Role of Class B Scavenger Receptor Type I in Phagocytosis of Apoptotic Rat Spermatogenic Cells by Sertoli Cells*

Akiko Shiratsuchi‡§, Yuki Kawasaki‡, Mamoru Ikemoto¶, Hiroyuki Ara†, and Yoshinobu Nakanishi‡§

From the ‡Faculty of Pharmaceutical Sciences and the §Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Ishikawa 920-0934, Japan and the ¶Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan

Rat Sertoli cells phagocytose apoptotic spermatogenic cells, which consist mostly of spermatocytes, in primary culture by recognizing phosphatidylserine (PS) exposed on the surface of degenerating spermatogenic cells. We compared the mode of phagocytosis using spermatogenic cells at different stages of spermatogenesis. Spermatogenic cells were separated into several groups based on their ploidy, with purities of 60–90%. When the fractionated spermatogenic cell populations were subjected to a phagocytosis assay, cells with ploidies of 1n, 2n, and 4n were almost equally phagocytosed by Sertoli cells. All the cell populations exposed PS on the cell surface, and phagocytosis of all cell populations was similarly inhibited by the addition of PS-containing liposomes. Class B scavenger receptor type I (SR-BI), a candidate for the PS receptor, was detected in Sertoli cells. Moreover, phagocytosis of spermatogenic cells by Sertoli cells was inhibited in the presence of an anti-SR-BI antibody. Finally, the addition of high density lipoprotein, a ligand specific for SR-BI, decreased both phagocytosis of spermatogenic cells and incorporation of PS-containing liposomes by Sertoli cells. In conclusion, SR-BI functions at least partly as a PS receptor, enabling Sertoli cells to recognize and phagocytose apoptotic spermatogenic cells at all stages of differentiation.

Most physiological cell death is caused by apoptosis, and apoptotic cells are believed to immediately undergo heterophagic elimination by surrounding phagocytic cells, such as macrophages (reviewed in Refs. 1–3). However, the molecular basis underlying the phagocytosis of apoptotic cells remains to be clarified.

Apoptosis and subsequent phagocytosis also occur in areas where macrophages do not infiltrate, such as the brain and the testis. In the testis, spermatogenic cells die, probably by apoptosis, before they mature into spermatozoa (reviewed in Refs. 4–8), although the mechanism and meaning of this phenomenon are unknown. The occurrence of spermatogenic cell apoptosis at various stages of differentiation has been reported (Refs. 9–13 and reviewed in Refs. 7 and 8). Only a limited number of apoptotic spermatogenic cells, however, are detectable when testis sections are histochemically examined. This may be due to the elimination of degenerating spermatogenic cells by testicular phagocytes at the early stage of apoptosis. Electron microscopic studies with rodent testis sections have shown that degenerating spermatogenic cells are engulfed by Sertoli cells, a type of testicular somatic cell (14–17). Some murine Sertoli cell lines show phagocytic activity against latex beads (18–20). Sertoli cells are thus likely to be responsible for eliminating apoptotic spermatogenic cells in the testis (21). However, little is known about the regulation of this Sertoli cell function.

We previously reported that primary cultured rat spermatogenic cells of 20-day-old rats, which mostly consisted of spermatocytes, underwent apoptosis and were phagocytosed by Sertoli cells (22). In the apoptotic spermatogenic cells, phosphatidylserine (PS), which is otherwise confined to the inner leaflet of the membrane bilayer, is translocated to the outer leaflet, and phagocytosis is inhibited in the presence of liposomes containing PS (23). In the present study, we examined whether spermatogenic cells at various stages of differentiation are phagocytosed by Sertoli cells in a manner similar to spermatocyte phagocytosis and tried to identify the PS receptor presumed to be present on the surface of Sertoli cells and to be responsible for recognition and subsequent phagocytosis of apoptotic spermatogenic cells.

MATERIALS AND METHODS

Testicular Cell Preparation—Dispersed testicular cells were prepared from testes dissected from either 20- or 45-day-old Donryu rats as described previously (24, 25). The dispersed spermatogenic cells were separated through a 2–4% (v/v) density gradient formed on a 20% cushion of Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) using a Celsep apparatus (Brinkmann Instruments) (26) according to the protocol supplied by the manufacturer. From six 20-day-old rats, 2–3 × 10^7 4n-rich cells and 6–10 × 10^6 2n-rich cells were obtained; and from 45-day-old rats, 1–2 × 10^7 1n-rich cells (first elution), 6–10 × 10^6 2n-rich cells, and 6–10 × 10^5 1n-rich cells (second elution) were obtained. Sertoli cells were isolated as described (23).

Phagocytosis Assay—Phagocytosis of spermatogenic cells by Sertoli cells was performed as described previously (23) with a few modifications. In brief, spermatogenic cells that had been cultured without Sertoli cells for 14–15 h were labeled with biotin (NHS-LS-Biotin, 1 The abbreviations used are: PS, phosphatidylserine; FITC, fluorescein isothiocyanate; HDL, high density lipoprotein; PC, phosphatidylcholine; SR-BI, class B scavenger receptor type I; Ad4BP, Ad4-binding protein.
Fierce) and added to Sertoli cell cultures maintained either in 96-well plates (Corning, Cambridge, MA) for phagocytosis of fractionated spermatogenic cells or in Lab-Tek Chamber Slides (Nalge Nunc, Naperville, IL) for phagocytosis of unfractionated spermatogenic cells of 20-day-old rats at a ratio of 1:1 spermatogenic cells:Sertoli cells. The culture was kept at 32.5 °C for 2 h, and unreacted spermatogenic cells were removed first by pipetting with phosphate-buffered saline and then by trypsin (0.5 mg/ml) treatment for 3 min at room temperature. The remaining cells were fixed, supplemented with fluorescein isothiocyanate (FITC)-labeled avidin (fluorescein-avidin D, Vector Labs, Inc., Burlingame, CA), and examined under a fluorescence/phase-contrast microscope (IX70, Olympus, Tokyo, Japan). The ratio of the phagocytosed cells having fluorescent signals to total Sertoli cells was determined in each microscopic field. Six to eight fields from different culture wells were examined in each experiment, and the results were analyzed statistically. The means ± S.D. of a typical example from at least two independent experiments were taken as the phagocytic index.

Since Sertoli cell-derived cell lines only weakly attached to the culture container, the phagocytosis assay with these cells was slightly modified. The phagocytic cells and cultured spermatogenic cells were mixed and maintained at 32.5 °C for 2–3 h, and the cells were all detached from the culture container by treatment with 0.1% (w/v) trypsin and 0.02% (w/v) EDTA, placed on poly-d-lysine-coated glass slides, and further treated as described above.

Lipoprotein and High Density Lipoprotein (HDL) Preparation—Lipoproteins were prepared as described previously (28). PS-containing lipoproteins were composed of phosphatidylcholine (PC) and PS at a molar ratio of 7:3. Fluorescence-labeled lipoproteins were prepared with N-(lissamine rhodamine B sulfonyl)-L-somes were prepared as described previously (28). PS-containing liposomes were prepared in the density range 1.063–1.21 g/ml from plasma by ultracentrifugation according to standard procedures (29).

Northern Blots—Total RNA was prepared using acid guanidinium thiocyanate (30), and poly(A)-containing RNA was enriched by affinity chromatography (oligo(dT)-cellulose type 2, Collaborative Biomedical Products, Bedford, MA). The RNA was separated on a formaldehyde-containing 1% (w/v) agarose gel and blotted onto a nitrocellulose membrane (BA85, Schleicher & Schuell, Dassel, Germany). The cells negative for propidium iodide staining, which were considered to retain integrity of the plasma membrane, were gated and finally with an anti-Ad4BP antibody (37) for 1 h. To detect the transcription factor Ad4-binding protein (Ad4BP) (37), the fixed Sertoli cells were further treated with 0.1% Triton X-100/phosphate-buffered saline, then with methanol for permeabilization of the plasma membrane, and finally with an anti-Ad4BP antibody (37) for 1 h at room temperature. The cells were supplemented with an FITC-conjugated anti-rabbit IgG antibody (Bio-Rad) and subjected to a chemiluminescence reaction using the Immun-Star system (Bio-Rad).

Immunohistochemistry—Cultured cell lines or Sertoli cells of 20-day-old rats were fixed with 4% paraformaldehyde/phosphate-buffered saline for 20 min at room temperature and blocked with 3% bovine serum albumin for 1 h at room temperature. The fixed cells were then mixed with an anti-SR-BI antibody and left at room temperature for 1 h. To transfect the separation apparatus. The morphology of the separated cells was examined by microscopy, and cells with similar morphology were combined. When the combined cells were subjected to DNA flow cytometry, cell populations with distinct ploidy were found at 60–90% purity (Fig. 1A). The cells were eluted from the gradient in the order of 4n-, 1n-, 2n-, and 1n-rich populations; 1n-rich cells were recovered in two different populations. 2n- and 4n-rich cell populations were obtained from 20-day-old rats, and 1n- and 2n-rich cell populations from 45-day-old rats. The separated cell populations were distinctive in appearance, and morphological examination allowed us to identify particu- ular spermatogenic cell types, i.e. 1n-rich cells (first elution) consisted of round spermatids, 1n-rich cells (second elution) contained many elongated spermatids, most 2n-rich cells were spermatogonia, and 4n-rich cells were spermocytes (Fig. 1B). Testicular somatic cells, including Sertoli cells, were not recovered as a major population in any fraction, and they probably remained attached to the chamber wall of the separation apparatus.

Phagocytosis of Fractionated Spermatogenic Cells by Sertoli Cells—We first determined how efficiently the fractionated spermatogenic cells were phagocytosed by Sertoli cells. The cells were cultured without Sertoli cells for 14–15 h to remove residual contamination of somatic cells that adhered to the culture container. The cultured spermatogenic cells, the viability of which was 80–90% as determined by trypan blue exclu-
tion, were subjected to a phagocytosis assay with isolated rat Sertoli cells. Sertoli cells prepared from 20-day-old rats phagocytosed 2n- and 4n-rich cells of 20-day-old rats as efficiently as they did unfractionated spermatogenic cells (Fig. 2A). Similarly, 1n- and 2n-rich cells prepared from 45-day-old rats were almost equally phagocytosed by Sertoli cells from 20-day-old rats (Fig. 2B). These results showed that spermatogenic cells at various stages of differentiation were phagocytosed by Sertoli cells with similar efficiencies.

Involvement of PS in Phagocytosis of Fractionated Spermatogenic Cells—We previously showed that PS was translocated from the inner to the outer leaflet of the membrane bilayer of unfractionated spermatogenic cells of 20-day-old rats during culture without Sertoli cells and that liposomes containing PS inhibited phagocytosis of the spermatogenic cells by Sertoli cells (23). We thus examined whether PS externalization occurs in fractionated spermatogenic cells. Translocation of PS to the surface of the spermatogenic cells was determined using flow cytometry with FITC-labeled annexin V, which specifically binds to PS (38). All spermatogenic cell populations examined showed the presence of annexin V-bound cells, although the content differed among the populations (Fig. 3A), indicating that PS externalization occurs in apoptotic spermatogenic cells at all stages of differentiation.

We then examined the effect of PS-containing liposomes on phagocytosis of fractionated spermatogenic cells by Sertoli cells. As shown in Fig. 3B, the addition of PS-containing liposomes significantly reduced phagocytosis of 1n- and 2n-rich cells of 45-day-old rats as well as of 4n-rich cells of 20-day-old rats, whereas liposomes composed of PC alone had little effect. The inhibition by PS-containing liposomes of phagocytosis of 2n-rich cells from 20-day-old rats was not significant, but a decrease in the phagocytic index in the presence of the lipo-
somes was reproducibly observed. These results indicated the involvement of PS in the phagocytosis of all apoptotic spermatogenic cells by Sertoli cells.

Identification of Sertoli Cell PS Receptor—The above results suggested the presence of a molecule(s) that recognizes PS and induces phagocytosis on the surface of Sertoli cells. SR-BI (reviewed in Ref. 39) is a strong candidate for such a PS receptor since it recognizes PS (40, 41) and is present in the testis (32, 42–44). We first examined whether Sertoli cells contain SR-BI mRNA. Oligo(dT)-selected RNA from Sertoli cells and spermatogenic cells of 20-day-old rats was blot-hybridized with a hamster SR-BI cDNA probe. The RNA from both cell types showed a discrete signal whose size roughly corresponded to that of the rat ovary SR-BI mRNA (45) (Fig. 4). This indicated that rat Sertoli cells express a gene coding for SR-BI. We then isolated an SR-BI cDNA by screening a library prepared from Sertoli cell mRNA using the hamster SR-BI cDNA as a hybridization probe. Three positive clones were obtained, and sequence analyses of two clones revealed one to be a part of the other. The longer clone included the entire coding region, and the primary sequence of rat Sertoli SR-BI was found to be identical to that of rat ovary SR-BI (45).

To determine the function of Sertoli cell SR-BI, the cDNA was introduced into Sertoli cell-derived cultured cell lines 15P-1 and TM4. From the cells that remained alive in G418-containing medium, 14 clones of 15P-1 and 30 clones of TM4 cells were isolated. They were first examined for the ability to incorporate fluorescence-labeled PS-containing liposomes by flow cytometry. The five 15P-1 clones and the 10 TM4 clones tested incorporated PS-containing liposomes more efficiently than the corresponding parental cells (Fig. 5A). The SR-BI mRNA was detectable in both parental cell lines (Fig. 5B). When parental cells and transfectants were immunohisto-

2 The nucleotide sequence for the rat Sertoli cell SR-BI cDNA has been deposited in the DDBJ/GenBank/EMBL nucleotide sequence data bases with accession number AB002151.

SR-BI as Sertoli Cell PS Receptor

Fig. 3. PS-mediated phagocytosis of fractionated spermatogenic cells by Sertoli cells. A, exposure of PS on the cell surface. Spermatogenic cell populations were analyzed by flow cytometry for the binding of FITC-labeled annexin V. Numbers are the percentages of annexin V-bound cells, indicated by horizontal bars. B, effect of PS-containing liposomes on phagocytosis. Phagocytosis assays were performed in the presence of liposomes composed of either PC only (PC) or a mixture of PC and PS (PS). Phagocytic activity was measured relative to that in a reaction with no added liposomes, which was taken as 100. Significance was determined using Student’s t test. *, p < 0.002; **, p < 0.001.

Fig. 4. Presence of SR-BI mRNA in Sertoli cells. Total RNA (lanes 1 and 4) and poly(A)-containing RNA (lanes 2, 3, 5, and 6) (2.5 μg each) from Sertoli (lanes 1, 2, 4, and 5) and spermatogenic (lanes 3 and 6) cells of 20-day-old rats were analyzed on Northern blots with probes of the hamster SR-BI cDNA (lanes 1–3) and human β-actin pseudogene DNA (lanes 4–6). The positions of 28S and 18S rRNAs are shown with arrowheads.

chemically examined for the presence of SR-BI with anti-SR-BI-110, extranuclear localization of the protein was observed in all of them, and all the transfectants examined were found to contain more SR-BI than the corresponding parental cells (Fig. 5C). These cell clones were then subjected to a phagocytosis assay with apoptotic spermatogenic cells. Both parental cell lines possessed activity for phagocytosing spermaticogenic cells in a PS-dependent manner (Fig. 5D). When the selected transfectants were tested, all showed higher activity levels of PS-mediated phagocytosis than the parental cells; the extent of the increase in the activity caused by SR-BI expression was not
large, but was significant (Fig. 5D). The levels of activity of engulfing latex beads were comparable among parental cells
and transfectants (data not shown). The above results indicated that overexpression of SR-BI confers the PS-mediated
phagocytic activity of apoptotic spermatogenic cells on Sertoli cell-derived cell lines.

We next studied whether SR-BI is actually involved in spermatogenic cell phagocytosis by Sertoli cells. Sertoli cells were
first examined for the presence of SR-BI protein. Membrane fractions prepared from primary cultured Sertoli cells and
spermatogenic cells of 20-day-old rats were analyzed by Western blotting with anti-SR-BI-76 (Fig. 6A). A discrete signal with
a molecular mass of ~70 kDa was detected in Sertoli cell proteins, and it disappeared in the presence of the antigen peptide, but not a peptide corresponding to another region of SR-BI. Moreover, the migration of the signal was almost the
same as that of a signal detectable with rat liver proteins. SR-BI protein seemed much less abundant in spermatogenic cells than in Sertoli cells. When Sertoli cells were immunohistochemically examined with anti-SR-BI-110, many of the cells showed extranuclear signals, but at differing intensities (Fig. 6B). On the other hand, the nuclei of Sertoli cells appeared to be uniformly stained with an antibody specific to the transcription factor Ad4BP, which exists in Sertoli and Leydig cells of the testis (37). Treatment with control normal rabbit IgG did not produce signals (data not shown). These results indicated that SR-BI exists in rat Sertoli cells. The antibodies used here do not distinguish SR-BII (46) from SR-BI. Most of the signal

**FIG. 6.** Effect of anti-SR-BI antibody on phagocytosis by Sertoli cells. A, Western blotting of SR-BI protein. Membrane fractions from liver (50 μg of protein) (lanes 1, 4, and 6), primary cultured Sertoli cells (10 μg of protein) (lanes 2, 5, and 7), and spermatogenic cells (10 μg of protein) (lane 3) of 20-day-old rats were analyzed by Western blotting with anti-SR-BI-76. Peptides (7 pmol) corresponding to amino acid residues 76–95 (lanes 4 and 5) or amino acid residues 110–132 (lanes 6 and 7) of SR-BI were added to the reaction simultaneously with the primary antibody. Shown on the left are the positions of size markers. B, immunohistochemical analysis of Sertoli cells. Primary cultured Sertoli cells of 20-day-old rats were immunohistochemically examined with anti-SR-BI-110 or an anti-Ad4BP antibody. Fluorescence and phase-contrast microscopic views are shown. Scale bar = 10 μm. C, effect of an anti-SR-BI antibody and PS-containing liposomes on phagocytic activity of Sertoli cells. Left panel, phagocytosis of apoptotic spermatogenic cells by Sertoli cells of 20-day-old rats was performed in the presence of anti-SR-BI-76 or normal rabbit IgG. The extent of phagocytosis was measured and is shown relative to that in a reaction with no added antibodies, which was taken as 100. Right panel, anti-SR-BI-76 (3 μg) and PS-containing liposomes (0.5 mM) were added to the phagocytosis reaction either by themselves or simultaneously. Significance was calculated using Student’s t test. *, p < 0.001.

**FIG. 7.** Effect of HDL on phagocytosis and liposome incorporation by Sertoli cells. A, effect of HDL on phagocytic activity of Sertoli cells. The phagocytosis reaction of apoptotic spermatogenic cells by Sertoli cells of 20-day-old rats was conducted in the presence of the indicated concentrations of HDL, and the extent of the reaction is shown relative to that in a control reaction with no added HDL, taken as 100. Vertical bars indicate the standard error. B, effect of HDL on incorporation of PS-containing liposomes by Sertoli cells. Incorporation of fluorescence-labeled PS-containing liposomes by Sertoli cells of 20-day-old rats was examined in the presence of HDL or unlabeled PS-containing liposomes (PS) (1 mM). Left panel, fluorescence microscopic views of liposome-incorporating Sertoli cells are shown. HDL was added at 800 μg/ml. Scale bar = 10 μm. Right panel, the level of the fluorescence signal was determined using a cooled charge coupled device camera and is shown relative to a control reaction, taken as 100.
was, however, likely to be derived from SR-BI since SR-BII is much less abundant than SR-BI in the testis (46). We then determined the effect of anti-SR-BI antibodies on phagocytosis of spermatogenic cells by Sertoli cells prepared from 20-day-old rats. The addition of anti-SR-BI-76 inhibited the phagocytosis reaction, whereas control normal rabbit IgG had a minimal effect (Fig. 6C). Anti-SR-BI-110 showed a similar inhibitory effect (data not shown). Significant levels of the phagocytic activity always remained in the presence of maximal amounts of the antibody, and the residual activity was reduced by the addition of PS-containing liposomes (Fig. 6D), indicating that some part of PS-mediated phagocytosis by Sertoli cells is resistant to anti-SR-BI antibodies. We next examined the effect of SR-BI, a member of the class B scavenger receptor family, is a

**Sertoli cell PS receptor responsible for phagocytosis of apopotic spermatogenic cells.** SR-BI has been shown to function as the PS-recognized phagocytosis receptor in some cultured cell lines such as Chinese hamster ovary cells (41) and a human monocyte-derived cell line, THP-1 (62). It is thus likely that this particular member of the scavenger receptor family is the phagocytosis-inducing PS receptor common to the non-macrophage-type phagocytic cells. Since antibody inhibition of phagocytic activity of Sertoli cells was only partial and the residual activity was inhibitable by PS-containing liposomes, the presence of another phagocytosis-inducing PS receptor is presumed. We previously showed that integrin is not involved in the phagocytosis reaction by Sertoli cells (23). Lectins are not likely to participate in the recognition between Sertoli cells and spermatogenic cells either since the addition of GlcNAc, GlcN, GalN, or Gla at 20 mM did not affect the phagocytosis reaction (data not shown). Other approaches should be taken to identify this additional phagocytosis receptor of Sertoli cells. The level of SR-BI expression did not appear to be uniform among Sertoli cells of 20-day-old rats. This suggests that their phagocytic activity varies at different spermatogenic stages.

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