Antitumor Activities in Mouse Xenograft Models of Canine Fibroblastic Tumor by Defucosylated Anti-Epidermal Growth Factor Receptor Monoclonal Antibody

Nohara Goto,1 Hiroyuki Suzuki,1 Tomokazu Ohishi,2 Akiko Harakawa,2 Guanjie Li,1 Masaki Saito,1 Junko Takei,3 Tomohiro Tanaka,3 Teizo Asano,3 Masato Sano,3 Manabu Kawada,2 Mika K. Kaneko,3 and Yukinari Kato1,3

The epidermal growth factor receptor (EGFR) is involved in tumor malignancy through gene amplification and/or protein overexpression. An anti-human EGFR (hEGFR) monoclonal antibody (clone EMab-134), which explicitly detects hEGFR and dog EGFR (dEGFR), was previously developed. The defucosylated mouse IgG2a version of EMab-134 (134-mG2a-f) exhibits antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) in dEGFR-overexpressed CHO-K1 (CHO/dEGFR) cells and antitumor activities in mouse xenografts of CHO/dEGFR cells. In this study, it was shown that 134-mG2a-f reacts with a canine fibroblastic tumor cell line (A-72) using flow cytometry and immunocytochemistry. Furthermore, 134-mG2a-f exerted ADCC and CDC on A-72 cell line. The administration of 134-mG2a-f significantly inhibited the A-72 xenograft growth. These results suggest that 134-mG2a-f exerts antitumor effects on dEGFR-expressing canine fibroblastic tumors.

Keywords: EGFR, monoclonal antibody, ADCC, CDC, canine fibroblastic tumor, antitumor activity

Introduction

A dog is an animal that spontaneously develops cancers, such as melanomas, breast cancers, and fibroblastic tumors.1–4 In terms of genetic, histological, and biological features, a dog has strong similarities to humans. Dogs share carcinogenic factors, such as aging, obesity, exposure to carcinogens, and environmental risk factors with humans.5,6 Dogs grow five to eight times faster than humans, making them suitable models for pathogenesis research and age-related gene profiling.4 Response to cancer treatment, the acquisition of drug resistance, and cancer metastasis are similarly observed in dogs and humans.7 Because of its similarity, human cancer research is expected to be applied to dogs.5,8 The inhibition of the programmed cell death-1 (PD-1) pathway has been reported to be one of the therapeutic strategies in canine metastatic oral malignant melanoma.9 Epidermal growth factor receptor (EGFR) is a type I transmembrane protein that forms a dimer by binding its ligand and regulating cell proliferation and survival. Overexpression and mutations of EGFR activate multiple intracellular cascades.10 EGFR is closely associated with different cancer development and malignancy in humans and dogs.11–15 EGFR expression also correlates with poor prognosis in canine mammary cancers.16 Human EGFR (hEGFR) and dog EGFR (dEGFR) have 91% amino acid homology.17 Therefore, the development of therapeutic methods targeting EGFR is suitable as a canine cancer treatment.

An anti-hEGFR monoclonal antibody (mAb), clone EMab-134 (mouse IgG1, kappa), was previously developed.18 The 134-mG2a, an IgG2a type of EMab-134, exerted antitumor activities in a model of human oral squamous cell carcinoma.19 In addition, the 134-mG2a-f, defucosylated type of 134-mG2a, possesses antitumor effects in mouse xenograft models of dEGFR-expressed cells.20

1Department of Molecular Pharmacology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan.
2Institute of Microbial Chemistry (BIKAKEN), Numazu, Microbial Chemistry Research Foundation, Numazu-shi, Shizuoka, Japan.
3Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan.
In this study, we investigated whether 134-mG2a-f possesses antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antitumor activities using a canine fibroblastic tumor xenograft model.

Materials and Methods

Cell lines, antibodies, and animals

A canine fibroblast cell line (A-72) was obtained from the American Type Culture Collection (Manassas, VA). A-72 was cultured in Dulbecco’s modified Eagle medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan), supplemented using 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2.

An anti-hEGFR mAb, EMab-134, was developed as previously described.(18) To generate 134-mG2a, VH complementary DNA (cDNA) of EMab-134 and CH mouse IgG2a was subcloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and VL and CL cDNAs of EMab-134 were subcloned into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation). The vectors of 134-mG2a were transfected into BINDS-09 cells (FUT8-deficient ExpiCHO-S cells) using the ExpiCHO expression system (Thermo Fisher Scientific Inc.). The resulting mAbs (134-mG2a,f) were purified using Protein G-Sepharose (GE Healthcare Biosciences, Pittsburgh, PA). Mouse IgG (cat. no. I8765) and mouse IgG2a (cat. no. M7769) were bought from Sigma-Aldrich (St. Louis, MO).

Female BALB/c nude mice (5 weeks old, weighing 14–17 g) were bought from Charles River Laboratories, Inc. The animal experiments were conducted following relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. The institutional committee for experiments of the Institute of Microbial Chemistry approved animal experiments for ADCC and antitumor activity (permit Nos. 2021-028 for ADCC assays and 2021-021 for antitumor experiments).

Mice were maintained in a specific pathogen-free environment (23°C±2°C, 55%±5% humidity) on 11 hours light/13 hours 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 weeks period of each experiment. The loss of original body weight to a point >25% and/or a maximum tumor size >3000 mm3 were determined as humane endpoints for euthanasia. Mice were euthanized by cervical dislocation; death was verified through respiratory and cardiac arrest.

Flow cytometry

A-72 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 (BSA) blocking buffer in phosphate-buffered saline (PBS), cells were treated with 1 μg/mL 134-mG2a,f or control blocking buffer for 30 minutes at 4°C. Then cells were incubated in Alexa Fluor 488-conjugated anti-mouse IgG at a dilution of 1:1000 (cat no. 4408S; Cell Signaling Technology, Inc., Danvers, MA) for 30 minutes at 4°C. Fluorescence data were obtained using the EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Determination of binding affinity

A-72 cells were suspended in 100 μL of serially diluted 134-mG2a-f (0.006–100 μg/mL) followed by Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.). Fluorescence data were obtained using the EC800 Cell Analyzer (Sony Corp.). The dissociation constant (Kd) was calculated by fitting binding isotherms to built-in one-site binding models in GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA).

Immunocytochemical analysis

Subconfluent A-72 cells cultured on acid-wash coverslips were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. After quenching with 50 mM NH4Cl in PBS containing 0.2 mM Ca++ and 2 mM Mg++ (PBS/c/m) for 10 minutes, the cells were blocked using a blocking buffer (PBS/c/m containing 0.5% BSA) for 30 minutes and incubated with 10 μg/mL of 134-mG2a-f or control blocking buffer for 1 hour. Then, the cells were labeled using Alexa Fluor 488-conjugated anti-mouse IgG for 45 minutes. The cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific Inc.). A fluorescence microscope BZ-X800 (Keyence, Osaka, Japan) was used to obtain fluorescence images.

ADCC

ADCC assay was previously reported.(20–36) In brief, spleen cells from five mice were used as the source of mononuclear cells to evaluate ADCC. After euthanasia by cervical dislocation, the spleens were eliminated aseptically and a syringe was used to force spleen tissue through a sterile cell strainer (352360; BD Falcon, Corning, New York, NY) and obtain single-cell suspensions. Erythrocytes were lysed by 10 seconds exposure to ice-cold distilled water. Splenocytes were washed using DMEM and resuspended in DMEM with 10% FBS to be used as effector cells.

A-72 cells were labeled with 10 μg/mL of Calcein AM (Thermo Fisher Scientific, Inc.) and resuspended in the same medium. A-72 cells (2×10^5 cells/well) were plated in 96-well plates and mixed with splenocytes (effector/target cells ratio, 50), 100 μg/mL of 134-mG2a-f or control mouse IgG2a. After 4.5 hours incubation at 37°C, the amount of calcein released into the supernatant was measured in each well. The fluorescence intensity was determined using a microplate reader (Power Scan HT; BioTek Instruments, Inc., Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Cytolytic activity (% lysis) was calculated as follows: % lysis = (E−S)/(M−S)×100, where “E” is the fluorescence measured in combined cultures of target and effector cells, “S” is the spontaneous fluorescence of target cells only, and “M” is the maximum fluorescence measured after the lysis of all cells with a buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4), and 10 mM EDTA.

CDC

CDC assay was previously reported.(20–36) In brief, A-72 cells were labeled with 10 μg/mL of Calcein AM (Thermo Fisher Scientific, Inc.) and resuspended in the same medium. A-72 cells were then plated in 96-well plates at 2×10^4
cells/well using rabbit complement (final dilution 1:10; Low-Tox-M Rabbit Complement; Cedarlane Laboratories, Hornby, Ontario, Canada) and 100 µg/mL of 134-mG2a-f or control mouse IgG2a. After 4 hours of incubation at 37°C, the amount of calcein released into the supernatant for each well was measured.

Cytolytic activity (% lysis) was calculated as follows: % lysis = (E - S)/(M - S) × 100, where “E” is the fluorescence measured in combined cultures of target and effector cells, “S” is the spontaneous fluorescence of target cells only, and “M” is the maximum fluorescence measured after the lysis of all cells with a buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4), and 10 mM EDTA.

Antitumor activity of 134-mG2a-f in xenografts of A-72 cells

Sixteen female BALB/c nude mice were used in experiments once they reached 7 weeks of age. A-72 cells (0.3 mL 1.33 × 10⁶ cells/mL in DMEM) were mixed with 0.5 mL of BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, San Jose, CA); 100 µL of this suspension (5 × 10⁶ cells) was injected subcutaneously into the left flanks of the mice.

On day 7 postinoculation, 100 µg of 134-mG2a-f (n = 8), or control mouse IgG (n = 8) in 100 µL of PBS was injected intraperitoneally. Additional antibody injections were administered on days 14 and 21. On day 25 after cell implantation, all mice were euthanized through cervical dislocation. Tumor diameters and volumes were determined as previously described.(20)

Statistical analyses

All data are expressed as mean ± standard error of the mean. Statistical analysis was conducted using Tukey’s test for ADCC and CDC and Welch’s t-test for tumor weight. Analysis of variance and Sidak’s multiple comparison tests were performed for tumor volume and mouse weight. All calculations were conducted using GraphPad Prism 8 (GraphPad Software, Inc.). A p-value of <0.05 was considered statistically significant.

Results

Flow cytometry analysis against canine fibroblastic tumor cell line, A-72 cells using 134-mG2a-f

In our previous study, an anti-hEGFR mAb, EMab-134, recognized CHO/dEGFR cells, showing that EMab-134 cross-reacts with dEGFR.(20) In this study, the defucosylated mouse IgG2a type of EMab-134 (134-mG2a-f) detected A-72 cells (Fig. 1A), showing that 134-mG2a-f detects endogenous dEGFR expressed on A-72 cells.

Determination of binding affinity

A kinetic analysis of the interactions of 134-mG2a-f with A-72 cells was conducted using flow cytometry. As shown in Figure 1B, the K_D for the interaction of 134-mG2a-f with A-72 cells was 1.1 × 10⁻⁹ M, suggesting that 134-mG2a-f shows a high affinity for A-72 cells.

Immunocytochemical analysis against A-72 cells using 134-mG2a-f

The 134-mG2a-f was applied to immunocytochemistry in A-72 cells. As shown in Figure 1C, 134-mG2a-f, but not buffer control, visualized dEGFR in A-72 cells, suggesting that 134-mG2a-f recognizes endogenous dEGFR in A-72 cells.
134-mG2a-f-mediated ADCC and CDC in A-72 cells

Next, it was investigated whether 134-mG2a-f has ADCC against A-72 cells. As indicated in Figure 2A, 134-mG2a-f showed ADCC (15.8% cytotoxicity) against A-72 cells, which is more potent than mouse IgG2a (5.6% cytotoxicity) and PBS (4.0% cytotoxicity).

It was then investigated whether 134-mG2a-f has CDC against A-72 cells. As shown in Figure 2B, 134-mG2a-f elicited a higher degree of CDC (43.2% cytotoxicity) in A-72 cells than that elicited by mouse IgG2a (36.1% cytotoxicity) and PBS (31.8% cytotoxicity). These results showed that 134-mG2a-f exerts ADCC and CDC against dEGFR-expressing A-72 cells.

Antitumor activities of 134-mG2a-f in the mouse xenografts of A-72 cells

In the A-72 xenograft models, 134-mG2a-f (100 µg) and control mouse IgG (100 µg) were injected intraperitoneally into mice on days 7, 14, and 21 after the injection of A-72 cells. The tumor volume was measured on days 7, 11, 14, 18, 21, and 25 postinoculation. The administration of 134-mG2a-f caused a significant reduction in tumor development on days 11 ($p < 0.01$), 14 ($p < 0.01$), 18 ($p < 0.01$), 21 ($p < 0.01$), and 25 ($p < 0.01$) compared with that of the control mouse IgG (Fig. 3A).

The administration of 134-mG2a-f caused 52% reduction in tumor volume compared with that of the control mouse IgG on day 25 postinjection. Furthermore, the tumor weight of the 134-mG2a-f-treated mice was significantly lower than that of IgG-treated mice (48% reduction; $p < 0.01$, Fig. 3B). The total body weights of the two groups did not significantly change (Fig. 3C). Altogether, these results indicate that the administration of 134-mG2a-f effectively suppresses tumor growth of A-72 xenografts.

**FIG. 2.** Evaluation of ADCC and CDC elicited by 134-mG2a-f. (A) ADCC elicited by 134-mG2a-f, mouse IgG2a, or PBS targeting A-72 cells. Asterisks indicate statistical significance (*$p < 0.05$, n.s.; Tukey’s test). (B) CDC elicited by 134-mG2a-f, mouse IgG2a, or PBS targeting A-72 cells. Values are means ± SEM. Asterisks indicate statistical significance (**$p < 0.01$, *$p < 0.05$, n.s.; Tukey’s test). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; n.s., not significant; PBS, phosphate-buffered saline; SEM, standard error of the mean.

**FIG. 3.** Evaluation of antitumor activity of 134-mG2a-f in A-72 xenografts. (A) A-72 cells were subcutaneously injected into the left flank. On day 7, 100 µg 134-mG2a-f or mouse IgG in 100 µL PBS was injected intraperitoneally into mice; additional antibodies were then injected on days 14 and 21. The tumor volume was measured on days 7, 11, 14, 18, 21, and 25 after the injection. Values are means ± SEM. Asterisks indicate statistical significance (**$p < 0.01$, n.s.; ANOVA and Sidak’s multiple comparisons test). (B) A-72 xenografts were resected from 134-mG2a-f and mouse IgG groups. Tumor weight on day 25 was measured. Values are means ± SEM. Asterisk indicates statistical significance (**$p < 0.01$, Welch’s t-test). (C) Body weights of mice implanted with A-72 xenografts were recorded on days 7, 11, 14, 18, 21, and 25 (n.s.). ANOVA, analysis of variance; n.s., not significant.
Discussion

EGFR is an essential oncoprotein, which promotes tumor development.\(^{(10)}\) Therefore, therapeutic agents targeting EGFR have been developed.\(^{(37,38)}\) The EGFR-targeted antibody drugs, including panitumumab and cetuximab,\(^{(39,40)}\) have similar binding affinities to EGFR but different epitopes on the EGFR.\(^{(41-43)}\) These mAbs can inhibit the EGFR signaling pathways and induce apoptosis in EGFR-expressing cancer cells and exhibit therapeutic potential for patients with advanced colorectal cancer.\(^{(34,45)}\)

Approved drugs for humans are often used for canine cancer treatment due to their similarities.\(^{(5,46)}\) Because EGFR is involved in canine cancer malignancy and poor prognosis, EGFR-targeting drugs are considered an important therapeutic strategy. It has been previously shown that the 134-mG2a-f has an antitumor effect against a dEGFR-overexpressed CHO-K1 model.\(^{(20)}\) This study also demonstrated antitumor activities of a defucosylated IgG2a type of anti-hEGFR mAb (134-mG2a-f) in vitro and in vivo on canine fibroblastic tumor cells expressing endogenous dEGFR (Figs. 2 and 3).

The combination therapy of cetuximab and pembrolizumab is successful for human head and neck squamous cell carcinomas.\(^{(47)}\) Pembrolizumab is an immune checkpoint inhibitor that recognizes immunosuppressive molecule PD-1 expressed on T cells and has been expanded to various human cancers recently.\(^{(48)}\) Immune checkpoint inhibitors and chimeric antigen receptor-T cell therapy are also used to treat canine cancers.\(^{(49)}\) Therefore, the combination of anti-EGFR mAb therapy and immune checkpoint inhibitors is expected to be more effective in canine cancer treatment. Further studies are needed to investigate the antitumor activity of 134-mG2a-f to spontaneously develop canine cancers.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported, in part, by Japan Agency for Medical Research and Development (AMED) under Grant Numbers: JP21am0401013 (to Y.K.) and JP21am0101078 (to Y.K.).

References


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

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

Received: November 25, 2021
Accepted: January 25, 2022