

Characterization of Anti-podoplanin Monoclonal Antibodies: Critical Epitopes for Neutralizing the Interaction Between Podoplanin and CLEC-2

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Abstract

Podoplanin (Aggrus) is a mucin-type sialoglycoprotein that is known as a useful marker for lymphatic endothelium and tumor-initiating cells (TICs). Interaction between podoplanin and C-type lectin-like receptor-2 (CLEC-2) is reported to be critical for podoplanin-induced platelet aggregation and cancer metastasis. Recently, several anti-human podoplanin antibodies have been created; however, these anti-podoplanin antibodies have not been well characterized. Five anti-podoplanin antibodies (NZ-1, D2-40, AB3, 18H5, and a rabbit polyclonal antibody) were investigated using ELISA, Western blot, and flow cytometry with synthesized podoplanin peptides and deletion mutants of recombinant podoplanin. The epitope of NZ-1 is platelet aggregation-stimulating (PLAG) domain-2/3; the epitope of D2-40, AB3, and 18H5 is PLAG1/2. The epitopes of D2-40 and AB3 are quite similar, although 18H5 is different from D2-40 and AB3. Using flow cytometric analysis, NZ-1 partially inhibited the interaction between podoplanin and CLEC-2, although other antibodies did not. In conclusion, the two most frequently used anti-podoplanin antibodies, D2-40 and AB3, have similar properties, although several studies have reported differences. NZ-1 neutralizes the interaction between podoplanin and CLEC-2, which may lead to the development of therapeutic antibodies against podoplanin-dependent cancer metastasis.

Introduction

PODOPLANIN (ALSO KNOWN AS Aggrus), a membranous 44 and 36 kDa sialoglycoproteins in cancer cells of mice and humans, respectively, aggregates platelets in the absence of plasma components.⁽¹⁾ Podoplanin has been described as a lymphatic specific marker,⁽²⁾ yet its expression has been reported in many tumor cells, including squamous cell carcinomas,⁽³⁾ mesothelioma,⁽⁴⁾ testicular seminoma,⁽⁵⁾ and brain tumors.^(3,6,7) Recent investigations have suggested that expression of podoplanin is associated with tumor metastasis or progression.⁽⁷⁻⁹⁾

Podoplanin belongs to the family of type I transmembrane sialomucin-like glycoproteins that comprise an extracellular domain with abundant Ser and Thr residues as potential O-glycosylation sites, a single transmembrane portion, and a short cytoplasmic tail with putative sites for protein kinase C and cAMP phosphorylation.⁽¹⁰⁻¹²⁾ We previously showed that the segment of EDxxVTPG in the extracellular domain,

designated as a platelet aggregation-stimulating (PLAG) domain, is critical for the activity of podoplanin.⁽¹⁾ In particular, this motif, which is highly conserved across species, is triplicated in tandem.⁽¹¹⁾ In a study of targeted mutagenesis of podoplanin molecules, we obtained evidence that Thr residues in the PLAG domain play an important role in platelet aggregation.⁽¹⁾ Furthermore, the unique characteristics of Chinese hamster ovary (CHO) mutant cell lines, Lec1 (*N*-glycan-deficient), Lec2 (CMP-sialic acid transporter-deficient), and Lec8 (UDP-galactose transporter-deficient), revealed that sialylated *O*-glycan is critical for the platelet aggregation-inducing activity of podoplanin.⁽¹²⁾ Recently, we purified human podoplanin from the glioblastoma cell line LN319 cells and from podoplanin-transfected CHO cells⁽¹⁰⁾ and showed that podoplanin possesses a disialyl-core1 structure at Thr52 in the PLAG domain. Furthermore, we identified CLEC-2 as an endogenous receptor of podoplanin.⁽¹³⁾ CLEC-2 may have pathophysiological importance through its role in podoplanin-dependent metastasis.⁽⁸⁾

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In this study, we identified the epitopes of the anti-podoplanin antibodies D2-40, AB3, 18H5, and NZ-1 using ELISA and Western blot analysis. Furthermore, we compared the reactivity of these antibodies using flow cytometry to clarify the differences between these monoclonal antibodies.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO) cells transfected with human podoplanin (CHO/hPod) were established as described previously.⁽¹⁾ CHO/hPod cells were cultured in RPMI 1640 medium (Wako, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma, St Louis, MO), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 100 U/mL of penicillin, 100 µg/mL of streptomycin (Invitrogen), and 1 mg/mL of geneticin (G418, Sigma) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. 293T-REx/CLEC-2 cells were established as described previously,⁽¹⁴⁾ and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Wako) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL of penicillin, 100 µg/mL of streptomycin, 100 µg/mL of Zeocin (Invitrogen), and 5 µg/mL blasticidin (Invitrogen). Expression of CLEC-2 was induced by the addition of 1 µg/mL of doxycycline (Takara Bio, Shiga, Japan) to the medium 24 h before experimentation. 293T-REx/CLEC-2 cells without doxycycline were used as a control.

Antibodies

The anti-human podoplanin antibodies are summarized in Table 1. NZ-1, a rat anti-human podoplanin monoclonal antibody, was previously generated.⁽³⁾ D2-40, AB3 (AngioBio, No. 11-003), and 18H5, mouse anti-human podoplanin monoclonal antibodies, were purchased from Dako (Glostrup, Denmark), AngioBio (Del Mar, CA), and Abcam (Tokyo, Japan), respectively. The concentration of D2-40 and AB3 is not clarified in the data sheet; therefore, we measured the concentration of antibodies and used them in the most appropriate concentration. A polyclonal anti-human podoplanin antibody (r2336) was prepared by immunizing a rabbit with a synthetic oligopeptide ASTGQPEDDTETT corresponding to amino acids 23–36 position of the human podoplanin sequence, plus C-terminal cysteine.

Expression and purification of podoplanin-Fc fusion proteins

cDNA of human podoplanin containing the extracellular domain was obtained by PCR, as described previously.⁽⁸⁾ PCR was performed using HotStarTaq polymerase (Qiagen, Hilden, Germany). The following oligonucleotides were used as primers (the EcoRV or EcoRI site in the forward primer and the BglIII site in the reverse primer are underlined): Pod25-128 (forward: gcgatattcAGAAGGAGCCAGCACAGG, reverse: ggcagatctTGTTGACAAACCATCTTTC); Pod25-103 (forward [EcoRI-hPod.F1]: acgaattcgATGTGGAAGGTGTC-AGCTCT, reverse: acagatctGTTTGAGGCTGTGGCGCTTG); Pod25-80 (forward: EcoRI-hPod.F1, reverse: acagatctGATGCGAATGCCTGTTACAC); Pod25-57 (forward: EcoRI-hPod.F1, reverse: acagatctTTCGCTGGTTCCTGGAGTCA); Pod55-128 (forward: cgaattcAACCAGCGAAGACCGCTA-TAAGT, reverse [hPod-R384-BglIII]: acagatctTGTTGACAA-ACCATCTTTC); and Pod81-128 (forward: cgaattcCGAG-GATCTGCCAACTTCAGAAA, reverse: hPod-R384-BglIII). The PCR products were purified by using a Rapid PCR Purification Kit (Marligen Bioscience, Ijamsville, MD), digested with EcoRV or EcoRI and BglIII, purified again, and ligated into the pFUSE-hFc2 (IL2ss) vector (InvivoGen, San Diego, CA), which contains human IgG Fc after the ligation site and a interleukin 2 signal sequence (IL2ss) before the ligation site, to allow secretion of Fc-fusion proteins. CHO cells were transfected with plasmids using LipofectAMINE 2000. To purify the fusion proteins, the conditioned culture medium from the transfected CHO cells was centrifuged, and the supernatant was applied to a column of protein A-Sepharose (Pierce, Rockford, IL). After extensive washing with phosphate-buffered saline (PBS), the fusion proteins were eluted by 0.1 M glycine and 0.15 M NaCl (pH 2.8) and then neutralized with 1 M Tris (pH 10.0). The proteins were dialyzed against PBS. Expression and purity of the proteins were confirmed by SDS-PAGE using 10–20% gradient gels (Wako).

Synthesis of peptides

Human podoplanin peptides (hpp) were purchased from Operon Biotechnologies (Tokyo, Japan). The sequences of the human podoplanin peptides are summarized in Table 2.

Enzyme-linked immunosorbent assay

Synthetic peptides corresponding to amino acids of the human podoplanin sequence and podoplanin-Fc chimeras were immobilized on 96-well plates at 10 µg/mL for 1 h, respectively. After blocking with 1% BSA in PBS, the plates

TABLE 1. ANTI-PODOPLANIN ANTIBODIES USED IN THIS STUDY

Designation	Source	Species	Mono/poly	Antigen
NZ-1	AngioBio	Rat	Monoclonal	Peptide (38 aa–51 aa)
D2-40	Dako	Mouse	Monoclonal	Dysgerminoma tissue
AB3 (No. 11-003)	AngioBio	Mouse	Monoclonal	Not clarified
18H5	Abcam	Mouse	Monoclonal	Recombinant protein expressed in MDCK cells
r2336	Generated in this study	Rabbit	Polyclonal	Peptide (23 aa–36 aa)

TABLE 2. SYNTHETIC PEPTIDES USED IN THIS STUDY

Peptide name	Sequence
hpp23-36 (14 mer)	ASTGQPEDDTETT
hpp29-37 (9 mer)	EDDTETTGL
hpp29-39 (11 mer)	EDDTETTGLEG
hpp29-41 (13 mer)	EDDTETTGLEGGV
hpp29-43 (15 mer)	EDDTETTGLEGGVAM
hpp29-45 (17 mer)	EDDTETTGLEGGVAMPG
hpp29-47 (19 mer)	EDDTETTGLEGGVAMPGAE
hpp31-47 (17 mer)	DTETTGLEGGVAMPGAE
hpp33-47 (15 mer)	ETTGLEGGVAMPGAE
hpp35-47 (13 mer)	TGLEGGVAMPGAE
hpp37-47 (11 mer)	LEGGVAMPGAE
hpp39-47 (9 mer)	GGVAMPGAE
hpp38-43 (6 mer)	EGGVAM
hpp38-45 (8 mer)	EGGVAMPG
hpp38-47 (10 mer)	EGGVAMPGAE
hpp38-49 (12 mer)	EGGVAMPGAEDD
hpp38-51 (14 mer)	EGGVAMPGAEDDVV
hpp40-51 (12 mer)	GVAMPGAEDDVV
hpp42-51 (10 mer)	AMPGAEDDVV
hpp44-51 (8 mer)	PGAEDDVV
hpp46-51 (6 mer)	AEDDVV

were incubated with NZ-1 (1 $\mu\text{g}/\text{mL}$), D2-40 (2 $\mu\text{g}/\text{mL}$), AB3 (2 $\mu\text{g}/\text{mL}$), 18H5 (1 $\mu\text{g}/\text{mL}$), and r2336 (2 $\mu\text{g}/\text{mL}$) for 1 h, respectively. After washing with 0.05% Tween-20 in PBS, the plates were incubated with peroxidase-conjugated anti-rat IgG, anti-mouse IgG, or anti-rabbit IgG antibodies (Dako) for 1 h, respectively. After further washing, the enzymatic reaction substrate solution containing TMB-Ultra (Pierce) was added. The reaction was stopped with 1 M H_2SO_4 , and the optical density was measured at 450 nm with an auto-plate reader. These reactions were performed with a volume of 50 μL at room temperature.

Western blot analysis

Proteins were electrophoresed under non-reducing conditions on 10–20% polyacrylamide gels (Wako). The separated proteins were transferred to a PVDF membrane. After blocking with 4% skim milk in PBS, the membrane was incubated with NZ-1 (1 $\mu\text{g}/\text{mL}$), D2-40 (10 $\mu\text{g}/\text{mL}$), AB3 (10 $\mu\text{g}/\text{mL}$), 18H5 (1 $\mu\text{g}/\text{mL}$), and r2336 (10 $\mu\text{g}/\text{mL}$), and then with peroxidase-conjugated anti-rat IgG, anti-mouse IgG, or anti-rabbit IgG antibodies (Dako) and developed for 1 min with ECL reagents (GE Healthcare UK, Buckinghamshire, United Kingdom) using Kodak X-Omat AR film.

Flow cytometry

Expression of CLEC-2 was induced by the addition of 1 $\mu\text{g}/\text{mL}$ doxycycline to 293T-REx/CLEC-2 cells 24 h before experimentation. CHO/hPod cells or 293T-REx/CLEC-2 cells were harvested by brief exposure to 1 mM EDTA. After washing with PBS, the cells were treated with primary antibodies for 1 h at 4°C followed by treatment with FITC-conjugated anti-human Fc, anti-rat IgG, anti-mouse IgG, or anti-rabbit IgG antibodies (Immuno-Biological Laboratories, Gunma, Japan), as appropriate. Fluorescence data were col-

lected using a FACS Calibur flow cytometer (BD Biosciences, Brea, MA).

Results

Determination of the epitope of the anti-podoplanin antibody NZ-1

We previously immunized rats with the PLAG (platelet aggregation-stimulating) domain of human podoplanin and produced a novel anti-podoplanin antibody (NZ-1).⁽³⁾ Podoplanin-induced platelet aggregation was specifically neutralized by NZ-1, indicating that NZ-1 recognizes the platelet aggregating-epitope of human podoplanin. We first produced several deletion mutants of human podoplanin as Fc chimeras in CHO cells. As shown in Figure 1A, NZ-1 recognized four types of C-terminal deletion mutants (Pod25-128, Pod25-103, Pod25-80, and Pod25-57) that include a PLAG domain in Western blot analysis; however, it did not react with N-terminal deletion mutants (Pod55-128 and Pod81-128) that lack the PLAG domain. We obtained the same results using ELISA (data not shown). Next, we synthesized seven peptides of human podoplanin and investigated the binding activity of NZ-1 with ELISA (Fig. 1B). NZ-1 recognized hpp38-51 strongly and showed a weaker reaction with hpp29-47, suggesting that NZ-1 recognized the peptide including PLAG domain-2 (PLAG2) and PLAG domain-3 (PLAG3). To identify the minimum epitope of NZ-1, we further synthesized nine peptides in the PLAG2/3 and investigated the binding activity of NZ-1. As shown in Figure 2, NZ-1 showed the strongest reaction with hpp40-51, hpp38-51, and hpp38-49, with weaker recognition of hpp42-51 and hpp38-47. In contrast, hpp44-51, hpp46-51, hpp38-45, and hpp38-43 were not detected by NZ-1. These results indicated that the minimum epitope of NZ-1 is six amino acids (AMPGAE), but 10 amino acids (GVAMPGAEDD) are necessary for strong binding by NZ-1.

Determination of the epitopes of additional anti-podoplanin antibodies

We next moved on to determine the epitope of the other anti-podoplanin antibodies, which are commercially available and have been discussed in many papers.⁽¹⁵⁾ As shown in Figure 1A, D2-40, AB3, and 18H5 reacted with four types of C-terminal deletion mutants and did not react with N-terminal deletion mutants (Pod55-128 and Pod81-128), similar to NZ-1. In ELISA using seven peptides of podoplanin, D2-40, AB3, and 18H5 strongly recognized hpp29-47; however, only 18H5 reacted with hpp38-51, albeit weakly (Fig. 1B), suggesting that they recognized the peptide including PLAG domain-1 (PLAG1) and PLAG domain-2 (PLAG2). To identify the minimum epitope of these antibodies, we further synthesized nine peptides in the PLAG1/2 and investigated the binding activities. As shown in Figure 2, D2-40 and AB3 produced similar results: hpp33-47, hpp31-47, hpp29-47, and hpp29-45 were strongly recognized by D2-40 and AB3, whereas hpp35-47 was weakly recognized by D2-40 and AB3. By contrast, other peptides were not detected by D2-40 and AB3. 18H5 reacted with hpp29-43, which was not recognized by D2-40 and AB3, indicating that the epitope of 18H5 differs from that of D2-40 and AB3. As expected, r2336 polyclonal anti-podoplanin antibody recognized only hpp23-36 among all of the peptides tested.

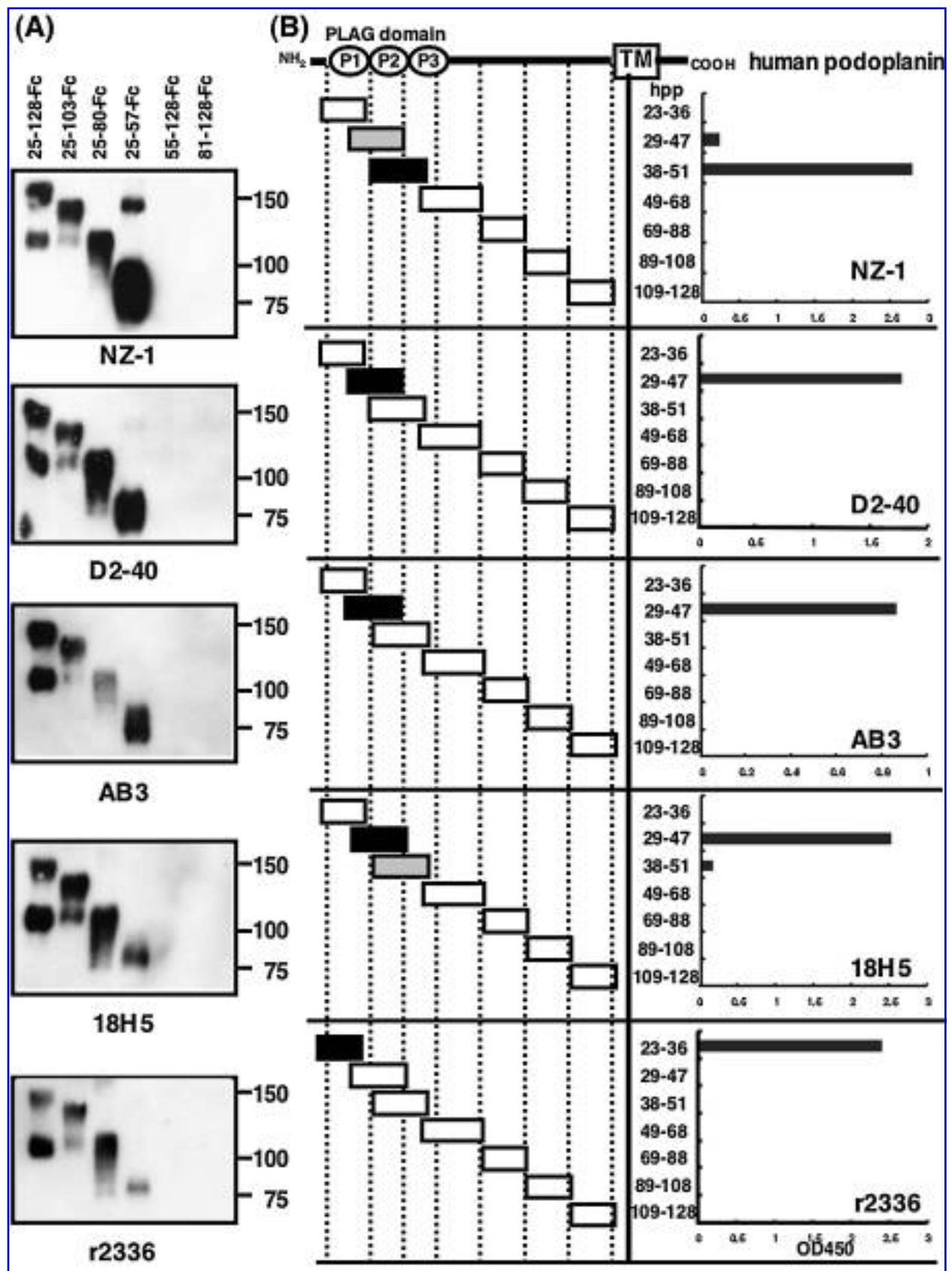


FIG. 1. Investigation of the epitope of five anti-podoplanin antibodies. (A) Western blot analysis of five anti-podoplanin antibodies. Purified podoplanin-Fc chimeras (0.1 μ g/lane) were electrophoresed under non-reducing conditions using 10–20% gels and Western-blotted with five anti-podoplanin antibodies. (B) Seven synthetic peptides corresponding to amino acids of the human podoplanin were immobilized on 96-well plates. After blocking with 1% BSA in PBS, the plates were incubated with anti-podoplanin antibodies, followed by peroxidase-conjugated anti-rat IgG, anti-mouse IgG, or anti-rabbit IgG antibodies. The enzymatic reaction was conducted with a substrate solution containing TMB. After the reaction was stopped with 1 M H₂SO₄, the optical density was measured at 450 nm. The reactivity was shown schematically. Solid bars, strong binding; gray bar, weak binding; open bars, no binding.

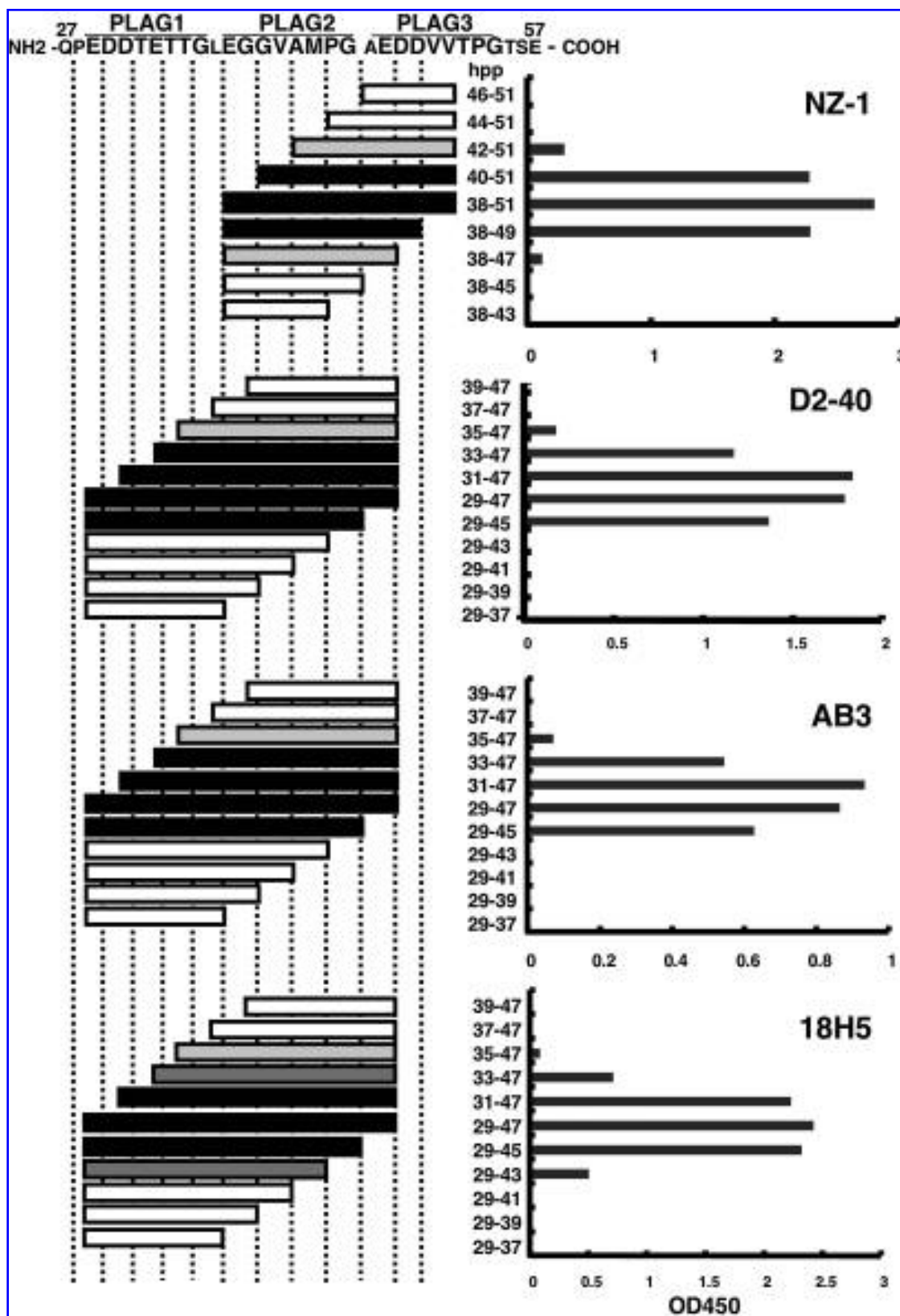


FIG. 2. Determination of the minimum epitope of the anti-podoplanin antibodies. Nine synthetic peptides (for NZ-1) or 11 synthetic peptides (for D2-40, AB3, and 18H5) corresponding to amino acids of the human podoplanin were immobilized on 96-well plates. After blocking with 1% BSA in PBS, the plates were incubated with anti-podoplanin antibodies, followed by peroxidase-conjugated anti-rat IgG or mouse IgG antibodies. The enzymatic reaction was conducted with a substrate solution containing TMB. After the reaction was stopped with 1 M H₂SO₄, the optical density was measured at 450 nm. The reactivity was shown schematically. Solid bars, strong binding; gray bar, weak binding; open bars, no binding.

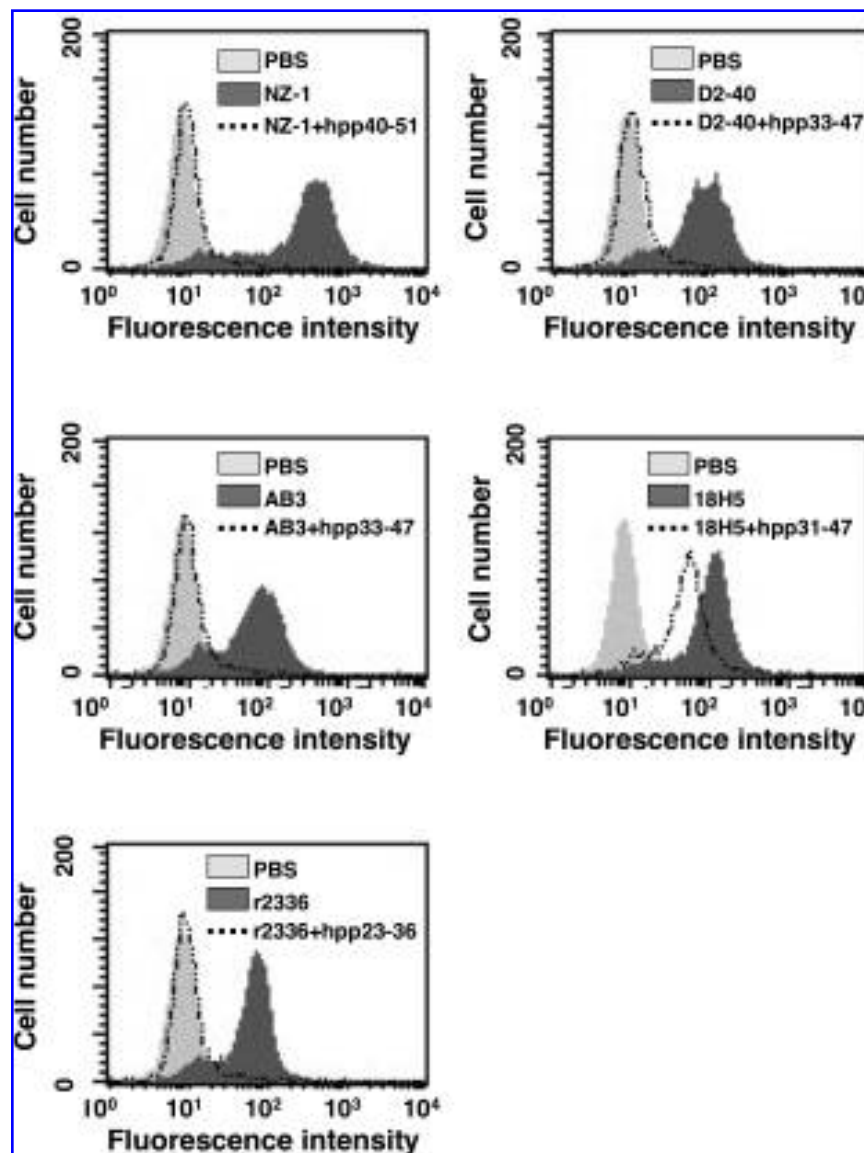


FIG. 3. Inhibition of anti-podoplanin antibody recognition of podoplanin by peptides. CHO/hPod cells were harvested by brief exposure to 1 mM EDTA. After washing with PBS, cells were treated with podoplanin antibodies (1 $\mu\text{g}/\text{mL}$) + peptides (100 $\mu\text{g}/\text{mL}$) or podoplanin antibodies (1 $\mu\text{g}/\text{mL}$) + PBS for 1 h at 4°C followed by treatment with FITC-conjugated anti-rat IgG, anti-mouse IgG, or anti-rabbit IgG antibodies. Fluorescence data were collected using a FACS Calibur flow cytometer.

Inhibition of anti-podoplanin antibody recognition of podoplanin by peptides

To confirm the specific epitope of anti-podoplanin antibodies, we performed flow cytometry using CHO transfected with human podoplanin (CHO/hPod). As shown in Figure 3, peptides containing each epitope could inhibit anti-podoplanin antibody recognition. Of the five anti-podoplanin antibodies, NZ-1 reacted most strongly with CHO/hPod, and the reaction was completely inhibited by the hpp40-51 peptide. The reactivity of D2-40 and AB3 to CHO/hPod was completely suppressed by the hpp33-47 peptide. On the other hand, the hpp31-47 peptide only partially inhibited the binding of 18H5 to CHO/hPod.

Inhibition of the podoplanin-CLEC-2 interaction by anti-podoplanin antibodies

We finally investigated the neutralizing activities of the five anti-podoplanin antibodies. As shown in Figure 4, NZ-1 partially inhibited the interaction between podoplanin and CLEC-2 expressed on 293T-Rex/CLEC-2 cells, whereas other antibodies did not. We have shown that podoplanin has twelve *O*-glycan attachment sites using Edman degradation and the total glycan structure of podoplanin was reported to be disialyl-core1 using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).⁽¹⁰⁾ Therefore, neutralization of the PLAG domain by NZ-1 is not sufficient to completely neutralize the interaction between podoplanin and CLEC-2, although we

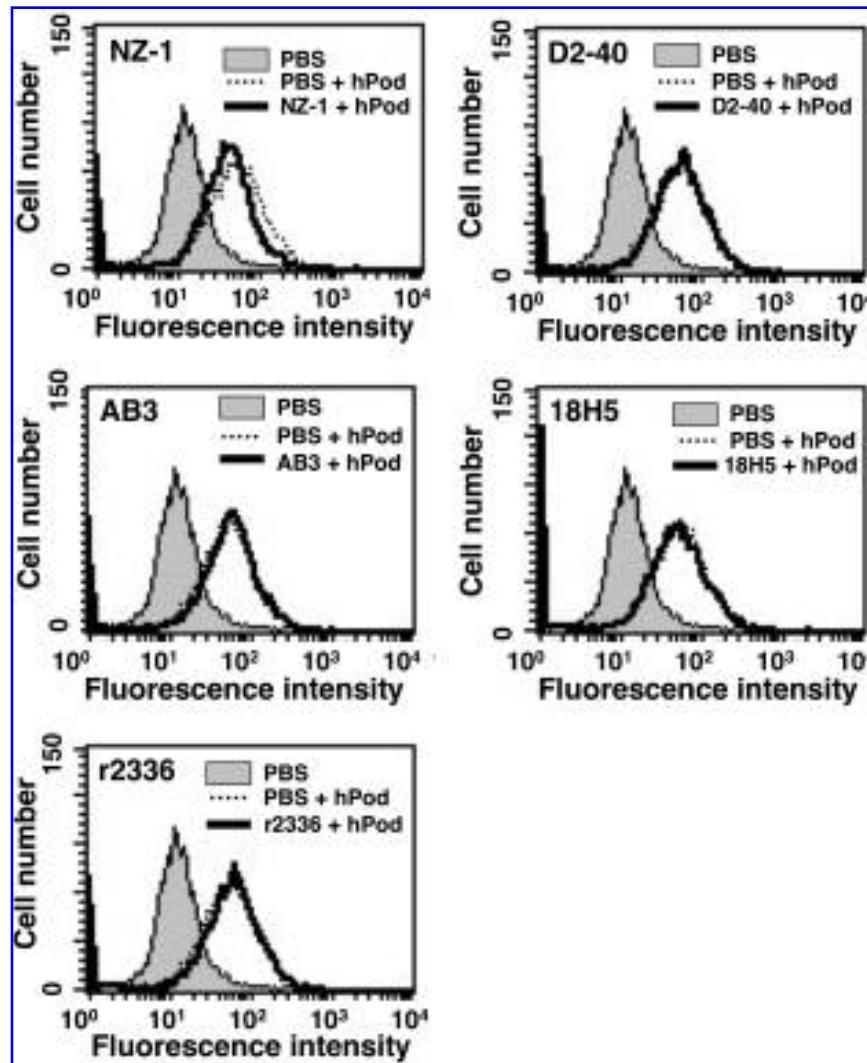


FIG. 4. Inhibition of the podoplanin-CLEC-2 interaction by podoplanin antibodies. Expression of CLEC-2 was induced by addition of 1 $\mu\text{g}/\text{mL}$ doxycycline to 293T-REx/CLEC-2 cells 24 h before experimentation. Cells were harvested by brief exposure to 1 mM EDTA. After washing with PBS, cells were treated with podoplanin antibodies (100 $\mu\text{g}/\text{mL}$) + hPod28-128-Fc (1 $\mu\text{g}/\text{mL}$) or PBS for 1 h at 4°C followed by treatment with FITC-conjugated anti-human Fc antibody (0.5 $\mu\text{g}/\text{mL}$). Fluorescence data were collected using a FACS Calibur flow cytometer.

previously reported that NZ-1 completely inhibited podoplanin-induced platelet aggregation.⁽³⁾ In this experiment, we could not show a difference between D2-40 and AB3, and neither antibody could neutralize the interaction of podoplanin and CLEC-2.

Discussion

Until recently, the absence of specific markers that can distinguish lymphatic from blood vascular endothelium has been a significant hindrance to the study of lymphangiogenesis. The identification of lymphatic vessels in routine histological sections has been a diagnosis of exclusion that relies on the absence of erythrocytes in the vessel lumen. Ultrastructurally, lymphatic vessels are identified by the presence of overlapping intercellular junctions, an absent or discontinuous basement membrane, and lack of pericytes.⁽¹⁶⁾

More recently, antibodies directed against Prox-1, podoplanin, and LYVE-1 have been established. Several anti-podoplanin antibodies are now commercially available; however, these antibodies have not been fully characterized.

We previously generated a novel anti-human podoplanin antibody (NZ-1), although at the time the minimum epitope was not defined.⁽³⁾ Other anti-podoplanin monoclonal antibodies, such as clones D2-40, AB3, and 18H5 are commercially available and have been used in many studies. For immunohistochemistry, various conditions have been used with the different monoclonal antibodies such as D2-40 and AB3; therefore, various results are reported.⁽¹⁵⁾ D2-40 was recently identified as anti-human podoplanin antibody.⁽³⁾ AB3 is commercially available from AngioBio (No. 11-003) and has been called merely an anti-podoplanin antibody. However, the identity of the antigen of D2-40 was unknown for some time (previously known as M2A antigen), and the clone

designation of AB3 is not shown in the AngioBio catalog.^(15,17) Several studies have discussed the difference between D2-40 and AB3, and the results have been controversial.^(17–23) In this study, we identified the epitope of these anti-podoplanin antibodies using ELISA, Western blot, and flow cytometry in an attempt to clarify the differences between these monoclonal antibodies.

We first produced six different podoplanin-Fc chimeras and synthesized 21 peptides (Table 2). NZ-1 recognized PLAG2/3 (Fig. 1). The minimum epitope of NZ-1 is six amino acids (AMPGAE), but 10 amino acids (GVAMP-GAEDD) are necessary for strong binding by NZ-1 (Fig. 2). On the other hand, D2-40, AB3, and 18H5 recognized PLAG1/2 (Fig. 1). The minimum epitope of D2-40 and AB3 is 11 amino acids (TGLEGGVAMPG), but 13 amino acids (ETTGLEGGVAMPG) are necessary for strong binding by D2-40 and AB3 (Fig. 2). The minimum epitope of 18H5 is nine amino acids (TGLEGGVAM), but 15 amino acids (DTETTGLEGGVAMPG) are necessary for strong binding by 18H5 (Fig. 2). These results suggest that D2-40 and AB3 have quite similar epitopes, which are different from the epitopes recognized by 18H5 and NZ-1. Although the NZ-1 epitope is a very small peptide (six amino acids), the minimum epitopes of other antibodies are larger (nine to 11 amino acids), probably because the immunizing antigens of D2-40, AB3 (not clarified), and 18H5 were endogenous antigens or recombinant proteins rather than synthesized peptides.

To compare the reactivity of D2-40 and AB3 in immunohistochemical reactions, lymphatic endothelial cells were stained with these antibodies using identical procedures (data not shown). Both D2-40 and AB3 showed similar staining of lymphatic endothelial cells without antigen retrieval. Several papers reported a difference in immunoreactivity between D2-40 and AB3, probably because D2-40 was usually used after antigen retrieval⁽¹⁵⁾; on the other hand, it has been reported that antigen retrieval was not necessary for staining with AB3.⁽⁴⁾

In summary, the epitope of NZ-1 is PLAG2/3; the epitope of D2-40, AB3, and 18H5 is PLAG1/2. The epitopes of D2-40 and AB3 are quite similar. In flow cytometry assays, NZ-1 partially inhibited the interaction between podoplanin and CLEC-2, while the other antibodies did not. The most frequently used two anti-podoplanin antibodies, D2-40 and AB3, have similar properties, although many studies have reported that they differ. NZ-1 neutralizes the interaction between podoplanin and CLEC-2, which may lead to the development of therapeutic antibodies against tumor-initiating cells (TICs)²⁴ or podoplanin-dependent cancer metastasis.

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