MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 34, Number 5, 2015

DOI: 10.1089/mab.2015.0022

Monoclonal Antibody LpMab-9 Recognizes *O*-glycosylated N-Terminus of Human Podoplanin

Mika K. Kaneko,[1*] Hiroharu Oki,[1,2*] Yasukazu Hozumi,[3] Xing Liu,[1,2]
Satoshi Ogasawara,[1] Michiaki Takagi,[2] Kaoru Goto,[3] and Yukinari Kato[1]

Podoplanin (PDPN) induces cell invasion and cancer metastasis, and its expression in cancer cells or cancer-associated fibroblasts has been reported to be involved in poor prognosis of several cancers including malignant gliomas and lung cancers. PDPN is also expressed in normal cells such as lymphatic endothelial cells, lung type I alveolar cells, and kidney podocytes. Many anti-PDPN monoclonal antibodies (MAbs) have been established; however, almost all anti-PDPN MAbs recognize a platelet aggregation-inducing (PLAG) domain, because the PLAG domain is known to be highly immunogenic. Here, we developed and characterized LpMab-9, a novel anti-PDPN MAb. LpMab-9 reacted with LN319 glioblastoma cells, but did not react with LN319/PDPN knockout cells. LpMab-9 showed slight reaction with sialylated *O*-glycan-deficient PDPN. We identified the minimum epitope of LpMab-9 as Thr25–Asp31, which is the N-terminus of human PDPN, using Western blot analysis. Furthermore, Thr25, Gly26, Gln27, and Pro28 were shown to be critical for LpMab-9-binding to PDPN using flow cytometry. Antibody-overlay lectin microarray using LpMab-9 demonstrated that PDPN reacts with sialic acid ± core1 binders and sialo-mucin binders. Taken together, these results indicate that LpMab-9 recognizes *O*-glycosylation of Thr25 in the N-terminus of PDPN. LpMab-9 could be useful for uncovering the physiological function of *O*-glycosylated N-terminus of human PDPN.

Introduction

Podoplanin (PDPN)/Aggrus is a platelet aggregation-inducing glycoprotein.[1,2] Expression of PDPN has been reported in malignant brain tumors, esophageal cancers, lung cancers, malignant mesotheliomas, bladder cancers, testicular tumors, and osteosarcoma.[1,3–14] Moreover, PDPN expression in cancer-associated fibroblasts (CAFs) is involved in poor prognosis of cancers.[15–19] We recently performed comparative crystallographic studies of PDPN in complex with C-type lectin-like receptor-2 (CLEC-2).[20] The interaction with CLEC-2 was mainly observed at Glu47 and Asp48 in the PLAG domain and the α2-6 linked sialic acid at Thr52 of PDPN. Anti-PDPN monoclonal antibodies (MAbs) with high sensitivity and specificity are necessary to investigate the physiological function of PDPN in cancers and normal tissues. Almost all anti-PDPN MAbs such as NZ-1 and D2-40 react with a platelet aggregation-inducing (PLAG) domain of human PDPN, because the PLAG domain is highly immunogenic.[7,21–23]

We recently established the platform to produce cancer-specific MAbs (CasMabs).[24] The newly established LpMab-2 MAb recognized both an aberrant *O*-glycosylation and a Thr55-Leu64 peptide from human PDPN. Because LpMab-2 reacted with PDPN-expressing cancer cells but not with normal cells, it is an anti-PDPN CasMab that is expected to be useful for molecular targeting therapy against PDPN-expressing cancers. Using CasMab methods, we also developed an anti-PDPN MAb, LpMab-7, which is more sensitive than NZ-1 MAb in immunohistochemistry.[25] We identified the minimum epitope of LpMab-7 as Arg79–Leu83 of human PDPN, which is different from PLAG domain. Immunohistochemical analysis using LpMab-7 showed that PDPN expression at metastatic lesions was higher compared with primary lesions.[26] We produced another anti-PDPN MAb, LpMab-3, the epitope of which is a sialylated glycopeptide of PDPN.[27] We identified the minimum epitope of LpMab-3 as Thr76–Glu81 of human PDPN. These results demonstrate that anti-PDPN MAbs against non-PLAG domain are also useful for analyzing function or expression of PDPN in cancers and normal tissues. Here, we developed and characterized a novel anti-PDPN MAb, LpMab-9, the epitope of which is the *O*-glycosylated N-terminus of human PDPN.

[1]Department of Regional Innovation, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan.
[2]Department of Orthopaedic Surgery, [3]Department of Anatomy and Cell Biology, Yamagata University Faculty of Medicine, Yamagata, Japan.
*These authors contributed equally to this work.

Materials and Methods

Cell lines

LN229, NCI-H226, HEK-293T, Chinese hamster ovary (CHO)-K1, glycan-deficient CHO cell lines (Lec1, Lec2, and Lec8), and P3U1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Human lymphatic endothelial cells (LEC) were obtained from Cambrex (Walkersville, MD). The human glioblastoma cell line LN319 was donated by Dr. Webster K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA). LN229, CHO-K1, Lec1, Lec2, and Lec8 were transfected with human PDPN plasmids (LN229/hPDPN, CHO/hPDPN, Lec1/hPDPN, Lec2/hPDPN, Lec8/hPDPN) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.[24] LN319/PDPN knock-out (KO) cells were produced by transfecting CRISPR/Cas plasmids, which target PDPN (Sigma-Aldrich, St. Louis, MO), using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Philadelphia, PA). CHO-K1, Lec1, Lec2, Lec8, NCI-H226, and P3U1 were cultured in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan), and LN229 and LN319 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Nacalai Tesque), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine (Thermo Fisher Scientific), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Thermo Fisher Scientific) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. L-proline (0.04 mg/mL) was added for Lec1, Lec2, and Lec8. LEC was cultured in endothelial cell medium EGM-2MV supplemented with 5% FBS (Cambrex).

Antibodies

LpMab-7 (mouse IgG_1, kappa) and RcMab-1 (rat IgG_{2a}, kappa) were developed previously in our labs.[7,21,24,28] Anti-FLAG tag MAb (1E6) and anti-β-actin MAb (AC15) were purchased from Wako Pure Chemical Industries and Sigma-Aldrich, respectively.

Production of PDPN mutants

The amplified human PDPN cDNA was subcloned into a pcDNA3 vector (Thermo Fisher Scientific) and a FLAG epitope tag was added at the C-terminus. Substitution of amino acids to alanine in PDPN was performed using a QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA).[24,29] CHO-K1 cells were transfected with the plasmids using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories).

Hybridoma production

BALB/c mice were immunized by intraperitoneal (i.p.) injection of LN229/hPDPN cells (1×10^8) together with Imject Alum (Thermo Fisher Scientific). After several additional immunizations, a booster injection was given i.p. 2 days before spleen cells were harvested. The spleen cells were fused with P3U1 cells using GenomONE-CF (Ishihara Sangyo Kaisha, Osaka, Japan). The hybridomas were grown in RPMI medium with hypoxanthine, aminopterin, and thymidine selection medium supplement (Thermo Fisher Scientific). The culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA) for binding to recombinant human PDPN purified from LN229/hPDPN cells.

Western blot analyses

Cell lysates (10 μg) were boiled in SDS sample buffer (Nacalai Tesque).[30] The proteins were electrophoresed on 5–20% polyacrylamide gels (Wako Pure Chemical Industries, Osaka, Japan) and were transferred onto a PVDF membrane (EMD Millipore, Billerica, MA). After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific), the membrane was incubated with primary antibodies (1 μg/mL) and then with peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark; 1:1000 diluted), and developed with the Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific) using a Sayaca-Imager (DRC Co., Tokyo, Japan).

Flow cytometry

Cell lines were harvested by brief exposure to 0.25% Trypsin/1 mM EDTA (Wako Pure Chemical Industries).[31] After washing with phosphate buffered saline (PBS), the cells were treated with primary antibodies (1 μg/mL) for 30 min at 4°C, followed by treatment with Alexa Fluor 488 conjugated goat anti-mouse IgG or Oregon Green 488 conjugated goat anti-mouse IgG (Life Technologies). Fluorescence data were collected using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

Immunocytochemical analyses

Cultured cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min at room temperature. Cells were then treated with 10% normal goat serum in PBS (NGS/PBS) to block non-specific binding sites, and were incubated with 5 μg/mL of LpMab-7, LpMab-9, or control (PBS) for 1 h at 4°C in a moist chamber. They were incubated with Alexa Flour 488 conjugated goat anti-mouse IgG (dilution 1:200; Thermo Fisher Scientific) for 30 min at room temperature. Cells were also treated with DAPI (Thermo Fisher Scientific) to stain the cell nuclei. They were examined using confocal laser-scanning microscopy (LSM700; Carl Zeiss, Jena, Germany). Then fluorescent images were processed using image-processing software (Adobe Photoshop, Adobe Systems, San Jose, CA).

Affinity determination by surface plasmon resonance

To determine the affinity, recombinant PDPN-Fc was immobilized on the surface of chips for analysis using the BIAcore 3000 system (GE Healthcare, Piscataway, NJ). The running buffer was 10 mM HEPES, 150 mM NaCl, and 0.005% v/v Surfactant P20 (pH 7.4; GE Healthcare, BR-1003-68). LpMab-9 was passed over the biosensor chip, and the affinity rate constants (association rate constant, k_{assoc}, and disassociation rate constant, k_{diss}) were determined by nonlinear curve-fitting using the Langmuir one-site binding model of the BIAevaluation software (GE Healthcare). The affinity constant (K_A) at equilibrium was calculated as $K_A = k_{assoc}/k_{diss}$, and the dissociate constant (K_D) was determined as $1/K_A$.

Lectin microarray

PDPN from CHO/hPDPN cells was solubilized using 1% Triton-X100 in PBS (PBST) and was purified using a FLAG-

tag system (Sigma-Aldrich). Then, 100 μL of purified PDPN (31.25–2000 ng/mL) were applied to a lectin array (LecChip v1.0; GlycoTechnica, Hokkaido, Japan), including triplicate spots of 45 lectins in each of seven divided incubation baths on the glass slide. After incubation at 20°C for 17 h, 4 μL of human IgG (5 mg/mL; Sigma-Aldrich) was applied to each well. After incubation at 20°C for 30 min, the glass slide was washed three times with PBST; 60 μL of biotinylated LpMab-9 (1 μg/mL) in PBS was applied to the array and then incubated at 20°C for 3 h. After washing three times with PBST, Cy3-labeled streptavidin (Thermo Fisher Scientific) was added to the array and then incubated at 20°C for 30 min. The glass slide was scanned using a GlycoStation Reader 1200 (GlycoTechnica). Abbreviation of lectins are: LTL, *Lotus tetragolonobus* lectin; PSA, *Pisum sativum* agglutinin; LCA, *Lens culinaris* agglutinin; UEA I, *Ulex europaeus* agglutinin *I*; AOL, *Aspergillus oryzae L-fucose-specific* lectin; AAL, *Aleuria aurantia* lectin; MAL I, *Maackia amrensis* lectin I; SNA, *Sambucus nigra* lectin; SSA, *Sambucus sieboldiana* agglutinin; TJA-I, *Trichosanthes japonica* agglutinin I; PHA-L, *Phaseolus vulgaris* leucoagglutinin; ECA, *Erythrina cristagalli* agglutinin; RCA120, *Ricinus communis*

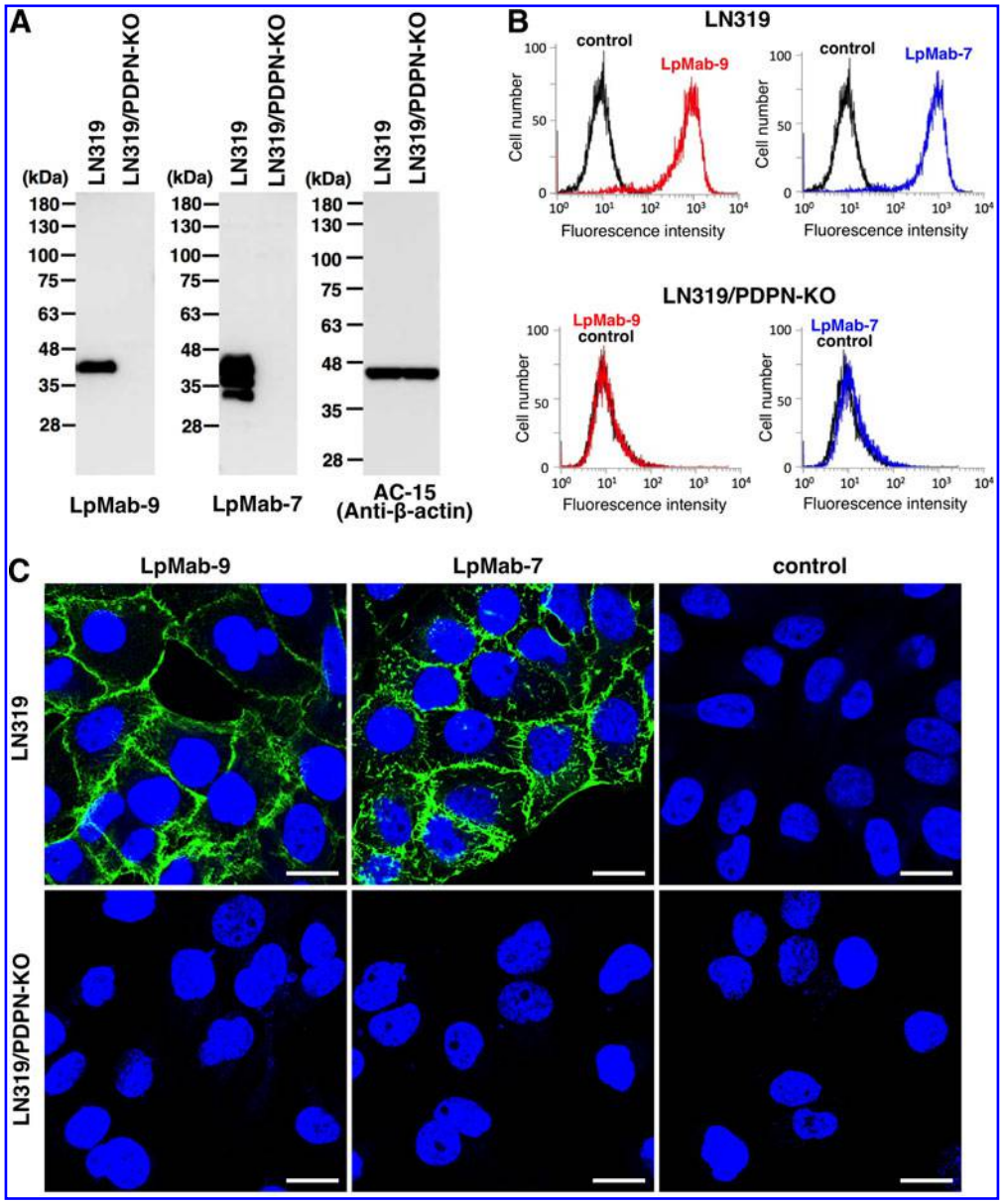

FIG. 1. (**A**) Western blot analysis. The membrane was incubated with 1 μg/mL of LpMab-9, LpMab-7, and anti-β-actin, and then with peroxidase-conjugated anti-mouse IgG; the membrane was detected using a Sayaca-Imager. (**B**) Flow cytometric analysis. LN319 and LN319/PDPN-KO cells were treated with LpMab-9 and LpMab-7 (1 μg/mL) for 30 min at 4°C, followed by treatment with Alexa Fluor 488 conjugated goat anti-mouse IgG. Fluorescence data were collected using a Cell Analyzer EC800. (**C**) Immunocytochemical analysis. LN319 and LN319/PDPN-KO cells were incubated with 5 μg/mL of LpMab-9, LpMab-7, or PBS for 1 h at 4°C in a moist chamber. They were incubated with Alexa Fluor 488 conjugated goat anti-mouse IgG for 30 min at room temperature. Cells were also treated with DAPI to stain the cell nuclei. Scale bars, 20 μm.

agglutinin 120; PHA-E, *Phaseolus vulgaris* erythroagglutinin; DSA, *Datura stramonium* agglutinin; GSL-II, *Griffonia simplicifolia* lectin II; NPA, *Narcissus pseudonarcissus* agglutinin; ConA, *Concanavalin A*; GNA, *Galanthus nivalis* agglutinin; HHL, *Hippeastrum hybrid* lectin; ACG, *Agrocybe cylindricea* galectin; TxLCI, *Tulipa gesneriana* lectin; BPL, *Bauhinia purpurea alba* lectin; TJA-II, *Trichosanthes japonica* agglutinin; EEL, *Euonymus europaeus* lectin; ABA, *Agaricus bisporus* agglutinin; LEL, *Lycopersicon esculentum* lectin; STL, *Solanum tuberosum* lectin; UDA, *Urtica dioica* agglutinin; PWM, pokeweed mitogen; PNA, peanut agglutinin; WFA, *Wisteria floribunda* agglutinin; ACA, *Amaranthus caudatus* agglutinin; MPA, *Maclura pomifera* agglutinin; HPA, *Helix pomatia* agglutinin; VVA, *Vicia villosa* agglutinin; DBA, *Dolichos biflorus* agglutinin; SBA, soybean agglutinin; PTL I, *Psophocarpus tetragonolobus* lectin I; MAH, *Maackia amurensis* hemagglutinin; WGA, wheat germ agglutinin; GSL-I, *Griffonia simplicifolia* lectin I.

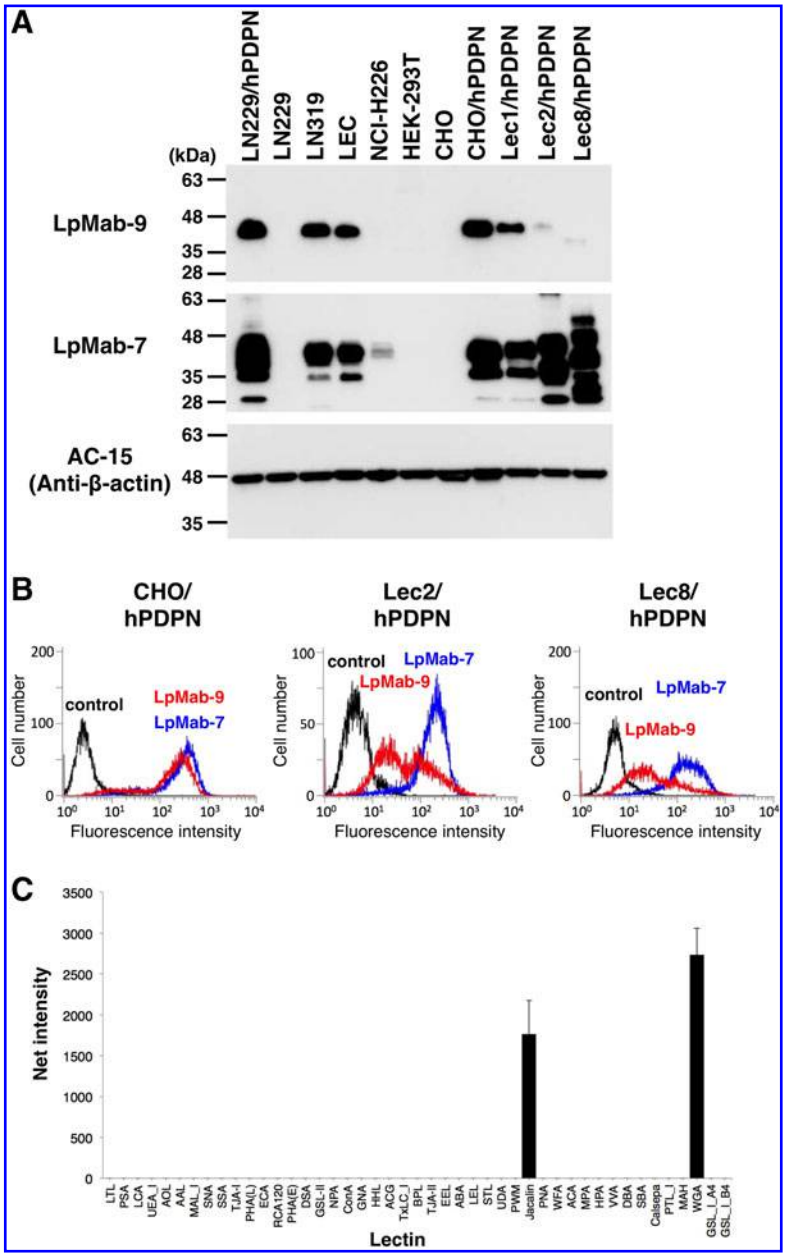

FIG. 2. (**A**) Western blot analysis. The membrane was incubated with 1 μg/mL of LpMab-9, LpMab-7, and anti-β-actin, and then with peroxidase-conjugated anti-mouse IgG; the membrane was detected using a Sayaca-Imager. (**B**) Flow cytometric analysis. CHO/hPDPN, Lec2/hPDPN, and Lec8/hPDPN were treated with LpMab-9 and LpMab-7 (1 μg/mL) for 30 min at 4°C, followed by treatment with Oregon Green 488 conjugated goat anti-mouse IgG. Fluorescence data were collected using a Cell Analyzer EC800. (**C**) Glycan profiling of PDPN using antibody-overlay lectin microarray. Purified PDPN was applied to a lectin array including 45 lectins. After blocking, biotinylated LpMab-9 was applied, and Cy3-labeled streptavidin was added. Signal was scanned using a GlycoStation Reader 1200.

Results

Production and characterization of novel anti-PDPN monoclonal antibody LpMab-9

To develop novel anti-PDPN MAbs, we immunized mice with LN229/hPDPN cells. The culture supernatants were screened using ELISA for binding to recombinant human PDPN purified from LN229/hPDPN cells. After limiting the dilution of the hybridomas, LpMab-9 (IgG_1, kappa) was established. LpMab-9 reacted with LN319 cells, not with LN319/PDPN knock-out (KO) cells in Western blot analysis (Fig. 1A), flow cytometry (Fig. 1B), and immunocytochemical analysis (Fig. 1C). LpMab-7, which was used as a positive control, reacted with PDPN of LN319 (Fig. 1A–C). Furthermore, LpMab-9 detected endogenous PDPN, which is expressed in a lymphatic endothelial cell (LEC) (Fig. 2A).

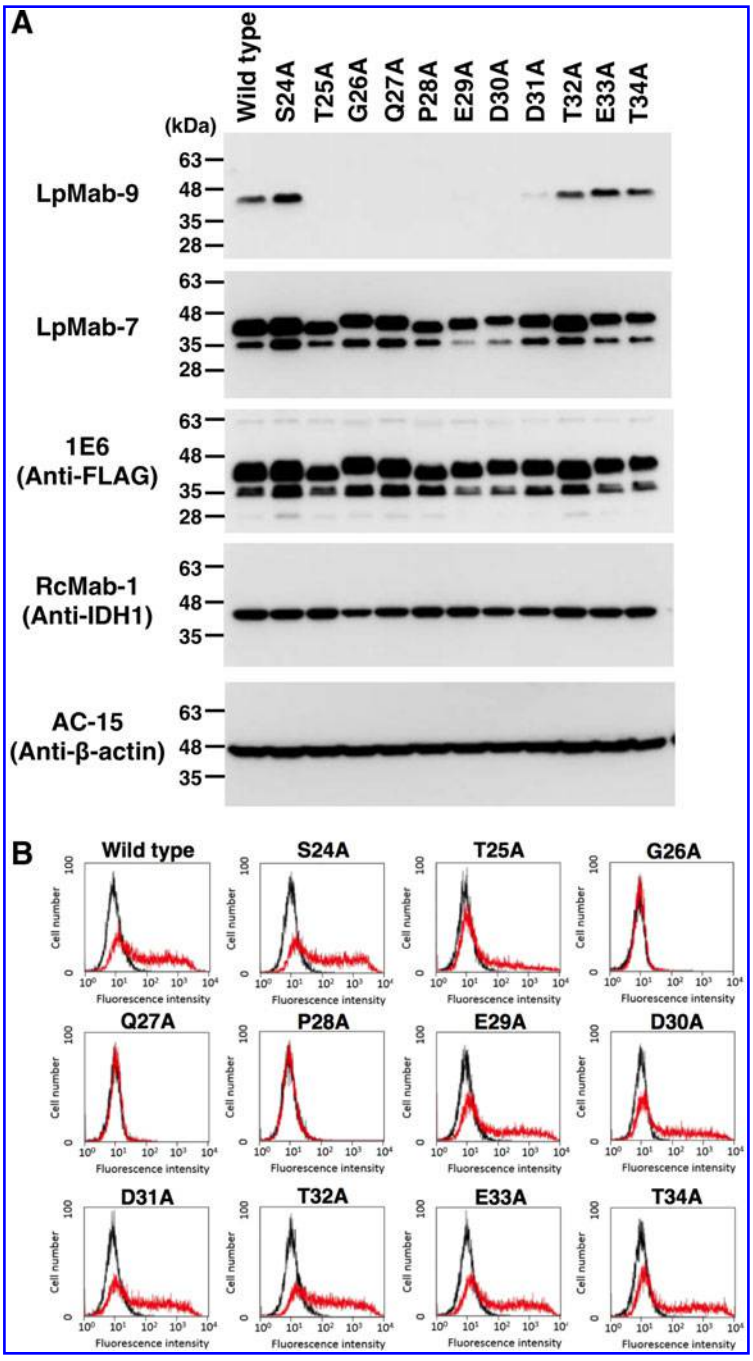

FIG. 3. Epitope mapping of LpMab-9 by Western blot and flow cytometry. (**A**) Western blot analysis. The membrane was incubated with 1 μg/mL of LpMab-9, LpMab-7, 1E6 (anti-FLAG), RcMab-1 (α-IDH1), and AC-15 (anti-β-actin) and then with peroxidase-conjugated secondary antibodies. The membrane was detected using a Sayaca-Imager. (**B**) Point mutants of human podoplanin were treated with LpMab-9 (1 μg/mL) for 30 min at 4°C, followed by treatment with Alexa Fluor 488 conjugated goat anti-mouse IgG. Fluorescence data were collected using a Cell Analyzer EC800.

LpMab-9 faintly reacted with Lec2/hPDPN (sialic acid deficient) and Lec8/hPDPN (*O*-glycan deficient) cells, although it strongly reacted with CHO/hPDPN and Lec1/hPDPN (*N*-glycan deficient) in Western blot analysis (Fig. 2A). Flow cytometric analysis also showed that the reaction of LpMab-9 against Lec2/hPDPN and Lec8/hPDPN decreased compared with CHO/hPDPN, indicating that the epitope of LpMab-9 includes sialylated *O*-glycan of PDPN. In contrast, LpMab-7 strongly detected all types of PDPN (Fig. 2A, B). We next performed lectin microarray analysis of PDPN. We previously established antibody-overlay lectin microarray.[7,32] Here, we applied LpMab-9 to this system. As shown in Figure 2C, PDPN reacted with Jacalin, one of sialic acid ± core1 binders and wheat germ agglutinin (WGA), one of sialomucin binders. We performed a kinetic analysis of the interaction of LpMab-9 with a recombinant PDPN using surface plasmon resonance (BIAcore). Determination of the association and dissociation rates from the sensorgrams revealed that a k_{assoc} of 6.79×10^3 (mol/L-s)$^{-1}$ and a k_{diss} of 2.21×10^{-3} s^{-1}. The K_A at binding equilibrium, calculated as $K_A = k_{assoc}/k_{diss}$, was 3.07×10^6 (mol/L)$^{-1}$, $K_D = 1/K_A = 3.25 \times 10^{-7}$ M.

Epitope mapping by Western blot analysis and flow cytometry

To determine the LpMab-9 epitope, we first performed Western blot analysis. LpMab-9 reaction was lost in point mutations of Thr25-Asp30 (Fig. 3A). The faint band was observed in D31A. This epitope includes Thr25, the only Ser/Thr residue, indicating that Thr25 is *O*-glycosylated and is essential for LpMab-9 recognition. In contrast, LpMab-7 reaction was observed in all point mutants. LpMab-9 detected only one band (40 kDa), although both LpMab-7 and anti-FLAG MAb detected two bands (40 kDa and 35 kDa). We next performed flow cytometric analysis using LpMab-9 against the same point mutants of PDPN. The results revealed that LpMab-9 weakly reacted with T25A and did not react with G26A, Q27A, and P28A (Fig. 3B), indicating that the *TGQP* sequence is the critical epitope of LpMab-9.

Discussion

LpMab-9 possesses a unique epitope, which is totally different from previously reported anti-PDPN MAbs such as NZ-1, D2-40, LpMab-2, LpMab-3, and LpMab-7.[21,24–27] The epitope of LpMab-9 needs *O*-glycosylation of Thr25. Previous reports showed that Thr25 is not *O*-glycosylated because *O*-glycan was not detected using several methods,[24,33] indicating that site-specific MAbs against glycopeptides are more advantageous for detecting *O*-glycan compared with mass spectrometry or Edman sequencing. Therefore, LpMab-9 is useful for distinguishing Thr25-glycosylated PDPN with Thr25-nonglycosylated one. The staining patterns of LpMab-7 and LpMab-9 look slightly different (Fig. 1C). We showed that LpMab-9 recognizes only sialylated PDPN (Fig. 2A, B); therefore, not all fractions of PDPN are detected by LpMab-9. In contrast, LpMab-7 can react with all types of PDPN independently of glycosylation, which might lead to the immunocytochemical difference. Antibody-overlay lectin microarray was previously developed.[32] Specific and sensitive monoclonal antibodies are necessary for this system. In this study, we applied LpMab-9 to this system for investigating the glycan profiling of PDPN. Our previous study using NZ-1 showed that PDPN reacted strongly with sialic acid ± core1 binders (ABA, Jacalin, ACA, and MPA), sialo-mucin binders (MAH and WGA), and alpha-GalNAc binder (HPA). In contrast, using LpMab-9, PDPN reacted with only Jacalin and WGA (Fig. 2C). This inconsistency may be derived from the different binding epitopes and binding affinities of anti-PDPN MAbs. Both LpMab-7 and anti-FLAG MAbs detect two bands (40 kDa and 35 kDa); in contrast, LpMab-9 detected only one band (40 kDa). Our previous study showed that another anti-PDPN MAb, clone NZ-1, also detects only one band (40 kDa), although the epitope of NZ-1 does not include glycosylation.[7,24] Therefore, we cannot conclude that 35-kDa band is a non-glycosylated form. In the near future, we should determine what kind of post-translational modification is involved in this specific 35 kDa isoform of PDPN. The binding affinity of LpMab-9 was shown to be low (K_D, 3.25×10^{-7} M) using BIAcore analysis. However, we previously demonstrated that K_D of LpMab-9 is 1.6×10^{-8} M using flow cytometry against LN229/hPDPN, indicating that the binding affinity of LpMab-9 against PDPN is much higher in cell-based assay.[24] We performed immunohistochemical analyses using LpMab-9 against several normal tissues and cancers; however, LpMab-9 did not stain any PDPN-positive cells probably because of the low binding affinity of LpMab-9 or the loss of sialic acid on PDPN in paraffin-embedded tissues.

PDPN is highly expressed not only in cancer cells but also in normal tissues.[34,35] The activation of CLEC-2 by PDPN rearranges the actin cytoskeleton in dendritic cells to promote efficient motility along stromal surfaces.[36] In contrast, the signal from CLEC-2 to PDPN controls the contractility of fibroblastic reticular cells and lymph node microarchitecture.[37] The physical elasticity of lymph nodes is maintained by PDPN of stromal fibroblastic reticular cells.[38] PDPN-CLEC-2 interaction is also important for embryonic blood-lymphatic vascular separation using platelet aggregation.[39] To investigate these complicated functions of PDPN, different epitope-possessing anti-PDPN MAbs are necessary because site-specific glycosylation is observed in pathological conditions. Until now, we established different epitope-possessing anti-PDPN MAbs such as NZ-1, LpMab-2, LpMab-3, LpMab-7, and LpMab-9, and LpMab.[21,24–27,40,41] Among those clones, LpMab-2, LpMab-3, and LpMab-9 detect site-specific *O*-glycosylation: LpMab-2 against Thr55/Ser56, LpMab-3 against Thr76, and LpMab-9 against Thr25. Different epitope-possessing anti-PDPN MAbs should be further developed for analyzing function of PDPN in cancers and normal tissues in the future.

Acknowledgments

We thank Yuta Tsujimoto, Takuro Nakamura, Noriko Saidoh, and Kanae Yoshida for their excellent technical assistance. This work was supported in part by the Platform for Drug Discovery, Informatics, and Structural Life Science (PDIS) from Japan Agency for Medical Research and Development, AMED (Y.K.); by the Basic Science and Platform Technology Program for Innovative Biological Medicine from AMED (Y.K.); by the Regional Innovation Strategy Support Program from the Ministry of Education,

Culture, Sports, Science and Technology (MEXT) of Japan (Y.K.); and by, and by JSPS KAKENHI Grant Number 25462242 (Y.K.) and Grant Number 26440019 (M.K.K.).

Author Disclosure Statement

The authors have no financial interests to disclose.

References

1. Kato Y, Fujita N, Kunita A, Sato S, Kaneko M, Osawa M, and Tsuruo T: Molecular identification of Aggrus/T1alpha as a platelet aggregation-inducing factor expressed in colorectal tumors. J Biol Chem 2003;278:51599–51605.
2. Kaneko MK, Kato Y, Kitano T, and Osawa M: Conservation of a platelet activating domain of Aggrus/podoplanin as a platelet aggregation-inducing factor. Gene 2006;378: 52–57.
3. Kato Y, Sasagawa I, Kaneko M, Osawa M, Fujita N, and Tsuruo T: Aggrus: a diagnostic marker that distinguishes seminoma from embryonal carcinoma in testicular germ cell tumors. Oncogene 2004;23:8552–8556.
4. Martin-Villar E, Scholl FG, Gamallo C, Yurrita MM, Munoz-Guerra M, Cruces J, and Quintanilla M: Characterization of human PA2.26 antigen (T1alpha-2, podoplanin), a small membrane mucin induced in oral squamous cell carcinomas. Int J Cancer 2005;113:899–910.
5. Kato Y, Kaneko M, Sata M, Fujita N, Tsuruo T, and Osawa M: Enhanced expression of Aggrus (T1alpha/podoplanin), a platelet-aggregation-inducing factor in lung squamous cell carcinoma. Tumor Biol 2005;26:195–200.
6. Yuan P, Temam S, El-Naggar A, Zhou X, Liu D, Lee J, and Mao L: Overexpression of podoplanin in oral cancer and its association with poor clinical outcome. Cancer 2006;107: 563–569.
7. Kato Y, Kaneko MK, Kuno A, Uchiyama N, Amano K, Chiba Y, Hasegawa Y, Hirabayashi J, Narimatsu H, Mishima K, and Osawa M: Inhibition of tumor cell-induced platelet aggregation using a novel anti-podoplanin antibody reacting with its platelet-aggregation-stimulating domain. Biochem Biophys Res Commun 2006;349:1301–1307.
8. Mishima K, Kato Y, Kaneko MK, Nakazawa Y, Kunita A, Fujita N, Tsuruo T, Nishikawa R, Hirose T, and Matsutani M: Podoplanin expression in primary central nervous system germ cell tumors: a useful histological marker for the diagnosis of germinoma. Acta Neuropathol (Berl) 2006; 111:563–568.
9. Mishima K, Kato Y, Kaneko MK, Nishikawa R, Hirose T, and Matsutani M: Increased expression of podoplanin in malignant astrocytic tumors as a novel molecular marker of malignant progression. Acta Neuropathol (Berl) 2006;111: 483–488.
10. Abe S, Morita Y, Kaneko MK, Hanibuchi M, Tsujimoto Y, Goto H, Kakiuchi S, Aono Y, Huang J, Sato S, Kishuku M, Taniguchi Y, Azuma M, Kawazoe K, Sekido Y, Yano S, Akiyama S, Sone S, Minakuchi K, Kato Y, and Nishioka Y: A novel targeting therapy of malignant mesothelioma using anti-podoplanin antibody. J Immunol 2013;190:6239–6249.
11. Takagi S, Oh-hara T, Sato S, Gong B, Takami M, and Fujita N: Expression of Aggrus/podoplanin in bladder cancer and its role in pulmonary metastasis. Int J Cancer 2014;134:2605–2614.
12. Kunita A, Kashima TG, Ohazama A, Grigoriadis AE, and Fukayama M: Podoplanin is regulated by AP-1 and promotes platelet aggregation and cell migration in osteosarcoma. Am J Pathol 2011;179:1041–1049.
13. Chandramohan V, Bao X, Kato Kaneko M, Kato Y, Keir ST, Szafranski SE, Kuan CT, Pastan IH, and Bigner DD: Recombinant anti-podoplanin (NZ-1) immunotoxin for the treatment of malignant brain tumors. Int J Cancer 2013; 132:2339–2348.
14. Kato Y, Vaidyanathan G, Kaneko MK, Mishima K, Srivastava N, Chandramohan V, Pegram C, Keir ST, Kuan CT, Bigner DD, and Zalutsky MR: Evaluation of anti-podoplanin rat monoclonal antibody NZ-1 for targeting malignant gliomas. Nucl Med Biol 2010;37:785–794.
15. Kawase A, Ishii G, Nagai K, Ito T, Nagano T, Murata Y, Hishida T, Nishimura M, Yoshida J, Suzuki K, and Ochiai A: Podoplanin expression by cancer associated fibroblasts predicts poor prognosis of lung adenocarcinoma. Int J Cancer 2008;123:1053–1059.
16. Hoshino A, Ishii G, Ito T, Aoyagi K, Ohtaki Y, Nagai K, Sasaki H, and Ochiai A: Podoplanin-positive fibroblasts enhance lung adenocarcinoma tumor formation: podoplanin in fibroblast functions for tumor progression. Cancer Res 2011;71:4769–4779.
17. Schoppmann SF, Jesch B, Riegler MF, Maroske F, Schwameis K, Jomrich G, and Birner P: Podoplanin expressing cancer associated fibroblasts are associated with unfavourable prognosis in adenocarcinoma of the esophagus. Clin Exp Metastasis 2013;30:441–446.
18. Shindo K, Aishima S, Ohuchida K, Fujiwara K, Fujino M, Mizuuchi Y, Hattori M, Mizumoto K, Tanaka M, and Oda Y: Podoplanin expression in cancer-associated fibroblasts enhances tumor progression of invasive ductal carcinoma of the pancreas. Mol Cancer 2013;12:168.
19. Inoue H, Tsuchiya H, Miyazaki Y, Kikuchi K, Ide F, Sakashita H, and Kusama K: Podoplanin expressing cancer-associated fibroblasts in oral cancer. Tumour Biol 2014;35: 11345–11352.
20. Nagae M, Morita-Matsumoto K, Kato M, Kaneko MK, Kato Y, and Yamaguchi Y: A platform of C-type lectin-like receptor CLEC-2 for binding O-glycosylated podoplanin and nonglycosylated rhodocytin. Structure 2014;22:1711–1721.
21. Ogasawara S, Kaneko MK, Price JE, and Kato Y: Characterization of anti-podoplanin monoclonal antibodies: critical epitopes for neutralizing the interaction between podoplanin and CLEC-2. Hybridoma 2008;27:259–267.
22. Takagi S, Sato S, Oh-hara T, Takami M, Koike S, Mishima Y, Hatake K, and Fujita N: Platelets promote tumor growth and metastasis via direct interaction between Aggrus/podoplanin and CLEC-2. PLoS One 2013;8:e73609.
23. Marks A, Sutherland DR, Bailey D, Iglesias J, Law J, Lei M, Yeger H, Banerjee D, and Baumal R: Characterization and distribution of an oncofetal antigen (M2A antigen) expressed on testicular germ cell tumours. Br J Cancer 1999;80:569–578.
24. Kato Y, and Kaneko MK: A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. Sci Rep 2014;4:5924.
25. Oki H, Kaneko MK, Ogasawara S, Tsujimoto Y, Liu X, Sugawara M, Takakubo Y, Takagi M, and Kato Y: Characterization of a monoclonal antibody LpMab-7 recognizing non-PLAG domain of podoplanin. Monoclon Antib Immunodiagn Immunother 2015;34:174–180.
26. Kaneko MK, Oki H, Ogasawara S, Takagi M, and Kato Y: An anti-podoplanin monoclonal antibody LpMab-7 detects

metastatic legions of osteosarcoma Monoclon Antib Immunodiagn Immunother 2015;34:154–161.
27. Oki H, Ogasawara S, Kaneko MK, Takagi M, Yamauchi M, and Kato Y: Characterization of monoclonal antibody LpMab-3 recognizing sialylated glycopeptide of podoplanin. Monoclon Antib Immunodiagn Immunother 2015;34: 44–50.
28. Kato Y: Specific monoclonal antibodies against IDH1/2 mutations as diagnostic tools for gliomas. Brain Tumor Pathol 2014;32:3–11.
29. Kaneko MK, Tian W, Takano S, Suzuki H, Sawa Y, Hozumi Y, Goto K, Yamazaki K, Kitanaka C, and Kato Y: Establishment of a novel monoclonal antibody SMab-1 specific for IDH1-R132S mutation. Biochem Biophys Res Commun 2011;406:608–613.
30. Kaneko MK, Kunita A, Abe S, Tsujimoto Y, Fukayama M, Goto K, Sawa Y, Nishioka Y, and Kato Y: Chimeric anti-podoplanin antibody suppresses tumor metastasis through neutralization and antibody-dependent cellular cytotoxicity. Cancer Sci 2012;103:1913–1919.
31. Kato Y, Kaneko MK, Kunita A, Ito H, Kameyama A, Ogasawara S, Matsuura N, Hasegawa Y, Suzuki-Inoue K, Inoue O, Ozaki Y, and Narimatsu H: Molecular analysis of the pathophysiological binding of the platelet aggregation-inducing factor podoplanin to the C-type lectin-like receptor CLEC-2. Cancer Sci 2008;99:54–61.
32. Kuno A, Kato Y, Matsuda A, Kaneko MK, Ito H, Amano K, Chiba Y, Narimatsu H, and Hirabayashi J: Focused differential glycan analysis with the platform antibody-assisted lectin profiling for glycan-related biomarker verification. Mol Cell Proteomics 2009;8:99–108.
33. Kaneko MK, Kato Y, Kameyama A, Ito H, Kuno A, Hirabayashi J, Kubota T, Amano K, Chiba Y, Hasegawa Y, Sasagawa I, Mishima K, and Narimatsu H: Functional glycosylation of human podoplanin: glycan structure of platelet aggregation-inducing factor. FEBS Lett 2007;581: 331–336.
34. Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, and Kerjaschki D: Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am J Pathol 1997;151:1141–1152.
35. Schacht V, Dadras SS, Johnson LA, Jackson DG, Hong YK, and Detmar M: Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. Am J Pathol 2005;166:913–921.
36. Acton SE, Astarita JL, Malhotra D, Lukacs-Kornek V, Franz B, Hess PR, Jakus Z, Kuligowski M, Fletcher AL, Elpek KG, Bellemare-Pelletier A, Sceats L, Reynoso ED, Gonzalez SF, Graham DB, Chang J, Peters A, Woodruff M, Kim YA, Swat W, Morita T, Kuchroo V, Carroll MC, Kahn ML, Wucherpfennig KW, and Turley SJ: Podoplanin-rich stromal networks induce dendritic cell motility via activation of the C-type lectin receptor CLEC-2. Immunity 2012; 37:276–289.
37. Astarita JL, Cremasco V, Fu J, Darnell MC, Peck JR, Nieves-Bonilla JM, Song K, Kondo Y, Woodruff MC, Gogineni A, Onder L, Ludewig B, Weimer RM, Carroll MC, Mooney DJ, Xia L, and Turley SJ: The CLEC-2-podoplanin axis controls the contractility of fibroblastic reticular cells and lymph node microarchitecture. Nat Immunol 2015;16:75–84.
38. Acton SE, Farrugia AJ, Astarita JL, Mourao-Sa D, Jenkins RP, Nye E, Hooper S, van Blijswijk J, Rogers NC, Snelgrove KJ, Rosewell I, Moita LF, Stamp G, Turley SJ, Sahai E, and Reis e Sousa C: Dendritic cells control fibroblastic reticular network tension and lymph node expansion. Nature 2014;514:498–502.
39. Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M, Chen CY, Xu B, Lu MM, Zhou D, Sebzda E, Santore MT, Merianos DJ, Stadtfeld M, Flake AW, Graf T, Skoda R, Maltzman JS, Koretzky GA, and Kahn ML: Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. Blood 2010;116:661–670.
40. Ogasawara S, Oki H, Kaneko MK, Hozumi Y, Liu X, Honma R, Fujii Y, Nakamura T, Goto K, Takagi M, and Kato Y: Development of monoclonal antibody LpMab-10 recognizing non-PLAG1/2 domain including Thr34 of human podoplanin. Monoclon Antib Immunodiagn Immunother 2015;34:318–326.
41. Kato Y, Kunita A, Abe S, Ogasawara S, Fujii Y, Oki H, Fukayama M, Nishioka Y, and Kaneko MK: The chimeric antibody chLpMab-7 targeting human podoplanin suppresses pulmonary metastatis via ADCC and CDC rather than via its neutralizing activity. Oncotarget 2015; DOI: 10.18632/oncotarget.5339

Address correspondence to:
Yukinari Kato
Department of Regional Innovation
Tohoku University Graduate School of Medicine
2-1 Seiryo-machi, Aoba-ku
Sendai, Miyagi 980-8575
Japan

E-mail: yukinari-k@bea.hi-ho.ne.jp

Received: April 13, 2015
Accepted: June 12, 2015