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Antitumor effects of a novel monoclonal antibody with high binding affinity to ganglioside GD3

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Abstract. Ganglioside GD3, which is one of the major gangliosides expressed on the cell surface human tumors of neuroectodermal origin, has been studied as a target molecule for passive immunotherapy. We established ten kinds of anti-GD3 monoclonal antibodies (mAb) of the mouse IgG3 subclass by immunization with purified GD3 and melanoma cells. One of the established mAb, KM641, showed major reactivity with GD3 and minor reactivity with GQ1b out of 11 common gangliosides in an enzyme-linked immunosorbent assay. Immunostaining of gangliosides, separated on thin-layer chromatography plates, using KM641 revealed that most of the melanoma cell lines contained immunoreactive GD3 and GD3-lactone at a high level, but only the adrenal gland and the urinary bladder out of 21 human normal tissues had immunoreactive GD3. In immunofluorescence, KM641 bound to a variety of living tumor cell lines especially melanoma cells, including some cell lines to which another anti-GD3 mAb R24, established previously, failed to bind. High-affinity binding of KM641 to a tumor cell line was quantified by Scatchard analysis ($K_d = 1.9 \times 10^{-8}$ M). KM641 exerted tumor-killing activity in the presence of effector cells or complement against melanoma cells expressing GD3 at a high level. Not only natural killer cells but also polymorphonuclear cells were effective as the effector cells in antibody-dependent cellular cytotoxicity. Intravenous injection of KM641 markedly suppressed the tumor growth of a slightly positive cell line, C24.22 (7.2×10^5 binding sites/cell), as well as a very GD3-positive cell line, G361 (1.9×10^7 binding sites/cell), inoculated intradermally in nude mice. KM641, characterized by a high binding affinity for GD3, has the potential to be a useful agent for passive immunotherapy of human cancer.

Key words: Ganglioside – Melanoma – Monoclonal antibodies

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Introduction

Gangliosides, which constitute a class of cell membrane constituent glycolipids, are molecules composed of a carbohydrate chain with sialic acid at the cell surface and a hydrophobic ceramide in the lipid bilayers [31]. Ganglioside expression differs with cell type, kind of organs and animal species. It has been known that quantitative and qualitative changes occur in the expression of gangliosides through the oncogenic transformation of cells [9].

Human tumors of neuro-ectodermal origin, such as melanoma, glioma, and neuroblastoma, express large amounts of GM2, GD2 or GD3, which are minor gangliosides in normal tissues [26, 39] and the relation to clinical features has been hinted at [5, 15, 20, 25, 27, 28, 34]. 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 [6, 17]. Regression of tumors was also observed in patients with cutaneous melanoma after intralesional treatments with human IgM anti-GM2 and anti-GD2 mAb [18, 19]. One of the mouse anti-GD3 mAb, R24 [7], has been intensely studied for its antitumor effects and applied in clinical tests, resulting in partial responses in some cases [35]. In order to obtain better clinical efficacy with mouse mAb, two major problems, human antimouse antibody and a short serum half-life, have had to be solved. Humanization of antibodies by genetic engineering, producing human/mouse chimeric antibodies and complementarity dermining region (CDR)-grafting antibodies, have brought a satisfactory solution to these problems [38]. However, the original mouse mAb have to have a high antigen-binding affinity and sensitive cell-binding ability because the genetic engineering of antibodies usually reduces their binding affinity to some extent [21]. We therefore attempted to develop mouse anti-GD3 mAb that have superior characteristics in terms of binding and antitumor effects. One of the anti-GD3 mAb, KM641, developed in this study, reacts with GD3 and the relevant ganglioside (GD3-lactone) with high affinity and binds to many GD3-expressing tumor cells. KM641 also

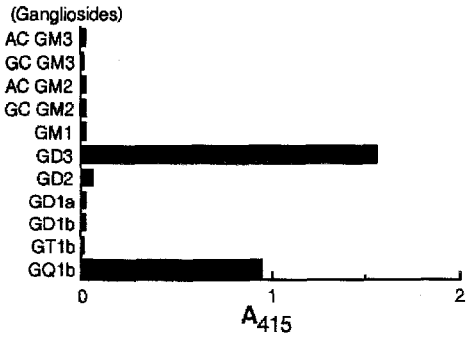

Fig. 1. Reaction of KM641 with gangliosides in enzyme-linked immunosorbent assay (ELISA). After various gangliosides had been coated on the microtiter plates, KM641 (2.5 μg/ml) was reacted for 2 h. *AC GM3*, N-acetyl-GM3; *GC GM3*, *N*-glycolyl-GM3; *AC GM2*, *N*-acetyl-GM2; *GC GM2*, *N*-glycolyl-GM2

exerts strong antitumor effects in vitro and especially in vivo. KM641 should become an ideal mouse anti-GD3 mAb for humanization.

Materials and methods

Cell lines. The following human melanoma cells were used as target cells: C24.22 (provided by Dr. Ishihara of the Japanese National Cancer Center Research Institute), G361 (provided by the Japanese Cancer Research Resources Bank), HMV-1 (purchased from Riken Cell Bank), KHm-1/4 (provided by Dr. Kanzaki of Kitazato University), Mela-3 (provided by Dr. Okabe of Tokyo University), SK-MEL-28 (purchased from ATCC), WM266-4 (purchased from ATCC). Human neuroblastoma cells IMR-32 (purchased from ATCC), human glioblastoma cells P122 (provided by Dr. Kishimoto from Osaka University) and human small-cell lung cancer cells Lu-135 (purchased from Riken Cell Bank) were used as control cells.

Gangliosides and mAb. Gangliosides were prepared as previously described [11, 13]. Anti-GD3 mAb R24 (mouse IgG3) was purchased from Signet Laboratories Inc. (Massachusetts, USA) and anti-(sialyl-Le^a) mAb KM231 (mouse IgG1) was produced in our laboratory [12].

Hybridoma. GD3-containing liposomes and SK-MEL-28 cells were used as immunogens. Balb/c mice were immunized several times by intravenous injection of GD3 liposomes (5 μg GD3/0.5 μmol dipalmitoylglycerophosphocholine/0.5 μmol cholesterol/0.05 μmol dipalmitoylglycerol 3-phosphoric acid/0.5 μg lipid A) and intraperitoneal injection of the tumor cells (1×10^7 cells). The spleen cells were fused with P3-X63.Ag8-U1 (P3U1) and underwent hypoxanthine/aminopterin/thymidine selection [10]. The hybridoma supernatants were assayed for reactivity with GD3 by enzyme-linked immunosorbent assay (ELISA) described below. The subclass of mAb was determined using a typing kit (Zymed Labs., California, USA). Large amounts of purified mAb were produced from serum-free culture medium with protein-A-Sepharose gel.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed by the previously reported method [13]. Briefly, gangliosides (2 nmol) were dissolved in 2 ml ethanol containing phosphatidylcholine (5 ng) and cholesterol (2.5 ng). Aliquots (20 μl) were then put in each well of microtiter plates (Flow Laboratories, Virginia, USA) 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 peroxidase-labeled rabbit anti-(mouse immunoglobulin) (Dakopatts, Glostrup, Denmark). The color development of the substrate (2,2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium) was measured at 415 nm.

Immunostaining of thin-layer chromatography (TLC) and immunofluorescence. Gangliosides spotted on high-performance TLC (HPTLC) plates (Whatmann, Maidstone, UK) were developed with chloroform/methanol/0.25% $CaCl_2$ in water (50/40/10). The immunostaining of TLC plates was performed as previously described [13]. The intensity of the immunoreaction was quantified by photodensitometer scanning (Atto, Tokyo, Japan). In indirect immunofluorescence, the tumor cells (1×10^6 cells) were incubated with purified mAb (10 μg/ml) for 45 min at 4° C. After the reaction with a fluorescein-isothiocyanate-labeled second antibody (Kirkegaard & Perry Laboratories, Maryland, USA) the reactivity was analyzed by flow cytometry (JASCO, Tokyo, Japan) [13].

Scatchard analysis. The purified mAb was labeled with Na^{125}I using Iodo-beads (Piece, Illinois, USA); the specific activity was 26 KBq/μg protein. The tumor cells (2×10^4 cells) were reacted with 200 μl of various dilutions of the ^{125}I-labeled mAb with or without a large excess of unlabeled mAb for 120 min on ice. The cell-bound radioactivity was measured and analyzed by the method of Scatchard as previously described [30].

Internalization. Tumor cells (1×10^6 cells) were cultured in the presence of ^{125}I-mAb for 90 min on ice and then moved to a CO_2 incubator for 180 min at 37° C after washing. The cells were washed on ice with 0.2 M acetis acid (pH 2.5)/0.14 M NaCl to remove the cell-surface antibodies and the radioactivity in the cells was measured.

Complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). The tumor cells (1×10^6 cells) were labeled with 3.7 MBq $Na_2{}^{51}CrO_4$ for 1 h at 4° C and kept for 30 min 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 mAb at various dilutions in the presence of 50 μl ninefold diluted rabbit complement (Cedarlane Laboratories, Ontario, Canada) for 1 h at 37° C for CDC or with 50 μl effector cells at the indicated effector cell/target cell ratio (E/T ratio) for 4 h at 37° C for ADCC. Peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN) were separated from the peripheral blood of a healthy donor and used as the effector cells using Leucoprep (Becton Dickinson, California, USA) and Polymorphoprep (Nycomed, Oslo, Norway) respectively. Monocytes were separated as dish-adherent cells. After centrifugation, the released ^{51}Cr in the supernatant was counted. Percentage specific lysis was calculated from the experimentally released radioactivity (cpm), the total release (cpm), and the spontaneous release (cpm).

Therapeutic effects on transplanted tumors. The tumor cells (2×10^7 cells) were intradermally transplanted in Balb/c *nu/nu* mice, and mAb were intravenously administrated five times. The tumor size (volume) was calculated by the following equation:

$$\text{tumor size (mm}^3) = 0.4 \times (\text{major axis}) \times (\text{minor axis})^2$$

Results

Specificity of mAb

We developed ten IgG3-type mAb that reacted with GD3 but not with GM3. Five mAb showed major reactivity with GD3 out of 11 common gangliosides in ELISA and the remaining mAb cross-reacted with GD1a or GD1b. KM641 was chosen because its major reaction was found to be restricted to GD3 except for a minor reaction with GQ1b (Fig. 1), and the binding avidity to GD3 was higher than that of R24 (Fig. 2). Detailed analysis by immuno-

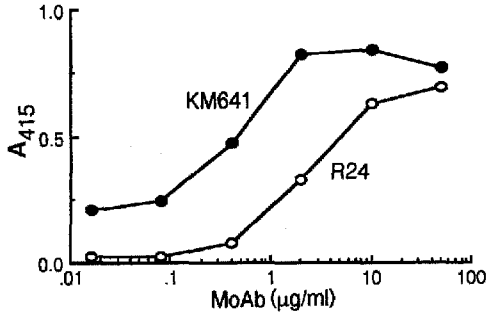

Fig. 2. Comparison of the reactivity between KM641 and R24 in ELISA. After GD3 had been coated on the microtiter plates, diluted mAb were reacted for 2 h

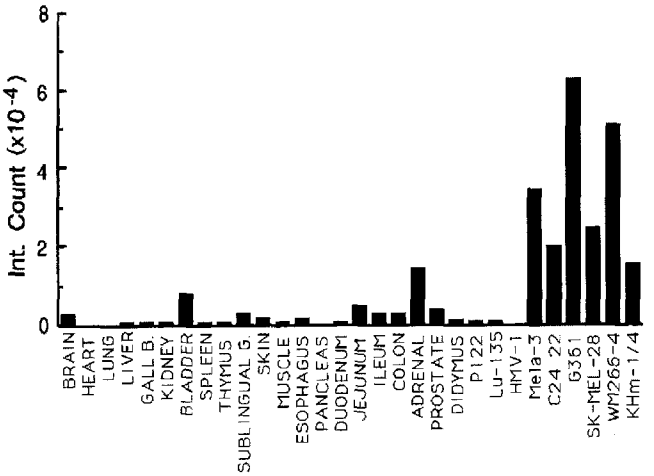

Fig. 3. Reaction of KM641 with glycolipids extracted from human normal tissues and cell lines. Glycolipids were prepared by extraction with chloroform/methanol (2:1) and Folch partition, using a previously reported method [13]. Aliquots (20 μl) of the glycolipid extracts (1 ml from 1 g wet tissues or cells) were spotted on high-pressure thin-layer chromatography (HPTLC) plates and developed with chloroform/methanol/0.25% $CaCl_2$ in water (50/40/10). After immunostaining with KM641 (10 μg/ml), the intensity of the immunoreaction was quantified by photodensitometer scanning. *Int. Count*, integral counts

staining of gangliosides separated on TLC plates demonstrated that KM641 also recognized the GD3-lactone formed by inducing an intramolecular ester in GD3. KM641 preferred GD3 having two *N*-acetyl sialic acids to that with two *N*-glycolyl sialic acids or the combination of an *N*-acetyl sialic acid and an *N*-glycolyl sialic acid (data not shown).

Distribution of KM641-reactive GD3 in human tumor cell lines and normal human tissues

KM641 detected GD3 in the melanoma cell lines (12/14), the neuroblastoma cell lines (3/6), the glioma cell lines (2/3), the leukemia cell lines (8/18), the lymphoma cell lines (3/6), the osteosarcoma cell lines (1/2), and the lung cancer cell lines (4/28) by TLC immunostaining. To determine the antigen distribution, glycolipids were extracted from 21 normal adult tissues and analyzed on TLC plates.

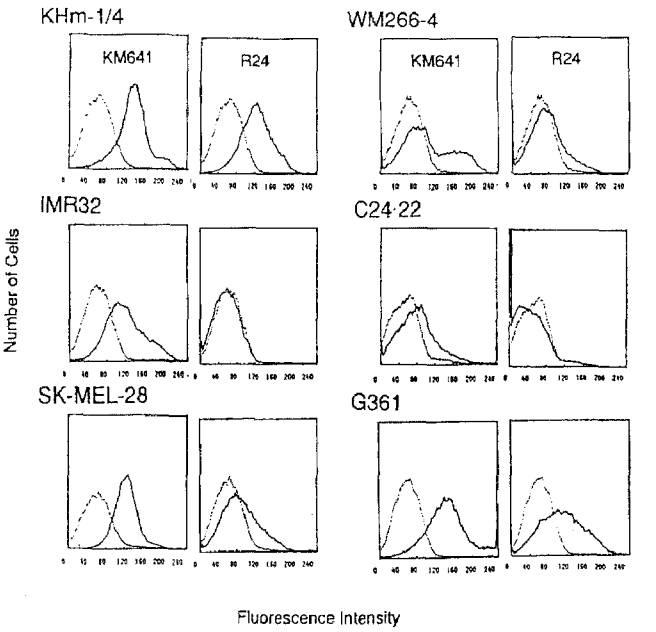

Fig. 4. Flow-cytometric analysis of the cell binding of KM641 and R24. The live cells (1×10^6) were reacted with purified mAb (10 μg/ml). The *left panels* show the staining patterns of KM641 and the *right panels* show that of R24 in each cell. ..., Negative control (no antibody)

The tissue samples were taken from several portions of each tissue. The brain sample was taken from the cerebral cortex. The adrenal tissue and the bladder contained KM641-reactive GD3, but at a relatively low level compared with the content in melanoma cell lines (6/7) (Fig. 3).

Binding to human tumor cells and internalization

We examined the binding of KM641 to living tumor cells by immunofluorescence, comparing the results with those for R24. KM641 positively bound to all cell lines examined, including five melanoma cell lines and a neuroblastoma cell line, while R24 failed to bind to 2 out of 6 cell lines (Fig. 4). Scatchard analysis was performed using G361 and C24.22 cells which showed high and low fluorescence intensity, respectively, in immunofluorescence (Fig. 5). Analysis based on the binding of ^{125}I-labeled KM641 revealed that KM641 had sufficient binding affinity for both the cell lines ($K_d = 1.9 \times 10^{-8}$ M). The number of binding sites was 1.9×10^7/cell on G361 and 7.2×10^5/cell on C24.22 cells. Internalization of ^{125}I-labeled KM641 into G361 cells took place when the culture was shifted from ice to a temperature of 37° C. The antibody bound on the cell surface was gradually incorporated into the cell, reaching 20% internalization after 180 min culture.

In vitro antitumor effects

CDC of KM641 on melanoma cells was studied in the presence of rabbit complement. KM641 killed SK-MEL-

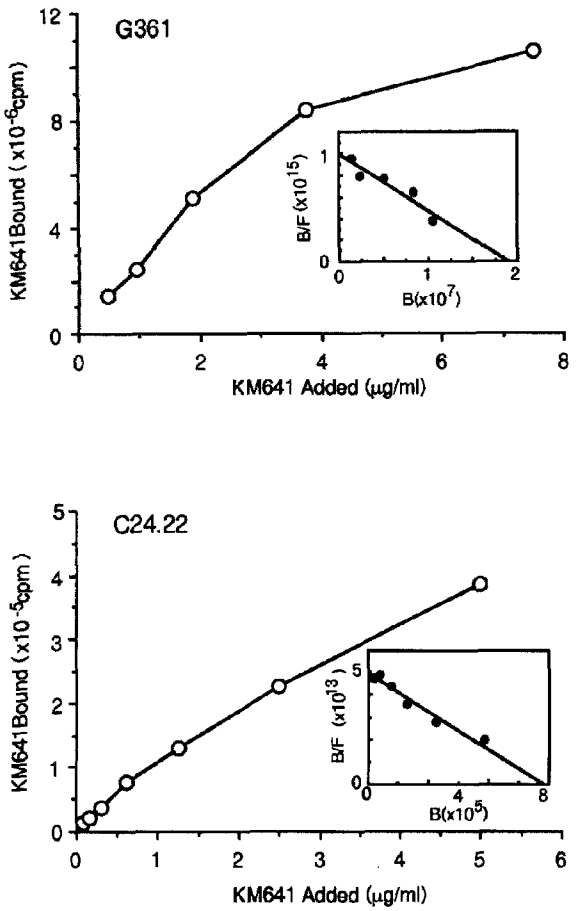

Fig. 5. Scatchard analysis of KM641 binding with G361 cells and C24.22 cells. Target cells (2×10^4) were reacted with various concentrations of ^{125}I-labeled KM641 with or without non-labeled KM641. The number of binding sites was calculated to be 1.9×10^7/cell on G361 cells and 7.2×10^5/cell on C24.22 cells. K_d was 1.9×10^{-8} M for both types of cell. B(mol/cell), number of molecules bound to each target cell; F, concentration of free ^{125}I-labeled KM641

Table 1. Complement-dependent cytotoxicity of KM641

Target	[mAb] (μg/ml)	Lysis (%)	
		+complement	–complement
SK-MEL-28	50	16.3	0.9
	5	32.4	0.0
	0.5	18.6	0.0
	0.05	2.7	0.0
G361	50	23.5	5.8
	5	47.0	1.2
	0.5	32.4	2.0
	0.05	15.2	2.7
C24.22	50	0.0	0.0
	5	0.0	0.2
	0.5	0.0	0.0
	0.05	0.0	0.0

28 cells and G-361 cells in a dose-dependent manner in the range 0.05–5 μg/ml, but did not kill C24.22 cells, which expressed the antigen at a low level (Table 1). KM641 exerted ADCC in almost the same manner as CDC, in that two cell lines, SK-MEL-28 and G-361, were killed dose-dependently by KM641 at the range 0.05–5 μg/ml in the presence of human PBMC, but not C24.22 cells (Table 2). Comparison of the effector function was made among human PBMC, PMN and monocytes. PBMC and PMN gave rise to killing of G-361 cell at an E/T ratio of 100:1 and 50:1. Monocytes failed to kill cells at the low E/T ratios of 10:1 and 5:1 (Table 3). We did not try the ADCC with monocytes at a high E/T ratio because of lack of monocytes.

Table 2. Antibody-dependent cellular cytotoxicity of KM641

Target	[mAb] (μg/ml)	Lysis (%)	
		+PBMC[a]	–PBMC
SK-MEL-28	50	43.2	0.0
	5	41.8	0.0
	0.5	16.3	0.0
	0.05	8.9	0.1
	–	7.5	
G361	50	28.0	0.0
	5	46.0	0.0
	0.5	22.9	0.0
	0.05	16.0	0.0
	–	20.5	
C24.22	50	14.5	0.8
	5	15.1	1.4
	0.5	16.0	0.3
	0.05	16.4	0.4
	–	14.6	

[a] PBMC, peripheral blood mononuclear cells; E/T ratio = 100:1

Table 3. Function of effector cells in antibody-dependent cellular cytotoxicity of KM641

Effector cell	E/T ratio	Lysis (%) for [mAb] (μg/ml) of:		
		50	5	–
PBMC	100:1	40.0	39.2	6.1
	50:1	12.4	22.2	6.1
PMN[a]	100:1	50.6	32.9	10.5
	50:1	25.4	25.4	8.4
Monocytes	10:1	8.4	9.0	12.6
	5:1	7.2	9.8	8.7

[a] PMN, polymorphonuclear cells

In vivo antitumor effects

Nude mice intradermally transplanted with 2×10^7 G-361 or C24.22 cells/animal were treated with five injections of KM641 from day 0. Treatments with KM641 (20 μg or 100 μg/injection) markedly suppressed the growth of G-361 cells through the experimental period of 65 days in the comparison with the control mAb KM231 (Fig. 6). The tumor growth of C24.22 cells, which was resistant to in vitro cytotoxicity, was also inhibited by the injection of 100 μg or 200 μg KM641 for 50 days (Fig. 6). All the above effects of KM641 were statistically significant by

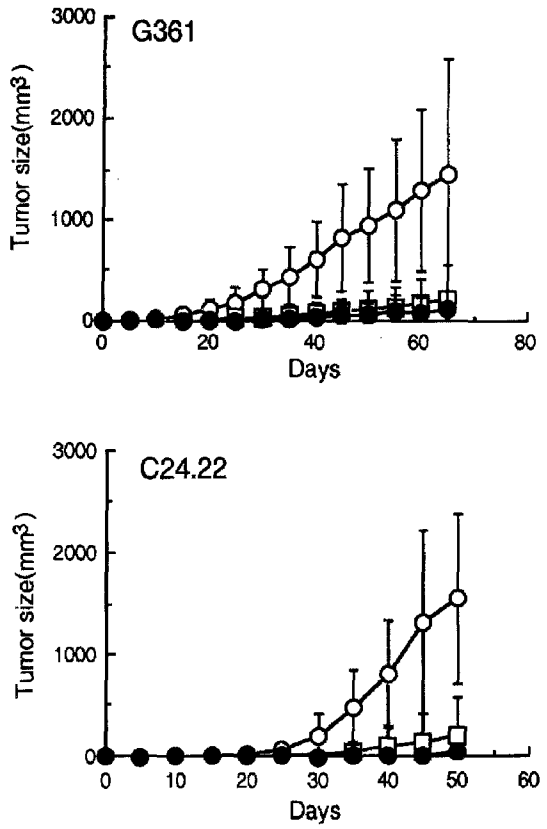

Fig. 6. Antitumor effects of KM641 on transplanted tumor in nude mice. The tumor cells were inoculated (2×10^7) i.d. and mAb were injected i.v. on day 0, 1, 2, 3 and 4. Each *point* represents the mean and SD from seven animals. In the G361 cell system (*upper figure*): ●, KM641 100 μg; □, KM641 20 μg; ○, KM231 100 μg. In the C24.22 cell system (*bottom figure*): ●, KM641 200 μg; □, KM641 100 μg; ○, KM231 100 μg. KM231, an anti-sialyl Le^a mAb, was used as a negative control antibody

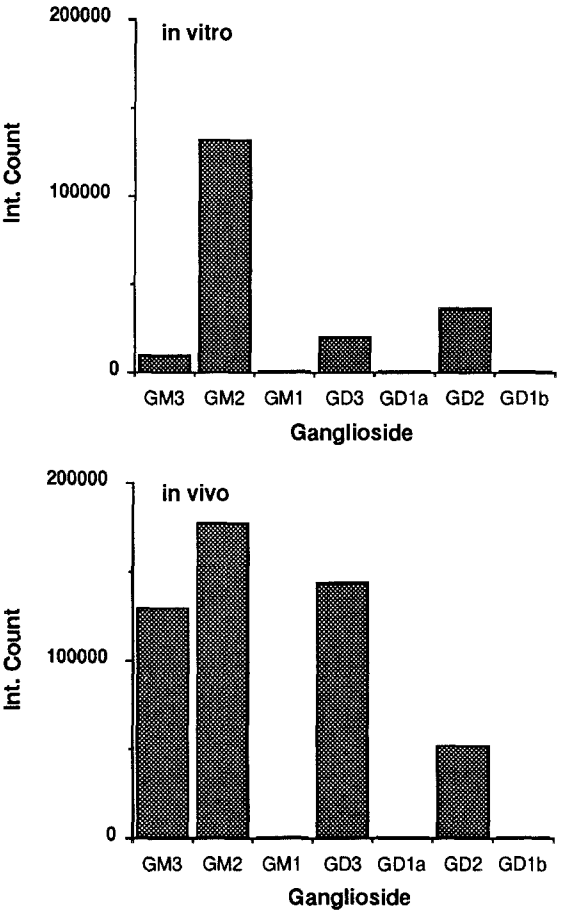

Fig. 7. Gangliosides in C24.22 cells cultured in vitro and grown in vivo. Glycolipids were extracted from the cultured cells (in vitro) or the tumor mass (in vivo) by the same method as that in Fig. 3. Aliquots (20 μl) of the extracts (1 ml from 1 g wet tissues or cells) were spotted on HPTLC plates and developed with chloroform/methanol/0.25% $CaCl_2$ in water (50/40/10). After the gangliosides had been stained with resorcinol, the intensity of the staining was quantified by photodensitometer scanning. *Int. Count*, integral counts

Student's *t*-test. Significant prolongation of life was also observed in the case of both G-361 and C24.22 (data not shown). The gangliosides were extracted from the tumor mass resected from the nude mice and compared with those from cultured C24.22 cells (Fig. 7). The amounts of GM3 and GD3 in the tumor increased approximately 13-fold and 7-fold above those in the cultured cell. The amounts of GM2 and GD2 in the tumor slightly increased compared with those in the cultured cells.

Discussion

In the present study, we attempted to develop mouse anti-GD3 mAb that have a strong binding affinity for the antigen in both the purified and the cell-surface-expressed form. KM641 was chosen from ten mouse anti-GD3 mAb on these criteria in this study. Several anti-GD3 mAb have been produced so far [2, 5, 7, 14, 15, 25, 27, 36, 37] and R24 is the only well-studied and clinically used anti-GD3 mAb among them. The binding specificity of KM641 was almost the same as that of R24 in that the both mAb had weak cross-reactivity with GQ1b [2, 36]. There was, however, a difference between the two mAb in the sialic acid composition of the immunoreactive GD3; KM641 demanded two *N*-acetyl sialic acids, but R24 allowed the combination of *N*-acetyl and *N*-glycolyl sialic acids [32]. KM641 was the only one known so far that required two *N*-acetyl sialic acids for its reaction with GD3. The GD3-specific mouse mAb species recently reported were of the IgM class [36], and the IgG-class mAb reported so far have had some cross-reactivity with GD1b, GD2, GT1a or GQ1b. The comparison between KM641 and R24 in the reaction with GD3 on ELISA plates revealed that KM641 bound more readily to the antigen. Chapman et al. reported that R24 bound to itself in a homophilic manner on solid phase [3]. We examined the homophilic binding of KM641, but did not observe this characteristic (data not shown). Light-chain variants of R24 have been reported to reduce its binding activity [34], but it was not clear that the preparation of R24 used in this study contained such variants.

Distribution of the antigen in human normal tissues is one of the critical factors when one considers the application of the mAb to human therapy. Some reports have

presented the immunohistochemical results of the antigen distribution of anti-GD3 mAb species. Garin-Chesa reported wide distribution of R24 antigen in normal tissues [8], while in two other reports the distribution of the antigen tended to be restricted to a few tissues such as the adrenal gland and the connective tissue [2, 29]. In this study the distribution of KM641 antigen was analyzed quantitatively on TLC plates. We found that the expression of the antigen was restricted to a few tissues such as the bladder and the adrenal gland, and the quantity was low compared with that in most melanoma cell lines. Holm et al. reported the expression of GD3 in the retina, which could not be examined in this study [16]. In the light of side-effects in passive immunotherapy, the reactivity with normal tissues is very important. More detailed immunohistological studies are under way using frozen tissue sections (the results will be published later).

We demonstrated that the high binding affinity of KM641 to GD3 was advantageous for the detection of the antigen expressed on living cells in flow-cytometric analysis (Fig. 4). Combination of the results of the flow cytometry and the results of the Scatchard analysis gave us the information that KM641 required a minimum number of binding sites, around 10^4/cell, for its positive detection in flow cytometry, because C24.22 cells expressing 7×10^5 binding sites/cell shifted slightly into the positive zone and G361 cells, having 2×10^4 binding sites/cell, showed a considerable shift into the positive zone in flow cytometry.

KM641 was found to cause in vitro cytotoxicity by both CDC and ADCC against the high-antigen-expressing cell lines, G361 and SK-MEL-28, while the mAb failed to kill the low-antigen-expressing cell line, C24.22. Few publications have dealt with the quantitative relationship between the number of binding sites and in vitro cytotoxicity. Mujoo et al. demonstrated that an immunotoxin composed of anti-GD2 mAb and gelonin effectively killed a tumor cell line having binding sites at the 10^6 level, but was not effective with a tumor cell line having binding sites at the 10^5 level [24]. The result that PMN were effective as well as PBMC as effector cells in ADCC with KM641 was coincident with the results using anti-GD2 mAb [1, 22]. The effector function of monocytes was unclear because experiments with a high E/T ratio could not be done for lack of cells.

In contrast to its failure to kill C24.22 cells in vitro, KM641 exerted potent tumor-growth suppression in vivo on C24.22 cells as well as on G361 cells. We expected that KM641 would potentiate the antitumor activity in vivo by inhibiting cell-cell interaction and tumor spreading at the site of inoculation in the nude mice in synergy with CDC and ADCC, as pointed out in the case of other anti-GD2/GD3 mAb [23]. Another explanation for the suppression of the tumor growth of C24.22 cells could be that GD3 on the cells transplanted in nude mice increased approximately seven times more than that on cells in vitro as shown in Fig. 7. The marked elevation of GD3 would lead to greater suppression of the growth of C24.22 in vivo after KM641 treatment compared to that in vitro. The results in this study coincide well with the results of Tsuchida et al. [33]. They demonstrated that amounts of GM3 and GD3 in surgical specimens of melanoma markedly decreased in the autologous tissue culture, but returned to the original high levels when the autologous cell lines were grown in athymic nude mice. The change in the expression of GM2 showed an inverse relation with that of GM3 and GD3 in their study.

This study has demonstrated that KM641 has sufficient binding ability and antitumor effects for the immunotherapy of human tumor. Efforts are being made to humanize KM641, and a study of its antitumor effects is in progress.

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