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PMab-52: Specific and Sensitive Monoclonal Antibody Against Cat Podoplanin for Immunohistochemistry

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Podoplanin (PDPN) is expressed in several normal tissues, such as lymphatic endothelial cells, podocytes of renal glomerulus, and type I alveolar cells of lung. PDPN activates platelet aggregation by binding to C-type lectin-like receptor-2 (CLEC-2) on platelet. Although monoclonal antibodies (mAbs) against human PDPN, mouse PDPN, rat PDPN, rabbit PDPN, dog PDPN, and bovine PDPN have been established, anticat PDPN (cPDPN) mAbs have not been developed. In this study, we immunized mice with Chinese hamster ovary (CHO)-K1 cell lines expressing cPDPN, and developed anti-cPDPN mAbs. One of the clones, PMab-52 (IgM, kappa), detected cPDPN specifically in flow cytometry and Western blot analysis. PMab-52 is also useful for detecting feline squamous cell carcinoma cells in immunohistochemical analysis. PMab-52 is expected to be useful for investigating the function of cPDPN in feline carcinomas.

Keywords: cat podoplanin, PDPN, monoclonal antibody, immunohistochemistry

Introduction

PODOPLANIN (PDPN), also known as T1α/Aggrus,[1–4] is a type I transmembrane glycoprotein. PDPN activates platelet aggregation by binding to C-type lectin-like receptor-2 (CLEC-2) on platelet.[5–8] The interaction between PDPN and CLEC-2 facilitates blood/lymphatic vessel separation.[9] PDPN is also expressed in human fetal rib and chondrocytes of the proliferative and hypertrophic regions of the growth plate.[10] The expression of human PDPN has been reported in many malignant tumors such as oral squamous cell carcinomas,[11] malignant brain tumors,[12–15] lung cancers,[16] esophageal cancers,[17] malignant mesotheliomas,[18,19] testicular tumors,[20] osteosarcomas,[21–23] and chondrosarcomas.[22] PDPN expression is also associated with malignant progression and cancer metastasis.[5,12,24]

Although we previously established monoclonal antibodies (mAbs) against human,[25] mouse,[26] rat,[27] rabbit,[28] dog,[29] and bovine PDPN,[30] anticat PDPN (cPDPN) mAbs have not been developed yet. To investigate the expression and function of cPDPN, specific and sensitive mAbs against cPDPN are required. In this study, we immunized mice with cPDPN and developed anti-cPDPN mAbs.

Materials and Methods

Cell lines, feline tissues, and animals

Chinese hamster ovary (CHO)-K1 and P3U1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Feline kidney cell line, CrFK, was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). CHO-K1, stable CHO transfectants, and P3U1 were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), and CrFK was cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (FBS; 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% CO_2 and 95% air. Female BALB/c mice (4 weeks old) were purchased from CLEA Japan (Tokyo, Japan). Feline tissues were obtained from North Lab (Hokkaido, Japan). Animals were housed under specific pathogen-free conditions. The Animal Care and Use Committee of Tohoku University approved the animal experiments described herein.

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Hybridoma production

The cPDPN with N-terminal MAP tag (MAP-cPDPN) was inserted into pCAG-zeo vector (Wako Pure Chemical Industries Ltd., Osaka, Japan). MAP tag consists of 12 amino acids (GDGMVPPGIEDK).[31] CHO-K1 was transfected with pCAG-zeo/MAP-cPDPN using Lipofectamine LTX reagent (Thermo Fisher Scientific, Inc.). Stable transfectant of CHO/cPDPN was established by limiting dilution. BALB/c mice were immunized by intraperitoneal (i.p.) injection of CHO/cPDPN cells together with Imject Alum (Thermo Fisher Scientific, Inc.). After several additional immunizations of CHO/cPDPN cells, a booster injection of CHO/cPDPN cells 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, Inc.). The culture supernatants were screened using flow cytometry for the binding to CHO/cPDPN and CHO-K1.

Flow cytometry

Stable transfectants [CHO/PA-dog PDPN-RAP-MAP (CHO/dPDPN), CHO/PA-bovine PDPN-RAP-MAP (CHO/bovPDPN), CHO/human PDPN-FLAG (CHO/hPDPN), CHO/mouse PDPN-FLAG (CHO/mPDPN), CHO/rat PDPN-His (CHO/rPDPN), and CHO/PA-rabbit PDPN (CHO/rabPDPN)] were previously established.[1,27–30] Cells were harvested by brief exposure to 0.25% trypsin/1 mM EDTA (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin/phosphate-buffered saline, the cells were treated with a primary mAb (1 μg/mL) for 30 minutes at 4°C followed by treatment with Alexa Fluor 488-conjugated antimouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using EC800 or SA3800 Cell Analyzers (Sony Corp.).

Western blot analysis

Cell lysates (10 μg) of CHO/cPDPN, CHO/dPDPN, CHO/bovPDPN, CHO/hPDPN, CHO/mPDPN, CHO/rPDPN, CHO/rabPDPN, and CHO-K1 were boiled in SDS sample buffer (Nacalai Tesque, Inc.). The proteins were electrophoresed on 5–20% polyacrylamide gels (Wako Pure Chemical Industries Ltd.) and were transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), the membrane was incubated with 1 μg/mL of PMab-52, anti-β-actin (clone AC-15; Sigma-Aldrich Corp., St. Louis, MO), anti-IDH1 (clone RMab-3), and then with peroxidase-conjugated antimouse IgG (1:1000 diluted; Agilent Technologies, Inc., Santa Clara, CA), and developed with ImmunoStar LD (Wako Pure Chemical Industries Ltd.) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Determination of the binding affinity using flow cytometry

CHO/cPDPN or CrFK (2×10^5 cells) was suspended in 100 μL of serially diluted PMab-52, followed by addition of Alexa Fluor 488-conjugated antimouse IgG (1:200; Cell Signaling Technology, Inc.). Fluorescence data were collected using EC800 Cell Analyzer. 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, Inc., La Jolla, CA).

Immunohistochemical analyses

Four micrometer thick histological sections were deparaffinized in xylene and rehydrated, and were autoclaved in citrate buffer (pH 6.0; Agilent Technologies, Inc.) for 20 minutes. Sections were incubated with 1 μg/mL of PMab-52 for 1 hour at room temperature followed by treatment with Envision+ kit for 30 minutes (Agilent Technologies, Inc.). Color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 2 minutes, and then the sections were counterstained with hematoxylin (Wako Pure Chemical Industries Ltd.).

Results

Production of mAbs against cPDPN

We immunized mice with CHO/cPDPN cells, and performed flow cytometric screening. Culture supernatants of 1100 wells were mixed with CHO/cPDPN cells and CHO-K1 cells, and 84 wells (84/1100; 7.6%) that were positive against CHO/cPDPN cells and negative against CHO-K1 cells were selected. Of these 84 wells, 43 (51%) were positive against a feline kidney cell line, CrFK cells, in flow cytometry, indicating that about half of the wells reacted with endogenous cPDPN. We further performed Western blot analysis using CHO/cPDPN and CrFK cells. After limiting dilution of several wells that displayed specific and sensitive signals in Western blot analysis, one of the clones, PMab-52 (IgM, kappa), was established.

Specificity of PMab-52 against cPDPN in Western blot and flow cytometry

In flow cytometry, PMab-52 recognized only cPDPN from CHO/cPDPN, and did not react with PDPNs of CHO/dPDPN, CHO/bovPDPN, CHO/hPDPN, CHO/mPDPN, CHO/rPDPN, or CHO/rabPDPN cells (Fig. 1A). Furthermore, PMab-52 displayed the ~40-kDa band of CHO/cPDPN, but did not give bands with CHO/dPDPN, CHO/bovPDPN, CHO/hPDPN, CHO/mPDPN, CHO/rPDPN, or CHO/rabPDPN cells in Western blot analysis (Fig. 1B). These results indicate that PMab-52 specifically detects cPDPN.

Determination of the binding affinity of PMab-52

We then performed a kinetic analysis of the interaction of PMab-52 with CHO/cPDPN or CrFK using flow cytometry. The dissociation constant K_D of PMab-52 was determined to be 4.6×10^{-8} M against CHO/cPDPN (Fig. 1C) and 3.6×10^{-8} M against CrFK (Fig. 1D), indicating that PMab-52 possesses moderate affinity for CHO/cPDPN and CrFK.

Immunohistochemical analysis

Finally, we investigated the immunohistochemical utility of PMab-52 in feline squamous cell carcinomas. As shown in Figure 2A and B, PMab-52 stained membranes of cancer cells in the squamous cell carcinomas. PMab-52 also stained lymphatic endothelial cells (Fig. 2A, C), indicating that

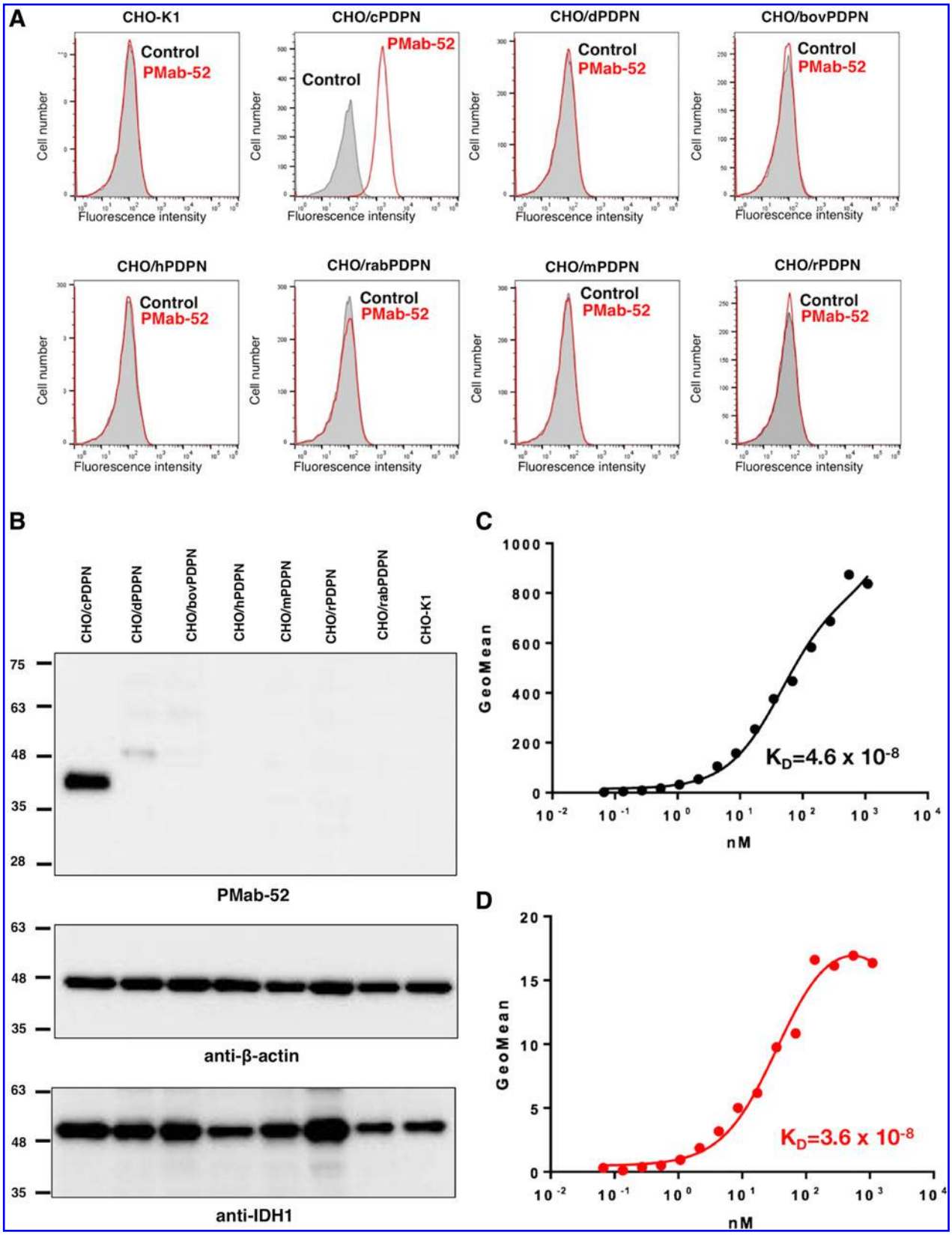

FIG. 1. Characterization of PMab-52. **(A)** Flow cytometry with PMab-52. Cells were treated with 1 μg/mL of PMab-52 followed by treatment with Oregon green-conjugated antimouse IgG. Black line: negative control. **(B)** Western blot with PMab-52. Cell lysates (10 μg) were electrophoresed and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μg/mL of PMab-52, anti-β-actin (AC-15), and anti-IDH1 (clone RMab-3), and then incubated with peroxidase-conjugated antimouse IgG. **(C, D)** Determination of the binding affinity of PMab-52 using flow cytometry. CHO/cPDPN **(C)** and CrFK **(D)** were suspended in 100 μL of serially diluted PMab-52 (6 μg/mL–100 μg/mL), followed by addition of secondary antimouse IgG. Fluorescence data were collected using a cell analyzer. CHO, Chinese hamster ovary; cPDPN, cat PDPN; PDPN, podoplanin; PVDF, polyvinylidene difluoride.

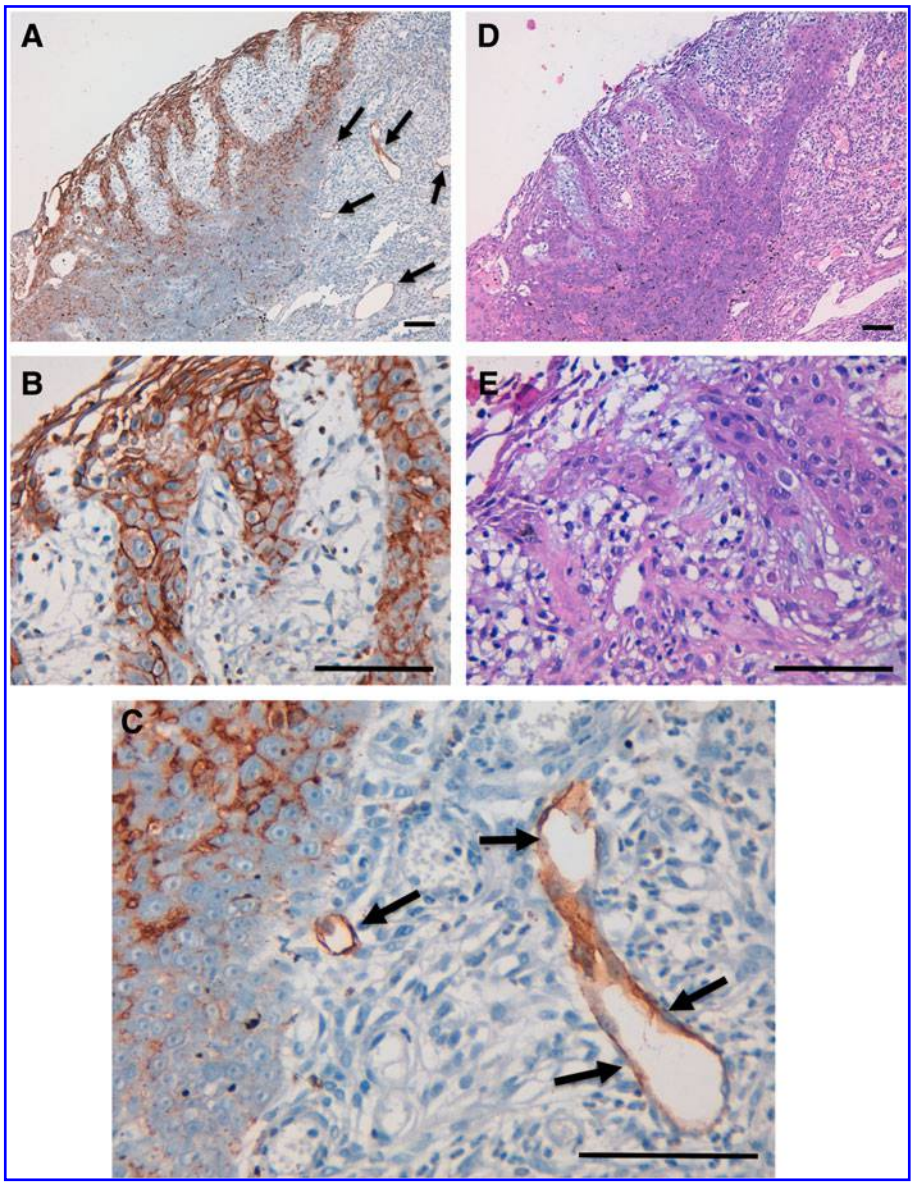

FIG. 2. Immunohistochemical analysis by PMab-52. **(A–C)** Sections of feline squamous cell carcinomas were autoclaved in citrate buffer (pH 6.0). After blocking, they were incubated with 1 μg/mL of PMab-52, followed by EnVision+ kit; color was developed using DAB and counterstained with hematoxylin. **(D, E)** Hematoxylin and eosin staining was performed against serial sections. Arrows: lymphatic endothelial cells; scale bar: 100 μm. DAB, 3,3-diaminobenzidine tetrahydrochloride.

PMab-52 is useful for immunohistochemistry using paraffin-embedded tissues.

Discussion

In recent studies, we successfully developed specific and sensitive mAbs against dPDPN[29] and bovPDPN,[30] and investigated the expression of PDPN in canine and bovine normal tissues, respectively. Furthermore, we investigated the expression of dPDPN in canine cancers, such as canine squamous cell carcinomas[32] and melanomas.[33] Until now, anti-cPDPN mAbs have not been developed; therefore, the expression of cPDPN has not been clarified in immunohistochemical analysis.

Our previous reports revealed that PMab-38 (anti-dPDPN mAb, mouse IgG_1),[29] PMab-44 (anti-bovPDPN mAb, mouse IgG_1),[30] LpMab-12 (anti-hPDPN mAb, mouse IgG_1),[34] PMab-1 (anti-mPDPN mAb, rat IgG_{2a}),[35] PMab-2 (anti-rPDPN mAb, mouse IgG_1),[27] and PMab-32 (anti-rabPDPN mAb, mouse IgG_1)[28,36] specifically react with CHO/dPDPN, CHO/bovPDPN, CHO/hPDPN, CHO/mPDPN, CHO/rPDPN, and CHO/rabPDPN, respectively. Many other anti-hPDPN mAbs also specifically detect hPDPN.[14,15,19,21,37–48] In this study, we immunized mice with CHO/cPDPN cells, and developed several anti-cPDPN mAbs including PMab-52. By using CHO/cPDPN cells for immunization and first screening, we did not require purifying recombinant cPDPN proteins. This method may be applicable for developing mAbs, especially against membrane proteins that cannot be easily purified. PMab-52 was determined to be IgM class although previously established anti-PDPN mAbs were determined to be IgG class, indicating that

immunization with CHO/cPDPN might not be enough for a class switch and affinity maturation. Indeed, the binding affinity of PMab-52, which was determined by flow cytometry, was not high (Fig. 1C, D).

PMab-52 could be useful for uncovering the pathophysiological function of cPDPN in feline tissues. PMab-52 might also be applicable for antibody-based therapy against feline tumors after producing cat–mouse chimeric antibodies.

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Author Disclosure Statement

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