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Establishment of Monoclonal Antibody PMab-202 Against Horse Podoplanin

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Podoplanin (PDPN), a type I transmembrane glycoprotein, is expressed in several body tissues, including podocytes of renal glomerulus, type I alveolar cells of lung, and lymphatic endothelial cells. PDPN activates platelet aggregation by binding to C-type lectin-like receptor-2 (CLEC-2) presented on platelets. Monoclonal antibodies (mAbs) against human-, mouse-, rat-, rabbit-, dog-, bovine-, and cat-PDPN have already been established. However, anti-horse PDPN mAbs have not yet been developed. In this study, we immunized mice with synthetic horse PDPN peptides and developed anti-horse PDPN mAbs. One of the established mAbs, PMab-202 (IgG_1, kappa), was specifically able to detect horse PDPN in Chinese hamster ovary/horse PDPN (CHO/horPDPN) cells in flow cytometry experiments. PMab-202 was also able to detect endogenous horse PDPN expressed in and a horse kidney cell line, FHK-Tcl3.1, in flow cytometry and Western blot analyses. PMab-202 is expected to prove useful in investigating the function of horse PDPN.

Keywords: horse podoplanin, PDPN, PMab-202

Introduction

Podoplanin (PDPN)/T1α/Aggrus, a type I transmembrane glycoprotein, induces platelet aggregation by binding to the C-type lectin-like receptor-2 (CLEC-2)[1–8] 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 several malignant tumors, such as lung cancers,[11] esophageal cancers,[12] malignant mesotheliomas,[13,14] testicular tumors,[15] osteosarcomas,[16–18] chondrosarcomas,[17] oral squamous cell carcinomas,[19] and malignant brain tumors.[20–23] PDPN expression is also associated with malignant progression and cancer metastasis.[5,20,24]

Previously we established monoclonal antibodies (mAbs) against human,[25] mouse,[26] rat,[27] rabbit,[28] dog,[29] bovine,[30] and cat PDPN.[31] However, anti-horse PDPN mAbs have not been developed yet. To investigate the expression and function of horse PDPN, sensitive and specific mAbs against horse PDPN are necessary. In this study, we immunized mice with synthesized peptides of horse PDPN and developed anti-horse PDPN mAbs.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3U1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The horse kidney cell line, FHK-Tcl3.1,[32] was established in Yamaguchi University. Horse PDPN (horPDPN) bearing an N-terminal PA16 tag (PA16-horPDPN) was inserted into pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The PA16 tag consists of 16 amino acids (GLEGGVAMPGAEDDVV).[33] CHO-K1 was transfected with pCAG-Ble/PA16-horPDPN using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were selected by limiting dilution and cultivated in a medium containing 0.5 mg/mL of zeocin (InvivoGen, San Diego, CA). CHO-K1, CHO/horPDPN, and P3U1 were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) and FHK-Tcl3.1

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was cultured in DMEM medium (Nacalai Tesque, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc), 100 units/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.

Hybridoma production

Female BALB/c mice (4 weeks 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 the animal experiments described herein. BALB/c mice were immunized against the synthesized horse PDPN peptides (horPP6279) corresponding to amino acids 62–79 of horse PDPN, which were administered intraperitoneally (i.p.) together with Imject Alum (Thermo Fisher Scientific, Inc.). After several additional immunizations, a booster injection was administered 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 supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). The culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA) for assessing binding to horPP6279.

Enzyme-linked immunosorbent assay

The culture supernatants were screened using ELISA for analyzing their binding to horPP6279, which was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at 1 μg/mL for 30 minutes. After blocking nonspecific binding sites with SuperBlock T20 (phosphate-buffered saline [PBS]) Blocking Buffer (Thermo Fisher Scientific, Inc.), the plates were incubated with culture supernatant followed by incubation with 1:1000 diluted peroxidase-conjugated anti-mouse IgG (Agilent Technologies, Inc., Santa Clara, CA). The enzymatic reaction was conducted with a 1-Step Ultra TMB-ELISA instrument (Thermo Fisher Scientific, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Flow cytometry

Cells were harvested after a brief exposure to 0.25% trypsin/1 mM EDTA (Nacalai Tesque, Inc.). After washing with 0.1% BSA/PBS, the cells were treated with a primary mAb 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). For the peptide blocking assay, cells were treated with PMab-202 (10 μg/mL) plus peptides (1 μg/mL) for 30 minutes at 4°C, followed by secondary antibodies. Fluorescence data were collected using SA3800 Cell Analyzers (Sony Corporation, Tokyo, Japan).

Western blot analysis

Cell lysates (10 μg) were boiled in SDS sample buffer (Nacalai Tesque, Inc.). The proteins were electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and separated proteins were transferred onto a polyvinylidene difluoride membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), the membrane was incubated with 1 μg/mL of PMab-202, and subsequently with peroxidase-conjugated anti-mouse IgG (1:1000 diluted; Agilent Technologies, Inc.). The immune reaction was observed with ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Determination of the binding affinity using flow cytometry

CHO/horPDPN or FHK-Tcl3.1 (2×10^5 cells) was suspended in 100 μL of serially diluted PMab-202, followed by addition of Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.). Fluorescence data were collected using EC800 Cell Analyzer (Sony Corporation). The dissociation constants (K_D) were obtained by fitting the binding isotherms using built-in one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Results and Discussion

Mice were immunized against horPP6279, corresponding to amino acids 62–79 of horse PDPN (NCBI Reference Sequence: XM_014737713). ELISA screening indicated positive signals in 21 out of 960 wells (2.2%). Among the 21 wells, 6 wells (29%) tested positive against CHO/horPDPN in flow cytometry analysis. One clone among the six positive clones, namely, PMab-202 (IgG_1, kappa), was established by limiting dilution.

PMab-202 recognized horse PDPN in CHO/horPDPN and did not react with CHO-K1 cells in flow cytometry analysis (Fig. 1A). Furthermore, PMab-202 reacted with the horse kidney cell line FHK-Tcl3.1, indicating the ability of PMab-202 to recognize endogenous horse PDPN. PMab-202 reacted with FHK-Tcl3.1 cells in a dose-dependent manner (Fig. 1B). Flow cytometry was used to perform a kinetic analysis of the interaction of PMab-202 with CHO/horPDPN or FHK-Tcl3.1 cells. The dissociation constant (K_D) of PMab-202 against CHO/horPDPN and FHK-Tcl3.1 was determined to be 8.7×10^{-9} M and 2.1×10^{-6} M, respectively, indicating a moderate affinity of PMab-202 toward CHO/horPDPN and a very low affinity of PMab-202 toward FHK-Tcl3.1.

PMab-202 was able to identify horse PDPN as a 35 kDa band in FHK-Tcl3.1 cells in Western blot analysis (Fig. 1C). This signal was blocked by horPP6279, but not by horPP2336, which is corresponding to amino acids 23–36 of horse PDPN (data not shown), indicating that PMab-202 specifically recognized horse PDPN. Furthermore, several high-molecular bands were observed at weak intensities (Fig. 1C). These bands were also blocked by horPP6279, but not by horPP2336, suggesting that these bands might be aggregation products of horse PDPN proteins.

In flow cytometry, the PMab-202 reaction with FHK-Tcl3.1 was blocked by horPP6279, but not by horPP2336 (Fig. 2), also indicating that PMab-202 specifically recognized horse PDPN.

Taken together, PMab-202 could prove useful for elucidating the pathophysiological functions of horse PDPN. Unfortunately, PMab-202 was not found useful for

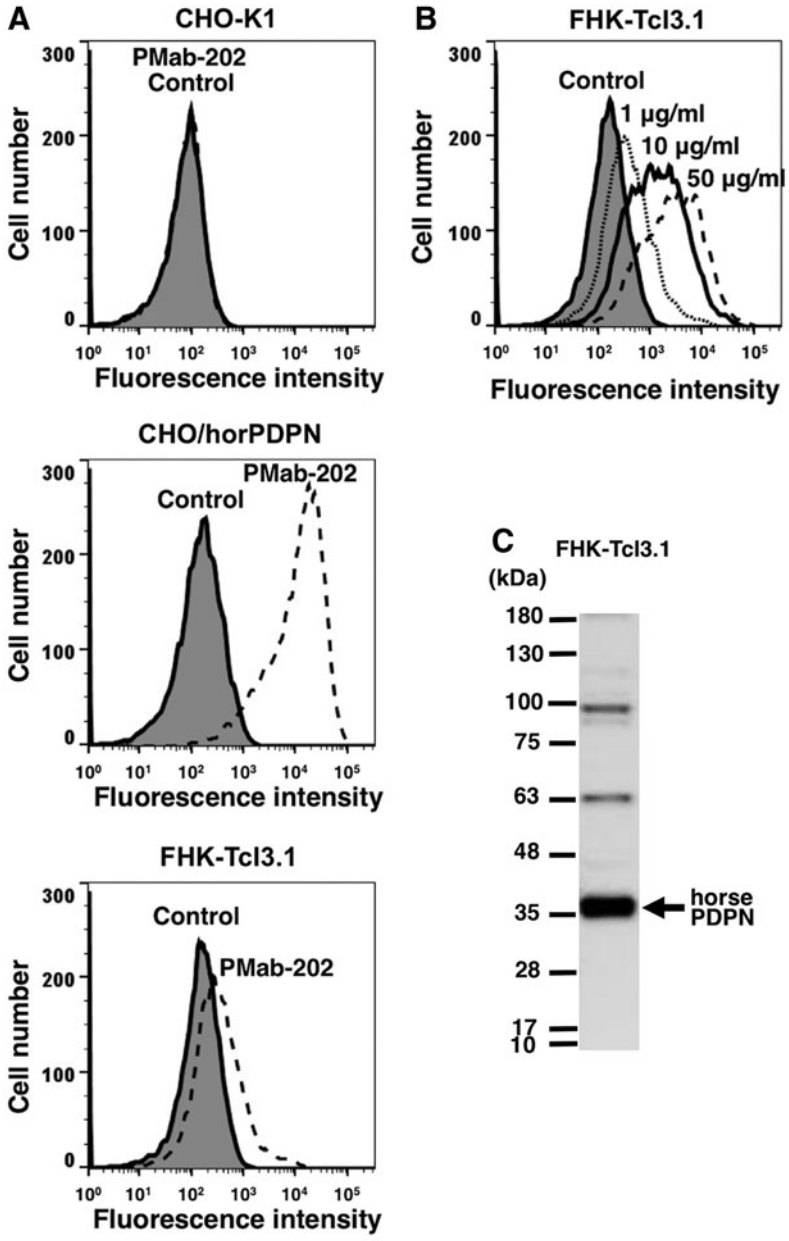

FIG. 1. Detection of horse PDPN by flow cytometry using PMab-202. **(A)** CHO-K1, CHO/horPDPN, and FHK-Tcl3.1 cells were treated with PMab-202 at a concentration of 1 μg/mL (dashed line) or control (gray shade) for 30 minutes at 4°C followed by the addition of secondary antibodies. **(B)** FHK-Tcl3.1 cells were treated with PMab-202 at a concentration of 1 μg/mL (dotted line), 10 μg/mL (solid line), and 50 μg/mL (dashed line) for 30 minutes at 4°C followed by the addition of secondary antibodies. **(C)** Cell lysates of FHK-Tcl3.1 (10 μg) were electrophoresed and transferred onto a PVDF membrane. The membrane was incubated with 1 μg/mL of PMab-202 and subsequently with peroxidase-conjugated anti-mouse IgG. CHO, Chinese hamster ovary; PDPN, podoplanin; PVDF, polyvinylidene difluoride.

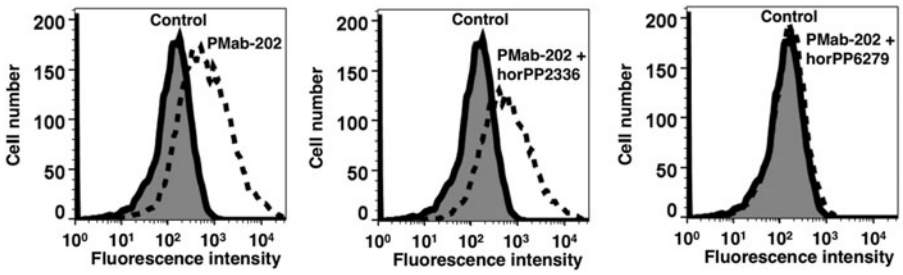

FIG. 2. Blocking assay of PMab-202 by horse PDPN peptide. PMab-202 (10 μg/mL) and horse peptides (1 μg/mL) were mixed (dashed line) and allowed to react with FHK-Tcl3.1 cells for 30 minutes at 4°C, followed by addition of secondary antibodies. Gray shade, control (without a primary mAb).

immunohistochemical analysis within this study probably due to a very low affinity of PMab-202 toward endogenous horse PDPN. Anti-horse PDPN mAbs, which can be utilized for immunohistochemical analysis, should be developed using other current mAb-producing methods such as Cell-Based Immunization and Screening (CBIS) method[33] in future study.

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Author Disclosure Statement

No competing financial interests exist.

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