Title: Generation and characterization of a human–mouse chimeric antibody against the extracellular domain of claudin-1 for cancer therapy using a mouse model

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Abstract

Claudin-1 (CLDN-1), an integral transmembrane protein, is an attractive target for drug absorption, prevention of infection, and cancer therapy. Previously, we generated mouse anti-CLDN-1 monoclonal antibodies (mAbs) and found that they enhanced epidermal absorption of a drug and prevented hepatitis C virus infection in human hepatocytes. Here, we investigated anti-tumor activity of a human–mouse chimeric IgG1, xi-3A2, from one of the anti-CLDN-1 mAbs, clone 3A2. Xi-3A2 accumulated in the tumor tissues in mice bearing with human CLDN-1–expressing tumor cells. Xi-3A2 activated Fc γ receptor IIIa–expressing reporter cells in the presence of human CLDN-1–expressing cells, suggesting xi-3A2 has a potential to exhibit antibody-dependent cellular cytotoxicity against CLDN-1 expressing tumor cells. We also constructed a mutant xi-3A2 antibody with Gly, Ser, and Ile substituted with Ala, Asp, and Arg at positions 236, 239, and 332 of the Fc domain. This mutant antibody showed greater activation of Fc γ receptor IIIa and in vivo anti-tumor activity in mice bearing human CLDN-1-expressing tumors than xi-3A2 did. These findings indicate that the G236A/S239D/I332E mutant of xi-3A2 might be a promising lead for tumor therapy.

Keywords: claudin-1, chimeric antibody, anti-cancer agent, Fc γ receptor IIIa

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CLDN, claudin; Fc γ RIIIa, Fc γ

receptor IIIa; HCV, hepatitis C virus; Luc, luciferase; mAb, monoclonal antibody; NFAT, nuclear factor of activated T cells

Introduction

Epithelium separates the internal environment of the body from the external environment. Most malignant tumors are derived from epithelium, and pathological microorganisms frequently invade into the body through the epithelium. Therefore, epithelium is an important target for drug development, including drug delivery, tumor therapy, and infectious disease treatment [1].

Claudins (CLDNs) are tetratransmembrane proteins that are components of the tight junctions that seal the paracellular space between adjacent epithelial cells [2]. CLDNs comprise a family consisting of 27 members [3].

Currently CLDN-1 is the most promising CLDN family member for drug development. CLDN-1-deficient mice show loss of epidermal barrier function [4]; overexpression of CLDN-1 frequently occurs in colorectal carcinoma, breast cancers, and gastric cancers [5-7]; and CLDN-1 is a co-receptor for hepatitis C virus (HCV) [8]. Therefore, CLDN-1 has been studied as a target for enhancing epidermal absorption of drugs, treating cancers, and preventing HCV infection, respectively. Both our group and another group have generated anti-CLDN-1 monoclonal antibodies (mAbs) recognizing the extracellular domain of CLDN-1 and have found that such mAbs enhance epidermal absorption of solutes and prevent HCV infection [9, 10]. However, it remains unclear whether CLDN-1 is a useful target for cancer therapy. Here, we investigated the effect of human-mouse chimeric anti-CLDN-1 mAb (clone 3A2) on tumor therapy using a mouse model.

Materials and methods

Animals

Female Balb/c nu/nu mice (6 weeks old) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). All animals were maintained under controlled conditions of a 12:12-h light:dark cycle and 23 ± 1.5 °C. Animal experiments were performed according to the ethics guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

Cells

HT-1080 cells stably expressing human CLDNs (HT-1080/hCLDNs cells) and L cells stably expressing mouse CLDNs (L/mCLDNs cells) were developed as described previously [9]. Jurkat cells expressing the human Fc γ receptor IIIa (Fc γ RIIIa) and the luciferase (Luc) reporter under the control of the nuclear factor of activated T cells (NFAT) promoter (Jurkat/Fc γ RIIIa/NFAT-Luc) were used to assess the activation of Fc γ RIIIa [11]. HT-1080/hCLDNs cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 100 U/mL penicillin, 100 μ g/mL streptomycin (Nacalai Tesque, Kyoto, Japan) and 500 μ g/mL G418 (Nacalai Tesque). Jurkat/Fc γ RIIIa/NFAT-Luc cells were maintained in RPMI1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin.

Flow cytometric analysis

To analyze the binding activity of mAbs against HT-1080/hCLDNs and L/mCLDNs, cultured cells were detached with trypsin, incubated with various concentrations of antibodies, and then stained with fluorescein-conjugated goat anti-mouse or anti-human IgG (Jackson ImmunoResearch, West Grove, PA). The fluorescence intensity of stained cells was analyzed by using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Preparation of human–mouse IgG1 chimeric anti-CLDN-1 monoclonal antibodies

Human–mouse chimeric antibodies were prepared as described previously [12]. Briefly, cDNA encoding the heavy-chain and light-chain variable domains of 3A2 (anti-CLDN-1) were grafted onto the pFUSE-CHlg-hG1 and pFUSE2-CLlg-hk vectors (InVivoGen, San Diego, CA), respectively. To increase the antibody-dependent cellular cytotoxicity (ADCC) activity of the resultant antibody, site-directed mutagenesis of the 3A2 vector was performed to produce the vector 3A2 (G236A/S239D/I332E).

Human–mouse chimeric mAbs were produced by using the FreeStyle MAX CHO Expression System (Invitrogen, Carlsbad, CA) as described previously [12]. The resultant chimeric mAbs in the medium were purified by using a Protein G Sepharose 4 Fast Flow column (GE Healthcare, Milwaukee, MI). The concentrations of the purified chimeric mAbs in phosphate-buffered saline

were determined by measuring the absorbance at 280 nm (NanoVue, GE Healthcare).

Tumor-targeting activity of chimeric 3A2

To follow the biodistribution of the chimeric 3A2 wild type (xi-3A2) and control human IgG (BD Biosciences), the antibodies were labeled with fluorescent dye CF750 by using a XenoLight CF750 labeling kit (Caliper Life Science, Hopkington, MA). To prepare the tumor xenograft model mouse, Balb/c nu/nu mice were subcutaneously injected with HT-1080/hCLDN-1 (1×10^7 cells) in 100 μ L of phosphate-buffered saline into the left flank. When the tumor size reached 200 mm³, each tumor-bearing mouse was intraperitoneally injected with 20 μ g of CF750-labeled antibody (xi-3A2 or control human IgG). Seventy-two hours after the injection, the mice were killed and tissues were harvested. Whole-body and ex-vivo imaging were performed by using a Maestro EX in vivo imaging system, version 2.10.0 (Cambridge Research & Instrumentation, Cambridge, MA).

Measurement of the antigen binding-dependent activation of Fc γ RIIIa

To assess the level of ADCC activity of xi-3A2 and the G236A/S239D/I332E mutant of xi-3A2, a Fc γ RIIIa reporter assay was performed as described previously [12]. Briefly, HT-1080/hCLDN-1 cells were seeded onto a 96-well plate (Thermo Fisher Scientific, Waltham, MA) at 1.0×10^4 cells/well and incubated for 24 h, and then the medium was changed to Opti-MEM I Reduced-Serum Medium (Invitrogen) containing Jurkat/Fc γ RIIIa/NFAT-Luc cells (1.0×10^5 cells/well) and various

concentrations of prepared mAbs. After 5 h of incubation in a 5% CO₂ air incubator at 37 °C, the activated cells were assessed for luminescence using ONE Glo Luc assay reagent (Promega, Madison, WI). The Luc activities were measured with an EnSpire Multimode Plate Reader (PerkinElmer, Foster City, CA)..

Evaluation of anti-tumor activity in vivo

To study the anti-tumor activity of prepared antibodies, tumor xenograft model mice were prepared as described above. After the inoculation of HT-1080/hCLDN-1 cells into the mice, 0.1 mg/kg of prepared mAbs or control human IgG were intraperitoneally injected twice weekly for 4 weeks. Body weight and tumor size were measured prior to each injection. The tumor volume was calculated according to the following equation: tumor volume (mm³) = length × width² /2.

Statistical analysis

Data were analyzed by the Student's t-test followed by post-hoc pairwise comparisons. The statistical significance for all comparisons was set at $P < 0.05$.

Results and Discussion

We previously generated 4 clones of anti-CLDN-1 mAbs [13]; here we focused on the clone 3A2, which has the highest affinity against CLDN-1 in the binding assay using flow cytometry (Suppl. Fig. 1). Most therapeutic antibodies for tumor therapy are human IgG1 because human IgG1 has the potential to induce ADCC [14, 15]. Therefore, we used a human–mouse chimeric IgG1 of 3A2 by genetically grafting the constant region of human IgG1 onto 3A2 [16]. As previously described, the resultant chimeric antibody, xi-3A2, showed similar CLDN-1 binding specificity to that of the parental 3A2 (Fig. 1A) [12, 13]. We next investigated the tissue distribution of xi-3A2 in mice bearing hCLDN-1–expressing fibroblastoma (HT-1080/hCLDN-1) xenografts. In vivo imaging analysis using XenoLight CF750-labeled antibodies showed accumulation of xi-3A2, but not control IgG, in the tumor (Fig. 1B). The accumulation in the tumor was significantly higher for xi-3A2 (55.0% injected dose [ID]/g tissue) than for the control IgG (12.7% ID/g tissue) at 72 h after antibody injection (Fig. 1C). These findings indicate that xi-3A2 might be a potential therapeutic probe targeting for CLDN-1-overexpressing tumor cells.

Effector activity, including ADCC activity, is frequently associated with anti-tumor activity of therapeutic mAbs [17]. Substitution of Gly, Ser, and Ile with Ala, Asp, and Arg at positions 236, 239, and 332 amino acids of the Fc domain of IgG1, respectively, has been shown to enhance the affinity of IgG1 to FcγRIIIa, thereby increasing ADCC activity [18, 19]. Therefore, to enhance the

anti-tumor activity of xi-3A2, the above series of mutations were genetically introduced into the Fc domain of xi-3A2 (Suppl. Fig. 2). The resultant mutant antibody, G236A/S239D/I332E, and xi-3A2 shared similar affinity to CLDN-1-expressing cells (Fig. 2A).

Tada et al. previously developed a simple system to assay the effector activities of therapeutic mAbs, including ADCC, by monitoring Luc activity driven by NFAT, which is a well-known transcription factor associated with FcγRIIIa activation [11, 20]. The G236A/S239D/I332E mutant activated FcγRIIIa more than xi-3A2 did (Fig. 2B), suggesting that the G236A/S239D/I332E mutant exhibits stronger ADCC activity against CLDN-1 expressing cells than xi-3A2.

To evaluate *in vivo* anti-tumor activity, mice bearing HT-1080/hCLDN-1 xenografts were intraperitoneally injected with xi-3A2, G236A/S239D/I332E, or control IgG (0.1 mg/kg) twice a week for 4 weeks. The G236A/S239D/I332E mutant attenuated tumor growth more than xi-3A2 or control IgG did at day 30 after antibody administration (Fig. 3A–C). These results suggest that ADCC is one of the mechanisms of action of antitumour activity of anti-CLDN-1 mAb (3A2), and the G236A/S239D/I332E mutant with higher ADCC activity may be a promising lead for CLDN-1-targeted tumor therapy.

CLDN-1 is widely expressed in the whole body, especially in the liver and kidney [21]. Therefore, safety evaluation is a major criterion for the clinical application of any anti-CLDN-1 mAb. However, we could not evaluate whether xi-3A2 or its mutant derivative G236A/S239D/I332E were toxic to normal tissues in the mouse xenograft model because these antibodies bind to human CLDN-1, but

not mouse CLDN-1 (Fig. 1A). CLDNs are incorporated into tight junctions in the cell–cell contact between adjacent cells. The intercellular space in tight junctions is 0.4 nm, and the size of the mAb is 10 nm [22, 23]. Thus, anti-CLDN mAbs may have difficulty accessing the CLDNs in the tight junctions [24]. Indeed, the administration of 3A2 to human liver chimeric mice did not induce toxicity in the humanized liver [13].

Patients with chronic HCV infection have an increased tendency to develop liver fibrosis and hepatocellular carcinomas [25]. A drug with therapeutic effects against both HCV infection and hepatocellular carcinoma is an ideal option for hepatocellular carcinoma therapy [26]. In our previous study, 3A2 prevented HCV infection [13]. The expression of CLDN-1 is higher in hepatocellular carcinoma cells than in normal hepatocytes, and CLDN-1 is highly expressed in many other aggressive carcinomas [5-7, 27, 28]. Thus, anti-CLDN-1 mAb might be a therapeutic molecule for HCV-related hepatocellular carcinoma, and anti-CLDN-1 mAb 3A2 might be a potent lead for cancer therapy.

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Figure legends

Figure 1 Tumor-targeting activity of chimeric anti-CLDN-1 mAb derived from clone 3A2.

(A) The CLDN-binding specificity of xi-3A2 was assessed by flow cytometric analysis. HT-1080 cells expressing human CLDNs (HT-1080/hCLDNs) and L cells stably expressing mouse CLDNs (L/mCLDNs) were incubated with phosphate-buffered saline (vehicle) or 5 µg/mL of xi-3A2 and then treated with fluorescein-conjugated secondary antibody. (B) Whole-body in vivo or (C) ex vivo near-infrared imaging of CF750-labeled xi-3A2 or control human IgG. Mice bearing HT-1080/hCLDN-1 tumors were intraperitoneally injected with 20 µg/mouse of CF750-labeled control human IgG or xi-3A2. Tissue accumulation of antibodies was quantified based on the photon count determined from regions of interest analysis. Data in (C) are presented as means ± SD (n = 3). *, $P < 0.05$.

Figure 2 Activation of Fcγ receptor IIIa by chimeric anti-CLDN-1 mAbs derived from clone 3A2.

(A) The CLDN-1 binding activity of xi-3A2 and G236A/S239D/I332E was analyzed by flow cytometry. HT-1080/hCLDN-1 cells were incubated with the indicated concentrations of mAbs and then treated with fluorescein-conjugated secondary antibody. (B) The degree of activation of Fcγ receptor IIIa was analyzed by using the luciferase reporter assay. HT-1080/hCLDN-1 cells were treated with the indicated concentrations of xi-3A2 or G236A/S239D/I332E in the presence of

Jurkat/FcγRIIIa/NFAT-Luc cells. At 5 h after the cultivation, cells were lysed, and the luciferase activity was measured as described in the Materials and Methods section. Data are presented as means $\pm$ SD (n = 3).

Figure 3 In vivo anti-tumor effect of chimeric anti-CLDN-1 mAbs derived from clone 3A2.

HT-1080/hCLDN-1 cells were inoculated into the left flank of nude mice (day 0). Commencing on day 1, the mice were intraperitoneally injected with control human IgG (A), xi-3A2 (B) or G236A/S239D/I332E (C) at a dosage of 0.1 mg/kg twice a week for 4 weeks, and the tumor volume of each mice was monitored. Each line represents an individual mouse.

Figure 1. Hashimoto et. al.,

(A)

Figure 1. Hashimoto et. al.,

(B)

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Figure 2. Hashimoto et. al.,

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Figure 3. Hashimoto et. al.,