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Epitope Mapping of Monoclonal Antibody PMab-48 Against Dog Podoplanin

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Podoplanin (PDPN), a type I transmembrane sialoglycoprotein, is expressed on normal renal podocytes, pulmonary type I alveolar cells, and lymphatic endothelial cells. Increased expression of PDPN in cancers is associated with poor prognosis and hematogenous metastasis through interactions with C-type lectin-like receptor 2 (CLEC-2) on platelets. We previously reported a novel PMab-48 antibody, which is an anti-dog PDPN (dPDPN) monoclonal antibody (mAb) recognizing PDPN expressed in lymphatic endothelial cells. However, the binding epitope of PMab-48 is yet to be clarified. In this study, an enzyme-linked immunosorbent assay and flow cytometry were used to investigate epitopes of PMab-48. The results revealed that the critical epitope of PMab-48 comprises Asp29, Asp30, Ile31, Ile32, and Pro33 of dPDPN.

Keywords: dog podoplanin, dPDPN, monoclonal antibody, epitope mapping

Introduction

PODOPLANIN (PDPN) is expressed in normal tissues such as renal podocytes, pulmonary type I alveolar cells, lymphatic endothelial cells, chondrocytes, myofibroblasts, and mesothelial cells.[1] However, higher expression of PDPN has been observed in different tumor types, including squamous cell carcinoma,[2] melanoma,[3] glioblastoma,[4] and mesothelioma.[5] Recent clinical studies have provided evidence of associations between increased PDPN expression, poor prognosis, and cancer metastasis,[6] indicating that the establishment and production of anti-PDPN monoclonal antibodies (mAbs) are necessary for developing novel therapeutic strategies against cancer development and progression to metastasis.[7]

Dog podoplanin (dPDPN) was first reported as gp40.[8] Recently, we developed two mAbs, PMab-38[2,3,9] and PMab-48,[10] both of which specifically recognize dPDPN. Tyr67 and Glu68 were determined to be the critical features of the epitope of PMab-38.[11] Using point mutants of dPDPN, the present study aimed to investigate the epitope of PMab-48 by enzyme-linked immunosorbent assay (ELISA) and flow cytometry.

Materials and Methods

Cell lines

CHO-K1 cells were transfected with the dPDPN-MAP tag plasmid using Lipofectamine LTX (Thermo Fisher Scientific, Inc., Waltham, MA).[9] CHO/dPDPN cells were cultured in an RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (Nacalai Tesque, Inc.), and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Enzyme-linked immunosorbent assay

Synthesized dPDPN peptides (PEPScreen; Sigma-Aldrich Corp., St. Louis, MO) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at 5 μg/mL for 30 minutes. After blocking with SuperBlock T20 (phosphate-buffered saline [PBS]) Blocking Buffer (Thermo Fisher Scientific, Inc.), the plates were incubated with purified PMab-48 (10 μg/mL), followed by a 1:2000 dilution of peroxidase-conjugated anti-mouse IgG (Agilent Technologies, Inc., Santa

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Clara, CA). The enzymatic reaction was conducted 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 with a total sample volume of 50–100 μL.

Flow cytometry

Cells were harvested after brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin in PBS, the cells were treated with PMab-48 (10 μg/mL) or PMab-48 (10 μg/mL) plus peptides (10 μg/mL) for 30 minutes at 4°C, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were acquired using the cell analyzer SA3800 (Sony Corp., Tokyo, Japan).

Results

We previously developed a novel PMab-48 mAb that exhibits high specificity and sensitivity against dPDPN and is highly suitable for detection of lymphatic endothelial cells in immunohistochemical analysis.[10] PMab-48 can be used to detect dPDPN expressed by squamous cell carcinomas.[10] These findings suggest that PMab-48 epitope mapping could be a promising target for PDPN-based diagnosis and cancer therapy.

In this study, we synthesized a series of point mutants of dPDPN peptides from the 26th to the 40th amino acid (Table 1). Using ELISA, PMab-48 detected V26A, R27A, P28A, G34A, V35A, E36A, D37A, S38A, V39A, and V40A; in contrast, PMab-48 did not react with D29A, D30A, I31A, I32A, or P33A, indicating that the DDIIP sequence is a critical epitope of PMab-48.

Next, we performed a blocking assay using flow cytometry. PMab-48 reacted with the CHO/dPDPN cell line

Table 1. Determination of PMab-48 Epitope by Enzyme-Linked Immunosorbent Assay

Mutation	*Sequence*	*PMab-48*
Wild type	VRPDDIIPGVEDSVV	+++
V26A	ARPDDIIPGVEDSVV	++
R27A	VAPDDIIPGVEDSVV	+
P28A	VRADDIIPGVEDSVV	+++
D29A	VRPADIIPGVEDSVV	—
D30A	VRPDAIIPGVEDSVV	—
I31A	VRPDDAIPGVEDSVV	—
I32A	VRPDDIAPGVEDSVV	—
P33A	VRPDDIIAGVEDSVV	—
G34A	VRPDDIIPAVEDSVV	++
V35A	VRPDDIIPGAEDSVV	+++
E36A	VRPDDIIPGVADSVV	+++
D37A	VRPDDIIPGVEASVV	+++
S38A	VRPDDIIPGVEDAVV	+
V39A	VRPDDIIPGVEDSAV	+++
V40A	VRPDDIIPGVEDSVA	+++

+++, OD655 ≧ 0.6; ++, 0.4 ≦ OD655 < 0.6; +, 0.2 ≦ OD655 < 0.4; —, OD655 < 0.2.

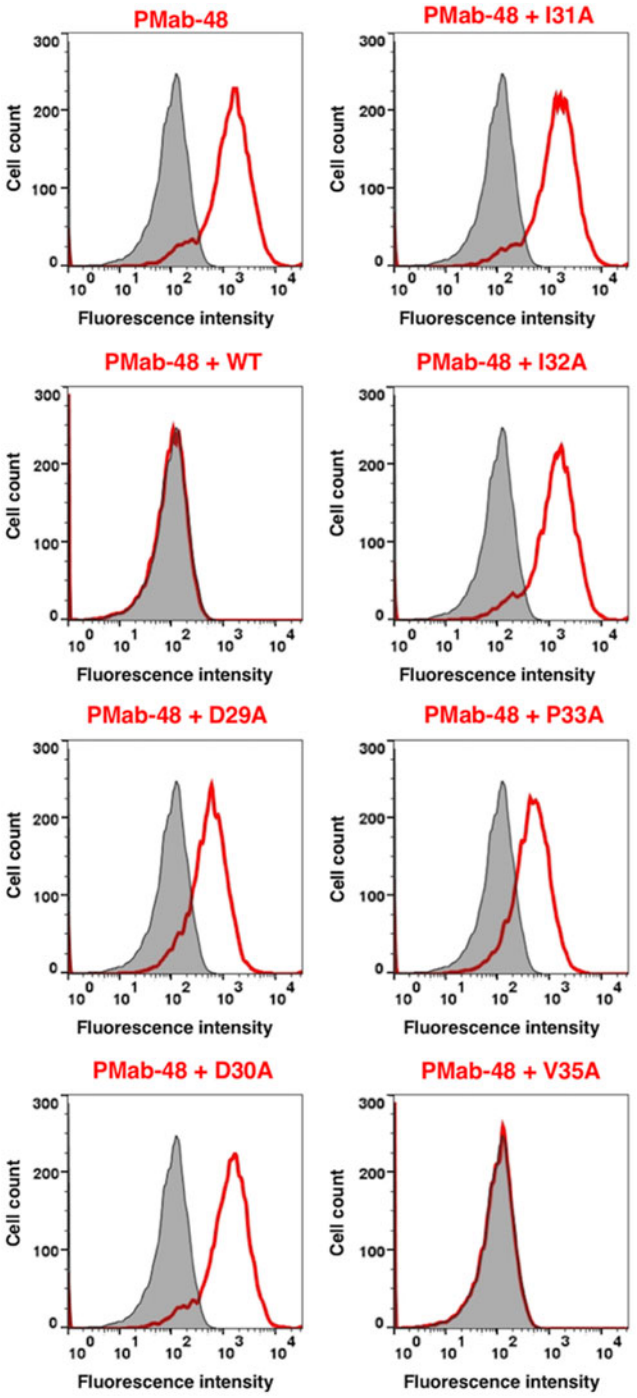

FIG. 1. Flow cytometry using PMab-48 and point mutants of dPDPN. PMab-48 or PMab-48 + peptides (WT, D29A, D30A, I31A, I32A, P33A, or V35A) were treated with CHO/dPDPN cells for 30 minutes at 4°C, followed by addition of secondary antibodies. dPDPN, dog podoplanin; WT, wild type; gray peak, negative control.

(Fig. 1). This reaction was completely neutralized by the wild-type peptide (VRPDDIIPGVEDSVV) and V35A; in contrast, D29A, D30A, I31A, I32A, and P33A did not block the reaction of PMab-48 with CHO/dPDPN, indicating that the DDIIP sequence is a critical epitope of PMab-48 (Fig. 2).

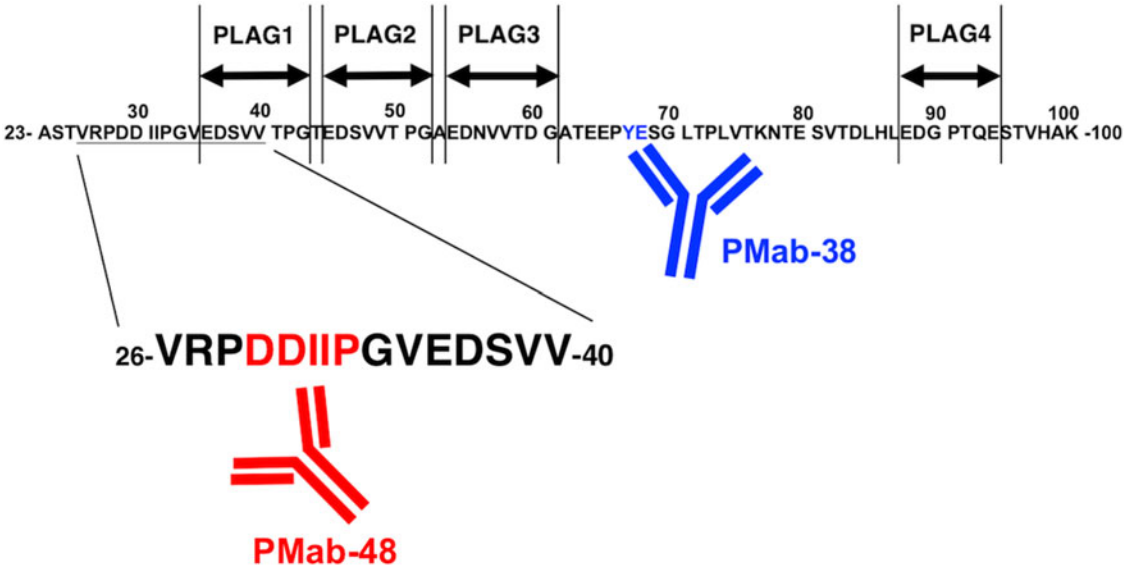

FIG. 2. Schematic illustration of the epitope recognized by PMab-48. PLAG, platelet aggregation-stimulating.

Discussion

Previously, a critical epitope of another anti-dPDPN mAb, PMab-38, was determined to comprise Tyr67 and Glu68,[11] indicating that epitopes of PMab-48 and PMab-38 are different (Fig. 2). PMab-48 reacted with lymphatic endothelial cells in immunohistochemistry,[10] although PMab-38 did not,[9] demonstrating that the N-terminal region is a more adequate epitope for detecting dPDPN of lymphatic endothelial cells probably because N-terminus might not be *O*-glycosylated.[12]

In contrast, PMab-38 showed cancer specificity in immunohistochemistry using canine tissues[2] in the same pattern with anti-human PDPN (hPDPN) cancer-specific mAbs, such as LpMab-2[7,13] and LpMab-23.[6,14,15] We previously showed that Thr55-Leu64 peptide of hPDPN, especially *O*-glycan attached in Thr55 and Ser56 of hPDPN, is a critical epitope of LpMab-2.[7] We further showed that Gly54-Leu64 peptide of hPDPN is a critical epitope of LpMab-23.[15] The epitope of PMab-38 for dPDPN is similar to those of LpMab-2 and LpMab-23 for hPDPN. Therefore, this kind of epitope mapping provides important evidence for clinical application of anti-PDPN mAbs.

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Author Disclosure Statement

No competing financial interests exist.

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