Interaction of Platelet Endothelial Cell Adhesion Molecule (PECAM) with α2,6-Sialylated Glycan Regulates Its Cell Surface Residency and Anti-apoptotic Role*

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Background: N-Glycan α2,6-sialylation regulates the cell surface residency of an anti-apoptotic molecule, platelet endothelial cell adhesion molecule (PECAM).

Results: An α2,6-sialylated oligosaccharide inhibited the homophilic PECAM interaction and a cluster-type α2,6-sialyl N-glycan probe bound to PECAM-immobilized beads.

Conclusion: PECAM is a weak sialic acid binding lectin.

Significance: There is a possibility of using a glycan-based method to modulate angiogenesis.

The luminal sides of vascular endothelial cells are heavily covered with a so-called glycocalyx, but the precise role of the endothelial glycocalyx remains unclear. Our previous study showed that N-glycan α2,6-sialylation regulates the cell surface residency of an anti-apoptotic molecule, platelet endothelial cell adhesion molecule (PECAM), as well as the sensitivity of endothelial cells toward apoptotic stimuli. As PECAM itself was shown to be modified with biantennary N-glycans having α2,6-sialic acid, we expected that PECAM would possess lectin-like activity toward α2,6-sialic acid to ensure its homophilic interaction. To verify this, a series of oligosaccharides were initially added to observe their inhibitory effects on the homophilic interaction. To this binding property supports endothelial cell survival. Notably, our findings that PECAM is a sialic acid binding lectin and that α2,6-sialyated glycans enhance the internalization of PECAM as well as the sensitivity to apoptotic stimuli. Collectively, these findings suggest that PECAM is a sialic acid binding lectin and that this binding property supports endothelial cell survival. Notably, our findings that α2,6-sialylated glycans influence the susceptibility to endothelial cell apoptosis shed light on the possibility of using a glycan-based method to modulate angiogenesis.

Vascular endothelial cells are covered with a glycocalyx. Accumulating evidence emphasizes the importance of endothelial glycans for physiological and pathological angiogenesis. Because endothelial heparan sulfate proteoglycan binds to several pro-angiogenic factors such as VEGF (1), FGF2 (2), and PDGF (3) and facilitates endothelial growth and migration (4), heparan sulfate is considered to be a target for antiangiogenic cancer therapy (5–7). In addition, critical roles of endothelial O-glycans in the segregation of blood and lymphatic vessels during embryonic and postnatal development have become recognized after analyses of several mutant mouse strains lacking galactosyltransferase for Tn antigen, T-synthase (8), or core 1 O-glycan-specific molecular chaperone, Cosmc (9). Furthermore, a recent study showed that the interaction of Galectin-1 and VEGF receptor 2 preserves tumor angiogenesis (10).

Endothelial N-linked glycoproteins are differently modified with α2,3- or α2,6-sialic acid. For instance, von Willebrand factor produced by endothelial cells has α2,3-sialic acid, and its deficiency by deleting sialyltransferase ST3Gal IV leads to rapid clearance of von Willebrand factor by hepatic asialoglycoprotein receptors (11, 12). The occurrence of α2,6-sialic acid in vascular endothelial cells and its cytokine-dependent up-regulation have been well-studied (13–15). We previously showed that α2,6-sialic acid is necessary for the homophilic interaction of platelet endothelial cell adhesion molecule (PECAM) (16). Indeed, PECAM has diverse roles in vascular biology (17, 18), including roles in angiogenesis (19), mechanosensing of endothelial cell responses to fluid shear.

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1 The abbreviations used are: PECAM, platelet endothelial cell adhesion molecule; HUVEC, human umbilical vein endothelial cell; SHP2, Src homology 2 domain-containing protein-tyrosine phosphatase 2; PA, pyridylaminated; NeuGc, N-glycolyl neuraminic acid; Siglec, sialic acid binding immunoglobulin-like lectin.
stress (20), anti-apoptosis (21–23), transmigration of lymphocytes (24), and vascular modeling (25), and the homophilic interaction of PECAM appears to be a prerequisite for signal transduction to the cells (17). We further found that endothelial cells lacking α2,6-sialic acid were more sensitive to mitochondria-dependent apoptotic stimuli, similar to the case for cells lacking PECAM (16, 21). Based on our previous findings that PECAM itself has α2,6-sialic acid and that sialidase treatment abolished the homophilic PECAM interaction, we expected that PECAM would possess a lectin activity to recognize α2,6-sialic acid-bearing N-glycans. Regarding sialic acid binding molecules, sialic acid binding immunoglobulin-like lectins (Siglecs) are the best-studied immunoglobulin-type (I-type) lectins. Among them, CD22, a well-characterized B-cell inhibitory receptor, binds to α2,6-sialic acid ligands (26). Several important features are shared between CD22 and PECAM. Both are members of the Ig gene superfamily, undergo glycosylation, and possess two immunoreceptor tyrosine inhibitory motifs in their cytoplasmic domain, and ligation of these molecules induces phosphorylation in the immunoreceptor tyrosine inhibitory motifs, leading to recruitment of phosphatases, such as SHP1/2 (17, 18, 26, 27). Lack of α2,6-sialic acid abolishes the proper localization of both CD22 and PECAM to cell-cell contact sites (16, 28).

In this study we dissected the sialic acid-dependent interaction of PECAM and found that PECAM is a weak, but α2,6-sialic acid-specific, lectin. Our finding that the addition of an α2,6-sialylated oligosaccharide to the culture media of endothelial cells increased the cellular sensitivity to apoptotic stimuli extends the possibility of glycan-based anti-angiogenic drug development by inducing endothelial apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The sources of the materials used in this study were as follows: tissue culture media and reagents, including DMEM and Dynabeads protein G from Invitrogen, *Arthrobacter ureafaciens* sialidase from Nacalai Tesque, *Vibrio cholera* sialidase and recombinant peptide N-glycosidase F from New England Biolabs, recombinant mouse PECAM from R&D Systems, human PECAM-His from Reprokine, nickel-Sepharose and Sephadex G-10 from GE Healthcare, protein molecular weight standards from Bio-Rad, and all other chemicals from Sigma or Wako Chemicals. Protein concentrations were determined with BCA protein assay reagents (Thermo Scientific). For detection of proteins after SDS-PAGE, 2D-Silver Stain-II (Cosmo Bio) was used. α2,3- and α2,6-linked sialyllactose reagents were purchased from Sigma. α2,6-Sialylated biantennary N-glycan, purchased from Fushimi Pharmaceutical Co., was treated with 25 mM HCl at 80 °C for 1 h and separated by gel filtration (Sephadex G-10) to obtain the asialo-type N-glycan. A series of oligosaccharides used for inhibition assays were chemically synthesized as 2-((trimethylsilyl)ethyl glycosides as previously described (29) and quantified by gravimetric means. NeuAcα2,3-, NeuAcα2,6-, and asialo-type biantennary N-glycan cluster probes, labeled with nitrobenzoxadiazole, were chemically synthesized as previously described (30). All animal experiments were performed in compliance with the Institutional Guidelines for Animal Experiments of RIKEN.

### TABLE 1

**Analysis of sialic acids**

| Sample          | Gc content (%, NeuGc/(NeuAc + NeuGc)) |
|-----------------|--------------------------------------|
| Mouse liver     | 80.3 ± 0.3                           |
| Mouse lung      | 67.4 ± 2.3                           |
| Pure PECAM      | 84.8 ± 0.4                           |

**Expression Plasmids**—A human PECAM cDNA was amplified by PCR from cDNA derived from human vein endothelial cells (HUVECs). Fc-tagged mouse PECAM (Fc-mPECAM) was generated as previously described (16). Fc-tagged human PECAM (Fc-hPECAM) was generated by inserting human PECAM amino acids 1–600 into the EcoRl and SpeI sites of pEF encoding the hinge and constant region (Fc) of human IgG1 in-frame. A series of Fc-tagged mutant mouse PECAMs was generated using a QuikChange site-directed mutagenesis kit (Stratagene).

**Cell Culture**—HUVECs (TaKaRa) were cultured in EBMTM-2 (TaKaRa) containing 2% FBS and EGM™-2 SingleQuots™ (TaKaRa) and used within four passages. COS-7 cells were cultured in DMEM containing 10% FBS.

**Immunofluorescence**—HUVECs grown on 8-well chamber slides coated with type I collagen were washed 3 times with Opti-MEM, incubated with *V. cholera* sialidase in Opti-MEM (10 or 50 milliunits/ml) for 30 min at 37 °C, washed with Opti-MEM and PBS, and fixed with 4% paraformaldehyde in PBS for 30 min. For glycan treatment, HUVECs grown on type I collagen-coated chamber slides were treated with 2 mM lactose, α2,3- or α2,6-sialylated lactose, or α2,6-sialylated- or asialoglycan for 18 h and fixed with 4% paraformaldehyde. The cells were permeabilized in 1% Triton X-100 in PBS for 30 min if needed. After blocking with 5% goat serum in PBS, the cells were incubated with rabbit anti-PECAM (H-300; 1:50 dilution; Santa Cruz Biotechnology), rabbit anti-cleaved caspase-3 (1:50 dilution; Cell Signaling Technology) antibodies, and mouse anti-early endosome antigen 1 (1:50 dilution; BD Transduction Laboratories) followed by Alexa Fluor 546-conjugated anti-rabbit IgG (1:100 dilution; Invitrogen) and Alexa Fluor 488-conjugated anti-mouse IgG (1:100 dilution; Invitrogen). DAPI (Wako Pure Chemical Industries) was used as a counterstain for nuclei. To investigate the binding of a series of nitrobenzoxadiazole-labeled N-glycan glycocluster probes, HUVECs were prefixed with 4% paraformaldehyde in PBS for 30 min, labeled with DAPI, and incubated with 10 μM probes at 25 °C for 45 min. After a single brief wash with PBS, the samples were mounted in CC/Mount (Diagnostic BioSystems) and observed using an FV1000-D laser scanning confocal microscope (Olympus).

**N-Glycan Profile of Mouse PECAM**—For purification of mouse PECAM, a microsome fraction (~50 mg of protein) was prepared by ultracentrifugation of mouse lung homogenates, solubilized in 1% Triton X-100 in TBS containing a protease inhibitor mixture, diluted with TBS until the final concentration of Triton X-100 was 0.3%, and subjected to immunoaffinity chromatography in which a de-N-glycosylated anti-PECAM antibody (200 μg; M-20; Santa Cruz Biotechnology) by peptide N-glycosidase F was coupled to 60 mg of tosylated Dyna-
PECAM Is a Sialic Acid-specific Lectin

beads M-280 (Invitrogen). After non-specifically bound proteins were eluted with a non-related peptide solution (100 μg/ml), the PECAM immunocomplex was digested with trypsin and chymotrypsin (Naciali Teseque) in 50 mM ammonium bicarbonate at 37 °C for 16 h, boiled for 10 min, and treated with 30 μl of peptide N-glycosidase F at 37 °C for 16 h. N-Glycans released from 5 μg of pure PECAM were enriched with Oasis HLB Extraction Cartridges (Waters) and pyridylaminated as described previously (31, 32). The pyridylaminated (PA) glycans were loaded on a TSKgel DEAE-5PW column (7.5 × 75 mm; Tosoh) equilibrated with buffer A (10% acetonitrile, 0.01% tritiethylamine). Each glycan was separated with an increasing gradient of buffer B (10% acetonitrile, 7.4% triethylamine, 3% acetic acid) according to the sialic acid content. Each separated glycan was evaporated and applied to an amide-silica column (TSKgel Amide-80; 4.6 × 250 mm; Tosoh) (32). The PA-labeled glycans were desalted using NuTip (Carbon + C18; Glygen) (33) and subjected to liquid chromatography/multiple-stage mass spectrometry (LC/MSn) using a Fourier transform ion cyclotron resonance/ion trap-type mass spectrometer (LTQ-FT, Thermo Fisher Scientific) connected to an UltiMate 3000 RSLCnano LC system (Dionex) with a graphitized carbon column (Hypercarb; 0.075 × 150 mm, 5 μm; Thermo Fisher Scientific). The PA-labeled glycans were eluted with 5 mM ammonium acetate (pH 8.6) containing 2% acetonitrile (buffer C) and 5 mM ammonium acetate (pH 8.6) containing 80% acetonitrile (buffer D) with a linear gradient of 5–70% buffer D over 50 min at a flow rate of 300 nl/min. The MS conditions were as follows: electrospray voltage of 2.5 kV in the positive ion mode; capillary temperature of 200 °C; and collision energy of 35% for MSn. Accurate masses were assigned with possible monosaccharide compositions using the GlycoWorkbench tool (34), and the proposed glycan structures were further verified through annotation using a fragmentation mass matching approach based on the MS/MS data.

Analysis of N-Acetyl Neuraminic acid (NeuAc) and N-Glycolyl Neuraminic Acid (NeuGc)—Sialic acids were released from mouse tissue lysates (10 μg protein) or immunopurified PECAM (200 ng of protein) by acid hydrolysis and labeled with 1,2-diamino-4,5-methylenedioxybenzene using a sialic acid fluorescence labeling kit (TaKaRa). The 1,2-diamino-4,5-methylenedioxybenzene-labeled sialic acids were quantitatively analyzed by HPLC according to the manufacturer’s protocol.

In Vitro PECAM Pulldown Assay—Fc-PECAM (0.1–0.5 μg) absorbed onto 50 μl of Dynabeads protein G was incubated with PECAM-His (0.5 μg) in PBS in the presence of a series of oligosaccharides (0, 0.2, 1, or 5 mM) at 37 °C for 1 h. After the incubation the proteins absorbed on the Dynabeads were analyzed by Western blotted with an anti-His, antibody (Roche Applied Science) for His-tagged PECAM or an anti-human IgG antibody (SouthernBiotech) for Fc-PECAM (16). HRP-conjugated secondary antibodies and a SuperSignal chemiluminescent substrate (Thermo Fisher Scientific Inc.) were used for detection. The detected bands were quantified using a Lumino-image Analyzer LAS-1000 PLUS (Fuji Film). For binding experiments with nitrobenzoxadiazole-labeled glycocluster probes, 5 μg of recombinant human PECAM that was immobilized on 20 μl of nickel-Sepharose and treated with 20 milliunits of A. ureafaciens sialidase was incubated for 45 min at 25 °C with a series of cluster probes at 10 μM. After a brief wash, the beads were observed by fluorescence and phase-contrast microscopy.

In Vitro Induction and Quantification of Apoptosis—Confluent HUVECs grown on 96-well plates (2 × 105 cells/well) were treated with V. cholerae sialidase (2.5–50 milliunits/ml) for 7 h. To see the effect of glycans, HUVECs were treated with 0.2 μM staurosporine plus 2 mM lactose and sialylated or asialoglycan for 7 h to induce apoptosis (21). The cells were measured for their caspase-3/7 activities using a Caspase-Glo 3/7 assay kit (Promega) as previously described (16).

RESULTS

α2,6-Sialylated Pentasaccharide Inhibits the Homophilic PECAM Interaction—Even though PECAM is considered to have dual roles through homophilic and heterophilic interactions, it is generally accepted that the principal ligand for PECAM is PECAM itself (17). Our previous finding that removal of sialic acid on mouse PECAM abolished the homophilic PECAM interaction (16) suggests that PECAM itself has the property of binding to sialic acid. In our initial trial to directly observe the glycan binding activity by virtue of a glycan array, we found that CD22 bound to α2,6-sialylated oligosaccharide, but we failed to detect the glycan binding activity of mouse PECAM, suggesting that the binding property of PECAM could be very weak. We then decided to take another approach in which a series of sialyl- or asialo-oligosaccharides were used to examine the inhibitory effect on the homophilic mouse PECAM interaction. We found that a series of sialylated pentasaccharides (glycan iv–viii) significantly inhibited the recruitment of PECAM-His to protein G-immobilized PECAM-Fc in a dose-dependent manner (Fig. 1, A and B). In the case of shorter oligosaccharides, such as α2,3-sialylactose (i) and α2,6-sialyllactose (ii), inhibitory effects were not clearly observed even at a higher dose (5 mM). It should be noted that even though α2,3-sialylated-pentasaccharide (iv) and α2,6-sialylated pentasaccharide (v) have an identical backbone structure, α2,6-sialylated pentasaccharide exhibited a significantly higher inhibitory potency (IC50 1.9 mM) than α2,3-sialylated pentasaccharide (IC50 4.9 mM). Meanwhile, asialo-type oligosaccharide (iii) had a negligible effect. By comparing the inhibitory effects of NeuAcα2,6-containing oligosaccharides with different core structures, we found that a pentasaccharide containing a type-1 moiety, Galβ1,3-GlcNAc structure, had a higher inhibitory potency (IC50 1.3 mM) as compared with a pentasaccharide containing a type-2 moiety, Galβ1,4-GlcNAc structure (IC50 1.9 mM). Furthermore, the inhibitory potency of NeuGc (v, IC50 0.48 mM) was markedly higher than that of NeuAc (vi, IC50 1.9 mM).

PECAM Binds to α2,6-Sialylated Glycocluster Probes—Multivalent glycan ligands are powerful tools for detecting weak lectin-glycan interactions (35, 36). Even though we failed to detect binding of PECAM to monovalent oligosaccharides that have been widely used in glycan array analyses, the above results raised our expectation that multivalent probes might allow us to prove that PECAM possesses lectin activity. We, therefore, used fluorescently labeled dendrimer-type glycocluster probes in which NeuAcα2,3-, NeuAcα2,6-, or asialo-type biantennary
N-glycans (16-mers) were each attached to a polylysine-based dendrimer template (Fig. 2A) (30) for binding assays with recombinant human PECAM or human endothelial cells. First, we used His-tagged human PECAM immobilized to nickel-Sepharose for binding assays with the glycocluster probes. As compared with asialo-type and NeuAc<sub>2,3</sub>-type probes, NeuAc<sub>2,6</sub>-type probe clearly bound to PECAM-immobilized beads (Fig. 2B). These results demonstrate for the first time that PECAM is indeed a lectin that prefers α<sub>2,6</sub>-sialic acid. Next, we performed cell-based assays using fixed HUVECs to prevent the internalization of PECAM, which would otherwise make the obtained results difficult to interpret. We found that NeuAc<sub>2,6</sub>-type but neither asialo-type nor NeuAc<sub>2,3</sub>-type probes bound to the surface of the cells (Fig. 2C, arrowheads).

FIGURE 1. Sialylated oligosaccharides inhibit the homophilic mouse PECAM interaction. A, PECAM-His and PECAM-Fc pulled down by Dynabeads protein G in the presence of a series of oligosaccharides (2-(trimethylsilyl)ethyl (SE) glycoside forms, 0, 0.2, 1, and 5 mM) were evaluated by Western blot analysis. WB, Western blot. B, the relative levels of PECAM-His bound to immobilized PECAM-Fc in the presence of a series of oligosaccharides (0.2, 1, and 5 mM) were quantified by Western blot analysis. The data are shown as the means ± S.E. when the level of PECAM-His in the absence of an oligosaccharide was set at 100% (n = 3).
PECAM Itself Has α2,6-sialylated Biantennary N-Glycans—

PECAM has seven potential N-glycosylation sites. First, to investigate the sites that were actually N-glycosylated, equivalent levels of a series of PECAM mutants in which Asn was replaced with Ala in each of the seven potential N-glycosylation sites were prepared and analyzed by SDS-PAGE. We found that all of the mutants except PECAMN540A showed faster migration than wild-type PECAM in the SDS-PAGE gel (Fig. 3A), indicating that most of the potential N-glycosylation sites of PECAM were fully occupied. Compared with wild-type PECAM and other PECAM mutants, the expression level of PECAMN540A was markedly lower (50%), indicating that this mutation caused protein unfolding and degradation. Given that recognition of 2,6-sialic acid by PECAM was found to ensure its homophilic interaction, we expected that PECAM would have 2,6-sialylated N-glycans, which are recognized by PECAM itself. Although we previously showed that the α2,6-sialic acid-specific lectin Sambucus sieboldiana agglutinin binds to PECAM, we decided to characterize the N-glycan profile of PECAM in more detail. Using anti-PECAM antibody-coupled magnetic beads, we purified PECAM from mouse lung tissues (Fig. 3B). First, we performed a sialic acid analysis of the purified PECAM as well as mouse liver tissues by measuring the NeuGc/NeuAc ratio (Table 1). We found that mouse lungs had a lower ratio of NeuGc (67.4 ± 0.3%) than mouse livers (80.3 ± 0.3%) but that the PECAM purified from lung tissues contained a much higher NeuGc ratio (84.8 ± 0.4%). Next, N-glycans liberated from 5 μg of pure PECAM were fluorescently labeled with PA (31, 32). The PA glycans were separated by DEAE-anion exchange chromatography based on the number of sialic acids (Fig. 3C). The flow-through fraction of the DEAE-anion exchange chromatography contained unreacted fluorescent reagents as well as neutral glycans, which were found to be mostly high mannose-type glycans. By MS analyses of all the significant fluorescent peaks, we found that the samples eluted at the positions corresponding to monosialylated, trisialylated, and tetrasialylated glycans did not contain detectable levels of oligosaccharides and that disialylated glycans could be solely detected. Even though the amount of N-glycans prepared from PECAM was not sufficient to carry out quantitative analyses for each N-glycan, both (NeuGc)/(NeuGc)-type disialylated biantennary complex-type glycan and hybrid-type biantennary glycan with a linear NeuGc-NeuGc structure were deduced by the
MS2 spectrum of the fragment ion at m/z 1241.0 [M+2H]2+, as shown in Fig. 4A. Although fucose migration on glycans has been reported (37), clear diagnostic ions at m/z 649.47 and m/z 937.47, respectively, most likely reveal the presence of core fucose. In the MS2 spectrum from a precursor ion at m/z 1167.9 [M+2H]2+, the fragment ions at m/z 1151.66 and m/z 1823.61 clearly show the presence of hybrid-type glycan (Fig. 4B).

Therefore, the non-fucosylated hybrid-type biantennary glycan with a linear NeuGc-NeuGc structure was deduced. Fig. 4C shows an MS2 spectrum acquired from a precursor ion at m/z 1263.5 [M+H+Na]2+ (C) are shown in the positive ion mode.

PECAM Is a Sialic Acid-specific Lectin

Endocytosis of PECAM Is Enhanced by α2,6-Sialylated Glycan—Because we previously observed that more PECAM undergoes endocytosis in α2,6-sialic acid-deficient endothelial cells (16), we expected that cancellation of the sialic acid-dependent PECAM interaction would result in enhanced internalization of PECAM. To prove this hypothesis, we took two different approaches, sialidase treatment and the addition of sialyl oligosaccharides. Immunostaining analysis of HUVECs revealed that PECAM was localized at the cell borders in addition to perinuclear regions (Fig. 5A). After sialidase treatment, junctional PECAM staining was abolished, and more internalized PECAM was observed. Actually, we found that endothelial cells were very sensitive to sialidase treatment, which is rou-
PECAM Is a Sialic Acid-specific Lectin

A

- Sialidase
+ Sialidase (10 mU/ml)
+ Sialidase (50 mU/ml)

B

PECAM / EEA1 / DAPI

No glycan
3'-Sialyllactose
Asialoglycan

Lactose
6'-Sialyllactose
Sialyl glycana

FIGURE 5. Sialidase or α2,6-sialylated oligosaccharide treatment causes PECAM internalization in endothelial cells. A, HUVECs were treated with V. cholera sialidase, fixed, and stained with anti-early endosome antigen 1 (EEA1; green) and PECAM (red) antibodies and DAPI (blue). B, HUVECs were incubated with 2 mM lactose, α2,3- or α2,6-sialylated lactose, or α2,6-sialylated or asialo-biantennary N-glycan for 18 h, fixed, and stained with PECAM (red) antibody. Scale bar, 20 μm.

PECAM was shown for the first time that PECAM has a weak but significant lectin activity with a preference for α2,6-sialic acid. Our MS/MS analysis revealed that PECAM itself has a NeuGcα2,6-bearing disialylated biantennary N-glycan, a preferred glycan ligand of PECAM. Notably, sialylated oligosaccharides were shown to have inhibitory activity toward the homophilic PECAM interaction in vitro, raising the possibility that the sialic acid-dependent PECAM-PECAM interaction can be greatly attributed to the cell adhesion property of PECAM (Fig. 7). Moreover, sialylated oligosaccharides as well as sialidase treatment enhanced both the internalization of endothelial PECAM and the sensitivity of endothelial cells to apoptotic stimuli, suggesting that the lectin property of PECAM is critical for transducing survival signals to endothelial cells. It has been shown that tyrosine phosphorylation within the immunoreceptor tyrosine inhibitory motifs of PECAM, which leads to recruitment of Src homology 2 domain-containing protein-tyrosine phosphatase 2 (SHP2), is critical for the transduction of antiapoptotic signals (21, 22). Importantly, however, our previous study using α2,6-sialic acid-deficient cells indicated that only cell surface PECAM can transduce signals via recruitment of SHP2 (16).

Vascular homeostasis is maintained by responses of mechanosensory complexes to fluid shear stress, and these complexes are composed of PECAM, vascular endothelial cell-cadherin, and VEGF receptor 2 (20). Because other endothelial glycoproteins, such as vascular endothelial-cadherin, were also shown to have α2,6-sialic acid (40), heterophilic interactions of PECAM with other endothelial surface molecules via the lectin property of PECAM could contribute to the maintenance of cell survival (Fig. 7). Recently, DeLisser et al. (41) showed that anti-PECAM monoclonal antibody therapy suppressed late-stage metastatic progression in tumor-bearing mice. Our finding that endothelial cells treated with sialylated oligosaccharides were susceptible to apoptotic stimuli raises the new possibility of glycan-based antiangiogenic strategies for cancer therapeutics. Regarding the vulnerability of endothelial cells to sialidase treatment, a previous report showed that sialidase
administration to mice prevented *in vitro* attachment of lymphocytes to high endothelial venules in peripheral lymph nodes (42). Taken together, virus- or bacteria-derived sialidase may diminish cell surface PECAM complexes, thereby affecting endothelial functionality.

Even though inhibitory roles of a series of oligosaccharides for the homophilic PECAM interaction have been extensively studied for mouse PECAM, the aspect of whether human PECAM prefers NeuGc(2,6)-bearing oligosaccharides, similar to mouse PECAM, remains an interesting project for future studies. Humans are genetically unable to synthesize NeuGc (43, 44) but can incorporate metabolically processed NeuGc into endothelial cells (45). Our NeuAc/NeuGc analyses of purified mouse PECAM and lung tissues as the starting material for PECAM purification revealed significantly higher accumulation of NeuGc in PECAM, possibly because NeuGc-bearing PECAM tends to strongly associate with PECAM, thereby leading to enhanced cell surface PECAM stability. In our LC/MS analyses of PECAM glycans, we found a NeuGc-NeuGc linear disialic acid on hybrid-type N-glycans. A linear disialic acid has recently been found in serum glycoproteins, such as plasminogen and vitronectin (46), but its biological property remains to be defined. The aspect of whether this disialic acid is a preferred ligand for PECAM is another interesting project for future studies.

Although PECAM and CD22 share similar specificity for α2,6-sialylated oligosaccharides, PECAM exhibits lower specificity for α2,6-sialic acid and much lower affinity than CD22. A glycan microarray, in which the glycan specificity of CD22 was successfully determined (47), failed to detect the glycan binding activity of PECAM. It is known that a V-set domain in the N-terminal Ig domain is a key for determining the specificity of Siglecs, including CD22 (26). Our informatics analysis using the primary sequence suggests that the N-terminal Ig domain of PECAM is most likely the I-set domain (48), indicating that the binding of PECAM to sialic acid is mediated by a novel mechanism rather than a typical Siglec-sialic acid interaction mode. Clarification of the three-dimensional PECAM structure in future studies will enable us to compare the glycan binding motifs between PECAM and CD22. In this study the sialylated N-glycan cluster probes enabled us to detect the lectin property of PECAM. Application of multivalent glycan probes can be

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**FIGURE 6. Sialidase- or α2,6-sialylated oligosaccharide-treated endothelial cells are susceptible to apoptotic stimuli.** A, HUVECs were treated with *V. cholera* sialidase, fixed, and stained with an anti-cleaved caspase-3 antibody (red) and DAPI (blue). Arrowheads show typical prominent signals of cleaved caspase-3. B, HUVECs were treated with *V. cholera* sialidase (0, 2.5, 10, 25, and 50 miliunits/ml) for 7 h, and their caspase-3/7 activities were measured. The data are presented as the means ± S.E. (n = 3), *, p < 0.05; **, p < 0.01. C, HUVECs were treated with 2 mM glycans (lactose, sialylated glycan, and asialoglycan) for 18 h, fixed, and stained with an anti-cleaved caspase-3 antibody (red) and DAPI (blue). Scale bar, 200 μm. D, HUVECs were treated with staurosporine (STSP) plus 2 mM NeuGcα2,6-lacto-N-tetraose silyl glycoside (SE) or its asialo form for 8 h and then measured for their caspase-3/7 activities. The data are presented as the means ± S.E. (n = 3), *, p < 0.01.
important to discover weak, but functionally important, glycan-binding molecules that have been overlooked.

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PECAM Is a Sialic Acid-specific Lectin

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