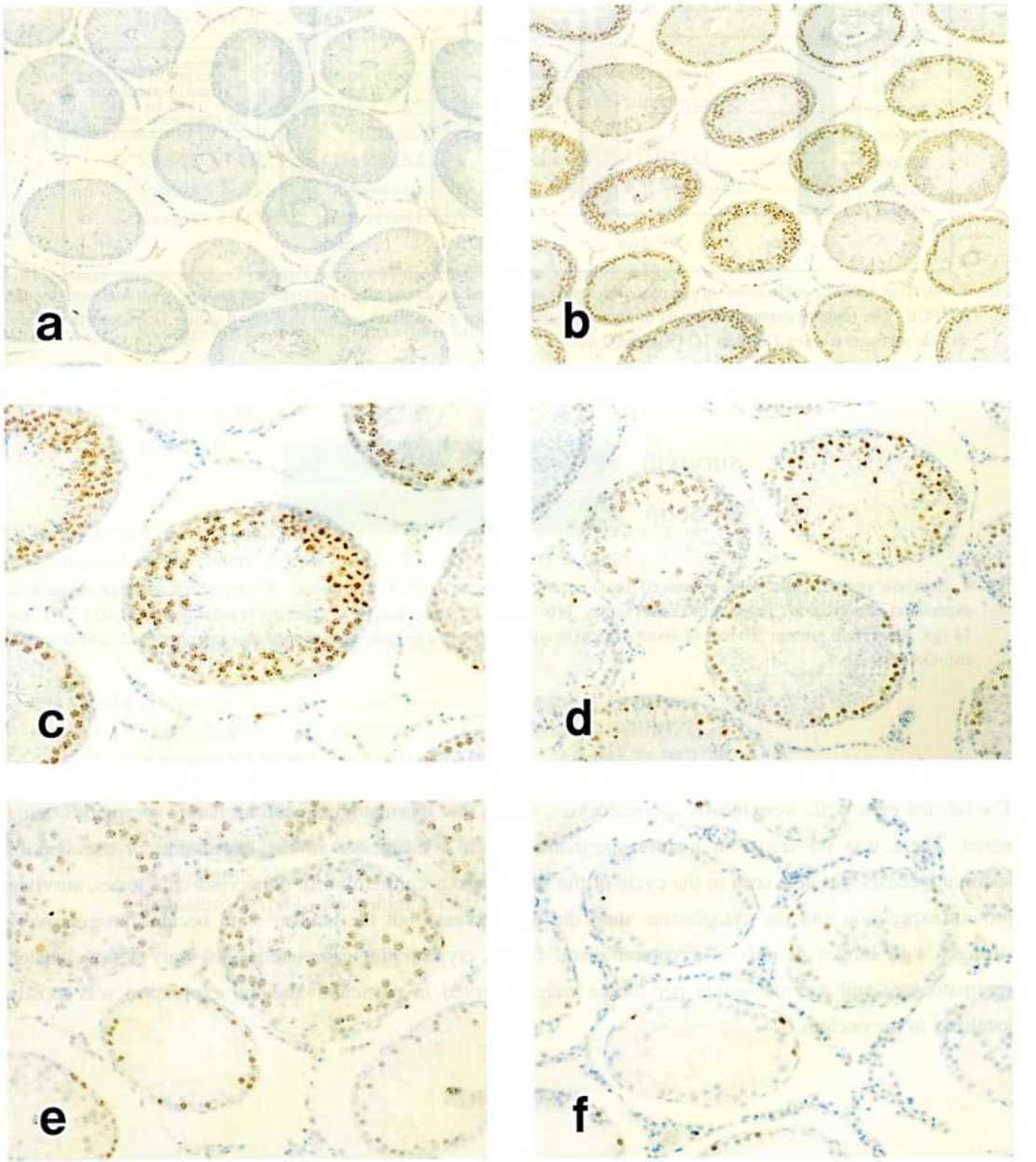

Fig. 5. Immunohistochemical analysis of survivin expression and localization. The sections of the normal control testes were used as a negative control. In the negative control sections, survivin blocking peptide was added to the primary Ab. In normal control sections, abundant expression was observed. 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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