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Development of a Monoclonal Antibody PMab-295 Against Elephant Podoplanin

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Podoplanin (PDPN) is an essential marker of lung type I alveolar cells, kidney podocytes, and lymphatic endothelial cells. Monoclonal antibodies (mAbs) that can specifically recognize PDPN in immunohistochemistry are important to analyze the development of tissues and the pathogenesis of diseases, including cancers. We have developed anti-PDPN mAbs against many animal species; however, mAbs that can recognize elephant-derived membrane proteins and distinguish the specific cell types in immunohistochemistry are limited. In this study, a novel anti-elephant PDPN (elePDPN) mAb, PMab-295 (IgG$_1$, kappa), was established using the peptide immunization method. PMab-295 recognized both elePDPN-overexpressed Chinese hamster ovary (CHO)-K1 cells and endogenous elePDPN-expressed LACF-NaNaI cells by flow cytometry and western blotting. Kinetic analyses using flow cytometry showed that the K_D of PMab-295 for CHO/elePDPN was 1.5×10^{-8} M. Furthermore, PMab-295 detected elePDPN-expressing cells using immunohistochemistry. These results showed the usefulness of PMab-295 to investigate the molecular function of elePDPN and the pathogenesis of diseases.

Keywords: elephant podoplanin, monoclonal antibody, immunohistochemistry

Introduction

PODOPLANIN (PDPN) IS a mucin-type type I transmembrane glycoprotein and is known as an important marker of lung type I alveolar cells,[1,2], kidney podocytes,[3] and lymphatic endothelial cells.[4,5] In humans, PDPN has been reported to be overexpressed in cancers and plays crucial roles in cancer cell migration, invasion, and metastasis.[6] Therefore, anti-PDPN monoclonal antibodies (mAbs) are useful to distinguish the specific cell types and investigate the PDPN-expressing cancer cells.

We have developed anti-PDPN mAbs for cat,[7] tiger,[8] horse,[9] pig,[10] goat,[11] alpaca,[12] Tasmanian devil,[13] bear,[14] whale,[15] sheep,[16] California sea lion,[17] golden hamster,[18,19] and ferret[20] using the Cell-Based Immunization and Screening (CBIS) method. These mAbs are available for flow cytometry, western blotting, and immunohistochemistry. However, an anti-elephant PDPN (elePDPN) mAb (clone PMab-265), which was established by the CBIS method, can be applicable only for flow cytometry, but not for the other applications.[21] In this study, we used the pep-

tide immunization methods to obtain novel mAbs against elePDPN and investigated the several applications, including flow cytometry, western blotting, and immunohistochemistry.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). African savanna elephant fibroblast-like cells (LACF-NaNaI) were obtained from the RIKEN BioResource Center (Ibaraki, Japan).[21] The elePDPN ORF with N-terminal RIEDL tag (RIEDL-elePDPN) was expressed in CHO-K1 cells, and previously named as CHO/elePDPN.[21] P3U1, CHO-K1, and CHO/elePDPN cells were cultured in RPMI-1640 (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.) and were incubated at 37°C in a

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humidified atmosphere containing 5% CO_2. LACF-NaNaI cells were cultured in minimum essential medium (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.), 5 μg/mL Plasmocin (InvivoGen), and 1 mM L-sodium pyruvate (Nacalai Tesque, Inc.) and were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Hybridoma production

Female BALB/c mice (6-week old) were purchased from CLEA, Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions. The animal care and use committee of Tohoku University approved all animal experiments. The elePDPN peptide immunization method was used to develop mAbs against elePDPN. Briefly, two mice were immunized, using three keyhole limpet hemocyanin-conjugated elePDPN peptides (100 μg of each peptide), including $_{38}$-EGGMVIPGVEDNMV-$_{51}$, + C-terminal cyste-ine. The administration was conducted through the intraperitoneal route with an Imject Alum (Thermo Fisher Scientific, Inc.). The procedure included three additional immunization procedures (100 μg of each peptide), followed by a final booster intraperitoneal injection (100 μg of each peptide) 2 days before its spleen cells were harvested. The harvested spleen cells were subsequently fused with P3U1 cells, using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN). Then, hybridomas were grown in an RPMI medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). Cultured supernatants were finally screened using enzyme-linked immunosorbent assay (ELISA) and flow cytometric analysis.

Enzyme-linked immunosorbent assay

Synthesized elePDPN peptides ($_{38}$-EGGMVIPGVEDN MV-$_{51}$, + C-terminal cysteine) were immobilized on Nunc MaxiSorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at a concentration of 1 μg/mL for 30 minutes at 37°C.
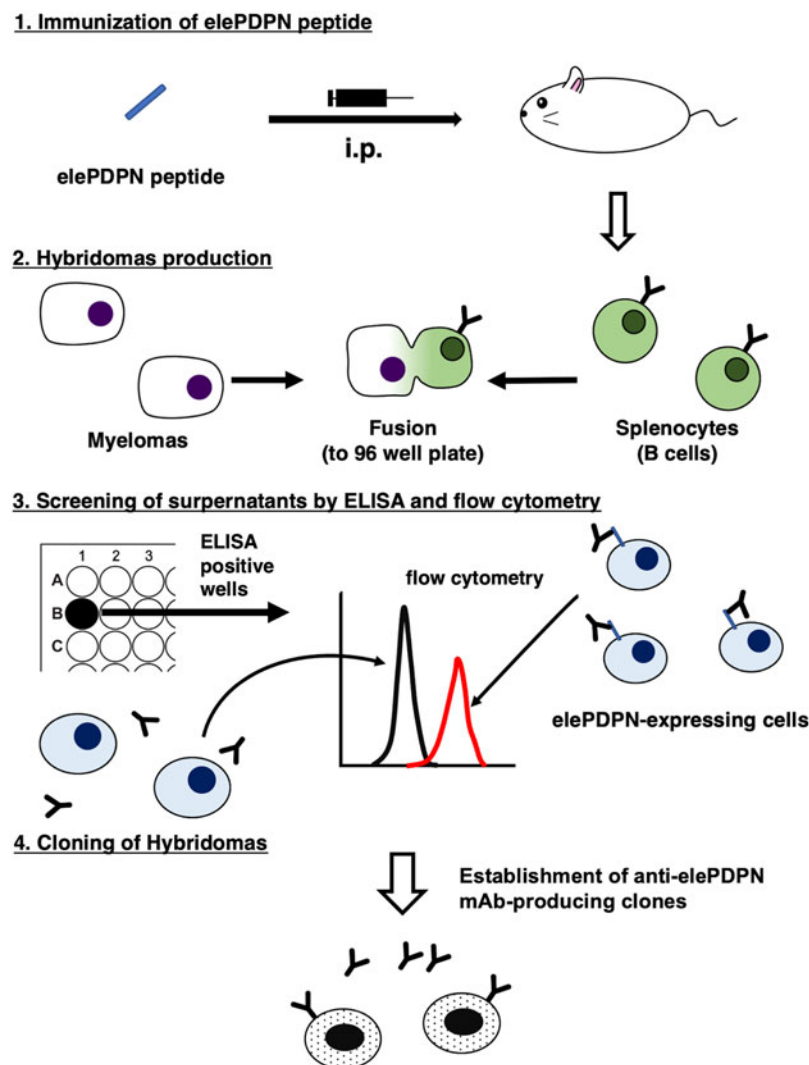

FIG. 1. A schematic procedure of anti-elePDPN mAb production. Mice were intraperitoneally immunized with the elePDPN peptide. The screening was then conducted by ELISA and flow cytometry. elePDPN, elephant PDPN; ELISA, enzyme-linked immunosorbent assay; mAbs, monoclonal antibodies.

After washing with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (Nacalai Tesque, Inc.), wells were blocked with 1% bovine serum albumin (BSA)-containing PBST for 30 minutes at 37°C. Culture supernatants were added to each well, followed by peroxidase-conjugated anti-mouse immunoglobulins (1:2000 diluted; Agilent Technologies, Inc., Santa Clara, CA). Afterward, enzymatic reactions were conducted, using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.), followed by the measurement of the optical density at 655 nm, using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Flow cytometry

Cells (2×10^5 cells/mL) were harvested after brief exposure to 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). After washing with 0.1% BSA (Nacalai Tesque, Inc.) in PBS (Nacalai Tesque, Inc.),

cells were treated with PMab-295 (1 μg/mL) for 30 minutes at 4°C, followed by Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; product no. 4408; Cell Signaling Technology, Inc., Danvers, MA). Finally, fluorescence data were collected using SA3800 Cell Analyzer (Sony Biotechnology Corp., Tokyo, Japan).

Determining the binding affinity of cells

Cells were suspended in 100 μL serially-diluted PMab-295 (0.006–100 μg/mL), followed by a subsequent suspension in an Alexa Fluor 488-conjugated anti-mouse IgG solution (1:200; Cell Signaling Technology, Inc.). Then, fluorescence data were collected, using the BD FACSLyric (BD Biosciences, Franklin Lakes, NJ). By fitting binding isotherms to built-in one-site binding models in GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA), the dissociation constant (K_D) was finally calculated.
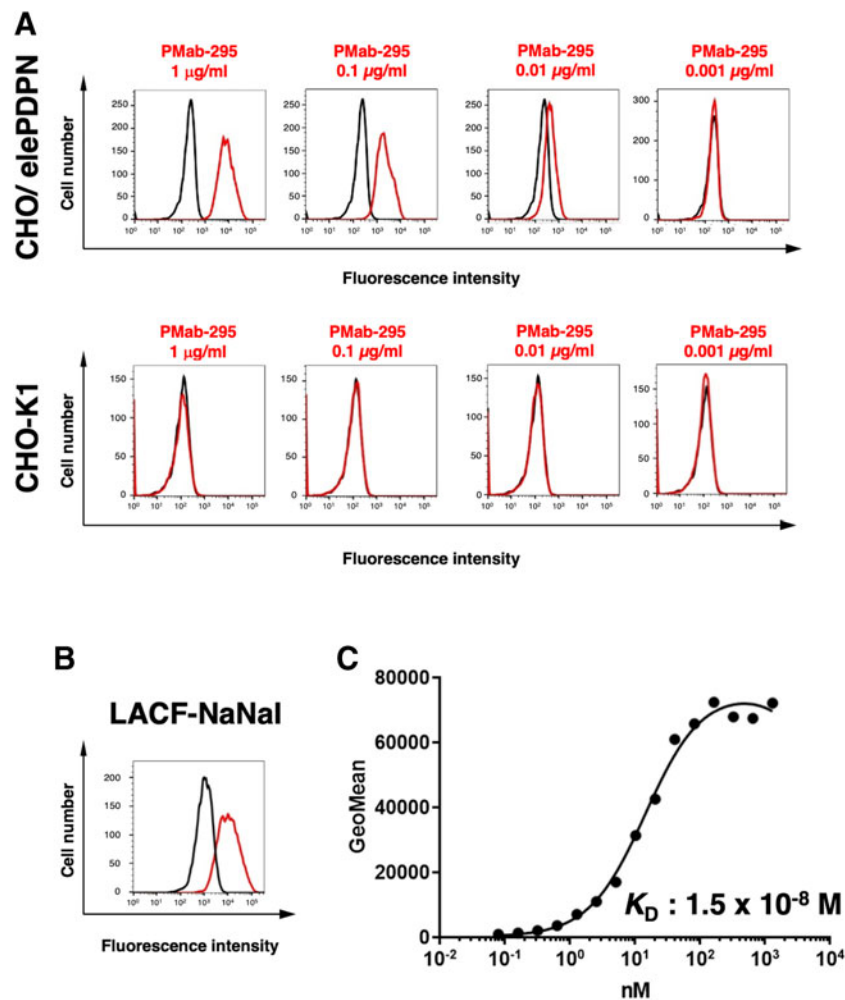

FIG. 2. Flow cytometry using anti-elePDPN mAb, PMab-295. **(A)** CHO-K1 and CHO/elePDPN cells were treated with 1–0.001 μg/mL of PMab-295, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. The black line represents the negative control. **(B)** LACF-NaNaI cells were treated with 1 μg/mL of PMab-295, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. The black line represents the negative control. **(C)** CHO/elePDPN cells were suspended in 100 μL serially-diluted PMab-295 (100–0.0006 μg/mL), following subsequent treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were subsequently collected using a BD FACSLyric, after which we calculated the dissociation constant (K_D) using GraphPad PRISM 8. CHO, Chinese hamster ovary.

Western blotting

Cell lysates (10 μg) were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.), after which proteins were separated on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and transferred to polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.) in 0.05% Tween 20-containing PBS, membranes were incubated with 1 μg/mL of PMab-295 or an anti-isocitrate dehydrogenase 1 (IDH1) mAb (RMab-3).[22–25] Then, they were incubated again with peroxidase-conjugated anti-mouse immunoglobulins (diluted 1:1000; Agilent Technologies, Inc.). Finally, protein bands were detected using ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) and a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Immunohistochemical analysis

Cell blocks were produced using iPGell (Genostaff Co., Ltd., Tokyo, Japan)[13] and processed to make 4 μm thick paraffin-embedded cell sections that were directly autoclaved in a citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. These tissue sections were blocked using the SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), incubated with PMab-295 (5 μg/mL) for 1 hour at the room temperature, and then treated with the EnVision+Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3,3′-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

Results

Establishment of anti-elePDPN mAbs

To develop anti-elePDPN mAbs, we used the elePDPN peptide immunization method. We designed the peptide in the platelet aggregation-stimulating domain of elePDPN since we could successfully generate anti-PDPN mAbs in various species using this strategy.[26–37] Hybridoma screening was conducted using ELISA and flow cytometry (Fig. 1). Mice were immunized with elePDPN synthetic peptides. Subsequently, hybridomas were seeded into 96-well plates, after which ELISA was used to extract positive wells for elePDPN peptides, followed by the selection of CHO/elePDPN-reactive and CHO-K1-nonreactive supernatants using flow cytometry. Finally, we established seven clones, which are applicable for flow cytometry. Among them, we selected PMab-295 (mouse IgG$_1$, kappa) for further analysis.

Flow cytometric analyses

Flow cytometric analyses were conducted using PMab-295 with CHO/elePDPN and CHO-K1 cells. PMab-295 recognized CHO/elePDPN dose dependently. In contrast, it did not react with CHO-K1 cells (Fig. 2A). PMab-295 also reacted with endogenous elePDPN, which is expressed in an African savanna elephant fibroblast-like cell line, LACF-NaNaI (Fig. 2B). Furthermore, kinetic analysis of PMab-295 interactions with CHO/elePDPN cells was conducted using flow cytometry. As indicated in Figure 2C, the K_D for PMab-

295 interactions with CHO/elePDPN cells was 1.5×10^{-8} M, suggesting that PMab-295 exhibited a moderate affinity against elePDPN.

Western blotting

Western blotting was conducted to further assess the sensitivity of PMab-295. For this assessment, lysates of CHO-K1, CHO/elePDPN, and LACF-NaNaI cells were probed. As demonstrated in Figure 3, PMab-295 detected double bands (between 35 and 48 kDa) of elePDPN in lysates from CHO/elePDPN cells, whereas these bands were absent in lysates from CHO-K1 cells. Furthermore, PMab-295 detected the endogenous elePDPN (about 40 kDa) in lysates from LACF-NaNaI cells. These results indicated that PMab-295 specifically detected both exogenous and endogenous elePDPN by western blotting.

Immunohistochemical analyses

We next investigated whether PMab-295 can be used for immunohistochemical analyses using paraffin-embedded cell (CHO-K1, CHO/elePDPN, and LACF-NaNaI cells) sections. PMab-295 strongly stained the CHO/elePDPN cell-embedded section, but not CHO-K1-embedded section (Fig. 4A, B).
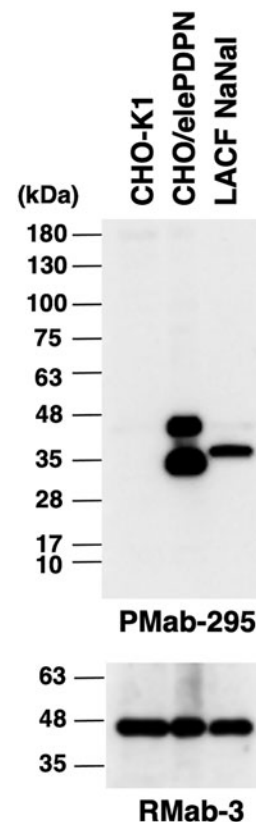

FIG. 3. Western blotting using PMab-295. Cell lysates (10 μg) of CHO-K1 and CHO/elePDPN and LACF-NaNaI cells were electrophoresed, after which proteins were transferred to PVDF membranes. After blocking, membranes were subsequently incubated with 1 μg/mL of PMab-295 or anti-IDH1 (RMab-3), following incubation with peroxidase-conjugated anti-mouse immunoglobulins. IDH1, isocitrate dehydrogenase 1; PVDF, polyvinylidene difluoride.
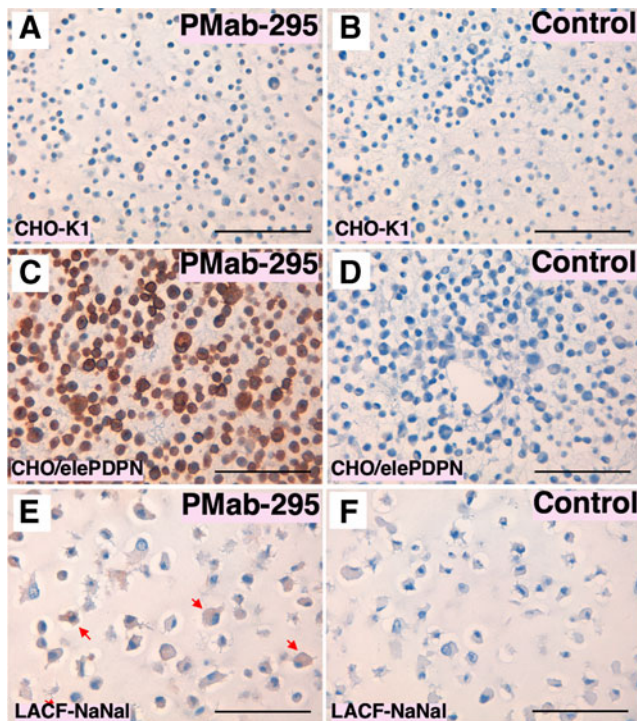
FIG. 4. Immunohistochemical analyses. The paraffin-embedded cell sections of CHO-K1 (**A, B**) and CHO/elePDPN (**C, D**) and LACF-NaNaI (**E, F**) cells were incubated with 5 μg/mL of PMab-295 (**A, C, E**) or with blocking buffer control (**B, D, F**), followed by that with the En-vision+Kit. Arrows show elePDPN-expressing LACF-NaNaI cells. Scale bar = 100 μm.

Moreover, PMab-295 weakly stained the LACF-NaNaI cell-embedded section (Fig. 4C). These results indicate the use-fulness of PMab-295 for detecting elePDPN-positive cells in paraffin-embedded cell samples.

Discussion

Previously, we established an anti-elePDPN mAb (clone PMab-265), which is only applicable for flow cytometry.[21] In this study, we established a novel anti-elePDPN mAb (clone PMab-295), which is useful for flow cytometry, western blotting, and immunohistochemistry. Especially, PMab-295 could stain paraffin-embedded cells (Fig. 4). Therefore, PMab-295 can be used to detect and analyze the development and pathogenesis of elephant tissues. Further studies are required to show whether PMab-295 can identify the elephant lung type I alveolar cells, kidney podocytes, and lymphatic endothelial cells.

Recently, the pathogenesis of elephant endotheliotropic herpesvirus-hemorrhagic disease has been reported as the most profound viral infectious disease in young Asian elephants (*Elephas maximus*).[38–41] These studies investigated the pathogenesis of the affected tissues, including heart, liver, lung, kidney, and intestine by hematoxylin and eosin staining and immunohistochemistry. Especially a study showed the increases in platelet endothelial cell adhesion molecules-1, and von Willebrand factor positive cells were significantly observed in injured blood vessels.[40] PMab-295 could con-tribute to the analyses of the pathogenesis as a lymphatic

endothelial marker. Furthermore, PMab-295 will contribute to the analyses of the injured lung and kidney in the future.

The incidence of elephant cancer is known to be extremely rare. However, about 3% of elephants develop cancer.[42,43] PDPN is overexpressed in many human cancers, including squamous cell carcinomas (head and neck,[44] lung,[45] uter-ine,[46] oral,[47] and esophageal[48] carcinomas), malignant mesotheliomas,[49–55] sarcomas,[56,57] and malignant glio-mas.[26,58–65] Recent study reported the reproductive tract neoplasia in 80 adult female Asian elephant mortalities in the United States from 1988 to 2019. Neoplasms occurred in 64/80 (80%) of cases. Most were in the uterus (63/64; 98%) with only a single case of ovarian neoplasia. This study also in-vestigated the expression of pan-cytokeratin, vimentin, and estrogen receptor in the uterine carcinomas by immunohis-tochemistry.[66] Therefore, the analysis of elephant carcino-mas by PMab-295 will help the elucidation of the mechanism of cancer development and the classification of neoplasia.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

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