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Anti-Adhesive Property of P-Selectin Glycoprotein Ligand-1 (PSGL-1) Due to Steric Hindrance Effect

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ABSTRACT

P-selectin glycoprotein ligand-1 (PSGL-1) is an adhesive molecule that is known to be a ligand for P-selectin. An anti-adhesive property of PSGL-1 has not been previously reported. In this study, we show that PSGL-1 expression is anti-adhesive for adherent cells and we have elucidated the underlying mechanism. Overexpression of PSGL-1 induced cell rounding and floating in HEK293T cells. Similar phenomena were demonstrated in other adherent cell lines with overexpression of PSGL-1. PSGL-1 overexpression inhibits access of antibodies to cell surface molecules such as integrins, HLA and CD25. Cells transfected with PSGL-1 deletion mutants that lack a large part of the extracellular domain and chimeric construct expressing extracellular CD86 and intracellular PSGL-1 only showed rounded morphology, but there are no floating cells. These results indicated that PSGL-1 causes steric hindrance due to the extended structure of its extracellular domain that is highly *O*-glycosylated, but intracellular domain also has some effect on cell rounding. This study implies that PSGL-1 has Janus-faced functions, being both adhesive and anti-adhesive. J. Cell. Biochem. 114: 1271–1285, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: PSGL-1; ANTI-ADHESIVE; INTEGRIN; ECM

P-selectin glycoprotein ligand-1 (PSGL-1) is a ligand for several adherent molecules, such as P-selectin, E-selectin, and L-selectin. PSGL-1 is expressed mainly by hematopoietic cells, including myeloid, lymphoid, and, in some circumstances, endothelial cells [Laszik et al., 1996; da Costa Martins et al., 2007]. PSGL-1 expressed on leukocytes functions as an adhesive molecule by binding to P-selectin or other adhesive molecules on endothelial cells during inflammation, and participates in tethering [Cummings, 1999]. In PSGL-1^{null} mice, leukocyte infiltration in the chemical peritonitis model was delayed, and leukocyte rolling in vivo was markedly decreased, after trauma [Yang et al., 1999]. On the other hand, it was recently reported that PSGL-1-deficient T cells from PSGL-1$^{-/-}$ mice showed increased homotypic adhesion compared with wild-type cells [Matsumoto et al., 2009]. This result indicates that PSGL-1 might function as an anti-adhesive molecule, in addition to its known function as an adhesive molecule.

One other molecule, CD43, has been previously reported to have a Janus-faced function, being both adhesive and anti-adhesive [Ostberg et al., 1998]. Thus, whereas CD43 binds to its receptor on a neighboring cell, peripheral B cells from CD43-B cell transgenic mice showed a decreased ability to homotypically aggregate [Ostberg et al., 1996], indicating that CD43 has two opposite effects; an adhesive and an anti-adhesive effect. Matsumoto et al. [2009] showed that the loss of CD43, in addition to loss of PSGL-1, leads to a further increase in T cell adhesion.

Cell detachment caused by anti-adhesive activity is an important step in the process by which cancer cells start to leave the primary tumor site for metastasis. In addition to CD43 as described above,

Abbreviations used: PSGL-1, P-selectin glycoprotein-1; ECM, extracellular matrix.
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many other molecules have been reported to have an anti-adhesive function, and most of them induce cell rounding and cell detachment from culture dishes after transfection into adherent cells. MUC1 and MUC21/Epiglycanin overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components [Wesseling et al., 1995; Yi et al., 2010]. Sialomucin complex (SMC)/MUC4 overexpression disrupts cell–cell or cell–ECM interactions [Komatsu et al., 1997]. Of these proteins, MUC1 is aberrantly overexpressed in human breast carcinomas and may relate to malignancy and poor prognosis in prostate, lung, and thyroid cancers [Lapointe et al., 2004; Wreesmann et al., 2004; Khodarev et al., 2009]. MUC4 expression is upregulated in carcinomas of the pancreas, gall bladder, ovary, breast and lung, and was shown to be associated with the development of metastases [Komatsu et al., 2000].

In this study, we analyzed the anti-adhesive properties of PSGL-1 and elucidated their mechanism.

MATERIALS AND METHODS

CELL CULTURE

The B16F10 (mouse melanoma) and P3U1 (mouse myeloma) cell lines were maintained in R10 complete medium (RPMI1640 supplemented with 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol). The retroviral packaging cell line PLAT-E and PLAT-gp [Morita et al., 2000], HEK293T [DuBridge et al., 1987], HeLa, Mandin–Darby canine kidney (MDCK) and HEK 293 Tet-On® advanced cell lines were grown in D10 complete medium (DMEM supplemented with the same reagents as R10). The Chinese hamster ovary (CHO) cell line was maintained in DMEM/Ham's F-12 culture medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. These cell lines were cultured at 37°C in a humidified incubator with 5% CO_2 in air. The CHO, HeLa, and B16F10 cell lines were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). The PLAT-E cell line was kindly provided by Dr. Toshio Kitamura (University of Tokyo, Japan). The HEK 293 Tet-On® Advanced cell line was purchased from Clontech Laboratories, Inc. (Mountain View, CA).

CONSTRUCTION OF PSGL-1 EXPRESSION VECTORS

To construct a vector that expresses human PSGL-1 (hPSGL-1), hPSGL-1 was amplified by PCR using HL60 cDNA as a template and the primers YTM407 and YTM408 (Supplementary Table I). PCR amplifications were performed in a final volume of 25 μl containing a pair of primers (0.3 μM each), 1 μl cDNA, 1× PCR buffer for KOD Plus, 0.2 mM dNTP, 2.5 mM $MgSO_4$ and 1.0 unit of KOD Plus (TOYOBO, Osaka, Japan). After incubation at 94°C for 2 min, PCR was carried out under the following conditions: denaturation at 98°C for 10 s, annealing at 56°C for 30 s, and polymerization at 68°C for 1.5 min. After 35 cycles, the polymerization step was extended at 68°C for 10 min. The amplified PCR products were subcloned into the *Sma*I site of the pBluescript SK(−) vector, and the insert was cut with *Xho*I and *Not*I for insertion into the retroviral expression vectors pMXs-IG or pMXs-IP (kindly provided by Dr. T. Kitamura), generating pMx-IG-hPSGL-1#1 or pMx-IP-hPSGL-1.

To construct a vector that express mouse PSGL-1 (mPSGL-1), mPSGL-1 was excised from pEF-mPsgl-1 [Nishimura et al., 2009] using *Bam*HI and *Not*I and was cloned into the same sites of pMXs-IG or pMXs-IP (pMx-IG-mPSGL-1 or pMx-IP-mPSGL-1, respectively).

To construct expression vectors encoding hPSGL-1 deletion mutants, sequential PCR amplification was performed as follows: The first PCR was performed using pMx-IG-hPSGL-1#1 as a template with a 5′ primer (YTM178) and one of a number of 3′ primers (either YTM469, YTM473, YTM477, YTM535, YTM537, or YTM539) to amplify the hPSGL-1 region upstream of the deleted domain. The second PCR was performed using pMx-IG-hPSGL-1#1 as a template with one of a number of 5′ primers (YTM468, YTM472, YTM476, YTM534, YTM536, or YTM538) and a 3′ primer (YTM179) to amplify the region downstream of the deleted domain. For the third PCR amplification, the appropriate overlapping PCR products were used as templates and were amplified using two cycles of denaturation at 94°C for 15 s, annealing at 50°C for 30 s, and polymerization at 68°C for 1.5 min, after which the primers YTM178 and YTM179 were added and PCR was continued for an additional 25 cycles to generate hPSGL-1 deletion constructs. These PCR products were cleaved with *Xho*I and *Not*I and subcloned into the pMXs-IG vector to generate pMx-IG-hPSGL-DM1, pMx-IG-hPSGL-DM3, pMx-IG-hPSGL-DM5, pMx-IG-hPSGL-DM8, pMx-IG-hPSGL-DM9, and pMx-IG-hPSGL-DM10, which had truncations of 69 bp (23 amino acids), 450 bp (150 amino acids), 129 bp (43 amino acids), 54 bp (18 amino acids), 525 bp (175 amino acids), 210 bp (70 amino acids) and 81 bp (27 amino acids), respectively in pMx-IG-hPSGL-1#1 (Fig. 6A).

To construct CD86 expression plasmids, mouse CD86 was amplified using mouse spleen cDNA as a template and the primers YTM618 and YTM619 (Supplementary Table I). PCR amplifications were performed as described above. The amplified PCR products were subcloned into the *Sma*I site of the pBluescript SK (−) vector. Mouse CD86 was amplified using the primers YTM620 and YTM627 to add *Mlu*I and *Hpa*I sites. The 5′ part of hPSGL-1 was amplified using the primers YTM178 and YTM622 to add an *Mlu*I site to the 3′ end. The 3′ part of hPSGL-1 was amplified using the primers YTM628 and YTM179. To construct an hPSGL-1 and mCD86 chimera (Fig. 7A), mouse CD86, the 5′ part of hPSGL-1 and the 3′ part of hPSGL-1 were cut with *Mlu*I and *Hpa*I, *Xho*I and *Mlu*I, and *Not*I, respectively, and these fragments were ligated into the *Xho*I and *Not*I sites of pMXs-IG (pMx-IG-hPSGL-mCD86#1).

To construct the canine CD25 expression plasmid that simultaneously expresses EGFP under the control of IRES, canine CD25 was excised from pMx-IP-cCD25 [Mizuno et al., 2009] and cloned into the *Eco*RI and *Not*I sites of pMXs-IG, generating pMx-IG-cCD25#1.

To construct an expression plasmid containing a fusion of EGFP and canine CD25, canine CD25 was excised from pMx-IP-cCD25 and cloned into the *Eco*RI and *Xho*I sites of pcDNA3.1+ vector, and the insert was cut with *Nhe*I and *Xho*I for insertion into the expression vectors pEGFP-N1, generating pEGFP-N1-cCD25.

To construct a Tet-on inducible vector, pMx-IG-hPSGL-1#1 was cleaved with *Bam*HI and *Not*I and subcloned into the pTRE-2pur vector to generate pTRE-2pur-hPSGL-1.

To construct the CD29 expression plasmids, pCMV-SPORT6 inserted with CD29 cDNA (HDG1180) (Health Science Research

Resources Bank (HSRRB), Osaka, Japan) was digested with *Nco*I, blunted with the Klenow fragment, and digested with *Sal*I, followed by ligation to the *Xho*I and *Sna*BI sites of the pMxs-IB vector, generating pMx-IB-ITGB1.

To construct the CD49e expression plasmids, CD49e cDNA was amplified by PCR using pOTB7 inserted with CD49e (HDG1353) (HSRRB) as a template and the primers YTM910 and YTM911 (Supplementary Table I). The PCR product was incubated with Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA), and ligated into pCR2.1-TOPO vector (Invitrogen Life Technologies), generating pCR-ITGA5#2. This plasmid was cut with *Bam*HI and *Xho*I, *Xho*I and *Not*I, and these fragments were ligated into the *Bam*HI and *Not*I sites of pMXs-IP (pMx-IP-ITGA5#4).

The nucleotide sequences of all of the clones were confirmed by sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit (PerkinElmer, Inc., Waltham, MA), followed by sequencing using the ABI PRISM3100-Avant sequencer (Applied Biosystems, Foster City, CA).

TRANSFECTION

HEK293T cells (2.5×10^5) or other adherent cell lines (1.0×10^5) were seeded in a 6-well dish 1 or 2 days before transfection. Transfection of these cells was performed using TransIT-LT1 (Mirus Bio LLC, Madison, WI) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were incubated for the indicated times and then used for further analysis.

RETROVIRAL TRANSDUCTION

Retroviral transduction using PLAT-E cells was done as described previously [Umeki et al., 2011]. When PLAT-gp cells were used as a packaging cell, pCMV-VSVG was cotransfeted with the retroviral vector. The viral supernatant were concentrated 10-fold with PEG-*it* Virus Precipitation Solution (System Biosciences, Mountain View, CA) only when P3U1 cells were transduced with pSIREN-RetroQ retroviral vectors (QIAGEN, Valencia, CA). After transfection, P3U1 or MDCK cells were cultured in the presence of 4 μg/ml of puromycin (Sigma–Aldrich Japan K.K., Tokyo, Japan) to obtain stably transduced cells.

TET-ON INDUCIBLE SYSTEM

The Tet-dependent inducible PSGL-1 expression plasmid, pTRE-2pur-hPSGL-1, was transfected into HEK 293 Tet-On® Advanced cell lines using TransIT-LT1. Twenty-four hours after transfection, transfected cells were cultured in the presence of 2 μg/ml of puromycin (Sigma–Aldrich Japan K.K.) to select colonies, which stably expressed Tet-responsive hPSGL-1. Once the colonies were obtained, we cloned each colony by applying the limiting dilution method and further analyzed these clones to confirm the expression of hPSGL-1. The resulting clone that was used for further experiments was termed HEK293/Tet-ON/hPSGL-1.

PSGL-1 KNOCKDOWN

Two siRNAs against mouse PSGL-1 sequences and the negative control siRNA were designed by QIAGEN. Based on these siRNA sequences, oligonucleotides for each siRNA were designed, annealed and cloned into the *Bam*HI and *Eco*RI sites of the pSIREN-RetroQ Retroviral vector (Clontech Laboratories, Inc.). pSIREN-NC#2 (NC#2) was used as a negative control while pSIREN-mSleplg-2#1 (2#1) and pSIREN-mSleplg-3#3 (3#3) were used as vector of siRNA of mouse PSGL-1.

FLOW CYTOMETRY

Flow cytometric analysis was done as previously described [Umeki et al., 2011]. Primary antibodies were follows: anti-human PSGL-1 (PL-1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-mouse PSGL-1, anti-mouse CD86-FITC (all from BD Biosciences, San Diego, CA); anti-CD49a, anti-CD49b, anti-CD49d (all from Millipore, Bedford, MA); anti-CD49c, anti-CD29, anti-CD49e, anti-CD49f, anti-CD51 (all from Biolegend, San Diego, CA); anti-HLA-ABC Biotin and anti-mouse MHCI biotin (eBioscience, San Diego, CA) and anti-CD25-PE (Dako, Carpinteria, CA). The appropriate secondary antibodies were follows: anti-rat IgG-RPE (Southern Biotech, Birmingham, AL), anti-mouse IgG-PE (Santa Cruz Biotechnology, Inc.), anti-mouse IgM-PE (Southern Biotech) or Streptavidin-PE (eBioscience). For intracellular staining, the cells were fixed and permeabilized using the fixation and permeabilization solution (BD Biosciences) according to the manufacturer's instructions, and were stained with anti-PSGL-1 antibody (C-19) (Santa Cruz Biotechnology, Inc.) followed by staining with anti-goat IgG-PE (Abcam, Cambridge, UK).

The samples were analyzed using CyFlow space (Partec, Munster, Germany), and the results obtained were analyzed using FlowJo software (Treestar, Inc., San Carlos, CA).

CONFOCAL MICROSCOPY

HEK293/Tet-ON/hPSGL-1 cells were transfected with pEGFP-N1-cCD25. Twenty-four hours after transfection, 1 μg/ml of doxycycline was added to the culture to induce the expression of PSGL-1. A further 24 h incubation after addition of doxycycline, the cells were stained with anti-PSGL-1 antibody (PL-1) as primary antibody for 30 min at 4°C. After two washes at 4°C, the cells were incubated with an Alexa Fluor 633-conjugated goat anti-mouse antibody (Invitrogen Life Technologies) for 30 min at 4°C. The samples were analyzed by confocal microscopy, a LSM 510 META (Carl Zeiss, Jena, Germany).

WESTERN BLOTTING

Cells were lyzed at 4°C for 30 min in ice-cold CHAPS buffer (3% CHAPS, 30 mM Tris–HCl (pH 7.5), 150 mM NaCl) or NP40 buffer (1% NP40, 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA) supplemented with complete, Mini EDTA-free protease inhibitor mixture (Roche Diagnostics K.K., Tokyo, Japan). SDS–PAGE and Western blotting were done as described previously [Umeki et al., 2011]. Primary antibodies were follows: anti-hPSGL-1 (PL-1) and anti-actin (all from Santa Cruz Biotechnology, Inc.); anti-mouse PSGL-1 (BD Biosciences); anti-CD29, anti-CD49d, anti-CD49e, anti-CD51, anti-Akt antibody, anti-cyclin D1 antibody, anti-cyclin D2 antibody, and anti-cyclin D3 antibody (all from Cell Signaling Technology, Inc., Danvers, MA); anti-CD49f (GeneTex Inc.); anti-HLA-ABC Biotin (eBioscience); anti-Bcl-2 antibody (Biolegend). For detection, horseradish peroxidase (HRP)-conjugated Streptavidin

(Sigma–Aldrich) or with the appropriate HRP-conjugated secondary antibody, anti-rabbit IgG antibody, anti-mouse IgG antibody, anti-rat IgG antibody (all from Zymed Laboratories), or anti-goat IgG antibody (Bethyl Laboratories, Inc., Montgomery, TX) were used.

CELL COUNTING OF DETACHED AND ATTACHED CELLS

For examination of PSGL-1-induced anti-adhesive properties, the number of detached or attached cells was counted after induction of PSGL-1. Suspended cells were collected and counted at the indicated time point. Subsequently, adherent cells were trypsinized and counted. For cell counting using a transient transfection system, suspended cells were collected for counting at the indicated time point after transfection, and were used for flow cytometric analysis of the percentage of EGFP-positive cells. EGFP-positive adherent cells were counted under a fluorescent microscope to analyze the numbers of adherent cells showing non-rounded or rounded-shapes. For counting P3U1 cells stably overexpressing mPSGL-1, the cells were seeded in 6-well plate at 2.0×10^5 per well. Twenty-four hours after seeding, floating and adherent cells in P3U1 were counted.

COUNTING OF ECM-ATTACHED CELLS

Ten microgram per milliliter (μg/ml) of collagen type I-C (Cellmatrix, Nitta Gelatin, Osaka, Japan), fibronectin (Roche Diagnostics K.K.) or laminin (Roche Diagnostics K.K) were seeded in a 24-well plate and incubated overnight at 4°C. Ten microgram per milliliter (μg/ml) of Poly-L-lysine (Sigma–Aldrich) was incubated in a 24-well plate for 5 min at RT and then dried for 2 h at RT. Subsequently, these wells were blocked with 3% BSA in PBS for 3 h at RT. Twenty-four hours after treatment with doxycycline, either floating or the remaining attached HEK293/Tet-ON/hPSGL-1 cells were collected, and seeded at 8.0×10^4 per well. P3U1 cells were seeded at 1.3×10^5 per well. After incubation for 1 h at 37°C, the supernatant was removed. The remaining attached cells were counted using a hemocytometer after trypsin treatment.

ANOIKIS ASSAY

Polyhydroxyethylmethacrylate (poly-HEMA; Sigma–Aldrich Japan K.K.) was dissolved at 120 mg/ml in 95% ethanol and diluted 1:10 with 95% ethanol [Folkman and Moscona, 1978]. Then, 96-well plate was coated three times with poly-HEMA and washed twice with PBS before use. MDCK cells stably expressing hPSGL-1 (MDCK/pMx-IP-hPSGL-1) or empty vector (MDCK/pMxs-IP) were established. Trypsinized MDCK/pMxs-IP and floating fraction of MDCK/pMx-IP-hPSGL-1 cells were seeded in poly-HEMA coated 96-well plates at a density of 1.0×10^5 cells/ml. The cells were stained with 40 μg/ml propidium iodide (Nacalai Tesque, Kyoto, Japan) or 2 μg/ml Hoechst 33342 (Sigma–Aldrich) at indicated time points and counted under a fluorescent microscope to analyze the number of dead cells.

RESULTS

OVEREXPRESSION OF PSGL-1 INDUCED CELL DETACHMENT IN SEVERAL ADHERENT CELL LINES

During analysis of canine PSGL-1 function in previous report [Umeki et al., 2011], we observed that PLAT-E cells overexpressing flag-tagged canine PSGL-1 had a round morphology and detached from the culture dish. This result prompted us to analyze the potential anti-adhesive properties of PSGL-1 in adherent cells. We next expanded our investigation of PSGL-1-induced cell detachment to human and mouse PSGL-1 in HEK293T cells. Transient transfection of the EGFP-expressing empty vector or unrelated single-spanning transmembrane protein, canine CD25 (cCD25) [Mizuno et al., 2009], -expressing vector did not induce any morphological change in the EGFP-positive HEK293T cells (Fig. 1A). However, when hPSGL-1- or mPSGL-1-expressing plasmids were transfected, the EGFP-positive HEK293T cells started to round up and float at 18 h after transfection and most of them were floated at 48 h after transfection, whereas cells weakly expressing EGFP tended to remain attached (Fig. 1A–C). This result indicated that PSGL-1 exerted anti-adhesive activity irrespective of the species origin of PSGL-1. Based on this result, we decided to further investigate the anti-adhesive properties of hPSGL-1.

We therefore further tested the anti-adhesive effect of PSGL-1 following transfection into other adherent cell lines; HeLa, B16F10 or CHO cells, which did not show any endogenous PSGL-1 expression (data not shown). Cell lines transfected with the empty EGFP-expressing vector or cCD25-expressing vector did not show any morphological change, but overexpression of hPSGL-1 in the same vector induced rounding and floating of the EGFP-expressing cells (Supplementary Fig. S1A). These results indicated that adherent cell lines displayed altered morphology and decreased attachment after overexpression of PSGL-1, independent of the type of cell line used.

INDUCIBLE EXPRESSION OF PSGL-1 RESULTED IN CELL DETACHMENT

To analyze the time dependent kinetics of PSGL-1-induced morphological changes, a tetracycline-inducible system was established, in which hPSGL-1 expression was reversibly regulated by addition or removal of doxycycline.

Addition of doxycycline at concentrations ranging from 0.001 to 0.1 μg/ml induced a dose-dependent expression of hPSGL-1 by flow cytometry (Fig. 2A) and Western blotting (Supplementary Fig. S2A). In the absence of doxycycline, no rounded or floating cells were observed under this condition (Fig. 2B). Following 0.1 μg/ml treatment, only 10% of the cells were attached to the dish without any noticeable morphological change. The remaining cells were rounded, and most of these cells were floating (Fig. 2B). Ten micrograms per milliliter of doxycycline did not induce any morphological change in parental HEK293/Tet-ON cells (Supplementary Fig. S2B,C).

Time-course experiments showed that PSGL-1 was already strongly expressed, and could be easily detected 9 h after the addition of doxycycline (Supplementary Fig. S3A,B). PSGL-1 expression was further enhanced at 18 h and subsequently did not change dramatically up to 72 h. At 18 h after induction, more than half of the cells had detached from the dishes and were floating (Supplementary Fig. S3C). The number of floating cells gradually increased over time up to 72 h, at which time most of the cells were floating.

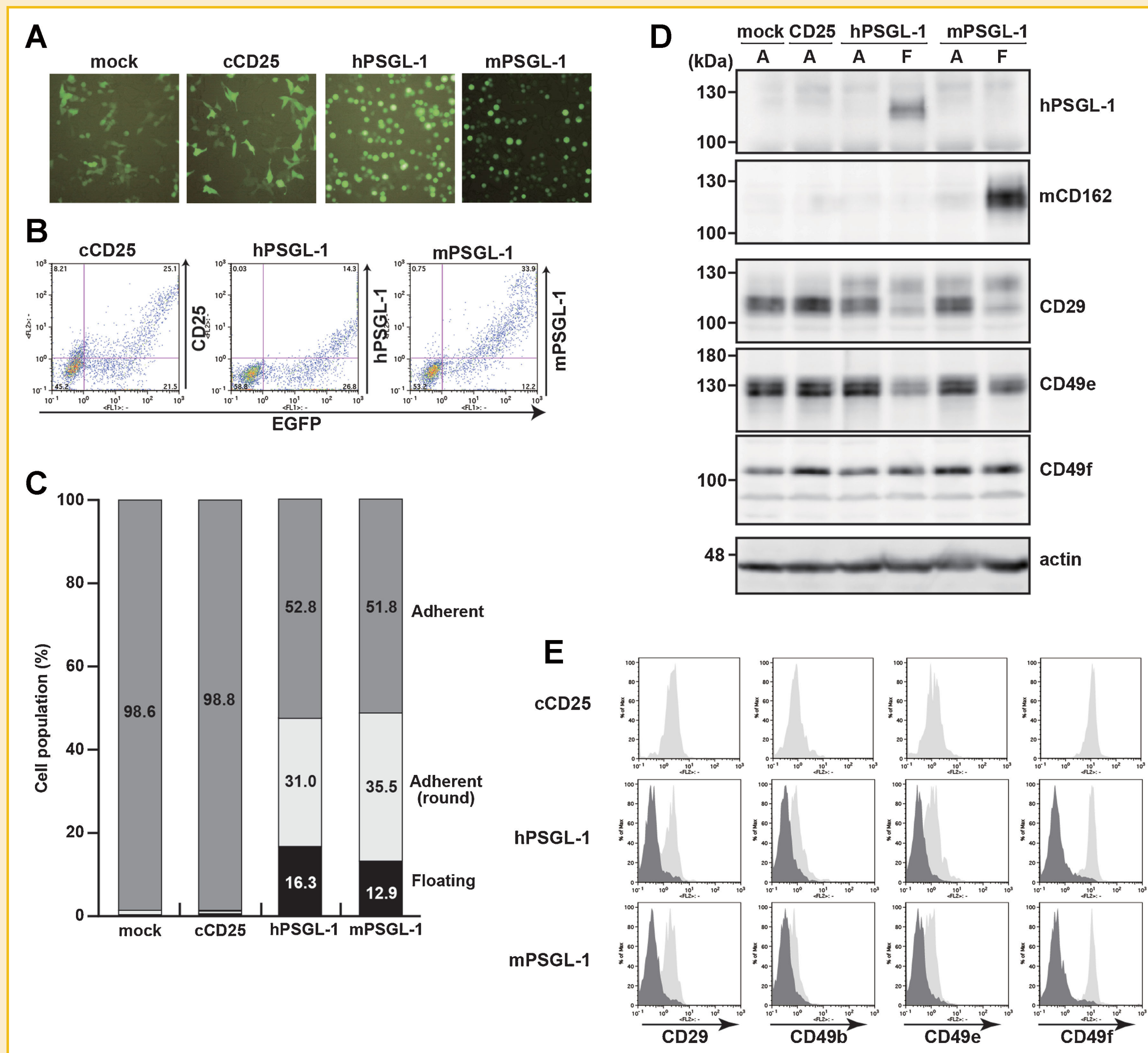

Fig. 1. Adherent cells were detached after PSGL-1 overexpression. A: HEK293T cells were transfected with pMXs-IG, pMx-IG-cCD25, pMx-IG-hPSGL-1#1, or pMx-IG-mPsgl-1. The cells were observed by phase contrast and fluorescent microscopy 48 h after transfection. Each Photo was overlayed. These results are representative results of two experiments. Magnification, 200×. B: Forty-eight hours after transfection, the cells were collected and analyzed by flow cytometry using the anti-CD25, anti-human PSGL-1 or anti-mouse PSGL-1 antibody. The result is representative of two experiments that showed a similar trend. C. Forty-eight hours after transfection, adherent cells, adherent cells but with a round shape (adherent (round)), and floating cells were counted as described in Materials and Methods Section. D: Forty-eight hours after transfection, adherent cells (including adherent with round shape; A) or floating cells (F) were collected and analyzed by Western blotting using the anti-human PSGL-1, anti-mouse PSGL-1 (mCD162) and anti-integrin antibodies (CD29, CD49e, and CD49f). E: Adherent cells (light gray shades) and floating cells (dark gray shades) were individually stained with the anti-integrin antibodies (CD29, CD49b, CD49e, and CD49f), and were analyzed using flow cytometry at 48 h after transfection.

FLOATING CELLS COULD SURVIVE IN LONG-TERM CULTURE AND RE-ATTACH TO DISHES UPON PSGL-1 DOWNREGULATION

After addition of doxycycline, cell death was not observed in detached and floating cells and these floating cells were shown to survival by trypan blue dye exclusion. Furthermore, in the presence of doxycycline, detached, suspended cells grew well until Day 19 when we stopped the culture (Fig. 2C). To clarify whether cell cycle progression factors such as cyclin D1, D2, D3, and survival factor Bcl-2 are induced by PSGL-1 in floating cells, Western blotting was performed. In HEK293/Tet-ON/hPSGL-1 treated with doxycycline, the levels of cyclin D1, D2, D3, and Bcl-2 were continuously detected and were not different as compared with those in control cells, except Bcl-2 was transiently increased in PSGL-1-induced floating cells 48 h after induction (Supplementary Fig. S4). Moreover, we examined whether anoikis was inhibited in PSGL-1-induced floating cells. MDCK cell line, sensitive to anoikis, expressing empty vector showed time dependent increment of dead cells, but floating MDCK cell line, which was induced by hPSGL-1 overexpression showed less amount of cell death at 24 and 48 h after culture (Supplementary Fig. S5).

We next determined if the floating cells could reversibly attach to the dish after floating in culture for several days. Following culture for 7 days in the presence of doxycycline, doxycycline was then removed. Approximately half of the cells showed a rounded

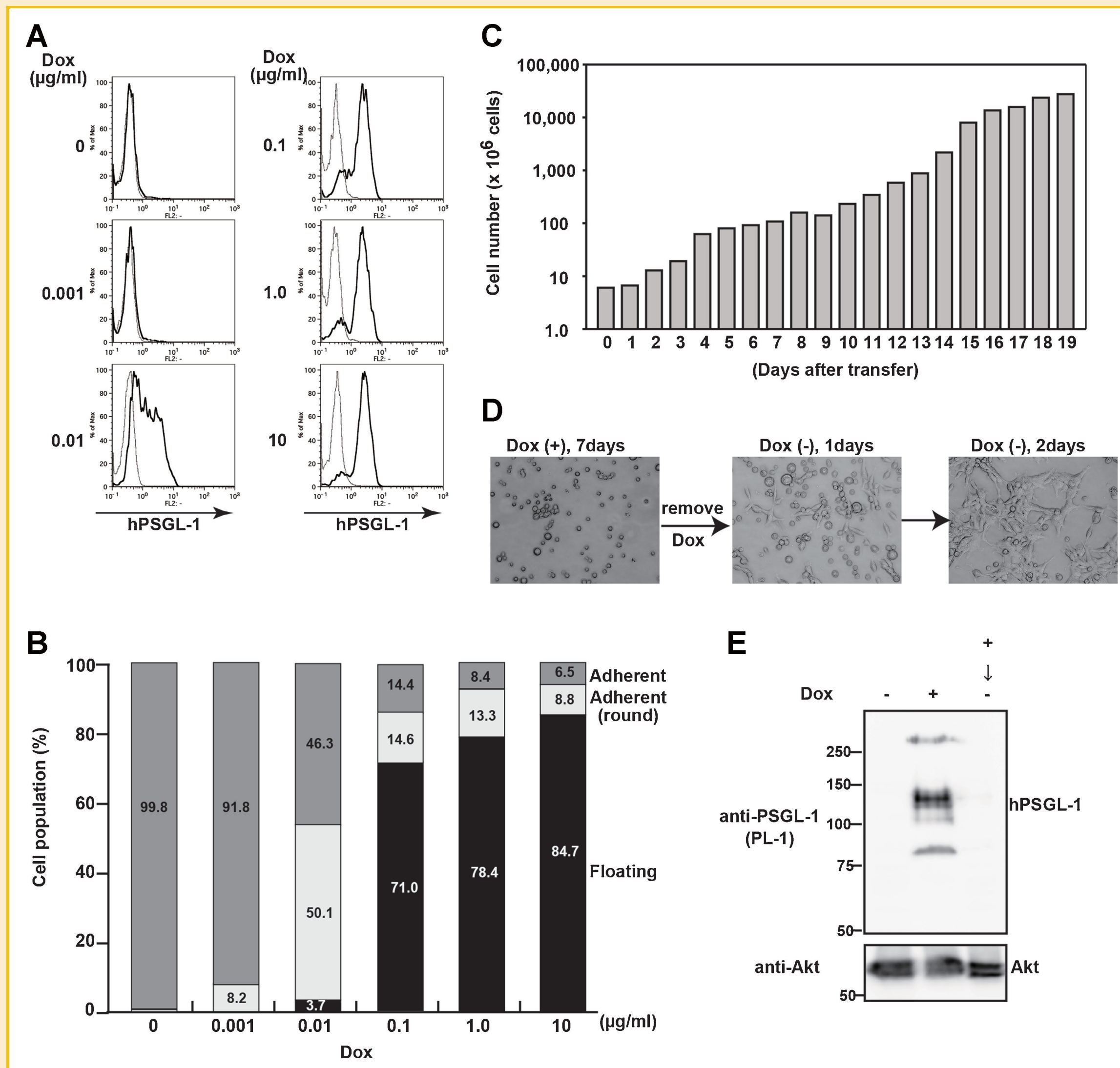

Fig. 2. PSGL-1 was induced by doxycycline (Dox) in a dose-dependent manner in HEK293/Tet-ON/hPSGL-1 cells. HEK293/Tet-ON/hPSGL-1 cells were cultured in the presence of 0.001–10 µg/ml of doxycycline (A and B) or 1 µg/ml of doxycycline (C–E) for indicated time. A: Fourty-eight hours after incubation, the collected cells were stained with the anti-human PSGL-1 antibody (black line) or isotype antibody (gray line), and were analyzed using flow cytometry. B: Fourty-eight hours after incubation, the cells of different morphology were counted as described in Materials and Methods Section. Each result is representative of two experiments that showed a similar trend. C: The cell number was counted every day. Every 3 days, the cells were diluted to a concentration of 6.0×10^4 cells/ml using fresh D10, containing doxycycline. D: At the indicated time point after start of the culture, some of the cells were collected, washed with fresh media and transferred to a new well to start a doxycycline minus subculture. At the indicated time points, cells were photographed using phase-contrast microscopy. Each result is a representative result of two experiments, which showed a similar trend. E: The cells were collected before (−), 8 days after Dox treatment (+), or 8 days after Dox treatment followed by 4 days after of Dox removal (+→-) and analyzed by Western blotting using the anti-human PSGL-1 antibody (PL-1). The blot was re-probed with an anti-Akt antibody, which was used as a loading control. Each result is a representative result of two experiments, which showed a similar trend.

morphology and remained floating, but the other half of the cells started to attach to the dish over the next 24 h, showing a fibroblast-like morphology (Fig. 2D). At 48 h after the removal of doxycycline, during which time the cells were continuously cultured in the absence of doxycycline, most of the cells were attached to the dish (Fig. 2D). PSGL-1 expression was detected in the floating cells at Day 8 after Dox treatment, but, after further 4 days culture without Dox, most of cells were reattached, in which PSGL-1 expression was lost (Fig. 2E).

PSGL-1 OVEREXPRESSION INHIBITED CELL ADHESION TO THE ECM AND ANTIBODY ACCESS TO THE INTEGRINS

Adherent cells attach to the dish via interaction between adherent molecules such as integrins on the cells and the extracellular matrix (ECM). The type of ECM molecule that was responsible for binding of HEK293T cells to the dishes and that is affected by PSGL-1 induction was examined. Approximately half of the HEK293/Tet-ON/hPSGL-1 cells were attached to collagen, fibronectin, or laminin after 1 h incubation without doxycycline (Fig. 3A).

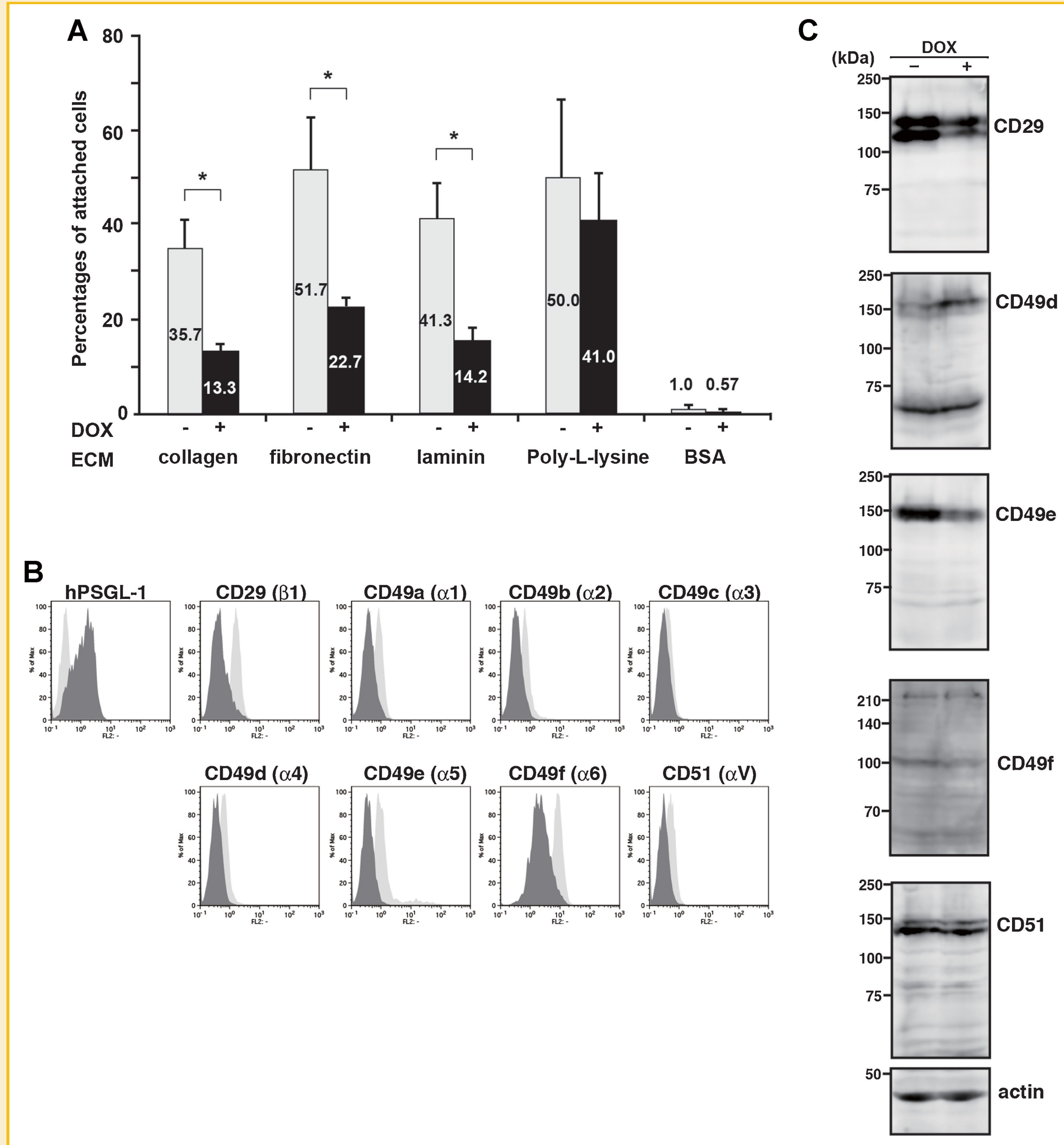

Fig. 3. PSGL-1-expressing floating cells have decreased attachment to the ECM due to ablated cell surface expression of integrins. A: Doxycycline-treated floating cells (black bars) or doxycycline-untreated attached cells (gray bars) were transferred to ECM-coated or positive (Poly-L-lysine-coated) or negative (BSA-coated) control wells. After incubation for 1 h, attached cells were counted and their number was calculated as a percentage of inoculated cells. The results are expressed as means ± SD of three independent experiments. $^{*}P < 0.05$. B and C: HEK293/Tet-ON/hPSGL-1 cells were incubated with or without doxycycline (1 μg/ml) for 48 h. The cells incubated with (dark gray shades) or without (light gray shades) doxycycline were individually stained with the indicated anti-integrin antibodies, and were analyzed using flow cytometry (B). Collected cells were analyzed by Western blotting using anti-human integrin antibodies (C).

However, doxycycline-induced PSGL-1 overexpression significantly reduced the number of attached cells. This result indicated that PSGL-1 expression inhibited the interaction between cell surface molecules and the ECM on the dish.

Adherent cells attach to cell dishes through an interaction between the ECM and cell surface integrins. HEK293/Tet-ON/hPSGL-1 cells expressed several integrins: CD29, CD49a, CD49b, CD49d, CD49e, CD49f, and CD51 but minimal CD49c on the cell surface (Fig. 3B). Whereas doxycycline treatment of HEK293/Tet-ON/hPSGL-1 induced the expression of PSGL-1, it reduced the reactivity of each antibody with its cognate integrin (Fig. 3B). We next determined the protein expression level of each integrin using Western blotting. The expression levels of CD29 and CD49e were reduced by doxycycline treatment, but the expression level of CD49f and CD51 was similar before and after doxycycline treatment and CD49d integrin expression was increased after treatment (Fig. 3C). These results indicated that the level of cell surface integrins that was detected was lower than that before induction of PSGL-1

expression. For some, but not all, integrins this decreased detection was due to reduced protein expression.

Furthermore, we determined the expression levels of integrins in adherent and floating cells in HEK293T transiently transfected with pMxs-IG (mock), pMx-IG-cCD25 (cCD25), pMx-IG-hPSGL-1#1 (hPSGL-1) or pMx-IG-mPSGL-1 (mPSGL-1) (Fig. 1D,E). The reactivity of integrin antibodies against CD29, CD49e, and CD49f in floating cells were completely lost after induction of human or mouse PSGL-1 (Fig. 1D). On the other hand, Western blotting showed that the protein expression levels of CD29 and CD49e in floating cells were lower than that in adherent cells (Fig. 1E), but the expression level of CD49f was not altered in adherent and floating cells. These results are consistent with the results of doxycycline treatment of HEK293/Tet-ON/hPSGL-1 (Fig. 3B,C).

To prove that the reduced expression of CD29 and CD49e shown by Western blotting not contributed to the cell detachment of hPSGL-1-induced cells, HEK293T cells were tritransfected with pMx-IB-ITGB1 (CD29), pMx-IP-ITGA5#4 (CD49e), and either of pMxs-IG (mock), pMx-IG-cCD25 (cCD25), or pMx-IG-hPSGL-1#1 (hPSGL-1) (Fig. 4A,B). Even if CD29 and CD49e were overexpressed together with PSGL-1, cell floating and rounding were induced (Fig. 4B). This result suggested that downregulated integrin proteins were not major concerns of cell detachment induced by PSGL-1.

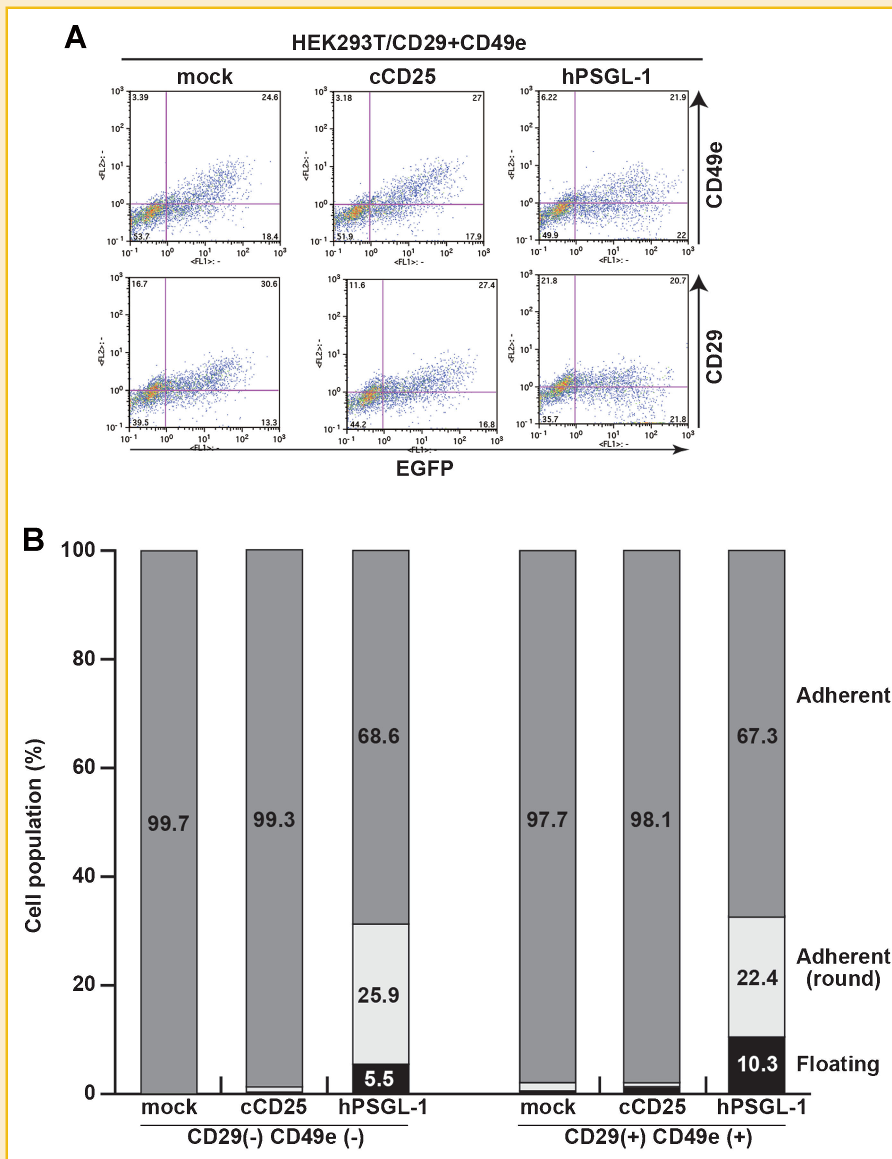

Fig. 4. Overexpression of CD29 and CD49e in HEK293T cells did not prevent cell detachment induced by PSGL-1 overexpression. A and B: HEK293T cells were transfected with pMx-IB-ITGB1 (CD29), pMx-IP-ITGA5#4 (CD49e), and either of pMxs-IG (mock), pMxs-IG-cCD25 (cCD25) or pMx-IG-hPSGL-1#1 (hPSGL-1). Fourty-eight hours after transfection, the cells of different morphology were counted as described in Materials and Methods Section (B), and remaining of cells were collected and stained with the anti-integrin antibodies (CD49e and CD29) (A).

INCREMENT OF PSGL-1 EXPRESSION INHIBITED THE ACCESSIBILITY OF OTHER MOLECULES

Since PSGL-1 has been reported to be a rod-shaped extended protein [Li et al., 1996], which possibly sterically hinders the access of antibody to cell surface molecules, the effect of PSGL-1 expression on the reactivity of an antibody to another cell surface molecule was determined. Cell surface expression of HLA-ABC was detected by flow cytometry before induction of PSGL-1 in HEK293/Tet-ON/hPSGL-1 cells, but a dose-dependent reduction in detectable HLA-ABC expression was observed after induction of PSGL-1 (Fig. 5A). However, the total level of HLA-ABC protein expression, as determined by Western blotting, was not different in cells expressing different levels of PSGL-1 (Fig. 5B).

We next examined whether overexpressed PSGL-1 sterically hinders antibody detection of an exogenously expressed molecule. Canine CD25 was transfected into HEK293/Tet-ON/hPSGL-1 cells and PSGL-1 expression was induced by doxycycline (Fig. 5C). The EGFP gate in flow cytometric analysis indicated that, in CD25-transfected cells, the detection of cell surface expression of CD25 was clearly reduced in PSGL-1-expressing cells (Fig. 5C). We performed the confocal microscopy to examine CD25 distribution before or after PSGL-1 overexpression. CD25 and PSGL-1 were equally and similarly distributed in the floating cells as well as adherent cells (after trypsinized), even after both molecules were overexpressed (Supplementary Fig. S6).

These data indicated that overexpressed PSGL-1 sterically inhibited antibody access to cell surface molecules, and that the

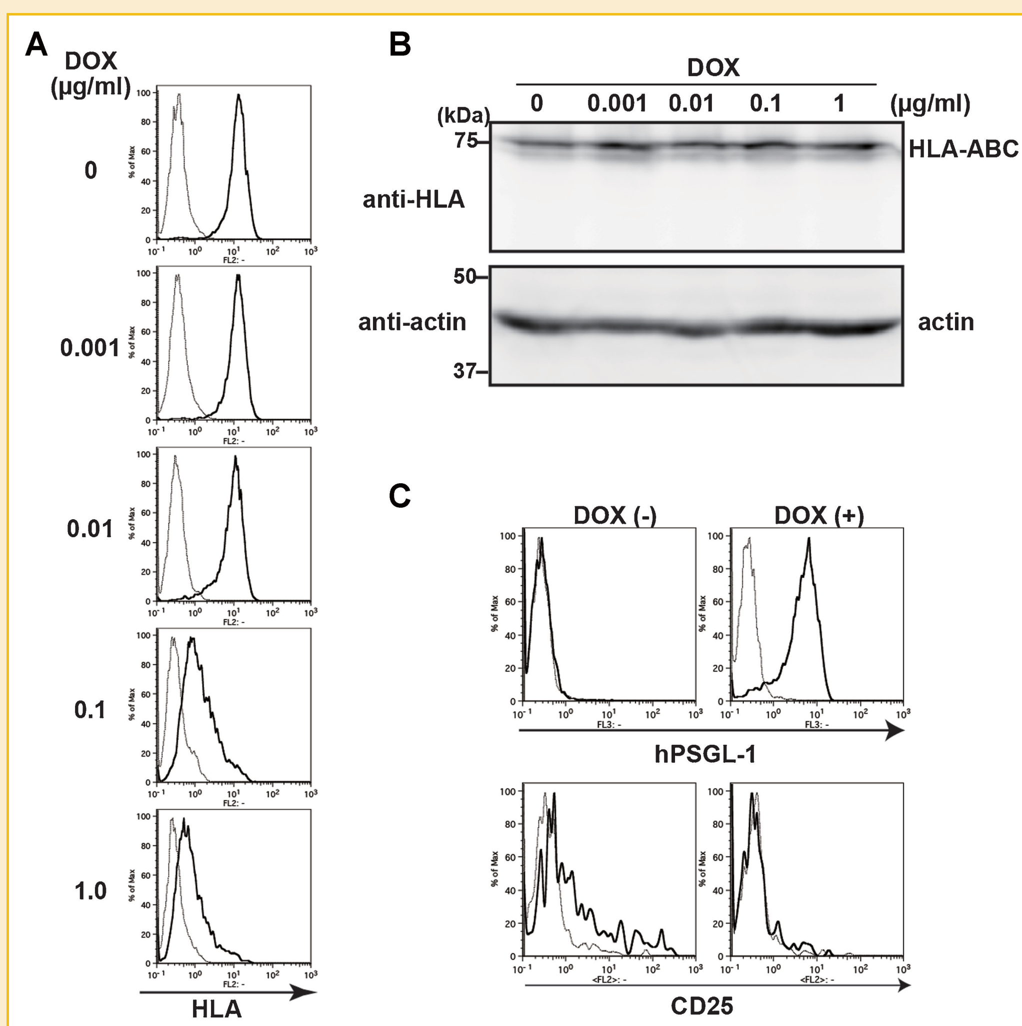

Fig. 5. Overexpression of PSGL-1 inhibited detection of cell surface HLA and CD25 by their cognate antibodies. A and B: HEK293/Tet-ON/hPSGL-1 cells were treated with doxycycline at the indicated concentration for 24 h. After incubation, the cells were stained with anti-human HLA-ABC antibody (black line) or appropriate isotype antibody (gray line), and were subjected to flow cytometric analysis (A). Protein extracts of the cells were Western blotted using an anti-human HLA-ABC antibody. The blot was re-probed with an anti-actin antibody, which was used as a loading control (B). C: HEK293/Tet-ON/hPSGL-1 cells were transfected with pMx-IG-cCD25#1 and incubated for 48 h. After incubation, 1 μg/ml of doxycycline was added to the culture to induce the expression of PSGL-1. Forty-eight hours after induction, the cells were collected and stained with the anti-PSGL-1 antibody (PL-1) or anti-CD25 antibody, followed by flow cytometric analysis. Each result is representative of two experiments that showed a similar trend.

phenomenon of floating may occur as a result of inhibition of the interaction between integrins and ECM molecules.

THE EXTRACELLULAR DOMAIN OF PSGL-1 IS IMPORTANT FOR CELL DETACHMENT

To determine the region of PSGL-1 that is responsible for cell detachment, several deletion mutants that lack specific regions of the extracellular domain were constructed (Fig. 6A). All deletion mutants were expressed at a similar level in HEK293T cells as judged by intracellular flow cytometry (Supplementary Fig. S7A) and Western blotting (data not shown). Floating cells accounted for approximately 30% of the total number of cells in HEK293T cells transfected with hPSGL-1 WT, and almost all of these cells were EGFP positive (Fig. 6B). Of the adherent cells that expressed EGFP, half of the cells were rounded and the other half showed the original morphology. The EGFP signal of the rounded adherent cells was brighter than that of the non-round adherent cells. The proportion of EGFP-positive cells that were floating, rounded adherent or non-round adherent cells was similar for most of the deletion mutants; pMx-IG-hPSGL-DM1, -DM5, -DM9, and -DM10. In contrast, cells transfected with either pMx-IG-hPSGL-DM3 or -DM8 did not show any floating cells and EGFP-positive cells were composed only of

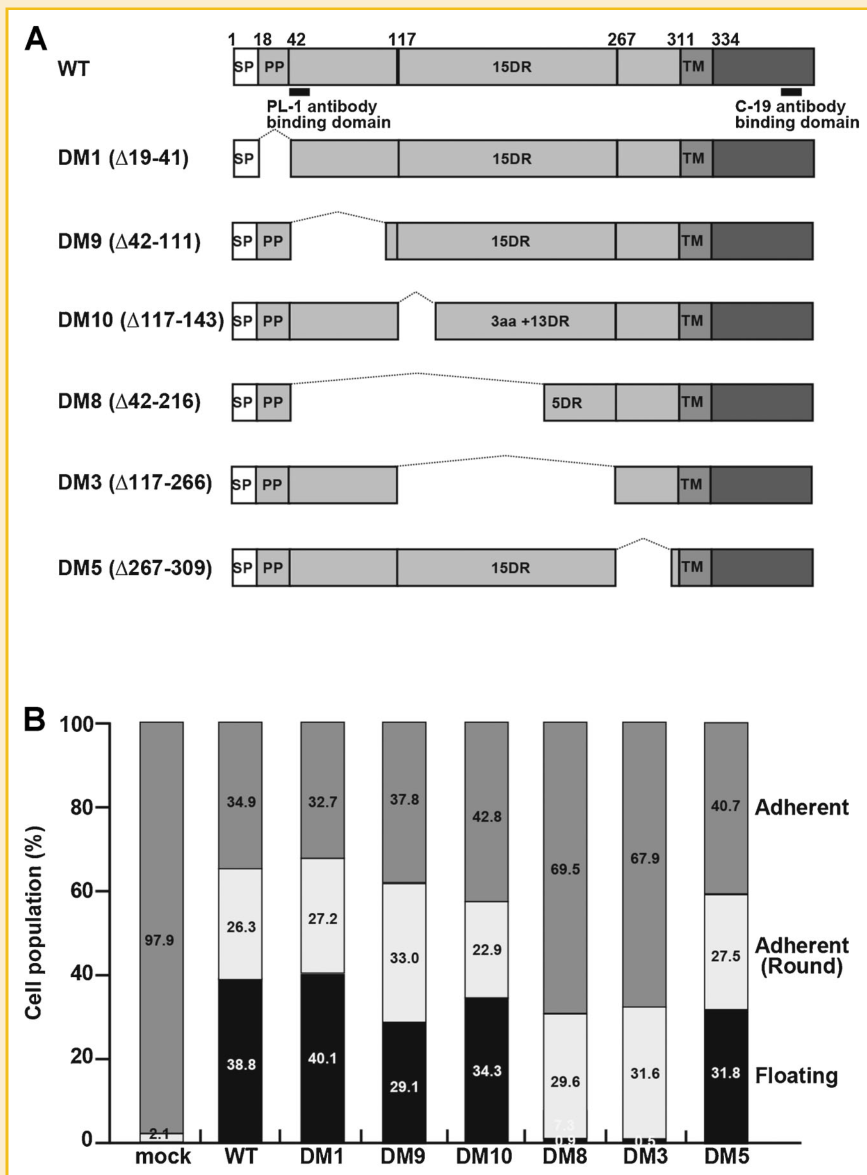

Fig. 6. Specific PSGL-1 deletion mutant clones abrogated PSGL-1-induced cell detachment. A: Schematic representation of the deletion mutants of hPSGL-1 constructed. DM1, DM9, DM10, DM8, DM3, and DM5 have deletions of amino acids 19–41, 42–111, 117–143, 42–216, 117–266, and 267–309, respectively. SP, signal peptide domain; PP, propeptide domain; DR, decameric repeat domain; TM, transmembrane domain B: Forty-eight hours after transfection, adherent cells, adherent cells but with a round shape (adherent (round)), and floating cells were counted as described in Materials and Methods Section. The result is representative of three experiments that all showed a similar trend.

rounded and non-round adherent cells (Fig. 6B). The percentage of rounded adherent cells was fairly similar to that in the other mutants.

Since DM3 and DM8 lack a large part of the extracellular domain of PSGL-1, which has many *O*-glycosylation sites, we investigated the contribution of *O*-glycosylation to PSGL-1-mediated effects. We therefore constructed a plasmid that encodes a chimeric protein in which 202 amino acids in the extracellular domain of hPSGL-1 were replaced with 205 amino acids of the extracellular domain of mouse CD86 (Fig. 7A). This region of mouse CD86 has a similar number of amino acids as the region of the PSGL-1 extracellular domain it replaces, but has a lower number of *O*-glycosylation sites. Flow cytometric analysis of HEK293T cells overexpressing this chimeric protein showed that the fluorescent intensity of PL-1 bound to hPSGL-1 and hPSGL-mCD86 expressed in HEK293T cells was similar (Supplementary Fig. S8A). Interestingly, when hPSGL-mCD86 was transfected into HEK293T cells, EGFP-positive cells showed a rounded morphology that was similar to that of hPSGL-1-transfected HEK293T cells, but none of the cells were floating (Fig. 7B). This result may suggest that the region of PSGL-1 that was replaced with the region of mCD86 contributes to the anti-adhesive property of PSGL-1, but is not related to events that resulted in a rounded morphology.

INCREMENT OF PSGL-1 EXPRESSION INHIBITED THE ACCESSIBILITY OF CELL SURFACE MOLECULES AND INCREASED THE FLOATING CELLS IN P3U1 CELLS

To extend our observation that PSGL-1 has steric hindrance effect in adherent cells, we tested if PSGL-1 has similar effect in non-adherent, but semi-adherent rounded cell line, mouse myeloma P3U1 cell line, which constitutively expressed PSGL-1 on the cell surface. We explored the alteration of PSGL-1 expression on cell surface could change the accessibility of other molecules. Mouse PSGL-1 was retrovirally transduced into P3U1 cell line, resulting in P3U1/mPSGL-1. P3U1/mPSGL-1 expressed more PSGL-1 on their cell surface than parental P3U1 cell line (Fig. 8A). Cell surface expression of CD49f was detected by flow cytometry in P3U1 cell line, but was decreased in the P3U1/mPSGL-1 cell line (Fig. 8A).

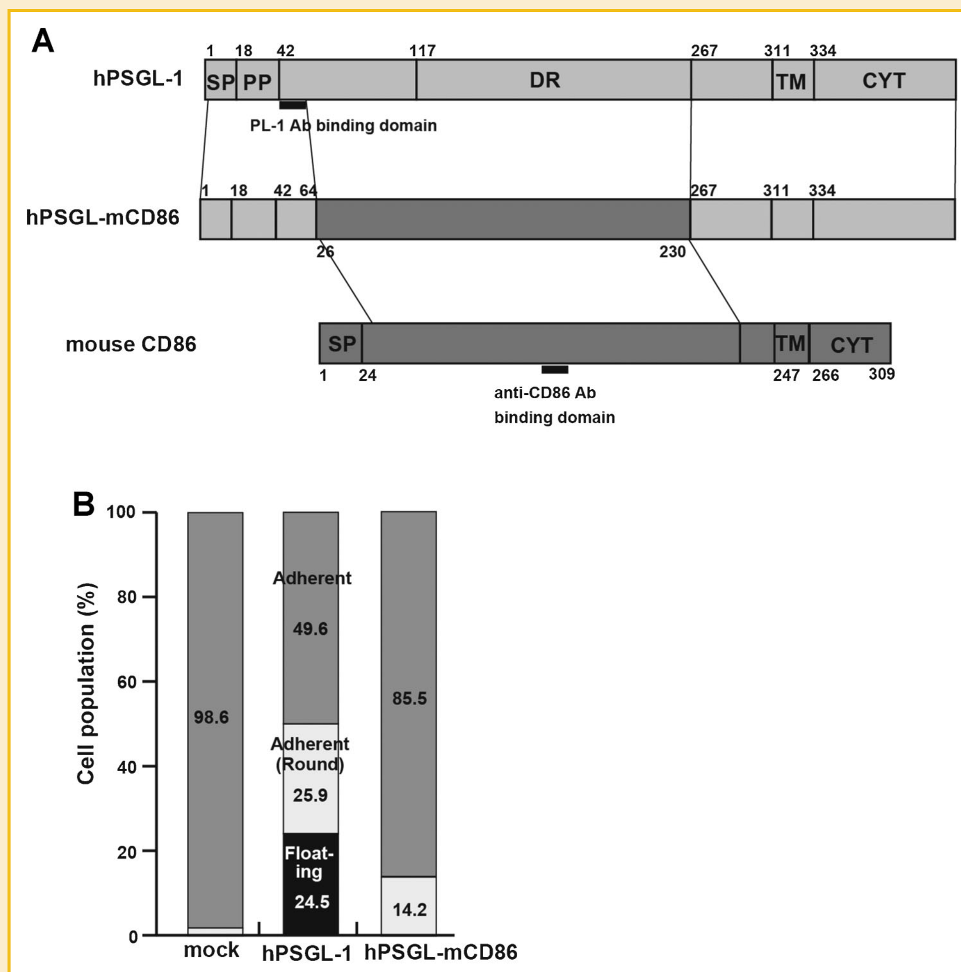

Fig. 7. A chimeric protein of hPSGL-1 and mouse CD86 induced cell rounding of HEK293T cells. A: Schematic representation of the hPSGL-1/mouse CD86 chimera. SP, signal peptide domain; PP, propeptide domain; DR, decameric repeat domain; TM, transmembrane domain; CYT, cytoplasmic domain B: pMXs-IG (mock), pMx-IG-hPSGL-1#1, or pMx-IG-hPSGL-mCD86 was transfected into HEK293T cells. Forty-eight hours after transfection, the cells with different morphologies were counted.

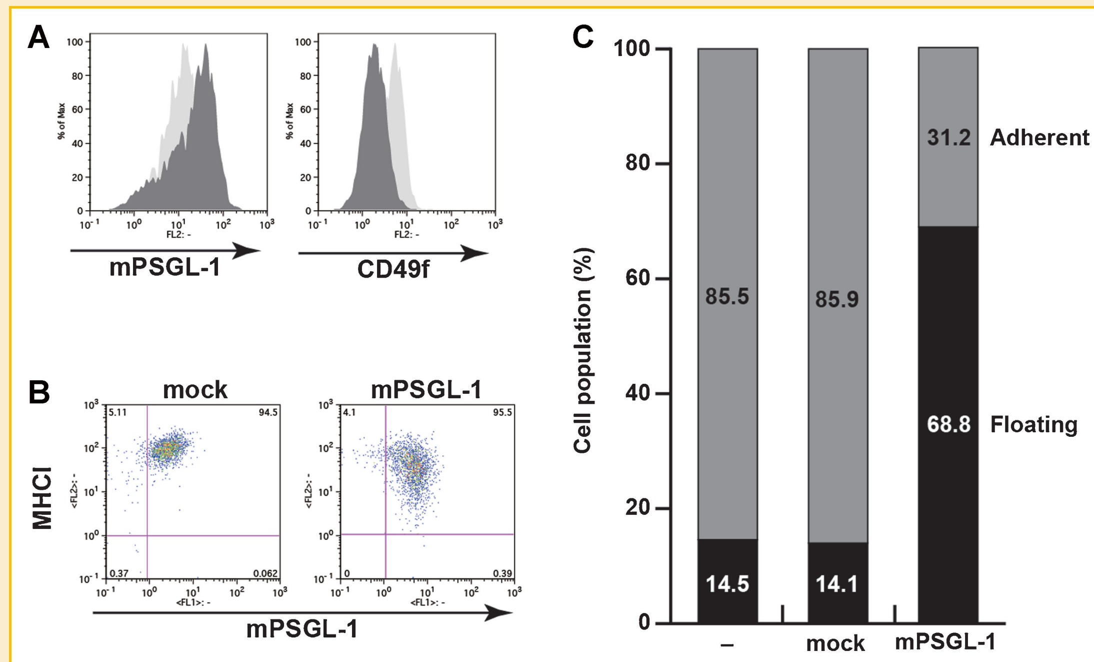

Fig. 8. Overexpression of PSGL-1 inhibited detection of cell surface CD49f and MHCI by their cognate antibodies in P3U1 cells. P3U1 cells stably expressing mPSGL-1 was established by retroviral techniques. A: P3U1/mPSGL-1 cells (dark gray shades) and P3U1/pMxs-IP cells (light gray shades) were stained with anti-mPSGL-1 or anti-CD49f antibodies, followed by flow cytometric analysis. B: P3U1/mPSGL-1 cells (right) and P3U1/pMxs-IP cells (left) were stained with anti-mPSGL-1 and anti-MHCI antibodies, followed by flow cytometric analysis. C: Twenty-four hours after seeding, adherent and floating cells in P3U1 cells (–), P3U1/pMxs-IP cells (mock) and P3U1/mPSGL-1 (mPSGL-1) were counted as described in Materials and Methods Section.

Furthermore, in P3U1/mPSGL-1 cell line, MHCI expression was decreased as compared to empty vector-transduced P3U1 cells (Fig. 8B). Although P3U1 cells were semi-adhesive cells expressing endogenous PSGL-1, additional overexpression of PSGL-1 induced cell detachment in more than half of the cells (Fig. 8C). This result indicated that steric hindrance effect by PSGL-1 expression on cell surface is not limited to the adherent cell line, but is observed even in the cells, which already expressed PSGL-1 to some extent.

Finally, we examined the loss of function of PSGL-1 in P3U1 cells on cell adherence and accessibility of other molecules. PSGL-1 expression was reduced in P3U1 transduced with 2#1 or 3#3, but not in NC#2 (Supplementary Fig. S9A) by flow cytometry. Reactivities of MHCI and CD49f antibodies were not altered in PSGL-1-knockdowned P3U1 cells (Supplementary Fig. S9A). PSGL-1-knockdowned P3U1 cells did not show any increment of binding to ECM proteins, collagen, fibrinogen and lamina, as compared to mock or NC#2 transduced P3U1 cells (Supplementary Fig. S9B).

DISCUSSION

In this study, we found that expression of exogenous PSGL-1 induced morphological changes, such as cell rounding, in adherent cells in which there is no endogenous expression of PSGL-1. This cell morphological change led to cell floating in some cases. PSGL-1 has been considered as an adherent molecule and as a ligand for P-selectin, but our observations indicate that PSGL-1 also has the opposite property of anti-adhesion when it is ectopically expressed in adherent cells, such as HEK293T, HeLa, B16F10, or CHO cells. PSGL-1 is expressed mainly in hematopoietic cells [Laszik et al., 1996] and not in other kinds of tissues. Therefore, it is unclear if PSGL-1 is ectopically expressed under any condition in cell types other than hematopoietic cells. A previous study reported that PSGL-1 was expressed in the prostate cancer cell line, MDA PCa 2b, and that metastatic bone tissues of prostate cancer, but normal prostate tissue or benign prostate tumor, expressed PSGL-1 [Dimitroff et al., 2005]. These results suggested that PSGL-1 expression in metastatic prostate cancer cells is important as a ligand for E-selectin in bone tropism of prostate tumor cells, but that the anti-adhesive property of PSGL-1 may also be an important characteristic of cancer cells in these metastatic processes. Most of experiments in this study have been done using HEK293 cell line, because this cell line was easy to be transfected and expresses high level of protein. Furthermore, HEK293T cell was used in many previous studies, in which function of the other anti-adhesive molecules were examined [Van Brocklyn et al., 1999; Kinoshita et al., 2001; Lawrenson et al., 2002; Yi et al., 2010]. But, the increased expression of PSGL-1 even in the cells expressing endogenous PSGL-1, such as leukocytes, may contribute to adhesive function of cells to ECM, as shown in P3U1 cell line (Fig. 8). On the other hands, we did not elucidate a functional role of PSGL-1 in physiological condition in this study, because overexpression of PSGL-1 in the cell lines was an artificial system. Further investigations were required to prove the physiological importance of PSGL-1-induced cell detachment.

One mechanism by which cells detach from culture dishes is considered to be a decreased interaction between ECMs on the dish surface and cell surface integrins. PSGL-1-overexpressing HEK293T cells showed lower binding to each of the ECM proteins collagen, fibrinogen and laminin that we tested, whereas PSGL-1-expressing and non-expressing HEK293T cells bound similarly to a Poly-L-lysine-coated dish (Fig. 3A). This result is consistent with the fact that integrin expression was reduced after PSGL-1 induction, as detected by flow cytometry. However, unexpectedly, some of the integrins (CD29 and CD49e) were downregulated at the protein level whereas others were not (Fig. 3C). We did not determine the mechanism by which the protein levels of these integrins were decreased during PSGL-1 induction. In contrast, the protein level of CD49d was actually increased following PSGL-1 induction despite the fact that no CD49d was detected by flow cytometry. Furthermore, exogenous transduction of CD29 and CD49e to compensate the reduced expressions of both proteins still induced the PSGL-1-induced cell-floating phenomenon (Fig. 4). These results indicated that, although some integrins might be downregulated at the protein level during PSGL-1 induction, this decrease was not the main reason for the lower expression of all of the integrins tested in this study that was detected by flow cytometry in PSGL-1-overexpressing cells compared with PSGL-1-non-expressing cells (Fig. 3B). Similar results were observed for HLA-ABC or CD25 molecules. The reduced detection of HLA-ABC by flow cytometry, but an unaltered level of protein expression as shown by Western blotting. PSGL-1 also inhibits the access of antibody to the exogenously transduced CD25. These results indicated the possibility that PSGL-1 overexpression might inhibit the access of antibodies to cell surface molecules. These results further encouraged our speculation that antibody detection of cell surface molecules was inhibited by PSGL-1 overexpression due to steric hindrance effect.

PSGL-1 is a highly *O*-glycosylated protein, which forms a rod-like structure that extends over 50 nm from the cell surface [Li et al., 1996]. This highly *O*-glycosylated sites expanded in the extracellular domain of PSGL-1, containing various number of tandem repeats. Because human and mouse PSGL-1 have many tandem repeats similarly in the extracellular domain despite that the numbers of repeats were variable [Yang et al., 1996], transduction of human and mouse PSGL-1 caused cell detachment similarly (Fig. 1A,C). The length of most cell surface molecules does not exceed 35 nm [Ostberg et al., 1998]. This long structure seen in PSGL-1-overexpressing cells may inhibit the access of other molecules such as antibodies to cell surface molecules, resulting in poor detection of the surface molecule by antibody. This phenomenon is known for other molecules and steric hindrance has been reported to be a feature of other cell surface molecules such as MUC1 [Gendler, 2001], Sialomucin complex (SMC)/MUC4 [Komatsu et al., 1997] and Mucin21/Epiglycanin [Yi et al., 2010]. We did not evaluate whether the degycosylated form of PSGL-1 alters cell detachment properties, because treatment with *O*-sialoglycoprotein endopeptidase during cell culture induced cell death of HEK293T cells. Transfection of deletion mutants of PSGL-1 in HEK293T cells showed that the propeptide (PP) domain, the domain between PP and the decameric repeat (DR) domain, and the domain between the DR and the transmembrane (TM) domain was not essential for the anti-adhesive property of PSGL-1. However, the deletion mutants, DM3 and DM8, which lacked large part of the extracellular domain, had fewer floating cells, although a similar number of rounded attached cells were still observed. DM3 and DM8 had a much smaller molecular weight than the other deletion mutants (data not shown), indicating that both deletion mutants lacked a large amount of glycosylation, which occurs in the deleted region. This result indicates that an extended structure that is heavily *O*-glycosylated may contribute to the anti-adhesive property of PSGL-1.

Cells overexpressing a hPSGL-mCD86 chimeric protein showed fewer floating cells, but a similar number of rounded cells as cells overexpressing WT hPSGL-1 (Fig. 7B). The cell surface expression of this hPSGL-mCD86 protein was similar to that of hPSGL-1 when overexpressed in HEK293T cells, as shown by flow cytometry. The region of PSGL-1 that was replaced with mCD86 had a similar number of amino acids as the substituted mCD86 region, but had more potential *O*-glycosylation (53 vs. 28 serine/threonine residues), and fewer potential *N*-glycosylation (2 vs. 7) sites. The hPSGL-mCD86 protein migrated as a 140 kDa dimer in a non-reducing gel analyzed by Western blotting, whereas the hPSGL WT dimer has a molecular weight of 250 kDa. These data indicate that hPSGL-mCD86 did have less *O*-glycosylation than hPSGL-1 WT, as was expected based on the amino acid sequence of the substituted mCD86 region. Transfection of DM3, DM8, hPSGL-mCD86 into HEK293T cells induced fewer floating cells. However, most of the cells showed a round shape, although they still attached to the culture dishes. We did not determine how cell rounding occurred without induction of cell floating in these hPSGL-1 mutant-transfected cells. It is indicated that transmembrane and cytoplasic domains of PSGL-1 might be sufficient to induce cell-rounding property in adherent cells.

Floating cells induced by PSGL-1 overexpression survived for more than 2 weeks in the presence of doxycycline (Fig. 2C). This was an unexpected result. It was previously reported that sphingosine 1-phosphate (S1P) induced cell floating, similar to PSGL-1, but these floating cells died by apoptosis [Van Brocklyn et al., 1999]. Usually adherent cells receive growth signal from adherent molecules such as integrins. Once cells start to float, this means that there is no growth signal from adherent molecules, which results in cell death. However, the number of PSGL-1-induced floating cells actually increased over time, and cell death was not observed in this study. This observation means that PSGL-1-expressing cells could survive for a while after detachment, which is a very important phenomenon. Thus, if PSGL-1 was ectopically expressed in adherent cells in some pathological situation, detached PSGL-1-expressing cells could circulate and survive for long enough to find a new place to reside in. Furthermore, PSGL-1 overexpression in MDCK cell line, which was shown to be anoikis-sensitive [Emoto, 2008], inhibited the cell death as compared to control at 24 and 48 h after culture (Supplementary Fig. S5). Cell survival and resistance of anoikis observed in PSGL-1-indced cell lines may be important mechanism for cancer cell survival. But we could not identify the proteins participating in these mechanisms in this study (Supplementary Fig. S4). Therefore further investigation may need to clarify this in detail. There has been no report of cancer cells that express PSGL-1, except

for human metastatic prostate tumor cells in bone marrow [Dimitroff et al., 2005]. In that study, normal, benign and malignant prostatic tissues were shown to be largely negative for PSGL-1 expression, but PSGL-1 expression was higher in prostate tumor cells in bone metastasis. A survey of the expression of PSGL-1 in cancer tissues therefore needs to be carried out, especially in metastatic tissues.

We also observed effect of PSGL-1 in P3U1 cells, which are semi-adherent and express PSGL-1 constitutively. PSGL-1 overexpression in P3U1 cells decreased antibody accessibility to HLA or CD49f and attached cell (Fig. 8). Against our expectations, PSGL-1 knockdown in P3U1 cells did not increase antibody accessibility to HLA or CD49f and the binding to the ECM (Supplementary Fig. S9). These results indicate that overexpression of PSGL-1 induced steric hindrance effect in the rounded cells such as P3U1 as well as adherent cells, but knockdown of PSGL-1 did not show the inhibition of steric hindrance effect. As shown by flow cytometry (Supplementary Fig. S9), the reduction efficiency of PSGL-1 expression by knockdown may not be sufficient to increase the antibody accessibility to HLA or CD49f. Or, the floating cells in origin, such as leukocytes, express not only PSGL-1 but other surface molecules such as CD43, which have long structure as similar as PSGL-1 [Ardman et al., 1992] and may contribute to the remaining function of steric hindrance effect.

In addition to the property of decreased attachment, a second possible important effect of steric hindrance is escape from immunosurveillance during metastasis, since it has been previously shown that MUC1 and MUC4 inhibited tumor killing by LAK cells [van de Wiel-van Kemenade et al., 1993; Komatsu et al., 1999]. Although P3U1 constitutively and highly expressed PSGL-1 on their cell surface, increment of PSGL-1 in P3U1 showed less binding property of anti-CD49f antibody and anti-MHCI antibody of cell surface molecule and increase of the floating cells (Fig. 8). This means that alteration of PSGL-1 expression on cells, which constitutively expressed PSGL-1, such as lymphocytes, change accessibility by other cell surface molecules on the neighbor cells. This may be one of the mechanisms of increased homotypic aggregation in PSGL-1-deficient T cells from PSGL-$1^{-/-}$ mice in previous report [Matsumoto et al., 2009]. Our findings in this study thus indicate that PSGL-1 might be new key factor for cancer biology.

In this study, we found important cell biological phenomenon caused by PSGL-1. Cell detachment of adherent cells, reduction of antibody accessibility, enhanced survival and resistance of anoikis, which were observed in PSGL-1-transduced cell lines, were new findings. Since PSGL-1 was exogenously overexpressed into several cell lines but not primary cells, physiological importance of these phenomenon were not elucidated. But further investigation may lead to the demonstration of importance of PSGL-1-induced steric hindrance effect in physiological status.

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