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Involvement of Fas in the apoptosis of mouse germ cells induced by experimental cryptorchidism

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Abstract The role of Fas in the apoptosis of testicular germ cells was investigated in BALB/c mice and Fas-deficient *lpr*/*lpr* mice. Spontaneous apoptosis of germ cells was observed in the testes of 40-day-old BALB/c mice, and experimentally induced cryptorchidism increased this apoptosis to such an extent that there was a decrease in the weight of the testis. Flow cytometry and immunohistochemistry using a Fas-specific monoclonal antibody demonstrated expression of Fas on germ cells including spermatogonia, spermatocytes, and spermatids. Furthermore, analysis by flow cytometry suggested that Fas expression on germ cells was increased following cryptorchidism. However, spontaneous and cryptorchidism-induced apoptosis of germ cells were also observed in 40-day-old Fas-deficient *lpr*/*lpr* mice. Moreover, testis weight also decreased following cryptorchidism in the mutant mice. The present results may indicate that the expression of Fas on germ cells does not correlate with spontaneous apoptosis or apoptosis induced by cryptorchidism. However, on the contrary, this study shows that Fas are partly involved in cryptorchidism-induced apoptosis, because the decrease in testis weight of *lpr*/*lpr* mice was less than that in BALB/c mice.

Key words Cryptorchidism · Apoptosis · Fas · Mouse · Testis

Introduction

Loss of germ cells during normal spermatogenesis [1, 4, 8, 9, 13] has been estimated to cause a decrease in sperm production by 25–75% [4, 8, 13]. A further loss of germ cells occurs under pathologic conditions such as withdrawal of stimulatory pituitary hormones (FSH and LH) or androgens, or following increases in testicular temperature caused by cryptorchidism [6, 10, 12, 15]. Recent studies [2, 3, 7, 16, 18] have revealed that spontaneous germ cell death as well as induced pathologic germ cell death occur via apoptosis. However, the mechanisms controlling germ cell apoptosis have not been elucidated.

Fas is a type I membrane protein that belongs to the tumor necrosis factor (TNF)/nerve growth factor receptor family and mediates apoptosis upon binding to Fas ligand, a type II membrane protein that is a member of the TNF family [11]. Since the testis expresses Fas mRNA [17], the present study investigated whether Fas plays a role in the spontaneous and experimental cryptorchidism-induced apoptosis of germ cells. These experiments were performed in *lpr*/*lpr* mice, which have a loss-of-function mutation in the Fas gene [11, 20].

Materials and methods

Mice

Five-week-old BALB/c and MRL/Mpj-*lpr*/*lpr* male mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan) and Japan Clea (Osaka, Japan), respectively. Forty-day-old mice were used for experiments. Mice were kept at 25°C under a controlled light regime (12 h light/12 h darkness) and allowed free access to water and pellet food. The use of mice was under the careful supervision of a person adequately trained and at all times the mice were treated humanely. The Guiding Principles in the Care and use of Animals approved by the Council of the American Physiological Society were followed.

Experimental cryptorchidism

Forty-day-old mice were anesthetized by an intraperitoneal injection of pentobarbital (20 mg/kg body weight), and a small incision was made in the abdomen. The gubernaculum was cut on one side to displace the testis into the abdomen. Testis descent was prevented by suturing one side of the inguinal canal. The contralateral testis was sham-operated. The day of surgery was designated day 0.

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Mice were killed by cervical dislocation on days 0, 1, 2, 4 and 7 after surgery, and the testes removed and weighed.

Histology and in situ DNA 3′-end labeling

The testes were fixed in 4% phosphate (0.01 M)-buffered paraformaldehyde (pH 7.4), embedded in paraffin, and cut into 3 μm thick sections. Sections were either mounted on noncoated glass slides for hematoxylin-eosin (H&E) staining or on poly-L-lysine coated glass slides for in situ DNA 3′-end labeling and immunohistochemistry. The method of in situ DNA 3′-end labeling of apoptotic cells was performed as described by Gavrieli et al. [5]. Briefly, terminal deoxynucleotidyl transferase and biotin-16-dUTP (Boehringer Mannheim Yamanouchi, Tokyo, Japan) were used at concentrations of 0.1 e.u./μl and 10 μM, respectively, and extra-avidin peroxidase (Sigma, St. Louis, MO.) was diluted 1:20 in distilled water. An aminoethylcarbazol substrate kit (Dako Japan, Kyoto, Japan) was used for staining. Approximately 1000 cells were examined per slide, and the percentage of stained cells (apoptotic index) was determined. Since the degree of apoptosis varied among cross-sections of seminiferous tubules, the apoptotic index was determined from the average in multiple seminiferous tubules.

Flow cytometry

Immunofluorescent staining with a rat anti-mouse Fas monoclonal antibody (MBL, Nagoya, Aichi, Japan) or hamster anti-mouse *Bcl-2* monoclonal antibody (Pharmigen, San Diego, Calif.) was performed according to the method described by Oda et al. [14]. The fresh testis was cut into small pieces in RPMI 1640 medium, and cells were separated by several gentle pipettings with a bore-reduced Pasteur pipette. Separated cells were then filtered through two 200-μm nylon filters to remove connective tissue and cell clusters, and suspended in 0.01 M phosphate-buffered saline (pH 7.2) (PBS) at a concentration of 10^6/ml. The cells were divided into four parts (samples 1–4). Sample 1 was incubated with the *Bcl-2* antibody (10 μg/ml PBS) and sample 3 was incubated with the Fas antibody (5 μg/ml PBS) for 45 min at room temperature. Samples 2 and 4 were incubated with control antibody and were used as controls for samples 1 and 3, respectively. After several washes in PBS, samples 1 and 2 were incubated with fluorescein-conjugated rabbit anti-hamster IgG (Organ Technika, Durham, N.C.), while samples 3 and 4 were incubated with fluorescein-conjugated rabbit anti-rat IgG (Organon Technika) for 45 min at room temperature. Cells were then washed again with PBS and fixed in 70% ethanol on ice. After incubation in an RNase solution (0.25 mg DNase-free RNase/ml PBS) for 1 h at 37°C, cells were incubated in a propidium iodide (PI) solution (50 mg PI/ml PBS) for 10 min on ice. Normal mouse thymocytes were used as a standard for PI labeling intensity of diploid cells. Epics Profile (Coulter, Hialeah, Fla.) was used for cell analysis. The photomultiplier was arranged to maintain the percentages of positive cells in the control samples at approximately 2%.

Immunohistochemistry

After deparaffinization, tissue sections were treated with 2% H_2O_2 in 50% methanol for 15 min to eliminate endogenous peroxidase. These sections were then heated in 10 mM citrate buffer (pH 6.0) for 5 min in a microwave oven (Toshiba, Tokyo, Japan) at 810 W five times to facilitate antigen retrieval and were incubated with normal goat serum for 30 min at room temperature. Subsequently, the sections were incubated with rat anti-mouse Fas monoclonal antibody at room temperature for 1 h and at 4°C overnight. Thereafter, the sections were incubated with biotinylated goat anti-rat IgG (1:100 dilution; Vector, Peterborough, UK) for 30 min at room temperature. The immunoreaction was visualized with diaminobenzidine tetrahydrochloride (DAB). The sections were lightly counterstained with hematoxylin. For a negative control, the incubation step with the primary antibody was omitted.

Statistical analysis

Statistical significance ($P < 0.05$) was determined by Student's *t*-test using Stat View version 4.0 (Abacus Concepts, Berkeley, Calif.).

Results

Forty-day-old BALB/c mice underwent surgery to establish unilateral cryptorchidism, and the contralateral testes were sham-operated. The weight of the cryptorchid testes decreased rapidly after surgery, whereas only slight decreases in the weights of the sham-operated testes were observed on day 7 after surgery (Table 1). As compared with the sham-operated testes, the weights of the cryptorchid testes on days 1, 2, 4, and 7 after surgery had decreased by 6%, 6%, 22%, and 45%, respectively. Histological examination of the cryptorchid testes on day 7 showed a marked decrease in the number of germ cells in seminiferous tubules (data not shown). Apoptosis in the seminiferous tubules was examined by the in situ DNA 3′-end labeling method, and the degree of apoptosis was calculated by the apoptotic index (percentage of apoptotic cells relative to total cells) (Table 1). Since the degree of apoptosis varied among seminiferous tubules, the apoptotic index was determined from the average in multiple seminiferous tubules. Although apoptosis was sometimes detected in seminiferous tubules of the sham-operated testes, the average apoptotic index in sham-operated testes was less than

Table 1 Effects of unilateral cryptorchidism in BALB/c mice on testis weights and the apoptotic index in seminiferous tubules. Forty-day-old normal mice underwent surgery to establish unilateral cryptorchidism, and the contralateral testis was sham-operated. The day of surgery for cryptorchidism was designated day 0. Numbers of mice are given in *parentheses*

Day after surgery for cryptorchidism	Testis weight (mg/testis)[a]		Apoptotic index (%)[a]	
	Sham-operated	Cryptorchid	Sham-operated	Cryptorchid
Day 0	78.5 ± 1.5 (5)	–	2.9 ± 0.2 (5)	–
Day 1	78.5 ± 1.5 (5)	73.6 ± 0.8[b] (5)	2.9 ± 0.2 (6)	5.6 ± 0.6[b] (6)
Day 2	79.1 ± 1.6 (6)	72.8 ± 1.8[b] (5)	2.8 ± 0.2 (7)	4.7 ± 0.3[b] (7)
Day 4	76.8 ± 1.2 (9)	59.8 ± 1.2[b] (9)	2.8 ± 0.2 (8)	5.2 ± 0.3[b] (8)
Day 7	71.3 ± 2.2 (7)	37.9 ± 3.9[b] (7)	2.8 ± 0.2 (5)	5.0 ± 0.3[b] (5)

[a] Mean ± SE
[b] $P < 0.05$, significant difference from the value of the sham-operated testis

3%. Cryptorchidism increased the percentage of seminiferous tubules with apoptotic cells, and furthermore increased the apoptotic index significantly as early as day 1 after surgery (Table 1, Fig. 1).

DNA content and Fas expression on testicular cells from the sham-operated and cryptorchid testes were examined on day 4 after surgery by flow cytometry. Testicular cells were divided into three groups (1C, 2C, and 4C) based on DNA content as assessed by PI labeling intensity (Fig. 2). Normal diploid thymocytes were in group 2C. Percentages (means ± SE, $n = 3$) of Fas-positive testicular cells from sham-operated and cryptorchid testes were 3.8 ± 0.8 versus 5.2 ± 0.5 in group 1C, 2.7 ± 0.5 versus 10.9 ± 2.2 in group 2C, and 6.3 ± 1.2 versus 19.3 ± 3.2 in group 4C, respectively. The percentages of Fas-positive testicular cells in groups 2C and 4C were significantly higher in cryptorchid testes than in the sham-operated testes. Expression of *Bcl-2* protein was not detected in testicular cells from either the sham-operated or cryptorchid testes (data not shown).

Immunohistochemical analysis of Fas expression using an anti-mouse Fas monoclonal antibody revealed that Fas was expressed on germ cells including spermatogonia, spermatocytes, and spermatids (Fig. 3). Fas was expressed predominantly in the cell membrane of spermatogonia and spermatocytes, and in both the cell membrane and cytoplasm of spermatids.

We observed normal spermatogenesis in the testes of 40-day-old *lpr*/*lpr* mice (data not shown), which are deficient in Fas expression. As in the BALB/c mice, we observed decreases in testicular weights following experimentally induced cryptorchidism (Table 2). The weights of the cryptorchid testes decreased significantly by 23% and 31% on days 4 and 7, respectively, as compared with the weights of the sham-operated testes. Additionally, in situ DNA 3′-end labeling showed that cryptorchidism increased the percentage of cross-sections of seminiferous tubules with apoptotic cells, and also increased the apoptotic index on days 1, 4, and 7 from approximately 2% to 5%.

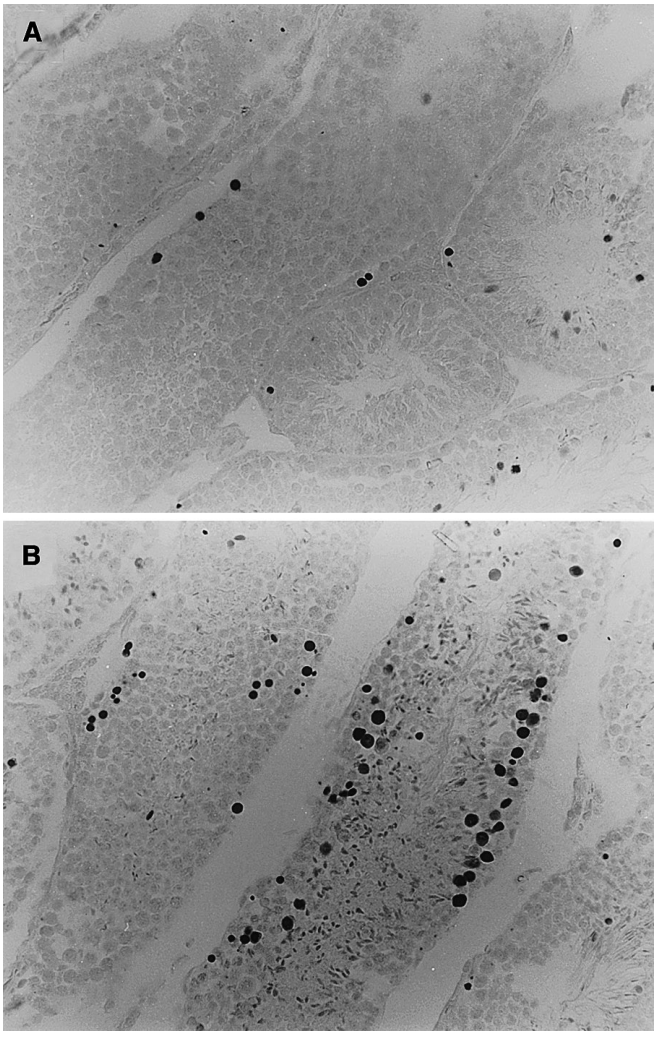

Fig. 1 In situ DNA 3′-end labeling of intact (**A**) and cryptorchid (**B**) testis. ×60. Forty-day-old BALB/c mice underwent surgery to establish unilateral cryptorchism. **A** In situ DNA 3′-end labeling of the testis prior to surgery. **B** In situ DNA 3′-end labeling of the cryptorchid testis on day 4 following surgery

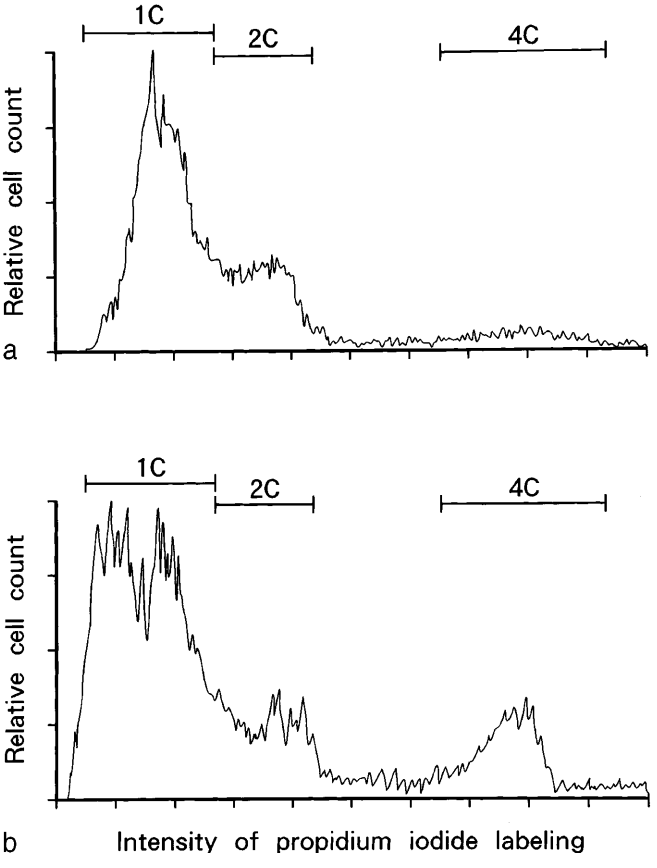

Fig. 2a, b Analysis of testicular cell populations by flow cytometry. Testicular cells from intact (**a**) and cryptorchid (**b**) testis were divided into three populations according to DNA content i.e., 1C, 2C, and 4C. Normal diploid thymocytes belong to group 2C

Table 2 Effects of cryptorchidism in *lpr*/*lpr* mice on testis weights and the apoptotic index in seminiferous tubules. Forty-day-old *lpr*/*lpr* mice underwent surgery to establish unilateral cryptorchidism, and the contralateral testis was sham-operated. The day of surgery for cryptorchidism was designated day 0. Numbers of mice are given in *parentheses*

Day after surgery for cryptorchidism	Testis weight (mg/testis)[a]		Apoptotic index (%)[a]	
	Sham-operated	Cryptorchid	Sham-operated	Cryptorchid
Day 0	85.6 ± 2.1 (4)	–	1.9 ± 0.2 (6)	–
Day 1	86.3 ± 1.3 (4)	86.2 ± 4.4 (4)	2.2 ± 0.1 (6)	6.0 ± 0.2[b] (6)
Day 4	82.2 ± 1.2 (6)	63.5 ± 3.9[b] (6)	1.3 ± 0.1 (9)	3.6 ± 0.2[b] (9)
Day 7	82.6 ± 1.5 (7)	57.0 ± 1.9[b] (5)	2.2 ± 0.3 (6)	5.0 ± 0.3[b] (5)

[a] Mean ± SE
[b] $P < 0.05$, significant difference from the value of the sham-operated testis

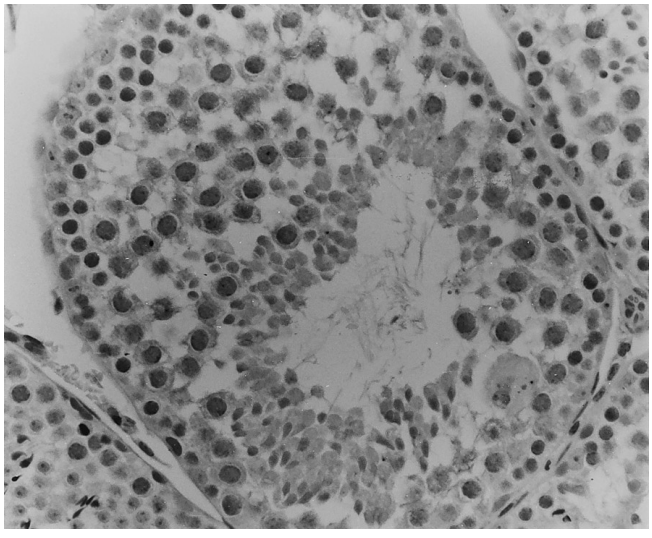

Fig. 3 Immunohistochemical detection of Fas expression in 40-day-old testis from BALB/c mice. ×360. Spermatogonia, spermatocytes, and spermatids stained positively for Fas in the cell membrane and cytoplasm

Discussion

Germ cell deletion during normal spermatogenesis [1, 4, 8, 9, 13] has been estimated to cause a decrease in sperm production by 25–75% [4, 8, 13]. Moreover, it is known that the increase in testicular temperature brought about by cryptorchidism causes a further loss of germ cells [6, 10, 12, 15]. A recent study has demonstrated that cryptorchidism-induced testis degeneration is associated with apoptotic DNA fragmentation of germ cells [16]. However, the mechanisms controlling this phenomenon are poorly understood.

Fas is a cell surface protein with a molecular size of 45 kDa and encodes a single transmembrane domain [21]. Fas is a member of the TNF/nerve growth factor receptor family. It is reported that Fas mRNA is expressed in various tissues [17], and that the expression has a role in the apoptotic process. The present study investigated the role of Fas in the spontaneous and experimentally induced loss of germ cells.

In this study, we demonstrated the expression of Fas protein on testicular cells by flow cytometry, in agreement with a previous report assessing Fas transcription in the testis [17]. Our immunohistochemical analyses revealed Fas expression on germ cells including spermatogonia, spermatocytes, and spermatids.

Testicular cells were divided into three groups according to DNA content, i.e., 1C, 2C, and 4C as reported by Troiano et al. [19]. Since normal diploid mouse thymocytes belong to group 2C, group 4C likely contains primary spermatocytes, and G_2 phase spermatogonia and non-germ cells; group 2C contains spermatogonia, secondary spermatocytes, Sertoli cells, Leydig cells, and non-germ cells; while group 1C contains spermatids. Cryptorchidism significantly increased the percentages of Fas-positive cells in groups 2C and 4C. Although groups 2C and 4C contain a minority of cells that are not germ cells, these results suggest that Fas expression is enhanced following cryptorchidism.

By assessing fragmented DNA on agarose gels [2] or by in situ DNA 3′-end labeling [7], it has been shown that spontaneous apoptosis of germ cells in the testes of adult rats is more frequent in seminiferous tubules of specific stages (I, IX–XI, XII–XIV). Furthermore, Brinkworth et al. [3] have reported that spontaneous apoptosis of germ cells in the testes of adult rats was found at specific stages (I, IX–XIV) as assessed by an in situ DNA 3′-end labeling method. In the present study, also using an in situ DNA 3′-end labeling method, spontaneous apoptosis was not found in all cross-sections of seminiferous tubules. This is likely due to the described differences in apoptosis in seminiferous tubules of distinct stages. Henriksen et al. (7) have shown that cryptorchidism in adult rats increases apoptosis in all stages except VI and VIII, and Shikone et al. [16] have reported that it increases the percentage of seminiferous tubules containing apoptotic cells. We also found that both the percentage of cross-sections of seminiferous tubules with apoptotic cells and apoptotic indices in cross-sections of seminiferous tubules increased following experimentally induced cryptorchidism. Henriksen et al. (7) revealed that the cell type affected by cryptorchidism is mainly primary spermatocytes. However, we could not discriminate specific types of germ cells by in situ DNA 3′-end labeling

method. The positively stained ratio of different germ cell types remained unclear.

Spontaneous apoptosis of germ cells was observed in both BALB/c and *lpr*/*lpr* mice, and cryptorchidism induced further apoptosis of germ cells in both strains of mice. Since *lpr*/*lpr* mice are deficient in Fas [11, 20], these results indicate that Fas may not be involved in the spontaneous and cryptorchidism-induced apoptosis of germ cells. However, on the contrary, this study shows that Fas are partly involved in cryptorchidism-induced apoptosis, because the decrease in testis weight of *lpr*/*lpr* mice was less than that in BALB/c mice. As compared with the sham-operated testes, the weights of the cryptorchid testes in *lpr*/*lpr* and BALB/c mice on day 7 after surgery had decreased by 31% and 45%, respectively. Although other factors such as vascularity, endocrine environment, and nutrition may be different between the two, the difference in the decrease in testis weight may indicate involvement of Fas. Since Fas and the other processes were involved in the cryptorchidism-induced apoptosis in BALB/c mice, the decrease in testis weight might be more severe than that in *lpr*/*lpr* mice which were deficient in Fas expression. Another reason suggesting involvement of Fas is that Fas protein was shown to be expressed on testicular cells in BALB/c mice by flow cytometry and immunohistochemistry. Although high level of Fas ligand mRNA was reported to be expressed in the testes [17], the roles of Fas and Fas ligand in the testis and germ cells remain to be delineated.

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