MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 34, Number 6, 2015

DOI: 10.1089/mab.2015.0041

Development of Sensitive Monoclonal Antibody PMab-2 Against Rat Podoplanin

Hiroharu Oki,[1,2*] Ryusuke Honma,[1,2,*] Satoshi Ogasawara,[1,*] Yuki Fujii,[1] Xing Liu,[1,2] Michiaki Takagi,[2] Mika K. Kaneko,[1] and Yukinari Kato[1]

Podoplanin (PDPN) is a platelet aggregation-inducing factor, which is known as an endogenous ligand of C-type lectin-like receptor-2 (CLEC-2). PDPN is also expressed in several normal tissues, such as lung type I alveolar cells and kidney podocytes. Although many monoclonal antibodies (MAbs) against human PDPN (hPDPN) or mouse PDPN (mPDPN) have been established, anti-rat PDPN (rPDPN) MAbs, especially against platelet aggregation-stimulating (PLAG) domain (29-54 amino acids) of rPDPN, have not been developed. Therefore, functional analysis of rPDPN in normal tissues has been limited. Here, we immunized mice with rPDPN peptides (38-51 amino acids) and developed a novel mouse anti-rPDPN MAb, PMab-2 (IgG_1, kappa), which possesses high affinity compared with anti-hPDPN or mPDPN MAbs. The K_D of PMab-2 was determined to be 5.9×10^{-10} M. PMab-2 is useful, not only in flow cytometry and Western blot analysis against endogenous rPDPN, which is expressed in rat dermal fibroblast, but also in immunohistochemistry against normal tissues. PMab-2 showed extraordinarily high sensitivity in immunohistochemistry, indicating that PMab-2 is very advantageous for functional analysis of rPDPN.

Introduction

Podoplanin (PDPN/PA2.26/T1α/Aggrus) is a type I transmembrane *O*-glycoprotein that possesses platelet aggregating activity.[1–4] We recently performed crystallographic studies of human PDPN (hPDPN) in complex with C-type lectin-like receptor-2 (CLEC-2).[5] The interaction with CLEC-2 was mainly observed at Glu47 and Asp48 in the platelet aggregation-stimulating (PLAG) domain of hPDPN (29-54 amino acids) and the α2-6 linked sialic acid at Thr52 of hPDPN. The platelet aggregation via PDPN-CLEC-2 interaction is critical for embryonic blood-lymphatic vascular separation.[6] In adult tissues, PDPN is very useful for detecting lymphatic endothelial cells.[7] PDPN also possesses many important functions. Interestingly, both PDPN-CLEC-2 axis and CLEC-2-PDPN axis have been reported. PDPN binds to CLEC-2 and rearranges the actin cytoskeleton in dendritic cells to promote efficient motility along stromal surfaces.[8] The development of ectopic lymphoid follicles is dependent on Th17-expressing PDPN.[9] The signal from CLEC-2 to PDPN controls the contractility of fibroblastic reticular cells and lymph node microarchitecture.[10] The physical elasticity of lymph nodes is maintained by PDPN of stromal fibroblastic reticular cells.[11] Expression of PDPN has also been reported in many cancers, including oral cancers, malignant brain tumors, esophageal cancers, lung cancers, malignant mesotheliomas, testicular tumors, and osteosarcomas.[3,12–23] Moreover, PDPN expression in cancer-associated fibroblasts (CAFs) is involved in poor prognosis of cancers.[24–28] PDPN is also reported in advanced atherosclerotic lesions of human aortas.[29] Although anti-PDPN MAbs with high sensitivity and specificity are necessary to investigate the physiological function of PDPN in normal tissues, almost all anti-PDPN MAbs have been developed against hPDPN[17,30–39] and mouse PDPN (mPDPN),[40–42] not against rat PDPN (rPDPN).[1] Therefore, we immunized mice with PLAG domain of rPDPN and developed high-sensitive MAbs against rPDPN.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3U1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Rat dermal fibroblast was obtained from Cell Applications (San Diego, CA). CHO-K1 was transfected with rPDPN, hPDPN, or mPDPN plasmids (CHO/rPDPN, CHO/hPDPN, CHO/mPDPN) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA).[3] CHO-K1, CHO/rPDPN,

[1]Department of Regional Innovation, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Miyagi, Japan.
[2]Department of Orthopaedic Surgery, Yamagata University Faculty of Medicine, Yamagata, Japan.
*These authors contributed equally to this work.

CHO/hPDPN, CHO/mPDPN, and P3U1 were cultured in RPMI 1640 medium including RPMI 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/mL penicillin, 100 µg/mL streptomycin, and 25 µg/mL amphotericin B (Nacalai Tesque), and rat dermal fibroblast was cultured in Fibroblast Growth Medium 2, including FBS, basic fibroblast growth factor (1 ng/mL), and insulin (5 µg/mL; PromoCell, Heidelberg, Germany) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Animals

Female BALB/c mice (four-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.

Hybridoma production

BALB/c mice were immunized by intraperitoneal (i.p.) injection of 100 µg of synthetic peptides (rpp3851: GDDMV NPGLEDRIEC), conjugated with KLH 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 rpp3851 peptides.

ELISA

Synthetic peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific) at 1 µg/mL for

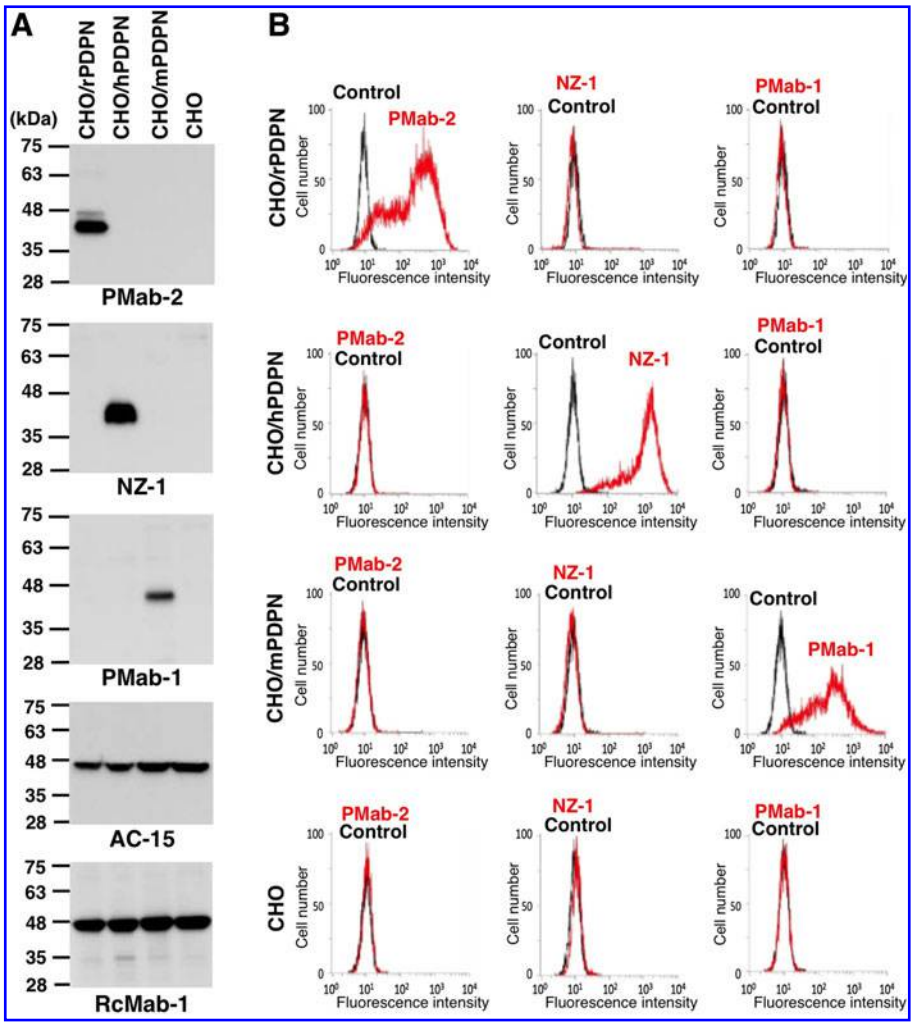

FIG. 1. Specific binding of anti-PDPN MAbs. (**A**) Western blot analysis by PMab-2, NZ-1, and PMab-1. AC-15 (anti-β-actin) and RcMAb-1 (anti-IDH1) were used as internal control. Total cell lysate were electrophoresed on 5–20% polyacrylamide gels and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 0.1 µg/mL of MAbs (PMab-2, NZ-1, PMab-1) or 1 µg/mL of MAbs (AC-15 and RcMAb-1), and then with peroxidase-conjugated secondary antibodies; the membrane was detected using a Sayaca-Imager. (**B**) Flow cytometry. Cells were harvested by brief exposure to 0.25% Trypsin/1 mM EDTA. After washing with PBS, cells were treated with PMab-2, NZ-1, and PMab-1, followed by treatment with secondary antibodies. Fluorescence data were collected using a Cell Analyzer EC800.

30 min. After blocking with SuperBlock T20 (PBS) Blocking Buffer, the plates were incubated with culture supernatant followed by 1:1000 diluted peroxidase-conjugated anti-mouse 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, Philadelphia, PA). 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 4% skim milk (Nacalai Tesque), the membrane was incubated with primary antibodies,[43] and then with peroxidase-conjugated secondary antibodies (Dako; 1:1000 diluted), and developed with Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific) or ImmunoStar LD Chemiluminescence Reagent (Wako Pure Chemical Industries) using a Sayaca-Imager (DRC Co., Tokyo, Japan).

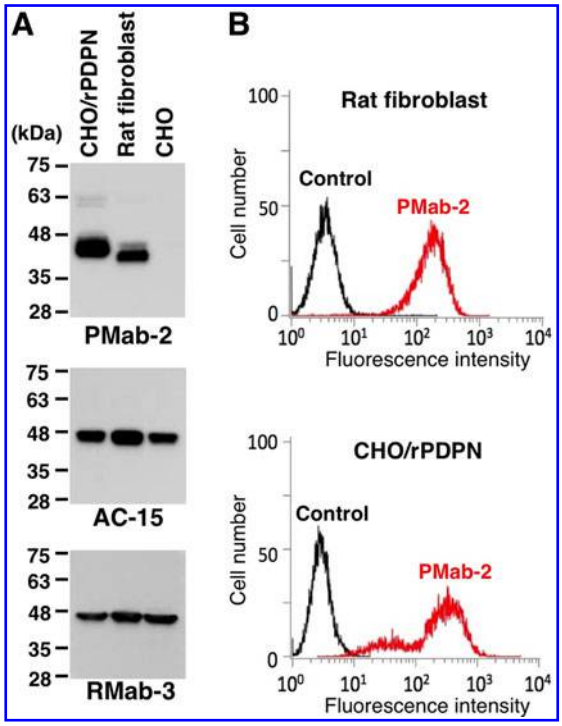

FIG. 2. Endogenous rPDPN is detected by PMab-2. (**A**) Western blot analysis. AC-15 (anti-β-actin) and RcMAb-1 (anti-IDH1) were used as internal control. Total cell lysate were electrophoresed on 5–20% polyacrylamide gels and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 µg/mL of MAbs, and then with peroxidase-conjugated secondary antibodies; the membrane was detected using a Sayaca-Imager. (**B**) Flow cytometry. Cells were harvested by brief exposure to 0.25% Trypsin/1 mM EDTA. After washing with PBS, the cells were treated with PMab-2, followed by treatment with secondary antibodies. Fluorescence data were collected using a Cell Analyzer EC800.

Flow cytometry

Cells were harvested by brief exposure to 0.25% Trypsin/1 mM EDTA (Nacalai Tesque). After washing with PBS, the cells were treated with primary MAbs (1 µg/mL) for 30 min at 4°C, followed by treatment with Oregon green-conjugated anti-mouse IgG or anti-rat IgG (Thermo Fisher Scientific). Fluorescence data were collected using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

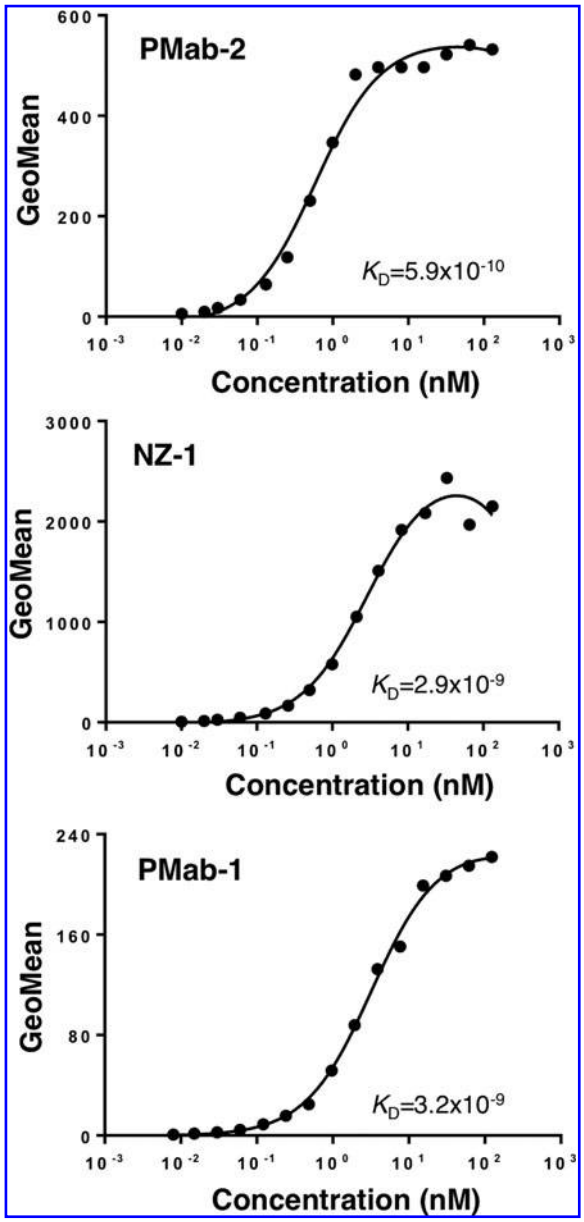

FIG. 3. Determination of binding affinity using flow cytometry. CHO = rPDPN, CHO = hPDPN, and CHO = mPDPN (2 × 10s cells) were re-suspended at 100 µL of serially diluted PMab-2, NZ-1, and PMab-1 (0.02–100 µg/mL, respectively. Fluorescence data were collected using a Cell Analyzer EC800. K_D was obtained by fitting binding isotherms using built-in one-site binding models in Prism software.

Determination of binding affinity by flow cytometry

Binding affinity was determined as described previously.[44] Briefly, cells (2×10^5 cells) were resuspended at 100 μL of serially diluted antibodies (0.02–100 μg/mL), followed by secondary antibodies (Thermo Fisher Scientific). Fluorescence data were collected using a Cell Analyzer EC800. The dissociation constants (K_D) were obtained by fitting the binding isotherms using the built-in one-site binding models in GraphPad PRISM 6 (GraphPad Software, La Jolla, CA).

Immunohistochemical analyses

Four-μm-thick histologic sections were deparaffinized in xylene and rehydrated. Sections were incubated with PMab-2 for 1 h at room temperature followed by treatment with Envision+ kit (Dako). Color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako) for 5 min, and then the sections were counterstained with hematoxylin (Wako Pure Chemical Industries).

Results

Production of monoclonal antibody against rPDPN

We first immunized mice with a synthetic peptide of rat PDPN; then ELISA screening was performed. Among 23 ELISA-positive wells, 11 wells reacted with CHO/rPDPN in flow cytometry. Furthermore, three wells recognized a specific band in Western blot analysis. After limiting dilution, clone PMab-2 (mouse IgG_1, kappa) was established. PMab-2 detected about 40 kDa band of rPDPN-transfected CHO cells (CHO/rPDPN), not with hPDPN-transfected CHO cells (CHO/hPDPN), mPDPN-transfected CHO cells (CHO/mPDPN), and CHO cells in Western blot analysis (Fig. 1A). In contrast, NZ-1 and PMab-1 detected about 40 kDa bands of CHO/hPDPN and CHO/mPDPN, respectively, indicating that three MAbs are species-specific. PMab-2 reacted with CHO/rPDPN, not with CHO/hPDPN, CHO/mPDPN, and CHO cells in flow cytometry (Fig. 1B). NZ-1 and PMab-1 specifically reacted with CHO/hPDPN and CHO/mPDPN, respectively. Taken together, PMab-2 is a specific MAb against rPDPN, and is very useful in

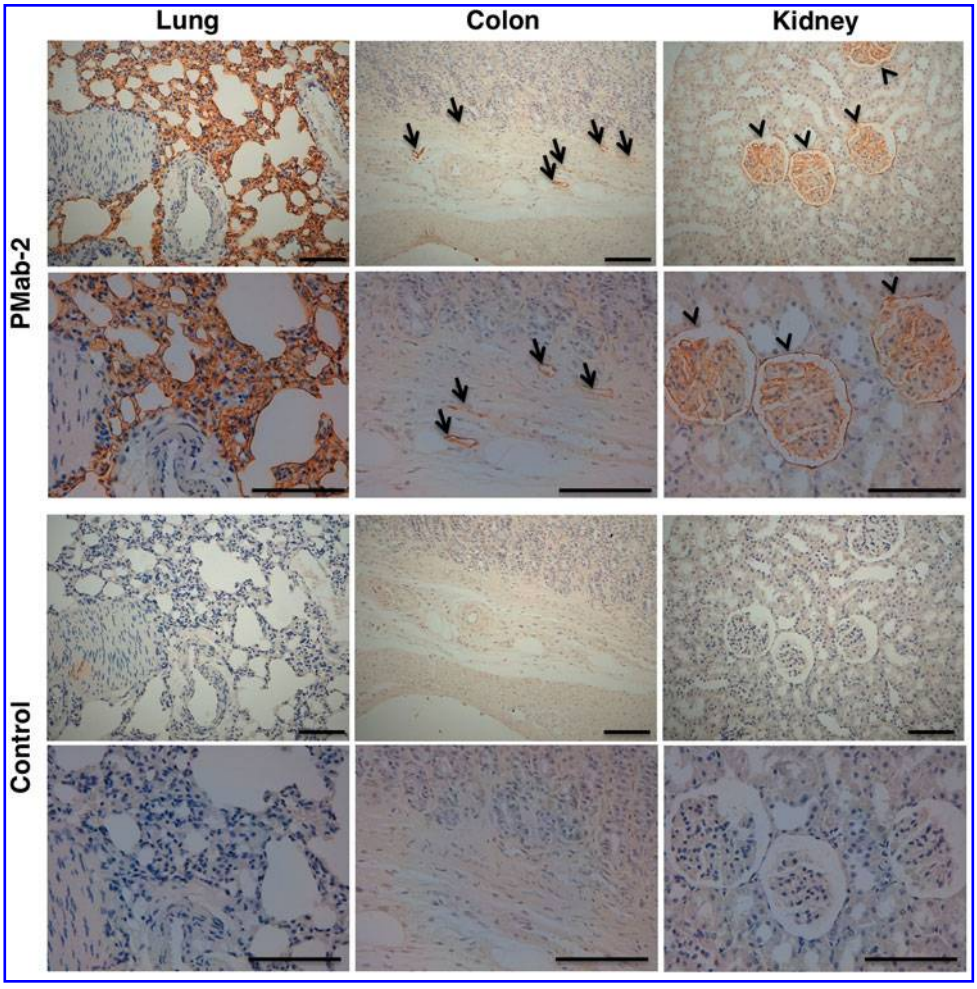

FIG. 4. Immunohistochemical analysis by PMab-2. Sections of rat lung, colon, and kidney were incubated with 0.5 μg/mL of PMab-2 or control PBS, followed by Envision+ kit, and color was developed using DAB and counterstained with hematoxylin. Arrows, lymphatic vessels; arrowheads, glomerulus. Scale bar, 100 μm.

Western blot and flow cytometric analyses. We next investigated whether PMab-2 can detect endogenous rPDPN. Because PDPN has been reported to be expressed in mouse or human fibroblast cells, we checked the PMab-2 reactivity against rat dermal fibroblast. As shown in Figure 2A, PMab-2 detected not only exogenous rPDPN of CHO/rPDPN but also an endogenous one, which is expressed in rat dermal fibroblast. PMab-2 also reacted with rat fibroblast in flow cytometry (Fig. 2B). These results indicate that PMab-2 is useful for detecting endogenous rPDPN. We further performed a kinetic analysis of the interaction of PMab-2 with CHO/rPDPN using flow cytometry. As shown in Figure 3, K_D of PMab-2 was determined to be 5.9×10^{-10} M using CHO/rPDPN. In contrast, K_D of NZ-1 was determined to be 2.9×10^{-9} M using CHO/hPDPN and K_D of PMab-1 was determined to be 3.2×10^{-9} M using CHO/mPDPN, indicating that the binding affinity of PMab-2 is much higher than that of NZ-1 and PMab-1.

Immunohistochemical analysis using PMab-2

We next investigated whether PMab-2 can detect rPDPN of normal tissues in immunohistochemistry. PMab-2 reacted with type I alveolar cells of lung, lymphatic endothelial cells of colon, podocytes, and Bowman's capsule epithelial cells of kidney (Fig. 4). Because PMab-2 showed high affinity against rPDPN, we next checked the dose dependency of PMab-2 in immunohistochemistry using three different tissues. As shown in Figure 5, PMab-2 detected rPDPN of lung type I alveolar cells at 0.001 μg/mL, rPDPN of colon lymphatic endothelial cells at 0.005 μg/mL, and rPDPN of kidney podocytes at 0.01 μg/mL, indicating that PMab-2 is very sensitive against rPDPN.

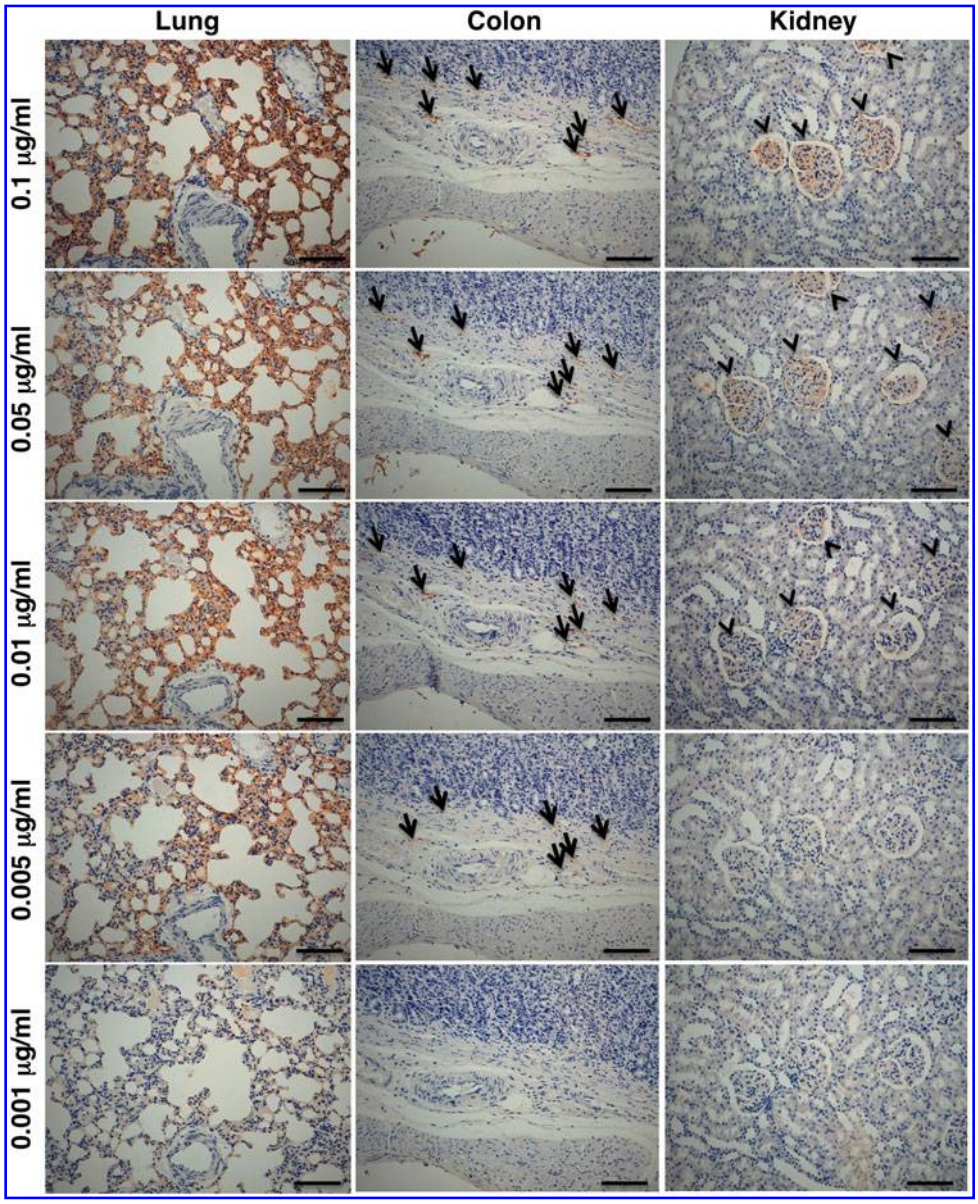

FIG. 5. Immunohistochemical analysis by PMab-2 in a dose-dependent manner. Sections of rat lung, colon, and kidney were incubated with 0.001-0.1 μg/mL of PMab-2, followed by Envision+ kit, and color was developed using DAB and counterstained with hematoxylin. Arrows, lymphatic vessels; arrowheads, glomerulus. Scale bar, 100 μm.

Discussion

In this study, we immunized mice with a synthetic peptide (rpp3851), which is included in platelet aggregation-stimulating (PLAG) domain of rPDPN. We previously produced an anti-hPDPN MAb (NZ-1)[17] and an anti-mPDPN MAb (PMab-1)[39] by immunizing rats with PLAG domain of hPDPN and mPDPN, respectively. Because these MAbs are not only very sensitive and specific but also very useful for almost all experiments including flow cytometry, Western blot, immunoprecipitation, immunocytochemistry, and immunohistochemistry, MAbs against PLAG domain of rPDPN was thought to be advantageous. As expected, established PMab-2 is a specific MAb against rPDPN, and is very useful in Western blot and flow cytometric analyses. Furthermore, PMab-2 detected not only exogenous rPDPN but also an endogenous one of dermal fibroblast in both Western blot and flow cytometry, which leads to the advantage in immunohistochemistry. By kinetic analysis of the interaction of PMab-2 using flow cytometry, K_D of PMab-2 was determined to be 5.9×10^{-10} M, indicating that the binding affinity of PMab-2 is extraordinarily high. In rat normal tissues, rPDPN was reported to be highly expressed in lymphatic endothelial cells, lung type I alveolar cells, or kidney podocytes.[1] Expectedly, PMab-2 reacted with type I alveolar cells of lung, lymphatic endothelial cells of colon, and podocytes of kidney in immunohistochemistry. A previous study showed that PMab-1 detects mPDPN in immunohistochemistry; however, antigen retrieval procedure was necessary.[39] Furthermore, NZ-1 detects hPDPN in immunohistochemistry without antigen retrieval; however, it does not react with type I alveolar cells of lung and podocytes of kidney.[20] In contrast, PMab-2 does not need antigen retrieval and detects normal rPDPN, which is expressed in various tissues. Surprisingly, PMab-2 detected rPDPN of lung type I alveolar cells at a low concentration of 1 ng/mL, indicating that PMab-2 is extraordinarily sensitive against rPDPN.

Taken together, PMab-2 is very useful for all experiments such as flow cytometry, Western blot, and immunohistochemistry. Furthermore, the epitope of PMab-2 is PLAG domain of rPDPN, indicating that PMab-2 is advantageous for investigating the platelet aggregation activity of rPDPN or rPDPN-CLEC-2 interaction.

Acknowledgments

We thank Takuro Nakamura, Noriko Saidoh, Hazuki Kanno, 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 JSPS KAKENHI Grant Number 26440019 (M.K.K.) and Grant Number 25462242 (Y.K.).

Author Disclosure Statement

The authors have no financial interests to disclose.

References

1. 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.
2. Gandarillas A, Scholl FG, Benito N, Gamallo C, and Quintanilla M: Induction of PA2.26, a cell-surface antigen expressed by active fibroblasts, in mouse epidermal keratinocytes during carcinogenesis. Mol Carcinog 1997;20: 10–18.
3. 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.
4. 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.
5. 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.
6. 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.
7. Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, and Kerjaschki D: Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. Am J Pathol 1999;154:385–394.
8. 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.
9. Peters A, Pitcher LA, Sullivan JM, Mitsdoerffer M, Acton SE, Franz B, Wucherpfennig K, Turley S, Carroll MC, Sobel RA, Bettelli E, and Kuchroo VK: Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation. Immunity 2011;35:986–996.
10. 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.
11. 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.
12. Ochoa-Alvarez JA, Krishnan H, Pastorino JG, Nevel E, Kephart D, Lee JJ, Retzbach EP, Shen Y, Fatahzadeh M,

Baredes S, Kalyoussef E, Honma M, Adelson ME, Kaneko MK, Kato Y, Young MA, Deluca-Rapone L, Shienbaum AJ, Yin K, Jensen LD, and Goldberg GS: Antibody and lectin target podoplanin to inhibit oral squamous carcinoma cell migration and viability by distinct mechanisms. Oncotarget 2015;6:9045–9060.
13. 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.
14. 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.
15. 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.
16. 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.
17. 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.
18. 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 2006;111:563–568.
19. 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 2006;111:483–488.
20. 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.
21. 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.
22. 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.
23. 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.
24. 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.
25. 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.
26. 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.
27. 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.
28. 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.
29. Hatakeyama K, Kaneko MK, Kato Y, Ishikawa T, Nishihira K, Tsujimoto Y, Shibata Y, Ozaki Y, and Asada Y: Podoplanin expression in advanced atherosclerotic lesions of human aortas. Thromb Res 2012;129:e70–76.
30. 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.
31. 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.
32. 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.
33. Kato Y, and Kaneko MK: A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. Sci Rep 2014;4:5924.
34. 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.
35. Kaneko MK, Oki H, Ogasawara S, Takagi M, and Kato Y: Anti-podoplanin monoclonal antibody LpMab-7 detects metastatic legions of osteosarcoma. Monoclon Antib Immunodiagn Immunother 2015;34:154–161.
36. 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.
37. 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 a monoclonal antibody LpMab-10 recognizing non-glycosylated PLAG1/2 domain including Thr34 of human podoplanin. Monoclon Antib Immunodiagn Immunother 2015;34:318–326.
38. Kaneko MK, Oki H, Hozumi Y, Liu X, Ogasawara S, Takagi M, Goto K, and Kato Y: Monoclonal antibody LpMab-9 recognizes O-glycosylated N-terminus of human podoplanin. Monoclon Antib Immunodiagn Immunother 2015;34:310–317.

39. 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 metastasis via ADCC and CDC rather than via its neutralizing activity. Oncotarget, 2015;6:36003–36018.
40. Watanabe M, Okochi E, Sugimoto Y, and Tsuruo T: Identification of a platelet-aggregating factor of murine colon adenocarcinoma 26: Mr 44,000 membrane protein as determined by monoclonal antibodies. Cancer Res 1988;48:6411–6416.
41. Kaji C, Tsujimoto Y, Kato Kaneko M, Kato Y, and Sawa Y: Immunohistochemical examination of novel rat monoclonal antibodies against mouse and human podoplanin. Acta Histochem Cytochem 2012;45:227–237.
42. Farr A, Nelson A, and Hosier S: Characterization of an antigenic determinant preferentially expressed by type I epithelial cells in the murine thymus. J Histochem Cytochem 1992;40: 651–664.
43. Kato Y: Specific monoclonal antibodies against IDH1/2 mutations as diagnostic tools for gliomas. Brain Tumor Pathol 2015;32:3–11.
44. Stein RA, Wilkinson JC, Guyer CA, and Staros JV: An analytical approach to the measurement of equilibrium binding constants: application to EGF binding to EGF receptors in intact cells measured by flow cytometry. Biochemistry 2001;40:6142–6154.

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: July 2, 2015
Accepted: September 15, 2015