Abstract

The thymus contains many apoptotic cells that arise from the process of positive and negative selection. Both thymic macrophages and thymic nurse cells/nursing thymic epithelial cells (nursing TECs), non-professional phagocytes, recognize and ingest apoptotic cells without inflammation or tissue damage. Previously we reported that human scavenger receptor class B (SR-B1) is involved in recognition of apoptotic thymocytes by nursing TECs. In this study, we examined the expression and role of a phosphatidylserine receptor (PSR). This receptor is believed to participate in the clearance of apoptotic cells. PSR was strongly expressed in nursing TECs. Transforming growth factor-β augmented the expression of PSR leading to enhanced binding of apoptotic cells to nursing TECs. In nursing TECs, suppressed expression of human SR-B1 with anti-PSR antibody decreased binding of apoptotic thymocytes to nursing TECs. Our results suggest that both PSR and SR-B1 are expressed in nursing TECs and these receptors appear to play a major role in the clearance of apoptotic cells from the thymus.
one TEC and many thymocytes, enclosed by epithelial cytoplasmic processes (Hiramine et al. 1990, 1996).

We have demonstrated that TNCs/nursing TECs in vivo exhibit phagocytic activity. The presence of phagolysosomes with acid phosphatase activity in these cells showed that they participated in the digestion and clearance of apoptotic thymocytes (Hiramine et al. 1996). We also reported that the human scavenger receptor-B1 (hSR-B1) may play a major role in clearance of apoptotic cells from the thymus (Imachi et al. 2000). hSR-B1 is believed to facilitate nursing TEC recognition and ingestion of apoptotic thymocytes. However, nursing TECs transfected with the antisense oligonucleotide against hSR-B1 showed only a 40% inhibition in binding of apoptotic thymocytes. Therefore, we postulate that another receptor or receptors may be involved in the binding of apoptotic thymocytes to nursing TECs. Consistent with this idea, recent findings of Fadok et al. (2000) showed that the PS receptor (PSR) may be involved in specific clearance of apoptotic cells. In the present study, we have examined the role of PSR in the human nursing TEC recognition of apoptotic thymocytes.

Materials and methods

Cell culture

Human monocyte-derived THP-1 and the human T-cell line, Jurkat cells, (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 (Life Technologies, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Life Technologies)/antibiotics in a humidified atmosphere containing 5% CO2, TNF-α, TGF-β1 and IL-1β were obtained from R&D Systems Inc. (Minneapolis, MN, USA).

Establishment of nursing TEC line

An intact thymus was obtained from a 15-year-old female patient with Hodgkin’s disease during a surgical procedure. A human nursing TEC line was established from the lymphoma-free thymus, as described (Hiramine et al. 1990, 1995). The cells were initially maintained in Eagle’s MEM-d-valine medium (Life Technologies) supplemented with 10% FBS, and subsequently the nursing TECs were maintained at 37°C in 5% CO2 in air at 100% humidity in DMEM medium (Life Technologies) supplemented with d-valine (9.2 mg/100 ml) (Wako, Osaka, Japan), 10% FBS and antibiotics.

Immunohistochemical staining

Cells were plated on glass coverslips, incubated at 37°C for 24 h, and then fixed in acetone. These cells were permeabilized with 0.1% NP-40 and incubated with anti-keratin 8 (diluted 1:100) (Dako, Carpinteria, CA, USA) or anti-keratin 5 (diluted 1:100) (Progen, Heidelberg, Germany). Sections were rinsed in PBS, incubated with a biotinylated goat anti-guinea pig IgG (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA, USA) in 1% normal goat serum in PBS, rinsed in PBS, and incubated with avidin-biotinylated peroxidase complex (Vectastain Elite Kit) in PBS according to the manufacturer’s instructions. Antibody binding was visualized with the diaminobenzidine reaction, and sections were counterstained with Mayer’s hematoxylin.

RT-PCR

Total RNA was isolated from nursing TECs as described previously (Chomczynski & Sacchi 1987). PSR mRNAs were detected by PCR analysis of the reverse-transcribed RNA as described previously (Mizobuchi et al. 1994). The primer sequences for hSR-B1 (Calvo & Vega 1993) and PSR were as follows: hSR-B1; sense primer 5'-ATGATCGTGCTTCGTGA TGGTGCCGTC-3' and antisense primer: 5'-ACTGAACCTGACTGCTGA-3'; PSR: sense primer: 5’-CACAAGGACCAAGGCGGA-3' and antisense primer: 5’-AACCACGGGCTTGTAAG-3'. β-Actin served as the control and was amplified and analyzed under identical conditions using previously described primers (Fujita et al. 1994).

Western blot analysis

Cells were washed, scraped in PBS and lysed as described previously (Guo et al. 1996). The proteins were resuspended under reducing conditions and 10 μg were fractionated by size on 7.5% SDS-polyacrylamide gel, and transferred to
polyvinylidene difluoride (PVDF) membranes for immunoblotting. The membranes were incubated for 1 h at 4 °C with 0.2% Tween 20 in PBS containing anti-PSR antibody (3 µg/ml) (Cascade BioScience, Winchester, MA, USA) or anticyclophilin A antibody (diluted 1/1000) (Biomol Research, Plymouth Meeting, PA, USA) or anti-hSR-B1 antibody (diluted 1/1000) as described previously (Murao et al. 1997)). Antibody binding was visualized using a chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech).

**Antisense oligonucleotide treatment**

The oligonucleotide sequences used corresponded to the 5'-ends of the respective mRNA. The hSR-B1 sense oligomer used was 5'-CGCGCAG ACATGGGCTGCTGCCGC-3'; the antisense oligomer was 5'-GGCGGAGCAGCAGCCC ATGTCTGCGCG-3'. Phosphorothioate oligonucleotides were purchased from Nippon Bioservice (Kanagawa, Japan). They were synthesized by a modification of the H-phosphonate procedure and purified by ion-exchange chromatography and ethanol precipitated. The cells were treated with 0.8 µM oligonucleotide every 24 h for 5 days. The proteins were extracted 24 h after the last oligonucleotide treatment as described (Takeuchi et al. 1994, Okada et al. 1997).

**Flow cytometric analysis**

Nursing TECs exposed to the oligonucleotide of interest were washed twice with analysis buffer (PBS containing 0.1% BSA) and then resuspended at a concentration of 1 × 10^7 cells/ml in the same buffer. These cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-SR-B1 antibody (1:100 dilution), as described previously (Murao et al. 1997) on ice for 1 h and then washed twice with analysis buffer prior to resuspending in the same buffer. Cells that were incubated with FITC-conjugated IgG (Dako) served as control. Cells were analyzed on a FACScan machine (Becton Dickinson, San Jose, CA, USA) as described previously (Imachi et al. 2003).

**Statistical analysis**

Statistical comparisons were made by one-way ANOVA and Student’s t-test, with P<0.05 considered significant.

**Results**

**Expression of PSR in human nursing TECs**

A human nursing TEC line, H-1, was established as described in our previous reports (Hiramine et al. 1990, 1995, Imachi et al. 2000). The H-1 cells showed contact inhibition leading to a cobblestone-like arrangement with desmosomes and tonofilament fibers, which were specific for epithelial
cells. Figure 1 shows that the established nursing TECs stained positively for anti-keratin 8 (K8) but not with anti-keratin 5 (K5). This finding raised the possibility that these nursing TECs have the major cortical TEC K8+K5− subset (Klug et al. 1998). The cells were negative for macrophage makers, Mac-1 and CD14 (data not shown). These findings show clearly that the established cell line is not of macrophage lineage and has characteristics of epithelial cells.

Next, we probed for the expression of PSR in the nursing TEC line using RT-PCR and Western blot analysis (Fig. 2A and B respectively). Results showed the presence of PSR cDNA product from RT-PCR that included RNA from THP-1 and nursing TECs (Fig. 2A). Additionally, the PSR protein was also detected in the nursing TECs and THP-1 cells treated with 10 nM transforming growth factor (TGF)-β, serving as a positive control, but not in Jurkat cells as a negative control (Fadok et al. 2000). The data in this section show that both PSR mRNA and protein are present in human nursing TECs.

Role of PSR in binding of apoptotic thymocytes to human nursing TECs

The potential role of PSR in the binding of apoptotic thymocytes to human nursing TECs was examined with an approach similar to that used for hSR-B1. In these prior studies, an inhibitor or antibody against hSR-B1 blocked binding of apoptotic cells to the treated cells. Accordingly, we exposed nursing TECs to an anti-PSR antibody. This treatment reduced the binding of apoptotic cells to human nursing TECs (Fig. 3, column 5). As expected, exposure of nursing TECs to high concentrations of HDL, a ligand of hSR-B1, also reduced the binding of apoptotic thymocytes to the nursing TECs in a dose-dependent manner (columns 2, 3). The combined use of both anti-PSR antibody and HDL showed an additive reduction in the binding of apoptotic thymocytes to the nursing TECs (column 6). Together these findings suggest that both PSR and hSR-B1 participate in the process of binding of apoptotic thymocytes to human nursing TECs.

Cytokines increase both PSR and hSR-B1 expression and binding to thymocytes

Previous studies reported by other investigators and those from our laboratory have shown that several cytokines may modulate the function of receptors which recognize PS (Fadok et al. 2000, Imachi et al. 2001). For example, TGF-β up-regulates expression of PSR in macrophages (Fadok et al. 2000). Therefore, we tested the effect of several cytokines: tumor necrosis factor (TNF)-α, TGF-β1 and interleukin (IL)-1β (Fig. 4A, lanes 2–4 respectively) on expression of PSR and hSR-B1 using Western blot analysis. Pretreatment of the cells with TGF-β1 for 24 h increased expression of PSR in human nursing TECs (Fig. 4A, lane 3). This stimulatory effect of TGF-β was dose-dependent with maximal response at 10 nM TGF-β (data not shown). In contrast, TNF-α (Fig. 4A, lane 2) had no effect on PSR expression, but it increased the expression of hSR-B1 in nursing TECs (Fig. 4A, lane 2).

To assess activity of the nursing TECs following 24 h of exposure to cytokines, confluent monolayers of these cells were incubated with viable or apoptotic thymocytes. Figure 4B shows that treatment with the cytokines tested did not affect binding of the viable
thymocytes to the nursing TECs. In contrast, the results showed that both TGF-β and TNF-α increased the binding of apoptotic thymocytes to the nursing TECs. Furthermore, treatment with both TNF-α and TGF-β increased the expression of SR-B1 and PSR respectively, leading to an additive effect of apoptotic thymocyte binding activity. These results suggest that the cytokines increased expression of PSR or/and hSR-B1 and augmented the binding of apoptotic cells to the nursing TECs.

Antisense oligonucleotides against hSR-B1 decrease nursing TEC binding to apoptotic thymocytes

If increasing either hSR-B1 or PSR enhances activity of the nursing TECs, then decreasing their expression should have the opposite effect. To test this hypothesis, we used antisense oligonucleotides against hSR-B1. Results (Fig. 5A) show that exposure of cells to this oligomer suppressed surface expression of the receptor in nursing TECs as reflected in flow cytometric analysis. Consistent with our hypothesis, TECs transfected with antisense oligonucleotide for SR-B1 cells showed a marked decrease in binding of apoptotic thymocytes (Fig. 5B, compare sense with antisense). Next, we attempted to suppress the expression of PSR by treating the cells with several antisense

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**Figure 2** Expression of PSR in human nursing TECs. (A) RT-PCR of hSR-B1 in nursing TECs. Lane M, markers; lane 1, THP-1 cells as a positive control; lane 2, nursing TECs; lane 3, negative control without reverse transcriptase. (B) Western blot analysis. Total cell protein extracted with 1% NP-40 from untreated THP-1 cells treated with TGF-β, human nursing TECs, and Jurkat cells were separated by SDS-PAGE, transferred to PVDF membrane and blotted with anti-PSR antibody. Each lane contained 10 µg protein. Lane 1, THP-1 cells with TGF-β as a positive control; lane 2, human nursing TECs; lane 3, Jurkat cells as a negative control.

**Figure 3** Effects of various competitors on binding of apoptotic thymocytes to nursing TECs. Apoptotic thymocytes were added to the nursing TECs treated with various competitors. The binding of thymocytes by the nursing TEC was determined as described under Methods. Results are the means±S.E. of three experiments. Lane 1, no treatment; lane 2, nursing TECs treated with 0.5 µg/ml HDL; lane 3, nursing TECs treated with 1 µg/ml HDL; lane 4, nursing TECs treated with 200 µg/ml mouse IgM as a negative control for anti-PSR antibody; lane 5, nursing TECs treated with 200 µg/ml anti-PSR antibody; lane 6, nursing TECs treated with both 1 µg/ml HDL and 200 µg/ml anti-PSR antibody. *Significantly different (P<0.005).
oligonucleotides for PSR, but unfortunately the studies were unsuccessful. Therefore, we used an antibody that inhibits the binding of apoptotic cells to PSR specifically. The addition of an antibody against PSR to cells treated with antisense against hSR-B1 led to a further decrease in nursing TEC binding to apoptotic thymocytes. As expected, the addition of HDL to mask the actions of hSR-B1 (column 3) caused no further decrease in binding of the nursing TECs to apoptotic thymocytes as compared with that of cells treated with hSR-B1 antisense alone (column 2). These findings suggest that the antisense oligomer had already decreased expression of SR-B1. Together, these results show that both hSR-B1 and PSR participate in the binding of nursing TECs to apoptotic thymocytes.

**Discussion**

Efficient removal of apoptotic cells prior to lysis is of particular importance in the thymus, where many millions of cells are eliminated on a daily basis. This function is carried out by thymic macrophages and TNCs/nursing TECs (Hiramine et al. 1996). The mechanism(s) by which apoptotic

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**Figure 4** Effect of cytokines on PSR and hSR-B1 expression and on binding of apoptotic thymocytes. (A) Nursing TECs treated for 24 h without (lanes 1, 5) or with 10 nM TNF-α (lane 2), 10 nM TGF-β (lane 3), 100 units/ml IL-1β (lane 4) and 10 nM TNF-α plus 10 nM TGF-β (lane 6) were separated by SDS-PAGE, transferred to PVDF membrane and blotted with anti-PSR or anti-hSR-B1 antibody. Each lane contained 10 µg protein. (B, C) Effect of cytokines on binding of viable thymocytes (B) or apoptotic thymocytes (C). Nursing TECs were pretreated for 24 h without (control) or with 10 nM TNF-α (TNF), 10 nM TGF-β (TGF), 100 units/ml IL-1β (IL-1), and 10 nM TNF-α pulse 10 nM TGF-β (TGF+TNF). Then the binding experiment was carried out. Results are the means±s.e. of five experiments. *Significantly different (P<0.005).
cells are recognized and removed have received considerable attention in the last few years because a number of receptors that may be involved in this process have recently been identified in vitro. The list includes: SR-A, CD36, hSR-B1, CD68 and CD14 (Savill et al. 1993, Pearson 1996, Krieger 1997). The only known receptor so far demonstrated to be definitely involved in this process is SR-A and this protein is abundantly expressed in thymic macrophages. The other receptors studied were expressed at much lower levels. Studies of Platt et al. (1996) showed that an antibody against murine SR-A inhibited by one-half the ability of thymic macrophages to phagocytose apoptotic thymocytes. Recently, we demonstrated that hSR-B1 was strongly expressed on human TNCs/nursing TECs and that antisense hSR-B1 oligonucleotides significantly inhibited binding of apoptotic thymocytes to nursing TECs (Imachi et al. 2000).

There is a considerable body of evidence showing that one mechanism by which macro-

![Graph](image-url)

**Figure 5** Effect of anti-PSR antibody on binding of apoptotic thymocytes in nursing TECs treated with antisense oligonucleotides against hSR-B1. (A) Flow cytometric analysis to detect surface expression of SR-B1 in nursing TECs treated with sense or antisense oligonucleotide against SR-B1. Cells were labeled with control IgG (control) or anti-SR-B1 (SR-B1) for the analysis. (B) Apoptotic thymocytes were added to the nursing TECs treated with the oligonucleotides and/or anti-PSR antibody. The binding of thymocytes by the nursing TECs was determined as described under Methods. Column (1), sense SR-B1 – sense oligonucleotide against SR-B1; column (2), antisense SR-B1 – antisense oligonucleotide against SR-B1; column (3), antisense SR-B1 and HDL – antisense oligonucleotide against SR-B1 and 1 µg/ml HDL; column (4), antisense SR-B1 and PSR-Ab – antisense oligonucleotide against SR-B1 and 200 µg/ml anti-PSR antibody. Results are the means±S.E. of six experiments. *Significantly different (P<0.005).
phages recognize apoptotic cells is mediated through recognition of excess PS on the plasma membrane (Fadok et al. 1992a,b, Savill et al. 1993). Exposure of PS on the surface of apoptotic cells is associated with macrophage phagocytosis of selected cell phenotypes (Savill et al. 1990, Fadok et al. 1992a, 1993). Loss of membrane phospholipid asymmetry and the presence of PS on the externally present surface are features of many asymmetry and the presence of PS on the externa

under active investigation (Fadok mechanism(s) mediating this membrane change are under active investigation (Fadok et al. 1992a, Mower et al. 1994, Martin et al. 1995). We showed that binding of apoptotic thymocytes to human nursing TECs was almost completely prevented by unilamellar liposomes containing PS but not by liposomes containing phosphatidylcholine (PC) (Imachi et al. 2000).

Although SR-B1 is now identified as a receptor for HDL (Acton et al. 1996, Murao et al. 1997), it was originally thought to belong to the SR family. This initial assignment was based on the ability of SR-B1 to bind anionic phospholipids (Rigotti et al. 1995) and modified forms of low-density lipoprotein (Acton et al. 1994), including OxLDL. SR-B1 has been shown to function as the receptor that recognizes PS in cell lines transfected with cDNA, including Chinese hamster ovary (Fukasawa et al. 1996) and human embryonic kidney cells (Murao et al. 1997). However, the nursing TECs transfected with antisense oligonucleotide against SR-B1 showed only 40% inhibition in binding of apoptotic thymocytes. One possible explanation for this observation is that receptor(s), yet unknown, may add to the net capacity of the entire cell to bind apoptotic thymocytes.

Recently Fadok et al. (2000) isolated PSR, a receptor that is PS-specific for the clearance of apoptotic cells. They cloned the PSR gene by using phage display and biopanning with the anti-PSR antibody. They have shown that this antibody almost completely inhibits the binding of apoptotic cells to PSR stably expressing cells. This observation prompted us to use the antibody in our blocking studies. However, the possibility that the antibody may also inhibit another apoptotic cell-recognition receptor cannot be excluded at this point. Our current study shows that PSR is strongly expressed on human nursing TECs and exposure of the cells to an antibody against the protein significantly inhibited binding of apoptotic thymocytes to these cells. This antibody had an additive effect to further inhibit binding of apoptotic cells to nursing TECs exposed to antisense oligonucleotide against hSR-B1. In other words, the ability of nursing TECs to bind apoptotic cells appeared to be the net effect of both hSR-B1 and PSR. Consistent with this idea, results in Fig. 4 show that TGF-β and TNF-α increased the binding capacity of apoptotic cells by increasing the expression of the receptors, PSR or SR-B1 respectively. The mechanism of cytokine effects on phagocytosis of apoptotic cells in the thymus remains to be explored.

In summary, we detected expression of PSR in human nursing TECs and showed that in addition to hSR-B1, PSR also plays a major role in nursing TEC binding to apoptotic thymocytes. The role of PSR as well as hSR-B1 in phagocytosis of apoptotic cells in the thymus in vivo remains to be explored.

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