Establishment of a monoclonal antibody PMab-233 for immunohistochemical analysis against Tasmanian devil podoplanin

Yoshikazu Furusawaa,b,c, Shinji Yamadaa, Shunsuke Itaiad, Takuro Nakamuraa, Junko Takeida, Masato Sanoa, Hiroyuki Harada, Masato Fukuc, Mika K. Kanekoa, Yukinari Katoa,b,*

a Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan
b New Industry Creation Hatchery Center, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
c ZENOAQ RESOURCE CO., LTD., 1-1 Tairanoue, Sasagawa, Asaka-machi, Koriyama, Fukushima, 963-0196, Japan
d Department of Oral and Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo, 113-8510, Japan

ARTICLE INFO

Keywords:
Tasmanian devil podoplanin
PDPN
PMab-233

ABSTRACT

Monoclonal antibodies (mAbs) against not only human, mouse, and rat but also rabbit, dog, cat, bovine, pig, and bovine podoplanin (PDPNs) have been established in our previous studies. PDPN is used as a lymphatic endothelial cell marker in pathological diagnoses. However, mAbs against Tasmanian devil PDPN (tasPDPN), which are useful for immunohistochemical analysis, remain to be developed. Herein, mice were immunized with tasPDPN-overexpressing Chinese hamster ovary (CHO)-K1 (CHO/tasPDPN) cells, and hybridomas producing mAbs against tasPDPN were screened using flow cytometry. One of the mAbs, PMab-233 (IgG1, kappa), specifically detected CHO/tasPDPN cells by flow cytometry and recognized tasPDPN protein by western blotting. Furthermore, PMab-233 strongly detected CHO/tasPDPN cells by immunohistochemistry. These findings suggest that PMab-233 may be useful as a lymphatic endothelial cell marker of the Tasmanian devil.

1. Introduction

Podoplanin (PDPN), a type I transmembrane glycoprotein, is expressed in many cell types, including lymphatic endothelial cells [1]. Therefore, PDPN is extremely useful to distinguish lymphatic endothelial cells from vascular endothelial cells in pathological diagnoses [2]. We previously reported that C-type lectin-like receptor-2 (CLEC-2) is an endogenous receptor of PDPN [3,4]. Importantly, the PDPN-CLEC-2 interaction has been shown to facilitate the separation of embryonic blood and lymphatic vessels [5]. The expression of human PDPN (hPDPN) has been reported in several malignant tumors, including malignant brain tumors [6–9], oral squamous cell carcinomas [10], pulmonary cancers [11], esophageal cancers [12], malignant mesotheliomas [13,14], osteosarcomas [15–17], chondrosarcomas [16], and testicular tumors [18]. The expression of hPDPN is associated with cancer metastasis and malignant progression [4,6,19]. To date, we have developed monoclonal antibodies (mAbs) against not only human [20] but also mouse [20], rat [21], rabbit [22], bovine [23], dog [24], cat [25], pig [26], and horse [27] PDPNs. Furthermore, an anti-cat PDPN mAb (PMab-52) cross-reacted with a tiger PDPN [28], and an anti-bovine PDPN mAb (PMab-44) cross-reacted with goat [29], sheep [30], and alpaca [31] PDPNs. However, anti-Tasmanian devil PDPN (tasPDPN) mAb has not yet been reported. In this study, we immunized mice with CHO/tasPDPN cells and established hybridomas that could produce mAbs against tasPDPN.

2. Materials and methods

2.1. Cell lines and animals

CHO-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The synthesized DNA of tasPDPN (accession No. XM_012545641.2) bearing an N-terminal PA16 tag (PA16-tasPDPN) was inserted into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) [32]. Importantly, the PDPN-CLEC-2 interaction has been shown to facilitate the separation of embryonic blood and lymphatic vessels [5]. The expression of human PDPN (hPDPN) has been reported in several malignant tumors, including malignant brain tumors [6–9], oral squamous cell carcinomas [10], pulmonary cancers [11], esophageal cancers [12], malignant mesotheliomas [13,14], osteosarcomas [15–17], chondrosarcomas [16], and testicular tumors [18]. The expression of hPDPN is associated with cancer metastasis and malignant progression [4,6,19]. To date, we have developed monoclonal antibodies (mAbs) against not only human [20] but also mouse [20], rat [21], rabbit [22], bovine [23], dog [24], cat [25], pig [26], and horse [27] PDPNs. Furthermore, an anti-cat PDPN mAb (PMab-52) cross-reacted with a tiger PDPN [28], and an anti-bovine PDPN mAb (PMab-44) cross-reacted with goat [29], sheep [30], and alpaca [31] PDPNs. However, anti-Tasmanian devil PDPN (tasPDPN) mAb has not yet been reported. In this study, we immunized mice with CHO/tasPDPN cells and established hybridomas that could produce mAbs against tasPDPN.

Received 25 February 2019; Received in revised form 25 March 2019; Accepted 25 March 2019
https://doi.org/10.1016/j.bbrep.2019.100631
© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA, USA).

The P3U1, CHO-K1, CHO/tasPDPN [34], CHO/mouse PDPN (mPDPN) [34], CHO/rabbit PDPN (rPDPN) [21], CHO/rat PDPN (rPDPN) [21], CHO/hamster PDPN (hPDPN) [26], CHO/horse PDPN (hPDPN) [32], CHO/tiger PDPN (tigPDPN) [28], CHO/alpaca PDPN (aPDPN) [31], CHO/bear PDPN (bPDPN) [26], CHO/goat PDPN (gPDPN) [29], CHO/sheep PDPN (sPDPN) [30], and CHO/whale PDPN (wPDPN) [26] were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), which was supplemented with 10% of heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc.), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). The cells were grown in an incubator at 37 °C with humidity and 5% CO2 and 95% air atmosphere. Female BALB/c mice (6 weeks of age) were purchased from CLEA Japan (Tokyo, Japan), which was supplemented with 10% of heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc.), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). The cells were grown in an incubator at 37 °C with humidity and 5% CO2 and 95% air atmosphere. Female BALB/c mice (6 weeks of age) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under specific pathogen-free conditions. The Animal Care and Use Committee of Tohoku University approved all animal experiments.

2.2. Hybridoma production

We employed a Cell-Based Immunization and Screening (CBIS) method [25,33,35,36] to develop sensitive and specific mAbs against tasPDPN. Briefly, two BALB/c mice were immunized with CHO/tasPDPN cells (1 × 10⁶) intraperitoneally (i.p.) together with the Imject Alum (Thermo Fisher Scientific Inc.). The procedure included three additional immunizations, followed by a final booster injection administered ip. 2 days prior to the harvest of spleen cells. Subsequently, these spleen cells were fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN, USA), and the hybridomas were grown in an RPMI medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific Inc.). The culture supernatants were screened by flow cytometry.

2.3. Flow cytometry

The cells were harvested following a brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). The cells were washed with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and treated with primary mAbs for 30 min at 4 °C. Thereafter, the cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA, USA) or Oregon Green anti-rat IgG (1:2000; Thermo Fisher Scientific Inc.). Then, fluorescence data were collected using the SA3800 Cell Analyzers (Sony Corp., Tokyo, Japan).
collected using the EC800 Cell Analyzer (Sony Corp.). The dissociation constant ($K_D$) was calculated by fitting the binding isotherms to built-in one-site binding models in the GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA, USA).

2.5. Western blotting

Cell lysates (10 μg) were boiled in a sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.). The proteins were electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and subsequently transferred onto a polyvinylidene

Fig. 3. Cross-reaction of PMab-233 to PDPNs of the other species by flow cytometry. CHO-K1 cells transfected with PDPNs of the other species were treated with PMab-233 (red line) or each positive control (blue line) at a concentration of 1 μg/mL or 0.1% BSA in PBS (gray) for 30 min, followed by incubation with secondary antibodies.


and subsequently, with peroxidase-conjugated anti-mouse or -rat IgG.

were incubated with l

were electrophoresed and transferred onto PVDF membranes. The membranes

blocking buffer (C, D), followed by that with the Envision + Kit. Scale

μg/mL of anti-PA16 tag (NZ-1), or 1

μg/mL of PMab-233, anti-PA16 tag (NZ-1), or

μg/mL of PMab-233 (A, B) or with

μg/mL of PMab-233 (Fig. 4) demonstrated

CHO/tasPDPN and negative for CHO-K1 were selected by

chose to tasPDPN. The identity of PDPN

mAbs will enable us to perform pathophysiological studies about the lymphatic metastasis or

In the present study, we employed the CBIS method to develop

sensitive and specific mAbs against tasPDPN to facilitate the

immunohistochemical analysis of paraffin-embedded tissue sections. Two

to CHO/tasPDPN cells and weak or no signals from CHO-K1 cells in 19 of the

960 wells (2.0%). After limiting dilution of 19 wells, we developed nine

PMab-233 recognized CHO/tasPDPN cells, but showed no reaction

with CHO-K1 cells, as assessed by flow cytometry (Fig. 2). PMab-233
did not react with human, mouse, rat, rabbit, dog, bovine, cat, pig,
horse, tiger, alpaca, bear, goat, sheep, or whale PDPNs (Fig. 3), which

indicates that PMab-233 is specific to tasPDPN. The identity of PDNP

amino acid sequence between tasPDPN and PDPNs of the other species

indicates that PMab-233 detects tasPDPN as a 40-kDa band in CHO/tasPDPN

cells. NZ-1, an anti-PA16 tag mAb also detected a 40 kDa band. The

PMab-233 for CHO/tasPDPN cells was determined to be

-6, indicating a low a

K₀ of PMab-233 for CHO/tasPDPN cells was determined to be

1.1 × 10⁻⁶, indicating a low affinity of PMab-233 for CHO/tasPDPN cells.

Western blotting performed using PMab-233 (Fig. 4) demonstrated

that PMab-233 detects tasPDPN as a 40-kDa band in CHO/tasPDPN

cells. NZ-1, an anti-PA16 tag mAb also detected a 40 kDa band. The

immunohistochemical analyses revealed that PMab-233 strongly

stained CHO/tasPDPN cells (Fig. 5A) and did not react with CHO-K1

cells (Fig. 5B). No staining was observed without primary antibodies

3. Results and discussion

Most cancers are somatic in origin, and only a few transmissible
cancers have been documented [37]. Transmissible cancers have been

reported only in natural cases, such as canine transmissible venereal
tumor in dogs [38] or devil facial tumor disease in Tasmanian devils
[39]. Tasmanian devils (Sarcophilus harrisii) are endangered owing to the

emergence of two clonally transmissible cancers: devil facial tumor
disease 1 (DFT1) and devil facial tumor disease 2 (DFT2). DFT1 and

DFT2 are infectious diseases that spread via biting [40]. DFT1 was first

discovered in northeastern Tasmania in 1996 and has since then spread to

more than 80% of the area across the island, causing a significant
decline in the population [41]. DFT2 was discovered in 2014 and is

currently restricted to a small region of southeastern Tasmania [42].

Although we had previously developed mAbs against human [20],
mouse [20], rat [21], rabbit [22], bovine [23], dog [24], cat [25], pig
[43], and horse [27] PDPNs, mAbs against tasPDPN has not yet been
developed. The development of anti-tasPDPN mAbs will enable us to

perform pathophysiological studies about the lymphatic metastasis or

lymphangiogenesis.

In the present study, we employed the CBIS method to develop

sensitive and specific mAbs against tasPDPN to facilitate the

immunohistochemical analysis of paraffin-embedded tissue sections. Two

mice were immunized with CHO/tasPDPN cells using an immunization

and screening procedure (Fig. 1). The developed hybridomas were seeded into 96-well plates and cultivated for 9 days. Wells positive for

CHO/tasPDPN and negative for CHO-K1 were selected by flow cyto-

metry. The screening approach identified strong signals from CHO/
tasPDPN cells and weak or no signals from CHO-K1 cells in 19 of the

960 wells (2.0%). After limiting dilution of 19 wells, we developed nine
clones. One of these nine clones, PMab-233 (IgG1, kappa), was

finally selected via immunohistochemistry against the paraffin-embedded

sections of CHO/tasPDPN cell.

PMab-233 recognized CHO/tasPDPN cells, but showed no reaction

with CHO-K1 cells, as assessed by flow cytometry (Fig. 2). PMab-233
did not react with human, mouse, rat, rabbit, dog, bovine, cat, pig,
horse, tiger, alpaca, bear, goat, sheep, or whale PDPNs (Fig. 3), which

indicates that PMab-233 is specific to tasPDPN. The identity of PDNP

amino acid sequence between tasPDPN and PDNP of the other species

is shown as below: 45% (vs. hPDPN), 41% (vs. mPDPN), 38% (vs.

amino acid sequence between tasPDPN and PDPNs of the other species

indicates that PMab-233 detects tasPDPN as a 40-kDa band in CHO/tasPDPN

cells. NZ-1, an anti-PA16 tag mAb also detected a 40 kDa band. The

PMab-233 for CHO/tasPDPN cells was determined to be

-6, indicating a low a

K₀ of PMab-233 for CHO/tasPDPN cells was determined to be

1.1 × 10⁻⁶, indicating a low affinity of PMab-233 for CHO/tasPDPN cells.

Western blotting performed using PMab-233 (Fig. 4) demonstrated

that PMab-233 detects tasPDPN as a 40-kDa band in CHO/tasPDPN

cells. NZ-1, an anti-PA16 tag mAb also detected a 40 kDa band. The

immunohistochemical analyses revealed that PMab-233 strongly

stained CHO/tasPDPN cells (Fig. 5A) and did not react with CHO-K1

cells (Fig. 5B). No staining was observed without primary antibodies

2.6. Immunohistochemical analyses

Cell blocks were produced using iPGell (Genostaff Co., Ltd., Tokyo,

Japan) and processed to make 4-μm thick paraffin-embedded cell sec-
tions that were directly autoclaved in a citrate buffer (pH 6.0; Nichirei
Biosciences, Inc., Tokyo, Japan) for 20 min. These tissue sections were

blocked using the SuperBlock T20 (PBS) Blocking Buffer (Thermo
Fisher Scientific Inc.), incubated with PMab-233 (1 μg/mL) for 1 h at
the room temperature, and then treated with the Envision + Kit
(Agilent Technologies Inc.) for 30 min. Color was developed using 3,3′-
diaminobenzidine tetrahydrochloride (Agilent Technologies Inc.) for
2 min, and counterstaining was performed using hematoxylin (FUJI-
ILM Wako Pure Chemical Corporation).

Fig. 4. Western blotting. Cell lysates of CHO-K1 and CHO/tasPDPN (10 μg)
were electrophoresed and transferred onto PVDF membranes. The membranes
were incubated with 1 μg/mL of PMab-233, anti-PA16 tag (NZ-1), or anti-β-actin
and subsequently, with peroxidase-conjugated anti-mouse or -rat IgG.

Fig. 5. Immunohistochemical analyses. Cell sections of CHO/tasPDPN (A, C)
and CHO-K1 (B, D) were incubated with 1 μg/mL of PMab-233 (A, B) or with
blocking buffer (C, D), followed by that with the Envision + Kit. Scale
bar = 100 μm.
In conclusion, we established an mAb, PMab-233, against tasPDPN, which is suitable for use in flow cytometry, Western blotting, and immunohistochemical analyses. The epitope of PMab-233 needs further investigation to clarify the sensitivity and specificity of PMab-233 against tasPDPN. We believe that PMab-233 should prove to be useful in elucidating the pathophysiological functions of tasPDPN in future studies.

Conflicts of interest
Y.K. received research funding from ZENOAQ RESOURCE CO., LTD. The other authors have no conflict of interest.

Acknowledgments
We thank Miyuki Yanaka, Kayo Hisamatsu, Saori Handa, and Yoshimi Nakamura for their excellent technical assistance. This research was supported in part by AMED under Grant Numbers: JP18am0101078 (Y.K.), JP18am0301010 (Y.K.), and JP18ae0101028 (Y.K.), and by JSPS KAKENHI Grant Number 17K07299 (M.K.K.) and JP18am0101078 (Y.K.), JP18am0301010 (Y.K.), and JP18ae0101028 (Y.K.).

References


