Recognition of Phosphatidylserine on the Surface of Apoptotic Spermatogenic Cells and Subsequent Phagocytosis by Sertoli Cells of the Rat*

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In a primary co-culture of spermatogenic and Sertoli cells of the rat, many spermatogenic cells die by apoptosis and are subsequently engulfed by Sertoli cells. We investigated the mechanism of this phagocytosis reaction. Testicular cells from 20-day-old rats were cultured, and spermatogenic cells and Sertoli cells were separated. When the recovered spermatogenic cells were maintained without Sertoli cells, the viability of the cells decreased and they became more susceptible to phagocytosis by Sertoli cells. Phagocytosis was severely impaired when liposomes containing acidic phospholipids, such as phosphatidylserine, phosphatidylinositol, and cardiolipin, were included in the reaction, whereas those consisting of neutral phospholipids showed little effect. Such anionic liposomes were more efficiently engulfed by Sertoli cells than were the other neutral liposomes. Also, the number of spermatogenic cells that exposed phosphatidylserine to the surface increased when cells were maintained in single culture. The results indicate that upon induction of spermatogenic cell apoptosis, phosphatidylserine and probably other acidic phospholipids, which are normally localized in the inner leaflet of the plasma membrane, translocate to the outer leaflet and serve as a signal for phagocytosis by Sertoli cells.

Most physiological cell death is caused by apoptosis, and apoptotic cells are believed to undergo heterophagic elimination by surrounding phagocytic cells such as macrophages (1, 2). However, the molecular basis underlying the phagocytosis of apoptotic cells remains to be clarified. One of the important questions to be answered is how phagocytes discriminate between target cells and other cells. Several approaches have been taken to solve this issue, and some clues have been obtained. Most of these studies were carried out using macrophages. It has been proposed that certain molecules present on the surface of macrophages are responsible for the recognition of apoptotic cells (3). Among these are lectins, αvβ3-integrin (vitronectin receptor)/CD36 complexes, and scavenger receptor-like molecules, all of which most likely capture target cells by binding to sugars, thrombospondin, and phosphatidylserine (PS), respectively. More recently, another macrophage protein, the ABC transporter ABC1, joined the candidates, although its ligand is unknown (4). However, characterization of these molecules has just begun, and further studies need to be made before reaching any conclusions as to their role in recognizing apoptotic cells.

Apoptosis and subsequent phagocytosis also occur in areas where macrophages do not infiltrate, such as the brain and the testis. In the testis, more than half of the differentiating spermatogenic cells die, probably by apoptosis, before they mature into spermatozoa (5–7). Only a limited number of apoptotic spermatogenic cells, however, are detectable when testis sections are histochemically examined. This may be explained by the fact that degenerating spermatogenic cells are eliminated at the early stages of their apoptotic death. Electron microscopic studies with rodent testis sections have shown that Sertoli cells, a testicular somatic cell, phagocytose degenerating spermatogenic cells (8–12). Sertoli cells are thus likely to be in control of the elimination of apoptotic spermatogenic cells in the testis (13). However, little is known about the regulation of this Sertoli cell function.

We previously established a primary culture of rat testicular cells (14, 15). During that culture, spermatogenic cells progress in their differentiation to some extent, depending upon their association with Sertoli cells (15, 16), and at the same time many of them undergo apoptotic death and are eliminated through phagocytosis by Sertoli cells (16). In the present study, the mechanism of this phagocytosis reaction was investigated.

MATERIALS AND METHODS

Testicular Cell Preparation—When testicular cells of 20-day-old Donryu rats were primary cultured at 32.5 °C as described previously (14), Sertoli cells adhered to the culture containers and spermatogenic cells attached lightly to the Sertoli cells. Spermatogenic and Sertoli cells were prepared in different ways. Spermatogenic cells were recovered by gentle pipetting from testicular cells co-cultured in collagen-coated multwell plates (Falcon 3046) for two days. On the other hand, Sertoli cells were obtained by removing the spermatogenic cells (by pipetting) from a co-culture of testicular cells maintained on Chamber Slide (Nunc). Most of the recovered spermatogenic cells were spermatocytes, and the Sertoli cell culture was about 90% pure, as described in the text.

Phagocytosis Assay—The recovered spermatogenic cells were maintained with no added cells for 2 days (unless otherwise stated), labeled with biotin (NHS-LS-Biotin; Pierce), and added back to the Sertoli cell culture maintained in Chamber Slide. Spermatogenic cells (about 2.5 × 105) were mixed with Sertoli cells (about 3 × 104) in 0.15 ml of medium, and the phagocytosis reaction was carried out at 32.5 °C for 2 h, except in the time course experiment. Phosphate-buffered saline was then added and unreacted spermatogenic cells washed out by pipetting 15 times. The mixture was further treated with trypsin (0.5

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1 The abbreviations used are: PS, phosphatidylserine; FITC, fluorescein isothiocyanate; PC, phosphatidylcholine; PI, phosphatidylinositol.
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mg/ml for 3 min at room temperature, after which those cells detached from the culture slides were removed. The remaining cells were fixed with 2% paraformaldehyde/0.1% glutaraldehyde/0.05% Triton X-100/ phosphate-buffered saline. The fixed cells were supplemented with fluorescein-avidin D (Vector) and kept for 20 min at room temperature. The biotinylated spermatogenic cells were detected under a fluorescence/phase-contrast microscope (BX50; Olympus). The ratio of the number of positively stained Sertoli cells to total Sertoli cells (100–150) was determined in each microscopic field. Eight to ten fields from different culture wells were examined in each experiment, and the results were statistically treated. The mean and standard deviations of a typical example from at least three independent experiments were presented as the phagocytic index. Under these conditions, we routinely obtained a phagocytic index of 13–20.

Liposome Preparation—Liposomes were prepared as described previously (17). In brief, dried lipid films containing various phospholipids (2 mmol) were swollen in 10 mL Tris-HCl (pH 7.4)/0.15 M NaCl and sonicated (Branson Sonifier model 250D) for 10 min on ice. The liposomes were composed of either phosphatidylcholine (PC) only or a combination of PC and another phospholipid at a molar ratio of 7:3. Sonicated (Branson Sonifier model 250D) for 10 min on ice. The liposomes were composed of either phosphatidylcholine (PC) only or a combination of PC and another phospholipid at a molar ratio of 7:3. Fluorescence-labeled liposomes were prepared as above in the presence of 1,2-β-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Avanti Polar Lipids) at 1% of total phospholipids. The engulfing of fluorescent liposomes by Sertoli cells was analyzed using a confocal laser microscope (MRC-1000; Bio-Rad).

Phagocytosis of spermatogenic cells by Sertoli cells. A, the morphology of spermatogenic (panels 1 and 3) and Sertoli (panels 2 and 4) cells was examined in phase-contrast (panels 1 and 2) or fluorescence (panels 3 and 4) microscopy after staining with Nile red. Scale bar = 10 μm. B, spermatogenic cells either adhering to (panel 1) or engulfed by (panels 2–4) Sertoli cells were visualized under a fluorescence/phase-contrast microscope. Scale bar = 10 μm. C, time course of the phagocytic reaction.

In our previous phagocytosis experiments (16), spermatogenic cells that adhered to Sertoli cells were not rigorously distinguished from those engulfed. In this experiment, we treated the phagocytosis reaction with trypsin to eliminate spermatogenic cells that attached to the surface of Sertoli cells. Even after extensive washing, a significant number of spermatogenic cells remained associated with Sertoli cells. These cells were distinguishable from phagocytosed cells when examined carefully under fluorescence/phase-contrast microscopy (Fig. 1B, panel 1). We speculate that the tight association of spermatogenic cells with Sertoli cells is an important step toward subsequent phagocytosis. Stained particles of various sizes were observed within the Sertoli cells; typical examples are shown in Fig. 1B, panels 2–4. We regarded these as phagocytosed spermatogenic cells and distinguished them from those cells adhering to the surface of Sertoli cells. It was unclear whether smaller stained particles represented phagocytosed apoptotic bodies or cell fragments processed after engulfment.

In order to analyze the phagocytosis reaction in a quantitative manner, we defined the phagocytic index as follows: the number of Sertoli cells positive for phagocytosis was determined as a percentage relative to the total number of Sertoli cells present in each microscopic field. We first determined a time course for the phagocytosis reaction. Spermatogenic cells that had been single-cultured for about 40 h were subjected to the phagocytosis reaction. As shown in Fig. 1C, the reaction seemed to continue during the first 2 h and reached a plateau at about index 20.

Phagocytosis of Dying or Dead Spermatogenic Cells—We previously showed that dead spermatogenic cells are eliminated when spermatogenic cells are cultured in association with Sertoli cells (16). This suggested that Sertoli cells selectively phagocytose degenerating spermatogenic cells in culture. To further examine this possibility, spermatogenic cells were singly cultured for 1 and 3 days and subjected to a phagocytosis assay. The phagocytic index increased as the culture continued, while viability of spermatogenic cells as assessed by trypan blue exclusion decreased (Fig. 2). These results support the above hypothesis that dying or dead spermatogenic cells are preferable targets for phagocytosis by Sertoli cells.

Inhibition of Phagocytosis by Liposomes Containing Acidic Phospholipids—For identifying the molecule(s) that participates in the cell-to-cell recognition between degenerating spermatogenic cells and Sertoli cells, we examined the effect of several compounds that are known to be recognized by the putative phagocytosis receptors present on the surface of
macrophages. The synthetic RGDS peptide, which contains an amino acid sequence bound by members of the integrin superfamily, was first tested, but the phagocytic index remained unchanged in the presence of this peptide (data not shown). We then examined whether phospholipids are involved in cell-to-cell recognition by adding liposomes that consist of various phospholipids to the phagocytosis reaction. As seen in Fig. 3A, liposomes containing PS, an acidic phospholipid, inhibited phagocytosis in a dose-dependent manner, whereas those containing neutral phospholipids, PC and phosphatidylethanolamine, had little effect. The inhibitory effect was not specific for PS but seemed to be common to the acidic phospholipid; the addition of liposomes containing either phosphatidylinositol (PI) or cardiolipin caused a significant reduction in the phagocytic index (Fig. 3B). Although the efficacy of inhibition somewhat varied among those anionic liposomes, the significance of this difference is not certain at the present time. These results suggest the involvement of acidic phospholipids in the phagocytosis of spermatogenic cells by Sertoli cells. Phosphoester compounds related to PS were examined next to determine whether they affected spermatogenic cell phagocytosis (Fig. 3C). The addition of glycerophosphoryl-L-serine led to a significant reduction of phagocytosis. Phospho-L-serine showed marginal inhibition at higher concentrations whereas phagocytosis was unaffected in the presence of its optical isomer, phospho-D-serine. These results suggest that the inhibitory effect of PS liposomes was executed through not only the serine residue but also through the more complicated structure of the phospholipid.

To examine the way these liposomes inhibit phagocytosis, fluorescence-labeled liposomes were used instead of spermatogenic cells as targets for Sertoli cells. Liposomes containing acidic phospholipids were more efficiently engulfed than were liposomes consisting of neutral phospholipids (Fig. 4). This coincided well with the results shown in Fig. 3, indicating that anionic liposomes competed with spermatogenic cells to be phagocytosed by Sertoli cells.

Exposure of PS to the Surface of Dying Spermatogenic Cells—Since the above results suggested that PS was recognized by Sertoli cells, we examined whether degenerating spermatogenic cells expose PS, which is normally restricted in the inner leaflet of the plasma membrane (21), to the cell surface. Spermatogenic cells were single-cultured for various periods and subjected to flow cytometric analysis with FITC-labeled annexin V, which specifically binds to PS (22) (Fig. 5). Since it was necessary to detect PS on the surface of spermatogenic cells, we analyzed only those cells whose plasma membrane remained intact. For that purpose, the cells were simultaneously treated with propidium iodide, which binds to DNA and stains the nucleus of cells whose plasma membranes are damaged and permeable, as described by Martin et al. (18, 19). We observed two distinct populations of spermatogenic cells in terms of their propidium iodide positivity (left panels, zones A and B); it was presumed that the cells with less staining possessed intact plasma membranes, while the membranes of cells that were more intensely stained with the reagent were damaged. The ratio of propidium iodide-positive cells (zone A) increased as the culture period was prolonged. This, in accord with the results shown in Fig. 2, indicates that spermatogenic cells degenerate during single culture.

When the binding of annexin V to cells that presumably possessed intact plasma membranes (zone B) was analyzed, two peaks were found, which probably represented annexin V-negative and -positive cell populations (right panels) (18, 19).
On the other hand, most of the propidium iodide-positive cells (zone A) appeared to be also positive with annexin V (left panels). It is likely that annexin V bound to PS present in both the outer and inner leaflets of the plasma membrane of damaged cells. As the culture continued, the relative number of cells that were propidium iodide-negative and FITC-positive gradually increased. These results suggest that spermatogenic cells that are dying but which still retain membrane integrity are bound by annexin V. From the above results, we presume that during the early stages of apoptotic death PS translocates from the inner leaflet to the outer leaflet of the spermatogenic cell membrane and serves as a signal for phagocytosis by Sertoli cells.

**DISCUSSION**

By adopting the phagocytic index it became feasible to quantitatively analyze spermatogenic cell phagocytosis by Sertoli cells. We showed that Sertoli cells are responsible, at least in culture, for heterophagic elimination of degenerating spermatocytes. As discussed below, Sertoli cells appeared to recognize and phagocyte apoptotic cells in a manner similar to that of macrophages. Macrophages attack a variety of cell types by infiltrating various areas. Sertoli cells, however, are presumed to phagocytose only testicular cells, since they are localized within the seminiferous tubules in the testis.

We tried several approaches in order to clarify the mechanism by which Sertoli cells selectively recognize and phagocytose apoptotic spermatogenic cells. αvβ3-integrin, which mediates phagocytosis of apoptotic neutrophils by macrophages (23), was seemingly not involved in spermatogenic cell phagocytosis, since the addition of the RGDS peptide, which binds to members of the integrin superfamily and inhibits their interaction with specific ligands, did not influence spermatogenic cell uptake by Sertoli cells. In contrast, acidic phospholipids, PS in particular, most likely act as a phagocytosis signal for spermatogenic cells. Liposomes containing acidic phospholipids, when present in the phagocytosis reaction, brought about a great reduction in the phagocytic index, whereas the addition of liposomes containing neutral phospholipids had little effect.

As degeneration of the cultured spermatogenic cells progressed, we observed a significant increase in the number of cells that exposed PS to the cell surface. These results suggest that the amount of cell surface PS, and probably other acidic phospholipids as well, increased during the apoptosis of spermatogenic cells and served as a signal for phagocytosis by Sertoli cells.

Membrane phospholipids of normal cells are localized asymmetrically with regard to the two leaflets of the membrane bilayer; that is, PC and sphingomyelin are mostly present in the outer leaflet, while other phospholipids including anionic PS, PI, and phosphatidic acid, are confined to the inner leaflet.
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It has been suggested that such asymmetry is lost upon induction of apoptosis and that all phospholipids are then redistributed evenly in the membrane bilayer (3, 21). This would result in the exposure of phospholipids that normally exist in the cytoplasmic side to the cell surface, and these phospholipids could help in the distinguishing of apoptotic cells from normal cells. Among those phospholipids, PS has been proposed to be a phagocytosis signal. Translocation of PS from the inner to the outer plasma membrane leaflet has been reported with a variety of apoptotic cells, including thymocytes (24), vascular smooth muscle cells (25), and cultured cell lines (18, 22, 26), as well as with aged red blood cells (27). We showed here that this is also the case with apoptotic spermatogenic cells.

Cell surface PS was shown to be responsible, at least in part, for thymocyte phagocytosis by macrophages (24) and homophagic elimination of vascular smooth muscle cells (25), since the addition of PS-containing liposomes significantly inhibited phagocytosis. However, unlike our findings, phagocytosis of those two cell types was unaffected by PI-containing liposomes. Moreover, Fadok et al. showed that phospho-L-serine stereospecifically inhibited phagocytosis of apoptotic thymocytes by macrophages as efficiently as glycero-phosphoryl-L-serine (24). PS thus most likely serves as a common phagocytosis signal for several different phagocytes such as macrophages, vascular smooth muscle cells, and Sertoli cells, but the modes of recognition of target cells by these phagocytes are presumably somewhat different. We speculate that phagocytosis receptors present on the surface of macrophages and vascular smooth muscle cells are related to but distinct from those of Sertoli cells. It has been proposed that scavenger receptors are responsible for recognition of cell surface PS. Among members of the scavenger receptor family CD36 (28), SR-BI (28, 29), and macroosomal (30, 31) bind to membrane acidic phospholipids including PS. Class B scavenger receptors CD36 (32) and SR-BI (29) were also shown to be involved in recognition and phagocytosis of apoptotic cells. However, only a limited number of examples have been reported, and the identity of the phagocytic receptor for PS is still a matter of conjecture. Whether or not Sertoli cells express any particular type of scavenger receptor is under investigation.

More than half of the differentiating spermatogonial cells undergo apoptosis and are eliminated through phagocytosis by Sertoli cells. It is not completely understood how and why such a great number of spermatogenic cells die before maturing into spermatozoa. We recently showed that Fas and its ligand (33) are differentially localized in the testis; Fas is present in spermatogonial cells and Fas ligand is present in Sertoli cells. It is thus possible that apoptosis of spermatogenic cells is induced by the binding of Fas ligand to Fas through interaction between Sertoli cells and spermatogenic cells. If this is the case, Sertoli cells are presumably responsible for both apoptosis induction and heterogeneous elimination of spermatogenic cells.

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