Epitope Mapping of Anti-Bear Podoplanin Monoclonal Antibody PMab-247

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Podoplanin (PDPN)/T1alpha is a type I transmembrane sialoglycoprotein, which is expressed on podocytes of the kidneys and type I alveolar cells of the lungs. PDPN is also known as Aggrus, a platelet aggregation-inducing factor, which comprises three platelet aggregation-stimulating (PLAG) domains (PLAG1, PLAG2, and PLAG3) in the N-terminus and PLAG-like domains (PLDs) in the middle of the PDPN protein. We have previously established a mouse anti-bear PDPN (bPDPN) monoclonal antibody (mAb) clone, PMab-247 using the Cell-Based Immunization and Screening (CBIS) method. PMab-247 is very useful in flow cytometry, Western blotting, and immunohistochemical (IHC) analyses; however, the binding epitope of PMab-247 has not been elucidated. In this study, we aimed to investigate the epitope of PMab-247 using enzyme-linked immunosorbent assay and IHC analyses. The results revealed that the critical epitopes of PMab-247 are Asp76, Arg78, Glu80, and Arg82 of bPDPN. The Glu80 and Arg82 are included in PLD of bPDPN. The findings of our study can be applied to the production of more functional anti-bPDPN mAbs.

Keywords: podoplanin, PDPN, PMab-247, epitope mapping

Introduction

PODOPLANIN (PDPN)/T1ALPHA/AGGRUS is a type I transmembrane sialoglycoprotein that is expressed in normal tissues, such as renal corpuscles, type I alveolar cells of lung, and lymphatic endothelial cells of every organ.[1] PDPN induces platelet aggregation by binding to C-type lectin-like receptor-2 (CLEC-2).[2] The interaction between PDPN on lymphatic endothelial cells and CLEC-2 on platelets facilitates the separation of embryonic blood/lymphatic vessels.[3] Human PDPN is overexpressed in several malignant tumors, such as malignant mesotheliomas,[4,5] oral squamous cell carcinomas,[6] malignant brain tumors,[7] and lung cancers.[8] PDPN expression has also been associated with malignant progression and cancer metastasis.[9]

In our previous study, we used the Cell-Based Immunization and Screening (CBIS) method to develop sensitive and specific monoclonal antibodies (mAbs) against bear

PDPN (bPDPN) to facilitate the immunohistochemical (IHC) analysis of paraffin-embedded tissue sections.[10] Flow cytometry revealed that PMab-247 (mouse IgG$_1$, kappa), one of the established clones, recognized CHO/bPDPN but did not react with CHO-K1. Western blotting demonstrated that PMab-247 could detect bPDPN as a 48-kDa band in CHO/bPDPN cells. IHC analyses revealed that PMab-247 strongly stained pulmonary type I alveolar cells, lymphatic endothelial cells, and podocytes of the kidney.[11] This study aimed to determine the binding epitope of PMab-247 to bPDPN using enzyme-linked immunosorbent assay (ELISA) and IHC analyses.

Materials and Methods

Enzyme-linked immunosorbent assay

Synthesized bPDPN peptides using PEPScreen (Sigma-Aldrich Corp., St. Louis, MO) were immobilized on Nunc

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TABLE 1. DETERMINATION OF PMAB-247 EPITOPE
BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Peptide	Sequence	PMab-247
18–37	DPAEGASTVRPEDDITPGVE	−
28–47	PEDDITPGVEDNMVTLGVED	−
38–57	DNMVTLGVEDNMVTAGTSEE	−
48–67	NMVTAGTSEEPYESGLTAAV	−
58–77	PYESGLTAAVPINREGVTDL	−
68–87	PINREGVTDLRLEDRTTEST	+++
78–97	RLEDRTTESTVRAEEESQST	−
88–107	VRAEEESQSTTTLNVVTSHP	−
98–117	TTLNVVTSHPTEKVGEDTET	−
108–127	TEKVGEDTETTVEKDGLATV	−

+++, OD655 ≥ 0.6; −, OD655 < 0.1.

Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham, MA) at 10 μg/mL for 30 minutes at 37°C. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), the plates were incubated with purified PMab-247 (1 μg/mL), followed by a peroxidase-conjugated anti-mouse IgG (dilution of 1:2000; Agilent Technologies Inc., Santa Clara, CA). Enzymatic reactions were performed using 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA). These reactions were performed at 37°C using a total sample volume of 50–100 μL.

Immunohistochemical analyses

Malayan Sun Bear (*Helarctos malayanus*) tissues were collected after autopsy at Hokkaido University, fixed in 10%

TABLE 2. DETERMINATION OF PMAB-247 EPITOPE
BY ENZYME-LINKED IMMUNOSORBENT ASSAY
USING POINT MUTANTS

Mutation	Sequence	PMab-247
P68A	AINREGVTDLRLEDRTTEST	+++
I69A	PANREGVTDLRLEDRTTEST	+++
N70A	PIAREGVTDLRLEDRTTEST	+++
R71A	PINAEGVTDLRLEDRTTEST	+++
E72A	PINRAGVTDLRLEDRTTEST	+++
G73A	PINREAVTDLRLEDRTTEST	+++
V74A	PINREGATDLRLEDRTTEST	+++
T75A	PINREGVADLRLEDRTTEST	+++
D76A	PINREGVTALRLEDRTTEST	−
L77A	PINREGVTDARLEDRTTEST	+++
R78A	PINREGVTDLALEDRTTEST	−
L79A	PINREGVTDLRAEDRTTEST	+++
E80A	PINREGVTDLRLADRTTEST	−
D81A	PINREGVTDLRLEARTTEST	+++
R82A	PINREGVTDLRLEDATTEST	+
T83A	PINREGVTDLRLEDRATEST	+++
T84A	PINREGVTDLRLEDRTAEST	+++
E85A	PINREGVTDLRLEDRTTAST	+++
S86A	PINREGVTDLRLEDRTTEAT	+++
T87A	PINREGVTDLRLEDRTTESA	+++

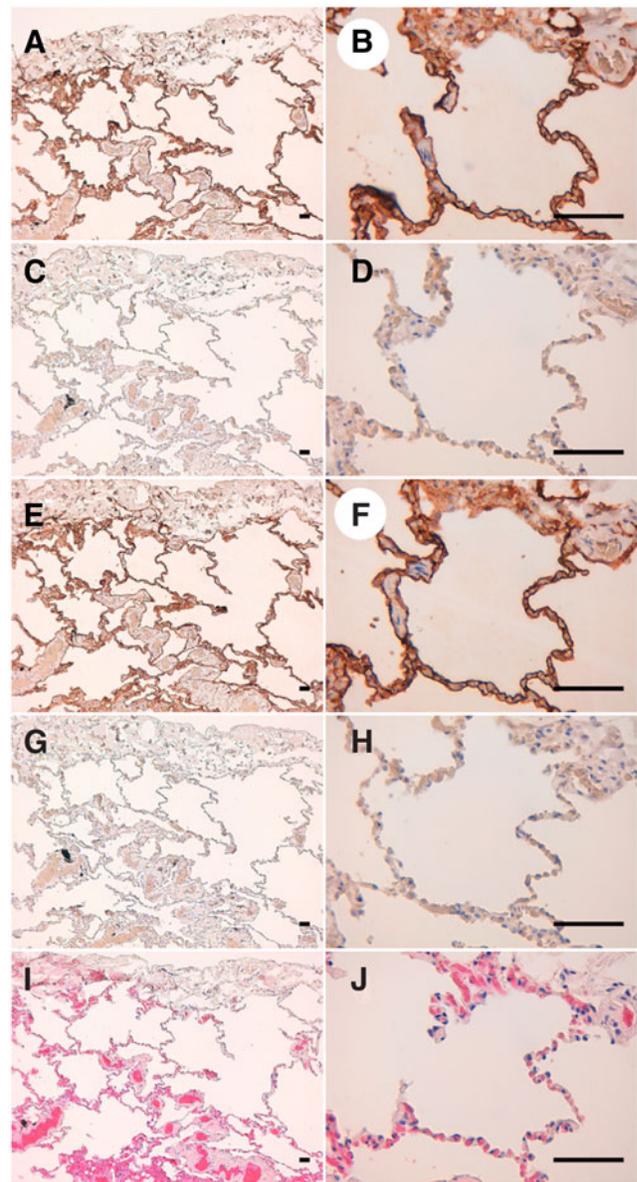+++, OD655 ≥ 0.6; +, 0.1 ≤ OD655 < 0.4; −, OD655 < 0.1.

FIG. 1. IHC analyses of bear lungs using PMab-247 and point mutants of bPDPN. Histological sections of the bear lungs were directly autoclaved in citrate buffer for 20 minutes. After blocking with SuperBlock T20 (PBS) Blocking Buffer, the sections were incubated with **(A, B)** PMab-247 (5 μg/mL), **(C, D)** PMab-247 (5 μg/mL) + P68A (50 μg/mL), **(E, F)** PMab-247 (5 μg/mL) + R78A (50 μg/mL), or **(G, H)** control (blocking buffer), followed by treatment with the Envision + Kit. **(I, J)** HE staining. Scale bar: 100 μm. bPDPN, bear podoplanin; HE, hematoxylin and eosin; IHCs, immunohistochemical.

neutral-buffered formalin, and routinely processed to make paraffin-embedded tissue sections. Histological sections (4-μm thick) were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. These tissue sections were then blocked with the SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), incubated with PMab-247 (5 μg/mL) or PMab-247 (5 μg/mL) + peptides (50 μg/mL) for 1 hour at room
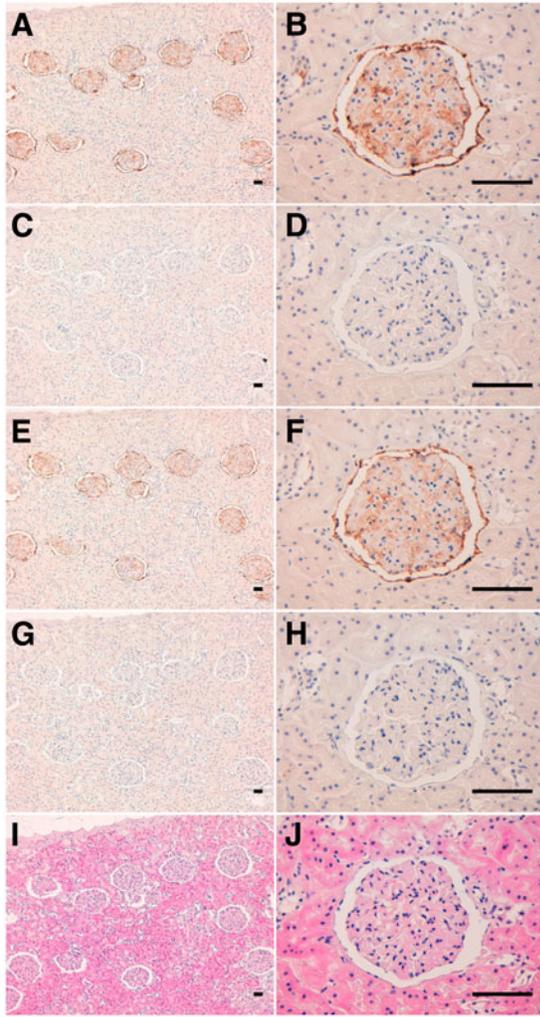
FIG. 2. IHC analyses of bear kidneys using PMab-247 and point mutants of bPDPN. Histological sections of the bear kidneys were directly autoclaved in citrate buffer for 20 minutes. After blocking with SuperBlock T20 (PBS) Blocking Buffer, the sections were incubated with **(A, B)** PMab-247 (5 μg/mL), **(C, D)** PMab-247 (5 μg/mL) + P68A (50 μg/mL), **(E, F)** PMab-247 (5 μg/mL) + R78A (50 μg/mL), or **(G, H)** control (blocking buffer), followed by treatment with the Envision + Kit. **(I, J)** HE staining. Scale bar: 100 μm.

temperature, and treated using an Envision + Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed with 3,3′-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Results and Discussion

We first synthesized a series of bPDPN peptides, which are summarized in Table 1. Using ELISA, PMab-247 detected the 68–87 peptide corresponding to the 68th–87th amino acids (AAs) of bPDPN. We then synthesized point mutants of 68–87 peptides (Table 2). Using ELISA, PMab-247 strongly detected P68A, I69A, N70A, R71A, E72A, G73A, V74A, T75A, L77A, L79A, D81A, T83A, T84A, E85A, S86A, and T87A, and weakly detected R82A. Conversely, it did not detect D76A, R78A, and E80A, indicating that Asp76, Arg78, Glu80, and Arg82 are the critical epitopes of PMab-247.

We then performed a blocking assay using IHC to confirm the PMab-247 epitope. PMab-247 reacted with lung type I alveolar cells (Fig. 1A, B). These reactions were completely neutralized by P68A (Fig. 1C, D). Conversely, R78A did not block these reactions of PMab-247 (Fig. 1E, F). Likewise, D76A, E80A, and R82A did not inhibit the PMab-247 reaction (Supplementary Fig. S1). Negative control (blocking buffer) did not show any staining (Fig. 1G, H). As shown in Figure 2A and B, PMab-247 reacted with the podocytes and Bowman's capsules of the kidneys. The reactions of PMab-247 in the kidneys were completely neutralized by P68A (Fig. 2C, D) although R78A did not block these reactions of PMab-247 (Fig. 2E, F). Negative control (blocking buffer) did not show any staining (Fig. 2G, H). These IHC results indicate that Asp76, Arg78, Glu80, and Arg82 of bPDPN are essential for PMab-247 binding to bPDPN.

In summary, the critical epitopes of PMab-247 are Asp76, Arg78, Glu80, and Arg82 of bPDPN. Among these AAs, Glu80 and Arg82 are included in PLAG-like domains of bPDPN, which is important for PDPN/CLEC-2 interaction (Fig. 3). These findings can be applied to the production of more functional anti-bPDPN mAbs.
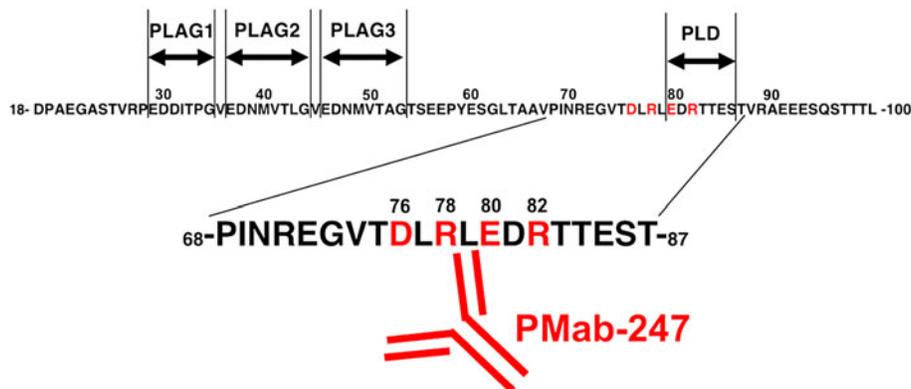

FIG. 3. Schematic illustration of the epitope recognized by PMab-247. PLAG and PLD. Red AAs indicate the critical epitope of PMab-247. AAs, amino acids; PLAG, platelet aggregation-stimulating; PLD, PLAG-like domain.

Supplementary Material

Supplementary Figure S1

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. 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.
3. 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.
4. Abe S, Kaneko MK, Tsuchihashi Y, Izumi T, Ogasawara S, Okada N, Sato C, Tobiume M, Otsuka K, Miyamoto L, Tsuchiya K, Kawazoe K, Kato Y, and Nishioka Y: Antitumor effect of novel anti-podoplanin antibody NZ-12 against malignant pleural mesothelioma in an orthotopic xenograft model. Cancer Sci 2016;107:1198–1205.
5. 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.
6. 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.
7. 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.
8. 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.
9. 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.
10. Furusawa Y, Takei J, Sayama Y, Yamada S, Kaneko MK, and Kato Y: Development of an anti-bear podoplanin monoclonal antibody PMab-247 for immunohistochemical analysis. Biochem Biophys Rep 2019;18:100644.
11. Takei J, Furusawa Y, Yamada S, Nakamura T, Sayama Y, Sano M, Konnai S, Kobayashi A, Harada H, Kaneko MK, and Kato Y: PMab-247 detects bear podoplanin in immunohistochemical analysis. Monoclon Antib Immunodiagn Immunother 2019;38:171–174.

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