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Chimeric Anti-Human Podoplanin Antibody NZ-12 of Lambda Light Chain Exerts Higher Antibody-Dependent Cellular Cytotoxicity and Complement-Dependent Cytotoxicity Compared with NZ-8 of Kappa Light Chain

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Podoplanin (PDPN), a type I transmembrane 36-kDa glycoprotein, is expressed not only in normal cells, such as renal epithelial cells (podocytes), lymphatic endothelial cells, and pulmonary type I alveolar cells, but also in cancer cells, including brain tumors and lung squamous cell carcinomas. Podoplanin activates platelet aggregation by binding to C-type lectin-like receptor-2 (CLEC-2) on platelets, and the podoplanin/CLEC-2 interaction facilitates blood/lymphatic vessel separation. We previously produced neutralizing anti-human podoplanin monoclonal antibody (mAb), clone NZ-1 (rat IgG_{2a}, lambda), which neutralizes the podoplanin/CLEC-2 interaction and inhibits platelet aggregation and cancer metastasis. Human–rat chimeric antibody, NZ-8, was previously developed using variable regions of NZ-1 and human constant regions of heavy chain (IgG_1) and light chain (kappa chain). Although NZ-8 showed high antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against human podoplanin-expressing cancer cells, the binding affinity of NZ-8 was lower than that of NZ-1. Herein, we produced a novel human–rat chimeric antibody, NZ-12, the constant regions of which consist of IgG_1 heavy chain and lambda light chain. Using flow cytometry, we demonstrated that the binding affinity of NZ-12 was much higher than that of NZ-8. Furthermore, ADCC and CDC activities of NZ-12 were significantly increased against glioblastoma cell lines (LN319 and D397) and lung cancer cell line (PC-10). These results suggested that NZ-12 could become a promising therapeutic antibody against podoplanin-expressing brain tumors and lung cancers.

Keywords: human podoplanin, chimeric antibody, monoclonal antibody, lambda chain, kappa chain

Introduction

A type I transmembrane 36-kDa glycoprotein, podoplanin (PDPN/Aggrus/T1α/gp36), is a platelet aggregation-inducing factor, and its expression has been reported in many cancers such as malignant brain tumors, mesotheliomas, and several squamous cell carcinomas.[1–10] Expression of podoplanin has been reported to be associated with cancer metastasis, epithelial-mesenchymal transition, malignant progression,[11–18] and clinical outcome.[19–21] Only a small and phenotypically distinct subset of cells in solid tumors is considered as tumor-initiating cells (TICs),[22] which are resistant to conventional therapies and responsible for relapse. Therefore, targeting TICs could be a useful approach for cancer therapy.[23] Podoplanin has been reported to be a TIC marker[24]; immunotherapy using specific antibodies reactive to podoplanin may eradicate TICs in cancers.

We previously produced a rat anti-human podoplanin monoclonal antibody (mAb), NZ-1.[5] NZ-1 possesses high specificity, high sensitivity, and high binding-affinity against podoplanin.[25,26] Because NZ-1 is highly internalized into glioma cell lines, and is also well accumulated into tumors *in vivo,* NZ-1 is a suitable candidate for therapy against malignant gliomas.[27] By its neutralizing activity, NZ-1 also inhibited the tumor cell-induced platelet aggregation and cancer metastasis.[12] We constructed a single-chain antibody variable region fragment using NZ-1 (NZ-1-scFv).[28] NZ-1-scFv was then fused to Pseudomonas exotoxin A (NZ-1-

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scFv-PE38). NZ-1-scFv-PE38 exhibited significant activity against glioblastoma and medulloblastoma cells, and demonstrated tumor growth delay in D2159MG and D283MED *in vivo* tumor models.

Furthermore, we produced a human–rat chimeric anti-podoplanin antibody (NZ-8) from NZ-1.[29] NZ-8 possesses high antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Furthermore, NZ-8 inhibits the growth of podoplanin-expressing tumors *in vivo*, and also suppresses hematogenous metastasis of podoplanin-expressing tumors. In contrast, the binding affinity of NZ-8 is lower than that of NZ-1.

Herein, we produced a novel human–rat chimeric antibody, NZ-12, the constant regions of which consist of IgG_1 heavy chain and lambda light chain.

Materials and Methods

Cell lines

CHO-K1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). LN319 and D397 were provided by Dr. Kazuhiko Mishima (Saitama Medical University, Saitama, Japan) and Dr. Darell D. Bigner (Duke University Medical Center, Durham, NC), respectively. PC-10 cells were purchased from Immuno-Biological Laboratories Co., Ltd. (Gunma, Japan). CHO-K1 and PC-10 were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), and LN319 and D397 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Antibodies

A rat anti-human podoplanin mAb (NZ-1) and a human–rat chimeric anti-human podoplanin antibody (NZ-8) were developed as described previously.[5,27,29,30] For the generation of human–rat chimera anti-human podoplanin (NZ-12), the appropriate V_H of a rat NZ-1 antibody and C_H of human IgG_1 were subcloned into the pCAG-Neo (Wako Pure Chemical Industries Ltd., Osaka, Japan), and V_L of a rat NZ-1 antibody and C_L of human lambda chain were subcloned into pCAG-Ble vectors (Wako Pure Chemical Industries Ltd.). Antibody expression vectors were transfected into CHO-K1 cells using Lipofectamin LTX (Thermo Fisher Scientific, Inc.). Stable transfectants of CHO/NZ-12 were selected by cultivating the transfectants in medium containing 1 mg/mL G418 (Wako Pure Chemical Industries Ltd.) and 0.5 mg/mL Zeocin (InvivoGen, San Diego, CA). CHO/NZ-12 cells were cultivated in CHO-S-SFM II medium (Thermo Fisher Scientific, Inc.). NZ-12 was purified using Protein G-Sepharose (GE healthcare Bio-Sciences, Pittsburgh, PA). Human IgG was purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Determination of the binding affinity by flow cytometry

LN319 cells (2×10^5 cells) were resuspended with 100 μL of serially diluted antibodies (NZ-1, NZ-8, or NZ-12; 0.02–100 μg/mL) followed by FITC-labeled anti-human IgG or Oregon green-labeled anti-rat IgG (Thermo Fisher Scientific, Inc.). Fluorescence data were collected using a cell analyzer (EC800; Sony Corp.). The dissociation constants (K_D) were obtained by fitting the binding isotherms using the built-in one-site binding models in Prism software.

Preparation of effector cells

The preparation methods of effector cells have been described previously.[31] Human peripheral blood mononuclear cells (MNCs) were obtained from leukocytes, which were separated from peripheral blood of healthy donors. The human study was approved by the Ethics Committee of Tokushima University.

Antibody-dependent cellular cytotoxicity

ADCC was determined with ^{51}Cr release assay.[31,32] Target cells were incubated with 0.1 μCi of ^{51}Cr-sodium chromate at 37°C for 1 hour. After washing with CRPMI1640 (RPMI1640 medium including 10% FBS) three times, ^{51}Cr-labeled target cells were placed in 96-well plates in triplicate. Effector cells and antibodies were added to the plates. After 6 hours incubation, ^{51}Cr release of the supernatant from each well (100 μL) was measured using a gamma counter (Perkin-Elmer, Waltham, MA). The percentage of cytotoxicity was calculated using the following formula: % Specific lysis = $(E-S)/(M-S) \times 100$, where E is the release in the test sample, S is the spontaneous release, and M is the maximum release.

Complement-dependent cytotoxicity

CDC was evaluated by ^{51}Cr release assay as described previously.[31,32] Target cells were incubated with ^{51}Cr-sodium chromate (0.1 μCi) for 1 hour at 37°C. After that, the cells were washed with CRPMI1640. ^{51}Cr-labeled cells were incubated with baby rabbit complement (Cedarlane, Ontario, Canada) at a dilution of 1:4 and antibodies (1 μg/mL) for 6 hours in 96-well plates. After incubation, the supernatant including ^{51}Cr was measured using a gamma counter. The percentage of cytotoxicity was calculated as described above.

Statistical analyses

The statistical significance of differences in *in vitro* data was analyzed by standard Student's *t*-test. In this study, *p*-values less than 0.05 were considered significant in all experiments.

Results and Discussion

Development of a novel human–rat chimeric anti-podoplanin antibody (NZ-12)

The sensitivity of NZ-8 is lower than that of NZ-1.[29] NZ-8 was generated by fusing the V_H of rat antibody (NZ-1) with C_H of human IgG_1 and fusing V_L regions of NZ-1 with C_L of human kappa because almost all chimeric antibodies and humanized antibodies are comprised of kappa light chains.[33,34] In contrast, NZ-1 possesses lambda light chain, the rare isotype found among rat IgGs (<5%).[35] Therefore, we subsequently generated the human–rat chimeric antibody (NZ-12) by fusing the V_H of rat antibody (NZ-1) with C_H of human IgG_1 and fusing V_L regions of NZ-1 with C_L of human lambda.

NZ-12 demonstrated high sensitivity against podoplanin-expressing cancer cell lines in flow cytometry, and its reactivity was shown to be much higher than that of NZ-8 (data

not shown). K_D of NZ-1 was determined to be 1.2×10^{-9} M by flow cytometry (Fig. 1). Using the same methods, K_D of NZ-8 was determined to be 7.3×10^{-7} M (Fig. 1), indicating that the binding affinity of NZ-8 was diminished following conversion from rat mAb to a human–rat chimeric antibody. In contrast, K_D of NZ-12 was determined to be 8.5×10^{-9} M by flow cytometry (Fig. 1), indicating that the binding affinity against a podoplanin-expressing cell line (LN319) became much higher following conversion from a human kappa light chain (NZ-8) to a human lambda light chain (NZ-12).

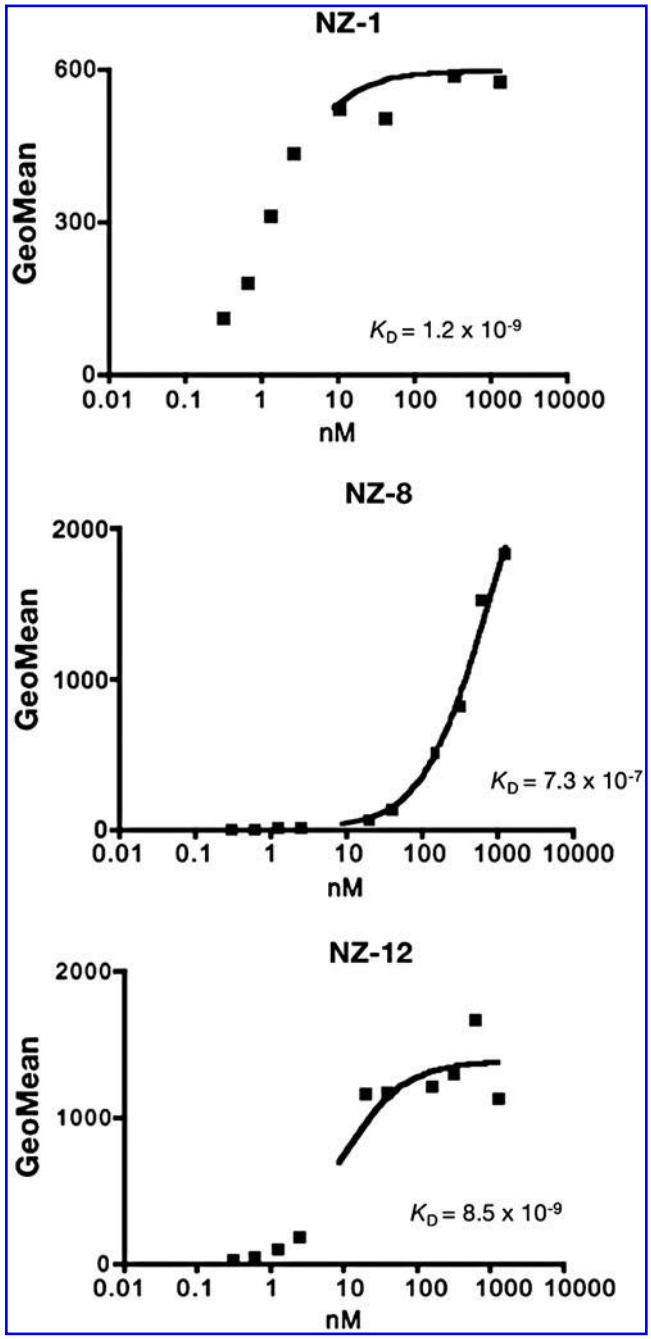

FIG. 1. Determination of binding-affinity using flow cytometry. LN319 (2×10^5 cells) was resuspended at 100 μL of serially diluted NZ-1, NZ-8, or NZ-12 (0.02–100 μg/mL).

ADCC and CDC mediated by anti-podoplanin antibodies

To apply targeted therapy to podoplanin, we further assessed whether anti-podoplanin antibodies could induce ADCC against podoplanin-expressing cell lines mediated by human MNC as effector cells. We compared the ADCC and CDC activities against NZ-1, NZ-8, and NZ-12 using LN319, D397, and PC-10 cell lines. ADCC against podoplanin-expressing cell lines was not exhibited by NZ-1 using human MNC, whereas NZ-8 and NZ-12 showed the induction of significant level of ADCC mediated by human MNCs against LN319 and D397 glioblastoma cells and PC-10 lung cancer cells (Fig. 2A). Furthermore, ADCC by NZ-12 was higher compared with NZ-8 particularly against LN319 and D397 glioblastoma cells. Interestingly, CDC was induced by

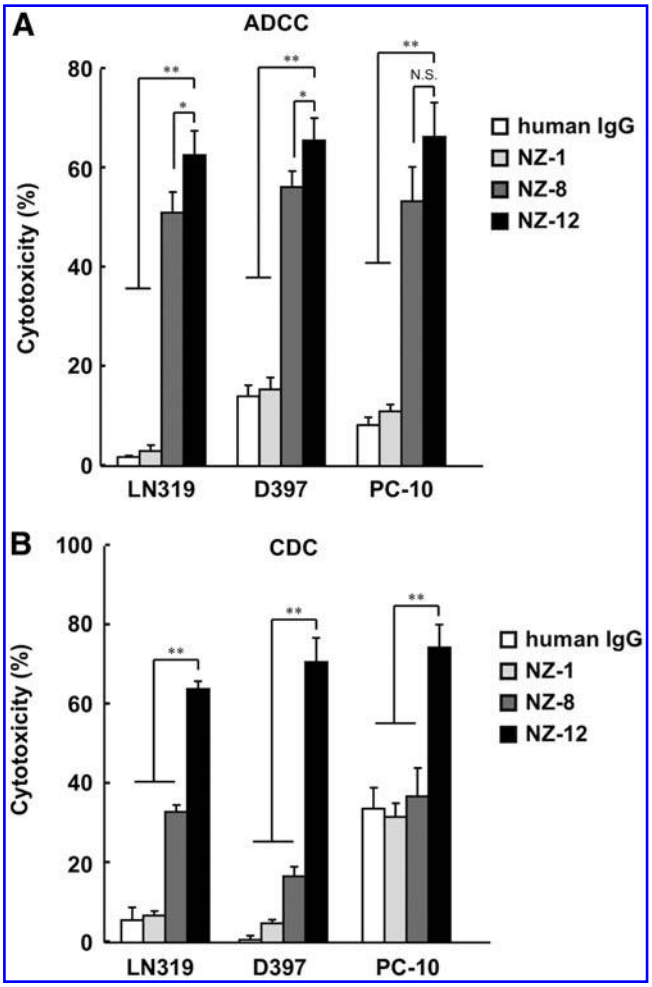

FIG. 2. ADCC and CDC activities of anti-podoplanin antibodies. **(A)** ADCC activities induced by human MNC against podoplanin-expressing cell lines LN319, D397, and PC-10 were determined with 6 hours ^{51}Cr release assay at the E/T ratio of 100 in the presence of 1 μg/mL of NZ-1, NZ-8, NZ-12, and human IgG. **(B)** CDC activities against podoplanin-expressing cell lines LN319, D397, and PC-10 were determined by ^{51}Cr release assay. $^{**}p < 0.01$, $^{*}p < 0.05$ versus control (values are means ± S.E.). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; MNC, mononuclear cell.

NZ-12 against all podoplanin-expressing cell lines, and this was significantly higher than that of NZ-8 (Fig. 2B).

Taken together, NZ-12 could be useful for antibody therapy against human podoplanin-expressing cancers. In the future, NZ-1/NZ-12 could be further applied to the novel anti-tumor reagents, including T cells and viruses.[36,37]

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Author Disclosure Statement

No competing financial interests exist.

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