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A Defucosylated Mouse Anti-CD10 Monoclonal Antibody (31-mG_{2a}-f) Exerts Antitumor Activity in a Mouse Xenograft Model of Renal Cell Cancers

Hiroki Kawabata,[1,*] Tomokazu Ohishi,[2,*] Hiroyuki Suzuki,[3] Teizo Asano,[4] Manabu Kawada,[2] Hiroyoshi Suzuki,[5] Mika K. Kaneko,[4] and Yukinari Kato[3,4]

CD10 is a cell surface metalloendopeptidase that cleaves and degrades many secreted physiologically active peptides by its enzymatic activity. Although CD10 expression has been found in various types of cells, its expression is increased in several cancers, including renal cancer. In this study, the antitumor activity of a novel anti-human CD10 monoclonal antibody (mAb) was investigated. A defucosylated mouse IgG_{2a} version of C_{10}Mab-31 (31-mG_{2a}-f) was created from an anti-CD10 mAb, C_{10}Mab-31 (IgG_1, kappa). Both C_{10}Mab-31 and 31-mG_{2a}-f specifically reacted with endogenous CD10 in renal cancer cells, VMRC-RCW, with the dissociation constant (K_D) values of 6.3×10^{-9} M and 1.1×10^{-9} M, respectively, indicating high binding affinity. To further examine the anti-CD10 mAb-mediated effector functions, the antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) were examined. The 31-mG_{2a}-f significantly exhibited ADCC and CDC against VMRC-RCW cells *in vitro*. Furthermore, 31-mG_{2a}-f exhibited antitumor activities in mouse xenografts of VMRC-RCW cells. These results suggest that 31-mG_{2a}-f exerts antitumor activities against CD10-expressing renal cancers and could be a valuable therapeutic candidate for treating them.

Keywords: CD10, monoclonal antibody, ADCC, CDC, antitumor activity

Introduction

Renal cell carcinomas (RCCs), which originate from the renal epithelium of the renal tubules, account for more than 90% of cancers in the kidney.[1] RCCs are the 6th most common cancer among men and the 9th most common cancer among women in the United States,[2] and the 14th most common cancer worldwide.[3] Treatment strategies for patients with RCCs have been expanding recently with the advent of immune checkpoint blockade by programmed cell death-1 (PD-1)/PD-1 ligand 1 (PD-L1) inhibitors or cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) inhibitors.[4,5]

Although cytotoxic T cells play a vital role in antitumor immunity, PD-1 negatively regulates the T cell function. Its ligand PD-L1 is highly expressed by cancer cells; the inhibition of the PD-1–PD-L1 axis causes cytotoxic T cell activation, which leads to tumor cell mortality. Similarly, CTLA-4 also degrades cytotoxic T cell function; the inhibition of its function causes the T cell activation.[1] However, their therapeutic efficacy is limited, and more effective treatment options are required.

CD10 is a member of cell surface glycoproteins known as membrane metalloendopeptidase, neprilysin, neutral endopeptidase, enkephalinase, EC3.4.24.11, and first identified as a tumor-specific antigen (common acute lymphoblastic leukemia antigen) in leukemia.[6–8] CD10 comprises the three following domains: a short cytoplasmic N-terminal domain, a transmembrane hydrophobic domain, and a large

[1]Research and Development Center, Fuso Pharmaceutical Industries, Ltd., Osaka, Japan.
[2]Institute of Microbial Chemistry (BIKAKEN), Numazu, Microbial Chemistry Research Foundation, Numazu-shi, Japan.
Departments of [3]Molecular Pharmacology and [4]Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.
[5]Department of Pathology and Laboratory Medicine, Sendai Medical Center, Sendai, Japan.
*These two authors contributed equally to this work.

extracellular domain with catalytic activity. It is a membrane-bound zinc-dependent metalloprotease that degrades many secreted physiologically active peptides, leading to the inactivation of several signaling pathways.[9] Altered expression of CD10 has been seen in some hematological malignancies, such as Burkitt lymphomas, acute lymphoblastic leukemia, T and B cell lymphomas, and solid tumors.[8,10–12] Therefore, CD10 has been used as useful diagnostic markers for leukemia, lymphoma, and various solid tumors.[8]

Accumulating evidence suggests a role for CD10 in the development and prognosis of various tumors. Elevated expression of CD10 has been reported in multiple tumors, including renal cancer,[13] lung cancer,[14] prostate cancer,[15,16] papillary thyroid cancer,[17] head and neck cancer,[18] colorectal cancer,[19] esophageal cancer,[20] ovarian cancer,[21] and melanoma.[22] In addition, high expression of CD10 was correlated with nodal metastasis, Gleason grade, and poor prognosis of prostate cancer patients in the comparative study.[15] Since CD10 is a cell surface metalloproteinase, increased expression of CD10 might facilitate the accumulation of inactivated peptides that are cleaved by CD10, modulating the proliferation of cancer cells by influencing the intracellular signal pathways in the tumor microenvironment.[23] In addition, CD10 also serves as a regulator in the immune system.[7] These results suggest that CD10 can be an attractive molecular target for cancer therapy.

In this study, a novel anti-human CD10 monoclonal antibody (mAb), C_{10}Mab-31 (IgG_1, kappa), was developed by immunizing one mouse with the CD10-overexpressed human renal cancer OUR-10 cells. From the C_{10}Mab-31, a defucosylated mouse IgG_{2a} version of C_{10}Mab-31 (31-mG_{2a}-f) was created. In this study, we evaluated whether 31-mG_{2a}-f induced antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or antitumor activity against RCC cells in a mouse xenograft model.

Materials and Methods

Cell lines

P3X63Ag8U.1 (P3U1) and CHO-K1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). OUR-10 and VMRC-RCW (renal cancer cell lines) were provided from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). The pCAG-Ble vector and the pCAG-Neo vector were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). OUR-10/CD10 and CHO/CD10 cells were established by transfecting pCAG-Ble/CD10-PAtag and pCAG-Neo/CD10-PAtag plasmids (Accession No. NM_000902.3) into OUR-10 cells and CHO-K1 cells, respectively.

BINDS-27 (VMRC-RCW/CD10-knockout [KO] cells) was established by transfecting CRISPR/Cas9 plasmids targeting human CD10 into VMRC-RCW cells (www.med-tohoku-antibody.com/topics/001_paper_cell.htm). Using TrueGuide gRNA tool, gRNA of CD10 was selected from GeneArt predesigned gRNA database (Thermo Fisher Scientific, Inc., Waltham, MA). P3U1, VMRC-RCW, BINDS-27, OUR-10, OUR-10/CD10, CHO-K1, and CHO/CD10 were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Hybridoma production

A female 4-week-old BALB/c mouse was purchased from CLEA Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions. The Animal Care and Use Committee of Tohoku University approved all animal experiments described in this study. A BALB/c mouse was immunized using intraperitoneal injections of OUR-10/CD10 (1×10^8 cells) together with Imject Alum (Thermo Fisher Scientific, Inc.). After several additional immunizations, a booster injection was intraperitoneally administered 2 days before harvesting spleen cells. Spleen cells were then fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The resulting hybridomas were grown in RPMI 1640 medium supplemented with hypoxanthine, aminopterin, and thymidine selection medium supplement (Thermo Fisher Scientific, Inc.). Culture supernatants were screened using flow cytometry (CHO-K1 vs. CHO/CD10). MAbs were purified from the supernatants of hybridomas, cultured in Hybridoma-SFM medium (Thermo Fisher Scientific, Inc.) using Protein G-Sepharose (GE Healthcare Biosciences, Pittsburgh, PA).

Production of the recombinant antibody

To generate 31-mG_{2a}, we subcloned V_H cDNA of C_{10}Mab-31 and C_H of mouse IgG_{2a} into the pCAG-Neo vector, along with cDNA of C_{10}Mab-31 light chain into the pCAG-Zeo vector, respectively. The vector of 31-mG_{2a} was transfected into BINDS-09 cells (FUT8-KO ExpiCHO-S cells) using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.). The resulting mAb, 31-mG_{2a}-f, was purified using Protein G-Sepharose.

Flow cytometry

Cells were harvested by brief exposure to 0.25% trypsin/1-mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin/phosphate-buffered saline (PBS), the cells were treated with 1 μg/mL of anti-CD10 (C_{10}Mab-31 and 31-mG_{2a}-f) for 30 minutes at 4°C and subsequently with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using EC800 Cell Analyzers (Sony Corp., Tokyo, Japan).

Animals for ADCC and antitumor activities

All animal experiments were performed following relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments for antitumor activity were approved by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (Permit No. 2021-045 for ADCC assay and 2021-030 for antitumor experiments). Mice were maintained in a specific pathogen-free environment (23°C ± 2°C, 55% ± 5% humidity) on an 11-hour light/13-hour dark cycle with food and water supplied *ad libitum* across the experimental period. Mice were monitored for health and weight every 2–5 days during the

3-week period of each experiment. We determined the loss of original body weight to a point >25% and/or a maximum tumor size >3000 mm^3 as humane endpoints for euthanasia. Mice were euthanized by cervical dislocation; death was verified by respiratory and cardiac arrest.

ADCC

Six 6-week-old female BALB/c nude mice were purchased from Charles River, and spleens were removed aseptically, and single-cell suspensions were obtained by dispersing the spleens using a syringe and pressing through stainless steel mesh. Erythrocytes were effectively lysed by 10-second exposure to ice-cold distilled water. Splenocytes were washed with Dulbecco's modified Eagle's medium (DMEM) and resuspended in DMEM with 10% FBS as effector cells. VMRC-RCW or BINDS-27 cells were labeled with 10 μg/mL Calcein AM (Thermo Fisher Scientific, Inc.) and resuspended in the medium. VMRC-RCW or BINDS-27 cells (2×10^4 cells/well) were placed in 96-well plates and mixed with effector cells, 31-mG_{2a}-f or control IgG (mouse IgG_{2a}; Sigma-Aldrich Corp., St. Louis, MO).

After a 4-hour incubation period, the Calcein release of supernatant from each well was measured. The fluorescence intensity was determined at an excitation wavelength of 485 nm and an emission wavelength of 538 nm using a microplate reader (Power Scan HT; BioTek Instruments, Winooski, VT). Cytolytic activity (as % of lysis) was calculated using the following formula: % lysis = (E − S)/(M − S) × 100 (where E is the fluorescence released in the

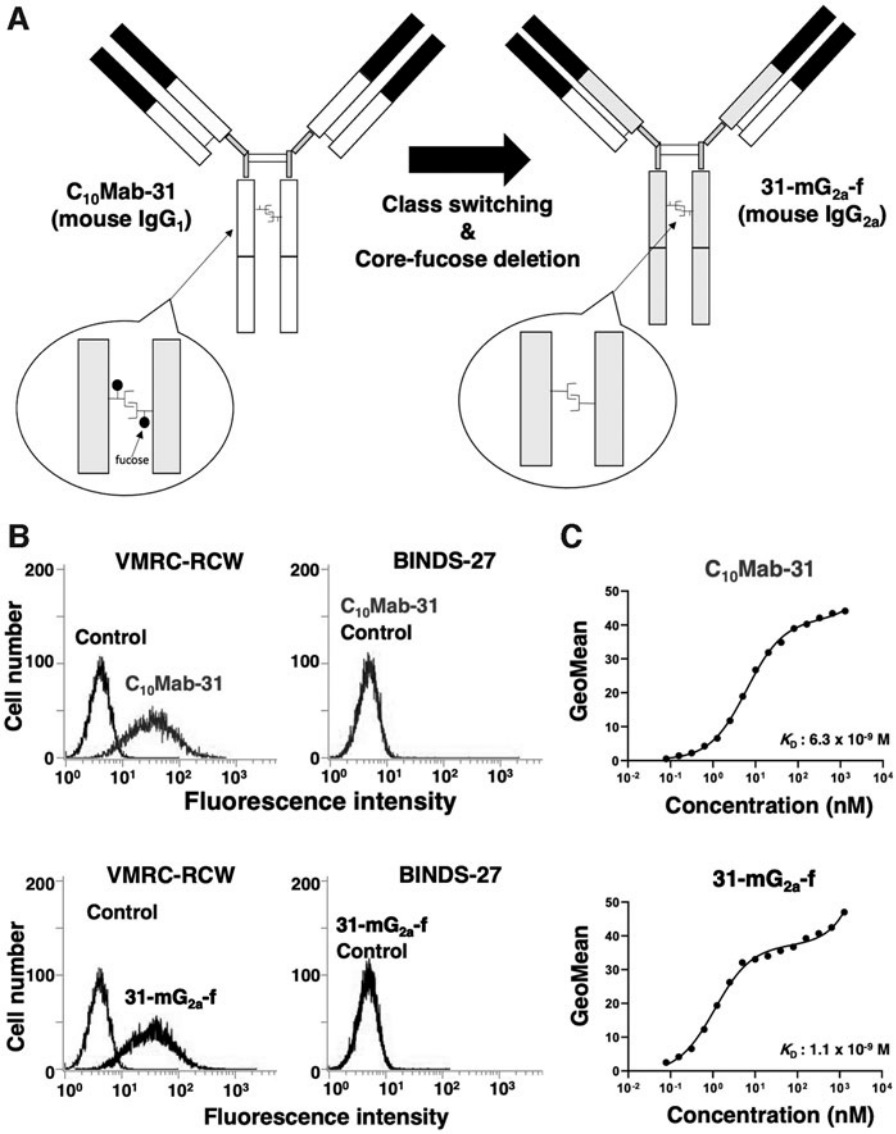

FIG. 1. Production of 31-mG_{2a} (mouse IgG_{2a}) and 31-mG_{2a}-f (defucosylated form) from an anti-CD10 mAb, C_{10}Mab-31 (mouse IgG_1). **(A)** Schematic illustration of 31-mG_{2a} and 31-mG_{2a}-f. **(B)** Flow cytometry using C_{10}Mab-31 and 31-mG_{2a}-f. VMRC-RCW and BINDS-27 cells were treated with C_{10}Mab-31, 31-mG_{2a}-f, or buffer control, followed by secondary antibodies. **(C)** Determination of the binding affinity of C_{10}Mab-31 and 31-mG_{2a}-f for VMRC-RCW cells using flow cytometry. VMRC-RCW cells were suspended in 100 µL serially diluted C_{10}Mab-31 and 31-mG_{2a}-f, followed by the addition of Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the EC800 Cell Analyzer. mAb, monoclonal antibody.

experimental cultures of target and effector cells, S is the spontaneous fluorescence released in cultures containing only target cells, and M is the maximum fluorescence obtained by adding a lysis buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4), and 10 mM of EDTA to the target cells to lyse all cells).

CDC

VMRC-RCW or BINDS-27 cells were labeled with 10 μg/mL Calcein AM (Thermo Fisher Scientific, Inc.) and resuspended in the medium. VMRC-RCW or BINDS-27 cells (2×10^4 cells/well) were placed in 96-well plates and 10% of rabbit complement (Low-Tox-M Rabbit Complement; Cedarlane Laboratories, Hornby, Ontario, Canada). After a 4-hour incubation period, the Calcein release of supernatant from each well was measured. Fluorescence intensity was calculated as described in the above ADCC section.

Antitumor activity of 31-mG$_{2a}$-f

Female BALB/c nude mice (5-week old) were purchased from Charles River (Kanagawa, Japan) and used in experiments when they were 7 weeks old. VMRC-RCW or BINDS-27 cells (0.3 mL of 1.33×10^8/mL in DMEM) were mixed with 0.5 mL of BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, San Jose, CA). A 100 μL suspension (containing 5×10^6 cells) was injected subcutaneously into the left flanks of nude mice. After day 6, 100 μg of 31-mG$_{2a}$-f or control mouse IgG (Sigma-Aldrich Corp.) in 100 μL PBS was injected into the peritoneal cavity of each mouse. Additional antibodies were then injected on day 15. The tumor diameter and volume were determined as previously described.[24] The mice were euthanized 23 days after cell implantation.

Statistical analyses

All data are expressed as mean ± standard error of the mean. Statistical analysis was conducted with Welch's *t*-test for ADCC, CDC, and tumor weight. ANOVA and Sidak's multiple comparisons tests were conducted for tumor volume and mouse weight. All calculations were performed using GraphPad Prism 8. A *p*-value of <0.05 was considered statistically significant.

Results

Development of anti-CD10 mAbs

In this study, one mouse was immunized with the OUR-10/CD10 cells. Flow cytometry was conducted to check reactions with CHO-K1 and CD10-overexpressed CHO-K1 (CHO/CD10) cells. A stronger reaction against CHO/CD10 was required compared with CHO-K1. One clone C_{10}Mab-31 of the IgG_1 subclass was obtained. The subclass of C_{10}Mab-31 was converted into mouse IgG_{2a} to add to the ADCC and CDC activities. In addition, a defucosylated anti-CD10 mAb (31-mG$_{2a}$-f) was produced using BINDS-09 cells (FUT8-KO ExpiCHO-S cells). This process is summarized in Figure 1A.

Flow cytometry analysis against a renal cell cancer cell line, VMRC-RCW cells, using anti-CD10 mAbs

First, anti-CD10 mAbs were characterized using flow cytometry. Both C_{10}Mab-31 and 31-mG$_{2a}$-f reacted with VMRC-RCW cells, and not with BINDS-27 cells (VMRC-RCW/CD10-KO cells), indicating that both C_{10}Mab-31 and 31-mG$_{2a}$-f are CD10-specific (Fig. 1B).

Kinetic analysis of the interactions of C_{10}Mab-31 and 31-mG$_{2a}$-f with VMRC-RCW cells was conducted using flow cytometry. As indicated in Figure 1C, the dissociation constant (K_D) for the interaction of C_{10}Mab-31 and 31-mG$_{2a}$-f with VMRC-RCW cells was 6.3×10^{-9} M and 1.1×10^{-9} M, respectively. This suggests that both C_{10}Mab-31 and 31-mG$_{2a}$-f exhibit high affinity for VMRC-RCW cells.

31-mG$_{2a}$-f-mediated ADCC and CDC in VMRC-RCW cells

Since the core-fucose-deficient anti-CD44 mAb (5-mG$_{2a}$-f) possesses ADCC and CDC activities in our previous study,[25] we next investigated whether 31-mG$_{2a}$-f was capable of

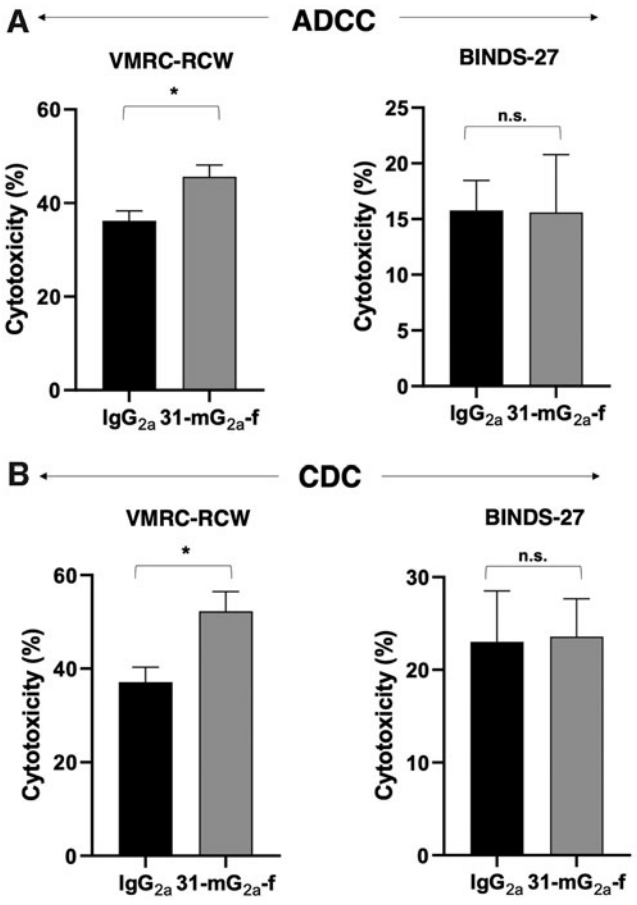

FIG. 2. Evaluation of ADCC and CDC elicited by 31-mG$_{2a}$-f. **(A)** ADCC elicited by 31-mG$_{2a}$-f or control mouse IgG_{2a} targeting VMRC-RCW or BINDS-27 cells. **(B)** CDC elicited by 31-mG$_{2a}$-f or control mouse IgG_{2a} targeting VMRC-RCW or BINDS-27 cells. Values are mean ± SEM. Asterisks indicate statistical significance (*$p<0.05$; n.s., not significant; Welch's *t*-test). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; SEM, standard error of the mean.

mediating ADCC against VMRC-RCW cells. As indicated in Figure 2A, 31-mG_{2a}-f showed ADCC (46% cytotoxicity) against VMRC-RCW cells more effectively than it did against control mouse IgG_{2a} (36% cytotoxicity; $p<0.05$). Contrastingly, 31-mG_{2a}-f did not exert ADCC against BINDS-27 cells.

Whether 31-mG_{2a}-f could mediate CDC against VMRC-RCW cells was then investigated. As indicated in Figure 2B, 31-mG_{2a}-f elicited a higher degree of CDC (52% cytotoxicity) in VMRC-RCW cells compared with that elicited by the control mouse IgG_{2a} (37% cytotoxicity; $p<0.05$). Contrastingly, 31-mG_{2a}-f did not exert CDC on BINDS-27 cells.

These results demonstrated that 31-mG_{2a}-f promoted significantly higher levels of ADCC and CDC against VMRC-RCW cells.

Antitumor activities of 31-mG_{2a}-f in the mouse xenografts of VMRC-RCW cells

In the VMRC-RCW xenograft models, 31-mG_{2a}-f (100 μg) and control mouse IgG (100 μg) were injected intraperitoneally into mice on days 6 and 15 following the injection of VMRC-RCW cells. The tumor volume was measured on days 6, 12, 15, 17, and 23 after the injection. The administration of 31-mG_{2a}-f resulted in a significant reduction in tumor development on days 15 ($p<0.05$), 17 ($p<0.01$), and 23 ($p<0.01$) compared with that of the control mouse IgG (Fig. 3A). The administration of 31-mG_{2a}-f resulted in 34% reduction in tumor volume compared with that of the control mouse IgG on day 23.

In the BINDS-27 xenograft models, 31-mG_{2a}-f (100 μg) and control mouse IgG (100 μg) were also injected intraperitoneally into mice on days 6 and 15 following the injection of BINDS-27 cells. The tumor volume of BINDS-27 cells between the 31-mG_{2a}-f-treated mice and control mouse IgG-treated mice showed no difference (Fig. 3B).

In the VMRC-RCW xenograft models, the tumor weight of the 31-mG_{2a}-f-treated mice was significantly lower compared with the control mouse IgG-treated mice (17% reduction; $p<0.05$, Fig. 3C). Contrastingly, the tumor weight between the 31-mG_{2a}-f-treated mice and control mouse IgG-treated mice indicated no difference in the BINDS-27 xenograft models (Fig. 3D).

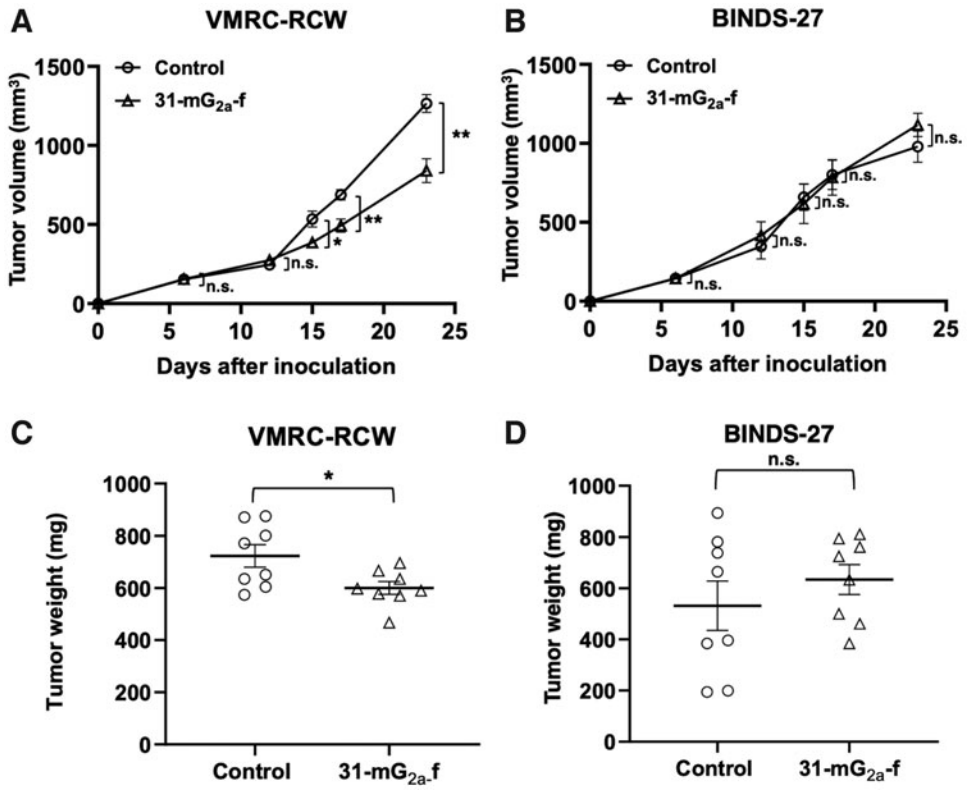

FIG. 3. Evaluation of the antitumor activity of 31-mG_{2a}-f in VMRC-RCW, or BINDS-27 xenografts. **(A)** VMRC-RCW cells (5×10^6 cells) were injected subcutaneously into the left flank. After day 6, 100 μg of 31-mG_{2a}-f or control mouse IgG in 100 μL PBS was injected intraperitoneally into mice; additional antibodies were then injected on day 15. The tumor volume was measured on days 6, 12, 15, 17, and 23 after the injection. Values are mean ± SEM. Asterisks indicate statistical significance (*$p<0.05$, **$p<0.01$, n.s., not significant; analysis of variance and Sidak's multiple comparisons test). **(B)** BINDS-27 cells (5×10^6 cells) were injected subcutaneously into the left flank. After day 6, 100 μg of 31-mG_{2a}-f or control mouse IgG in 100 μL PBS was injected intraperitoneally into mice; additional antibodies were then injected on day 15. The tumor volume was measured on days 6, 12, 15, 17, and 23 after the injection. Values are mean ± SEM. n.s., not significant (analysis of variance and Sidak's multiple comparisons test). **(C)** Tumors of VMRC-RCW xenografts were resected from 31-mG_{2a}-f and control mouse IgG groups. Tumor weight on day 23 was measured from excised xenografts. Values are mean ± SEM. Asterisk indicates statistical significance (*$p<0.05$, Welch's *t*-test). **(D)** Tumors of BINDS-27 xenografts were resected from 31-mG_{2a}-f and control mouse IgG groups. Tumor weight on day 23 was measured from excised xenografts. Values are mean ± SEM. n.s., not significant (Welch's *t*-test). PBS, phosphate-buffered saline.

Mouse body weight was not significantly different among the two groups in the VMRC-RCW or BINDS-27 xenograft models (Supplementary Fig. S1).

Altogether, these results indicate that the administration of 31-mG_{2a}-f effectively suppresses the tumor growth of VMRC-RCW xenografts.

Discussion

In immunotherapy, mAbs are recognized as ideal adjuvant therapeutic reagents for various human diseases.[26–28] Because RCCs are highly vascular tumors, angiogenic factors, such as vascular endothelial growth factor-A (VEGF-A), are overproduced in tumors.[29] Therefore, targeting the VEGF signaling axis is an effective option for treating RCCs.[30] The anti-VEGF-A mAb bevacizumab is used with interferon-α as a first-line option to treat patients with RCCs.[31,32] In addition, several studies have investigated combinations of anti-VEGF therapy with other immune checkpoint blockade like PD-1/PD-L1, or CTLA-4 inhibitors.[1]

CD10 is expressed in tumor cells and a wide variety of normal cells, including bone marrow stromal cells, granulocytes, a subset of B-cell progenitors, germinal center B cells, and fibroblasts.[33] In a previous study, cancer-specific mAbs (CasMabs) technology was established against cell surface proteins, and the usefulness of CasMabs against tumors was shown.[34,35] To prevent side effects of CD10-targeting antibody therapy, the development of CasMabs is thought to have a significant potential for an innovative therapeutic approach. Since CasMabs could attack only cancer cells by recognizing both target-derived peptide sequence and cancer-specific glycosylation, establishing CasMabs against human CD10 would be an essential way to develop novel CD10-targeting therapeutic antibodies without affecting normal cells.

Recently, Mizutani *et al.* reported that a mouse anti-CD10 mAb (JMAM-1) prolonged the survival in a tumor-bearing mice model.[36] They showed that JMAM-1-treated malignant mesothelioma cell line MSTO-211H increased cell cycle arrest, which is involved by cyclin-dependent kinase. However, the precise molecular mechanisms about how JMAM-1 affects antitumor activity remain unclear.

The main aim of this study was to investigate the antitumor activity of anti-CD10 mAb against RCC cells. The antitumor effects of mAbs are mediated by several mechanisms of action, including ADCC and CDC.[25,37,38] Particularly, the ADCC activity plays a critical role of mAbs for cancer therapy, and natural killer cells are involved in the immune system, exhibiting potent effector mechanisms against cancer cells in humans.[39] It was demonstrated that a novel anti-CD10 mAb, C_{10}Mab-31, and its defucosylated mouse IgG_{2a} version, 31-mG_{2a}-f, specifically react with RCC cells (Fig. 1). In addition, 31-mG_{2a}-f significantly exhibited ADCC and CDC in RCC cells *in vitro* (Fig. 2) and suppressed tumor growth of the RCC cell xenograft model *in vivo* (Fig. 3).

However, we could not determine whether 31-mG_{2a}-f affects the metalloprotease activity of CD10 and cross-reacts with other species due to amino acid sequence homology. In the future, we will examine the influence of our anti-CD10 mAbs on the CD10 enzymatic activity and determine the species cross-reactivity. Collectively, our findings in this study suggest that anti-CD10 mAbs could be a therapeutic candidate, and the combined use with immune checkpoint inhibitors might be a treatment option for patients with RCCs.

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Supplementary Material

Supplementary Figure S1

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Address correspondence to:
Yukinari Kato
Department of Molecular Pharmacology
Tohoku University Graduate School of Medicine
2-1, Seiryo-machi, Aoba-ku
Sendai 980-8575
Japan

E-mail: yukinarikato@med.tohoku.ac.jp

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