Ganglioside G\textsubscript{M3} Overexpression Induces Apoptosis and Reduces Malignant Potential in Murine Bladder Cancer

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ABSTRACT

We demonstrated previously (S. Kawamura et al., Int. J. Cancer, 94: 343–347, 2001) that large amounts of ganglioside G\textsubscript{M3} accumulate in superficial bladder tumor, compared with invasive bladder tumors and that exogenous G\textsubscript{M3} inhibits the invasive potential of tumor bladder cells. To apply the G\textsubscript{M3} overexpression system to bladder tumor therapy, direct evidence for the important role of G\textsubscript{M3} in bladder tumor invasion must be obtained through transfer of the gene responsible for G\textsubscript{M3} overexpression. To determine the most appropriate cancer cell line for elucidating the antitumor effect of ganglioside G\textsubscript{M3} overexpression, the present study examined glycolipid composition, enzyme activity, and mRNA expression of the glycosyltransferases responsible for G\textsubscript{M3} synthesis in the bladder tumor cell lines KK-47, J82, MGH-UI, YTS-1, and MBT-2. A murine bladder carcinoma cell line (MBT-2) was transfected with a G\textsubscript{M3} synthase (lactosylceramide α2,3-N-acetyl sialic acid transferase); sialyltransferase-I; SAT-I) cDNA, because this line does not naturally express G\textsubscript{M3}. Stable transfectants (MBT-2-SAT-I) that overexpressed G\textsubscript{M3} were characterized by a reduced potential for cell proliferation, motility, invasion, and xenograft tumor growth, and an increase in the number of apoptotic cells. In the proportion of synthetic S phase cells did not differ between MBT-2-SAT-I and mock-transfectant cells. These results suggest that the decreased proliferative potential related to G\textsubscript{M3} overexpression was attributable to the increased number of apoptotic cells. Although details of the mechanism of apoptosis remain unclear, the overexpression of G\textsubscript{M3} by gene transfer of SAT-I may present a novel therapeutic modality.

INTRODUCTION

The bladder is the most common site of urinary tract cancer. An estimated 50,500 new patients were diagnosed with bladder cancer in the United States during 1995 (1). Bladder cancers tend to occur in two principal forms: low-grade superficial tumors and high-grade invasive cancer. Most superficial tumors are papillary, often multifocal, and occasionally progress to invasive disease but have a good prognosis. Most invasive tumors are nodular, metastasize during the early phase, and have a poor prognosis (2). Although recent advances in chemotheraphy of invasive bladder tumors have improved the survival of patients with bladder tumor, the prognosis of invasive bladder tumor is still poor, and the therapy causes side effects. A novel therapeutic modality that could overcome these concerns is required.

Carbohydrates are major components of the cell membrane that undergo significant fluctuations in quantity and quality during differentiation and malignant transformation (3–5). Several glycosyltransferase genes have been cloned and gene-targeting technology has demonstrated the in vivo function of products of individual glycosyltransferases (6). The remodeling of cell surface carbohydrate structures by the gene transfer of specific glycosyltransferases has also demonstrated the important roles of carbohydrates in tumor metastasis (7, 8).

Among the various species of carbohydrates, gangliosides that are in general of low toxicity often have pronounced antiproliferative and differentiation-inducing properties (9). Ganglioside G\textsubscript{M3} shows promise as a novel antitumor agent against human brain tumors (10).

We indicated that a quantitative change of G\textsubscript{M3} expression in bladder tumors is a biochemical parameter associated with growth and invasiveness (11). Moreover, exogenous G\textsubscript{M3} inhibits bladder cancer cell invasion (12). Locally injected G\textsubscript{M3} also inhibits murine MBT-2 bladder tumor invasion and growth (11).

To apply the G\textsubscript{M3} overexpression system as a bladder cancer therapy, direct evidence supporting the notion that G\textsubscript{M3} overexpression has an antitumor effect is required, such as from the gene transfer of a cDNA of the glycosyltransferase responsible for G\textsubscript{M3} synthesis. Here, we present evidence showing that G\textsubscript{M3} synthase gene transfer has an antitumor effect on the murine bladder cancer MBT-2.

MATERIALS AND METHODS

Cell Lines. KK-47, J82, MGH-UI, and YTS-1 are human bladder tumor (transition cell carcinoma) cell lines. KK-47 was provided by Dr. Takashi Tatsuko (Department of Hygienic Chemistry, Department of Pharmacology, Tohoku University, Sendai, Japan). YTS-1 was a gift from Dr. Hiroshi Kakizaki (Department of Urology, Yamagata University, Yamagata, Japan). J82 and MGH-UI were obtained from the American Type Culture Collection. MBT-2 is mouse transitional cell carcinoma cell line induced by the carcinogen N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT; Ref. 13). MBT-2 cells resemble their human counterpart both grossly and histologically and have been used in an animal model to evaluate the efficacy of antitumor drugs in bladder cancer. MBT-2 was a gift by Dr. Katsunori Uchida (Department of Urology, Tsukuba University, Tsukuba, Japan). These cell lines were maintained in RPMI 1640 containing 10% FCS in a humidified 5% CO\textsubscript{2} atmosphere at 37°C.

Glycolipid Extraction and TLC. Glycolipids were extracted as described previously (11, 12). Briefly, total glycolipids were extracted from 300 μl of packed cells in chloroform/methanol [2:1, 1:1, and 1:2 (v/v)] followed by isopropanol/hexane:water [55:25:20 (v/v/v)]. After partition, the upper phase was dialyzed and separated into ganglioside and upper neutral fractions by DEAE Sephadex A 25 column chromatography. The lower neutral fraction was purified by acetylation and Florisil column chromatography. Samples of equivalent wet weight were applied to high-performance TLC plates (Baker, Philipsburg, NJ). The upper neutral and ganglioside phases were separated by chromatography in a solvent system of chloroform/methanol: 0.5% aqueous CaCl\textsubscript{2} [60:40:9 (v/v/v)], and the lower phase in chloroform/methanol:water [60:25:4 (v/v/v)]. The colorizing agent was orcinol-sulfuric acid.

Assays for Glycosyltransferase Activity. G\textsubscript{M3} and G\textsubscript{D3} synthase activities were assayed with modifications as described previously (11). Briefly, samples were homogenized with cell suspension solution [15 mM sodium cacydolate (pH 6.5), 5% glycerc, 1× Complete (Boehringer Mannheim), and 0.1% Lubrol], and the supernatants served as enzymatic source. For the G\textsubscript{M3} and G\textsubscript{D3} assay, lactosylceramide and G\textsubscript{M3} were used as acceptor, respectively, and \([\text{14C}]\text{CMP-}\text{N-acetyl neuraminic acid was used as a donor. SepPak C18 cartridge (Millipore) was used for the separation of radiolabeled reaction products. For the measurement of radioactivity of the radiolabeled product, the eluates were}

2 The abbreviations used are: TLC, thin-layer chromatography; SAT-I, lactosylceramide (α2,3-N-acetyl sialic acid transferase, sialyltransferase-I); BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

Received 12/26/01; accepted 4/22/02.

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dried up, developed on TLC with chloroform/methanol/0.5% CaCl₂ (60:40:9, by volume), and visualized with Fuji FLA-2000 Bio-imaging analyzer. All of the assays were carried out in triplicate.

**Northern Blotting.** A 1086-bp open reading frame fragment of the human \( G_{M3} \) synthase (SAT-I; Ref. 14) cDNA was gel-purified, labeled with [\( \alpha^{32} \)P]dCTP, and used as a probe. Total RNA from cultured cells was isolated following a standard protocol.

**Establishment of Stable Transfectant.** A cDNA encoding human SAT-I was subcloned to pMlKHygB vector containing hygromycin B-resistant gene, resulting in pMlK-hSAT-I. MBT-2 cells, which do not express \( G_{M3} \), were transfected with pMlK-hSAT-I using LipofectAMINE (Life Technologies, Inc.) as described in the protocol provided by the supplier. Transfected cells were cultured in RPMI 1640 containing 10% FCS and hygromycin B (300 \( \mu \)g/ml) for 2 weeks; then 20 individual clones were immunocytochemically screened for \( G_{M3} \) expression using the anti-\( G_{M3} \), monoclonal antibody, M2590 (mouse IgM; Japan Biotest Research Institute, Tokyo, Japan). Monodispersed cells were incubated with 10 \( \mu \)g/ml M2590 followed by FITC-conjugated goat-antimouse F(ab'2) fragment specific to mouse IgM (Cappel); and the \( G_{M3} \)-positive stable transfectants, MBT-2-SAT-I-1, -2, and -3 were established.

**Flow Cytometry.** MBT-2-SAT-I and mock-transfectant cells were assessed by fluorescence-activated cell sorting (FACS) analysis after incubations with M2590 followed by incubations with FITC-conjugated secondary antibody. Labeled cells were analyzed by FACS caliber flow cytometry using the CellQuest program (Becton-Dickinson).

**In Vitro Cell Proliferation Assay.** Cells were seeded in 96-well plates at a density of 10\(^3\) cells/ml in RPMI 1640 containing 10% FCS and 300 \( \mu \)g/ml of hygromycin B, and cultured for various periods. Cell proliferation was measured daily in triplicate cultures using a cell-counting kit (Wako Pure Chemical Industries, Tokyo, Japan).

**Tumor Formation in Mice.** Female C3H/He mice (6–8 weeks old) were inoculated with MBT-2-SAT-I and mock transfectants. Cell viability was assessed by trypan blue staining; then 2 \( \times \)10\(^5\) cells exhibiting >95% viability were suspended in 0.1 ml of serum-free RPMI 1640 and s.c. injected into the right hind limb. Tumor size was measured every other day for 10 days, and tumor volume was estimated as:

\[
\text{Tumor volume} = (\text{longest axis}) \times (\text{shortest axis}) \times (\text{height}) \times \frac{\pi}{6}
\]

**In Vitro Motility Assay and Invasion Assay.** Motility was assayed in vitro using Transwell cell culture chambers (Costar, Cambridge, MA; Refs. 11, 15) with some modifications. The top of the upper chamber was sealed with a polyvinylpyrrolidone-free polycarbonate filter, pore size 8 mm. The lower face was covered with 50 \( \mu \)g/ml fibronectin (Biomedical Technologies, Stoughton, MA). Cells (1 \( \times \)10\(^5\)) were plated into the upper chamber and incubated for 2 h, and the lower chamber was filled with serum-free RPMI 1640. Cells that did not migrate through the membrane were removed; then cells on the lower face of the membrane were fixed with methanol and visualized by Giemsa staining. Cells on the lower face were counted under a microscope, and the mean number of 10 fields were plotted. Motility assays were performed in triplicate, and the coefficient of variation values were always within 5%.

**Quantitation of Synthetic S-Phase Cell Fraction.** We evaluated the influence of \( G_{M3} \) overexpression on the cell cycle in S phase using Cell Proliferation ELISA BrdUrd kits (Roche Diagnostics, Mannheim, Germany). This assay is based on a colorimetric immunoassay of cells labeled with BrdUrd (16) and closely correlates with \( [^{3}H] \)thymidine assays.

**Detection of Apoptotic Cells.** Apoptotic cells cultured on cover slips were detected by TUNEL assays using the Tumor TACS In Situ Apoptosis Detection kit (R&D Systems, Minneapolis, MN). One thousand cancer cells were counted in five fields and the ratio (%) of positive cells (apoptotic cells) was determined.

**RESULTS**

**TLC Profile of Bladder Tumor Cell Lines.** A trace level of \( G_{M3} \) was expressed in all of bladder tumor cell lines examined except in KK-47 (data not shown). All of these human cell lines except KK-47 originated from invasive bladder tumors.

**Glycosyltransferase Activities of Bladder Tumor Cell Lines.** Despite the low amount of \( G_{M3} \) expression on TLC in J82, YTS-1, and MGH-UL, \( G_{M3} \) synthase activities in these cell lines were quite high. This is because the expression amount of \( G_{M3} \) can be regulated not only by SAT-I but also by \( G_{M2} \) and \( G_{D3} \) synthase (11). In contrast, MBT-2 cells were deficient in both \( G_{M2} \) and \( G_{D3} \) synthase activities (data not shown).

**Northern Blots.** Although the amount of \( G_{A43} \) synthase (SAT-I) mRNA expression in bladder tumor cell lines was not always consistent with that of \( G_{M3} \) on TLC, the SAT-I mRNA in MBT-2 was not detected (data not shown). These results indicated that the optimal cell line for elucidating phenotypic alteration caused by SAT-I gene transfer would be MBT-2.

**Changes in Phenotypes of MBT-2-SAT-I Cells.** The parent MBT-2 cells as well as mock transfectants were negative, whereas MBT-2-SAT-I cells were positive for anti-\( G_{M3} \) (M2590 staining; Fig. 1, A and B). Changes in the amount of \( G_{M3} \) expression were also confirmed by TLC (Fig. 1C). Morophologically, MBT-2 cells, as well as mock transfectants, are spindle shaped with long spicular protrusions, but MBT-2-SAT-I cells became cuboid (Fig. 2). The growth of...
MBT-2-SAT-I cells was remarkably slower than that of mock transfectants in vitro (Fig. 3). Motility and invasion potential were significantly suppressed in MBT-2-SAT-I cells (Figs. 4 and 5).

**Tumor Formation in Mice.** Mock transfectants that were s.c. inoculated into mice rapidly proliferated. However, MBT-2-SAT-I cells became smaller each day and impalpable by day 8 (Fig. 6).

**Quantitation of S-Phase Cell Fraction.** To evaluate the influence of G_m3 overexpression on the cell cycle, we quantified the S-phase fractions of cancer cells. The S-phase cell fractions did not differ between mock and MBT-2-SAT-I cells (Fig. 7).

**Detection of Apoptotic Cells.** TUNEL assays revealed that many apoptotic cells were observed in MBT-2-SAT-I, compared with mock transfectants (Fig. 8).

**DISCUSSION**

Invasion in bladder cancer represents the capability of a tumor to behave aggressively and lead to a life-threatening event. The ability to invade is attributable to the complement of biochemical pathways in tumor cells, as well as their inability to preserve or restore normal mucosal architecture. Although recent advances in molecular biology have deepened the understanding of the mechanism in bladder cancer initiation and its subsequent progression (17), epigenetic mechanisms that directly affect biological behavior of bladder cancer still remain unclear.

We determined the glycolipid composition of human bladder cancer cells and found a massive and specific accumulation of G_m3 in superficial papillary tumors (11). Exogenous ganglioside modulates the growth of many cell types (18); we found also that when G_m3 was added to the culture medium of the human bladder cancer cell lines KK-47 and T-24, invasive activity decreased (11). This antitumor effect of exogenous G_m3 has also been demonstrated in syngeneic xenografts of the murine bladder cancer, MBT-2 (12). We, therefore, hypothesized that the G_m3 overexpression could be a novel bladder tumor therapy.

Such application of GM3 overexpression should be supported by
direct evidence of its antitumor effect in a reconstituted system using gene transfer of glycosyltransferase responsible for $G_{M3}$ synthesis. MBT-2 is an excellent model of human invasive bladder tumors because of its aggressive properties (13) and glycolipid profile (12). As we demonstrated previously, the expression level of $G_{M3}$ can be regulated not only by SAT-I but also by $G_{M2}$, $G_{D3}$, and CTH ($G_{M3}$) synthases (11). In the present study, the enzymatic background of $G_{M3}$ and $G_{D3}$ synthases and the mRNA expression also supported the notion that MBT-2 is an excellent model for evaluating functional and phenotypic alterations caused by $G_{M3}$ overexpression. If MBT-2 has $G_{D3}$ synthase activity, $G_{M3}$ accumulation by gene transfer could be interfered with by the conversion of $G_{M3}$ to $G_{D3}$.

Various biological activities are associated with $G_{M3}$. The growth of HL-60 and U937 was significantly inhibited by $G_{M3}$, and they morphologically matured along monocytic lineage (19). The mechanism of this morphological change in MBT-2-SAT-I cells caused by $G_{M3}$ overexpression is unclear, but it may be the same as that in HL-60 and U937.

To understand the mechanism of inhibited motility and invasion potential associated with $G_{M3}$ overexpression, functional support of $G_{M3}$ in fibronectin-integrin interaction should be noted (11, 12, 20). The adhesion of FUA169 cells (a mouse mammary carcinoma mutant cell line) to fibronectin requires the presence of $G_{M3}$, which supports the function of the $\alpha 5\beta 1$ integrin receptor (20). Within an optimal concentration of $G_{M3}$, liposome adhesion to fibronectin-coated plates was significantly enhanced, whereas $G_{M3}$ concentrations above or below the optimal range decreased adhesion (20). The $G_{M3}$ concentration of mock transfectants in the present study may have been optimal or excessive in MBT-2-SAT-I cells for fibronectin-mediated cell attachment. Secondly, $G_{M3}$ has been identified as a cofactor of CD9 in regulating tumor cell motility in that CD9, complexed with $G_{M3}$, inhibits motility (21). CD9 was originally discovered as a motility-regulatory membrane receptor (22). Our previous data also indicated that $G_{M3}$ expression is significantly reduced in the invasive bladder cancer cell lines YTS-1 and J82, whereas CD9 is equally expressed in invasive and noninvasive cell lines (23). We assume that reduced $G_{M3}$ expression causes the loss of a cofactor required for the CD9-dependent inhibition of motility.

Apoptosis is also induced by $G_{M3}$ in thymocytes (24) and in proliferating neuronal cells (25). The cellular mechanisms through
which G\textsubscript{D3} induces apoptosis may be Fas mediated (27). G\textsubscript{D3} induces the mitochondrial permeability transition, and this event precedes apoptosis (26). Similar effects may be induced by GM\textsubscript{3} ganglioside.

Our studies showed that neither exogenous (11) nor endogenous GM\textsubscript{3} overexpression caused by gene transfer affected the S-phase cell population in cultured bladder tumor cell lines. The major focus of cisplatin activity is at the growth (G\textsubscript{1}) and S phases. Because cisplatin-based chemotherapy is a major therapeutic modality for invasive bladder tumors (28), the conserved S-phase cell fraction with GM\textsubscript{3} overexpression is beneficial for combination therapy with conventional chemotherapy using cisplatin.

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