Title: Development of an anti-claudin-3 and -4 bispecific monoclonal antibody for cancer diagnosis and therapy

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Abstract

Most malignant tumors are derived from epithelium, and claudin (CLDN)-3 and -4 are frequently overexpressed in such tumors. Although antibodies have potential in cancer diagnostics and therapy, development of antibodies against CLDNs has been difficult because the extracellular domains of CLDNs are too small and there is high homology among human, rat, and mouse sequences. Here, we created a monoclonal antibody that recognizes human CLDN-3 and -4 by immunizing rats with a plasmid vector encoding human CLDN-4. A hybridoma clone that produced a rat monoclonal antibody recognizing both CLDN-3 and -4 (clone 5A5) was obtained from a hybridoma screen by using CLDN-3- and -4-expressing cells; 5A5 did not bind to CLDN-1, -2, -5, -6, -7 or -9-expressing cells. Fluorescence-conjugated 5A5 injected into xenograft mice bearing human cancer MKN74 or LoVo cells could visualize the tumor cells. The human-rat chimeric IgG1 monoclonal antibody (xi5A5) activated FcγRIIIa in the presence of CLDN-3- or -4-expressing cells, indicating that xi5A5 may exert antibody-dependent cellular cytotoxicity. Administration of xi5A5 attenuated tumor growth in xenograft mice bearing MKN74 or LoVo cells. These results suggest that 5A5 shows promise in the development of a diagnostic and therapeutic antibody for cancers.
Introduction

Most malignant tumors are derived from epithelial tissues. A feature of normal epithelial cells is that they are polarized and spatially asymmetric (Nelson and Yeaman, 2001). In epithelial cells, the apical and basolateral membrane domains, which vary in protein and lipid content, are separated by specialized junctional complexes between adjacent cells, called tight junctions (TJs). TJs also act as a seal between adjacent cells to prevent large molecules from crossing the epithelial layer (Gumbiner, 1987). Malignant transformation of epithelium is accompanied by the loss of both cellular polarity and epithelial integrity leading to aberrant growth control, detachment of malignant cells from the primary tumor site, and formation of distant metastasis (Wodarz and Nathke, 2007). Dysregulation of these functions of TJ contributes to the initiation and progression of cancers (Marchiando et al., 2010; Martin and Jiang, 2009). However, the identification and characterization of novel targets for malignant tumors had been delayed due to our poor understanding of the biochemical structure of TJs.

Claudins (CLDNs) are tetra-transmembrane proteins with molecular masses of approximately 23 kDa and are major components of TJ-seals (Furuse et al., 1998). The CLDN multigene family consists of 27 members (Mineta et al., 2011). The expression profiles of the CLDNs vary among tissues, and CLDNs form homotypic and heterotypic strands on the lateral membrane. The CLDN strands between neighboring cells are associated with one another, forming TJ-seals that prevent the free movement of lipids and proteins on the membrane between the apical and basolateral domains and the free movement of solutes across the epithelial cell sheets (Anderson and Van Itallie, 2009; Furuse and Tsukita, 2006).
A series of pathological analyses revealed that numerous tumors, including gastric, colorectal, pancreatic, ovarian, breast, and prostate cancers, overexpress CLDN-3 and CLDN-4 (Ding et al., 2013; Neesse et al., 2012; Tsukita et al., 2008; Turksen and Troy, 2011). CLDN-3 and CLDN-4 are two of the known receptors for *Clostridium perfringens* enterotoxin (CPE), a food poison in humans (McClane and Chakrabarti, 2004; Sonoda et al., 1999). CPE has an N-terminal cytotoxic domain and a C-terminal receptor-binding domain (C-CPE) (McClane and Chakrabarti, 2004; Sonoda et al., 1999). CPE and C-CPE are the first identified CLDN-targeting toxin and CLDN binder, respectively. Intratumoral injection of CPE resulted in tumor suppression and necrosis in pancreatic or breast cancer cells without any observable adverse effects (Kominsky et al., 2004; Michl et al., 2001). Moreover, injection of fluorescence-dye conjugated C-CPE allowed visualization of pancreatic cancers in a xenograft model (Neesse et al., 2013). C-CPE also enhanced sensitivity to chemotherapy in ovarian cancers by modulating the TJ integrity in mice xenograft models of human cancer (Gao et al., 2011). Thus, CPE and C-CPE have contributed to the proof-of-concept for CLDN-3- and CLDN-4-targeted cancer diagnosis and therapy (Gao and McClane, 2012). However, CPE and C-CPE also bind to CLDN-6, -7, -8 and -14 (Fujita et al., 2000), and are immunogenic (Sugii, 1994; Suzuki et al., 2011). Overcoming these problems with the CPE technology is needed to develop CLDN-based cancer diagnosis and therapy.

Antibodies are potential therapeutics for CLDN-targeted cancer therapy because they have high antigen-specificity, high stability, and moderate (or low) immunogenicity (Espiritu et al., 2014; Stockwin and Holmes, 2003). However, the extracellular loop domains of CLDNs are very small (first loop, approximately 50 amino acids; second loop, approximately 15 amino acids), the loop domains show high similarity among the
human, mouse, and rat sequences (approximately 90% homology), and it is difficult to prepare recombinant proteins of CLDNs (currently, only CLDN-4 can be prepared as a recombinant protein) (Evans et al., 2007; Mitic et al., 2003). Therefore, attempts to produce anti-CLDN antibodies have had little success. In this study, we created a monoclonal antibody (mAb) to CLDN-3 and -4 by immunizing rats with a plasmid vector encoding CLDN-4; we then characterized this mAb.
Materials and methods

Mice

BALB/c male mice (6 weeks old) and BALB/c Slc-nu/nu female mice (6 weeks old) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), and were housed in an environmentally controlled room at 23 ± 1.5 °C with a 12-h light/12-h dark cycle. The mice had free access to water and commercial chow (Type MF, Oriental Yeast, Tokyo, Japan). Experimental protocols involving mice were performed according to the ethics guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

Cells

Mouse fibroblasts stably expressing murine CLDN-3 or -4 (mCLDN-3/L or mCLDN-4/L cells) were kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). Jurkat/FcγRIIIa/NFAT-Luc cells, in which the luciferase gene, transcriptionally controlled by the activation of FcγRIIIa, was used to evaluate the activation of FcγRIIIa (Tada et al., 2014). Human gastric cancer MKN74 cells and human colorectal cancer LoVo cells were obtained from ATCC (Manassas, VA). The mCLDN-3/L or mCLDN-4/L cells were maintained in modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and 500 μg/mL G418 (Nacalai Tesque, Kyoto, Japan). Jurkat/FcγRIIIa/NFAT-Luc cells were maintained in RPMI1640 medium supplemented with 10% FBS, 500 μg/mL G418, and 500 μg/mL hygromycin B (Nacalai Tesque). MKN74 cells were maintained in RPMI 1640 supplemented with 10% FBS. LoVo cells were maintained in modified HAM’s F12 supplemented with 20% FBS. All cells were
incubated in a 5% CO₂ atmosphere at 37 °C.

Establishment of stable CLDN transfectants

Human CLDN-1, -2, -3, -4, -5, -6, -7, and -9 cDNA was amplified by polymerase chain reaction, and the resultant cDNAs were cloned into pcDNA3.1(-) (Invitrogen, Carlsbad, CA). The CLDN-expression vectors were then transfected into HT1080 cells by using the X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Basel, Switzerland), and G418-resistant clones were isolated, resulting in the isolation of stable transfectants of each CLDN. CLDNs/HT1080 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS.

Isolation of anti-CLDN4 monoclonal antibodies

Six-week-old male Wistar rats were immunized with a eukaryotic expression vector encoding human CLDN-4 every two weeks for 2 months according to proprietary GENOVAC technology (GENOVAC GmbH, Freiburg, Germany). Lymphocytes were removed 7 days after the last immunization and fused with P3-UI cells in the presence of polyethylene glycol 1000, generating hybridoma cells. Hybridoma cells producing anti-CLDN-3 and -4 mAb were initially screened for the ability of their conditioned medium to bind to human CLDN4-transiently transfected 293T cells but not to wild-type 293T cells; the hybridoma cells producing anti-CLDN-4 mAb were then subsequently selected mAbs that interacted with human CLDN-3/HT1080 cells, resulting in the isolation of a hybridoma that produced a mAb to both human CLDN-3 and -4 (clone 5A5). The immunoglobulin class and subclass of 5A5 was determined by using a rat immunoglobulin isotyping enzyme-linked immunosorbent assay kit (BD
Surface plasmon resonance (SPR) analysis

Human CLDN-4 recombinant proteins were purified by using an Sf-9 cell expression system as described previously (Uchida et al., 2010). To determine the binding kinetics of 5A5 to human CLDN-4 protein, we performed an SPR analysis by using a Biacore T200 instrument (GE healthcare, Little Chalfont, UK) as previously described (Uchida et al., 2010). HBS-EP+ (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05 % Tween 20, pH 7.4) was used as the running buffer. Amine-coupling chemistry was used to immobilize the anti-rat mAb on a CM5 sensor chip (GE Healthcare) surface. The carboxymethyl surface of the CM5 chip was activated with a 1:1 ratio of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride : N-hydroxysuccinimide. Anti-rat mAb was diluted to 20 μg/mL in 10 mM acetate (pH 4.5) and injected over the surface. Excess activated groups were blocked by 1 M ethanolamine (pH 8.5). Approximately 5000 RU of anti-rat mAb was immobilized. Then, the culture supernatant of the mAb-producing hybridoma cells was injected for 2 min. Human CLDN-4 protein was serially diluted to 10, 100, 200, 300, and 500 nM in HBS-EP+. Within a single binding cycle, human CLDN-4 protein was injected sequentially in order of increasing concentration over both the ligand and the reference surfaces. The reference surface, an unmodified flow cell, was used to correct for systematic noise and instrumental drift. The sensorgrams were globally fitted by using a 1:1 binding model to determine $k_a$, $k_d$, and $K_D$ values with the Biacore T200 Evaluation Software.

Purification of the monoclonal antibody
Six-week-old female BALB/c Slc-nu/nu mice were intraperitoneally injected with the adjuvant pristane and $1 \times 10^7$ hybridoma cells producing mAb (clone 5A5). Ascites was collected and the mAb was purified by using Ab-Capcher ExTra (ProteNova, Kagawa, Japan) and a protein G column.

**Flow cytometric analysis**

To analyze the CLDN binding of the mAb, cells were detached and incubated with the mAb ($5 \mu g/mL$) and then treated with secondary fluorescein-conjugated goat anti-rat IgG or goat anti-human IgG for 5A5 or human-rat chimeric 5A5, respectively. mAb-bound cells were analyzed by using a FACSCalibur flow cytometer (BD Biosciences).

**Immunoblot analysis**

Cells were lysed in lysis buffer [20 mM Tris, pH 7.4; 150 mM NaCl; 1% Triton X-100; protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)], and cell lysates (10 μg protein) were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto a polyvinylidene difluoride membrane, and then immunoblotted with anti-CLDN-1, -2, -3, -4, and -5 Ab (Invitrogen), or with an anti-β-actin Ab (Sigma-Aldrich). The immunoreactive band was visualized by using ECL™ Western Blotting Detection Reagents (GE Healthcare).

**Cytotoxic Activity**

Cancer cells were seeded onto a 96-well plate at $1 \times 10^4$ cells/well. After 24 h of
culture, the cells were treated with the mAb for 48 h at the indicated concentrations. Cytotoxicity was assessed by using a WST-8 kit according to the manufacturer’s instructions (Nacalai Tesque).

**Preparation of CF750-conjugated monoclonal antibodies**

Rat IgG and 5A5 were labeled with the fluorescent dye CF750 by using a XenoLight CF750 rapid antibody-labeling kit (Caliper Life Sciences, Inc., Hopkinton, MA), in accordance with the manufacturer’s instructions. The concentrations of conjugated mAbs were calculated by using the following equation provided in the manufacturer’s protocol:

\[
\text{concentration (mg/mL)} = \left( \frac{\text{absorbance at } 280 \text{ nm} - \text{absorbance at } 755 \text{ nm} \times 0.3}{1.4} \right) \times \text{dilution factor}
\]

**5A5 distribution in a nude mouse xenograft tumor model**

Six-week-old female BALB/c Slc-nu/nu mice were injected subcutaneously into the right flank with cancer cells (1 × 10⁷ cells) in 100 μL of phosphate buffered saline (PBS). After 5 weeks, mAbs labeled with CF750 were intraperitoneally injected into the mice at 20 μg/100 μL of PBS per mouse. After 6, 24, 48, 72, or 96 h, the mice were anesthetized with thiamylal sodium and set under a Maestro EX in vivo imaging system, version 2.10.0 (Cambridge Research & Instrumentation Inc., Woburn, MA) to visualize CF750-conjugated mAbs. The imaging system was equipped with an excitation filter (wavelength 229 to 684 nm). Fluorescence was detected by use of a CCD camera equipped with a C-mount lens and a long-pass emission filter (745 nm). Spectral data “cubes” were created by the acquisition of a series of images obtained by using different wavelengths. The mice were then sacrificed under anesthesia with thiamylal sodium and
their lungs, liver, kidney, thyroid, and tumors were excised. The organs from each mouse were placed side-by-side and imaged at a 500 ms exposure time. In the spectral data cubes, each pixel is associated with a spectrum. Maestro software can be used to analyze these data; any autofluorescence can be identified, separated from the CF750 fluorescence, and removed. The signal detected intensity was set at $5 \times 10^{-5}$, and the resulting signals (counts) from each tissue were used to evaluate the mAb distributions. The levels of mAbs in each tissue, as percentages of the injected doses, were then calculated.

**Preparation of the human-rat chimeric IgG1 mAb**

The cDNA encoding the heavy-chain and light-chain variable domains of 5A5 were amplified by PCR, and the PCR products were subcloned into the pFUSE-CHIg-hG1 and pFUSE2-CLIg-hk vectors (InVivoGen, San Diego, CA), respectively. Human-rat chimeric 5A5 (xi5A5) was prepared by using the FreeStyle MAX CHO Expression System (Life Technologies, Carlsbad, CA). Briefly, CHO-S cells were co-transfected with pFUSE-CHIg and pFUSE-CLIg of 5A5 by using the FreeStyle MAX Reagent, and then the transfected cells were cultured for 6 days in FreeStyle CHO Expression Medium. The conditioned medium was recovered and applied to a protein G column. The column was washed with 20 mM sodium phosphate buffer (pH 6.8), and the mAb was eluted by using 0.1 M glycine-HCl (pH 3.0). The eluted fraction containing the mAb was neutralized with 1 M Tris-HCl (pH 8.0), and then desalted by using a PD-10 column (GE Healthcare) with PBS as the exchange buffer. The concentration of the purified antibody was determined by measuring the absorbance at 280 nm.
Measurement of FcγRIIIa activation

Cells were seeded onto a 96-well plate at $1 \times 10^4$ cells/well. After 24 h of culture, Jurkat/FcγRIIIa/NFAT-Luc cells ($1 \times 10^5$ cells/well) suspended in Opti-MEM I Reduced Serum Medium were added to the 96-well plate in the presence of the mAbs. After a 5-h incubation at 37 °C in a 5% CO₂ atmosphere, the luciferase activities were measured by using a commercially available luciferase assay kit as described previously (Tada et al., 2014).

In vivo antitumor activity

Six-week-old female BALB/c Slc-nu/nu mice were injected subcutaneously with cancer cells ($1 \times 10^7$) in 100 μL of PBS into the right flank on day 0. After the inoculation of the cells, the mAb was intraperitoneally injected into the xenograft tumor model at 1 mg/kg body weight twice a week. Body weight and tumor size were measured prior to each injection. The tumor volume was calculated by using the following equation: tumor volume = length × width² / 2.

Statistical analysis

Data were analyzed by the Student’s t-test or the one-way ANOVA followed by post hoc pairwise comparison. The statistical significance for all comparisons was set at $p < 0.05$. 
Results

Creation of a mAb to claudin-3 and -4

To create a mAb that recognizes CLDN-3 and -4, rats were immunized with a plasmid vector encoding human CLDN-4. B cells isolated from immunized rats were fused with mouse myeloma cells. The resultant hybridomas were screened by using CLDN-3- or -4-expressing cells, resulting in the identification of a hybridoma that produces a mAb (clone 5A5) that recognizes both human CLDN-3 and -4. Clone 5A5 bound to CLDN-3 and -4 but not to CLDN-1, -2, -5, -6, -7, or -9 exogenously expressed in HT1080 cells (Fig. 1). The subclass of 5A5 was IgG2a. To our knowledge, of the CLDN family, only recombinant CLDN-4 protein can be prepared. Therefore, we carried out an SPR analysis to determine the kinetics of 5A5 to only CLDN-4. The binding kinetics of 5A5 to CLDN-4 were as follows: $k_a$, 1.33 (1/Ms, × 10^4); $k_d$, 0.58 (1/s, × 10^{-4}); and $K_D$, 4.35 nM.

To evaluate the diagnostic and therapeutic potential of 5A5 for cancer therapy, we selected human gastric cancer MKN74 cells and human colonic cancer LoVo cells because these cells are reported to express CLDN-3 and -4 mRNA and they have been used effectively in mice xenograft tumor models (http://www.lsbm.org/site_e/index.html). CLDN-1, -4, and -5 were detected in MKN74 cells, and CLDN-1, -2, -4, and -5 (faint signal) were observed in LoVo cells (Fig. 2A). However, CLDN-3 protein was not detected in these cells. Flow cytometric analysis showed that 5A5 bound to both MKN74 cells and LoVo cells (Fig. 2B). Therefore, we used MKN74 cells and LoVo cells to further characterize the anti-tumor activity of 5A5.
In vivo imaging in xenograft models

To investigate whether 5A5 could be used as an in vivo targeting molecule for cancer, a fluorescent dye (XenoLight CF750) was conjugated with 5A5 via its reactive amine group because the fluorescent signal of CF750 can be non-invasively detected under an in vivo imaging system. CF750-conjugated 5A5 retained the ability to bind to CLDN-4-expressing cells (Suppl. Fig. 1). MKN74 cells were subcutaneously inoculated into BALB/c-nu/nu mice. Five weeks later, the conjugated 5A5 was injected into the xenograft tumor models at 20 μg/body. 5A5 distributed through the tumor tissue in a time-dependent manner, reaching a plateau at 72 h after injection (Fig. 3A and 3B); 5A5 was similarly distributed through the tumor tissue in mice bearing LoVo cells (Fig. 4A and 4B).

Preparation of human chimeric IgG1

Most therapeutic mAbs for cancer therapy are human IgG1 because human IgG1 has effector activities such as antibody-dependent cellular cytotoxicity (ADCC) (Houot et al., 2011; Shuptrine et al., 2012). Therefore, we next prepared a human-rat chimeric IgG1 mAb (xi5A5) by grafting the variable regions of the heavy and light chain of 5A5 (Fig. 5A). xi5A5 retained the CLDN specificity of the parental mAb (Suppl. Fig. 2). Activation of FcγRIIIa is involved in the activation of ADCC (Houot et al., 2011). Accordingly, we investigated the effects of xi5A5 on the activation of FcγRIIIa by using Jurkat/FcγRIIIa/NFAT-Luc reporter cells, in which luciferase expression was accompanied by activation of FcγRIIIa (Tada et al., 2014). Jurkat/FcγRIIIa/NFAT-Luc reporter cells were not activated by xi5A5 when co-cultured with CLDN-1/HT1080 cells (Fig. 5B); however, the reporter cells were activated when co-cultured with
CLDN-3/HT1080 or CLDN-4/HT1080 cells (Fig. 5C and 5D). The activation of the reporter cells by xi5A5 were also detected when co-cultured with MKN74 or LoVo cells (Fig. 6A and 6B), suggesting that xi5A5 activate FcγRIIIa in an antigen-binding dependent manner, and may exert ADCC activity against CLDN-3/CLDN-4 expressing tumor cells.

In vivo anti-tumor activity in a xenograft model

Finally, we investigated the anti-tumor activity of xi5A5 in mice bearing xenograft MKN74 or LoVo cells. After subcutaneous inoculation of mice with MKN74 or LoVo cells, xi5A5 was intraperitoneally injected twice a week at 1 mg/kg. xi5A5 suppressed tumor growth in both MKN74- and LoVo-bearing mice on days 7 and 14 of post-inoculation, respectively (Fig. 7A and 7C). Administration of xi5A5 caused no apparent adverse effects or weight loss (Fig. 7B and 7D). On the other hand, xi5A5 showed no cytotoxicity in MKN74 or LoVo cells in vitro even at 10 μg/mL (Suppl. Fig. 3). The anti-tumor activity of xi5A5 may, therefore, be due to effector activity such as ADCC.
Discussion

Recent advances in our understanding of the biochemical structures of TJs have provided us with new insights into targets for therapeutic intervention for cancer: the CLDNs (Ding et al., 2013; Lal-Nag and Morin, 2009; Morin, 2005; Neesse et al., 2012; Tsukita et al., 2008; Turksen and Troy, 2011). In the present study, we created a mAb (clone 5A5) that recognizes human CLDN-3 and -4. Although 5A5 bound to CLDN-3- and -4-expressing cells, it could not detect CLDN-3 and -4 in an immunoblot (data not shown), indicating that 5A5 recognizes the intact forms of human CLDN-3 and -4. We also found that 5A5 could serve as a diagnostic probe for tumor tissues and that a human chimeric IgG1 mAb (xi5A5) had in vivo anti-tumor activity in mice bearing MKN74 or LoVo xenograft tumors.

The CLDNs constitute an integral protein family of 27 members, which share approximately 50% homology among their extracellular domains (Kato-Nakano et al., 2010; Mineta et al., 2011; Morita et al., 1999). They are ubiquitously expressed in various normal tissues, exhibiting barrier- and fence-functions in epithelia (Anderson and Van Itallie, 2009; Furuse and Tsukita, 2006). Therefore, to avoid adverse effects caused by cancer therapies targeting CLDNs, the specificity of the CLDN member and ligands for the type of cancer being treated are critical. CLDN-3 and -4 are receptors for CPE, but CPE also binds to CLDN-6, -7, -8, and -14 (Fujita et al., 2000). While CPE- and C-CPE-based cancer therapy has established the proof-of-concept for CLDN-targeted cancer treatment, therapeutic interventions have been limited because of CLDN-member specificity and immunogenicity (Gao and McClane, 2012). Clone 5A5 showed binding specificity to CLDN-3 and -4. Thus, 5A5 may be superior to C-CPE as
a CLDN-targeted ligand in terms of specificity and immunogenicity.

CLDN-3 and -4 are both highly expressed in lung, intestine, pancreas, and kidney; CLDN-3 is also highly expressed in liver (Morita et al., 1999; Rahner et al., 2001). The safety of CLDN-3- and -4-based cancer therapy is very important for its clinical application. We previously found that the liver and kidney are two major sites of C-CPE distribution and that intravenous injection of a C-CPE-fused toxin led to hepatic but not renal injury (Li et al., 2014). The C-CPE mutant which lacks the CLDN-binding domain, distributed through the kidney with similar kinetics to those of C-CPE. CLDN-3 and CLDN-4 are expressed in the lateral membrane of the epithelium in Henle's loop, the distal tubule, and the collecting duct (Balkovetz, 2009). The CLDN-3- and CLDN-4-based TJMs regulate the paracellular transport of ions (Hou et al., 2010; Milatz et al., 2010), and the size of the CLDN-based pores in the TJMs has been estimated to be 4 nm (Van Itallie and Anderson, 2011). Therefore, a CLDN-targeted toxin with a molecular weight of approximately 60 kDa, and also 5A5 with a molecular weight of 150 kDa, could not easily access the CLDNs embedded in the renal TJMs. Indeed, intratumoral or intraperitoneal injection of CPE (35 kDa) attenuated tumor growth without apparent adverse effects on the xenograft tumor models (Kominsky et al., 2004; Michl et al., 2001; Santin et al., 2005).

CLDN-3-deficient mice are not available, but CLDN-4 knockout mice have been developed. Fujita et al. showed that CLDN-4-deficient mice develop normally until about 12 months of age, but then begin to exhibit urothelial hyperplasia and lethal hydronephrosis (Fujita et al., 2012). CLDN-4 deficiency did not affect epithelial TJ integrity in the nephrons or urothelium. An increase in the proliferation of the pelvic and ureteral urothelial cells was thought to be associated with the urothelial hyperplasia and
development of hydronephrosis. CLDN-4 hetero-deficient mice showed none of these abnormal phenotypes. Although the underlying mechanism for renal dysfunction in CLDN-4 homo-deficient mice is unclear, therapeutic and adverse effects might trade-off in CLDN-3- and -4-targeted cancer therapy at least when using mAbs because of their size advantage.

Currently, conjugation of mAbs with cytotoxic drugs or radionuclides is receiving much attention as new options for therapeutic mAbs (Lambert, 2005; Senter, 2009; Wu and Senter, 2005). It is critical for the conjugation that chemical modification of mAbs does not affect binding of mAbs to antigen and intracellular uptake of the drug-conjugated mAbs. Here, we chemically conjugated 5A5 with a fluorescent dye by reacting the dye with an amine group such as that of the lysine residue of 5A5. The conjugated 5A5 showed similar CLDN-binding tropism to that of the parental 5A5. Moreover, when MKN74 cells were treated with 5A5, 5A5 may appear to enter into the cells (Suppl. Fig. 4). These finding suggest that 5A5 could be developed as an antibody–drug conjugate because conjugation of drugs to a mAb takes place at a reactive amine group such as that of lysine (Panowksi et al., 2014).

In conclusion, we here developed a dual-specific mAb (clone 5A5) to human CLDN-3 and -4. Although the druggability of 5A5 requires the improvement through humanization and the safety evaluations in monkeys, 5A5 appears to be a promising lead mAb in the development of CLDN-based agents for the diagnosis and treatment of epithelium-derived malignant tumors.
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Authorship contribution

Participated in research design: Li, Iida, Tada, Kondoh

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Contributed new reagents or analytic tools: Watari, Tada, Yamashita, Ishii-Watabe, Uno, Fukasawa

Performed data analysis: Li, Iida, Tada, Ishii-Watabe, Kuniyasu, Yagi, Kondoh

Wrote or contributed to the writing of the manuscript: Li, Tada, Ishii-Watabe, Kondoh
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Footnotes

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Figure legends

Figure 1  Specificity of 5A5 assessed by using various CLDN transfectants.

CLDN-transfected cells were incubated with 5A5 at 5 μg/mL, and then reacted with fluorescence-conjugated goat anti-rat IgG (H+L). The antibody-bound cells were detected by using a flow cytometer as described in the Materials and Methods. Open and closed histograms represent vehicle- and 5A5-treated cells, respectively. hCLDN, human CLDN; mCLDN, mouse CLDN.

Figure 2  Interaction of 5A5 with CLDN-expressing cancer cells

A) Immunoblot analysis of MKN74 and LoVo cells. Lysates of MKN74 or LoVo cells (10 μg protein) were subjected to SDS-PAGE, followed by immunoblotting to detect CLDNs. Lysates of human CLDN-1, -2, -3, -4, and -5/HT1080 cells were used as positive controls (far right lane); β-actin as an endogenous control. B) Flow cytometric analysis of MKN74 and LoVo cells. Cells were collected, and the cell suspension was incubated with 5A5 at 5 μg/mL, and then reacted with fluorescence-conjugated goat anti-rat IgG (H+L). The antibody-bound cells were detected by using a flow cytometer. The open histogram represents vehicle-treated cells; the closed histogram represents 5A5-treated cells.

Figure 3  Tissue distribution of 5A5 in mice bearing xenograft MKN74 cancer cells.

MKN74 cells (1 × 10⁷ cells) were subcutaneously transplanted into BALB/c Slc-nu/nu mice. Five weeks after the inoculation, CF750-5A5 or CF750-rat IgG was intraperitoneally injected at 20 μg/mouse, and the fluorescence intensity was monitored.
in the mice (A) and in isolated tissues (B) at the indicated times. Circles indicate the transplanted tumors in panel A. The amount of mAb accumulated in each tissue was calculated as a percentage of the injected dose per gram of tissue. Data are means ± SEM (n = 3). %ID/g: percent of the injected dose per gram of tissue. *p < 0.05 vs. CF750-IgG.

Figure 4  Tissue distribution of 5A5 in mice bearing xenograft LoVo cancer cells. LoVo cells (1 × 10⁷ cells) were subcutaneously transplanted into BALB/c Slc-nu/nu mice. Five weeks after the inoculation, CF750-5A5 or CF750-rat IgG was intraperitoneally injected at 20 μg/mouse, and the fluorescence intensity was monitored in the mice (A) and in isolated tissues (B) at 72 h after the injection of the mAbs. Circles indicate the transplanted tumors in panel A. The amount of mAb accumulated in each tissue was calculated as a percentage of the injected dose per gram of tissue. Data are means ± SEM (n = 3). %ID/g: percent of the injected dose per gram of tissue. *p < 0.05 vs. CF750-IgG.

Figure 5  CLDN-3/4-specific activation of FcγRIIIa by the human-rat chimeric mAb (xi5A5)
A) Purification of xi5A5. xi5A5 was produced in CHO-S cells and was purified by using protein G as described in the Materials and Methods. The purified xi5A5 was subjected to SDS-PAGE followed by staining with coomassie brilliant blue. The left lane shows the molecular weight markers; the right lane shows xi5A5. The arrowhead indicates the purified xi5A5. B–D) Activation of FcγRIIIa by xi5A5. hCLDN-1 (B), hCLDN-3 (C), or hCLDN-4 (D) /HT1080 cells were seeded at 1 × 10⁴ cells/well in
After 24 h of cultivation, Jurkat/FcγRIIIa/NFAT-Luc cells were added to the 96-well plates at 1 \times 10^5 cells/well in the presence of rat IgG, human IgG, 5A5, or xi5A5 at the indicated concentrations. The cells were then incubated for an additional 5 h, and the luciferase activity in each well was measured as described in the Materials and Methods. Data are means ± SD (n = 3). *p < 0.05 vs. human IgG.

Figure 6  Activation of FcγRIIIa by xi5A5 bound to MKN74 or LoVo cells.

MKN74 (A) or LoVo (B) cells were seeded at 1 \times 10^4 cells/well in a 96-well plate and cultured for 24 h. Then, Jurkat/FcγRIIIa/NFAT-Luc cells were added at 1 \times 10^5 cells/well to the 96-well plate in the presence of rat IgG, human IgG, 5A5, or xi5A5 at the indicated concentrations. After a 5-h incubation, the luciferase activity was measured. Data are means ± SD (n = 3). *p < 0.05 vs. human IgG.

Figure 7  Anti-tumor activity of xi5A5 in mice bearing xenograft tumor cells.

LoVo cells (A and B) or MKN74 cells (C and D) were subcutaneously transplanted into BALB/c Slc-nu/nu mice. xi-5A5 was intraperitoneally injected at 1 mg/kg twice a week for 4 weeks. Tumor size (A and C) and the body weight of the mice (B and D) were monitored during xi5A5 treatment. Tumor volume was calculated by using the following equation: tumor volume = length × width^2 / 2. Data are means ± SEM (n = 5). *p < 0.05 vs. PBS.
Figure 4

A. IgG vs. 5A5

B. %ID/g

- Lung
- Liver
- Kidney
- Thyroid
- Tumor

* indicates significant difference.
Figure 5A and 5B
Figure 6

A. Luciferase activity (RLU/10^5 cells) versus mAb concentration (μg/mL) for different antibodies: Rat IgG, Human IgG, Rat 5A5, and Xi5A5.

B. Luciferase activity (RLU/10^5 cells) versus mAb concentration (μg/mL) for different antibodies: Rat IgG, Human IgG, Rat 5A5, and Xi5A5.
**C.**

Tumor volume (mm$^3$) over time (Day).

- PBS (open circles)
- Xi5A5 (closed circles)

**D.**

Body weight (g) over time (Day).

- PBS (open circles)
- Xi5A5 (closed circles)
Title: Development of an anti-claudin-3 and -4 bispecific monoclonal antibody for cancer diagnosis and therapy

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Figure legends for supplemental figures

Supplemental figure 1  Interaction of CF750-conjugated 5A5 with hCLDN-4/HT1080 cells.

hCLDN-4/HT1080 cells were recovered; the cell suspension was incubated with CF750-conjugated rat IgG (CF750-rat IgG) or 5A5 (CF750-5A5), and was then reacted with fluorescein-conjugated goat anti-rat IgG (H+L). The antibody-bound cells were detected by using a flow cytometer. Open and closed histograms represent CF750-rat IgG-treated and CF750-5A5-treated cells, respectively.

Supplemental figure 2  CLDN specificity of xi5A5.

hCLDN-1, -2, -3, -4, -5, -6, -7, or -9 stably transfected HT1080 cells were recovered; the cell suspension was incubated with xi5A5 at 5 μg/mL, and was then reacted with fluorescence-conjugated goat anti-human IgG. The antibody-bound cells were detected by using a flow cytometer. Open and closed histograms represent vehicle- and xi5A5-treated cells, respectively.

Supplemental figure 3  Cytotoxicity of xi5A5 in MKN74 and LoVo cells.

MKN74 cells (A) or LoVo cells (B) were seeded onto a 96-well plate at $1 \times 10^4$ cells/well. After 24 h of culture, the cells were treated with xi5A5 for 48 h at the indicated concentrations. Cytotoxicity was determined by using a WST-8 kit according to the manufacturer’s instructions (Nacalai Tesque, Kyoto, Japan). Viability was calculated as a percentage of the vehicle-treated cells. Data are means ± SD (n = 3).
Supplemental figure 4  Immunofluorescence analysis of 5A5 localization.

MKN74 cells were seeded onto a 12-well plate coated with 50 µg/mL collagen Type I (BD Biosciences, CA) at 1 × 10^5 cells/well. After 24 h of culture, the cells were treated with 5A5 at 5 µg/mL for 1 or 6 h. The cells were then treated with 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) and 0.1% (v/v) Triton-X/PBS. Goat-anti-rat IgG-FITC (KPL, MD) and 4’,6-Diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich, MO) were added and fluorescence was observed by use of a Leica TCS SP5 fluorescence confocal microscope (Leica Microsystems, Germany). The green and blue signals represent 5A5 and nuclei, respectively. Each pictures at 1 h are the imaged at different dimension. Bar = 50 µm.
Supplemental figure 1

CF750-IgG

CF750-5A5

Vehicle  CF750-conjugated antibodies
A. 

Relative viability (%) 

$xi5A5$ concentration ($\mu g/mL$) 

B. 

Relative viability (%) 

$xi5A5$ concentration ($\mu g/mL$)