Inhibition of tumor cell-induced platelet aggregation using a novel anti-podoplanin antibody reacting with its platelet-aggregation-stimulating domain

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Abstract

The mucin-type sialoglycoprotein, podoplanin (aggrus), is a platelet-aggregating factor on cancer cells. We previously described up-regulated expression of podoplanin in malignant astrocytic tumors including glioblastoma. Its expression was associated with tumor malignancy. In the present study, we investigated podoplanin expression and platelet-aggregating activities of glioblastoma cell lines. First, we established a highly reactive anti-podoplanin antibody, NZ-1, which inhibits podoplanin-induced platelet aggregation completely. Of 15 glioblastoma cell lines, LN319 highly expressed podoplanin and induced platelet aggregation. Glycan profiling using a lectin microarray showed that podoplanin on LN319 possesses sialic acid, which is important in podoplanin-induced platelet aggregation. Interestingly, NZ-1 neutralized platelet aggregation by LN319. These results suggest that podoplanin is a main reason for platelet aggregation induced by LN319. We infer that NZ-1 is useful to determine whether platelet aggregation is podoplanin-specific or not. Furthermore, podoplanin might become a therapeutic target of glioblastoma for antibody-based therapy.
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Recent discovery of lymphatic endothelium markers such as vascular endothelial growth factor (VEGF) receptors [1] and lymphatic vessel endothelial hyaluronan receptor (LYVE-1) [2] has facilitated identification of lymphatic vessels. However, some of these markers are not expressed exclusively on lymphatic vessels. A mucin-type transmembrane sialoglycoprotein—podoplanin—is a highly expressed lymphatic specific gene in cultured human lymphatic endothelial cells (LECs) [3–5]. In human tissues, podoplanin expression is apparent only in lymphatic endothelial cells: not in vascular endothelial cells. More recently, human podoplanin has been demonstrated to be recognized specifically using monoclonal antibody D2-40 [4], which was produced originally against M2A antigen expressed by testicular germ cell tumors [6].

Podoplanin is reportedly expressed in many tumor cells such as squamous cell carcinoma [4,7–9], malignant mesothelioma [10,11], Kaposi's sarcoma, angiosarcoma [3], hemangioblastoma [12], testicular seminoma [13], dysgerminoma [4], and brain tumors [14–16]. Recent investigations have reported that podoplanin expression might be associated with tumor invasion, metastasis, or malignant progression [9,16].

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Mouse podoplanin/aggrus, a 44-kDa sialoglycoprotein with platelet aggregation-inducing ability, is expressed on the surface of mouse colon adenocarcinoma cells [17]. Antibody against mouse podoplanin/aggrus inhibits lung metastasis of NL-17 colon carcinoma cells in vivo [18]. Cloning of cDNA revealed that human podoplanin is identical to human aggrus, a separately isolated protein that can also induce mouse and human platelet aggregation [19]. Therefore, podoplanin might be involved in tumor cell-induced platelet aggregation and metastasis. Unique characteristics of Chinese hamster ovary (CHO) mutant cell lines Lec1, Lec2, and Lec8 revealed that sialylated *O*-glycan is critical for platelet aggregation-inducing activity [5].

Furthermore, we showed previously that podoplanin was expressed in astrocytic tumors [16]. In that report, 11 of 43 anaplastic astrocytomas (Grade III in astrocytic tumors: 25.6%) and in 54 of 115 glioblastomas (Grade IV in astrocytic tumors: 47.0%), podoplanin was expressed on the surface of anaplastic astrocytoma cells and glioblastoma cells, especially around necrotic areas and proliferating endothelial cells. On the other hand, podoplanin expression was not observed in diffuse astrocytoma (Grade II in astrocytic tumors: 0/30, 0%). These data suggest that podoplanin expression might be associated with malignancy of astrocytic tumors. However, platelet-aggregating activity of podoplanin in glioblastoma and its contribution to tumor malignancy have not been elucidated because of the lack of neutralizing antibody of podoplanin-induced platelet aggregation.

In this study, we produced a novel monoclonal antibody (NZ-1) against human podoplanin by immunizing rats with a platelet-aggregation-stimulating (PLAG) domain of podoplanin to neutralize podoplanin-induced platelet aggregation. Using this neutralizing antibody, we can determine whether platelet aggregation by cancer cells might be podoplanin-specific or not. Furthermore, we investigated glycan profiling of podoplanin on glioblastoma cell lines using a lectin microarray.

Materials and methods

Animals and Cell lines. Female SD rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Chinese hamster ovary (CHO), P3U1, and 15 glioblastoma cell lines (LN18, LN215, LN229, LNZ308, LN319, LN340, LN428, LN464, U87, U178, U251, U373, A1207, SF763, and T98G) were obtained from the American Type Culture Collection (ATCC). These cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ and 95% air in RPMI 1640 medium (for CHO and P3U1) or Dulbecco's modified Eagle's medium (DMEM; for glioblastoma cell lines) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY), and 100 μg/ml of kanamycin (Sigma Chemical Co.).

Hybridoma production. SD rats were immunized by neck s.c. injections of the synthetic peptide EGGVAMPGAEDDVV (hpp3851), corresponding to amino acids 38–51 of human podoplanin with Freund's Complete Adjuvant (Difco Laboratories, Detroit, MI). One week later, secondary i.p. immunization was performed. The booster injection was given i.p. 2 days before spleen cells were harvested. The spleen cells were fused with mouse myeloma P3U1 cells using polyethylene glycol (M_r 4000); the hybridomas were grown in RPMI medium with hypoxanthine,

aminopterin, and thymidine selection medium supplement (Sigma Chemical Co.). The culture supernatants were screened by ELISA for the binding to the synthetic peptide.

Specimens and tissue microarrays. In this study, we used the 642 cases of human tumors (Table 1). Tissue microarrays of brain tumors (132 cases: 22 diffuse astrocytoma, 29 anaplastic astrocytoma, and 81 glioblastoma), lung carcinoma (129 cases: 24 squamous cell carcinomas, 51 adenocarcinomas, 23 large cell carcinomas, and 31 small cell carcinomas), testicular tumors (13 cases), malignant melanoma (37 cases), esophageal squamous cell carcinoma (65 cases), stomach adenocarcinoma (72 cases), colon adenocarcinoma (66 cases), and rectal adenocarcinoma (57 cases) were purchased from Cybrdi, Inc. (Frederick, MD). Clinical information of patients (age, sex, grade, and pathology diagnosis) is obtainable from its home page. Furthermore, this study included 11 seminoma patients and 59 lung cancer patients (28 squamous cell carcinomas and 31 adenocarcinomas) who underwent surgery during 1990–2003 at Yamagata University Hospital (Yamagata, Japan). Informed consent was obtained from each patient before specimens were taken. The tumor specimens were fixed routinely in 10% buffered formalin for 18–20 h at room temperature and processed using paraffin. Sections (5 μm thick) were cut and attached to poly-L-lysine-coated glass slides. Hematoxylin-eosin was used as a routine staining.

Flow cytometry. Expression levels of human podoplanin were compared for confirmation using flow cytometry. Glioblastomas and transfected CHO cells, which were collected by trypsin-EDTA treatment, were incubated with NZ-1 (0.1 μg/ml) for 1 h at 4 °C. Then the cells were incubated with Oregon green-conjugated antibodies (Invitrogen Corp., Carlsbad, CA), for 30 min. Flow cytometry was performed using FACS Calibur (Becton–Dickinson).

Western-blot analysis. The cell lines were solubilized with lysis buffer (1% Triton in PBS) and electrophoresed under reducing conditions on 10–20% polyacrylamide gels. The separated proteins were transferred to a PVDF membrane. After blocking with 4% skim milk in PBS, the membrane was incubated with NZ-1 (a rat monoclonal antibody: 0.1 μg/ml), D2-40 (a mouse monoclonal antibody, 1/40 diluted; Signet Laboratories, Inc., Dedham, MA) or anti-β-actin antibody (a mouse monoclonal antibody: 1 μg/ml; Sigma Chemical Co.), and then with peroxidase-conjugated anti-rat or mouse antibodies (1/1000 diluted; Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) and developed for 1 min with ECL reagents (Amersham Pharmacia Biotech) using Kodak X-Omat AR film.

Immunohistochemistry. Immunohistochemical staining was performed by the avidin-biotinylated immunoperoxidase method. Briefly, 5-μm sections were deparaffinized and rehydrated. All the tissues were then exposed to 3% hydrogen peroxidase for 5 min. NZ-1 (1 μg/ml) was added to the

Table 1
Results of podoplanin staining by NZ-1 (642 cases)

Tumor type	No. of cases	Podoplanin immunostaining			
		+++	++	+	−
Brain tumor	132				
Diffuse astrocytoma	22	0	0	0	22
Anaplastic astrocytoma	29	3	2	1	23
Glioblastoma	81	23	8	5	45
Lung carcinoma	188				
Squamous cell carcinoma	52	2	7	5	38
Adenocarcinoma	82	0	0	0	82
Large cell carcinoma	23	0	0	0	23
Small cell carcinoma	31	0	0	0	31
Esophageal squamous cell carcinoma	65	5	3	6	51
Stomach adenocarcinoma	72	0	0	0	72
Colon adenocarcinoma	66	0	0	0	66
Rectal adenocarcinoma	57	0	0	0	57
Testicular seminoma	24	24	0	0	0
Malignant melanoma	38	0	0	0	38

sections for 1 h at room temperature. Biotin-conjugated secondary anti-rat IgG (DakoCytomation, Glostrup, Denmark) was incubated for 30 min at room temperature followed by the peroxidase-conjugated biotin-streptavidin complex (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature. Color was developed using 3,3-diaminobenzidine tetrahydrochloride tablet sets (DakoCytomation) for 3 min. The sections were counterstained with Mayer's hematoxylin. Podoplanin expression was semi-quantitatively assessed from the percentage of tumor cells with membrane staining: 0, no staining; +, <10%; ++, 10–50%; and +++, >50%.

Quantitative real-time PCR. Total RNAs were prepared from glioblastoma cell lines using an RNeasy mini prep kit (Qiagen Inc., Hilden, Germany). The initial cDNA strand was synthesized using SuperScript III transcriptase (Invitrogen Corp.) by priming nine random oligomers and an oligo(dT) primer according to the manufacturer's instructions. We performed PCR using the human podoplanin sense (5′-GGAAGGTGTC AGCTCTGCTC-3′), human podoplanin antisense (5′-CGCCTTCCAA ACCTGTAGTC-3′), human β-actin sense (5′-ACTCTTCCAGCCTT CCTTCCTG-3′), and human β-actin antisense (5′-ATCTCCTTCTGC ATCCTGTCGG-3′) oligonucleotides. Real-time PCR was carried out using the QuantiTect SYBR Green PCR (Qiagen Inc.). The PCR conditions were 95 °C for 15 min (1 cycle), followed by 40 cycles of 94 °C for 15 s, 53 °C for 20 s, 72 °C for 10 s for podoplanin, or 94 °C for 15 s, 55 °C for 25 s, 72 °C for 20 s for β-actin. Subsequently, a melting curve program was applied with continuous fluorescence measurement. Standard curves for podoplanin and β-actin templates were generated by serial dilution of the PCR products (1×10^8 copies/μl to 1×10^2 copies/μl). The expression levels of podoplanin were normalized by estimating the quantity of the β-actin transcript.

Platelet aggregation assay by WBA Carna. Heparinized mouse whole blood (WB) was drawn from BALB/c mice. Platelet aggregation was measured according to the screen filtration pressure method using WBA Carna (M.C. Medical) [20]. Two hundred microliters each of mouse whole blood samples and NZ-1 or control rat IgG in four reaction tubes was stirred at 1000 rpm at 37 °C and pre-incubated for 2 min, followed by addition of 12 μl each of cells (2×10^7 cells/ml). Using a 3.7-mm-diameter syringe containing screen microsieves made of nickel, with 300 openings of 20×20 μm^2 in a 1-mm-diameter area, WB samples were sucked to detect aggregation pressure at a rate of 200 μl/6.4 s 1–5 min later. The final platelet aggregation pressure of each reaction tube was determined at the pressure rate (%) of a pressure sensor connected to the syringe.

Lectin microarray. Lectin microarray was performed basically as described by Kuno et al. [21]. Interaction of podoplanin with the lectin immobilized on the glass slide was detected using biotinylated NZ-1-Cy3-streptavidin method to profile glycans of podoplanin. Briefly, podoplanin on LN319 solubilized with 1% Triton X-100 in PBS (PBSTx) was immunoprecipitated using NZ-1 antibody and then released with 100 μl of elution buffer containing the synthetic peptide, hpp3851, in PBS. Then 10 ng of podoplanin was diluted to 60 μl with PBSTx and applied to the lectin array containing triplicate spots of 43 lectin (see Supplementary Table 1 and Fig. 2D) into each of 8-divided incubation baths on the glass slide. After incubation at 20 °C for 12 h, the reaction solution was discarded. The glass slide was washed three times with PBSTx; 60 μl of biotinylated NZ-1 antibody (0.17 μg/ml) in PBS was applied to the array and then incubated at 20 °C for 3 h. After washing three times with PBSTx, Cy3-labeled streptavidin (GE Healthcare, UK) was added to the array and then incubated at 20 °C for 30 min. The glass slide was rinsed with PBSTx and scanned using an evanescent-field fluorescence scanner (GTMASScan III; Nippon Laser and Electronics Lab, Nagoya, Japan).

Results and discussion

Production of a novel monoclonal antibody against podoplanin

By immunizing rats with platelet-aggregation-stimulating (PLAG) domain of podoplanin, we newly generated a monoclonal antibody, NZ-1, which can specifically recognize human podoplanin. The NZ-1 stained lymphatic vessels (Fig. 1A), esophageal squamous cell carcinoma (Fig. 1B), and testicular seminoma (Fig. 1C) without antigen retrieval. As summarized in Table 1, using 642 tumors, NZ-1 stained astrocytic tumors, lung or esophageal squamous cell carcinomas, and testicular seminomas. However, stomach adenocarcinoma, colorectal adenocarcinoma, and malignant melanoma were not stained by NZ-1. Western-blot analysis showed that NZ-1 recognized two bands: 36-kDa band with a strong signal and 25-kDa band with a weak signal; 25-kDa band might be de- or non-glycosylated form of podoplanin (Fig. 1D). In addition, D2-40, another anti-human podoplanin antibody, similarly detected 36-kDa band of podoplanin. The NZ-1 binding to human podoplanin was neutralized by the synthetic peptide hpp3851, whereas D2-40 binding was not (Fig. 1D). Furthermore, NZ-1 strongly recognized podoplanin expressed in podoplanin-transfected CHO cells (CHO/pod) using flow cytometric analysis (Fig. 1E).

In our previous studies, CHO/pod demonstrated its capability to induce platelet aggregation of both humans and mice, whereas CHO did not [19]. We determined the PLAG domain of podoplanin; PLAG domain was repeated three times [22]. Because NZ-1 antibody was produced by immunizing PLAG domain, we then checked the inhibitory effect of NZ-1 against podoplanin-induced platelet aggregation. Results showed that NZ-1 inhibited platelet aggregation by CHO/pod in a dose-dependent manner; 10 μg of NZ-1 suppressed its platelet aggregation completely (Fig. 1F), whereas control rat IgG did not (data not shown). Furthermore, another anti-podoplanin antibody, D2-40, did not neutralize the platelet aggregation by CHO/pod (data not shown), probably because D2-40 did not recognize PLAG domain including hpp3851 (Fig. 1D). Using this neutralizing antibody, it became possible to determine whether the platelet aggregation by cancer cells might be podoplanin-specific or not.

Expression of podoplanin in glioblastoma cell lines

In a previous study, podoplanin was expressed in malignant astrocytic tumors [16]. We performed Western-blot and flow cytometry using NZ-1 against 15 glioblastoma cell lines to investigate podoplanin expression in glioblastoma cell lines. Fig. 2A shows that podoplanin was highly expressed in one glioblastoma cell line, LN319, and slightly expressed in LN215, LNZ308, U87, U178, U251, and A1207. Mobility shift of podoplanin band was observed in Western-blot, probably because the glycosylation pattern varied among these cell lines. The podoplanin expression in Western-blot was consistent with those of flow cytometry, except for LN215 (Fig. 2B). The reason why podoplanin on LN215 was not detected by NZ-1 in flow cytometry has not been clarified, although it was detected in Western-blot; podoplanin might not be expressed properly on its cell membrane. Furthermore, real-time PCR
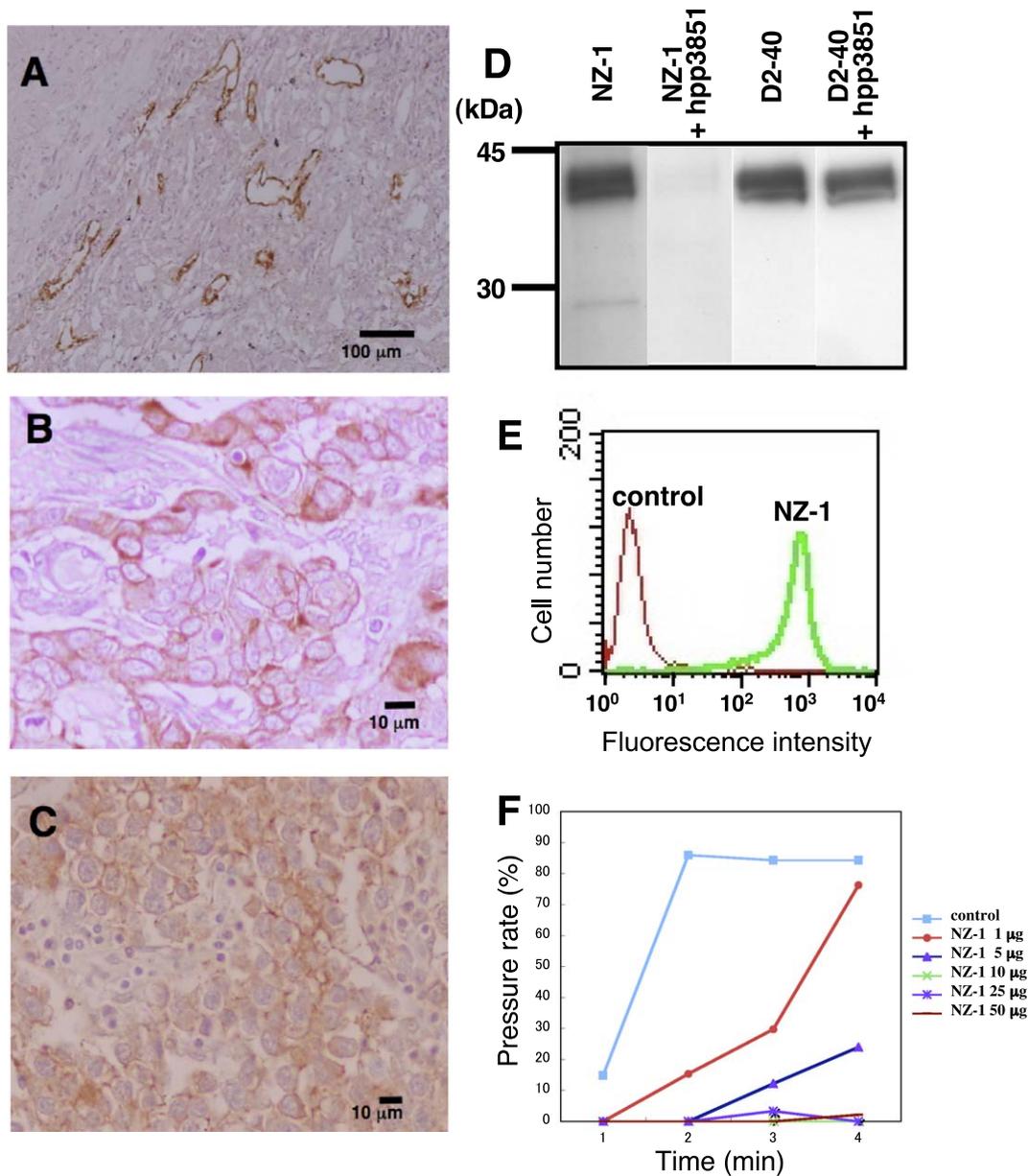
Fig. 1. Characterization of NZ-1, a novel monoclonal antibody against podoplanin. (A–C) Immunohistochemical staining with NZ-1 on human specimens derived from normal lung (A), esophageal carcinoma (B), and testicular seminoma (C). (D) The cell lysate of CHO-human podoplanin (CHO/pod) was electrophoresed and immunoblotted using NZ-1 (1 μg/ml), NZ-1 (0.1 μg/ml) + hpp3851 (100 μg/ml), D2-40 (1/40 diluted), or D2-40 (1/40 diluted) + hpp3851 (100 μg/ml). (E) Flow cytometric analyses of NZ-1 to CHO/pod. (F) Platelet aggregation assay. Heparinized mouse whole blood (WB) was drawn from BALB/c mice. Platelet aggregation was measured using WBA Carna with the screen filtration pressure method. Two hundred microliters each of mouse whole blood samples and NZ-1 (1, 5, 10, 25, or 50 μg/ml) was pre-incubated for 2 min, followed by the addition of 12 μl each of CHO/pod cells (2×10^7 cells/ml). Whole blood samples were sucked 1–5 min later to detect aggregation.

analysis was performed to confirm these results (Fig. 2C). Results showed that podoplanin mRNA was also expressed highly in LN319 and expressed slightly in LN215, LNZ308, LN428, U87, U178, U251, U373, and A1207.

Glycan profiling of podoplanin on LN319 using lectin microarray

Previously, podoplanin was expressed stably in a series of CHO cell mutants: *N*-glycan-deficient Lec1, CMP-sialic

acid transporter-deficient Lec2, and UDP-galactose transporter-deficient Lec8 [5]. Podoplanin on Lec1 cells induced platelet aggregation, but those on Lec2 and Lec8 cells did not. Furthermore, podoplanin expressed in CHO and Lec1 cells showed *Wheat-germ* agglutinin (WGA) and Jacalin bindings, but not *Vicia Villosa* lectin (VVA). On the other hand, podoplanin on Lec2 showed VVA and peanut agglutinin (PNA) binding, but not WGA binding. Lectin blotting results indicated that sialylated core1 structures, sialic acid + Galβ1,3GalNAc-Ser/Thr, were critical for platelet aggregation activity.
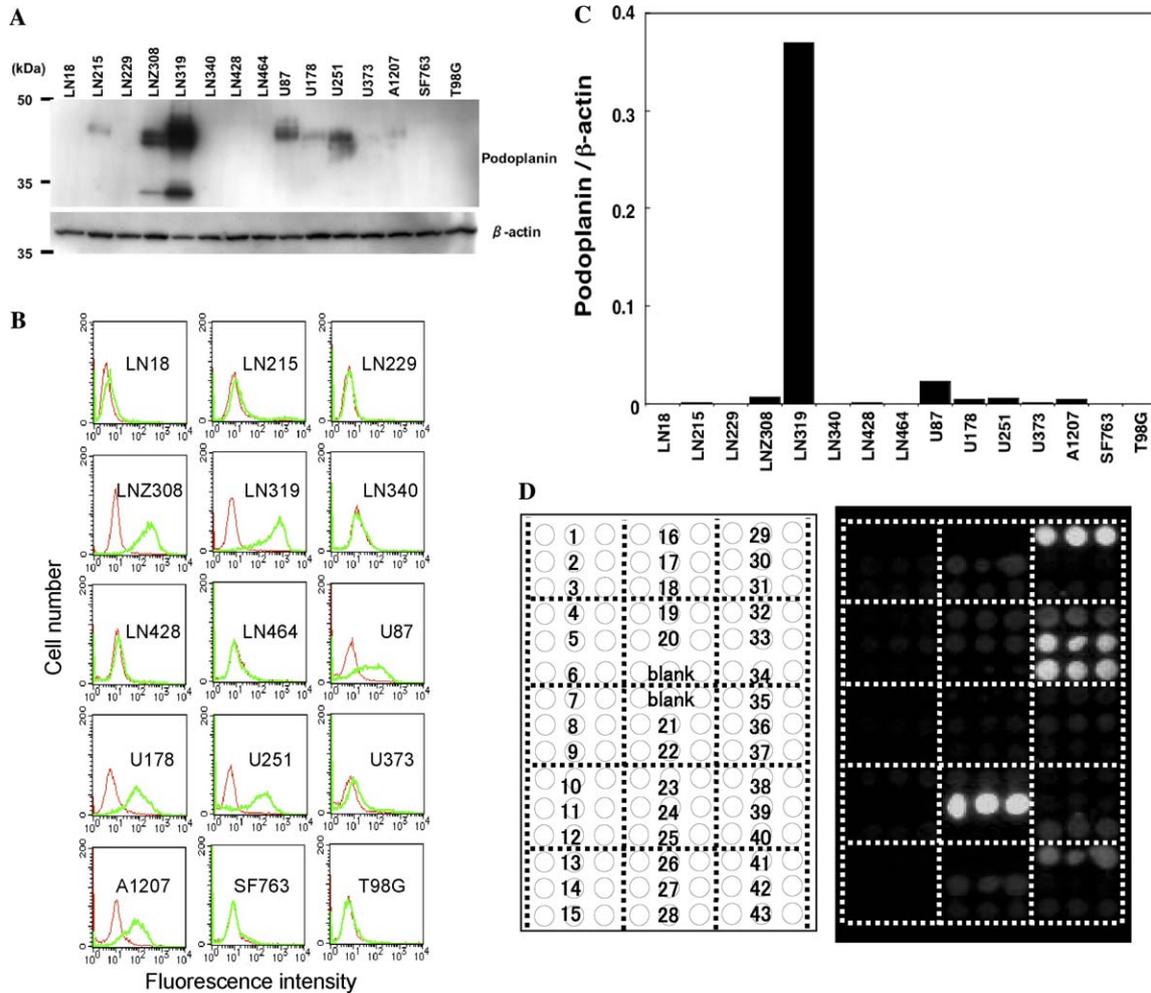
Fig. 2. (A) Western-blot analyses of glioblastoma cell lines using NZ-1. The cell lysates of 15 glioblastoma cell lines were electrophoresed and immunoblotted with NZ-1 and anti-β-actin. (B) Flow cytometric analyses of NZ-1 to glioblastoma cell lines. (C) Quantitative analysis of podoplanin transcripts in glioblastoma. First-strand cDNA samples derived from 15 glioblastoma cell lines were used as real-time PCR templates. Respective expression levels of podoplanin were normalized to that of the β-actin transcript, as described in Materials and methods. (D) Glycan profiling of LN319 using lectin microarray. Podoplanin on LN319 immunoprecipitated using NZ-1 antibody was applied to the lectin array containing triplicate spots of 43 lectins (Supplementary Table 1). The podoplanin that was bound to the immobilized lectins was detected using biotinylated NZ-1 antibody and Cy3-labeled streptavidin. Positive signals; 24, ABA; 29, Jacalin; 32, ACA; 33, MPA; 34, HPA; 40, MAH; and 41, WGA.

The present study investigated glycan profiling of podoplanin on LN319 using the lectin microarray (see Fig. 2D and Supplementary Table 1) with the detection method by biotinylated NZ-1 antibody and Cy3-labeled streptavidin. Fig. 2D shows that podoplanin on LN319 reacted strongly with core1 ± sialic acid binders (*Agaricus bisporus* agglutinin (ABA), Jacalin, *Amaranthus caudatus* agglutinin (ACA), and *Maclura pomifera* agglutinin (MPA)), sialo-mucin binders (*Maackia amurensis* hemagglutinin (MAH) and WGA), and alpha-GalNAc binder (*Helix pomatia* agglutinin (HPA)); it did not react with core1 binders (*Bauhinia purpurea alba* lectin (BPL) and PNA). On the other hand, in the case of asialo-podoplanin treated with sialidase A, the signals were observed on BPL and PNA spots instead of the loss of signals on MAH and WGA spots; HPA signals were increased after treatment of sialidase A (data not shown). These results indicate that

podoplanin on LN319 also possesses disialyl-T antigen, sialyl-T antigen, and/or sialyl-Tn antigen, and have the platelet-aggregating activity. Although a significant signal was observed on HPA spot, another Tn antigen binder, VVA, did not react with podoplanin. We recently confirmed that HPA reacts with Tn antigen much stronger than VVA on lectin microarray (data not shown). These data suggest that sialyl-Tn antigens on podoplanin expressed in LN319 might be partially de- or non-sialylated.

Platelet aggregation by glioblastoma cell lines

Tumor cells can activate platelets via several pathways: tumor cell-induced thrombin generation through a coagulation pathway [23], releasing ADP [24], thromboxane A_2 (TXA_2) [25], MMP-2 [26], and a membranous pro-
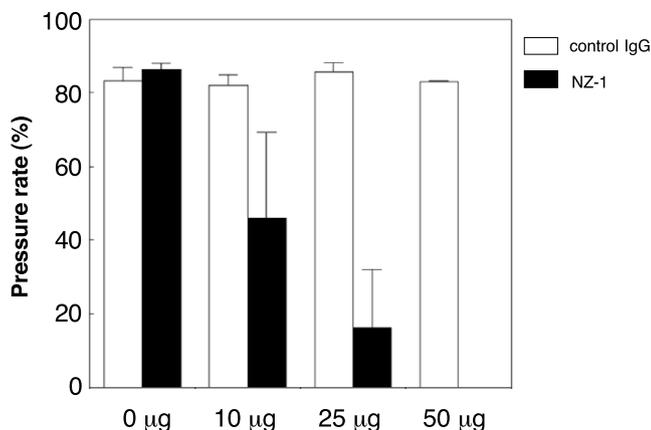
Fig. 3. Platelet aggregation assay. Heparinized mouse whole blood (WB) was drawn from BALB/c mice. Platelet aggregation was measured using WBA Carna with the screen filtration pressure method. Two hundred microliters each of mouse whole blood samples and NZ-1 or control IgG (0, 10, 25, or 50 μg/ml) was stirred at 1000 rpm at 37 °C, and pre-incubated for 2 min, followed by the addition of 12 μl each of LN319 cells (2×10^7 cells/ml). Whole blood samples were sucked 2 min later to detect aggregation. Data are means ± SD of three independent experiments.

tein Aggrus/podoplanin [19]. In our previous studies, CHO/pod was able to induce platelet aggregation, whereas CHO did not [19]. Fig. 2 shows that LN319 extraordinarily expressed podoplanin among 15 glioblastoma cell lines. Therefore, we investigated platelet-aggregating activity by LN319. Results showed that LN319 was able to induce platelet aggregation; this aggregation was inhibited by NZ-1 antibody in a dose-dependent manner, whereas control rat IgG did not (Fig. 3). These results indicate that platelet aggregation by LN319 was attributable to high expression of podoplanin on its cell membrane.

In summary, we produced a novel anti-podoplanin antibody that can specifically neutralize podoplanin-induced platelet aggregation. This neutralizing antibody enabled determination of whether platelet aggregation induced by cancer cells is podoplanin-specific or not. Using this antibody, we demonstrated that glioblastoma cells expressed podoplanin and possess high platelet-aggregating activity; they might be involved in tumor thrombosis. Podoplanin might become a promising therapeutic target for antibody-based therapy.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.08.171.

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