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A mouse/human chimeric anti-(ganglioside GD3) antibody with enhanced antitumor activities

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Abstract. Ganglioside GD3, which is one of the major gangliosides expressed on the cell surface of human tumors of neuroectodermal origin has been focused on as a target molecule for passive immunotherapy. We have cloned the cDNA encoding the immunoglobulin light and heavy chains of an anti-GD3 monoclonal antibody KM641 (murine IgG3, κ), and constructed the chimeric genes by linking the cDNA fragments of the murine light and heavy variable regions to cDNA fragments of the human κ and γ1 constant regions, respectively. The transfer of these cDNA constructs into SP2/0 mouse myeloma cells resulted in the production of the chimeric antibody, designated KM871, that retained specific binding activity to GD3. Indirect immunofluorescence revealed the same staining pattern for chimeric KM871 and the mouse counterpart KM641 on GD3-expressing melanoma cells. When human serum and human peripheral blood mononuclear cells were used as effectors in complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity respectively, the chimeric KM871 was more effective in killing GD3-expressing tumor cells than was the mouse counterpart KM641. Intravenous injection of chimeric KM871 markedly suppressed tumor growth in nude mice. The chimeric KM871, having enhanced antitumor activities and less immunogenicity than the mouse counterpart, would be a useful agent for passive immunotherapy of human cancer.

Key words: Ganglioside – Melanoma – Chimeric antibody

Introduction

Human tumors of neuro-ectodermal origin, such as melanoma, glioma, and neuroblastoma, express large amounts of gangliosides GM2, GD2 or GD3, which are minor gangliosides in normal tissues [26, 38], and there are hints of their relationship to clinical features [2, 11, 15, 24, 27, 28, 35]. Monoclonal antibodies (mAb) to these gangliosides have gained clinical interest, and treatments with these mAb have induced the regression of cutaneous melanoma and metastases of neuroblastoma in some clinical trials [3, 12–14]. One of the mouse anti-GD3 mAb, R24 [4], has been intensely studied for its antitumor effects and applied to clinical tests, resulting in partial responses in some cases [36]. We attempted to develop mouse anti-GD3 mAb that have superior characteristics to R24 in terms of binding and antitumor effects, in order to achieve better clinical efficacy for mAb. One of the anti-GD3 mAb developed, KM641, reacted with GD3 with higher affinity and bound to GD3-expressing tumor cells more widely than did R24. KM641 also exerted strong antitumor effects in vitro and especially in vivo. Moreover, we found that expression of KM641-reactive GD3 was rather restricted to a few normal tissues, such as the bladder and the adrenal gland, and the quantity was low compared with that in most melanoma cells [25]. It is likely that KM641 has the potential to be a useful agent for passive immunotherapy of human cancer if two major problems, human anti-(mouse Ig) antibody and short serum half-life, are solved [17]. Humanization of antibodies by genetic engineering, such as mouse/human chimeric antibodies and complementarity-determining-region-grafting antibodies, have brought about one solution to these problems [6, 20, 37].

In this study, we generated a mouse/human chimeric anti-(ganglioside GD3) antibody, KM871, by linking cDNA sequences encoding light(L)- and heavy(H)-chain variable (V) regions of KM641 with cDNAs encoding human κ and γ1 constant (C) regions. The chimeric KM871 reacted specifically with GD3 and exerted higher complement-mediated cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) than the mouse counterpart KM641 when human serum and peripheral blood mononuclear cells were used as effectors. Moreover, KM871 showed strong antitumor activity to xenograft tumors in nude mice.

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Materials and methods

Cell lines. Immunoglobulin-nonsecreting mouse myeloma cells SP2/0-Ag14 (ATCC CRL 1581) were used for all transfectants with chimeric antibody constructs. GD3-expressing human melanoma cells, G361 (provided by Japanese Cancer Research Resources Bank), and GD3-nonexpressing human glioblastoma cells, P122, kindly provided by Dr. Kishimoto (Osaka University, Osaka, Japan) were used as target cells for the chimeric antibody. A murine anti-GD3 mAb KM641 (IgG3, κ) established in our laboratories was used for the isolation of KM641 H- and L-chain cDNA. A cell line producing chimeric mouse/human anti-(phosphorylchorine) antibody, SP2-PC Chimera-1 [16], kindly provided by Dr. Kurosawa (Fujita-gakuen Health University, Aichi, Japan), was used for the isolation of human Cκ- and Cγ1-chain cDNA.

Molecular cloning and sequencing of the KM641 H- and L-chain cDNA. Poly(A)-rich RNA was isolated from KM641 hybridoma cells using a mRNA isolation kit, Fast Track (Invitrogen, San Diego, Calif.). cDNA was synthesized using a ZAP-cDNA synthesis kit, (Stratagene, La Jolla, Calif.), inserted into λZAP and packaged using the Stratagene Gigapack Gold in vitro packaging system. The cDNA library was screened for H- and L-chain clones with probes from the murine Cκ and Cγ1 genes respectively [31, 33]. The KM641 H- and L-chain genes were subcloned in pBluescript and the V regions were sequenced by the dideoxy-chain-termination method using a Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corporation, Cleveland, Ohio). Since the H-chain cDNA clone obtained lacks the first ATG sequence and several codons for the amino acid residues of the leader peptide, deleted amino acid residues were supplemented by linking the oligonucleotide 5′AATTCACCATGGAGTTTCTCAGCTGGCTTTTT3′ to the 5′ *Alu*I site of the cloned H-chain cDNA (Fig. 1B). This oligonucleotide contains the initiation codon, the *Eco*RI site at the 5′ side and the 3′ blunt end.

Molecular cloning and sequencing of human Cκ and Cγ1-chain cDNAs. A cDNA library was generated from SP2-PC Chimera-1 cells and screened for human Cκ- and Cγ1-chain clones with probes from the Vκ and VH genomic genes respectively, which code for the anti-PC antibody [16]. The human Cκ- and Cγ1-chain genes were subcloned in pBluescript and sequenced. The nucleotide sequence of the cloned human Cκ was identical to that of human Cκ genomic DNA reported by Hieter et al. [10]. In case of the cloned human Cγ1, only one codon (CGT: Arg) at position 321 (Kabat nomenclature) was different from the codon (CGG: Arg) at the corresponding position of human Cγ1 genomic DNA reported by Ellison et al. [5].

Construction of chimeric genes. A 0.8×10^3-base (0.8 kb) *Eco*RI-*Xba*I fragment containing chimeric L chain and a 1.6-kb *Eco*RI-*Xba*I fragment containing chimeric H chain were constructed by linking the cloned murine Vκ and VH cDNA to the appropriate human C region cDNA using the oligonucleotides as junctions (Figs. 2, 3). For the L chain fusion, an *Eco*RV site was created by an oligonucleotide (5′GATGAAGACAGATATCGCAGCCACAGTTC3′) using the Altered Site in vitro mutagenesis system (Promega Corporation, Madison, Wis.) near the 5′ side of the human Cκ cDNA. The *Eco*RI site at the 3′ site of the human Cκ cDNA was changed to the *Xba*I site using a linker. The murine Vκ *Eco*RI-*Hin*dIII fragment (Fig. 1A) was linked to the human Cκ *Eco*RV-*Xba*I fragment using the junction sequence for chimeric L chain described in Fig. 3. The natural *Sma*I site at the 3′ non-coding region of the human Cγ1 cDNA was changed to the *Xba*I site using a linker. The murine VH *Eco*RI-*Sty*I fragment (Fig. 1B) was linked to the human Cγ1 *Apa*I(natural site)-*Xba*I fragment using the junction sequence for chimeric H chain described in Fig. 3. In these constructions, none of the amino acid sequence of the murine Vκ and VH, and human Cκ and Cγ1 lesions was changed.

Expression vector construction. The structure of the chimeric L-chain (A) and H-chain (B) expression vectors, pChi641LGM4 and pChi641HGM4 respectively, is shown in Fig. 2. They consist of the following five DNA fragments: (a) a 0.63-kb *Xho*I-*Eco*RI fragment containing the ecotropic Moloney virus long terminal repeat (LTR) fragment, derived from ECO-MOL LTR [18] after changing the *Cla*I and *Kpn*I sites into *Xho*I and *Eco*RI sites, respectively, using linkers; (b) a 0.8-kb *Eco*RI-*Xba*I fragment containing chimeric L chain for pChi641LGM4 or a 1.6-kb *Eco*RI-*Xba*I fragment containing chimeric H chain for pChi641HGM4; (c) a 1.6-kb *Xba*I-*Cla*I(blunt) fragment containing the rabbit β-globin RNA processing signals for splicing and polyadenylation and the simian virus 40 RNA processing signal for polyadenylation from pAGE107 [21]; (d) a 2.7-kb *Xho*I(blunt)-*Cla*I fragment carrying a dihydrofolate reductase transcription unit from pSE1dhfr1A [22]; (e) a 4.4-kb *Cla*I-*Xho*I fragment containing an ampicillin-resistance gene and a G418-resistance gene from pAGE107.

Chimeric antibody expression and purification. Expression plasmids pChi641LGM4 and pChi641HGM4, 2 μg each, were transfected into SP2/0 cells using Somatic hybridizer SSH-1 (Shimadzu Seisakusyo, Kyoto, Japan) by the previously reported method [21]. Cells were selected in the presence of G418 at 0.5 mg/ml. After 14 days, the supernatants of G418-resistant transfectants were screened for antibodies by enzyme-linked immunosorbent assay (ELISA).

Transfectants producing antibodies were cultured in the presence of methotrexate from 50 nM to 500 nM and cloned by limiting dilution. The chimeric antibody was purified from the tissue-culture supernatants of the transfectants grown in bovine immunoglobulin-eliminated GIT medium (Nihon Seiyaku, Osaka, Japan) using an Affigel Protein A MAPS-II kit (Bio-Rad Laboratories, Richmond, Calif.).

Gangliosides and mAb. Gangliosides were prepared as previously described [7, 9]. Anti-GD3 mAb KM641 (mouse IgG3) [25], anti-sialyl-Le^a mAb KM231 (mouse IgG1) [8] and anti-(salmon growth hormone) mAb, KM737 (mouse IgG3) were produced in our laboratory.

ELISA analysis. ELISA was performed by the previously reported method [9]. Briefly, gangliosides (2 nmol) were dissolved in 2 ml ethanol containing phosphatidylcholine (5 ng) and cholesterol (2.5 ng). The aliquots (20 μl) were put in each well of microtiter plates (Flow Laboratories, Va.) and dried. After blocking with bovine serum albumin, mAb (50 μl) were reacted for 1–2 h at room temperature or overnight at 4° C. The intensity of the reaction was measured using biotinylated protein A (Vector Laboratories, Burlingame, Calif.) and peroxidase-labeled avidin (Vector). The color development of the substrate [1 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium solution (ABTS)] was measured at 415 nm.

Flow-cytometric analysis. In indirect immunofluorescence, the tumor cells (1×10^6 cells) were incubated with purified antibodies (50 μg/ml) for 45 min at 4° C. After the reaction with fluorescein-isothiocyanate-labeled protein A as second antibody (Boehringer Mannheim, Germany), the reactivity was analyzed in an EPICS Elite flow cytometer (Coulter Corporation, Hialeah, Fla.).

CDC assay. The CDC assay was performed by the previously reported method [25]. Briefly, the tumor cells (1×10^6 cells) were labeled with 3.7 MBq $Na_2{}^{51}CrO_4$ for 1 h at 37° C and kept for 30 min at 4° C to remove loosely bound ^{51}Cr after washing. Aliquots of the labeled cells were put in 96-well microtiter plates (2×10^5 cells/50 μl) and incubated with 50 μl antibody with various dilutions in the presence of 50 μl diluted human serum (final volume, 15%) as complement for 1 h at 37° C. After centrifuging, the released ^{51}Cr in the supernatant was counted. Percentage specific lysis was calculated from the experimental ^{51}Cr release, the total release, and the spontaneous release.

ADCC assay. ADCC assays were performed as previously reported [25]. Briefly, aliquots of the ^{51}Cr-labeled cells were put in 96-well microtiter plates (2×10^4 cells/50 μl) and incubated with 50 μl antibodies with various dilutions in the presence of 100 μl effector cells at the indicated effector-to-target ratio (E/T ratio) for 4 h at 37° C. Peripheral blood mononuclear cells were separated from healthy donor's peripheral blood as the effector cells using LeucoPREP (Becton Dickinson, Calif., USA). After centrifugation, the ^{51}Cr released in the supernatant was counted.

A. Vk

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                                  |→Leader peptide
                                  Met Met Ser Ser Ala Gln Phe Leu Gly
AATTCGGCAC GAGTCAGCCT GGAC ATG ATG TCC TCT GCT CAG TTC CTT GGT
|
EcoRI                                                |→FR1
Leu Leu Leu Leu Cys Phe Gln Gly Thr Arg Cys Asp Ile Gln Met Thr
CTC CTG TTG CTC TGT TTT CAA GGT ACC AGA TGT GAT ATC CAG ATG ACA
                                        |              |
                                      KpnI           EcoRV
Gln Thr Ala Ser Ser Leu Pro Ala Ser Leu Gly Asp Arg Val Thr Ile
CAG ACT GCA TCC TCC CTG CCT GCC TCT CTG GGA GAC AGA GTC ACC ATC

        |→CDR1                                              |→FR2
Ser Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr Leu Asn Trp Tyr Gln
AGT TGC AGT GCA AGT CAG GAC ATT AGT AAT TAT TTA AAC TGG TAT CAA

                                                   |→CDR2
Gln Lys Pro Asp Gly Thr Val Lys Leu Leu Ile Phe Tyr Ser Ser Asn
CAG AAA CCA GAT GGA ACT GTT AAA CTC CTG ATC TTT TAC TCA TCA AAT

            |→FR3
Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly Gly Gly Ser Gly Thr
TTA CAC TCG GGA GTC CCA TCA AGG TTC AGT GGC GGT GGG TCC GGG ACA


Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Pro Glu Asp Ile Ala Thr
GAT TAT TCT CTC ACC ATC AGC AAC CTG GAG CCT GAA GAT ATT GCC ACT

            |→CDR3                                  |→FR4
Tyr Phe Cys His Gln Tyr Ser Lys Leu Pro Trp Thr Phe Gly Gly Gly
TAC TTT TGT CAT CAG TAT AGT AAG CTT CCG TGG ACG TTC GGT GGA GGC
                                    |
                                 HindIII
                                  |→Mouse Ck
Thr Lys Leu Glu Ile Lys Arg
ACC AAG CTG GAA ATC AAA CGG
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B. VH

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          |→Leader peptide
          Met Glu Phe Leu Ser Trp Leu Phe Leu Val Leu Val Phe
AATTCACC ATG GAG TTT CTC AGC TGG CTT TTT CTT GTC CTT GTT TTC
|
EcoRI                    |→FR1
Lys Gly Val Gln Cys Glu Val Thr Leu Val Glu Ser Gly Gly Asp Phe
AAA GGT GTT CAG TGT GAA GTG ACG CTG GTG GAG TCT GGG GGA GAC TTT


Val Lys Pro Gly Gly Ser Leu Lys Val Ser Cys Ala Ala Ser Gly Phe
GTG AAA CCT GGA GGG TCC CTG AAA GTC TCC TGT GCA GCC TCT GGA TTC

            |→CDR1                |→FR2
Ala Phe Ser His Tyr Ala Met Ser Trp Val Arg Gln Thr Pro Ala Lys
GCT TTC AGT CAT TAT GCC ATG TCT TGG GTT CGC CAG ACT CCG GCG AAG

                              |→CDR2
Arg Leu Glu Trp Val Ala Tyr Ile Ser Ser Gly Gly Ser Gly Thr Tyr
AGG CTG GAA TGG GTC GCA TAT ATT AGT AGT GGT GGT AGT GGC ACC TAC

                              |→FR3
Tyr Ser Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala
TAT TCA GAC AGT GTA AAG GGC CGA TTC ACC ATT TCC AGA GAC AAT GCC


Lys Asn Thr Leu Tyr Leu Gln Met Arg Ser Leu Arg Ser Glu Asp Ser
AAG AAC ACC CTG TAC CTG CAA ATG CGC AGT CTG AGG TCT GAG GAC TCG

                              |→CDR3
Ala Met Tyr Phe Cys Thr Arg Val Lys Leu Gly Thr Tyr Tyr Phe Asp
GCC ATG TAT TTC TGT ACA AGA GTT AAA CTG GGA ACC TAC TAC TTT GAC

    |→FR4                                          |→Mouse Cr3
Ser Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser Ala
TCC TGG GGC CAA GGC ACC ACT CTC ACT GTC TCC TCA GCT
          |
        StyI
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Percentage specific lysis was calculated from the experimental ^{51}Cr release, the total release, and the spontaneous release.

Therapeutic effects on transplanted tumors. The tumor cells (2×10^7 cells) were intradermally transplanted in BALB/c *nu/nu* mice, and 100 μg antibodies was intravenously administrated five times. The tumor size (volume) was calculated by the following equation: tumor size (mm^3) = 0.4 × (major axis) × (minor axis)^2

Results

Expression of the chimeric mouse/human anti-GD3 antibody KM871

The nucleotide and amino acid sequences of the cloned Vκ and VH of the anti-GD3 antibody KM641 (mouse IgG3, κ) are shown in Fig. 1 A, B. Comparison of the amino acid sequence of the cDNAs to the N-terminal amino acid sequence of the purified KM641 (dashed lines in Fig. 1 A, B) showed that the cDNA represented the productive alleles. Vκ and VH belonged to the mouse κ-chain subgroup V and H-chain subgroup IIIA respectively. Since the H-chain cDNA clone obtained lacked the nucleotides to code for several amino acid residues in the leader peptide, codons for the deleted amino acid residues were supplemented by the oligonucleotide shown in Fig. 1 B.

Chimeric L- and H-chain cDNAs were constructed by linking the cloned murine Vκ and VH cDNA to the appropriate human C-region cDNA using the oligonucleotides as junctions (Figs. 2, 3). Expression vectors for chimeric L chain, pChi641LGM4, and H chain, pChi641HGM4, were constructed by linking the chimeric L- and H-chain cDNA to the expression vector having the ecotropic Moloney virus long terminal repeat as a promoter for the chimeric antibody, the G418-resistance gene for selection of transfectant and the dihydrofolate reductase gene for gene coamplification (Fig. 2). The two expression vectors were transfected into mouse SP2/0 cells by electroporation. The transfected cells were initially selected by G418 and screening was done with ELISA against GD3. Transfectants producing antibodies were cultured in the presence of methotrexate from 50 nM to 500 nM and cloned by limiting dilution. One of the stable clones secreted 2 μg/ml chimeric antibody. We designate the chimeric anti-GD3 antibody as KM871 to distinguish it from the mouse counterpart antibody KM641.

The chimeric antibody KM871 was purified from culture medium using protein A affinity chromatography. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions showed that the molecular masses of the chimeric L and H chains were about 25 kDa and 50 kDa respectively. From analysis by

Fig. 1. Nucleotide sequences and predicted amino acid sequences of KM641 Vκ (**A**) and V_H (**B**). The framework regions (*FR*) and complementarity-determining regions (*CDR*) are indicated. Amino acid residues matching those obtained from peptide sequencing are *underlined. Doubly underlined* amino acid residues at the leader peptide of V_H (**B**) were supplemented by the oligonucleotide as described in Materials and methods

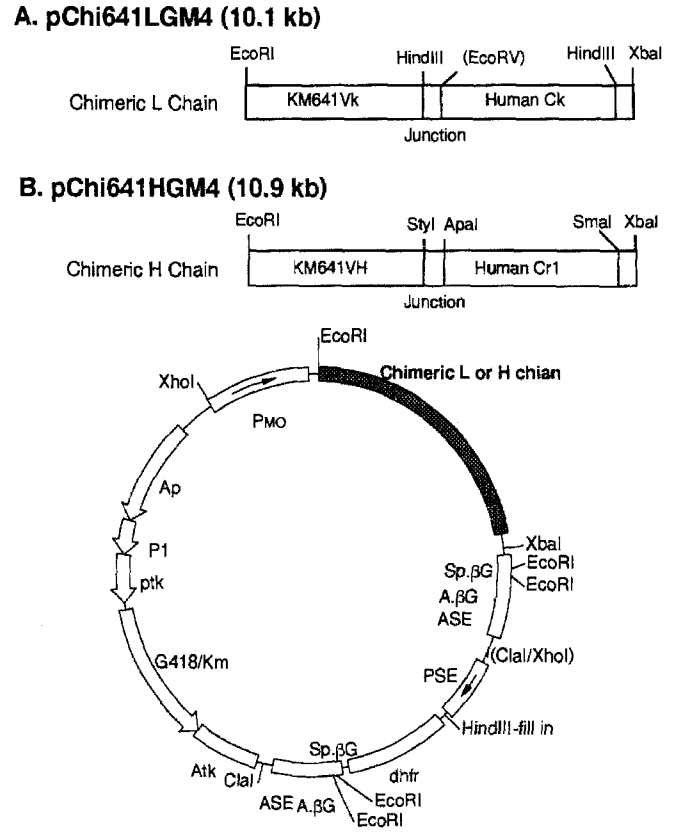

Fig. 2. Schematic diagram of the expression vectors pChi641LGM4 for chimeric light (*L*) chain (**A**) and pChi641HGM4 for chimeric heavy (*H*) chain (**B**). The sequences of junctions of murine V regions and human C regions are shown in Fig. 3. Details of vector construction are given in the text. The following abbreviations are used: *G418/Km*, Tn5-derived G418-resistance gene; *Ap*, ampicillin-resistance gene; *P1*, pBR322 P1 promoter; *Ptk*, the Herpes simplex virus thymidine kinase promoter; P_{MO}, the ecotropic Moloney virus long terminal repeat fragment; *Atk*, the polyadenylation signal from the Herpes simplex virus thymidine kinase gene; *ASE*, the polyadenylation signal from the simian virus 40 early gene; *AβG*, the polyadenylation signal from the rabbit β-globin gene; *Sp.βG*, the splicing signal from the rabbit β-globin gene

non-reducing SDS-PAGE, the molecular mass of chimeric KM871 was calculated to be around 150 kDa (data not shown) and it appeared that the L and H chains were assembled as the correct tetrameric molecule. The amino-terminal amino acid sequences of chimeric L and H chains were identical to those of their murine counterparts (data not shown).

Binding characteristics of chimeric antibody KM871

We examined the characteristics of chimeric antibody KM871 binding to gangliosides by ELISA. As shown in Fig. 4A, the chimeric antibody KM871 reacted with GD3 in a dose-dependent fashion and the binding to GD3 was almost the same as that of the murine counterpart KM641. KM871 showed specific reactivity with GD3 and weak cross-reactivity with GQ1b out of 11 common gangliosides in ELISA (Fig. 4B).

To analyze the binding activity of chimeric KM871 in more detail, we examined the binding of KM871 to living cells compared with that of its murine counterpart KM641. Both KM871 and KM641 reacted to the GD3-expressing melanoma cell line G361, but not to the GD3-nonexpressing glioblastoma cell line P122 (Fig. 5).

CDC and ADCC activities of chimeric antibody, KM871

CDC of chimeric KM871 and murine KM641 was tested with ^{51}Cr-labeled G361 melanoma cells in the presence of human serum as complement. Figure 6 shows that both KM871 and KM641 lysed G361 cells at the range 0.5–50 μg/ml in the presence of human serum, while control

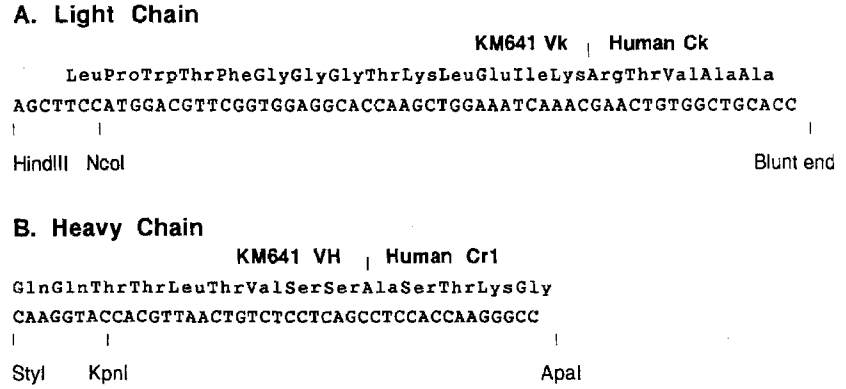

Fig. 3. Junctions between V and C regions for chimeric L- (**A**) and H-chain (**B**) cDNA

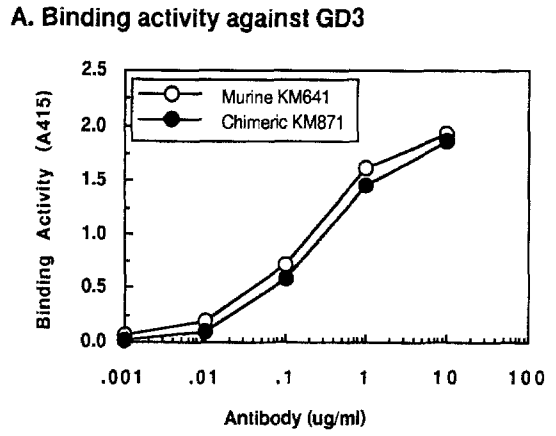

Fig. 4 A, B. Enzyme-linked immunosorbent assay analysis of chimeric KM871. **A** Comparison of the reactivity with GD3 between chimeric KM871 and murine KM641. After coating GD3 on the microtiter plates, diluted antibodies were reacted for 2 h. **B** Binding specificity of chimeric KM871 against various gangliosides. After coating various gangliosides on the microtiter plates, chimeric KM871 (10 μg/ml) was reacted for 2 h. *AcGM3*, *N*-acetyl-GM3; *GcGM3*, *N*-glycolyl-GM3; *AcGM2*, *N*-acetyl-GM2; *GcGM2*, *N*-glycolyl-GM2. The intensity of the reaction was measured using biotinylated protein A and peroxidase-labeled avidin

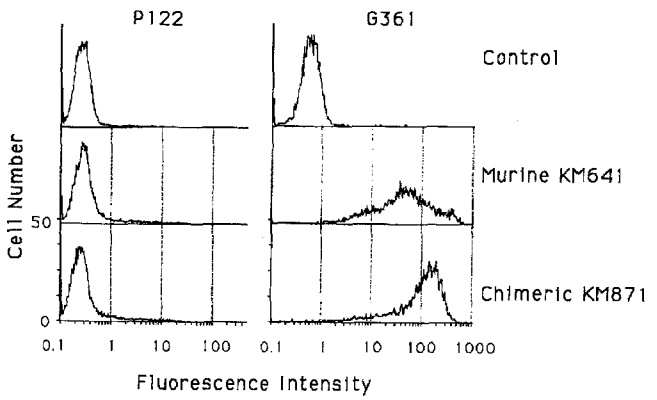

Fig. 5. Flow-cytometric analysis of the cell binding of chimeric KM871 and murine KM641. Neuroblastoma P122 cells and melanoma G361 cells were reacted with murine anti-(salmon growth hormone) mAb, KM737 (control antibody), murine KM641 and chimeric KM871. The cells were then stained with fluorescein-isothiocyanate-labeled protein A and analyzed by flow cytometry

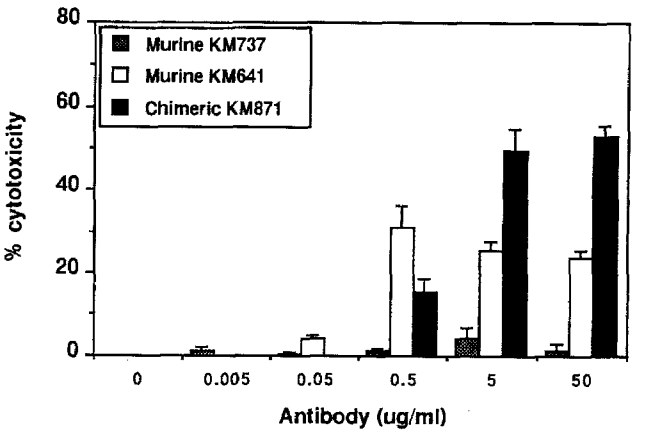

Fig. 6. Complement-dependent cytotoxicity (*CDC*) activity mediated by chimeric KM871 and murine KM641 using human serum as the source of complement. Lysis of melanoma G361 cells was measured in a 1-h ^{51}Cr-release assay

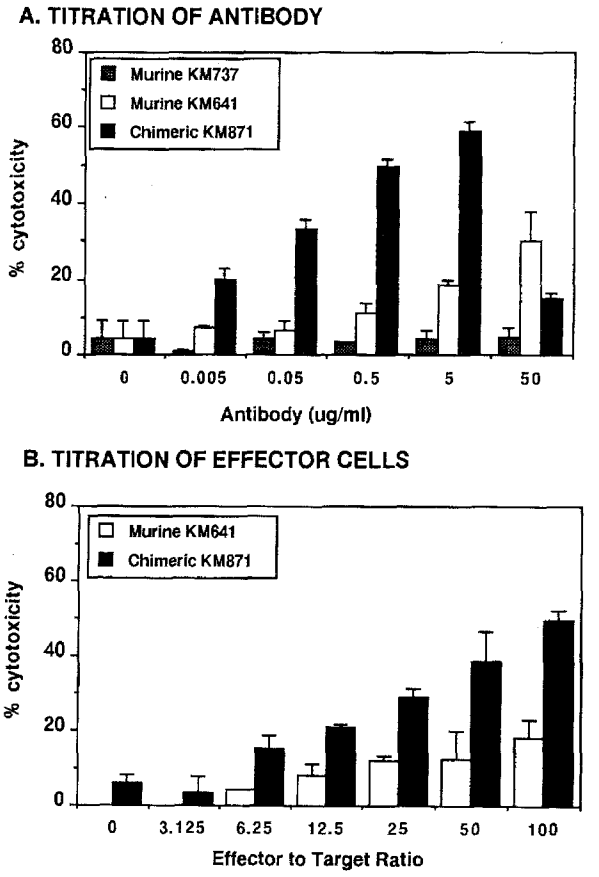

Fig. 7 A, B. Antibody-dependent cell cytotoxicity (*ADCC*) activity mediated by chimeric KM871 and murine KM641. **A** Titration of chimeric KM871 and murine KM641 in ADCC assays with human peripheral blood mononuclear cells (PBMC) as effectors (E/T ratio = 50:1). Murine KM737 was used as control antibody. **B** Titration of PBMC mediating ADCC activity in the presence of chimeric KM871 and murine KM641 (5 μg/ml). Lysis of melanoma G361 cells was measured in a 4-h ^{51}Cr-release assay

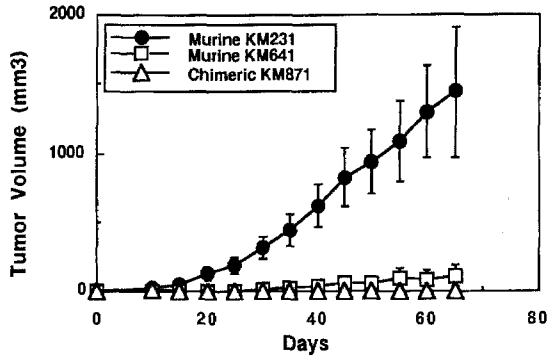

Fig. 8. Antitumor effects of chimeric KM871 and murine KM641 on transplanted tumor in nude mice. Human melanoma G361 cells were inoculated (2×10^7) i. d. and antibodies were injected i. v. on days 0, 1, 2, 3 and 4. Each point represents the mean and SD from five to seven animals. Murine KM231, anti-sialyl-Le^a mAb, was used as a negative control antibody

antibody KM737 did not show significant CDC activity. The experiment also revealed that chimeric KM871 gave higher CDC at 5 μg/ml and 50 μg/ml than its murine counterpart KM641.

ADCC of chimeric KM871 and murine KM641 was examined against ^{51}Cr-labeled G361 melanoma cells with human PBMC. Concentrations of the antibody ranging from 0.005 μg/ml to 50 μg/ml were tested at an E/T ratio 50/1 (Fig. 7 A). Chimeric KM871 demonstrated ADCC on G361 melanoma cells in a dose-dependent fashion in the range 0.005–5 μg/ml. Chimeric KM871 mediated a higher specific release than its murine counterpart KM641 and the amount of KM871 that was necessary to mediate specific lysis was almost 1000-fold less than that of KM641. A control antibody, murine anti-(salmon growth factor) mAb, KM737, did not show significant ADCC activity. An E/T ratio ranging from 3.125/1 to 100/1 was also tested at 5 μg/ml antibody (Fig. 7B). Chimeric KM871 showed ADCC against G361 cells that was dependent upon the E/T ratio, and significant ADCC was observed at an E/T ratio 6.25/1. Chimeric KM871 also mediated a higher specific release than its murine counterpart KM641 under these conditions and the amount of effector cells that was necessary to mediate specific lysis by KM871 was 15-times less than that by KM641.

In vivo antitumor effect of chimeric antibody, KM871

Nude mice intradermally transplanted with 2×10^7 G361 cells/animal were treated with five injections of chimeric KM871 and its murine counterpart KM641 from day 0. Treatments with KM871 and KM641 markedly suppressed the growth of G361 cells throughout the experimental period of 65 days by comparison with a control antibody, anti-sialyl-Le^a mAb, KM231 (Fig. 8). All the above effects of KM871 were statistically significant by Student's *t*-test.

Discussion

In the previous study [25], we established a mouse anti-GD3 mAb, KM641, which reacted with GD3 with higher affinity and bound to GD3-expressing tumor cells more widely than mAb, R24 [4]. GD3 has been assumed to be one of the ideal tumor antigens for passive immunotherapy of human cancer, especially melanoma, for the following reasons. (a) The distribution of GD3 is rather restricted to a few tissues such as the adrenal and the connective tissue [1, 29]. (b) Almost all the melanoma specimens contained large amounts of GD3 [35]. (c) One of the mouse anti-GD3 mAb, R24 [4], has been applied in clinical tests, resulting in partial responses in some cases [36]. (d) GD3 might play an important role in the adhesion of melanoma cells to substrate [2]. (e) The expression of GD3 was increased in the metastatic lesions of melanoma patients [32].

In the present study, we constructed mouse/human chimeric antibody, KM871, from mouse mAb KM641. The chimeric KM871 was made by joining cDNA sequences encoding the V regions of the L and H chains of the mouse mAb KM641 to cDNA sequences encoding the human κ chain and γ1C regions respectively. The expression vectors were transfected into SP2/0 cells and a complete immunoglobulin molecule of the chimeric KM871 was detected in the culture supernatant.

The chimeric KM871 was found to react with GD3 with similar binding activity to that of the mouse counterpart KM641, and showed specific reactivity with GD3 out of 11 common gangliosides in ELISA. There is a tendency for the cross-reactivity of chimeric KM871 with GQ1b to appear less than that of murine KM641. Rinfret et al. reported the possibility that the conformation of certain idiotypes was modulated by the isotype change of the CH1 domain [30]. It is likely that a class switch from mouse IgG3 to human IgG1 affected the binding specificity of KM871. Indirect immunofluorescence revealed the same staining pattern for chimeric KM871 and the mouse counterpart KM641 on GD3-expressing melanoma cells. KM871 gave a more uniform binding pattern in flow-cytometric analysis than did KM641, which might be due to the minor change of binding character of KM871. Immunostaining analysis on TLC plates showed that the expression of KM871-reactive GD3 was rather restricted to a few tissues such as the bladder and the adrenal (data not shown).

Effector functions of antibodies such as CDC and ADCC, which are dependent on isotypes of the C regions, are critical in the passive immunotherapy of cancer patients. Steplewski et al. compared the biological activity of mouse/human IgG1, IgG2, IgG3, and IgG4 chimeric antibodies having anti-(colorectal cancer) specificity and found that chimeric IgG1 was superior in its antitumor activity [34]. We found that the chimeric KM871 was more effective in killing GD3-expressing tumor cells than the mouse counterpart KM641, when human serum and human PBMC were used as effectors in CDC and ADCC respectively. In the case of ADCC, the amount of KM871 that was necessary to mediate specific lysis was almost 1000-fold less than that of KM641, and the amount of effector cells necessary to mediate specific lysis with KM871 was as little as 15-fold less than that required with

KM641. Consistent with the results reported in the present paper, Müeller et al. observed that the mouse/human chimeric anti-GD2 IgG1 antibody, ch.14.18, was able to mediate ADCC with human peripheral blood mononuclear cells 50- to 100-fold more efficiently than did the mouse counterpart IgG2a antibody, 14.G2a [23]. Liu et al. showed that murine mAb L6, IgG2a, mediated ADCC and CDC with human components, but a chimeric L6 having a human IgG1 constant region was much more active than murine L6 [19].

We found that the chimeric KM871 was as effective as its mouse counterpart KM641 in its ability to inhibit growth of human tumor xenografts in nude mice. CDC and ADCC are considered to be main mechanisms for suppression of tumor growth; inhibition of cell adhesion may be another mechanism. At present, however, we do not know exactly what mechanisms are involved in the in vivo antitumor effect of the chimeric KM871. Interestingly, Steplewski et al. also showed that the IgG1 and IgG4 chimeric antibodies were as effective as the parental mouse IgG2a, 17-1A, in inhibiting tumor growth in nude mice. They speculated that chimeric IgG1 and IgG4 had abilities to activate mouse effector cells, such as macrophages [34].

Since the majority of the sequences of the chimeric antibody are derived from the human sequences, the chimeric KM871 is expected to be less immunogenic and to have a longer serum half-life in patients than its murine counterpart KM641 from previous studies. In a clinical study, LoBuglio et al. demonstrated that only one of ten patients who had been given a human γ1κ chimeric form of murine mAb 17-1A generated an anti-17-1A idiotype response, while 70% of patients who had been treated with murine mAb 17-1A gave rise to an immune response to the mAb and 25% of the patients had an anti-idiotype response. Moreover, serum half-life of the chimeric 17-1A was reported to be six times as long as that of the murine counterpart [19]. Hakimi et al. demonstrated that usage of humanized anti-Tac antibody caused reduction of immunogenicity and improvement of pharmacokinetics in comparison with its murine counterpart in cynomolgus monkeys [6].

In conclusion, the mouse/human chimeric anti-GD3 antibody KM871, having enhanced antitumor activities and less immunogenicity than the mouse counterpart, would be a useful agent for passive immunotherapy of human cancer.

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References

1. Bernhard H, Bauerschmitz J, Büschenfelde K-H, Dippold W (1992) Immunorecognition of different ganglioside epitopes on human normal and melanoma tissues. Int J Cancer 51: 568
2. Cheresh DA, Harper JR, Schulz G, Reisfeld RA (1984) Localization of the gangliosides GD2 and GD3 in adhesion plaques and on the surface of human melanoma cells. Proc Natl Acad Sci USA 81: 5767
3. Cheung NV, Lazarus H, Miraldi FD (1987) Ganglioside GD2 specific monoclonal antibody 3F8: a phase-I study in patients with neuroblastoma and malignant melanoma. J Clin Oncol 5: 1430
4. Dippold W, Lloyd KO, Li LTC, Ikeda H, Oettgen HF, Old LJ (1980) Cell-surface antigens of human malignant melanoma-definition of six antigenic systems with mouse monoclonal antibodies. Proc Natl Acad Sci USA 77: 6114
5. Ellison JW, Berson BJ, Hood LE (1982) The nucleotide sequence of a human immunoglobulin Cγ1 gene. Nucleic Acids Res 10: 4071
6. Hakimi J, Chizzonite R, Luke DR, Familletti PC, Bailon P, Kondas JA, Pilson RS, Lin P, Weber DV, Spence C, Mondini LJ, Tsien W-H, Levin JL, Gallati VH, Korn L, Waldmann TA, Queen C, Benjamin WR (1991) Reduced immunogenicity and improved pharmacokinetics of humanized anti-Tac in cynomolgus monkeys. J Immunol 147: 1352
7. Hanai N, Nores GA, MacLeod C, Torres-Mendez C-R, Hakomori S (1988) Ganglioside-mediated modulation of cell growth. J Biol Chem 263: 10915
8. Hanai N, Furuya A, Shitara K, Oda S, Yoshida H (1988) Comparative studies on monoclonal antibodies raised against human gastric cancer for application to serum diagnosis of cancer. Anticancer Res 8: 329
9. Hanai N, Shitara K, Furuya A, Yoshida H, Dohi T, Nudelman E, Hakomori S, Satoh S (1990) Detailed characterization of reactivities of anti-gastric cancer monoclonal antibodies to carbohydrate antigen. Anticancer Res 10: 1579
10. Hieter PA, Max EE, Seidman JG, Maizel JV Jr, Leder P (1980) Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. Cell 22: 197
11. Hellström I, Brankovan V, Hellström KE (1985) Strong antitumor activities of IgG3 antibodies to a human melanoma-associated ganglioside. Proc Natl Acad Sci USA 82: 1499
12. Houghton AN, Mintzer D, Cordon-Cardo C, Welt S, Fliegel B, Vadham S, Carswell E, Melamed MR, Oettgen H, Old LJ (1985) Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: a phase-I trial in patients with malignant melanoma. Proc Natl Acad Sci USA 82: 1242
13. Irie RF, Morton DL (1986) Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside. Proc Natl Acad Sci USA 83: 8694
14. Irie RF, Matsuki T, Morton DL (1989) Human monoclonal antibody to ganglioside GM2 for melanoma treatment. Lancet I: 786
15. Jennemann R, Rodden A, Bauer BL, Mennel H-D, Wiegandt H (1990) Glycosphingolipids of human gliomas. Cancer Res 50: 7444
16. Kameyama K, Imai K, Itoh T, Taniguchi M, Miura K, Kurosawa Y. Convenient plasmid vectors for construction of chimeric mouse/human antibodies. (1989) FEBS Lett 244: 301
17. Khazaeli MB, Saleh MN, Wheeler RH, Huster WJ, Holden H, Carrano R, LoBuglio AF (1988) Phase I trial of multiple large doses of murine monoclonal antibody CO17-1A. II. Pharmacokinetics and immune response. JNCI 80: 937
18. Kuwana Y, Asakura Y, Utsunomiya N, Nakanishi M, Arata Y, Itoh S, Nagase F, Kurosawa Y (1987) Expression of chimeric receptor composed of immunoglobulin-derived V lesions and T-cell receptor-derived C lesions. Biochem Biophys Res Commun 149: 960
19. Liu AY, Robinson RR, Hellstrom KE, Murray ED Jr, Chang CP, Hellstrom I (1987) Chimeric mouse-human IgG1 antibody that can mediate lysis of cancer cells. Proc Natl Acad Sci USA 84: 3439
20. LoBuglio AF, Wheeler RH, Trang J, Haynes A, Rogers K, Harvey EB, Sun L, Ghrayeb J, Khazaeli MB (1989) Mouse/human chimeric monoclonal antibody in man: kinetics and immune response. Proc Natl Acad Sci USA 86: 4220
21. Miyaji H, Mizukami T, Hosoi S, Sato S, Fujiyoshi N, Itoh S (1990) Expression of human beta-interferon in Namalwa KJM-1 which was adapted to serum-free medium. Cytotechnology 3: 133
22. Miyaji H, Harada N, Mizukami T, Sato S, Fujiyoshi N, Itoh S (1990) Efficient expression of human beta-interferon in Namalwa KJM-1 cells adapated to serum-free medium by a dhfr gene coamplification method. Cytotechnology 4: 173

23. Müeller BM, Romerdahl CA, Gillies SD, Reisfeld RA (1990) Enhancement of antibody-dependent cytotoxicity with a chimeric anti-GD2 antibody. J Immunol 144: 1382
24. Nudelman E, Hakomori S, Kannagi R, Levery S, Yeh M-Y, Hellström KE, Hellström I (1982) Characterization of a human melanoma-associated ganglioside antigen defined by a monoclonal antibody, 4.2. J Biol Chem 257: 12752
25. Ohta S, Honda A, Tokutake T, Yoshida H, Hanai N (1993) Antitumor effects of a novel monoclonal antibody with high binding-affinity to ganglioside GD3. Cancer Immunol Immunother (in press)
26. Portoukalian J, Zwingelstein G, Dore J (1979) Lipid composition of human malignant melanoma tumors at various levels of malignant growth. Eur J Biochem 94: 19
27. Pukel CS, Lloyd KO, Travassor R, Dippold WG, Oettgen HF, Old LJ (1982) GD3, a prominent ganglioside of human melanoma. J Exp Med 155: 1133
28. Ravindranath MH, Tsuchida T, Morton DL, Irie RI (1991) Ganglioside GM3 :GD3 ratio as an index for the management of melanoma. Cancer 67: 3029
29. Real FX, Houghton AN, Albino AP, Cordon-Cardo C, Melamed MR, Oettgen HF, Old LJ (1985) Surface antigens of melanomas and melanocytes defined by mouse monoclonal antibodies: specificity analysis and comparison of expression in cultured cells and tissues. Cancer Res 45: 4401
30. Rinflet A, Horne C, Bour H, Markers A, Dorrington KJ, Klein M (1990) Isotype modulation of idiotypic expression in recombinant isotype variants of MOPC 315. J Immunol 145: 925
31. Roeder W, Maki R, Traunecker A, Tonegawa S (1981) Linkage of the four γ subclass heavy chain genes. Proc Natl Acad Sci USA 78: 474
32. Rosenberg JM, Sander DJ, Derango RE, Cheresh DA (1988) Enzymatic basis for increased expression of GD3 on human melanoma cells derived from metastatic lesions. J Clin Lab Anal 2: 91
33. Sakano H, Huppi K, Heinrich H, Tonegawa S (1979) Sequences at the somatic recombination sites of immunoglobulin light-chain genes. Nature 280: 288
34. Steplewski Z, Sun LK, Shearman CW, Ghrayeb J, Daddona P, Koprowski H (1988) Biological activity of human-mouse IgG1, IgG2, IgG3, and IgG4 chimeric monoclonal antibodies with antitumor specificity. Proc Natl Acad Sci USA 85: 4852
35. Tsuchida T, Saxton RE, Morton DL, Irie RF (1987) Gangliosides of human melanoma. JNCI 78: 45
36. Vadhan-Raj S, Cordon-Cardo C, Carswell E, Mintzer D, Dantis L, Duteau C, Templeton MA, Oettgen HF, Old LJ, Houghton AN (1988) Phase-I trial of a mouse monoclonal against GD3 ganglioside in patients with melanoma: induction of inflammatory responses at tumor sites. J Clin Oncol 6: 1636
37. Winter G, Milstein C (1991) Man-made antibodies. Nature 349: 293
38. Yates AJ, Thompson PK, Boesel CP, Albrightson C, Hart RW (1979) Lipid composition of human neural tumors. J Lipid Res 20: 428