Mouse-Human Chimeric Anti-Tn IgG1 Induced Anti-tumor Activity against Jurkat Cells in Vitro and in Vivo

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Tn-antigen (α-N-acetyl-galactosamine(GalNAc)-Ser/Thr) is a cancer-associated carbohydrate antigen expressed in various epithelial and hematological cancers, and although a number of anti-Tn IgG and IgM antibodies have been generated, they have not been fully validated for cancer immunotherapy. In this study, we generated a novel murine anti-Tn IgG1 monoclonal antibody, KM3413, by immunization of mucins purified from a culture supernatant of LS180: a human colon cancer cell line. The binding of KM3413 was detected against consecutive Tn-antigens (Tn3 and Tn2), but not against monovalent antigens (Tn1). The affinity (Kd) of KM3413 was determined to be about 10–7 M with BIAcore. Cross-reactivity against type-A blood antigen, which shares a sugar residue, α-linked GalNAc, with Tn-antigen, was not detected. Next, we generated mouse-human chimeric IgG1 of KM3413 (cKM3413) and evaluated its anti-tumor activities against Jurkat: a human T-lymphoid leukemia cell line. In vitro assay revealed that cKM3413 induced antibody-dependent cellular cytotoxicity (ADCC) and direct killing activity with cross-link antibody. Furthermore, treatment of cKM3413 (1 or 10 mg/kg) showed significantly better survival of Jurkat-inoculated C.B-17/Id-cd Jcl mice compared with controls using PBS treatment (p<0.001). These results suggest that humanized antibody against clustered Tn-antigens is a promising therapeutic antibody against Tn-positive cancers.

Key words Tn-antigen; carbohydrate antigen; monoclonal antibody; antibody-dependent cellular cytotoxicity; cancer therapy

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MATERIALS AND METHODS

Blood Donors Blood donors were randomly selected from healthy volunteers registered at the BioFrontier Laboratory, Kyowa Hakko Kogyo Co., Ltd. All donors gave written informed consent prior to participation.

Animals All experiments were performed in conformity with institutional guidelines and in compliance with national laws and policies. C.B-17/Id-cd Jcl (SCID) male mice (5—6 weeks old) were purchased from CLEA Japan, Inc. (Tokyo) and maintained under pathogen-free conditions.

Cell Lines For the production of mouse-human chimeric antibodies and hybridoma screening, theCHO cell line, DG44, was kindly provided by Dr. Lawrence Chasin (Columbia University). CHO cell line, Lec8 (ATCC CRL-1737), for the hybridoma screening, murine myeloma cell line P3-U1 (ATCC CRL-1597), human colon cancer cell line LS180 (ATCC CL-187), and human acute T-cell leukemia cell line Jurkat (ATCC TIB-152) were purchased from the American

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Type Culture Collection (Rockville, MD, U.S.A.).

**Purification of Mucins** Culture supernatant containing mucins derived from LS180 was lyophilized and reconstituted in deionized water. The reconstituted solution was subjected to a Sepharose CL-4B column (Amersham Biosciences, Piscataway, NJ, U.S.A.) and the collected fractions containing Tn-antigens, were measured by RCAS1 enzyme-linked immunosorbent assay (ELISA) kit (MBL, Nagoya, Japan) containing 22-1-1 as the detection monoclonal antibody. The fractions were dialyzed in deionized water and used for immunization.

**Isolation of a Murine Anti-Tn Monoclonal Antibody**

Six-week-old female BALB/c mice (CLEA Japan, Inc.) were immunized four times with 50 μg of mucins with aluminum hydroxide and Bordetella pertussis adjuvant. The spleen was removed 3 d after final injection of the antigen, and 2×10^8 splenocytes fused with 2×10^7 P3-U1 in the presence of polyethylene glycol 1000 (Junsei, Tokyo, Japan). Cultured hybridoma cells in wells showing anti-Tn antibody activity were screened by 8200 cellular detection system (Applied Biosystems, Tokyo, Japan), using Lee8 and DG44 as target cells. After cloning twice with limited dilution, a stable clone was obtained (KM3413). The immunoglobulin class and subclass of KM3413 (IgG1) were determined with anti-mouse isotype-specific antibodies (Zymed, San Francisco, CA, U.S.A.).

**Establishment of Productive Chimeric Anti-Tn IgG1**

The heavy- and light-chain variable region cDNAs were prepared from hybridoma cells producing KM3413 by polymerase chain reaction (PCR) and cloned into the pKANTEX93 chimeric IgG1 antibody expression vector to obtain the cKM3413 expression vector. The vector was then introduced into DG44 cells via electroporation, and transfected cells selected for gene amplification in methotrexate containing medium.

**Production and Purification of Chimeric Anti-Tn IgG1**

A high-producing cell clone, determined by ELISA specific for human IgG1, was grown in serum-free EX-CELL301 (JRH Bioscience, Lenexa, KS, U.S.A.). Following manufacturer’s instructions, the culture supernatant was subjected to a Prosep-A column (Nihon Millipore, Tokyo, Japan), and the elution buffer changed into 10 mM citrate buffer (pH 6.5) containing 150 mM NaCl by gel filtration on an NAP-10 column (Nihon Millipore, Tokyo, Japan) containing 22-1-1 as the detection monoclonal antibody. The fractions were dialyzed in deionized water and used for immunization.

**Flow Cytometry (FCM) Analysis** Anti-Tn antibodies binding to cell surface molecules were analyzed by flow cytometry (FCM). A-positive bloods from healthy volunteers were identified by clotting using mouse anti-human blood group antigen-A antibody (Dako, Tokyo, Japan). Jurkat cells or type-A red blood cells were stained with KM3413 or cKM3413. Fluorescein isothiocyanate-conjugated (FITC-conjugated) goat anti-mouse IgG antibody (Dako) or FITC-conjugated mouse anti-human Ig antibody (Jackson Laboratory, Bar Harbor, ME, U.S.A.) was used as a secondary reagent. These stained cells were analyzed using an EPICS XL-MCL FCM (Beckman Coulter, Tokyo, Japan).

**Western Blot Analysis** Jurkat cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 2 mM MgCl2, 2 mM CaCl2, 0.1% NaN3, 5 μM phenylmethylsulfonyl fluoride, 100 μM dithiothreitol, 1% Triton X-100, 50 mM N-ethylmaleimide and 1 mg/ml leupeptin. The protein concentration was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA, U.S.A.). The lysate (5 μg) was solubilized in loading buffer containing 0.1 M Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol and 5% 2-mercaptoethanol, boiled for 5 min, and separated by 5—20% SDS-polyacrylamide gel electrophoresis. The proteins were then electrophotoretically transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 10% BSA and incubated with 2 μg/ml anti-CD43 monoclonal antibody, 1G10 (Becton Dickinson, Tokyo, Japan), or KM3413. In the inhibition experiment, 100 mM galactose (Nacalai Tesque, Kyoto, Japan) or N-acetyl-D-galactosamine (Sigma-Aldrich, St. Louis, MO, U.S.A.) were pre-incubated with KM3413. After washing, the membrane was exposed to
horseradish peroxidase-conjugated anti-mouse IgG (Zymed) and the bands were visualized with ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, U.K.).

**Direct Killing Assay** Aliquots of Jurkat cells (5×10^3 cells/well) were put into 96-well plates and incubated at 37 °C with cKM3413 with or without 20 μg/ml of cross-link antibody, F(ab’)_2 fragment goat anti-human IgG (Pierce, Rockford, IL, U.S.A.), for 48 h. Viable cells were detected with WST-1 (Roche Diagnostics, Tokyo, Japan), and the absorbance (450 nm) measured with a max precision microplate reader. Percentage-specific direct killing activity was calculated from sample counts according to the formula:

\[
\% \text{ cytotoxicity} = 100 \times \frac{1 - (EBL/nAb - BL)}{M - S}
\]

Where \(E\) is experimental absorbance (target cells incubated with antibodies), \(nAb\) is spontaneous absorbance (target cells incubated with medium alone), and \(BL\) is the medium-alone absorbance.

**ADCC** Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood of healthy donors using Lymphoprep (Fresenius Kabi, Oslo, Norway) and used as effector cells. Jurkat cells (1×10^5) were labeled with 3.7 MBq Na₂¹⁵²⁵⁰CrO₄ for 90 min at 37 °C and kept for 30 min at 4 °C to remove loosely bound ¹⁵²⁵⁰Cr after washing. Aliquots of the labeled cells (1×10⁴ cells/well) and effector cells (2×10⁵ cells/well, effector/target ratio is 20/1) were put into 96-well plates and incubated with various concentrations of cKM3413 or control human IgG (Sigma-Aldrich) for 4 h at 37 °C. After centrifugation, the released ¹⁵²⁵⁰Cr in the supernatant was counted. Percentage-specific lysis was calculated from sample counts according to the formula:

\[
\% \text{ cytotoxicity} = 100 \times \frac{E - S}{M - S}
\]

Where \(E\) is experimental release (cpm in the supernatant from target cells incubated with antibody and effector cells), \(S\) is spontaneous release (cpm in the supernatant from target cells incubated with medium alone), and \(M\) is the maximum release (cpm released from target cells lysed with 1 M HCl).

**In Vivo Assay** Jurkat cells (1.2×10^⁵) were injected intravenously (i.v.) into SCID mice treated the previous day with anti-asialo GM1 antibody (Wako) intraperitoneally (i.p.). After injection, cKM3413 (1 or 10 mg/kg) or PBS was injected i.p. twice a week for 4 weeks (total: 8 injections). The survival days of each mouse were measured (n=6).

**Statistical Analysis** Results are expressed as means or means±S.D. The statistical significance of differential findings between the experimental groups of animals was determined by log-rank test. Statistical analysis was performed using the SAS software program (Release 9.1.3, SAS Institute Inc., Cary, NC, U.S.A.).

**RESULTS**

**Isolation of Anti-Tn IgG1 Monoclonal Antibody, KM3413** In this study, we purified mucins containing Tn-antigen from the culture supernatant of LS180 and used these mucins as immunogen. BALB/c mice were immunized with the mucins, and then hybridoma cells generated by fusion of murine splenocytes and myeloma cells. To select anti-Tn monoclonal antibodies, we demonstrated cell-based screening with the Applied Biosystems 8200 cellular detection system. In the screening, DG44 and Lec8 cells were used as negative and positive targets, respectively. Lec8 cells stably expressed Tn-antigen on the cell surface, because they were unable to translocate UDP-galactose from the cytosolic compartment to the Golgi apparatus,23,24) thus resulting truncation of the Ser/Thr-linked oligosaccharides to a single residue of terminal α-linked GalNAc.25,26) Cross-reactivity against type-A blood antigen of selected candidates was further evaluated by FCM analysis using type-A red blood cells. Finally, we succeeded in isolating KM3413, which bound to Lec8 cells but not to DG44 or type-A red blood cells.

**The Binding Activity and Specificity of KM3413 against Clustered Tn-Antigens** Binding activity of KM3413 was measured by ELISA and BIAcore using synthetic Tn-antigens: Tn3, Tn2, Tn1 and Tn0-biotin. ELISA analysis showed dose-dependent binding of KM3413 against Tn3 and Tn2, but not Tn1 and Tn0 (Figs. 1A—D). BIAcore assay using an SA sensor chip bound Tn3-biotin revealed binding affinity (\(K_d\)) was 1.6×10⁻⁷ M \(\text{Ca}=1.8×10^6 \text{M}^{-1}\text{s}^{-1}\) and \(K_d=0.029 \text{s}^{-1}\) (Fig. 1E).

We evaluated the binding activity against Jurkat cells expressing Tn-antigen on the cell surface with FCM. Incubation with KM3413 (2 μg/ml) consequently showed significant binding of KM3413, but not against type-A red blood cells (Figs. 2A, B). According to the cross reactivity, ca. 500 μg/ml of KM3413 reacted with type-A red blood cells and did not indicate agglutination of red blood cells (data not shown).

We made a western blot analysis of Jurkat cell lysate using KM3413 and detected specific signals in the region of high molecular weight (>100 kDa) (Fig. 3). It is already known that Jurkat cells express Tn-antigen on CD4327) and our results indicate that KM3413 recognizes Tn-antigen on CD43. Furthermore, binding of KM3413 was inhibited by 100 mM GalNAc, but not by 100 mM galactose (Fig. 3).

These results suggest that KM3413 binding depends on carbohydrate residues and is specific to clustered Tn-antigens of more than two consecutive sequences, while the cross-reactivity to type-A red blood cells may not be a serious problem.

**Production of cKM3413** Using PCR, we cloned the variable regions of heavy and light chains from KM3413 hybridoma cells, and constructed the expression vector of cKM3413. DG44 cell line was used for production of cKM3413. FCM analysis showed significant binding of cKM3413 (2 μg/ml) to Jurkat cells, but not to type-A red blood cells (Figs. 2C, D). Specificity and affinity against clustered Tn-antigens were nearly equivalent to murine KM3413 (data not shown).

**In Vitro Anti-tumor Activity of cKM3413** We evaluated direct killing activity of cKM3413 against Jurkat cells, since it is already known that anti-CD43 antibodies induce apoptosis.23,24) Cells were incubated with cKM3413 and with or without the cross-link antibody for 48 h. Cytotoxicity was slightly detected (ca. 10%) in 10 μg/ml of cKM3413, and the activity significantly enhanced with cross-link antibody (Fig. 4).

Next, we measured the human PBMC-mediated ADCC of cKM3413 against Jurkat cells. Cells were labeled with ⁵¹Cr and then incubated with antibodies and human PBMC. Anti-
body-dose dependent ADCC activity was detected with a potency of 0.1—1 μg/ml (Fig. 5).

**In Vivo Anti-tumor Activity of cKM3413**

To evaluate the therapeutic potential of cKM3413, we examined in vivo anti-tumor activity against Jurkat-inoculated SCID mice. The cKM3413 treatment significantly improved survival compared with the control PBS treatment (Fig. 6) \( (p<0.001) \). Median survival results±S.D. were as follows: PBS treatment (38.3±8.5 d), 1 mg/kg of cKM3413 treatment (73.7±10.5 d) and 10 mg/kg of cKM3413 (82.5±10.9 d).

**DISCUSSION**

In this study, we isolated a novel murine anti-Tn IgG1, KM3413, bound to Tn-antigen by the immunization of mucins from the culture supernatant of a colon cancer cell. To generate carbohydrate-directed monoclonal antibodies, intact cancer cells or isolated glycopeptides as immunogens have already been used with some success.\(^{25}\) We also tried to use not only mucins but also Jurkat cells as immunogens and established that mucin antigen was more effective for isolat-
against type-A blood antigen. The affinity (\(K_d\)) of KM3413 was calculated as \(10^{-7}\) M and indicated rapid \(K_e/K_d\) against clustered Tn-antigens with BIAcore. For therapeutic application, affinity is not strong compared to antibodies such as Rituximab (anti-CD20), Trastuzumab (anti-Her2), and Erbitux (anti-EGFR), all with affinities of \(10^{-9}\) to \(10^{-10}\) M.\(^9\) We tried to select stronger binding antibodies via screening, but the candidates were excluded because of cross-reactivity against type-A red blood cells (data not shown). We believe that anti-carbohydrate antibodies, which recognize small, flexible structures, are difficult to achieve with high affinity and selectivity. KM3413 was the best candidate with a good balance between affinity and selectivity.

CD43 (Also known as leukosialin, sialophorin and leukocyte large sialoglycoprotein) is a mucin-like transmembrane protein expressed on almost all mature human leukocytes, except for a population of B cells.\(^23\) It has been shown to produce an apoptotic signal via CD43 to hematopoietic progenitor cells\(^23\) and T-lymphoblastoid cells.\(^22\) Therefore, we evaluated direct killing activity of cKM3413 against Jurkat cells, and cKM3413 induced strong cytotoxicity with a cross-link antibody. Similar direct killing activity with a cross-link has been reported for anti-CD20 antibodies and recognized as an important mechanism of therapeutic efficacy.\(^11\) In addition, cKM3413 containing human antibody constant region exerted ADCC activity. ADCC, a lytic attack on antibody-targeted cells by NK cells, is triggered after leukocyte receptors (Fc\(\gamma R\)) bind to Fc region of the antibody already bound to a target. ADCC of cKM3413 may be a central mechanism for therapeutic efficacy, since several clinical studies indicate ADCC is an important therapeutic mechanism of monoclonal antibody therapy for cancer immunotherapy.\(^29\)–\(^31\)

In this study, we evaluated in vivo activity of cKM3413 in Jurkat-inoculated mice and found significantly better survival rates. Although further examination is necessary to elucidate the underlying mechanism of in vivo efficacy, cKM3413-ADCC may be an important contributor, since several mouse studies indicate ADCC is an important therapeutic mechanism for in vivo efficacy.\(^52\) On the other hand, it is possible that direct killing activity with cross-link also may contribute to in vivo effects. We believe that a cross-linked physiological effect may occur with Fc-receptor expressing cells (macrophages, monocytes, dendritic cells).\(^33\)

As far as we know, this is the first report of in vivo anti-tumor activity for an anti-Tn monoclonal antibody. Our results show that chimeric or humanized anti-Tn monoclonal antibody may be a valuable therapeutic antibody against Tn-positive cancer. Furthermore, Tn-positive hematological cancers, including acute T-cell leukemia,\(^6\) Hodgkin’s lymphoma\(^7\) and B-cell chronic lymphocytic leukemia,\(^5\) may be promising targets for this antibody therapy.

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