

# Inhibition of sperm production in mice by annexin V microinjected into seminiferous tubules: possible etiology of phagocytic clearance of apoptotic spermatogenic cells and male infertility

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## Abstract

Many differentiating spermatogenic cells die by apoptosis during the process of mammalian spermatogenesis. However, very few apoptotic spermatogenic cells are detected by histological examination of the testis, probably due to the rapid elimination of dying cells by phagocytosis. Previous *in vitro* studies showed that Sertoli cells selectively phagocytose dying spermatogenic cells by recognizing the membrane phospholipid phosphatidylserine (PS), which is exposed to the surface of spermatogenic cells during apoptosis. We examined here whether PS-mediated phagocytosis of apoptotic spermatogenic cells occurs *in vivo*. For this purpose, the PS-binding protein annexin V was microinjected into the seminiferous tubules of normal live mice, and their testes were examined. The injection of annexin V caused no histological changes in the testis, but significantly increased the number of apoptotic spermatogenic cells as assessed by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay. The number of Sertoli cells did not change in the annexin V-injected testes, and annexin V itself did not induce apoptosis in primary cultured spermatogenic cells. These results indicate that annexin V inhibited the phagocytic clearance of apoptotic spermatogenic cells and suggest that PS-mediated phagocytosis of those cells occurs *in vivo*. Furthermore, the injection of annexin V into the seminiferous tubules brought about a significant reduction in the number of spermatogenic cells and epididymal sperm in anticancer drug-treated mice. This suggests that the elimination of apoptotic spermatogenic cells is required for the production of sperm.

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**Keywords:** annexin V; apoptosis; phagocytosis; spermatogenesis

**Abbreviations:** BSA, bovine serum albumin; PS, phosphatidylserine; SR-BI, class B scavenger receptor type I; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling

## Introduction

Mammalian spermatogenesis is a complex process during which spermatogonial stem cells undergo meiotic cell division and mature into spermatozoa. Although a large number of differentiating spermatogenic cells are considered to undergo apoptosis,<sup>1,2</sup> only a small fraction of cells appear to be apoptotic when normal mouse testes are histochemically examined by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method.<sup>3</sup> This is probably because most apoptotic spermatogenic cells are rapidly removed from the seminiferous tubules.

Cells undergoing apoptosis are efficiently eliminated from the organism by phagocytosis,<sup>4</sup> and this phenomenon is likely to be a part of self-defense mechanisms.<sup>5,6</sup> However, the mechanism and role of the phagocytic clearance of apoptotic cells are not fully understood. Phagocytes such as macrophages bind to apoptosing cells by recognizing phagocytosis markers that appear on the surface of target cells using specific receptors.<sup>6,7</sup> The membrane phospholipid phosphatidylserine (PS) is the best characterized phagocytosis marker.<sup>8–11</sup> Phospholipids are asymmetrically distributed in the plasma membrane bilayer, and PS is one of the phospholipids restricted to the inner leaflet.<sup>12</sup> The regulatory mechanism that defines the phospholipid localization in the plasma membrane appears to be altered in apoptotic cells,<sup>13,14</sup> and most phospholipids are believed to be redistributed evenly between the two layers. As a result, PS translocates to the outer membrane leaflet and becomes expressed on the surface of apoptotic cells. The externalized PS then serves as a phagocytosis marker by which apoptotic cells are recognized by phagocytes.<sup>8–11</sup> The occurrence of PS externalization in apoptotic cells or tissues has been examined by flow cytometry or histochemistry using the PS-binding protein annexin V as a probe, and PS externalization is now considered to be an event that occurs commonly in many types of apoptotic cells.<sup>8–11</sup>

Apoptosis and subsequent phagocytosis also occur in areas where macrophages do not infiltrate, such as in the brain and the seminiferous tubules of the testis. Our previous studies using primary cultured rat testicular cells showed that Sertoli cells, the testicular phagocytes present

within the seminiferous tubules, efficiently phagocytose spermatogenic cells undergoing apoptosis.<sup>15,16</sup> When rat spermatogenic cells were fractionated based on their DNA content and individually analyzed for the externalization of PS and susceptibility to phagocytosis by Sertoli cells, we found that spermatogenic cells at any differentiation stage undergo apoptosis and are efficiently phagocytosed by Sertoli cells in a PS-mediated manner.<sup>17</sup> Furthermore, we showed that class B scavenger receptor type I (SR-BI) is responsible for the recognition of PS expressed on the surface of apoptotic spermatogenic cells by Sertoli cells.<sup>17</sup> Since all these studies were done with primary cultured cells, it has been uncertain whether or not apoptotic spermatogenic cells are phagocytosed in the testis in a manner mediated by PS.

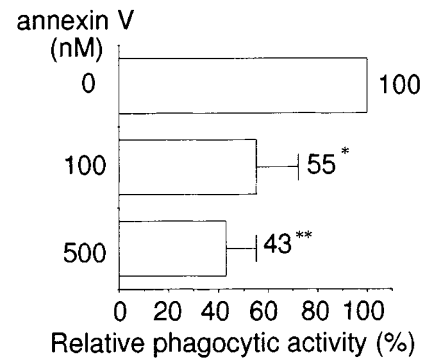
To obtain *in vivo* evidence of PS-mediated phagocytosis of apoptotic spermatogenic cells, we introduced an inhibitor of PS-mediated phagocytosis directly into the seminiferous tubules of live mice, adopting a microinjection technique that has been successfully used for the transplantation of male germ cells.<sup>18,19</sup> We used annexin V as an inhibitor since it inhibits PS-mediated phagocytosis reactions *in vitro*. The results from the histological and histochemical analyses of annexin V-injected testes revealed that PS-mediated phagocytosis of apoptotic spermatogenic cells is likely to occur *in vivo*, and suggested that the phagocytic elimination of dying spermatogenic cells is important for the production of sperm.

## Results

### Increase in number of TUNEL-positive spermatogenic cells in testes injected with annexin V

We previously established a phagocytosis assay in which rat spermatogenic cells are phagocytosed by Sertoli cells in a manner mediated by PS that is expressed on the surface of apoptotic spermatogenic cells.<sup>16</sup> The phagocytosis was significantly reduced by the addition of PS-binding protein annexin V at almost the same concentrations as used to inhibit macrophage phagocytosis of apoptotic cells<sup>20</sup> (Figure 1). We therefore decided to use annexin V to inhibit the phagocytosis of spermatogenic cells *in vivo*.

Testes of live adult mice were microinjected with annexin V and subjected to the TUNEL assay after various periods (Figure 2A). We found that the number of TUNEL-positive spermatogenic cells, which were rarely detectable in normal testes, increased after the injection of annexin V. The TUNEL positivity became apparent 1 day after injection and was maximized at day 3. We then conducted more detailed analyses with testes 3 days after injection of annexin V or control reagents (Figure 2B and C). The testes were first subjected to the histological examination by staining with hematoxylin and eosin. Annexin V appeared to exert no effect on the histology of testes. The number and morphology of spermatogenic cells were apparently normal, and there was no indication of inflammation. We found no appreciable changes in the weight of testes (data not shown) or gross morphology of the interstitial cell popula-

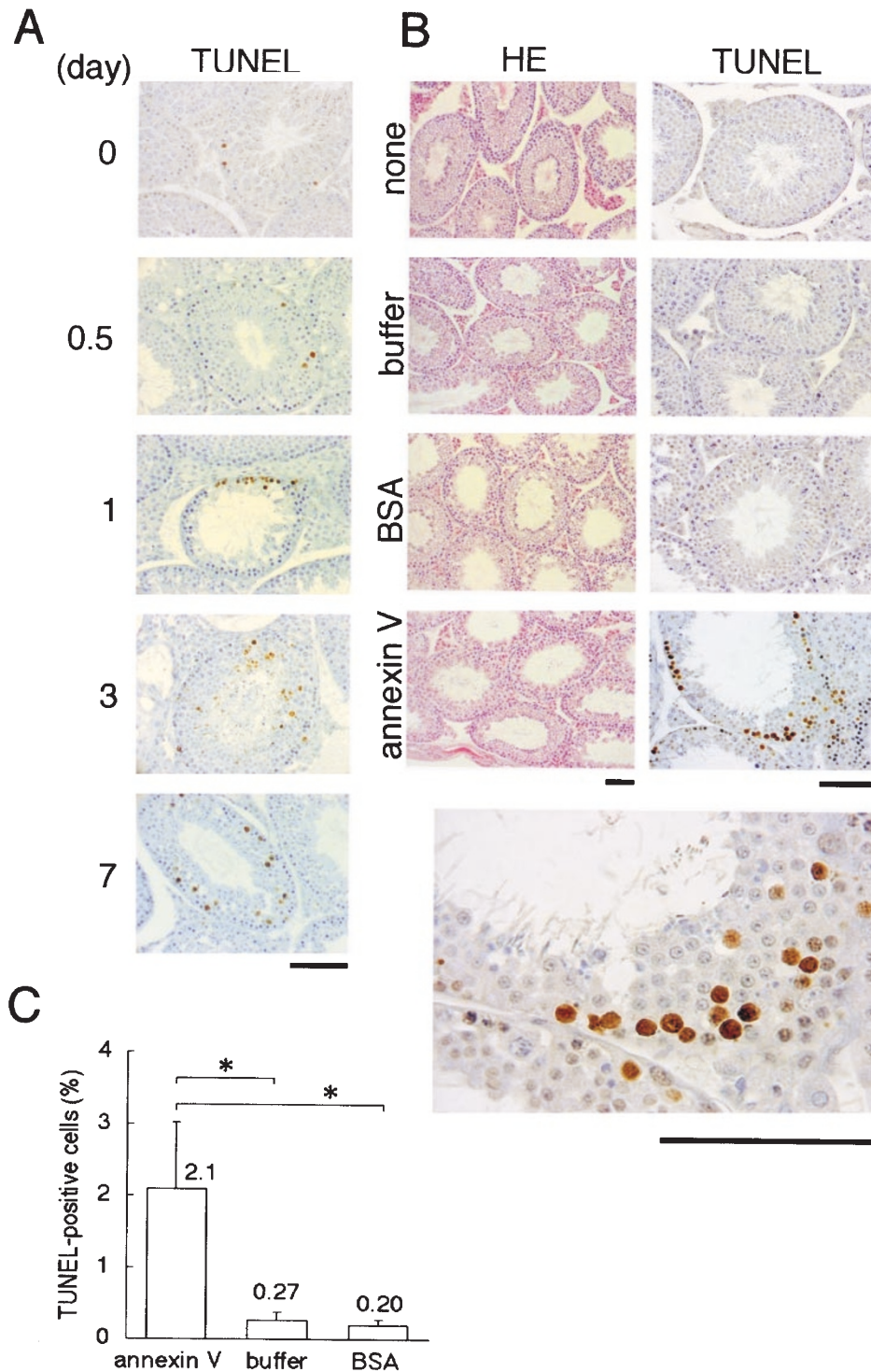


**Figure 1** Inhibition of phagocytosis of apoptotic spermatogenic cells by annexin V *in vitro*. Phagocytosis assays were conducted with primary cultured rat spermatogenic cells and Sertoli cells in the presence of various amounts of recombinant human annexin V. The extent of phagocytosis is shown relative to that with no added inhibitor, which was taken as 100. The mean and standard deviation from one of three experiments with similar results are presented. The mean phagocytic index in the control reaction was 9.1. Significantly different from the control with no inhibitor by Student's *t*-test (\* $P < 0.005$ , \*\* $P < 0.001$ ).

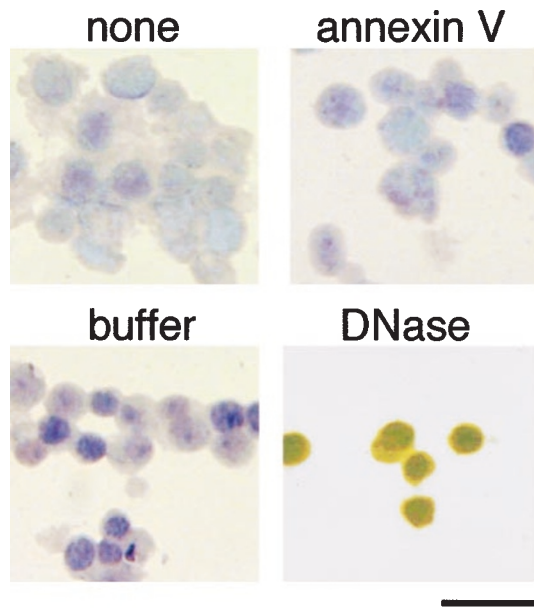
tions. In addition, the number of Sertoli cells in the testis of annexin V-injected mice ( $7.8 \pm 0.4$  per cross section of the seminiferous tubules) was close to that in the controls ( $7.9 \pm 0.6$  and  $6.9 \pm 1.0$  with buffer alone and bovine serum albumin (BSA), respectively) ( $n=25$ ). We then analyzed the testis sections for the presence of apoptotic spermatogenic cells by the TUNEL assay. The administration of annexin V caused a significant increase in the number of apoptotic spermatogenic cells, and this effect was not observed when buffer alone or BSA was injected. We sometimes observed TUNEL-positive shrunken cells, which were presumably at late stages of apoptosis. The TUNEL-positive spermatogenic cells included spermatogonia, spermatocytes, and spermatids, but no Sertoli cells or interstitial cells appeared to be apoptotic in the testes injected with annexin V. The mitotic and meiotic divisions of spermatogenic cells take place at distinct stages of the cycle of the seminiferous epithelium. In the mouse, there are 12 stages that repeat at an 8.6-day interval. Morphological determination of the spermatogenic stage of each seminiferous tubule section revealed that TUNEL-positive cells were enriched in stages IX–XII and VII–VIII. These stages almost correspond to those of the rat spermatogenic cycle during which apoptotic cells are detectable.<sup>1</sup>

### Lack of effect of annexin V on survival of cultured spermatogenic cells

To verify the possibility that annexin V directly induces apoptosis in spermatogenic cells, we treated testicular cells isolated from normal adult mice with annexin V and examined the occurrence of apoptosis (Figure 3). We carried out a 6-h incubation since the testicular cells underwent cell death when they were maintained in  $Ca^{2+}$ -containing phosphate-buffered saline for a longer period (data not shown). We found no evidence of apoptosis in spermatogenic cells present in the cultured testicular cells, while treatment with deoxyribonuclease I significantly increased the number of TUNEL-positive



**Figure 2** Increase in number of apoptotic spermatogenic cells by treatment with annexin V. **(A)** TUNEL assay of annexin V-injected testes. Testis sections of adult mice that had been microinjected with annexin V were subjected to the TUNEL assay after the indicated periods (in day). The TUNEL signals are shown in brown while nuclei are stained in blue with hematoxylin. Bar: 100  $\mu$ m. The results are from one experiment of two with similar results. **(B)** Histological and histochemical analysis of annexin V-injected testes. Testes that had not been microinjected (none), or injected with buffer alone, BSA, or annexin V were examined histologically by staining with hematoxylin and eosin (HE), and histochemically by the TUNEL assay. The bottom panel shows a higher-magnification view of the TUNEL result with annexin V-injected testes. Bar: 100  $\mu$ m. Each reagent was injected into five testes, and a typical example is shown. **(C)** Quantification of TUNEL-positive cells. The mean and standard deviation are presented. \*Significantly different by Welch's *t*-test ( $P < 0.05$ )



**Figure 3** Effect of annexin V on survival of primary cultured spermatogenic cells. Testicular cells of adult mice were incubated with the indicated reagents for 6 h and analyzed by the TUNEL assay. Treatment with deoxyribonuclease I (DNase) was done as a positive control. The TUNEL signals are shown in brown. Bar: 25  $\mu$ m

cells. Similarly, the addition of annexin V did not significantly increase the number of spermatogenic cells that were positive for the trypan blue staining or contained condensed chromatin (data not shown). These results indicate that annexin V does not induce apoptosis, at least in mouse spermatogenic cells. We therefore speculate that the increase in the number of TUNEL-positive spermatogenic cells in the testes of annexin V-microinjected mice is not due to an increase in the rate of apoptosis, but rather a consequence of the inhibition of phagocytic clearance of apoptotic spermatogenic cells.

### Inhibition of sperm production in annexin V-treated mice

The presence of annexin V in the seminiferous tubules of normal mice caused an increase in the number of TUNEL-positive spermatogenic cells, but spermatogenesis did not seem to be significantly affected (see Figure 2B). We thus intended to determine the effect of annexin V on the progress of spermatogenesis and the production of sperm using a more sensitive experimental system. The administration of the anticancer drug busulfan caused severe reduction in the number of spermatogenic cells within 8 weeks, but spermatogenesis recovered from the damage during the next 12 weeks (Figure 4A), as reported previously.<sup>21</sup> Annexin V or control reagents were microinjected into the seminiferous tubules 8 weeks after treatment with busulfan so that the reagents could affect spermatogenesis from the beginning of recovery, and the testes were analyzed for the occurrence of spermatogenesis at week 20 (Figure 4B). We found that the histology of the testis sections prepared from annexin V-injected mice was almost equal to that obtained at week 8.

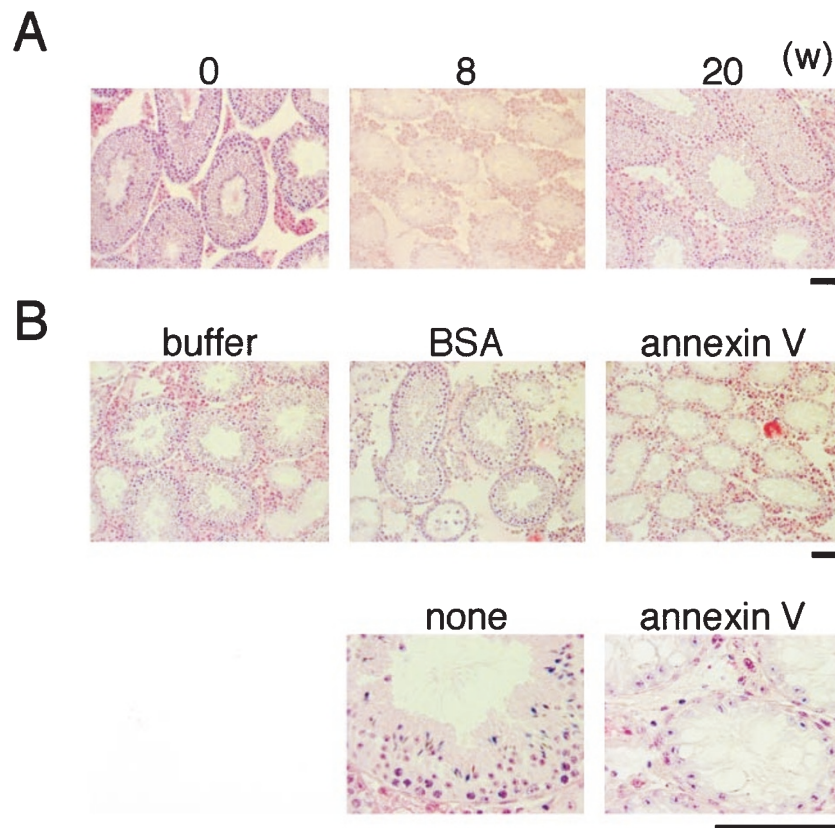
This indicates that spermatogenesis did not recover from the damage caused by busulfan when testes received microinjection of annexin V. Injection of either buffer alone or BSA did not influence the recovery of spermatogenesis. Unlike the results shown in Figure 2, TUNEL-positive spermatogenic cells were almost undetectable in testes of annexin V-injected mice (data not shown). This is probably because the injected annexin V inhibited spermatogenesis at the beginning of recovery. The inhibitory effect of annexin V was also observed at the level of the production of sperm; the number of sperm in the epididymis of the annexin V-injected mice at week 20 was enormously lower than that in the control mice with no change in the weight of the testes (Table 1). These results indicate that the presence of annexin V in the seminiferous tubules inhibited the recovery of spermatogenesis that had been impaired by the administration of busulfan. This suggests that the phagocytic elimination of apoptotic spermatogenic cells by Sertoli cells is needed for spermatogenic differentiation and thus for the production of sperm.

### Discussion

Microinjection of the PS-binding protein annexin V into the seminiferous tubules of live mice resulted in an increase in the number of apoptotic spermatogenic cells as assessed by the TUNEL assay. This result is most likely the consequence of inhibition by annexin V of phagocytic clearance of apoptotic spermatogenic cells, since annexin V did not induce apoptosis in primary cultured mouse spermatogenic cells and the number of Sertoli cells, testicular phagocytes, did not decrease in the testes of annexin V-treated mice. This is, to our knowledge, the first demonstration of the occurrence of PS-mediated phagocytosis of apoptotic cells *in vivo*. Furthermore, annexin V caused inhibition or at least a delay of the recovery of spermatogenesis in mice treated with the anticancer drug busulfan. This indicates that the phagocytic elimination of apoptotic spermatogenic cells is required for spermatogenesis, and suggests that dysfunction of this process could lead to male infertility associated with oligozoospermia or azoospermia.

As for the physiological role of phagocytosis of apoptotic cells, one reasonable hypothesis is that the removal of dying cells before their lysis protects the surrounding tissues from being exposed to the noxious contents that would otherwise be released from those cells.<sup>4</sup> Fadok, Henson, and colleagues have proposed a more active role of this event in the maintenance of tissue homeostasis. They showed that macrophages that had phagocytosed apoptotic cells suppressed inflammation by changing the production of both pro- and anti-inflammatory cytokines *in vitro*<sup>22,23</sup> and *in vivo*<sup>24</sup> experiments. Among other possible roles of phagocytosis of apoptotic cells are activation of the adaptive immune response and the clearance of pathogenic microbes from the organism. Dendritic cells cross-present viral or tumor antigens and activate CD8-positive T-lymphocytes after they phagocytose apoptotic virus-infected cells<sup>25,26</sup> or cancer cells.<sup>27–29</sup> On the other hand, macrophage phagocytosis of influenza virus-infected cells in an apoptosis-dependent manner leads to direct elimination of the virus.<sup>30</sup> Another role of the phagocytic clearance





**Figure 4** Effect of annexin V on recovery of spermatogenesis in busulfan-treated mice. **(A)** Impairment of spermatogenesis by busulfan. Testis sections prepared from busulfan-treated mice at the indicated periods (in week) were stained with hematoxylin and eosin. Bar: 100  $\mu$ m. **(B)** Inhibition of recovery of spermatogenesis by annexin V. Testes of busulfan-treated mice were microinjected with annexin V or control reagents at week 8, and analyzed as in (A) at week 20. Bar: 100  $\mu$ m

**Table 1** Effect of annexin V on the weight of testis and the number of epididymal sperm in busulfan-treated mice

Reagents injected <sup>a</sup>	Testis weight <sup>b</sup> (mg)	Sperm number <sup>b</sup> (log <sub>10</sub> (counts/ml))
none	52 ± 16	5.5 ± 0.7
buffer alone	58 ± 13	5.5 ± 0.8
BSA	40 ± 6	4.4 ± 2.0
annexin V	49 ± 16	2.1 ± 1.6 <sup>c</sup>

<sup>a</sup>The number of testes/epididymides and mice examined in each condition: none, 11 testes from 10 mice; buffer, 6 testes from 4 mice; BSA, 5 testes from 5 mice; annexin V, 8 testes from 6 mice. <sup>b</sup>Mean ± standard deviation. <sup>c</sup>Significantly different from the values in other conditions by Student's *t*-test ( $P < 0.0025$ )

of apoptotic cells could be the prevention of autoimmune diseases. Macrophages phagocytose some types of apoptotic cells in a complement-mediated manner.<sup>5</sup> Mice in which the gene coding for the complement C1q was disrupted showed decreased efficiency of phagocytosis and exhibited autoimmune disease-like symptoms.<sup>31,32</sup> In addition, the injection of apoptotic cells<sup>33</sup> or the disruption of a candidate phagocytosis-signaling gene<sup>34</sup> led to an increase in the production of autoantibodies. In contrast to the above observations, our results have provided evidence of the possible requirement of the phagocytic elimination of

apoptotic cells for a developmental phenomenon normally occurring in the organism.

The presence of annexin V in the seminiferous tubules of the busulfan-treated mice led to the inhibition of spermatogenesis. This suggests that PS-mediated phagocytosis of apoptotic spermatogenic cells is required for the efficient production of sperm. However, it is unclear at the present time how the phagocytic elimination of dying spermatogenic cells is involved in the process of spermatogenesis. The occurrence of inflammation is not the cause, since infiltration of inflammatory leukocytes in the seminiferous tubules of annexin V-injected mice was not evident. Apoptotic spermatogenic cells left unremoved could interrupt the progress of the differentiation of normal cells in two ways: apoptotic cells may occupy the space needed by normal cells or take away the support provided by Sertoli cells. Another fascinating hypothesis is that Sertoli cells secrete factors needed for differentiation of spermatogenic cells when they ingest apoptotic cells. In any case, the data suggest that impaired clearance of dead spermatogenic cells could cause certain kinds of male infertility.

Male germ cells undergo apoptosis during the development of the testis and spermatogenesis. Previous histological and histochemical analyses revealed that cells at various stages of spermatogenic differentiation are lost via apoptosis before maturing into sperm.<sup>1,2</sup> However, since

apoptotic cells are rapidly removed by phagocytosis, previous studies might have underestimated the frequency of apoptosis. Our study, however, eliminated this problem by selectively inhibiting the phagocytic clearance of apoptotic spermatogenic cells. When the testes of annexin V-injected mice were analyzed histochemically, spermatogenic cells positive in the TUNEL assay were found to be at various differentiation stages from spermatogonia to spermatids. This agrees well with the notion described above that apoptosis occurs at various stages, and it can thus be concluded that apoptosis is induced in male germ cells at all stages of spermatogenic differentiation. It is still totally unclear why such a large proportion, up to 75% of the potential number of mature sperm,<sup>1</sup> of spermatogenic cells need to die during differentiation. Massive apoptosis of germ cells also occurs in the ovary. Less than 0.1% of follicles present at the beginning of puberty develop into follicles capable of being ovulated.<sup>35,36</sup> Therefore, one can speculate that the production of germ cells in both males and females is regulated by apoptosis, and a very small fraction of the original germ cells are selected to mature into the gamete. Investigations of various possible mechanisms will be required to gain insight into this issue.

Although the occurrence of PS-mediated phagocytosis of apoptotic spermatogenic cells in live mice has been shown in the present study, whether SR-BI, a candidate PS-receptor of Sertoli cells,<sup>17</sup> participates in the reaction is not certain. Phagocytosis of apoptotic spermatogenic cells by Sertoli cells is inhibited by the addition of an anti-SR-BI antibody or high density lipoprotein, a ligand specific for SR-BI.<sup>17</sup> It will therefore be informative to microinject these reagents into the seminiferous tubules instead of annexin V.

## Materials and Methods

### Preparation of annexin V

Recombinant human annexin V was prepared as described previously.<sup>20,37</sup> In brief, cDNA coding for human placental annexin V was expressed in *E. coli*, and lysates were prepared by sonication. Annexin V was co-precipitated with liposomes containing PS (PS:phosphatidylcholine=2:1) in the presence of CaCl<sub>2</sub> (5 mM) and released from the liposomes with a buffer containing no CaCl<sub>2</sub>. The final preparation gave a single band on a sodium dodecyl sulfate-polyacrylamide gel stained with Coomassie brilliant blue.

### Animals

Thirteen 15-week-old ddY mice and 20 day-old Donryu rats were used throughout the study. The animals were maintained in an air-conditioned room at 22°C with a 12-h cycle of day and night. All experimental procedures were conducted with the approval of the Animal Ethics Committee of the University.

### Phagocytosis assay

Phagocytosis of rat spermatogenic cells by Sertoli cells was done as described previously.<sup>16</sup> In brief, primary cultures of testicular cells of 20 day-old Donryu rats were grown, and spermatogenic cells and Sertoli cells were isolated therefrom. The recovered spermatogenic

cells were further cultured without Sertoli cells for 2 days, labeled with biotin (NHS-LC-Biotin; Pierce, Rockford, IL, USA), and mixed with the Sertoli cells in a buffer consisting of 10 mM HEPES-NaOH (pH 7.4), 0.15 M NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, and 1.8 mM CaCl<sub>2</sub>. The mixture was incubated for 2 h and treated with trypsin to remove unreacted spermatogenic cells. The remaining cells were fixed, permeabilized with methanol, and treated with fluorescein isothiocyanate-labeled avidin. The spermatogenic cells that had been incorporated into Sertoli cells were visualized by fluorescence microscopy. The number of Sertoli cells containing engulfed spermatogenic cells was determined with eight different microscopic fields and expressed relative to the total number of Sertoli cells taken as 100, that is, the phagocytic index.

### Microinjection

Microinjection into the seminiferous tubules of live adult mice was carried out according to the method of Ogawa *et al.*<sup>19</sup> Phosphate-buffered saline (50–80  $\mu$ l) containing 2 mM CaCl<sub>2</sub>, 0.05% trypan blue, and 10  $\mu$ M annexin V or 10  $\mu$ M BSA (Fraction V; Sigma, St. Louis, MO, USA) was injected through the efferent duct. Trypan blue was included to monitor the distribution of the injected materials in the seminiferous tubules. In most experiments, about a half of the testis was stained blue shortly after injection, and thus the average concentration of the injected annexin V in the seminiferous tubules was estimated to be about 5  $\mu$ M.

### Histology and histochemistry of the testis

Testes were isolated from mice, fixed in Bouin's solution, and embedded with paraffin. Sections (5  $\mu$ m) were examined histologically after staining with hematoxylin and eosin. Cells containing DNA with 3'-OH ends, a hallmark of apoptosis, were detected in the TUNEL assay as follows. The paraffin sections were prepared on silanized slide glasses, dewaxed with xylene, and rehydrated with ethanol. After sections were washed with phosphate-buffered saline, they were incubated with proteinase K (5  $\mu$ g/ml) prior to the TUNEL assay using a commercial kit (ApopTag; Intergen, Purchase, NY, USA). The sections were then treated with a horseradish peroxidase-conjugated anti-digoxigenin antibody, and the signals were visualized by treatment with hydrogen peroxide and diaminobenzidine. Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted. At least 10<sup>4</sup> cells present in the seminiferous tubules were examined with sections prepared from five different testes, and the proportion of TUNEL-positive cells was determined and expressed as a percentage. The spermatogenic stage of each seminiferous tubules was morphologically determined.<sup>38</sup> Spermatogenic cell types and Sertoli cells were identified based on their cell shape, nuclear morphology, and location in the seminiferous tubules.

### Analysis of epididymal sperm

To deplete spermatogenic cells, mice were injected intraperitoneally with busulfan (Sigma) (40 mg/kg).<sup>21</sup> After 8 weeks, the mice were microinjected with annexin V, BSA or buffer alone. Epididymides were isolated, soaked in M2 medium (Sigma) supplemented with 0.5% BSA, and disrupted with forceps. The number of sperm released from the epididymides was determined with a hemacytometer.<sup>39</sup>

### Treatment of mouse testicular cells with annexin V

Testicular cells of adult mice were prepared and primary cultured as for the rat testicular cells, and incubated with phosphate-buffered

saline containing 2 mM CaCl<sub>2</sub> and 10 μM annexin V or control reagents at 32.5°C for 6 h in a humidified atmosphere with 5% CO<sub>2</sub> in air. The cells were then smeared on silanized slide glasses, fixed, permeabilized, and subjected to the TUNEL assay as described above except that treatment with proteinase K was omitted. The occurrence of apoptosis was also determined by staining cells with trypan blue (for integrity of the plasma membrane) or Hoechst 33342 (for chromatin condensation).

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