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Nobuo Hanai · Kazuyasu Nakamura · Kenya Shitara

Recombinant antibodies against ganglioside expressed on tumor cells

Abstract Several gangliosides such as GM2, GD2, and GD3 have been thought of as target molecules for active or passive immunotherapy of human cancers because of their dominant expression on the tumor cell surface, especially in tumors of neuroectodermal origin. We established a number of mouse or rat monoclonal antibodies (mAbs) to a series of gangliosides to investigate the nature of the molecules on the cell surface. Some of those mAbs were converted to chimeric or humanized mAbs with the aim of developing immunotherapy for human cancer. It is desirable for mAbs to remain on the cell surface for a long time so that they can exert effector functions such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). We found that mAbs to GM2, GD2, and GD3 remain on the cell surface for ≥60 min after binding, while mAbs to other types of carbohydrate such as sialy Le^a are quickly internalized. A chimeric mAb to GD3, KM871, was generated by linking cDNA sequences encoding light- and heavy-chain variable regions of mouse mAb KM641 with cDNAs encoding the constant region of human immunoglobulin γ1 (IgG-1). KM871 bound to a variety of tumor cell lines, especially melanoma cells, including some cell lines to which R24 failed to bind. In a preclinical study, intravenous injection of KM871 markedly suppressed tumor growth and radiolabeled KM871 efficiently targeted the tumor site in a nude mouse model. This chimeric mAb is being evaluated in a phase I clinical trial in melanoma patients. The chimeric mAb KM966 and humanized mAb KM8969 to GM2 originated from a mouse IgM mAb. When human serum and human peripheral blood mononuclear cells were used as effectors in CDC and ADCC, respectively, KM966 and KM8969 killed GM2-expressing tumor cells effectively. In addition, these mAbs may induce apoptosis of a small cell lung cancer cell line cultured under conditions mimicking physiological tumor conditions.

Key words Ganglioside · Antibody · Melanoma · Lung · Cancer

Introduction

Gangliosides are glycosphingolipids composed of a carbohydrate chain with sialic acid at the cell surface and a hydrophobic ceramide in the lipid bilayers [19]. Expression of gangliosides differs by cell type, organ, and animal species. Quantitative and qualitative changes are known to occur in the expression of gangliosides through the oncogenic transformation of cells [5]. Human tumors of neuroectodermal origin, such as melanoma, glioblastoma, and neuroblastoma, express large amounts of gangliosides GM2, GD2, or GD3, which are minor gangliosides in normal tissues and suggested to be associated with the clinical features of cancer [5]. Recently, these gangliosides have received attention due to their potential role in active or passive cancer immunotherapy.

Vaccination of GM2 bound to keyhole limpet hemocyanin or bacille Calmette-Guerin has been evaluated clinically, resulting in the production of antibodies to GM2 in melanoma patients [8, 10]. Another vaccine, an anti-idiotypic antibody called BEC2 that mimicks the GD3 melanoma antigen, is also under clinical evaluation [2]. Since the 1980s monoclonal antibodies (mAbs) to these gangliosides have attracted clinical interest. The antitumor effects of the mouse mAb to GD3, R24, have been studied intensively and in clinical trials in melanoma patients partial responses were achieved in some [11, 20]. A mouse mAb and chimeric mAb to GD2 have also been used in the treatment of melanoma and neu-

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N. Hanai (✉) · K. Nakamura · K. Shitara
Division of Immunology, Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machida-shi, Tokyo 194-8533, Japan
Tel.: +81 42 725 2555; Fax: +81 42 725 2559
e-mail: nhanai@kyowa.co.jp

roblastoma patients [3, 6]. In the case of mAbs to GM2, several derived from mice, rats, and humans have been established for passive immunotherapy [14], but all are in the immunoglobulin M class and are not feasible for clinical applications due to their physicochemical properties and because they lack one of the two important effector functions of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).

This paper describes the production of chimeric or humanized mAbs with high affinity for and a strong effector function on GD3 and GM2 using antibody engineering technology based on recombinant techniques [16]. A chimeric mAb to GD3, KM871 [17], was generated by linking the constant region of human immunoglobulin γ1 (IgG-1) to the variable region of the mouse mAb KM641, which has superior binding affinity over the intensively investigated R24 [15]. The pharmacokinetics, targeting ability, and toxicity of KM871 have been evaluated in a phase I clinical trial. The chimeric mAb [12] KM966 and humanized mAb KM8969 to GM2 exhibited CDC and ADCC effector functions in killing tumor cells. Interestingly, recent results have indicated that blocking functionally active GM2 with these mAbs leads to apoptosis of GM2-expressing tumor cells [13].

Materials and methods

Internalization

This study utilized the methods of Kusano et al. [9]. Briefly, tumor cells were reacted with mAb 10 μg/mL for 30 min on ice. The cells were maintained at 37 °C for 30 min and fixed in periodate 0.01 M-lysine 0.75 M-2% paraformaldehyde solution. After snap-freezing with OCT compound, 7 μm-thick sections were cut and reacted with a biotinylated anti-mouse immunoglobulin antibody, followed by reaction with an avidin-biotin-peroxidase complex. The sections were postfixed in 1% glutaraldehyde solution and stained with 0.5% diaminobenzidine solution. The sections were osmicated, dehydrated in ethanol, and embedded in Epon 812. Ultrathin sections were examined electron microscopically.

Incorporation of ^{125}I-labeled mAb

Tumor cells were cultured in the presence of ^{125}I-labeled mAb for 90 min on ice and rinsed with cold phosphate-buffered saline (PBS). The cells were incubated at 37 °C for a maximum of 60 min. The supernatant was assayed for the presence of free ^{125}I. The precipitated cells were washed with acetic acid 0.2 M-NaCl 0.14 M and centrifuged at 1500 rpm to remove the cell surface ^{125}I-mAb. The radioactivity in the supernatant and in the pellet was measured with a γ-counter.

Production of chimeric mAb and humanized mAb

The detailed methods for molecular cloning and construction of expression vector were reported by Shitara et al. [18] and Nakamura et al. [12]. Humanization steps were described in detail by Nakamura et al. [13]. Briefly, humanized mAb variants that had various amino acid residue mutations in their frameworks were constructed based on molecular modeling analysis of the antibody variable region, and their binding affinities to the antigen were evaluated using a transient expression system. Stably producing cells were established by transfection of the tandem vector, which consists of heavy- and light-chain gene transcription units, and a dihydrofolate reductase gene transcription unit, followed by repetitive cloning [18].

Immunostaining on thin-layer chromatography

Gangliosides on high-performance thin-layer chromatography (TLC) plates were developed with chloroform/methanol/0.25% $CaCl_2$ in water (50/40/10) following immunostaining of the TLC plates. The intensity of the immunoreaction was quantified by photodensitometer scanning.

Results

The cells chosen for the study of internalization of mAbs were: the mouse melanoma cell line B16.F10 for anti-GD3 mAb; small cell lung carcinoma cell line SBC-3 for anti-GM2 mAb; two melanoma cell lines, SK-MEL-28 and G361, for anti-GD3 mAb; melanoma cell line G361 and neuroblastoma cell line IMR-32, for anti-GD2 mAb; and colon cancer cell line SW1116, for anti-sialyl Le^a mAb. After culture at 37 °C for 30 min, numerous anti-GM3 mAbs remained on the cell surface, and a few endosomes containing the mAbs appeared. Anti-GM2 mAb was present on the cell surface, but no localization in the cytoplasm was observed. Anti-GD3 mAb was mainly retained on the cell surface of the two melanoma cell lines, and positively stained endozsomes were occasionally observed. Localization of anti-GD2 mAb was limited to the cell surface of the melanoma cell line and neuroblastoma cell line. In contrast to these anti-ganglioside mAbs, anti-sialyl Le^a mAb was localized mainly in endosomes and there was a remarkable decrease in mAb on the cell surface.

To investigate the kinetics of mAb internalization, ^{125}I-labeled anti-GD3 mAb and anti-sialyl Le^a mAb were incubated with the cells under the same conditions as for the immunocytochemical study. The ratio of intracellular counts to cell surface counts of anti-GD3 mAb gradually changed from 8:92 at 0 min to 13:87 at 60 min (Fig. 1a). Internalization was examined until 180 min and did not increase markedly. On the contrary, internalization of anti-sialyl Le^a mAb had already begun while the culture was being maintained on ice. The ratio of intracellular counts to cell surface counts of the ^{125}I-mAb was enhanced from 31:69 at 0 min to 54:46 at 60 min (Fig. 1b).

The chimeric mAb to GD3, KM871 (human IgG-1, κ), reacted with GD3 in a dose-dependent fashion, and its binding to GD3 was almost the same as that of its murine counterpart KM641. KM871 showed higher binding affinity to GD3 than did R24 [15]. KM871 showed specific reactivity with GD3 and weak cross-reactivity with GQ1b among 11 common gangliosides tested in enzyme-linked immunosorbent assay (ELISA). The CDC of KM871 was tested with ^{51}Cr-labeled G361 melanoma cells ranging from 0.5–50 μg/mL in the presence of human serum. The results showed that

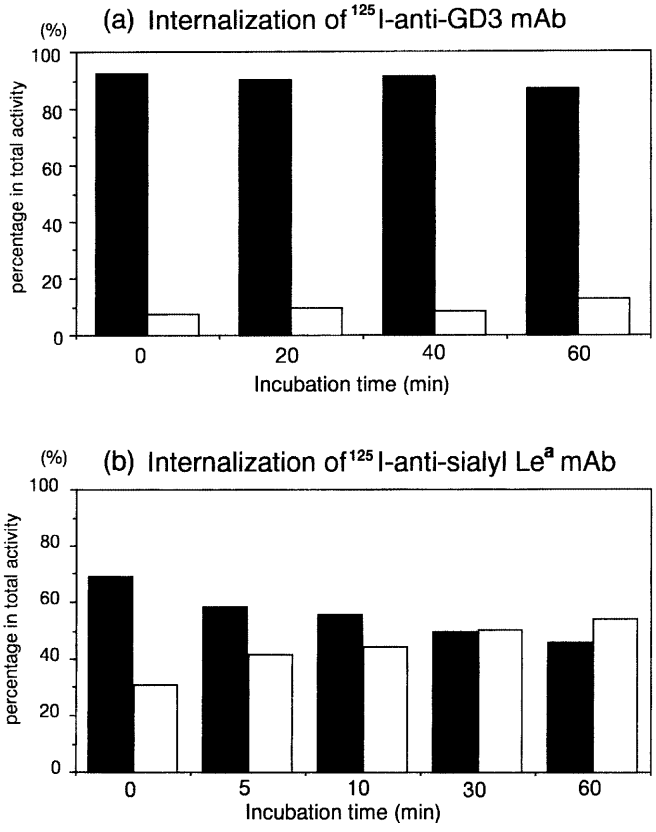

Fig. 1 Binding and internalization of ^{125}I-labeled mAb. The cells were preincubated on ice for 90 min with ^{125}I-labeled mAb and then placed in a CO_2 incubator at 37 °C. The radioactivity of the cell surface and intracellular region was measured. (a) G361 cells were incubated with ^{125}I-labeled mAb to GD3, KM641. (b) SW 1116 cells were incubated with ^{125}I-labeled mAb to sialyl Le^a, KM 231. ■ radioactivity of cell surface; □ intercellular radioactivity

KM871 had greater CDC than its murine counterpart KM641. The ADCC of KM871 was examined against ^{51}Cr-labeled G361 melanoma cells with human peripheral blood mononuclear cells (PBMCs). KM871 mediated a higher specific release than KM641, and the amount of KM871 required to mediate specific lysis was almost 1,000-fold less than that of KM641. The in vivo antitumor effect of KM871 was evaluated using nude mice transplanted intradermally with 2×10^7 G361 cells. Five consecutive injections of KM871 markedly suppressed tumor growth throughout the 65-day experimental period (Fig. 2).

The chimeric mAb KM966 and humanized mAb KM8969 showed high specificity to N-acetyl-GM2 and N-glycolyl-GM2, with low cross-reactivity with GD2 in ELISA. We also examined the binding specificity of the mAbs by immunostaining of gangliosides separated on TLC. The results indicated that the mAbs reacted with GM2 but not with GD2 and other gangliosides. KM966 and KM8969 exerted strong ADCC in a dose-dependent fashion in the range of 0.05 to 5 μg/mL. CDC activity of the mAbs was sufficient to kill GM2-expressing cells, but at higher concentrations than that required for ADCC

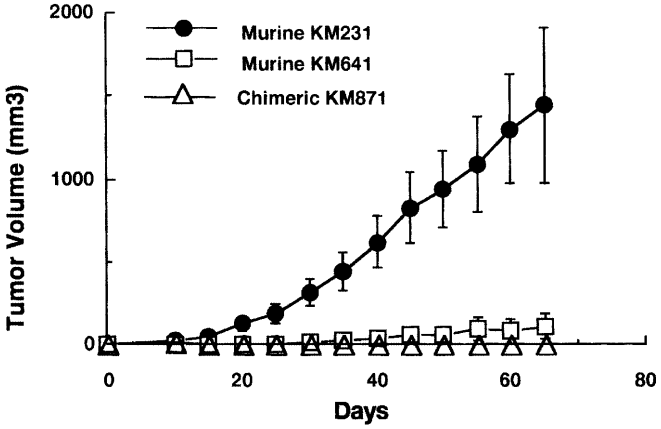

Fig. 2 Antitumor effects of chimeric KM871 and murine KM641 on transplanted tumors in nude mice. Human melanoma G361 cells 2×10^7 id were inoculated into each mouse and mAb 100 μg/day was injected intravenously on days 0, 1, 2, 3, and 4. Each point represents the mean and SD from five to seven animals. The anti-sialyl Le^a mAb KM231 was used as a negative control

activity. In addition to ADCC and CDC, KM8969 was found to induce apoptosis of small cell lung cancer cells cultured in the form of multicellular heterospheroids resembling physiological conditions. Nude mice that had received intradermal transplantation of 2×10^7 cells of SBC-3 cells were treated with five consecutive daily injections of KM966 from day 0 to day 5. Administration of KM966 markedly suppressed tumor establishment during the 53-day experimental period (Fig. 3).

The distribution of anti-GD_3 mAb-reactive GD3 and anti-GM_2 mAb-reactive GM2 in human tumor cell lines and normal human tissues was shown as the intensity of immunoreaction on TLC plates (Fig. 4).

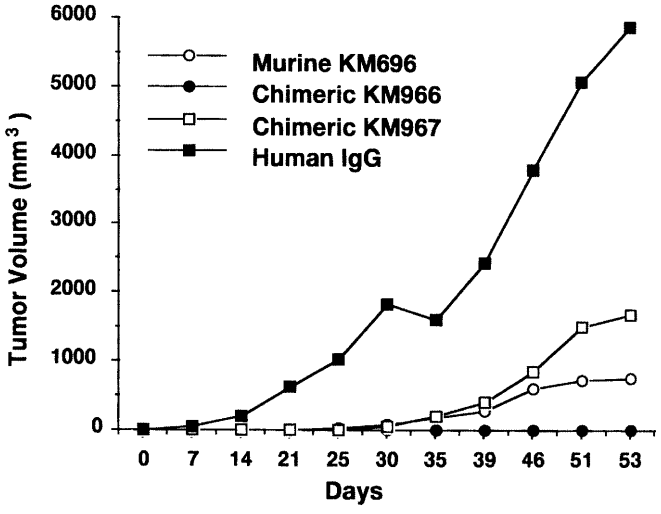

Fig. 3 Antitumor effects of chimeric mAb on transplanted tumors in nude mice. Human small cell lung carcinoma SBC-3 cells 2×10^7 id were inoculated into each mouse and mAb 100 μg/day iv was injected on days 0, 1, 2, 3, and 4. Each point represents mean tumor volume from 5 animals

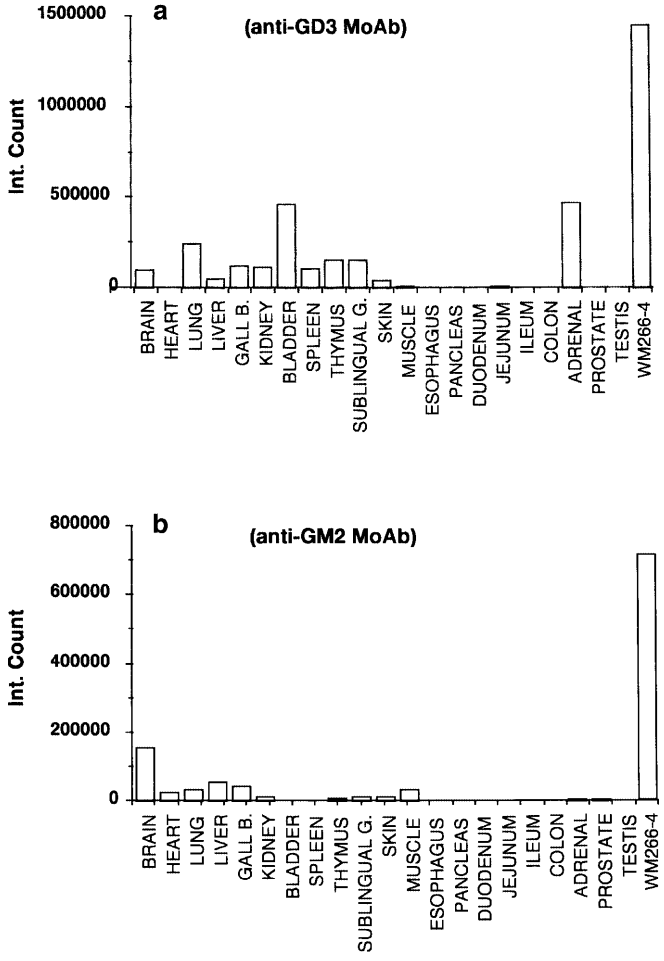

Fig. 4 Reaction of the mAb to GD3 (a) and mAb to GM2 (b) with glycolipids extracted from normal human tissues and cell lines. Glycolipids were prepared by extraction with chloroform/methanol (2:1) and Folch partition. Aliquots (20 μL) of the glycolipid extracts (1 mL from 1 g wet tissue or cells) were spotted on TLC plates and developed with chloroform/methanol/0.25% $CaCl_2$ in water (50/40/10). After immunostaining with the mAbs 10 μg/mL, the intensity of the immunoreaction was quantified by photodensitometer scanning

Discussion

The fate of mAbs after binding to the cell surface is important in the therapy of human cancer. From the results of this study, it is feasible for mAbs to gangliosides to have characteristics that enable them to exert effector functions, such as CDC and ADCC, since they remain for a long period on the cell surface of the target cells. Human IgG-1 class mAbs have an advantage in terms of ADCC function compared with the other classes of human immunoglobulins. The chimeric mAb to GD3, KM871, which has an IgG11 class heavy chain, was effective in killing GD3-expressing tumor cells. KM871 showed high tumor-killing activity and suppressed tumor growth markedly in early-stage models of nude mice inoculated with human tumor cells. Recently, we found that KM871 was effective even in late-stage models of some human melanoma cell lines.

Clinical trials of mAbs in melanoma were conducted with R24, a mouse mAb to GD3. Catimel et al. clearly demonstrated higher binding affinity of KM871 than of R24 using a biosensor (BIAcore, Pharmacia Biosensor, Uppsala, Sweden) in which GD3 was directly immobilized onto gold-carboxymethyldextran [1]. KM871 is under clinical evaluation in melanoma treatment, and clinical benefits are expected based on its weak antigenicity, long serum half-life, tumor-targeting ability due to its high binding affinity, and strong antitumor effector functions.

The chimeric mAb KM966 and humanized mAb KM896 have human IgG1 class heavy chains with effective ADCC and CDC functions. Our recent study suggested that GM2 plays a physiological role in the complicated structure of solid tumors, and blocking its function with mAb led to induction of apoptosis of GM2-expressing tumor cells [13]. The in vivo effect of KM966 has been studied extensively recently: a human solid tumor in nude mice derived from a doxorubicin-resistant clone of small cell lung cancer cells was effectively treated with KM966 [4]; and metastasis formation by small cell lung cancer cells was strongly inhibited by KM966 in natural killer cell-depleted severe combined immunodeficient mice [7]. In contrast to results showing that glycolipid extracts from normal human tissues contain GM2 only in a limited number of tissues (Fig. 4), an immunohistochemical study on tissue sections indicated that GM2 is expressed in many human cancers as well as in normal tissues [21]. The potent killing activity of KM966 and KM8969 is likely to be of benefit in the therapy of human cancer, although their interactions with normal tissues must be studied in detail to avoid unwanted side effects.

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