Interaction Between Src Homology 2 Domain Bearing Protein Tyrosine Phosphatase Substrate-1 and CD47 Mediates the Adhesion of Human B Lymphocytes to Nonactivated Endothelial Cells

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Interaction Between Src Homology 2 Domain Bearing Protein Tyrosine Phosphatase Substrate-1 and CD47 Mediates the Adhesion of Human B Lymphocytes to Nonactivated Endothelial Cells

Hitoshi Yoshida,* Yoshiaki Tomiyama,** Kenji Oritani,* Yoko Murayama,* Jun Ishikawa,* Hisashi Kato,* Jun-ichiro Miyagawa,* Nakayuki Honma, † Tetsuo Nishiura,* and Yuji Matsuzawa*

CD47 modulates a variety of cell functions such as adhesion, spreading, and migration. Using a fusion protein consisting of the extracellular region of Src homology 2 domain bearing protein tyrosine phosphatase substrate-1 (SHPS-1) and the Fe portion of human Ig (SHPS-1-Ig) we investigated the effects of SHPS-1 as a ligand for CD47 on B lymphocytes. Although SHPS-1-Ig binding to human B cell lines was solely mediated via CD47, their binding capacity for soluble and immobilized SHPS-1-Ig varied among cell lines irrespective of the similar expression levels of CD47, suggesting that distinctive affinity/avidity states exist during B cell maturation. NalM6 cell line and tonsilar B lymphocytes adhered to immobilized SHPS-1-Ig and showed polarization-like morphology. These effects of SHPS-1-Ig were blocked by anti-CD47 mAbs (B6H12 and SE5A5). Wortmannin, a phosphatidylinositol-3 kinase inhibitor, but not pertussis toxin significantly inhibited the polarization induced by the immobilized SHPS-1-Ig. Thus, SHPS-1 acts as an adhesive substrate via CD47 in human B lymphocyte. Immunohistochemical analyses indicated that SHPS-1 is expressed on high endothelial venule as well as macrophages in human tonsils. HUVECs also express SHPS-1 in the absence of any stimuli, and the adhesion of tonsilar B lymphocytes to nonactivated HUVECs was significantly inhibited by SE5A5, indicating that SHPS-1/CD47 interaction is involved in the adhesion. Our findings suggest that SHPS-1/CD47 interaction may contribute to the recruitment of B lymphocytes via endothelial cells under steady state conditions. The Journal of Immunology, 2002, 168: 3213–3220.

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Thrombospondin (TSP) is known to be a ligand for CD47 and involved in modulating the differentiation, proliferation, and migration of various cells (22, 23). The interaction between TSP and CD47 induces the activation of autocrine T lymphocytes and the spreading of platelets and melanoma cells on fibrinogen (24–26). Recently, Src homology 2 domain bearing protein tyrosine phosphatase substrate-1 (SHPS-1) has been shown to be a ligand for CD47 (27, 28). SHPS-1 is a glycosylated receptor-like protein with three Ig-like domains in its extracellular region and has two immunoreceptors with tyrosine-based inhibition motifs in its intracellular region (29). The intracellular region of SHPS-1 seems to act as a site for the recruitment of Src homology 2 domain containing phosphatases, leading to inhibition of signals evoked by cytokines and integrins that promote tyrosine kinase activity (30, 31). However, the biological roles of the extracellular region of SHPS-1 remain obscure. In this study, we have demonstrated the expression of SHPS-1 even on nonactivated endothelial cells including HEV in tonsils, and suggested a novel role of SHPS-1/CD47 interaction on B lymphocyte biology.

Materials and Methods

Abs and reagents

mAbs specific for CD47 (B6H12 and 2D3, IgG2b) were generously provided by Dr. E. Brown (Washington University, St. Louis, MO; Ref. 32). B6H12 has been reported to modulate the β2 integrin-mediated cell functions and inhibit the transmigration of neutrophils (8, 12, 14). In contrast, 2D3 has been shown to modulate the cell functions. An anti-CD47 mAb, SES5A5, which can specifically block the binding of SHPS-1 to CD47 was generously provided by Dr. H.-J. Bühring (Eberhard-Karls Universität, Tübingen, Germany; Ref. 28). MOPC195 (IgG2b) was purchased from Cappel Laboratories (Cochranville, PA). Wortmannin was purchased from Sigma Aldrich (St. Louis, MO). Rabbit anti-SHPS-1 polyclonal Ab was generously provided by Dr. T. Hirano (Osaka University, Osaka, Japan). Wortmannin was generously provided from Dr. T. Sudoh (Kirin Brewery Company, Gunma, Japan) and Dr. T. Matozaki (Osaka University; Ref. 37). A construct of Ig fusion protein of human SHPS-1 (SHPS-1-Ig/FEBOS) was produced as previously described (38). The extracellular domain of SHPS-1 was amplified from cDNA using sense primer (5′-GGATGCTTGATCCTAACAAGTTG-3′) and antisense primer (5′-GGACGTTAGGAGATGCCAG-3′) under the following conditions: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles. The amplified fragments digested with KpnI and XhoI and the 5′ fragments of SHPS-1/Tracer CMV vector digested with NotI and KpnI were ligated into the Ig/FEBOS vector (SHPS-1-Ig/FEBOS). The sequence of this vector was confirmed by using the DNA Sequencing Kit (PerkinElmer, Warrington, U.K.). SHPS-1-Ig and CD44-Ig (38) were purified by Immunopure protein A-Sepharose column (Pierce, Rockford, IL) from culture supernatants of 293T cells transfected with SHPS-1-Ig/FEBOS and CD44-Ig/FEBOS, respectively.

Flow cytometry

The surface phenotypes of cells were examined with indirect immunofluorescent method as previously described (21). Briefly, the aliquots (100 µl) of cell suspension (1 × 10⁵ cells/µl) were first incubated with an appropriate mAb (1 µg/ml) or rabbit polyclonal Ab (1 µg/ml) at 4°C for 30 min, and then rinsed and incubated with secondary FITC-conjugated goat anti-mouse IgG (BD Biosciences, Mountain View, CA) or goat anti-rabbit Ig (DAKO, Copenhagen, Denmark). To measure the binding of SHPS-1-Ig or CD44-Ig, the aliquots (100 µl) of cell suspension (1 × 10⁵/µl) were incubated with each fusion protein (1 µg/ml) in HBSS at 4°C for 30 min, and then rinsed and incubated with FITC-conjugated goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL). The analysis was performed using FACSsort (BD Biosciences).

Immobilization of fusion proteins, peptides, or mAbs

Immobilization of fusion proteins or mAbs was performed as previously described (21). In brief, 96-well microtiter plates were coated with 50 µl of SHPS-1-Ig, CD44-Ig (10 µg/ml), or 1% BSA at 37°C for 30 min, and washed with PBS. For blocking nonspecific-binding sites, 96-well microtiter plates were further incubated with 50 µl of 1% BSA at 37°C for 30 min. The wells were washed with PBS three times before cell culture. In some experiments, 96-well microtiter plates were coated with various mAbs (10 µg/ml) by the same procedure described above. ELISA using anti-human Ig or anti-mouse Ig showed that an equal amount of each fusion protein or mAb was coated on each well.

Polarization assay

The polarization assay was performed as described previously (21). The cells were washed with chemically defined medium (AIM-V medium, Life Technologies) and resuspended in AIM-V medium with 0.1% BSA. The aliquots (100 µl) of cell suspension (5 × 10⁵/µl) were added into the 96-well microtiter plates coated with SHPS-1-Ig, CD44-Ig, or mAb as described above. After a 2-h incubation, the number of a polarization-like morphology was counted in the indicated area under the phase-contrast microscopy. At least 300 cells/well were counted blindly. The polarization was scored according to the criteria by Wilkinson (39). The proportion of polarized cells was calculated using the following formula: the percentage of polarization = (polarized cell number/total cell number) × 100.

Adhesion assays

To measure the activity to adhere to SHPS-1-Ig, the adhesion assays using a crystal violet method were performed as previously reported (21). Briefly, 1 × 10⁵ cells treated with or without various mAbs in AIM-V medium were incubated for 1 h on the 96-well microtiter plates coated with SHPS-1-Ig or CD44-Ig. After washing the wells with the medium three times, adherent cells were fixed with 1% glutaraldehyde in PBS for 15 min and stained with 0.1% crystal violet solution for 30 min. Following the washing by distilled water for 15 min, the dyes were solubilized with 200 µl of distilled water containing 0.2% Triton X-100, and then the absorbance at 620 nm was measured by microphotometry. In some experiments, human tonsilar B cells (5 × 10⁵ cells/well) were incubated on HUVEC monolayer in 96 microtiter wells in the presence or absence of anti-CD47 mAb.

Phosphatidylinositol-3 kinase (PI3K) activity assay

PI3K activity assay was performed as previously described (40). After the incubation on immobilized SHPS-1-Ig or CD44-Ig for 15 min, Nalm6 cells were lysed in a lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 137 mM NaCl, 5 mM EDTA, 1% glycerol, and 1% Nonidet P-40) containing protease inhibitors, and insoluble material was removed by centrifugation at 10,000 × g at 4°C for 10 min. The cell lysates were subjected...
Immunofluorescence microscopy

The 4% paraformaldehyde-fixed cryostat sections (8 μm) of the tonsils were incubated with rabbit anti-SHPS-1 polyclonal Ab, followed by the incubation of biotinylated goat anti-rabbit Ig and alkaline phosphatase-conjugated streptavidin (DAKO). Reactions were visualized using the 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium kit (Promega, Madison, WI). Following that, the sections were incubated with mouse anti-PI9 mAb, MECA-79 (IgM; BD Pharmingen, San Diego, CA), and then incubated with FITC-conjugated anti-mouse Ig mAb (DAKO). As a negative control, the primary Ab was replaced with normal rabbit IgG (DAKO). The sections were counterstained with methyl green, followed by an appropriate FITC-conjugated secondary Ab (DAKO). As a negative control in the subsequent experiments. In Nalm6, BALL, RPMI8226, and OPM-2 than to ONHL-1 and RPMI8226 cells (1.7 0 92.3). Although SHPS-1-Ig bound to all B cell lines tested, the amounts of the bound SHPS-1-Ig did not correlate to the expression levels of CD47; larger amounts of SHPS-1-Ig bound to CD47 (Table I). Although SHPS-1-Ig bound to all B cell lines tested, the amounts of the bound SHPS-1-Ig did not correlate to the expression levels of CD47; larger amounts of SHPS-1-Ig bound to Nalm6 cells, while 2D3, a nonfunctional mAb, did not block its binding (Fig. 1B). B6H12 completely blocked the binding of SHPS-1-Ig to Nalm6 cells, while 2D3, a nonfunctional mAb, did not block its binding (Fig. 1B). B6H12 completely blocked the binding of SHPS-1-Ig to the other cell lines as well as Nalm6 cells (data not shown). The complete blockade of SHPS-1-Ig binding by B6H12 suggests that the binding of SHPS-1-Ig requires CD47. In addition, precubation of Nalm6 cells with SHPS-1-Ig significantly inhibited the binding of B6H12 (data not shown). Essentially the same results were obtained in other B cell lines (ONHL-1 and RPMI8226; data not shown). These data suggest that SHPS-1 binds to human B cells via CD47 and there are some differences in the affinity/avidity of CD47 for SHPS-1-binding between the types of human B lymphocytes.

Table I. The binding of SHPS-1-Ig to human B cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SHPS-1-Ig</th>
<th>MFI CD44-Ig</th>
<th>CD47</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-B</td>
<td>15.1</td>
<td>0.1</td>
<td>122.3</td>
</tr>
<tr>
<td>Nalm6</td>
<td>18.9</td>
<td>0</td>
<td>86.2</td>
</tr>
<tr>
<td>BALL</td>
<td>18.9</td>
<td>0</td>
<td>86.2</td>
</tr>
<tr>
<td>Mature B</td>
<td>5.6</td>
<td>0.1</td>
<td>81.8</td>
</tr>
<tr>
<td>ONHL-1</td>
<td>1.7</td>
<td>0</td>
<td>92.3</td>
</tr>
<tr>
<td>OPM-3</td>
<td>27.7</td>
<td>0</td>
<td>89.0</td>
</tr>
<tr>
<td>RPMI8226</td>
<td>14.2</td>
<td>0</td>
<td>86.5</td>
</tr>
</tbody>
</table>

*The binding of SHPS-1-Ig and the expression of CD47 were analyzed by flow cytometry. Cells (1 10^5) were incubated with SHPS-1-Ig, CD44-Ig, or anti-CD47 mAb (B6H12) (1 μg/ml), followed by an appropriate FITC-conjugated secondary Ab in HBSS. The complete blockade of SHPS-1-Ig binding by B6H12 suggests that the binding of SHPS-1-Ig requires CD47. In addition, precubation of Nalm6 cells with SHPS-1-Ig significantly inhibited the binding of B6H12 (data not shown). Essentially the same results were obtained in other B cell lines (ONHL-1 and RPMI8226; data not shown). These data suggest that SHPS-1 binds to human B cells via CD47 and there are some differences in the affinity/avidity of CD47 for SHPS-1-binding between the types of human B lymphocytes.

**Immunofluorescence microscopy**

We next examined the adhesion of human B cell lines to immobilized SHPS-1-Ig. Among B cell lines, pre-B cell lines (Nalm6 and BALL) and a lymphoma B cell line (ONHL-1) markedly adhered to immobilized SHPS-1-Ig in a dose-dependent manner (Fig. 2A). In contrast, two myeloma cell lines (RPMI8226 and OPM-2) showed the dose-dependent but modest adhesion to immobilized SHPS-1-Ig. A mature B cell line (OPM-3) scarcely adhered in any doses of immobilized SHPS-1-Ig performed in this study. Again, B6H12 completely inhibited the adhesion of Nalm6 to immobilized SHPS-1-Ig, while 2D3 did not (Fig. 2B). These findings suggest that SHPS-1 acts as an adhesive substrate via CD47 in certain human B cell lines.

**Immunofluorescence microscopy**

We have reported that immobilized B6H12 induces an elongated and polarized shape change (polarization) in pre-B cell lines.
SHPS-1 expression was examined in rat and cattle lymph nodes (Nalm6 and BALL) and mature B cell lines (ONHL-1 and OPM-3), but not in myeloma cell lines (RPMI8226 and OPM-2; Ref. 21). To extend our findings, we examined the morphological changes induced by immobilized SHPS-1-Ig. Among B cell lines tested, SHPS-1-Ig induced polarization only in Nalm6 cells, and the percentage of the polarized cells induced by immobilized SHPS-1-Ig was smaller than immobilized B6H12 (Fig. 3). Immobilized CD44-Ig did not significantly induce the polarization in Nalm6 cells. These data suggest that SHPS-1 provokes the signals to induce the cytoskeletal reorganization via CD47, at least in some pre-B cell lines.

PTK3 is involved in signals inducing SHPS-1/CD47 interaction

PTX-sensitive heterotrimeric guanosine triphosphate-binding protein (G protein) has been reported to be involved in the signaling via the TSP/CD47 interaction (41). Although the activity of PTX was confirmed by Chinese hamster ovary cell cytotoxicity assay (42), PTX could not significantly inhibit the immobilized SHPS-1-Ig-induced polarization of Nalm6 cells. In contrast, a specific PI3K inhibitor, wortmannin, completely inhibited the polarization of Nalm6 cells (97% decrease of polarization; Fig. 4A). Wortmannin also significantly inhibited the adhesion to immobilized SHPS-1-Ig (38% decrease of adhesion), while PTX did not affect the adhesion activity (Fig. 4B). To investigate whether PI3K is activated by SHPS-1/CD47 interaction, we performed PI3K activity assay. PI3K is constitutively activated. However, immobilized SHPS-1-Ig further activated the PI3K activity (Fig. 4C). Therefore, PI3K, but not PTX-sensitive G protein, is mainly involved in the signals through the interaction between SHPS-1 and CD47, at least in Nalm6 cells.

Human tonsil B cells adhere to SHPS-1-Ig via CD47

To further investigate the role of CD47/SHPS-1 interaction in B lymphocyte biology, we examined the effect of immobilized SHPS-1-Ig on the adhesion of normal B lymphocytes isolated from tonsils. As shown in Fig. 5A, enriched tonsil B lymphocytes significantly adhered to immobilized SHPS-1-Ig (percentage of adhesion of applied cells was 43.5 ± 0.7%; n = 3). The adhesion of human tonsil B lymphocytes to SHPS-1-Ig was again completely inhibited by B6H12. Moreover, after 1–2 h of incubation on immobilized SHPS-1-Ig, ~20% of tonsil B lymphocytes showed the polarization-like or dendritic shape change (Fig. 5, B and C). To confirm that the adhered cells were really B lymphocytes, we performed the immunofluorescence staining using anti-CD19 mAb. As shown in Fig. 5D, CD19 immunoreactivity was detected in most of the cells adhered to immobilized SHPS-1-Ig. In addition, the immunoreactivity for CD19 was also observed in the polarized cells. These findings indicate that interaction between SHPS-1 and CD47 may mediate normal B lymphocyte adhesion and subsequent their morphological change.

SHPS-1 is expressed on HEV in human tonsil

To further clarify the physiological roles of SHPS-1, we examined the expression and localization of SHPS-1 in human tonsil by immunohistochemical staining. SHPS-1 immunoreactivity was detected in macrophages in interfollicular space and was weakly detected in tingible macrophages (data not shown). In contrast, there was no detectable stain of SHPS-1 in the lymphocytes in tonsils. These data are consistent with previous reports, in which the SHPS-1 expression was examined in rat and cattle lymph nodes (43, 44). In addition to the expression on macrophages, we found
that SHPS-1 immunoreactivity was detected on endothelial cells in tonsils (Fig. 6A). It is noteworthy that immunoreactivity of PNAd, a specific marker of HEV, was detected in the endothelial cells (Fig. 6B). These results indicate that SHPS-1 is expressed on HEV.

**The interaction between SHPS-1 and CD47 is involved in the adhesion of human tonsilar B cells to HUVECs**

To investigate whether the interaction between SHPS-1 and CD47 may be involved in the adhesion of human tonsilar B cells to endothelial cells, we used HUVECs as a source of SHPS-1. As shown in Fig. 7A, HUVECs constitutively express SHPS-1 on their surface (mean fluorescence intensity (MFI) 6.8) as well as HEV cells under nonactivated conditions. Although the expression of ICAM-1 was significantly up-regulated (MFI 14.3 and 128.2, before and after 36 h treatment, respectively), the expression of SHPS-1 on HUVECs was scarcely changed by IL-1β, a proinflammatory cytokine, for up to 36 h (MFI 6.5, after treatment; Fig. 7A). We then performed the adhesion assay using enriched human tonsilar B cells and nonactivated HUVECs in static conditions in the presence or absence of anti-CD47 mAb (SE5A5). Human tonsilar B cells apparently adhered to nonactivated HUVECs, and SE5A5, which is a blocking mAb for the binding between SHPS-1 to CD47, but has no functional activity, significantly inhibited the adhesion of human tonsilar B cells to nonactivated HUVECs (45% decrease of adhesion; Fig. 7B). To exclude the possibility that the contaminated cells such as monocytes adhered to HUVECs, we performed flow cytometric analyses. Flow cytometric analyses showed that ~90% of the adhered cells to HUVECs were CD19 positive (data not shown). These data suggest that the interaction between SHPS-1 and CD47 may be involved in the adhesion of human B lymphocytes to nonactivated endothelial cells.

**Discussion**

SHPS-1 is a ligand for CD47 and its expression has been shown on neuronal and myeloid cells (27, 28). However, the physiological role of its extracellular domain remains elusive. In this study, we have newly demonstrated that SHPS-1 is constitutively expressed on endothelial cells including HEV in human tonsil. In addition, the interaction between SHPS-1 and CD47 was involved in the adhesion of B cells to nonactivated human endothelial cells and the induction of a polarized shape change in some tonsilar B cells and pre-B cell line, Nalm6. Thus, CD47 acts as a signaling molecule, at least in certain B cell subpopulations. Our findings newly suggest that SHPS-1/CD47 interaction may contribute to the recruitment of B lymphocytes via endothelial cells under steady state conditions.

CD47 has been reported to play important roles in the activation, adhesion, and migration of leukocytes in concert with integrins. Recently, we have demonstrated that CD47 transduces the signals involved in the cell migration through Cdc42, a member of Rho family GTPases (21). However, these results were obtained using ligand-mimic anti-CD47 mAb, B6H12. In this study, using a fusion protein composed of the extracellular domain of SHPS-1 and the Fc portion of human IgG, we have investigated the role of SHPS-1 as a physiological ligand for CD47. We revealed that SHPS-1-Ig acts as an adhesive substrate in human B lymphocytes. In addition, SHPS-1-Ig showed similar effects on certain human B lymphocytes to B6H12. B6H12 completely blocked the binding of SHPS-1-Ig to CD47, and SHPS-1-Ig partially blocked the B6H12 binding to CD47. These findings strongly suggest that the binding sites of SHPS-1 and B6H12 in CD47 are very close to each other, which may account for similar effects of these ligands on human B cells. Seiffert et al. (28) reported that extracellular signal-regulatory protein, which is identical to SHPS-1, cannot bind to the CD47-deficient human ovarian carcinoma cell line OV10, but to human CD47-transfected OV10. Taken together, it is strongly suggested that SHPS-1 exclusively bound to CD47 on human B lymphocytes. Although all B cell lines examined in this study expressed CD47, the binding capacity of soluble SHPS-1-Ig varied between B cell lines: pre-B cell lines (Nalm6 and BALL) and myeloma cell lines (RPMI8226 and OPM-2) bound larger amounts of SHPS-1-Ig than mature B cell lines (ONHL-1 and OPM-3). Of particular interest was that pre-B cell lines, but not myeloma cell lines, well-adhered to immobilized SHPS-1-Ig irrespective of the similar binding capacity for soluble SHPS-1-Ig. Our findings suggest that the affinity/avidity state of CD47 for SHPS-1 may change during B cell maturation. In addition, the intercellular signaling evoked by the CD47/SHPS-1 interaction may also change during B cell maturation. Because SHPS-1-Ig contains the Fc portion of human IgG1, there exists the possibility that FcR would be involved in the binding and the adhesion to SHPS-1-Ig. Flow cytometric analysis showed that the expression of FcγRII (CD32) was modestly expressed on Nalm6 and RPMI8226 cells and abundantly on OPM-2 (data not shown). However, there is no relationship between the SHPS-1-Ig function and the expression of FcγR. In addition, CD44-Ig, which also contains the Fc portion of IgG, did not bind to any human B cell lines in this study. These results suggest that SHPS-1-Ig binds to human B cell lines via CD47.

In this study, we investigated the signaling via SHPS-1/CD47 interaction in Nalm6 cells. Wortmannin, but not PTX, affected the adhesion to immobilized SHPS-1-Ig and the morphological change induced by SHPS-1 in Nalm6 cells. In contrast, it has been shown that PTX-sensitive heterotrimeric GTP-binding protein (G protein)
is involved in the signals evoked by TSP/CD47 interaction (10, 41). These results suggested that SHPS-1 and TSP may play differential roles in human B lymphocyte biology. In addition to marked inhibition of the polarized shape change and cell adhesion by wortmannin, SHPS-1/CD47 interaction activates PI3K activity in Nalm6 cells. Although the intracellular signaling cascade evoked by SHPS-1/CD47 interaction in B lymphocyte remains to be clarified, PI3K may be involved in a downstream effector of Cdc42 and Rac1 and regulate integrin-mediated cell motility, as suggested in mammary epithelial cells (45). Experiments of this regard are in progress.

The recruitment of lymphocytes is essential to the development and function of the immune system. Circulating lymphocytes continuously enter the peripheral lymphoid tissue by traversing endothelial cells under steady state conditions. After the differentiation and the maturation, memory and effector lymphocytes migrate from lymphoid tissue to the effector sites. The interaction between endothelial cells and lymphocytes is an essential step in this recruitment. It has been demonstrated that adhesion molecules play central roles in the extravasation of lymphocytes. Naive lymphocytes interact with PNAAd and MAdCAM-1 on HEV via L-selectin and integrin $\alpha_4\beta_7$, respectively (3, 4, 7). Memory and effector lymphocytes, which express little L-selectin but highly express $\alpha_4\beta_7$, mainly interact with MAdCAM-1 via integrin $\alpha_4\beta_7$ for the extravasation at effector sites, although the homing mechanism of memory/effectector lymphocytes is heterogeneous (3, 4, 46). In addition, the existence of another mechanism for the extravasation of

**FIGURE 5.** Human tonsilar B lymphocytes adhere to immobilized SHPS-1-Ig via CD47 and change their shape to the polarization-like morphology. Human tonsilar B lymphocytes were isolated as described in Materials and Methods. A, Human tonsilar B lymphocytes (1 x 10^5) were incubated on 96 microtiter wells immobilized SHPS-1-Ig or CD44-Ig (10 $\mu$g/ml) for 1 h with or without the preincubation of anti-CD47 mAb. The adhesive activity was measured by crystal violet method. Statistically significant differences from control values are indicated by asterisks (**, $p < 0.05$; □, CD44-Ig; ■, SHPS-1-Ig). The data are shown by the mean ± SD in triplicate. The representative data are shown in three independent experiments. B, Human tonsilar B lymphocytes isolated as described in Materials and Methods were incubated on immobilized SHPS-1-Ig or CD44-Ig (17 $\mu$g/ml) for 1 h. The proportions of polarization (percentage of polarization) are shown (**, $p < 0.05$; □, CD44-Ig; ■, SHPS-1-Ig). The data are shown by the mean ± SD in triplicates. C, Morphology of human tonsilar B lymphocytes on immobilized SHPS-1-Ig (left) or CD44-Ig (right) was photographed under the phase-contrast microscopy (bar, 10 $\mu$m). Similar results were obtained from another independent experiment. D, Immunofluorescence staining for human tonsilar B lymphocytes adhered to SHPS-1-Ig. The adhere tonsilar cells were stained with anti-CD19 mAb (left) and isotype-matched mouse IgG (right) followed by PE-conjugated goat anti-mouse Ig. The nucleus was visualized by 4’6-diamino-2-phenylindole dihydrochloride (bar, 10 $\mu$m). Similar results were obtained from another independent experiment.

**FIGURE 6.** SHPS-1 is expressed on the surface of HEV in human tonsil. Endothelial cells in human tonsil were stained with anti-SHPS-1 polyclonal Ab following alkaline phosphatase-conjugated secondary Ab (A), and concomitantly stained with anti-PNAd mAb (MECA-79) by FITC-conjugated secondary Ab (B). Arrowheads indicate HEV cells (bar, 30 $\mu$m).
Similar results were obtained in another independent experiment. Expression of SHPS-1 and ICAM-1 on HUVECs after a 36-h incubation in the absence or presence of IL-1β (10 ng/ml). HUVECs were incubated with anti-SHPS-1 Ab or anti-ICAM-1 mAb, followed by FITC-conjugated goat anti-rabbit Ig or FITC-conjugated goat anti-mouse Ig, respectively (solid line). Rabbit serum or isotype-matched mouse IgG was used as negative control (dotted line). Representative data are shown in two independent experiments. B, Human tonsilar B lymphocytes (5 × 10⁵) were applied on nonactivated HUVECs, which subconfluent grew on 96 microwell plates, in the presence or absence of anti-CD47 mAb, SE5A5 (10 µg/ml) for 1 h. The wells were washed, and then adhered cells were recovered by tripounization, followed by the count of the number of lymphocytes. Statistically significant differences from control values are indicated by asterisks (**, p < 0.05). The data are shown by the mean ± SD in triplicates. Similar results were obtained in another independent experiment.

memory/effector lymphocytes has been speculated, especially IgA-producing mucosal B lymphocytes (47). In this study, we demonstrated that SHPS-1 is expressed on human endothelial cells and HEV, an entrance of lymphocytes from blood stream, in human tonsil. Flow cytometric analysis also showed that primary cultured HUVECs constitutively express SHPS-1 and that the expression of SHPS-1 on HUVECs was not changed by the addition of IL-1β. In addition, anti-CD47 mAb SE5A5 significantly inhibited the adhesion of human tonsilar B lymphocytes to nonactivated HUVECs. From these findings, we suggest that SHPS-1/CD47 interaction may be involved in the extravasation of B lymphocytes under the conditions without inflammatory stimuli. Recently, Ticchioni et al. (48) demonstrated that CD47 contributes to the arrest of Jurkat cells (human T leukemia cell line) on HUVECs activated by TNF-α under flow.

In immune response, the contact between lymphocytes and APCs such as dendritic cells is the first step to initiate the immune response (49). Several reports have suggested that CD47 is involved in the activation and the differentiation of T lymphocyte (18–20, 50). Brooke et al. (44) demonstrated that SHPS-1 expressed on monocytes and dendritic cells mediates the binding to CD4-positive T lymphocytes in cattle. In B lymphocyte activation, cocultivation of dendritic cells with B lymphocytes induces the cell-cell interaction and enhances the Ig production (51). Although LFA-1/ICAM-1 interaction is partially involved in the cell-cell contact between dendritic cells and B lymphocytes (52), other mechanisms should exist. The morphological change, like polarization, may also facilitate the cell-cell contact between lymphocyte and APC. Because macrophages expressed SHPS-1 in human tonsils, it is also possible that SHPS-1/CD47 interaction may be involved in the cell-cell contact between B lymphocytes and macrophages or dendritic cells in human tonsils for the differentiation and the maturation of B lymphocytes.

In summary, we demonstrate that SHPS-1 acts as an adhesive substrate of human B lymphocyte via CD47, and that the affinity/avidity of CD47 may be regulated during the maturation of human B lymphocyte. We also show that SHPS-1/CD47 interaction may be involved in human B lymphocyte adhesion to nonactivated HUVEC. Our results suggest that SHPS-1/CD47 interaction may play important roles in the immune system. The investigations concerning the roles and signals through SHPS-1/CD47 interaction in human B lymphocytes would provide further understanding of the immune system.

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