Anti–Glypican 3 Antibody as a Potential Antitumor Agent for Human Liver Cancer

Takahiro Ishiguro,[1] Masamichi Sugimoto,[1] Yasuko Kinoshita,[1] Yoko Miyazaki,[1] Kiyotaka Nakano,[2] Hiroyuki Tsunoda,[2] Izumi Sugo,[3] Iwao Ohizumi,[1] Hiroyuki Aburatani,[5] Takao Hamakubo,[5] Tatsuhiko Kodama,[5] Masayuki Tsuchiya,[2] and Hisafumi Yamada-Okabe[1,4]

[1]Pharmaceutical Research Department, Chugai Pharmaceutical Co. Ltd., Kanagawa, Japan; [2]Genome Antibody Research Department, [3]Preclinical Research Department, and [4]Research Planning and Coordination Department, Chugai Pharmaceutical Co. Ltd., Shizuoka, Japan; and [5]Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan

Abstract

Human glypican 3 (GPC3) is preferentially expressed in the tumor tissues of liver cancer patients. In this study, we obtained a monoclonal antibody (mAb) against the COOH-terminal part of GPC3, which induced antibody-dependent cellular cytotoxicity (ADCC). The mAb, designated GC33, exhibited marked tumor growth inhibition of s.c. transplanted Hep G2 and HuH-7 xenografts that expressed GPC3 but did not inhibit growth of the SK-HEP-1 that was negative for GPC3. GC33 was efficacious even in an orthotopic model; it markedly reduced the blood α-fetoprotein levels of mice intrahepatically transplanted with Hep G2 cells. Humanized GC33 (hGC33) was as efficacious as GC33 against the Hep G2 xenograft, but hGC33 lacking carbohydrate moieties caused neither ADCC nor tumor growth inhibition. Depletion of $CD56^+$ cells from human peripheral blood mononuclear cells markedly abrogated the ADCC caused by hGC33. The results show that the antitumor activity of hGC33 is mainly attributable to ADCC, and in human, natural killer cell–mediated ADCC is one possible mechanism of the antitumor effects by GC33. hGC33 will provide a novel treatment option for liver cancer patients with GPC3-positive tumors. [Cancer Res 2008;68(23):9832–8]

Introduction

Glypican 3 (GPC3) is a heparan sulfate proteoglycan and locates on the cell surface by a mechanism involving the glycerophosphatidylinositol anchor (1). Loss-of-function mutation of GPC3 was identified in patients of Simpson-Golabi-Behmel syndrome (SGBS), an X-linked disorder characterized by prenatal and postnatal overgrowth with visceral and skeletal abnormalities, indicative of the *GPC3* gene as the causative gene for SGBS (2). Patients of SGBS have an increased risk of embryonal tumors, including Wilms' tumors. Mice carrying the homozygous deletion of the *GPC3* gene exhibited clinical features similar to SGBS, such as developmental overgrowth, perinatal death, cystic and dysplastic kidneys, and abnormal lung development (3).

GPC3 protein is expressed in a wide variety of tissues during development, but its expression in most adult tissues is suppressed, at least in part, by the methylation of DNA within the promoter region (4). GPC3 regulates cell growth positively and negatively depending on the cell type. Overgrowth of SGBS and GPC3 in knockout mice suggests a negative regulation of cell growth by GPC3. In fact, ectopic expression of GPC3 inhibited the growth of mesothelium, breast, ovary, and lung cancer cells (5–8) and induced apoptosis in a cell line–specific manner (9). Because GPC3 is a heparan sulfate proteoglycan, it may function as a coreceptor for fibroblast growth factor 2 (FGF2), and GPC3 has been shown to bind to FGF2 (10). In addition, GPC3 is required for the stimulation of the noncanonical Wnt pathway in some cell types; GPC3 stimulates the noncanonical Wnt pathway, which in turn suppresses canonical Wnt signaling in embryos and in mesothelioma cells (11). Furthermore, on insulin stimulation, GPC3 is physically associated with GLUT4 on the cell surface and enhances insulin-stimulated glucose uptake (12).

On the other hand, GPC3 is highly expressed in hepatocellular carcinoma (HCC) cells and tissues (13–18) and stimulates the growth of HCC cells by increasing autocrine/paracrine canonical Wnt signaling (19). Overexpression of the GPC3 protein in tumor tissues has been confirmed by immunohistochemistry in ~70% of HCC patients (17). In addition, GPC3 negatively regulates BMP7, a negative regulator of cell growth (16). GPC3 is processed by a furin-like convertase and the processing occurs in the cysteine-rich domain located in the middle of the protein (20). Curiously, neither heparin sulfate chains nor processing of GPC3 is required for binding to Wnts, stimulation of Wnt signaling, or growth of HCC cells *in vitro* and *in vivo* (19, 21). Furthermore, requirement of SULF2 for GPC3-mediated FGF2 signaling was also recently reported (22).

In this study, we show that a monoclonal antibody (mAb) against the COOH-terminal region of GPC3 induced antibody-dependent cellular cytotoxicity (ADCC) and elicited antitumor activity in an antigen-dependent manner. Marked inhibition of tumor growth by the anti-GPC3 mAb was observed in both ectopic and orthotopic tumor xenograft models. Experiments using a humanized anti-GPC3 mAb suggest that, in human, natural killer (NK) cells serve as major effectors for ADCC.

Materials and Methods

Cells. Human HCC cell lines SK-HEP-1 and HuH-7 were purchased from the American Type Culture Collection (ATCC) and Health Science Research Resources Bank Japan, respectively. These cells were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS). The human hepatoblastoma cell line Hep G2 was obtained from ATCC, and the cells were cultured in Eagle's MEM (Sigma) supplemented with 10% FBS, nonessential amino acids, and 1.0 mmol/L sodium pyruvate. The cell surface expression of the human GPC3 was confirmed by flow cytometry and quantified using a QIFI-Kit (DakoCytomation).

Requests for reprints: Hisafumi Yamada-Okabe, Research Planning and Coordination Department, Gotemba Research Laboratories, 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan. Phone: 81-550-87-6730; Fax: 81-550-87-3637; E-mail: okabehsf@chugai-pharm.co.jp.

doi:10.1158/0008-5472.CAN-08-1973

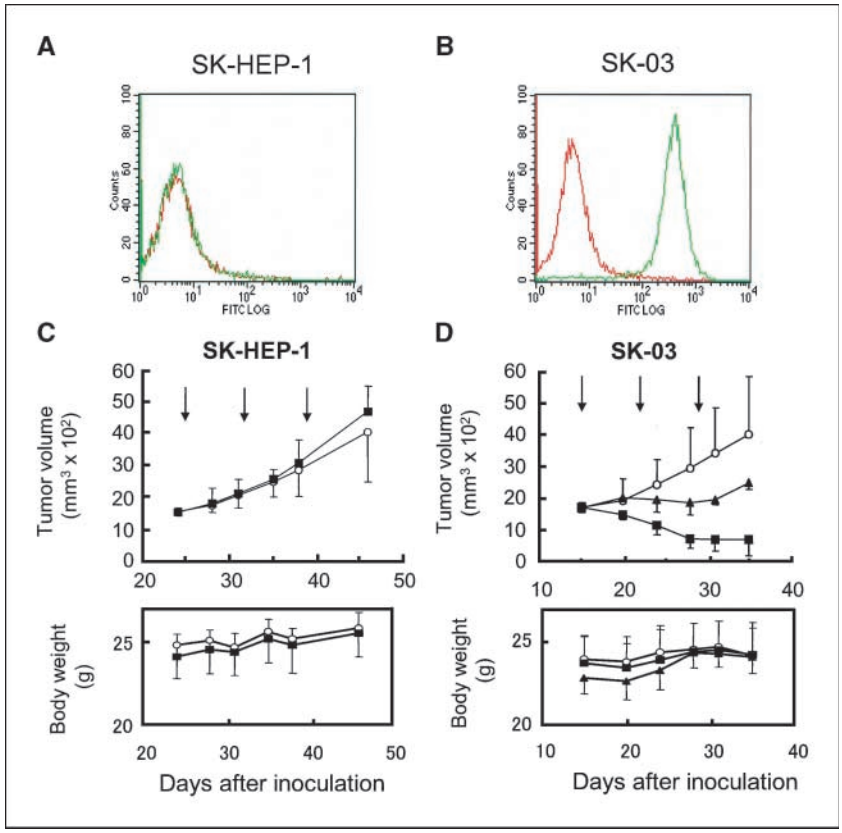

Figure 1. Antitumor activity of GC33 in SK-HEP-1 and SK-03 xenograft models. *A* and *B,* expression of GPC3 in SK-HEP-1 (*A*) and SK-03 (*B*) cells. Cell surface expression of the GPC3 protein was examined by flow cytometry. SK-03 was generated from SK-HEP-1 by the transfection of the human GPC3 cDNA. *C* and *D,* antitumor activity of GC33 against SK-HEP-1 (*C*) and SK-03 (*D*) xenografts. SCID mice were s.c. inoculated with 5×10^6 cells of SK-HEP-1 or SK-03. When tumors reached an average volume of 180 mm^3, mice were administered 1 mg/kg GC33 (▲), 5 mg/kg GC33 (■), or only PBS (○). GC33 and PBS were administered once a week for 3 wk. *Points,* mean of six animals; *bars,* SD. Tumor volume (*top*) and body weight (*bottom*) are indicated. *Arrows,* the days on which GC33 was administered.

Antibody and drugs. MRL/lpr mice were immunized with a GPC3 fragment containing the COOH-terminal region (amino acid positions between 524 and 563). The hybridoma clone that produced the mAb with the strongest binding to the antigen was selected for further characterization, and the mAb was designated GC33 (IgG2a, κ). Details of the generation of GC33 will be described in a separate article.[6] The murine antibody was collected from the culture medium of the hybridoma, and the humanized antibody was produced in Chinese hamster ovary (CHO) cells. The antibodies were purified by protein A affinity column chromatography (20) and diluted with PBS before use. Doxorubicin and mitoxantrone were obtained from Kyowa Hakko Kogyo Co. Ltd. and Takeda Pharmaceutical Co. Ltd., respectively, and were diluted with PBS before administration. Sorafenib was synthesized by Chugai Pharmaceutical Co. Ltd. and was diluted with 10% ethanol and 10% Cremophor EL in water before administration.

Transfection of DNA. The entire region of the human GPC3 cDNA (15) was cloned at the *Eco*RI sites of the pCXND2 expression vector, which is a derivative of pCXN (23) containing a CAG promoter and neomycin-resistant gene. The resulting plasmid, pCXND2, in which the human GPC3 cDNA was linked to the CAG promoter, was designated pCXND2/hGPC3(FL). To generate a cell line that stably overexpressed the human GPC3, SK-HEP-1 cells were transfected with pCXND2/hGPC3(FL) using FuGENE 6 (Roche Diagnostics Corp.) and selected with 1 mg/mL Geneticin (Invitrogen). Cells that grew even in the presence of G418 were collected, and the colonies were isolated by limiting dilution. One of the clones, SK-03, which expressed $\sim 2.1 \times 10^6$ molecules per cell of the human GPC3, was selected and used for the experiments.

Animals and drug administration. Six-week-old male CB-17 severe combined immunodeficient (SCID) mice were purchased from CLEA Japan, Inc. Just before tumor transplantation, the mice were i.p. injected with 200 μg of anti-asialo GM1 antibody (Wako). The mice were then s.c. inoculated with 5×10^6 cells of SK-03, SK-HEP-1, Hep G2, or HuH-7 suspended in 50% Matrigel (Becton Dickinson). When the tumor volume reached approximately 170 to 400 mm^3, the mice were i.v. administered the indicated doses of the anti-GPC3 antibody (GC33) once a week for 3 wk. The control mice received the same volume (10 mL/kg) of PBS. Day 0 represents the day of the first administration. For combination therapy with sorafenib, sorafenib was orally administrated 5 d a week for 3 wk. For combination therapy with doxorubicin and mitoxantrone, drugs were administered i.v. once a week for 3 wk starting day 0. Control mice were administrated with the vehicle, which was PBS(−) for GC33 and 10% ethanol and 10% Cremophor EL in water for sorafenib. Tumor volume was estimated using the equation $V = ab^2/2$, where a and b are tumor length and width, respectively.

An orthotopic tumor model was created by intrahepatically injecting 5×10^6 Hep G2 cells into 5-wk-old male BALB/c *nu/nu* (Charles River Japan, Inc.). Twenty-one days after tumor transplantation, mice whose serum α-fetoprotein (AFP) levels had reached between 10 and 100 ng/mL were i.v. administered 5 mg/kg anti-GPC3 antibody (GC33) or vehicle (PBS) 21 and 28 d after tumor transplantation.

Determination of the serum levels of AFP. AFP concentrations in sera were determined by ELISA using an Enzyme Immunoassay kit (Hope Laboratories, Inc.). Whole blood samples were collected from mice 21 and 35 d after tumor transplantation, and the concentration of AFP in the sera was determined according to the manufacturer's instruction.

Preparation of mouse and human effector cells. Mouse bone marrow cells (BMC) were obtained from the femurs of 6-wk-old male CB-17 SCID mice. After washing twice, the BMCs were cultured in RPMI 1640 supplemented with 10% FBS, antibiotic-antimycotic (Invitrogen), 50 ng/mL of recombinant human interleukin-2 (IL-2; PeproTech, Inc.),

[6] K. Nakano et al. Generation of anti-glypican 3 antibodies with the ability to induce antibody-dependent cellular cytotoxicity against human hepatocellular carcinoma cells, submitted.

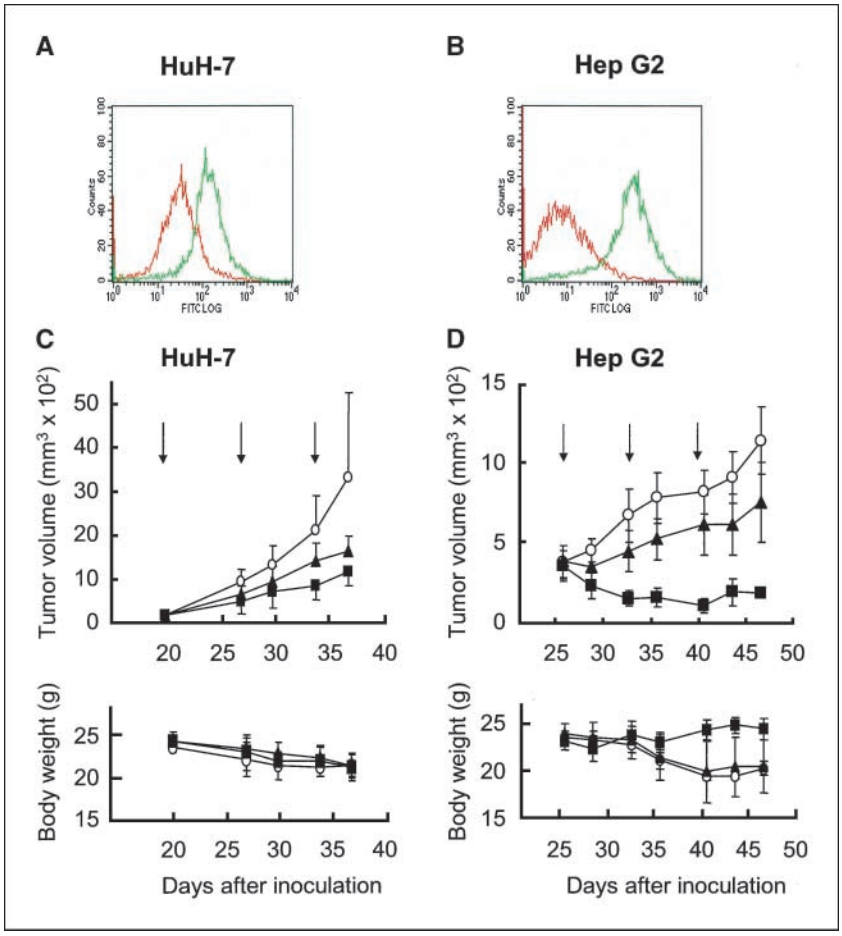

Figure 2. Antitumor activity of GC33 in HuH-7 and Hep G2 xenograft models. *A* and *B,* expression of GPC3 in HuH-7 (*A*) and Hep G2 cells (*B*). Cell surface expression of the GPC3 protein was examined by flow cytometry. *C* and *D,* antitumor activity of GC33 against HuH-7 (*C*) and Hep G2 (*D*) xenografts. SCID mice were s.c. inoculated with 5×10^6 cells of HuH-7 or Hep G2. When tumors reached an average volume of 170 mm^3 (for HuH-7) or 400 mm^3 (for Hep G2), mice were administered 1 mg/kg GC33 (▲), 5 mg/kg GC33 (■), or only PBS (○). GC33 and PBS were administered once a week for 3 wk. *Points,* mean of five animals; *bars,* SD. Tumor volume (*top*) and body weight (*bottom*) are indicated. *Arrows,* the days on which GC33 was administered.

and 10 ng/mL of recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF; PeproTech) for 5 d at 37°C and used as effector cells. Human peripheral blood mononuclear cells (PBMC) were purified from whole blood samples of healthy volunteers by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare) and were resuspended in PBS containing 0.5% bovine serum albumin and 2 mmol/L EDTA. $CD14^+$ and $CD56^+$ cells were depleted by MACS using CD14 and CD56 microbeads (Miltenyi Biotec), respectively. The resulting PBMCs depleted of $CD14^+$ or $CD56^+$ were washed with RPMI 1640 containing 10% FBS and used as human effector cells.

Determination of ADCC. ^{51}Cr-labeled Hep G2 cells were plated onto a 96-well plate and incubated with serial dilutions of antibodies in the presence of effector cells at an E:T ratio of 50:1. After incubation at 37°C for 4 h, the supernatants were collected, and radioactivities in the supernatants were counted with a gamma counter. Specific cell cytotoxicity was calculated according to the following formula: cell cytotoxicity (%) = $(A - C)/(B - C)$, in which *A, B*, and *C* represent the radioactivity of the target cells in the supernatant incubated with antibody and effector cells, lysed with 1% NP40, and cultured without antibody or effector cells, respectively.

Preparation of the humanized GC33. The humanized GC33 (hGC33) was generated by complementarity-determining region grafting as described (24). Details of the generation of the humanization of GC33 will be reported in a separate article. The cDNA for the heavy chain variable region (VH) of the hGC33 was cloned at the *Eco*RI-*Nhe*I sites in pBluescript KS (+) (Toyobo) carrying the constant region of human IgG1. The cDNA for the light chain variable region (VL) of the hGC33 was cloned at the *Xho*I-*Bsi*WI sites in pBluescript KS (+) harboring the constant region of the human κ chain. cDNAs for the hGC33 were cloned in pCXND, and the resulting plasmid DNA was transfected into CHO cells by electroporation. Transformants were selected by G418 and mAbs were purified by protein G-Sepharose affinity chromatography. The purified antibodies were dialyzed against and diluted with PBS before use. cDNA for the aglycosylated form of the hGC33 was generated by alanine substitution for asparagine at 297 within the Fc region that serves as the glycosylation by site-directed mutagenesis (25).

Results

Antitumor activity of the anti-GPC3 mAb. Mouse and human GPC3 protein are highly homologous (94% sequence identity) and human GPC3 might not be quite antigenic in mice. Therefore, we used MRL/lpr mice, which develop an autoimmune syndrome for immunization. Immunization of the MRL/lpr mice with the COOH-terminal region of GPC3 yielded a mAb that bound to CHO cells expressing the human GPC3.[6] This mAb, designated GC33, also induced ADCC in GPC3-expressing CHO cells but not in the parental CHO cells.[6] This prompted us to examine the antitumor activity of GC33 in human hepatoma xenograft models. GC33 was not efficacious against the SK-HEP-1 that did not express GPC3 even at 5 mg/kg (Fig. 1). Transfection of the human GPC3 cDNA into SK-HEP-1 cells resulted in the expression of the GPC3 protein as revealed by flow cytometry, and the clone designated SK-03, in which the amount of cell surface GPC3 protein was estimated to be $\sim 2.1 \times 10^6$ molecules per cell, was used for testing the antitumor activity of GC33. Administration of 1 mg/kg GC33 markedly

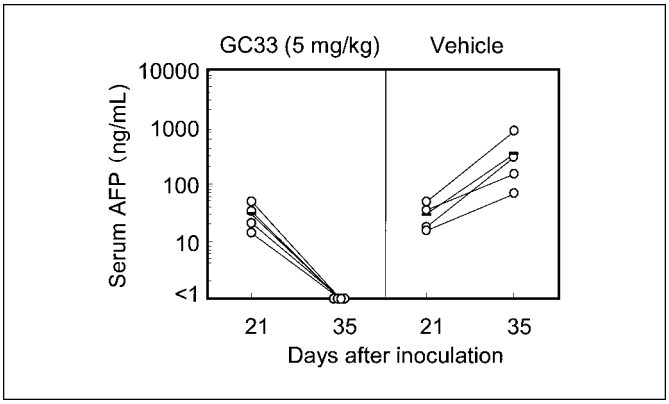

Figure 3. Efficacy of GC33 in an orthotopic Hep G2 xenograft model. Nude mice were intrahepatically inoculated 5×10^6 cells of Hep G2. Twenty-one days after the tumor inoculation, the serum AFP concentrations reached between 10 to 100 ng/mL and mice were divided into two groups: an anti-GC33 antibody group in which mice were administered 5 mg/kg GC33 and a vehicle group in which mice were administrated PBS (*Vehicle*). Each group consisted of five animals. PBS and GC33 were administered 21 and 28 d after the tumor inoculation. The serum AFP levels of each animal 21 and 35 d after tumor inoculation are shown. *Horizontal bars,* mean AFP level in each group.

inhibited the growth of the SK-03 xenograft, and the antitumor effect from GC33 became more profound at higher doses; GC33 at 5 mg/kg caused tumor remission (Fig. 1).

Next, we examined whether GC33 caused tumor growth inhibition in xenografts that expressed endogenous GPC3. Hep G2 and HuH-7 were selected because of the amount of GPC3 expressed on the cell surface (Fig. 2). The estimated amount of cell surface GPC3 protein was 1.5×10^6 molecules per cell in Hep G2 and 4.0×10^4 molecules per cell in HuH-7 cells (Fig. 2*A* and *B*). GC33 was efficacious against both Hep G2 and HuH-7 xenografts; at 5 mg/kg, GC33 strongly inhibited the growth of the HuH-7 xenograft and caused tumor remission in the Hep G2 xenograft model (Fig. 2). The antitumor effect of GC33 also attenuated body weight loss caused by the Hep G2 xenograft. In mice carrying the Hep G2 xenograft, body weight decreased as the tumor grew (after day 35), whereas it remained constant ($\sim$25 g) when mice were administered 5 mg/kg GC33 (Fig. 2*D*). The degree of tumor growth inhibition from GC33 seems to correlate with the level of cell surface antigens because the amount of cell surface GPC3 protein was highest in SK-03 and lowest in HuH-7 cells among SK-03, Hep G2, and HuH-7 (Figs. 1 and 2). We also confirmed that control human IgG had no significant effect on the growth of the tumor xenografts.

The above results clearly show the antitumor activity of GC33 in human hepatoma xenograft models, in which tumors were transplanted s.c. Accordingly, we asked whether GC33 elicits antitumor activity even when tumors have been transplanted orthotopically. After intrahepatic injection of Hep G2, serum AFP levels reached between 10 and 100 ng/mL 21 days after tumor transplantation (Fig. 3). Administration of GC33 caused marked decrease of serum AFP; serum AFP levels were <1 ng/mL in mice administered 5 mg/kg GC33, whereas the level of serum AFP continued to increase in mice that received only vehicle (PBS; Fig. 3). On day 35, there were no visible tumors in mice administered GC33, but multiple tumors were observed in vehicle-treated mice. All these results show that GC33 is efficacious even against the Hep G2 xenograft growing in the liver.

Antitumor activity in combination with the anti-GPC3 mAb and chemotherapeutic agents. Although not quite efficacious, chemotherapeutic agents such as doxorubicin, mitoxantrone, *cis*-platinum, and 5-fluorouracil have been used for the treatment of liver cancers. Recently, sorafenib, which preferably inhibits the tyrosine kinase activities of c-Raf, vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3, and platelet-derived growth factor receptor-β, was shown to prolong the survival of HCC patients (26). Therefore, we examined the antitumor activity of GC33 in combination with sorafenib. When administrated as single agents, GC33 at 1 mg/kg and sorafenib at the maximum tolerated dose (MTD) of 80 mg/kg inhibited the growth of Hep G2 xenografts, and tumor growth inhibition became more profound when mice received both GC33 and sorafenib (Fig. 4). Tumor

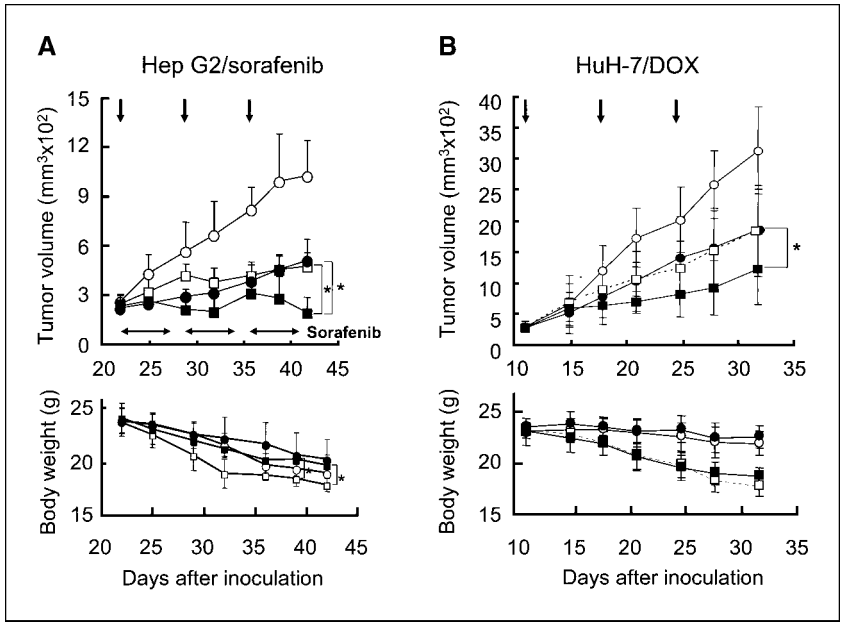

Figure 4. Antitumor activity of GC33 in combination with sorafenib or doxorubicin. *A,* antitumor activity of GC33 in combination with sorafenib. SCID mice were s.c. inoculated with 5×10^6 cells of Hep G2. When tumors reached an average volume of 230 mm^3, mice were administered 1 mg/kg GC33 (●), 80 mg/kg sorafenib (□), 1 mg/kg GC33 plus 80 mg/kg sorafenib (■), or only control (○). *B,* antitumor activity of GC33 in combination with doxorubicin (*DOX*). SCID mice were s.c. inoculated with 5×10^6 cells of HuH-7. When tumors reached an average volume of 270 mm^3, mice were administered 5 mg/kg GC33 (●), 3 mg/kg doxorubicin (□), 5 mg/kg GC33 plus 3 mg/kg doxorubicin (■), or only PBS (○). GC33, doxorubicin, and PBS were administered once a week for 3 wk. Sorafenib was orally administered 5 d a week for 3 wk. Tumor volume (*top*) and body weight (*bottom*) are indicated. *Arrows,* the days on which GC33, doxorubicin, sorafenib, and PBS were administered. *Points,* mean of six animals; *bars,* SD. *, $P < 0.05$ versus GC33 alone by Student's *t* test.

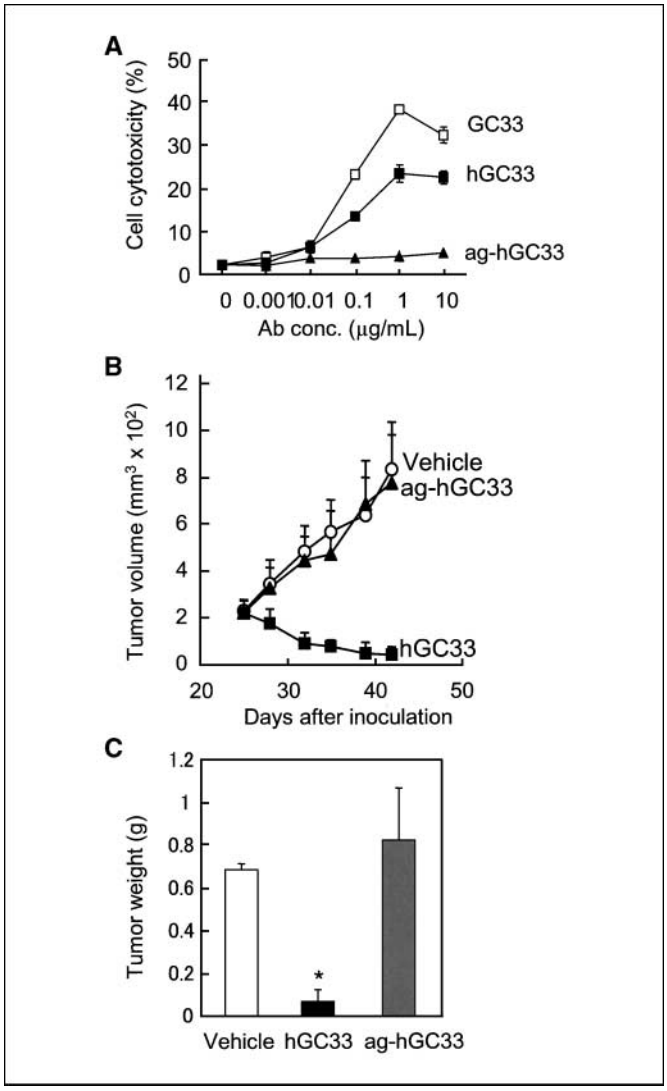

Figure 5. Effects of deglycosylation on the antitumor activity of GC33. *A,* ADCC of the GC33, hGC33, and aglycosylated mutant of the hGC33 (*ag-hGC33*). Hep G2 cells that had been labeled with ^{51}Cr and mouse BMCs that had been activated with IL-2 and GM-CSF were incubated at the E:T ratio of 50:1 in the presence of the indicated concentrations of the antibodies. Specific cell cytotoxicity was calculated from the radioactivities in the supernatants. Complete (100%) cell cytotoxicity represents the radioactivities in the supernatant of Hep G2 cells lysed with 1% NP40. *B* and *C,* antitumor activity of ag-hGC33 and hGC33. SCID mice were s.c. inoculated with 5×10^6 cells of Hep G2. When tumors reached an average volume of 220 mm^3, mice were administered 5 mg/kg hGC33, 5 mg/kg ag-hGC33, or only PBS. Antibodies and PBS were administered once a week for 3 wk. Tumor volume (*B*) and tumor weight 68 d after tumor inoculation (*C*) are shown. Values indicate the mean of five animals with SD. *C, asterisk,* significant difference ($P < 0.0001$) by Dunnett's test compared with vehicle control.

growth inhibition by the combination of 1 mg/kg GC33 and 80 mg/kg sorafenib was stronger than that attained by the same doses of each drug (Fig. 4*A*). In addition, GC33 significantly reduced the body weight loss caused by sorafenib (Fig. 4*A*). An additive effect on antitumor activity was also observed by the combination of GC33 with the MTD of doxorubicin (1 mg/kg) in the doxorubicin-sensitive HuH-7 xenograft model (Fig. 4*B*) and with the MTD of mitoxantrone (1 mg/kg) in the mitoxantrone-sensitive Hep G2 xenograft model (data not shown). These results support the possibility that GC33 can be safely combined with chemotherapeutic agents to elicit additive effects.

Mode of action of the anti-GPC3 mAb. Because GC33 induced ADCC in GPC3-positive hepatoma cells, tumor growth inhibition by GC33 might be attributable to ADCC. To confirm this possibility, we generated an aglycosylated mutant that lacked carbohydrates, thereby losing the ability to induce ADCC. In addition, a humanized antibody is appropriate for clinical application because of reduced antigenicity in human. Therefore, we generated hGC33 and then substituted alanine for asparagine at 297, which serves as the carbohydrate attachment site within the human IgG Fc region (25). Although the ADCC of hGC33 with mouse effector cells was somewhat weaker than that of GC33 at antibody concentrations higher than 0.1 mg/mL (Fig. 5*A*), hGC33 was highly efficacious against the Hep G2 xenograft and caused tumor remission at the dose of 5 mg/kg (Fig. 5*B* and *C*). An aglycosylated mutant of hGC33 had the same antigen-binding activity as GC33 did (K_d = 0.67 nmol/L) but failed to induce ADCC in the presence of mouse effector cells (Fig. 5*A*). The antitumor activity of the aglycosylated mutant of hGC33 was nearly completely diminished (Fig. 5*B* and *C*), showing that the antitumor activity of hGC33 is largely attributable to ADCC.

Next, we asked which effector cells are important for the ADCC activity of hGC33 in human. The human PBMCs used in this study contained 15.9% $CD14^+$ cells and 16.3% $CD56^+$ cells, and 99.3% of the $CD56^+$ cells and 99.9% of the $CD14^+$ cells were eliminated by MACS using anti-CD56 mAb beads and anti-CD14 mAb beads, respectively (data not shown). hGC33 strongly induced ADCC against Hep G2 cells in the presence of human PBMCs (Fig. 6). Depletion of $CD56^+$ cells from human PBMC markedly diminished the ADCC from hGC33 but depletion of $CD14^+$ cells only slightly attenuated the activity (Fig. 6). Because a large majority of $CD56^+$ cells and $CD14^+$ cells were NK cells and monocytes, respectively, NK cells may play an important role as effector cells for the ADCC of hGC33 in human.

Discussion

In this study, we showed that GC33, a mAb against the COOH-terminal region of the human GPC3, induced ADCC in GPC3-positive hepatoma cells and caused tumor growth inhibition in human liver cancer xenograft models. The antitumor activity of

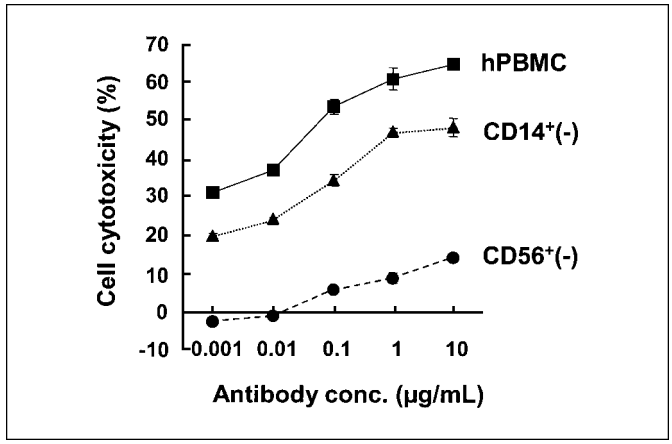

Figure 6. Effects of depletion of $CD56^+$ or $CD14^+$ cells from human PBMC on the ADCC activity of hGC33. ADCC from hGC33 in the presence of human PBMC (*hPBMC*), $CD56^+$ cell–depleted PBMC [*$CD56^+(-)$*], and $CD14^+$ cell–depleted PBMC [*$CD14^+(-)$*] was determined. *Points,* mean percentage of cell cytotoxicity from triplicate experiments; *bars,* SD.

GC33 is dependent on the antigen expression on the cell surface; GC33 strongly inhibited the growth of GPC3-positive tumors but did not affect the growth of GPC3-negative tumors. Furthermore, the degree of tumor growth inhibition by GC33 roughly correlated with the levels of cell surface antigens, suggesting that clinical efficacy may also correlate with the antigen expression levels within the tumor tissue. GC33 also restored body weight in mice bearing the Hep G2 xenograft. The reason for body weight loss by Hep G2 is not clear but Hep G2 might secrete cytokines that cause cachectic symptoms. It is possible that GC33 inhibits cytokine secretion from Hep G2. Although the sequences of GPC3 proteins are highly conserved among species, GC33 showed a similar binding activity to monkey GPC3 but did not react with mouse GPC3. Therefore, we administered GC33 to cynomolgus monkeys to assess the safety profile. Even after repeated administrations at a dose higher than 100 mg/kg, there were no abnormal clinical or pathologic signs in all the major tissues tested, including heart, lung, liver, and kidney. Nevertheless, adverse events should be carefully evaluated in clinical studies. Further details of the toxicity profile of GC33 will be published separately.

Although the ADCC from hGC33 in the presence of mouse effector cells was weaker than that from GC33, hGC33 was as efficacious as GC33 in Hep G2 xenograft models. Substitution of an alanine for asparagine at 297 almost completely diminished the ability to induce ADCC, presumably due to a lack of carbohydrate moieties. Consequently, the aglycosylated mutant of the hGC33 did not show antitumor activity *in vivo*. Furthermore, depletion of $CD56^+$ NK cell–rich cell fractions from human PBMC markedly decreased ADCC. On the other hand, the chimera GC33 carrying the Fc region derived from the human IgG1κ induced complement-dependent cellular toxicity (CDC) in CHO cells expressing the human GPC3 but not in Hep G2 and HuH-7 in the presence of human serum.[6] Although the reason why the chimera GC33 could not induce CDC against Hep G2 and HuH-7 remains unclear, these results suggest that the antitumor activity of hGC33 is largely attributable to ADCC and that NK cell–mediated ADCC is one possible mechanism of the antitumor effects in human. Nevertheless, we cannot rule out the possibility that Fc-mediated mechanisms other than ADCC are also involved in the antitumor activity of hGC33.

GPC3 expression is up-regulated in the majority of the HCC, colorectal carcinomas, and embryonal tumors, such as neuroblastoma, medulloblastoma, and Wilms' tumors. On the other hand, it is suppressed in other types of tumors and cells, such as breast cancer cells, ovarian cancer cells, and malignant mesothelioma cells, presumably due to the hypermethylation of DNA in its promoter region (5–7, 27). In addition, overexpression of GPC3 in certain cell types, such as human breast and ovarian cancer cell lines, causes apoptosis (5, 6, 9). Thus, GPC3 may positively and negatively modulate cell proliferation in a cell type–dependent manner. De Cat and colleagues (20) reported that GPC3 underwent endoproteolytic cleavage within the cysteine-rich domain, and this processing was essential for the apoptosis caused by the GPC3 overexpression. On the other hand, although SK-03 cells, a SK-HEP-1 derivative line transfected with human GPC3 cDNA, expressed a certain amount of GPC3 as determined by flow cytometry, their growth was not significantly different from that of the parent SK-HEP-1 cells. Because the NH_2-terminal region of GPC3 was detected under reduced conditions both in the culture supernatants of these cells and in the blood of the mice transplanted with these tumors (14, 28),[7] GPC3 protein seems to be processed also in SK-03, HuH-7, and Hep G2 cells. Further studies are necessary to understand the growth regulation of these cells from GPC3.

Recently, GPC3 was shown to be involved in atypical multidrug resistance in gastric cancer cells. Although the overexpression of GPC3 alone was not sufficient to confer resistance to mitoxantrone, gastric cells that became resistant to mitoxantrone expressed an increased level of GPC3, and suppression of GPC3 expression by an anti-GPC3 ribozyme not only restored sensitivity to mitoxantrone but also attenuated cross-resistance to etoposide (29). Furthermore, the degree of drug resistance correlated with the level of GPC3 mRNA. Although the molecular mechanism of atypical multidrug resistance by GPC3 remains unclear, the above facts raise the possibility that impairment of the GPC3 function by an anti-GPC3 antibody may also increase the susceptibility of liver cancers to chemotherapeutic agents. Additive antitumor effects from the combination of the anti-GPC3 antibody with doxorubicin, mitoxantrone, and sorafenib may also be a consequence of an increase in the susceptibility of cells to these drugs from the anti-GPC3 antibody.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 5/26/2008; revised 9/19/2008; accepted 9/22/2008.

Grant support: Program of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation; New Functional Antibody Technology Project of the New Energy and Industrial Technology Development Organization; and Special Coordination Fund for Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology (T. Kodama).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank F. Ford for proofreading of the manuscript.

[7] T. Ishiguro, unpublished observation.

References

1. Filmus J, Shi W, Wong ZM, Wong MJ. Identification of a new membrane-bound heparan sulphate proteoglycan. Biochem J 1995;311:561–5.
2. Pilia G, Hughes-Benzie RM, MacKenzie A, et al. Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome. Nat Genet 1996;12:241–7.
3. Cano-Gauci DF, Song HH, Yang, et al. Glypican-3-deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson-Golabi-Behmel syndrome. J Cell Biol 1999;146:255–64.
4. Huber R, Hansen RS, Strazzulo M, et al. DNA methylation in transcriptional repression of two differentially expressed X-linked genes, GPC3 and SYBL1. Proc Natl Acad Sci U S A 1999;96:616–21.
5. Lin H, Huber R, Schlessinger D, Morin PJ. Frequent silencing of the GPC3 gene in ovarian cancer cell lines. Cancer Res 1999;59:807–10.
6. Xiang YY, Ladeda V, Filmus J. Glypican-3 expression is silenced in human breast cancer. Oncogene 2001;20:7408–12.
7. Kim H, Xu GL, Borczuk AC, et al. The heparan sulate proteoglycan GPC3 is a potential lung tumor suppressor. Am J Respir Cell Mol Biol 2003;29:694–701.
8. Powell CA, Xu G, Filmus J, Busch S, Brody JS, Rothman PB. Oligonucleotide microarray analysis of lung adenocarcinoma in smokers and nonsmokers

identifies GPC3 as a potential lung tumor suppressor. Chest 2002;121:6–7S.
9. Gonzalez AD, Kaya M, Shi W, et al. OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell line-specific manner. J Cell Biol 1998; 141:1407–14.
10. Song HH, Shi W, Filmus J. OCI-5/rat glypican-3 binds to fibroblast growth factor-2 but not to insulin-like growth factor-2. J Biol Chem 1997;272:7574–7.
11. Song HH, Shi W, Xiang Y-Y, Filmus J. The loss of glypican-3 induces alterations in Wnt signaling. J Biol Chem 2005;280:2116–25.
12. Taguchi A, Emoto M, Okuya S, et al. Identification of glypican3 as a novel GLUT4-binding protein. Biochem Biophys Res Commun 2008;369:1204–8.
13. Zhu ZW, Friess H, Wang L, et al. Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut 2001;48: 558–64.
14. Sung YK, Hwang ST, Park MK, et al. Glypican-3 is overexpressed in human hepatocellular carcinoma. Cancer Sci 2003;94:259–62.
15. Nakatsura T, Yoshitake Y, Senju S, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003;306:16–25.
16. Midorikawa Y, Ishikawa S, Iwanari H, et al. Glypican-3, overexpressed in hepatocellular carcinoma, modulates FGF2 and BMP-7 signaling. Int J Cancer 2003;103: 455–65.
17. Capurro M, Wanless IR, Sherman M, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003;125: 89–97.
18. Yamauchi N, Watanabe A, Hishinuma N, et al. The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. Mod Pathol 2005; 18:1591–8.
19. Capurro MI, Xiang Y-Y, Lobe C, Filmus J. Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res 2005; 65:6245–54.
20. De Cat B, Muyldermans SY, Coomans C, et al. Processing by proprotein convertases is required for glypican-3 modulation of cell survival, Wnt signaling, and gastrulation movements. J Cell Biol 2003;163: 625–35.
21. Cappuro MI, Shi W, Sandal S, Filmus J. Processing by convertase is not required for glypican-3-induced stimulation of hepatocellular carcinoma growth. J Biol Chem 2005;280:41201–6.
22. Lai JP, Sandhu DS, Yu C, et al. Sulfatase 2 up-regulates glypican 3, promotes fibroblast growth factor signaling, and decreases survival in hepatocellular carcinoma. Hepatology 2008;47:1211–22.
23. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 1991;108:193–9.
24. Sato K, Tsuchiya M, Saldanha J, et al. Humanization of a murine anti-human interleukin-6 receptor antibody comparing two methods for selecting human framework region. Mol Immunol 1994;31:371–81.
25. Dorai H, Mueller BM, Reisfeld RA, Gillies SD. Aglycosylated chimeric mouse/human IgG1 antibody retains some effector function. Hybridoma 1991;10: 211–7.
26. Zhu AX. Development of sorafenib and other molecularly targeted agents in hepatocellular carcinoma. Cancer 2008;112:250–9.
27. Boily G, Z Saikali, Sinnett D. Methylation analysis of the glypican 3 gene in embryonal tumours. Br J Cancer 2004;90:1606–11.
28. Hippo Y, Watanabe K, Watanabe A, et al. Identification of soluble NH_2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res 2004;64:2418–23.
29. Wichert A, Stege A, Midorikawa Y, Holm PS, Lage H. Glypican-3 is involved in cellular protection against mitoxantrone in gastric carcinoma cells. Oncogene 2004; 23:945–55.