Development of Monoclonal Antibody LpMab-10 Recognizing Non-glycosylated PLAG1/2 Domain Including Thr34 of Human Podoplanin

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Podoplanin (PDPN) is a type-I transmembrane sialoglycoprotein that possesses a platelet aggregation-stimulating (PLAG) domain in the N-terminus. PLAG domain includes three tandem repeats of eight amino acids: PLAG1, PLAG2, and PLAG3. Among the three PLAG domains, *O*-glycan on Thr52 of PLAG3 is critical for binding with C-type lectin-like receptor-2 (CLEC-2) and is essential for platelet-aggregating activity of PDPN. In contrast, the glycosylation of Thr34 of PLAG1 of human PDPN remains to be clarified. Herein, we developed and characterized a novel anti-PDPN monoclonal antibody, LpMab-10, which targets PLAG1/2 domain. LpMab-10 detects endogenous PDPN of cancer cells and normal cells independently of glycosylation. The minimum epitope of LpMab-10 was identified as Glu33–Gly45 of PDPN using Western blot and flow cytometry. The Thr34 of PLAG1 is critical for LpMab-10 recognition, and *O*-glycan is not included in LpMab-10 epitope, indicating that Thr34 of PLAG1 is not *O*-glycosylated. In immunocytochemical and immunohistochemical analyses, LpMab-10 strongly detected PDPN-expressing tumor cells. By using monoclonal antibodies against different Ser/Thr, including epitopes of PDPN, it becomes possible to determine whether Ser/Thr residues of PDPN are *O*-glycosylated.

Introduction

PODOPLANIN (PDPN/AGGRUS), A PLATELET aggregation-inducing type-I transmembrane sialoglycoprotein, is highly expressed not only in cancer cells but also in normal tissues.[1,2] PDPN binds to CLEC-2 and rearranges the actin cytoskeleton in dendritic cells to promote efficient motility along stromal surfaces.[3] The signal from CLEC-2 to PDPN controls the contractility of fibroblastic reticular cells and lymph node microarchitecture.[4] The physical elasticity of lymph nodes is maintained by PDPN of stromal fibroblastic reticular cells.[5] For embryonic blood-lymphatic vascular separation, PDPN-CLEC-2 interaction is also important.[6] PDPN is also very useful for detecting lymphatic endothelial cells.[7] The development of ectopic lymphoid follicles is dependent on Th17-expressing PDPN.[8] Expression of PDPN has been reported in many tumors including malignant brain tumors, mesotheliomas, oral cancer, lung cancer, esophageal cancer, testicular tumors, and osteosarcoma.[9–22] To study these complicated functions of PDPN, different epitope-possessing anti-PDPN monoclonal antibodies (MAbs) are necessary because site-specific glycosylation is observed in patho-logical conditions. Almost all anti-PDPN MAbs react with platelet aggregation-inducing (PLAG) domain of human PDPN.[23] Rabbit polyclonal antibodies were previously produced by Matsui and colleagues and also recognized PLAG domain because PLAG domain was shown to be immunodominant antigenic sites.[24] Recently, we established the platform to produce cancer-specific MAbs (Cas-Mabs).[25] Using CasMab methods, we established different epitope-possessing anti-PDPN MAbs such as LpMab-2, LpMab-3, LpMab-7, and LpMab-9.[25–28] Interestingly, 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. In this study, we developed and characterized a novel anti-PDPN MAb, LpMab-10, which possesses a novel epitope of PDPN.

Materials and Methods

Cell lines, animals, and tissues

LN229, NCI-H226, HEK-293T, Chinese hamster ovary (CHO)-K1, glycan-deficient CHO cell lines (Lec1, Lec2, and Lec8), Met-5A, and P3U1 were obtained from the American

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Type Culture Collection (ATCC, Manassas, VA). Human lymphatic endothelial cells (LEC) were purchased 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). PC-10 cells were purchased from Immuno-Biological Laboratories (Gunma, Japan). 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.[25] LN319/PDPN-knock out (KO) cells were produced by transfecting CRISPR/Cas plasmids, which targets 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, PC-10, and P3U1 were cultured in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan), and LN229, LN319, and Met-5A were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Nacalai Tesque), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific), 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). MITO + serum extender (Thermo Fisher Scientific) was added for Met-5A cells. Female BALB/c mice (4 weeks old) were purchased from CLEA Japan (Tokyo, Japan). Animals were housed under pathogen-free conditions. The Animal Care and Use Committee of Tohoku University approved the animal experiments described herein. This study examined one seminoma patient[11] and one osteosarcoma patient[26] who underwent surgery at Yamagata University Hospital. The ethical committee of the Yamagata University Faculty of Medicine approved our study. Informed consent for obtaining samples and for subsequent data analyses was obtained from patients or the patient's guardian.

Hybridoma production

BALB/c mice were immunized by intraperitoneal (i.p.) injection of 1×10^8 LN229/hPDPN cells 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 PEG1500 (Roche Diagnostics, Indianapolis, IN). 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.

Enzyme-linked immunosorbent assay

Purified proteins or peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific) at 1 μg/mL for 30 min. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific), the plates were incubated with culture supernatant or purified MAbs (1 μg/mL) followed by 1:1000 diluted peroxidase-conjugated

anti-mouse IgG or anti-rat IgG (Dako, Glostrup, Denmark). The enzymatic reaction was conducted with a 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific). The optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories). These reactions were performed with a volume of 50 μL at 37°C.

Western blot analyses

Cell lysates (10 μg) were boiled in SDS sample buffer (Nacalai Tesque). 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, the membrane was incubated with primary antibodies (1 μg/mL) and then with peroxidase-conjugated secondary antibodies (Dako; 1:1000 diluted), and developed with the Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific) using a Sayaca-Imager (DRC, Tokyo, Japan).

Flow cytometry

Cell lines were harvested by brief exposure to 0.25% Trypsin/1 mM EDTA (Nacalai Tesque).[29] After washing with phosphate buffered saline (PBS, Nacalai Tesque), the cells were treated with primary antibodies (1 μg/mL) for 30 min at 4°C, followed by treatment with Oregon Green 488 goat anti-mouse IgG (Thermo Fisher Scientific). Fluorescence data were collected using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

Determination of binding affinity using flow cytometry

LN319 and PC-10 (2×10^5 cells) were resuspended with 100 μL of serially diluted antibody (0.02–100 μg/mL) followed by secondary anti-mouse IgG (Thermo Fisher Scientific). Fluorescence data were collected using the EC800 cell analyzer. The dissociation constants (K_D) were obtained by fitting the binding isotherms using the built-in one-site binding models in Prism software.

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) was 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-10 (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 incubated at 20°C for 30 min. The glass slide was scanned using a GlycoStation Reader 1200 (GlycoTechnica). Abbreviation of lectins: 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* 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.

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 or glycine in PDPN was performed using a QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). CHO-K1 cells were transfected with the plasmids using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories).

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 nonspecific binding sites, and were incubated with 1 μg/mL of LpMab-10 or control (PBS) for 1 h at 4°C in a moist chamber. They were incubated with goat anti-mouse IgG-Alexa 488 (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).

Immunohistochemical analyses

Four-μm-thick histologic sections were deparaffinized in xylene and rehydrated. They were then autoclaved in citrate buffer (pH 6.0; Dako) for 20 min. Sections were incubated with 5 μg/mL of primary antibodies overnight at 4°C followed by treatment with Envision+ kit (Dako). Color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako) for 10 min; then the sections were counterstained with hematoxylin (Wako Pure Chemical Industries).

Results

Development and characterization of novel anti-PDPN MAb LpMab-10

We immunized mice with LN229/hPDPN cells to develop novel anti-PDPN MAbs. Using ELISA, the culture superna-

tants were screened for binding to recombinant human PDPN purified from LN229/hPDPN cells. After the limiting dilution, LpMab-10 (mouse IgG$_3$, kappa) was established. LpMab-10 reacted with LN229/hPDPN, not with LN229, a PDPN-negative cell in Western blot analysis (Fig. 1A). LpMab-10 also recognized endogenous PDPN, which is expressed in a glioblastoma cell line LN319 and a lymphatic endothelial cell (LEC) in Western blot analysis (Fig. 1A) and flow cytometry (Fig. 1B). Although LpMab-10 did not react with a malignant mesothelioma cell line NCI-H226 and HEK-293T in Western blot analysis (Fig. 1A), it strongly recognized both cells in flow cytometry (Fig. 1B), indicating that LpMab-10 binds to native PDPN effectively. LN229 and CHO-K1, which do not express PDPN, were not detected by LpMab-10, indicating that LpMab-10 does not recognize non-related antigens in native condition (Fig. 1B). LpMab-10 also detects PDPN of a lung squamous cell carcinoma (SCC) cell line PC-10 and a normal mesothelial cell Met-5A (Fig. 1B), indicating that LpMab-10 is useful for detecting PDPN of both cancer cells and normal cells. We also performed Western blot analysis using LpMab-10 against several glycan-deficient PDPN transfectants, such as Lec1/hPDPN (*N*-glycan deficient), Lec2/hPDPN (sialic acid deficient), and Lec8/hPDPN (*O*-glycan deficient) (Fig. 1A). All transfectants of glycan-deficient PDPN were detected by LpMab-10, indicating that the epitope of LpMab-10 is independent of glycan. To investigate whether LpMab-10 reacts with synthetic peptides, we performed ELISA using several synthetic peptides of human PDPN (Fig. 1C). NZ-1 reacted with PLAG2/3 domain that corresponds to 38–51 amino acids of human PDPN (hpp38–51). In contrast, LpMab-10 strongly recognized PLAG1/2 domain that corresponds to 29–47 amino acids of human PDPN (hpp29–47). We next performed a kinetic analysis of the interaction of LpMab-10 with LN319 and PC-10 using flow cytometry. As shown in Figure 1D, K_D was determined to be 8.755×10^{-9} M using LN319 and 9.830×10^{-9} M using PC-10, indicating that LpMab-10 possesses high affinity against human PDPN. We further performed antibody-overlay lectin microarray analysis of PDPN using LpMab-10 (Fig. 1E). PDPN strongly reacted with a α 2–3 sialic acid binder ACG, a sialic acid ± core1 binder Jacalin, and a sialo-mucin binder WGA, moderately reacted with a core1 ± sialic acid binder MPA, and faintly reacted with a poly LacNAc binder STL, indicating that LpMab-10 is useful in antibody-overlay lectin microarray analysis and PDPN possesses sialylated core1 structure.

Immunocytochemistry and immunohistochemistry

We next investigated whether LpMab-10 is useful for immunocytochemical and immunohistochemical analyses. LpMab-10 clearly stained the membrane of CHO/hPDPN, whereas it did not react with CHO-K1 cells (Fig. 2). LpMab-10 also stained endogenous PDPN expressed on LN319 cells, whereas it did not react with LN319/PDPN knock-out cells, indicating that LpMab-10 specifically recognizes human PDPN. As shown in Figure 3, LpMab-10 stained seminoma cells (Fig. 3C, D) and osteosarcoma cells (Fig. 3G, H) in a membranous/cytoplasmic staining pattern, indicating that LpMab-10 is very useful in immunohistochemical analysis.
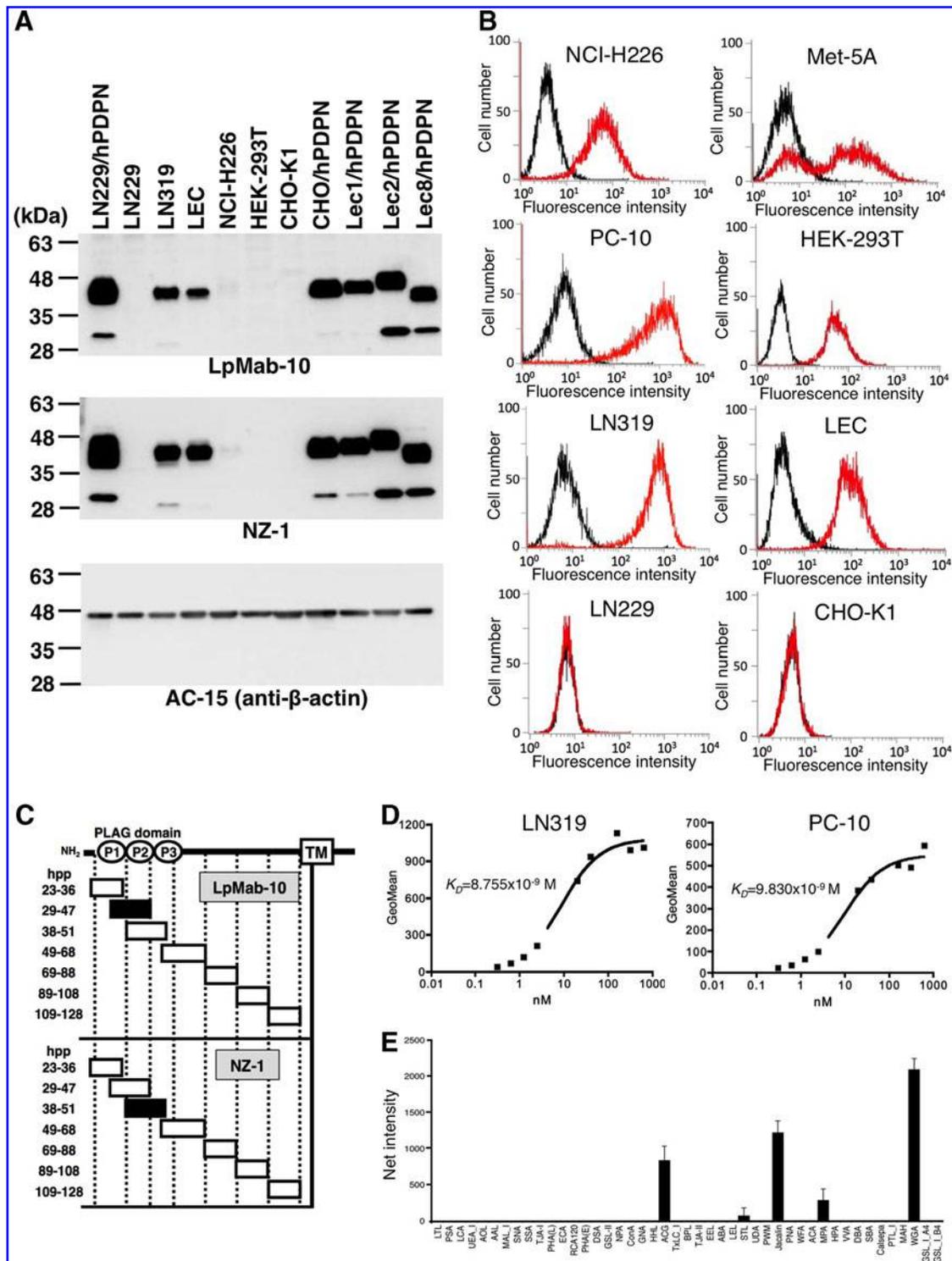
FIG. 1. Characterization of a novel anti-PDPN MAb, LpMab-10. (**A**) Western blot analysis by LpMab-10 and NZ-1. AC-15 (anti-β-actin) was used as internal control. Total cell lysate were electrophoresed on 5–20% polyacrylamide gels and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μg/mL of primary antibodies and then with peroxidase-conjugated secondary antibodies; the membrane was detected using a Sayaca-Imager. (**B**) Flow cytometric analysis by LpMab-10. Eight cell lines were treated with LpMab-10 (1 μg/mL; red) or control PBS (black) for 30 min at 4°C, followed by treatment with anti-mouse IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. (**C**) Epitope mapping of LpMab-10 and NZ-1 using ELISA. Peptides were immobilized at 1 μg/mL for 30 min. After blocking, the plates were incubated with LpMab-10 or NZ-1 (1 μg/mL) followed by 1:1000 diluted peroxidase-conjugated anti-mouse IgG or anti-rat IgG. The enzymatic reaction was conducted with a 1-Step Ultra TMB-ELISA. Black bar, positive; white bar, negative. P1, PLAG1; P2, PLAG2; P3, PLAG3; TM, transmembrane. (**D**) Determination of binding-affinity of LpMab-10 using flow cytometry. (**E**) Glycan profiling of PDPN using antibody-overlay lectin microarray. Purified PDPN was applied to a lectin array including 45 lectins. After blocking, biotinylated LpMab-10 was applied and Cy3-labeled streptavidin was added. Signal was scanned using a GlycoStation Reader 1200.
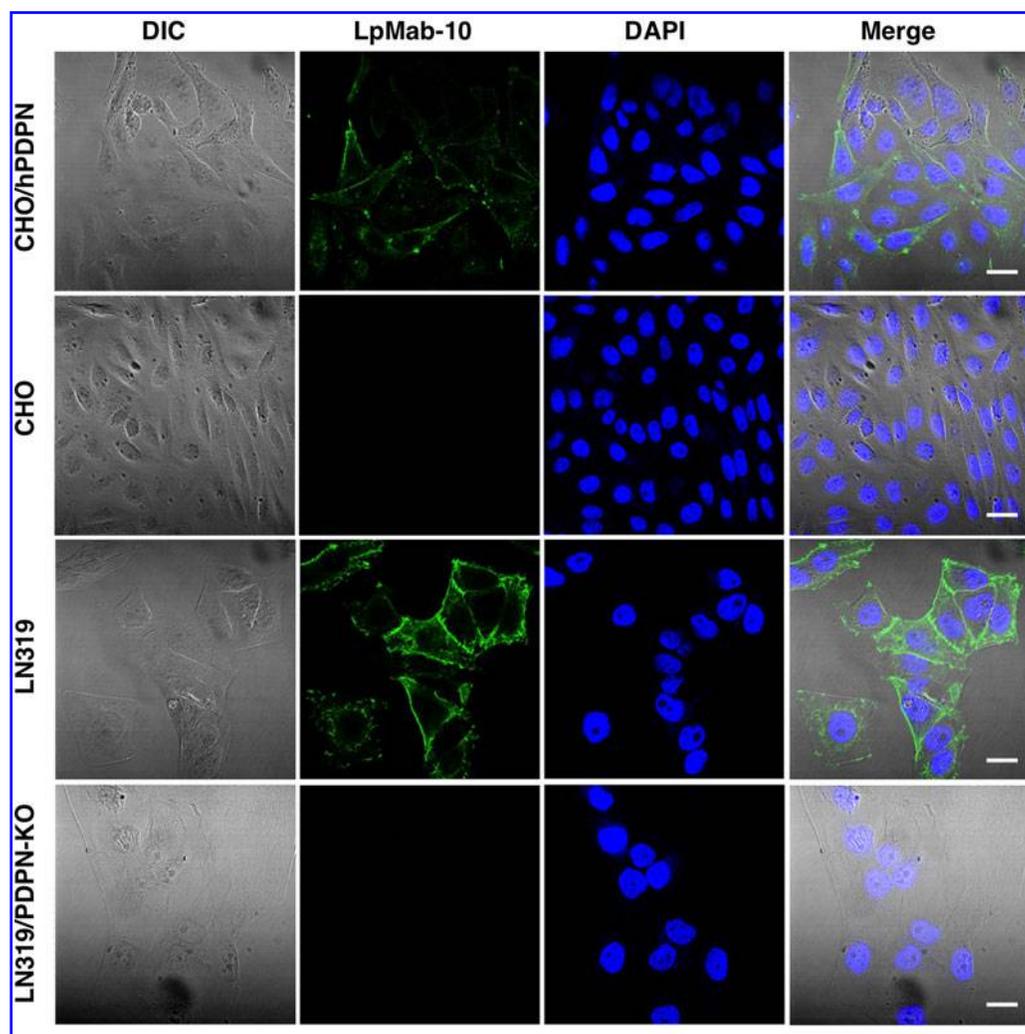
FIG. 2. Immunocytochemical analysis. Cells were incubated with 1 µg/mL of LpMab-10 for 1 h at 4°C in a moist chamber. They were incubated with goat anti-mouse IgG-Alexa 488 for 30 min at room temperature. Cells were also treated with DAPI to stain the cell nuclei. Scale bars, 20 µm.

Epitope-mapping Western blot analysis and flow cytometry

The epitope of LpMab-10 was shown to be PLAG1/2 in ELISA (Fig. 1C); therefore, we next performed Western blot analysis to determine the LpMab-10 epitope in details. Western blot analysis showed that LpMab-10 reaction was lost in point mutations of 33–45 amino acids (Fig. 4A). Flow cytometric analysis revealed that LpMab-10 did not react with L37A and E38A; weakly recognized E33A, T34A, and P44A; moderately reacted with G39A, G40A, V41A, and G45A, indicating that Leu37 and Glu38 are much more important for LpMab-10 (Fig. 4B). Taken together, the PDPN epitope of LpMab-10 is Glu33-Gly45, and Leu37 and Glu38 are the most important amino acids.

Discussion

Until now, ours and other groups have developed many anti-PDPN MAbs. To neutralize platelet aggregation activity by blocking the association between PDPN and CLEC-2, we produced NZ-1 MAb by immunizing rats with synthetic peptides of PLAG domain.[15] NZ-1 possesses very high binding affinity, which was clarified by several methods including ELISA, flow cytometry, Scatchard analysis, and BIAcore.[21,30] NZ-1 inhibited cancer-induced platelet aggregation and experimental metastasis by its neutralizing activity, indicating that NZ-1 is a candidate of anti-metastatic MAb.[15,29,31] In addition, NZ-1 was highly internalized into glioma cells and also accumulated efficiently into tumors *in vivo*.[21] NZ-1 and its rat-human chimeric anti-PDPN antibody (NZ-8) possess antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against PDPN-expressing glioblastoma or malignant mesothelioma cells.[18,31] Therefore, NZ-1 is known to be an extremely useful anti-PDPN MAb. In contrast, rat MAbs are often avoided in immunohistochemistry, especially in pathological diagnosis, because almost all secondary antibodies or commercially available kits for immunohistochemistry have been developed against mouse MAbs or polyclonal antibodies, although many rat MAbs possess high-binding affinity and specificity. LpMab-10 shows the same reaction pattern with NZ-1 in Western blot analysis (Fig. 1A) and flow cytometry (Fig. 1B), demonstrating that LpMab-10 could possess more advantages compared with NZ-1 in several
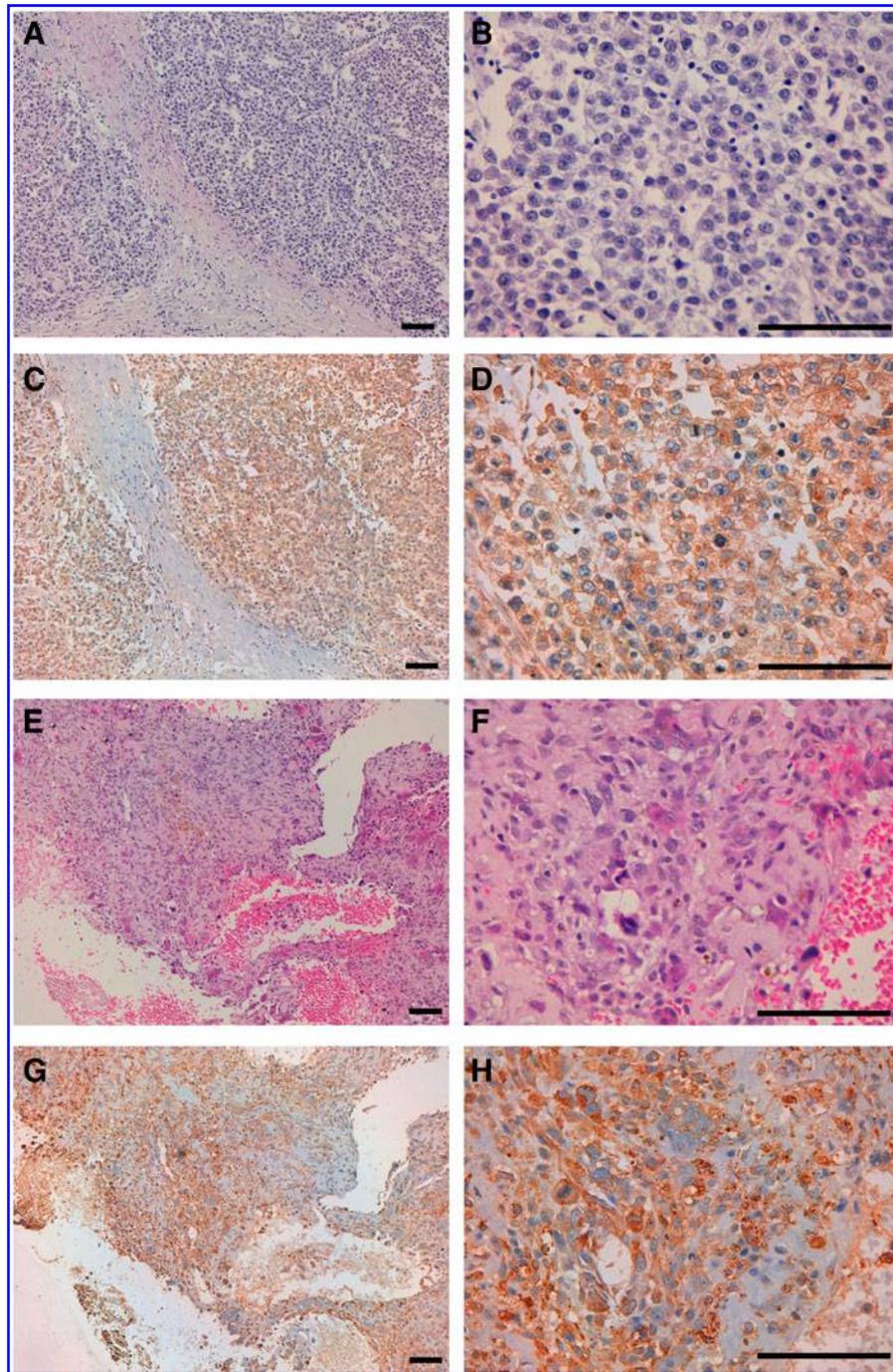
FIG. 3. Immunohistochemical analysis by LpMab-10 against seminoma (**A–D**) and osteosarcoma (**E, F**). Sections were incubated with 5 μg/mL LpMab-10, followed by Envision+ kit, and color was developed using DAB and counterstained with hematoxylin (**C, D, G, H**). The serial sections were also stained by hematoxylin and eosin (**A, B, E, F**). Original magnification: ×100 (**A, C, E, G**); ×400 (**B, D, F, H**). Scale bar, 100 μm.

applications. Indeed, we used Dako Envision+ kit in immunohistochemistry, which is not applied for rat MAbs, indicating LpMab-10 is more advantageous than NZ-1.

Figure 4C shows that LpMab-10 epitope is independent of previously established anti-PDPN MAbs such as LpMab-2, LpMab-3, LpMab-7, LpMab-9, and NZ-1.[25,27,28,32,33] Furthermore, the minimum epitope of LpMab-10 is ETT-GLEGGVAMPG, which consists of 13 amino acids, dem-

onstrating that LpMab-10 epitope is longer than those of anti-PDPN MAbs. As we previously reported, Thr34 of mouse, rat, and hamster PDPNs might be *O*-glycosylated and shows the critical role for platelet aggregation.[34] Although we also demonstrated that Thr34 of human PDPN might not be glycosylated, and is not involved in platelet-aggregating activity,[35] the possibility of glycosylation of Thr34 was suggested in platelet aggregation and in a tumor

FIG. 4. Epitope mapping of LpMab-10 using flow cytometry and Western blot analysis. (**A**) Total cell lysate of point mutants of human PDPN were electrophoresed on 5–20% polyacrylamide gels and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μg/mL of primary antibodies and then with peroxidase-conjugated secondary antibodies; the membrane was detected using a Sayaca-Imager. (**B**) Flow cytometric analysis by LpMab-10. Point mutants of human PDPN were treated with LpMab-10 (1 μg/mL) for 30 min at 4°C, followed by treatment with anti-mouse IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. (**C**) Comparison of epitopes of anti-PDPN MAbs. Minimum epitopes of LpMab-9, LpMab-10, NZ-1, LpMab-2, LpMab-3, and LpMab-7 are TGQPED, ETTGLEGGVAMPG, MPGAEDD, TSEDRYKSGL, VTGIRIE, and RIEDL, respectively. The residues of Thr25, Thr55, Ser56, and Thr76 were determined to be *O*-glycosylated using LpMab-9, LpMab-2, and LpMab-3, respectively. *O*-glycosylated Thr52 was detected by CLEC-2.

metastasis model in another study.[22] It was also suggested that *O*-glycosylation sites are predicted by the NetOGlyc prediction tool, and Thr32, Thr34, and Thr35 of PLAG1 might be *O*-glycosylated.[36] These studies did not investigate the *O*-glycosylation directly because there was no tool to detect the *O*-glycosylation of each Thr. As shown in Figure 4C, we have produced several site-specific MAbs

against each *O*-glycosylated Ser/Thr of human PDPN; therefore, we can directly detect *O*-glycosylation of Thr25, Thr34, Thr52, Thr55, Ser56, and Thr76. Because Thr34 of PLAG1 is critical for LpMab-10 recognition (Fig. 4A, B), and *O*-glycan is not included in LpMab-10 epitope (Fig. 1A), it is more likely that Thr34 of PLAG1 is not *O*-glycosylated.

In conclusion, LpMab-10 could be advantageous for investigating expression and function of PDPN in cancers and normal tissues. By using MAbs against different Ser/Thr-including epitopes of human PDPN, we can determine whether Ser/Thr residues of PDPN are *O*-glycosylated. Further, different epitope-possessing anti-PDPN MAbs should be established as powerful tools for investigating function of PDPN in cancers and normal tissues in the future.

Author Disclosure Statement

The authors have no financial interests to disclose.

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