Review

Phagocytic Removal of Apoptotic Spermatogenic Cells by Sertoli Cells: Mechanisms and Consequences

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More than half of differentiating spermatogenic cells undergo apoptosis before maturing into spermatozoa during mammalian spermatogenesis. These cells are selectively and rapidly eliminated through phagocytosis by Sertoli cells, a testicular somatic cell type possessing phagocytic activity. We have investigated the mechanism by which Sertoli cells specifically recognize and phagocytose apoptotic spermatogenic cells and the consequences of phagocytosis. We showed by *in vitro* as well as *in vivo* analyses that Sertoli cells recognize apoptotic spermatogenic cells through the binding of their surface receptor, class B scavenger receptor type I, to phosphatidylserine that is expressed on the surface of spermatogenic cells during apoptosis. The inhibition of phagocytosis in live animals resulted in a decrease in the number of epididymal sperm. These results suggest that phosphatidylserine-mediated phagocytosis of apoptotic spermatogenic cells by Sertoli cells is required for the efficient production of sperm.

Key words spermatogenesis; apoptosis; phagocytosis; scavenger receptor; phosphatidylserine externalization

1. INTRODUCTION

The mammalian spermatogenic pathway consists of a complex series of events, including proliferation and differentiation of spermatogonial stem cells, meiotic cell division of differentiating spermatogenic cells, and morphogenic maturation of spermatids.[1)] During spermatogenic differentiation, more than half of the differentiating spermatogenic cells die by apoptosis before they mature into spermatozoa,[2—7)] although the mechanism and functional significance of this phenomenon are unknown. The occurrence of apoptosis in spermatogenic cells at various stages of differentiation has been reported,[7—12)] but only a limited number of apoptotic spermatogenic cells are detectable when testis sections are examined histochemically. This is most probably due to the rapid elimination of apoptotic cells by phagocytosis, a common fate of cells undergoing apoptosis.[13—16)] Electron microscopic studies of rodent testis sections have revealed that degenerating spermatogenic cells are engulfed by Sertoli cells.[17—20)]

Cells undergoing apoptosis are efficiently eliminated from the organism by phagocytosis,[14)] and this phenomenon is likely to be a part of self-defense mechanisms.[15,16)] 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, which appear on the surface of target cells, using specific receptors.[16,21)] The membrane phospholipid phosphatidylserine (PS) is the best characterized phagocytosis marker.[22)] Phospholipids are asymmetrically distributed in the plasma membrane bilayer, and PS is one of the phospholipids restricted to the inner leaflet.[23)] The regulatory mechanism that defines the phospholipid localization in the plasma membrane appears to be altered in apoptotic cells,[24)] 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.[22,24)]

To clarify the mechanisms and consequences underlying the phagocytic clearance of differentiating spermatogenic cells, we have conducted both *in vitro* and *in vivo* experiments. The results collectively showed that Sertoli cells phagocytose apoptotic spermatogenic cells in a PS-dependent manner, and that the elimination of those cells is necessary for the efficient production of sperm.

2. INDUCTION OF APOPTOSIS IN SPERMATOGENIC CELLS

Throughout the course of mammalian spermatogenesis, differentiating spermatogenic cells remain in close contact with somatic Sertoli cells,[25,26)] and this association has been considered to be essential for the progression of spermatogenesis as well as for the cyclic function of Sertoli cells.[1,27,28)] We previously established a primary culture of rat testicular cells, in which both spermatogenic cells and testicular somatic cells, mostly Sertoli cells, are co-cultured, and spermatogenic differentiation proceeds at least to some extent.[29)] When spermatogenic cells that are lightly attached to Sertoli cells in culture were isolated and further maintained without Sertoli cells, they underwent cell death showing features typical of apoptosis such as fragmentation of DNA, condensation of chromatin, and externalization of PS.[30,31)] It was eventually found that spermatogenic cells apoptose in culture irrespective of the presence of Sertoli cells, and that phagocytic elimination of those cells by Sertoli cells makes the occurrence of apoptosis difficult to detect in co-cultures (see below). The cause of apoptosis in spermatogenic cells is still controversial, although Sertoli cells have been suggested to induce apoptosis in those cells. We showed that the apoptosis-inducing protein Fas-ligand and its receptor Fas are expressed in Sertoli cells and spermatogenic cells, respectively, and that neutralizing anti-Fas antibody reduces the level of apoptosis in spermatogenic cells when introduced into mouse testes.[32)]

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3. PHAGOCYTOSIS OF APOPTOTIC SPERMATOGENIC CELLS BY SERTOLI CELLS: *IN VITRO* STUDIES

During the course of analyses of the role of Sertoli cells in the survival and differentiation of spermatogenic cells, we found that the ratio of apoptotic spermatogenic cells increases when Sertoli cells are removed from primary co-cultures of rat testicular cells.[30] However, the presence of Sertoli cells did not seem to protect spermatogenic cells from undergoing apoptosis but instead decreased the number of apoptotic cells.[30] This finding indicated that apoptotic spermatogenic cells disappeared from the cultures in the presence of Sertoli cells.

In order to examine the possibility that Sertoli cells eliminate apoptotic spermatogenic cells by phagocytosis, we established an *in vitro* phagocytosis assay with spermatogenic cells and Sertoli cells that were individually isolated from primary co-cultures. Both cell types were prepared from the primary cultures with purity of higher than 80%.[31] By conducting phagocytosis reactions in a quantitative manner, we found that Sertoli cells more efficiently phagocytosed a spermatogenic cell population with a higher ratio of apoptotic cells, and that the reaction was specifically inhibited in the presence of liposomes containing PS.[31] Cell populations rich in spermatogenic cells at different meiotic phases were almost equally phagocytosed by Sertoli cells, all in a manner inhibitable by PS-containing liposomes, when they underwent apoptosis accompanied by the externalization of PS.[33] These results allowed us to conclude that apoptotic spermatogenic cells at all differentiation stages are phagocytosed by Sertoli cells, at least as examined *in vitro*, in a manner dependent on PS. We next searched for a receptor that is present on the surface of Sertoli cells and responsible for the recognition of PS-exposing spermatogenic cells by Sertoli cells. Class B scavenger receptor type I (SR-BI), which had been known as a receptor for high-density lipoprotein (HDL), appeared to be a strong candidate because this receptor was shown to bind to acidic liposomes or apoptotic cells by other investigators.[34—36] We obtained a body of evidence supporting this possibility: SR-BI was expressed in Sertoli cells but not in spermatogenic cells; a cultured cell line became able to bind to PS-exposing apoptotic cells upon forced expression of SR-BI; and Sertoli cell phagocytosis of apoptotic spermatogenic cells was inhibited by anti-SR-BI antibody.[33] Furthermore, we showed that SR-BI directly binds to PS, but not to other phospholipids, with a reasonably high affinity in a solid-phase binding assay.[37] From these results, we concluded that SR-BI is a phagocytosis-inducing PS receptor of Sertoli cells (Fig. 1).

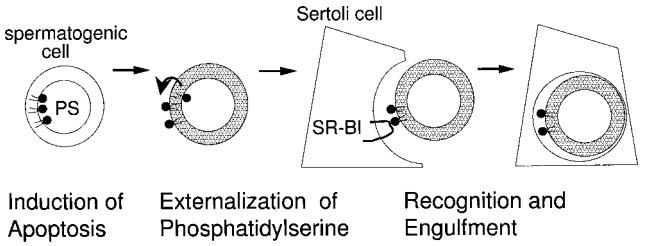

Fig. 1. Molecular Basis for Recognition of Apoptotic Spermatogenic Cells by Sertoli Cells

Sertoli cells recognize apoptotic spermatogenic cells through specific interaction between SR-BI and PS, the latter of which is externalized in spermatogenic cells during apoptosis. It is presumed that the binding of PS causes SR-BI to transmit signals leading to phagocytosis by Sertoli cells.

4. MECHANISMS AND CONSEQUENCES OF SERTOLI CELL PHAGOCYTOSIS OF SPERMATOGENIC CELLS: *IN VIVO* STUDIES

Tight junctional complexes that exist between Sertoli cells are responsible for the blood-testis barrier.[38] The tight junction of Sertoli cells divides the seminiferous epithelium into the basal and adluminal compartments, and cells at early stages of spermatogenesis, including spermatogonia, are localized in the basal compartment.[26,39] As the spermatogenic differentiation proceeds, those cells move to the adluminal compartment, where they continue to differentiate into spermatozoa.[26,39] We thus examined the presence and function of SR-BI in rat Sertoli cells in terms of the two distinct compartments. The localization of SR-BI was determined immunohistochemically in either cultured Sertoli cells with polarized membranes or the seminiferous tubules. To determine the function of SR-BI, the incorporation of lipids from HDL by Sertoli cells was examined either in culture or within the seminiferous tubules of live animals. In addition, the involvement of SR-BI in lipid incorporation by Sertoli cells was determined by using neutralizing antibody in both assays. The results collectively indicated that functional SR-BI, in terms of incorporating lipids from HDL, exists on both the basal and adluminal surfaces of polarized Sertoli cells (unpublished observation).

The occurrence of SR-BI-mediated phagocytosis of apoptotic spermatogenic cells was examined by microinjecting neutralizing antibody into the seminiferous tubules of live rats. Our anticipation was that the number of apoptotic spermatogenic cells, as determined by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay, would increase when phagocytosis was impeded. We found that the level of apoptotic spermatogenic cells was raised upon injection of monoclonal anti-SR-BI antibody (Fig. 2A). These results indicated that apoptotic spermatogenic cells are eliminated, at least in part, by Sertoli cells in a manner dependent on SR-BI. The dependency of phagocytosis on PS that is exposed on the surface of apoptotic spermatogenic cells was examined by introducing the PS-binding protein annexin V, which has been shown to inhibit the phagocytosis reaction *in vitro*,[40] into the seminiferous tubules of live mice. The results were quite similar to those observed in the experiment with microinjection of anti-SR-BI antibody (Fig. 2B),[40] indicating that PS-mediated phagocytosis of apoptotic spermatogenic cells occurs *in vivo*. All the above-described results collectively indicated that Sertoli cells recognize and phagocytose PS-exposing spermatogenic cells using SR-BI.

We next analyzed the consequences of phagocytic removal of apoptotic spermatogenic cells. Inhibition of phagocytosis by anti-SR-BI antibody or annexin V in animals did not seem to cause a significant defect in spermatogenesis when testis sections were examined histologically.[37,40] We therefore took an alternative approach: annexin V was injected into the seminiferous tubules of live mice that had been treated with an anticancer drug to transiently remove spermatogenic cells.

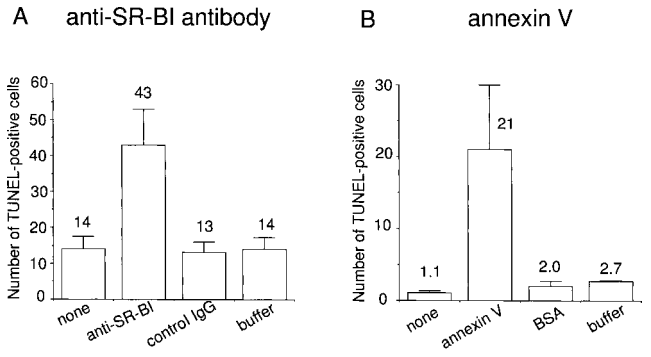

Fig. 2. Results Showing the Occurrence of PS- and SR-BI-Mediated Phagocytosis of Apoptotic Spermatogenic Cells *In Vivo*

The indicated reagents were injected into the seminiferous tubules of live animals, and testis sections were histochemically examined for the presence of apoptotic spermatogenic cells by the TUNEL assay. The number of TUNEL-positive spermatogenic cells in 100 cross-sections of the seminiferous tubules (A) or of 1000 spermatogenic cells (B) is shown. BSA, bovine serum albumin. The injection of antibody that neutralizes the activity of SR-BI (A) or the PS-binding protein annexin V (B) caused an increase of apoptotic spermatogenic cells. Panel B has been adopted with permission from Reference 40 after modification.

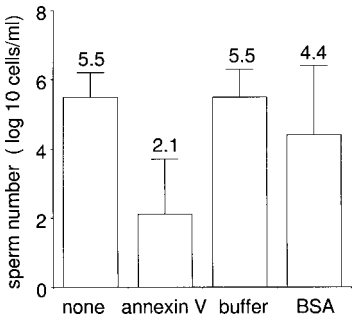

Fig. 3. Results Showing that Phagocytic Elimination of Apoptotic Spermatogenic Cells Is Needed for Efficient Production of Sperm

The indicated reagents were injected into the seminiferous tubules of anticancer drug-treated mice, and the number of sperm present in epididymides was determined. BSA, bovine serum albumin. The injection of annexin V but not other reagents caused a decrease of epididymal sperm. Adopted with permission from Reference 40 after modification.

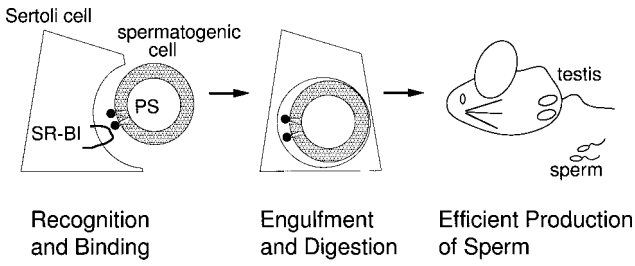

Fig. 4. Summary of These Findings

Apoptotic spermatogenic cells express PS on their surfaces and are recognized by Sertoli cells through specific binding of SR-BI to PS. This interaction induces Sertoli cells to phagocytose apoptotic spermatogenic cells. Phagocytic elimination of apoptotic spermatogenic cells might be needed for the progress of spermatogenesis and thus for the efficient production of sperm.

The presence of annexin V delayed the recovery of spermatogenesis in drug-treated mice and caused the number of sperm present in epididymides to decrease (Fig. 3).[40] These results suggest that phagocytic clearance of apoptotic spermatogenic cells is needed for the progress of spermatogenesis and thus for the efficient production of sperm (Fig. 4).

5. DISCUSSION AND PERSPECTIVES

The Sertoli cell is one of two testicular somatic cell types that actively regulate spermatogenesis: the other is the Leydig cell. Unlike interstitially located Leydig cells, Sertoli cells are present within the seminiferous tubules, being attached to spermatogenic cells at various differentiation stages. Questions that have been asked about the function of Sertoli cells and not yet completely answered include the following: how the adhesion between Sertoli cells and spermatogenic cells is maintained, how Sertoli cells help spermatogenic cells proceed through meiotic division and morphological changes, whether or not Sertoli cells control the life and death of spermatogenic cells, and how Sertoli cells selectively eliminate dead spermatogenic cells by phagocytosis. We have approached the last question by conducting *in vitro* as well as *in vivo* experiments.

The mechanism by which spermatogenic cells are induced to undergo apoptosis is not clear. In our primary culture of dispersed rat testicular cells, spermatogenic cells apoptosed when maintained without other cell types. The simple explanation that the absence of Sertoli cells causes apoptosis in spermatogenic cells appears not to be true, because the number of live spermatogenic cells remained the same in either the presence or absence of Sertoli cells in culture. We speculate that a portion of spermatogenic cells that had already been destined to die within the testes proceeded through the apoptosis process in culture. Those cells should be susceptible to phagocytosis *in vitro*, and we took advantage of this feature to identify molecules involved in the recognition of apoptotic spermatogenic cells by Sertoli cells. We found that SR-BI, a member of the scavenger receptor family of proteins, is present on the surface of Sertoli cells and serves as a receptor, and that its ligand is the membrane phospholipid PS, which is externalized and exposed on the surface of spermatogenic cells during apoptosis. To our knowledge, SR-BI is the only one of the candidate receptors involved in PS-mediated phagocytosis that has been shown to directly bind to PS. Furthermore, we showed that SR-BI- and PS-mediated phagocytosis of apoptotic spermatogenic cells occurs in the testes of live animals. SR-BI is presumably responsible for the phagocytic elimination of apoptotic spermatogenic cells at various differentiation stages, because this receptor was shown to be expressed in a functional form at both the basal and adluminal sides of polarized Sertoli cells. A further issue to be addressed is how the binding of PS to SR-BI induces Sertoli cells to phagocytose bound spermatogenic cells. Phagocytosis receptors are presumed to transmit signals that cause changes in the structure of the cytoskeleton in phagocytes. Candidate molecules involved in this event have been found in nematodes, and the existence of their mammalian homologues has been reported.[41] However, the precise nature of this signal transduction pathway remains to be determined. The fact that the intracellular region of SR-BI is very small makes us speculate that there are protein(s) that help SR-BI transduce the signal. The addition of PS-containing liposomes almost completely inhibits the phagocytosis of apoptotic spermatogenic cells by Sertoli cells *in vitro*, whereas inhibition of the same reactions by anti-SR-BI antibody is partial. We therefore speculate that apoptotic spermatogenic cells are phagocytosed by Sertoli cells mostly in a

PS-mediated manner, and multiple receptors including SR-BI present on the surface of Sertoli cells are involved in the reaction. Several molecules have been proposed as phagocytosis-inducing PS receptors,[16,21] and some of them could function, in addition to SR-BI, in Sertoli cell phagocytosis of apoptotic spermatogenic cells.

Our results suggest that the phagocytic elimination of apoptotic spermatogenic cells is necessary for healthy spermatogenic cells to proceed through spermatogenesis, though the underlying mechanism is not clear. Apoptotic cells lyse if they are not removed in time,[15] and phagocytes change their pattern of gene expression upon engulfment of apoptotic cells.[16] These facts lead us to propose two explanations for how the removal of apoptotic spermatogenic cells contributes to spermatogenesis: 1) apoptotic cells are removed before noxious contents of those cells leak out and poison healthy cells, and 2) phagocytosis endows Sertoli cells with ability to express genes whose products are needed for spermatogenesis. The relevance of the phagocytic clearance of dead materials to tissue functions in mammals has not been intensely analyzed. One exception is a study on phagocytosis of the rod outer segment by retinal pigment epithelial cells, the failure of which may lead to blindness,[42,43] and our study provides a second example.

It is unclear why a large proportion of differentiating spermatogenic cells are discarded. Possible explanations are either that the number of spermatogenic cells that Sertoli cells can support for maturation is limited and cells beyond this number are eliminated; or that spermatogenic cells that acquire some defects, such as aberrant DNA recombination, during meiosis are removed. Similar selection appears to occur during oogenesis; it is presumed that less than 1% of oocytes present in the embryo may eventually proceed through ovulation. Death of the majority of germ cells is thus an event common to the gametogenesis in both males and females. It is not known at all why such severe selection is needed for the production of gametes. We expect it will be a tough task to obtain a full answer to this question.

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