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Detection of Tiger Podoplanin Using the Anti-Cat Podoplanin Monoclonal Antibody PMab-52

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Podoplanin (PDPN) is expressed in type I alveolar cells of lung but not in type II alveolar cells. PDPN is also known as a specific lymphatic endothelial cell marker because PDPN is not expressed in vascular endothelial cells. PDPNs of several animals have been characterized using specific anti-PDPN monoclonal antibodies (mAbs): PMab-1, PMab-2, PMab-32, PMab-38, PMab-44, and PMab-52 for mouse, rat, rabbit, dog, bovine, and cat PDPNs, respectively. In this study, we investigated the possible crossreaction between these anti-PDPN mAbs and tiger PDPN. Flow cytometry and western blot analyses revealed that the anti-cat PDPN mAb PMab-52 (IgM, kappa) reacted with tiger PDPN, which is overexpressed in Chinese hamster ovary-K1 cells. Using immunohistochemical analysis, type I alveolar cells of the tiger lung were strongly detected by PMab-52. These results indicate that PMab-52 may be useful for the detection of tiger PDPN.

Keywords: tiger podoplanin, PDPN, PMab-52

Introduction

Podoplanin (PDPN) is a specific marker for lymphatic endothelial cells and type I alveolar cells of the lung.[1,2] Therefore, anti-PDPN monoclonal antibodies (mAbs) are useful in distinguishing lymphatic from vascular endothelial cells or type I from type II alveolar cells of the lung. We characterized the PDPNs of several animals using specific anti-PDPN mAbs, such as anti-mouse (PMab-1),[3] anti-rat (PMab-2),[4] anti-rabbit (PMab-32),[5] anti-dog [PMab-38[6] and PMab-48[7]], anti-bovine (PMab-44),[8] and anti-cat (PMab-52).[9] Furthermore, we developed many anti-human PDPN mAbs, including anti-pan human PDPN mAbs, such as NZ-1.2,[3] LpMab-7,[10,11] LpMab-10,[12] LpMab-13,[13] and LpMab-17[14]; anti-glycopeptide mAbs (GpMabs), such as LpMab-3,[10] LpMab-9,[10] LpMab-12,[15] LpMab-19,[16] and LpMab-21[17,18]; and cancer-specific mAbs (CasMabs), such as LpMab-2[10,19] and LpMab-23.[20,21]

PDPN of all species is a type I transmembrane sialoglycoprotein that induces platelet aggregation through the C-type lectin-like receptor-2 (CLEC-2) of platelets.[22] It comprises three platelet aggregation-stimulating (PLAG) domains, termed PLAG1–3 (EDxxVTPG sequence).[2] Previously, we have shown that PLAG3 is the most important domain for platelet aggregation by human PDPN.[2,23,24] Recently, PLAG4 (EDxxT sequence) was reported to be the other critical sequence for the PDPN–CLEC-2 interaction. However, PLAG4 may be categorized as ''PLAG-like domain'' because the original definition of PLAG domain is ''EDxxVTPG'' sequence.[25] These functional assays have been performed using their original anti-PDPN mAbs, the binding affinity of which may differ. Therefore, the most critical domain for the PDPN–CLEC-2 interaction remains controversial. Cancer-specific anti-PDPN mAbs should be used for PDPN-targeted therapy[10,19–21,26] because PDPN is expressed in many healthy tissues.

Until now, anti-tiger PDPN mAbs have not been reported. Although tiger tumors have been investigated in several studies, the presence of tiger PDPN in cancers or type I alveolar cells of the lung has not been investigated.[27–33]

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In the present study, we investigated the potential cross-reaction between our anti-PDPN mAbs for many species and tiger PDPN using flow cytometry, western blot, and immunohistochemical analyses.

Materials and Methods

Cell line

Chinese hamster ovary (CHO)-K1 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). CHO-K1 and transfectants were cultured in RPMI medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 25 μg/mL of amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air.

Overexpression of tiger PDPN in CHO-K1 cells

The synthesized DNA of tiger PDPN (accession no.: XM_007083790.2) plus the N-terminal LP tag (NSVTGIRIEDLPTSES), recognized by an anti-LP tag mAb [LpMab-17[14]], was subcloned into a pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Plasmids were transfected using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Inc.). Stable transfectants were selected by limiting dilution and cultivated in a medium containing 0.5 mg/mL of G418 (Nacalai Tesque, Inc.).

Flow cytometry

Cells were harvested after brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). After washing with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, the cells were treated with PMab-52 (1 or 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 Cell Analyzer SA3800 (Sony Corp., Tokyo, Japan).

Western blot analysis

Cell lysates (10 μg) were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.). The proteins were electrophoresed using 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred onto a polyvinylidene difluoride membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), the membrane was initially incubated with 1 μg/mL of PMab-52 or anti-β-actin (clone AC-15; Sigma-Aldrich, Corp., St. Louis, MO) and subsequently with peroxidase-conjugated anti-mouse IgG (Agilent Technologies, Inc., Santa Clara, CA; 1:1000 diluted) and developed using ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) and a Sayaca-Imager (DRC, Co., Ltd., Tokyo, Japan).

Immunohistochemical analyses

Tiger lungs were collected at autopsy in Yamaguchi University, fixed in 10% neutral-buffered formalin, and processed routinely to make paraffin-embedded tissue sections. Histological sections (4-μm thick) of tiger lungs were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), sections were incubated with PMab-52 (0.5 μg/mL) for 1 hour at room temperature and treated using Envision+ Kit (Agilent Technologies, Inc.) for 30 minutes. Next, color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed with Hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

Results and Discussion

We previously developed a mouse anti-cat PDPN mAb (PMab-52: IgM, kappa) using the Cell-Based Immunization

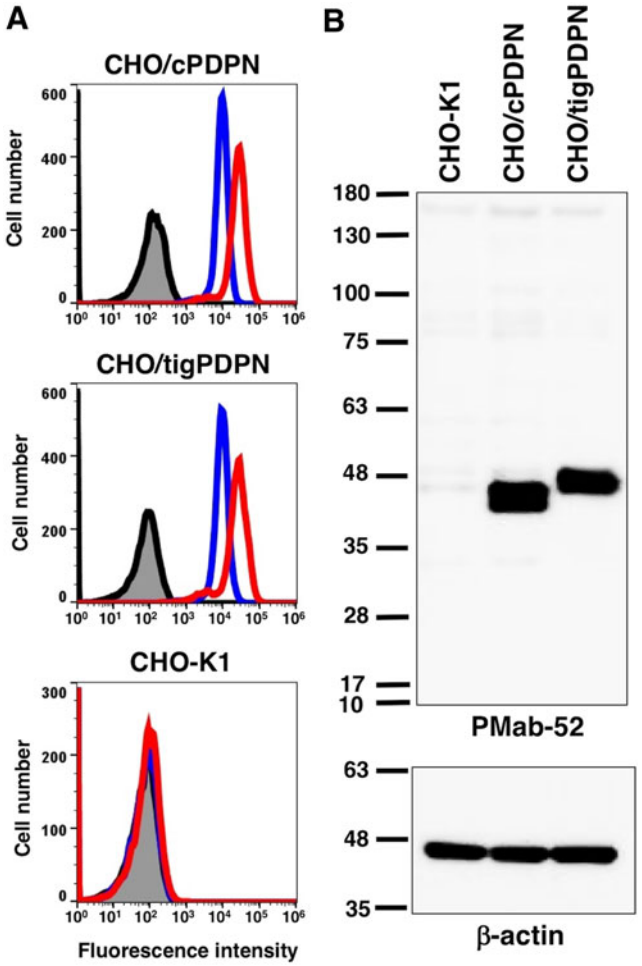

FIG. 1. Detection of tiger PDPN using PMab-52. **(A)** Flow cytometry. CHO/cPDPN and CHO/tigPDPN cells were treated with PMab-52 at a concentration of 1 μg/mL (blue) or 10 μg/mL (red) for 30 minutes at 4°C, followed by the addition of secondary antibodies. **(B)** Western blot with PMab-52. Cell lysates (10 μg) were electrophoresed and transferred onto a PVDF membrane. After blocking, the membrane was initially incubated with 1 μg/ml of PMab-52 or anti-β-actin (AC-15) and subsequently with peroxidase-conjugated anti-mouse IgG. CHO, Chinese hamster ovary; cPDPN, cat PDPN; IgG, immunoglobulin G; PDPN, podoplanin; PVDF, polyvinylidene difluoride; tigPDPN, tiger PDPN.

and Screening method.[9] PMab-52 specifically detects cat PDPN using flow cytometry[9] and successfully recognizes cat PDPN in feline squamous cell carcinomas.[34] Furthermore, a series of deletion or point mutants of cat PDPN were utilized to investigate the binding of PMab-52 epitopes using flow cytometry and western blotting.[35] The PLAG4 of cat PDPN was identified as a critical epitope of PMab-52. Comparison of amino acid sequences revealed that 99% homology is observed between the tiger and cat PDPNs (Supplementary Fig. S1). Therefore, in this study, we investigated the potential reaction between PMab-52 and tiger PDPN.

We initially produced a tiger PDPN-stable transfectant using CHO-K1 cells plus an N-terminal LP tag, recognized by an anti-LP tag mAb (LpMab-17). Flow cytometry revealed that PMab-52 reacted with CHO/cat PDPN (cPDPN) and CHO/tiger PDPN (tigPDPN) in a dose-dependent manner (Fig. 1A). The other anti-PDPN mAbs, namely, anti-mouse (PMab-1),[3] anti-rat (PMab-2),[4] anti-rabbit (PMab-32),[5] anti-dog [PMab-38[6] and PMab-48[7]], and anti-bovine (PMab-44),[8] did not react with CHO/tigPDPN (data not shown). Western blot analysis revealed that PMab-52 also detected specific bands of CHO/cPDPN and CHO/tigPDPN (Fig. 1B). These results indicate that PMab-52 is useful for the detection of tiger PDPN. Subsequently, we investigated the expression of tiger PDPN in the tiger lung. Previously, it was shown that PMab-52 reacted with type I alveolar cells of the feline lung.[9] Similarly, PMab-52 strongly stained type I alveolar cells of the tiger lung in a membrane-staining pattern (Fig. 2A, B).

In conclusion, PMab-52 is useful for the detection of tiger PDPN using flow cytometry, Western blot, and immunohistochemical analyses. Further studies are necessary to show that PMab-52 is able to detect tiger PDPN in other healthy tiger tissues or tiger cancers, such as mesotheliomas.[33]

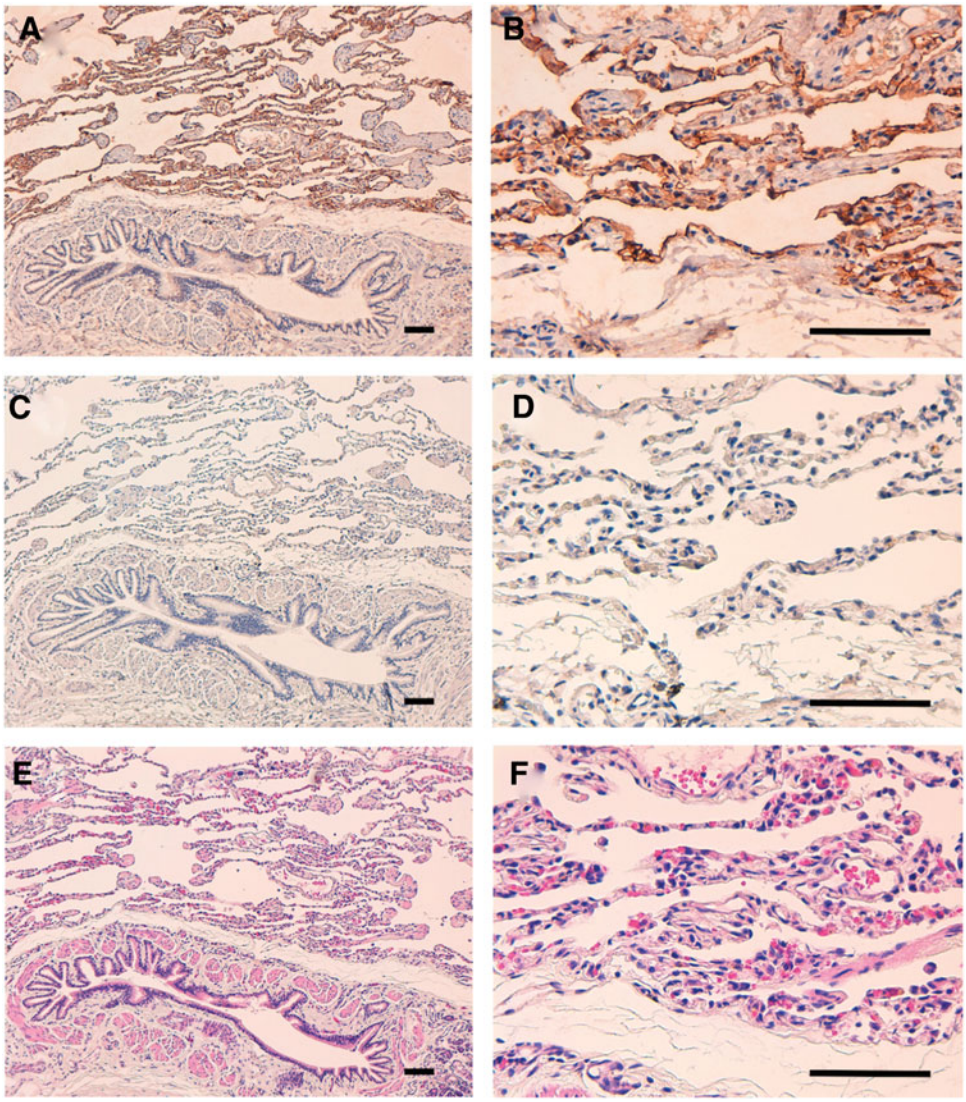

FIG. 2. Immunohistochemical analyses using tiger lung tissues. Histological sections of the tiger lung were directly autoclaved in citrate buffer for 20 minutes. After blocking, sections were incubated with 0.5 μg/mL of PMab-52 **(A, B)** or control phosphate-buffered saline **(C, D)**, followed by detection using Envision+ Kit. **(E, F)** Hematoxylin and Eosin staining. Scale bar = 100 μm.

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Author Disclosure Statement

No competing financial interests exist.

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