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Novel sugar-cholestanols as anticancer agents against peritoneal dissemination of tumor cells

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Abstract Chemically synthesized sugar-cholestanols with mono-, di-, and tri-saccharides attached to cholestanol showed strong inhibiting activity against the proliferation of colorectal and gastric cancer cells. In contrast, cholestanol without sugar moieties was totally ineffective. Furthermore, when cancer cells were exposed to GlcNAcRβcholestanol (R=(−) or β1-3Gal), the compound was rapidly taken up via the lipid rafts/microdomains on the cell surface. The uptake of sugar-cholestanol in mitochondria increased gradually and was followed by the release of cytochrome *c* from mitochondria and the activation of apoptotic signals through the mitochondrial pathway and the caspase cascade, leading to apoptotic cell death, characterized by DNA ladder formation and nuclear fragmentation. Additionally, the examination of GlcNAcRβcholestanol in a mouse model of peritoneal dissemination showed a dramatic reduction of tumor growth ($P<0.003$) and prolonged mouse survival time ($P<0.0001$). Based on these observations, we believe that the sugar-cholestanols described here have clinical potential as novel anticancer agents.

Keywords Sugar-cholestanols · Apoptosis · Peritoneal dissemination · Anticancer agent · Colorectal cancer

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Abbreviations

GlcNAc	*N*-acety-D-glucosamine
Gal	D-galactose
Fuc	L-fucose
Glc	D-glucose
GalNAc	*N*-acetyl-D-galactosamine
NeuAc	*N*-acetyl-neuraminic acid
Chol	cholestanol
Cer	ceramide
HPβCD	hydroxypropyl-β-cyclodextrin
Apaf-1	apoptotic protease-activating factor 1
PARP	poly (ADP-ribose) polymerase
FCS	fetal calf serum
HPLC	high performance liquid chromatography
DMSO	dimethylsulfoxide
CPI	cell proliferation inhibition
PBS	0.01 M phosphate-buffered saline, pH 7.0
SLX	sialyl Le^X
Bn	benzyl
PN	*p*-nitrophenyl

Introduction

Tumor cells aberrantly express various glycoconjugates on their surface to control different aspects of tumor behavior, such as proliferation, invasion, angiogenesis and metastasis [1–6]. Accordingly, the properties of aberrant glycoconjugates should guide the design of therapeutic interventions for the treatment of malignancy. This approach has led to significant advances in the diagnosis and treatment of cancer, particularly in patients in early stages of the disease. However, patients in advanced stages of cancer are still exposed to a significant risk of a recurrence after curative resection of tumors.

Peritoneal dissemination is a major form of recurrence in patients with advanced stages of gastric, colorectal, ovarian, and pancreatic cancers for which successful treatment awaits further progress [7–11]. Peritoneal dissemination reflects the behavior of cancer cells. In the most basic process, tumor cells exfoliate from the tumors to the abdominal cavity, adhere to the surface of the peritoneum to invade the basement membrane, and, in particular, adhere to the greater omentum and mesenterium [12–14]. It might be appropriate to say that the occurrence of peritoneal dissemination depends on the presence of invisible dissemination or micrometastasis at the time of surgery in at least half of the patients receiving curative operations whose tumors had already invaded up to the serosal surface [12–14].

Cell surface glycoconjugates have an essential role in cell-to-cell adhesion. Accordingly, cell surface glycoconjugates are involved in adhesion as well as the invasion and metastasis of cancer cells that lead to peritoneal dissemination [3, 7–10]. Based on this understanding, we recently developed anti-adhesion molecules consisting of chemically synthesized sugars against the peritoneal dissemination of cancer cells [12–14]. Likewise, using chemically synthesized oligosaccharides consisting of sugar moieties attached to hydrophobic aglycones, we found that cancer cells could utilize such oligosaccharides as substrates for cellular fucosyltransferases and this resulted in the suppression of tumor-specific fucosylated antigen expression along with cell-mediated priming of the oligosaccharides and an increase in the susceptibility of cancer cells to anticancer agents [5]. The same priming effect of chemically synthesized disaccharide attached to naphthalenemethanol was also reported on the suppression of the formation of the sialyl Le^X (SLX) antigen in cancer cells leading to the inhibition of selectin-mediated cell adhesion and their metastatic potential [15, 16]. When the concentrations of primers having sugar moieties attached to cholestanol were increased, the viability of cancer cells was dose- and time-dependently reduced [17]. Furthermore, sugar-cholestanols induced the apoptosis of esophageal cancer cells and specifically suppressed the expression of vascular endothelial growth factor A (VEGF-A) [18]. Therefore, the induction of cancer cell death by specific glycoconjugates attached to hydrophobic aglycones suggested that glycoconjugates could be candidates for developing novel anticancer medicines.

In this study, we synthesized a series of sugar-cholestanols as well as other sugar-sterols and tested their effectiveness against cancer cell proliferation. We were equally interested in learning how these sugar-cholestanols induced apoptotic cell death. All sugar-cholestanols of GlcNAc derivatives showed strong inhibiting effects against various cancer cells by inducing apoptotic cell death. Likewise, for the first time, these sugar-cholestanols were demonstrated to possess strong inhibiting activity against peritoneal dissemination in a mouse model. Taken together, our findings suggest that the sugar-cholestanols described here have clinical potential as a novel anticancer agent.

Materials and methods

Materials

Human gastric (MKN45) and colorectal (colo201, HT-29) and mouse colorectal (colon26) carcinoma cells were obtained from the American Type Culture Collection and cultured in an RPMI1640 medium (Sigma, MO) containing 10% FCS (Gibco RRL, Gaitherburg, MD), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The cell-counting kit was from Dojin (Tokyo). Antibodies against Bcl-2 family, Xiap, the caspase family, and PARP were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Anti-caveolin-1 rabbit antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa Fluor 555-conjugated cholera toxin subunit B and Alexa Fluor 488-conjugated streptavidin were obtained from Invitogen (Carlsbad, CA). Hydroxypropyl-β-cyclodextrin (HPβCD) was from BICO (Yokohama, Japan). GlcNAcβ1-3Gal, GlcNAcβ1-3GalβBn, GlcNAcβ1-3Galβ-naphthalenemethanol, GlcNAcβBn, and GlcNAcβpNP were kindly provided by Dr. K.L. Matta, Roswell Park Memorial Institute, NY, USA. GlcNAcβ1-3Galβ1-1GlcCer was provided by Dr. T. Taki, Otsuka Pharmaceutical, Co., Ltd., Tokushima, Japan.

Sugar-cholestanols, sugar-lanosterol, and sugar-farnesol

3β, 5α-Cholestanyl 2-acetamido-2-deoxy-β-D-glucopyranoside (GlcNAcβChol) [19], 3β, 5α-cholestanyl 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (GlcNAcβ1-3GalβChol) and 3β, 5α-cholestanyl-β-D-galactopyranoside (GalβChol) [20] were synthesized as described

previously. The remaining sugar-cholestanols (Fig. 1, Table 1) were synthesized by the methods described elsewhere (manuscript in preparation); briefly, 3β, 5α-cholestanyl β-D-glucopyranoside (GlcβChol) [19] and 3β, 5α-cholestanyl 3-*O*-(α-L-fucopyranosyl)-β-D-galactopyranoside (Fucα1-3GalβChol) [21] were synthesized according to the literature. 3β, 5α-Cholestanyl 6-*O*-(2-acetamido-2-deoxyl-β-D-gluco-pyranosyl)-β-D-galactopyranoside (GlcNAcβ1-6GalβChol) and 3β, 5α-cholestanyl 2-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (GlcNAcβ1-2GalβChol) were synthesized by the same method as described previously [20] using corresponding glycosyl donors and cholestanol. 3β,5α-Cholestanyl 3-*O*-{2-acetamido-2-deoxyl-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl}-β-D-galactopyranoside (Galβ1-4GlcNAcβ1-3GalβChol), 3β,5α-cholestanyl 3-*O*-{2-acetamido-2-deoxyl-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl}-β-D-galactopyranoside (Galβ1-3GlcNAcβ1-3GalβChol), 3β, 5α-cholestanyl 4-*O*-(2-acetamido-2-deoxyl-β-D-glucopyranosyl)-β-D-galactopyranoside (GlcNAcβ1-4Galβ Chol), 3β,5α-cholestanyl 3-*O*-(β-D-glucopyranosyl)-β-D-galactopyranoside (Glcβ1-3Galβ-Chol), 3β-lanostenyl 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (GlcNAcβ1-3Galβlanosterol) and (2*E*, 6*E*)-3,7.11-trimethyldodeca-2,6,10-trienyloxy 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (GlcNAcβ1-3Galβfarnesol) were synthesized by glycosylation between corresponding di- or tri-saccharide (glycosyl chlorides) and alcohols using AgOTf as an activator and several functional group transformations. 3β, 5α-Cholestanyl 3-*O*-(2-biotinoylamino-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (GlcN(biotin) β1-3GalβChol) was synthesized by the method described previously [22] using biotin-NHS.

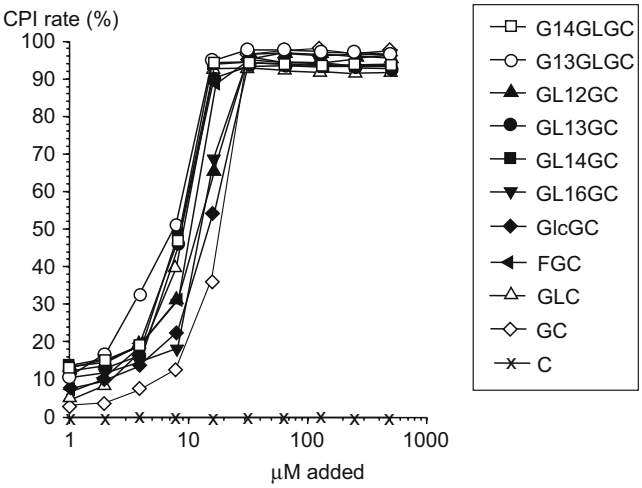

Fig. 1 Effect of various sugar-cholestanols consisting of mono-, di-, and tri-saccharide attached to cholestanol on colon26 cells proliferation. Colon26 cells (1×10^4) preincubated overnight were treated with various concentrations (1–500 μM) of sugar-cholestanols for 72 h. Cell proliferation inhibition (*CPI*) rate was calculated in each concentration and plotted as an average of triplicate tests. The structures of each compounds are shown in Table 1

Table 1 Cell proliferation inhibition of colon26 cells by various sugar-cholestanols

Compound	Abbreviation	CPI_{50} (μM)[a]
Galβ1-4GlcNAcβ1-3Ga1βChol	G14GLGC	8.57±2.28
Galβ1-3GlcNAcβ1-3Ga1βChol	G13GLGC	8.13±2.05
G1cNAcβ1-2GalβChol	GL12GC	11.38±0.39
G1cNAcβ1-3GalβChol	GL13GC	8.97±2.65
G1cNAcβ1-4GalβChol	GL14GC	8.29±1.71
G1cNAcβ1-6GalβChol	GL16GC	12.16±2.34
G1cβ1-3GalβChol	GlcGC	17.16±4.73
Fucα1-3GalβChol	FGC	10.38±2.62
G1cNAcβChol	GLC	9.17±0.14
Ga1βChol	GC	19.62±2.62
βChol	C	>1,000

[a] Concentration (μM) giving a 50% inhibition of cell proliferation

Preparation of inclusion complexation of sugar-cholestanols with cyclodextrin

Sugar-cholestanols are insoluble in aqueous solvents. To overcome this limitation, the compounds (up to 10 μmol/mL) were mixed with 40% HPβCD and stirred at 80°C for 30 min. The clathrate was then filtered through a 0.2 μm pore size membrane and then lyophilized and stored until use. The lyophilized preparations could be dissolved in distilled water for further experiments. GlcNAcβ1-3Galβ1-1GlcCer, GlcNAcβ1-3Galβ attached to cholesterol, lanosterol, farnesol, and naphthalene methanol were prepared with a similar procedure.

Cell proliferation inhibition assay

A cell proliferation inhibition (CPI) assay against various cancer cells was conducted in the presence of serially diluted HPβCD containing sugar-cholestanol and other sterols. Fifty microliters of a cell suspension (1×10^4) was seeded into each well of a 96-well microtiter plate (Falcon, Franklin Lakes, NJ) and incubated at 37°C overnight. Fifty microliters of an FCS-free RPMI medium containing various amounts of sugar-cholestanol or other sterols was then added to each well, and the plate was incubated at 37°C for 48 h. Cell viability was determined with the aid of a cell-counting kit according to the manufacturer's instructions. The cell proliferation rate was determined by measuring the absorbance of the cells at

450 nm with the reference wavelength at 650 nm [5]. The CPI rate was calculated according to the following formula.

$$\mathrm{CPI}(\%) = (1 - \text{optical density of the treated cells} / \text{optical density of the untreated cells}) \times 100$$

Each sample was assayed in triplicate.

DNA ladder and nuclear fragmentation assays

Cells (approx. 1×10^7) that had been treated with sugar-cholestanols for 16 h were harvested and washed in cold PBS. DNA was extracted using a DNA extraction kit (Stratagene) according to the manufacturer's instructions. Twenty micrograms of DNA extracted from the cells was applied to a 2% gel and stained with ethidium bromide. Similarly, for the nuclear fragmentation assay, cells (approx. 1×10^6) that had been treated with sugar-cholestanols for 16 h were harvested and fixed in 1% glutaraldehyde for 30 min at room temperature. After washing with 1 mL of 0.01 M phosphate-buffered saline, pH 7.0 (PBS), the fixed cells were suspended in 20 μL of PBS and 1 μL of Hoechest 33258 (Dojin, Tokyo) dissolved in PBS was added. The cells were then mounted and examined by fluorescent microscopy.

LC-MS analyses of sugar-sterols

LC/MS analyses of sugar-cholestanols were carried out using an LC/MS System, which consists of a Nanospace SI-2 (Shiseido, Tokyo) and a 4000QTRAP LC/MS/MS System, and a hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada) equipped with an atmospheric pressure chemical ionization source. HPLC on an XBridge C_{18} column (2.1×50 mm, 2.5 mm, Waters) was carried out at a flow rate of 0.25 mL/min and a column temperature of 40°C using running buffers formed by gradient solutions of water and acetonitrile, *i.e.*, 40% acetonitrile (0 to 2 min), 40 to 90% acetonitrile (2 to 3 min), 90% acetonitrile (3 to 6 min), 90% acetonitrile (6 to 8 min at a flow rate of 0.3 mL/min), 100% acetonitrile (8 to 9 min), and 40% acetonitrile (9 to 15 min) and then applying the initial condition. Samples separated by HPLC were then scanned under the selected ion monitoring condition in the negative-ion mode. The monitor ions of sugar-sterols (*m*/*z*) were determined as GlcNAcβ1-3Galβ-Chol (752.5), GlcNAcβChol (590.4), GlcNAcβ1-3Galβ-lanosterol (790.5), and digitoxin (internal standard, Sigma; 763.4). The sugar-sterols dissolved in *N*, *N*-dimethylformamide (DMF) and prepared with successive dilutions using DMF to obtain standard solutions with concentrations in the range of 0.25 to 50 μg/mL. After the addition of a given amount of mouse serum (Kitayama Lab., Nagano, Japan) or a culture medium containing 10% FCS, all test samples were deproteinized with acetonitrile and the supernatant obtained after centrifugation was analyzed. Standard curve fittings were accompanied with an Analyst Software (ver. 1.4.1, Applied Biosystem/MDS SCIEX). The concentration of each sugar-sterol was measurable from 0.1 to 20 μg/mL (r>0.99).

Isolation of detergent-insoluble membrane fractions

Detergent-insoluble membrane fractions were isolated from colon26 cells that had been treated with sugar-cholestanols as previously described [23, 24] with slight modification. Cells (approx. 5×10^6) that had been treated with sugar-cholestanols were washed twice in an ice-cold RPMI 1640 medium, scraped with 2 mL of a TNE buffer containing 20 mM Tris–HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 10 μg/mL aprotinin, 2% sucrose, and 0.5% Triton X-100, and then mixed vigorously. After centrifugation at 400×g for 5 min, the supernatant was mixed with 80% sucrose/0.15M NaCl to make a 40% part of a discontinuous sucrose gradient (total 15.5 mL) from 40 (4 mL), 35 (4 mL), 15 (2.5 mL), 5% (2.5 mL) and, DW (2.5 mL). After centrifugation at 146,000×g for 24 h, 16 fractions (1 mL/tube) based on the sucrose density were collected with the aid of a Piston Gradient fractionator (Biocomp model 152, BioComp Instruments Inc., New Bunswick, Canada) following the original method [25].

Western blot analyses and immunofluorescence labeling of caveolin-1 and G_{M1}

Western blot analyses and immunofluorescence labeling of caveolin-1 and G_{M1} in the detergent-insoluble cell fractions were conducted as follows. The distribution of caveolin-1 and G_{M1} as markers of lipid rafts/microdomains in the fractions obtained from the aforementioned ultra-centrifugation of cells was detected by Western blotting after electrophoresis on a 10–20% SDS-PAGE, and electroblotting was conducted on a nitrocellulose membrane using the anti-caveoline-1 antibody and the Alexa Fluor 555-conjugated cholera toxin B subunit. The chemiluminescence signals from the latter were detected using a Laser-base imaging system for fluorescent imaging (Typhoon 9400, Amersham Biosciences, Uppsala, Sweden). For fluorescence microscopy, cells were fixed with 4% paraformaldehyde in PBS for 30 min at 4°C [23] after being exposing to a biotinylated sugar-cholestanol, GlcN(biotin)-β1-3Galβcholestanol in place of GlcNAcβ1-3 Galβcholestanol. The cells were then incubated with Alexa Fluor 488-conjugated streptavidin and Alexa Fluor 555-conjugated cholera toxin subunit B and then examined with a Leica DMI6000B fluorescence microscope equipped with an appropriate set of filters for detecting biotinylated sugar-cholestanol and G_{M1} in the cells.

Isolation of cytosolic and mitochondrial fractions

For the isolation of cytosolic and mitochondrial fractions, cells (approx. 1×10^6) that had been treated with sugar-cholestanols were harvested, and their cytosolic and mitochondrial fractions were isolated following a slightly modified version of the method described in the manufacturer's instructions. After harvesting cells by centrifugation at 600×g for 5 min, 4°C, the pellets were washed in PBS and then re-suspended in 3 vol of a buffer containing 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 mM phenylmethylsulfonylfluoride, 10 μM leupeptin and 10 μM aprotinin in 250 mM sucrose. The cells were disrupted by 50 strokes of a glass homogenizer, and the homogenate was centrifuged at 600×*g* for 10 min, 4°C. The mitochondria from the supernatant were then precipitated by centrifugation at 8,000×*g* for 10 min.

Cytochrome *c* assay

The amounts of cytochrome *c* released in the cells, mitochondria, and culture medium were measured using a Cytochrome *c* ELISA kit (MBL, Nagoya, Japan) following the manufacturer's instructions. The ELISA kit is based on a sandwich ELISA consisting of the anti-cytochrome *c* monoclonal antibody and peroxides-conjugated anti-cytochrome *c* polyclonal antibody. The concentration of cytochrome *c* in each preparation was determined using a standard curve obtained from the standard. Each sample was assayed in triplicate.

Western blot analyses of apoptosis-related molecules

All cells were harvested at approx. 80% confluent growth. The cell lysate was prepared as above, and the protein concentration of the lysate was determined using a BCA protein assay kit (Pierce, Rockfold, IL) with BSA as a standard. Forty micrograms of protein was run on a 5–20% ReadyGel (Bio-Rad, Tokyo) and the gel was electro-transferred to a hybond-enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The expression levels of each molecule were determined using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) with β-actin as a loading control. The levels between treated and untreated cells were compared using the Photoshop software (Adobe) and analyzed using the Quantity software (Bio-Rad).

Mouse model for the peritoneal dissemination of cancer cells

The peritoneal dissemination of cancer cells was investigated in a mouse model [14], and this model was used to study the anticancer actions of sugar-cholestanols. Eight-week-old Balb/c female mice were intraperitoneally inoculated with 5×10^4 of colon26 cells in a volume of 200 μL. One-hundred microliters of HPβCD containing 2 μmol of sugar-cholestanol was administered intraperitoneally three times at 0, 24, and 48 h after inoculation of cancer cells. Mice were sacrificed on day 18, and the weights of tumor modules in the omentum and mesentery were measured. The survival of mice that had been treated with sugar-cholestanol was also monitored with untreated mice as controls.

Statistical analyses

Statistical analyses were performed using the Stat View software (ver.5.0, SAS Institute, Inc., NC).

Results

Effects of sugar-cholestanols on colon26 cancer cell proliferation

The effects of chemically synthesized sugar-cholestanols comprising mono-, di-, and tri-saccharides on the viability of colon26 cells were determined at 1–500 μM of the compound prepared in chrathlate forms using 40% HPβCD (Fig. 1). All sugar-cholestanols showed strong inhibitory activities against the proliferation of colon26 cells over a wide range of concentrations. However, βChol itself without sugar moieties up to 500 μM was completely ineffective. When cell proliferation inhibition (CPI) was compared on the basis of the CPI_{50} (the minimum concentration giving a 50% inhibition of cell proliferation), GlcNAc-derivatives appeared to possess stronger activities in the order of GlcNAcβ1-3(4)>GlcNAcβ1-2>GlcNAcβ1-6 and Glcβ1.3GalβChol or GalβChol seemed to possess weak activities compared with R′GlcNAcβRChol (R=βGal or (−), R′=(−) or Galβ) and Fucα1-3GalβChol (Table 1). When GlcNAcβ1-3Galβ disaccharide attached to lanosterol or farnesol was used for assaying CPI against colon26 cells, the inhibiting activity was found to be very low, and the CPI_{50} values were estimated to be 121.6 or 525.1 μM, respectively (Fig. 2). This indicated that GlcNAcβ1-3Galβ-Chol (CPI_{50}=8.97μM) was 13.6 fold more potent than GlcNAcβ1-3Galβlanosterol. In contrast, nearly equivalent activities were seen in GlcNAcβ1-3Gal disaccharide attached to both cholestanol and cholesterol throughout the range tested. Among the compounds having the same sugar moieties, GlcNAcβ1-3Galβnaphthalenemethanol and GlcNAcβ1-3Galβ1-1GlcCer showed very weak inhibiting activities and GlcNAcβpNP, GlcNAcβ1-3GalβBn,

GlcNAcβ1-3Gal or GlcNAcβpNP, up to 1mM, had no activity against colon26 cells (data not presented).

Morphological changes of cancer cells after treatment with sugar-cholestanols

When gastric and colorectal cancer cells were treated with sugar-cholestanols, morphological changes were clearly seen after 12 h, and cell death was induced in all cancer cells tests. However, no changes were seen in tests treated with βChol or with HPβCD during 72 h (Fig. 3).

DNA ladder formation and nuclear fragmentation

When the colon26 cells were treated with GlcNAcβ1-3GalβChol, or βChol for 16 h, DNA ladder formation was seen only with sugar-cholestanol but not with cholestanol (Fig. 4a,b). Similarly, nuclear fragmentation was clearly observed in colon26 cells under the same treatment with GlcNAcβ1-3GalβChol, but fragmentation did not occur with βChol (Fig. 4c). It was, therefore, obvious that treatment of cancer cells with sugar-cholestanols rapidly induced apoptotic cell death.

Incorporation of sugar-sterols into colon26 cells

Sugar-cholestanols added to the culture medium seemed to be taken up rapidly by colon26 cells, as described above. The amounts of sugar-cholestanols were determined by LC-MS analysis. The levels of GlcNAcβ1-3GalβChol in both the cells and the medium were changed in a time-dependent manner; incorporation into the cells was found to start earlier than 2 h after addition and continue up to 24 h, while the level in the medium decreased during the same period (Fig. 5a). The same distribution profile in both the cells and the culture medium was seen when GlcNAcβChol was added under identical conditions (data not presented). GlcNAcβ1-3Gal-βlanosterol, which had very weak inhibiting activity against the proliferation of colon26 cells was also measured in both fractions (Fig. 5b). Interestingly, the levels of GlcNAcβ1-3GalβChol and GlcNAcβ1-3Galβlanosterol in both fractions seemed to change similarly in a time-dependent manner.

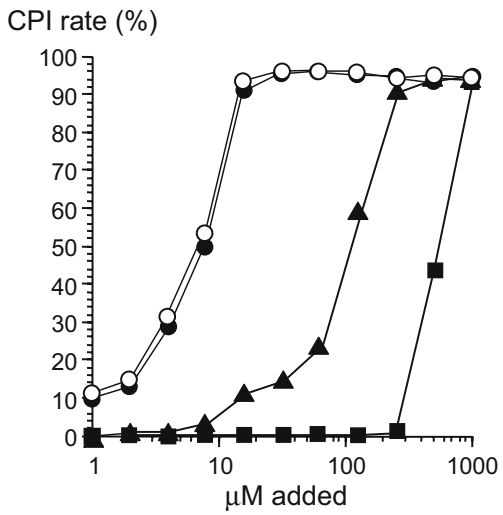

Fig. 2 Effect of various concentrations of GlcNAcβ1-3Galβ attached to different sterols on colon26 cell proliferation. Colon26 cells (1×10^4) preincubated overnight were treated with various concentrations (1–1,000 μM) of sugar-sterols for 72 h. GlcNAcβ1-3Galβcholestanol (*filled circles*), GlcNAcβ1-3Galβcholesterol (*empty circles*), GlcNAcβ1-3Galβlanosterol (*filled triangles*), and GlcNAcβ1-3Galβ-farnesol (*filled squares*). The cell proliferation inhibition (*CPI*) rate was calculated and is plotted as in Fig. 1

Colocalization of sugar-cholestanol and G_{M1} on the surface of colon26 cells

When colon26 cells were exposed to a biotin-labeled sugar-cholestanol, GlcN(biotin)β1-3GalβChol, morphological changes were induced and no clear difference was observed in the inhibiting activities between labeled and non-labeled sugar-cholestanols (data not presented). These findings suggest that sugar-cholestanols have high binding affinity for cellular cholesterol. Cholesterol is known to be distributed irregularly in lipid rafts/microdomains at the cell surface. Therefore, in order to test whether sugar-cholestanol is actively taken up by cells via lipid rafts/microdomains, we examined the localization of sugar-cholestanol and of other lipid rafts/microdomains components, as a function of time. No clear green signal indicating the localization of GlcN(biotin)β1-3GalβChol was seen after 1 h (Fig. 6a), but it appeared in the cell surface after 3 h (Fig. 6b) and throughout the cells after 17 h (Fig. 6c). The localization of G_{M1}, one of the markers for lipid rafts/microdomains detected by the occurrence of a complex with cholera toxin subunit B (in red signal), was clear during the early incubation periods but was distorted after 17 h when morphological changes and apoptotic cell death were seen in part of the cells. During these periods, the colocalization of GlcN(biotin)β1-3GalβChol and G_{M1} was observed only at 3 h, as shown in yellow (Fig. 6b, merged). The distribution of these two molecules changed in a time-dependent manner, suggesting that sugar-cholestanol had been taken up by the cells mainly via the lipid rafts/microdomains.

Sub-cellular fractionation of colon26 cells after treatment with sugar-cholestanol

Detergent-insoluble and detergent-soluble membrane fractions were isolated from colon26 cells after treatment with GlcNAcβ1-3GalβChol by ultra-centrifugation in the presence

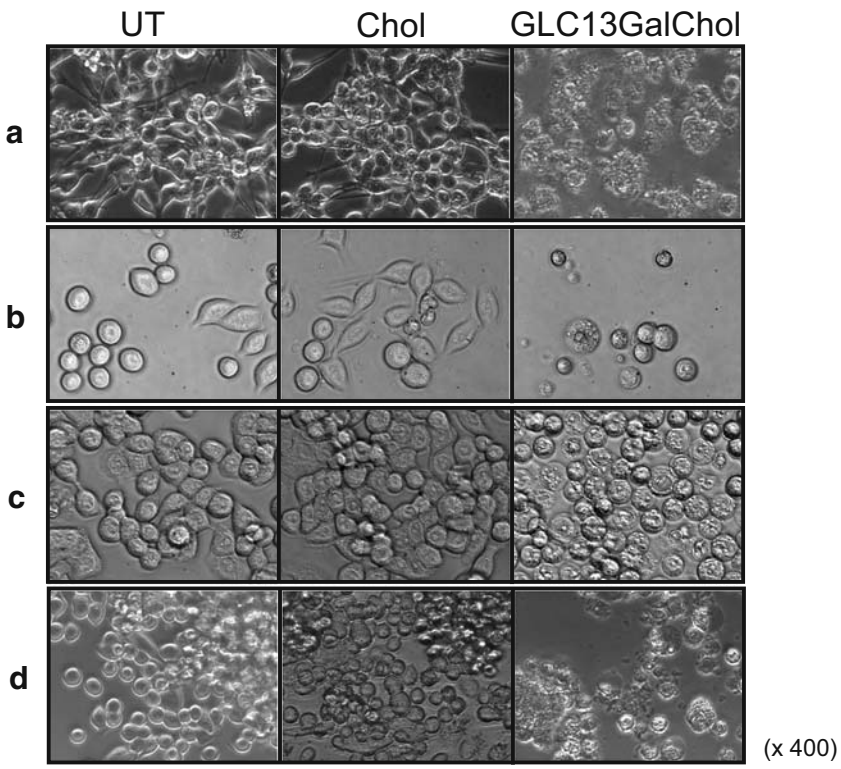

Fig. 3 Morphological changes of colorectal and stomach cancer cells after treatment with GlcNAcβ1-3Galβcholestanol. Cells treated with GlcNAcβ1-3Galβcholestanol and cholestanol (15.6 μM, each) for 3 days were photographed using a phase-contrast microscope (× 400). **a** colon26, **b** colon 201, **c** HT-29 and **d** MKN45 cells

of Triton X-100 and sucrose. Sugar-cholestanol was determined in each centrifugation fraction as shown above (Fig. 7). One major peak appeared in fractions 6 to 8, which were recovered at 15% sucrose density. Western blotting of caveolin-1 and G_{M1} as markers for cholesterol-rich lipid rafts/microdomains was performed in each fraction using anti-caveolin-1 and cholera toxin subunit B (Fig. 7, bottom). Signals for both molecules were found in the same fractions, appearing as two major peaks, which coincided with those of sugar-cholestanol. The first peak (fractions 6 to 8) appeared at a 15% sucrose density and contained small amounts of protein, whereas the second peak (fractions 11 to 14) appeared at a 35% sucrose density and contained a large amount of protein (data not presented). On the other hand, the levels of sugar-cholestanol as well as the two molecules of markers for lipid rafts/microdomains in the latter peak declined to less than 20% of the total amounts when each level was converted to a unit of protein (data not presented). Immunostaining of G_{M1} and/or caveolin-1 in the fixed cells and in the ultra-centrifugation fractions suggested that the sugar-cholestanol, which had been added to the colon26 culture medium, was taken up by the cells mainly via the cell lipid rafts/microdomains.

Release of mitochondrial cytochrome *c* from colon26 cells

As shown above, sugar-cholestanols, which had been added to the culture medium of colon26 cells, were rapidly taken up by the cells. The amounts of sugar-cholestanols were

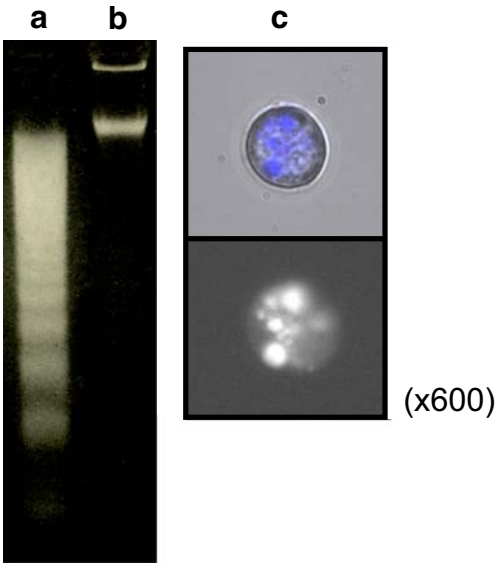

Fig. 4 Occurrence of DNA ladder formation and nuclear fragmentation in colon26 cells after treatment with GlcNAcβ1-3Galβcholestanol. The colon26 cells (1×10^7) were treated with GlcNAcβ1-3Galβcholestanol (**a**) and cholestanol (**b**; 50 μM) for 16 h. Twenty micrograms of DNA extracted from treated colon26 cells was applied to a 2% agarose gel and stained with ethidium bromide after electrophoresis. The colon26 cells (1×10^6) were also treated with GlcNAcβ1-3Galβcholestanol (**c**; 30 μM) for 16 h. Cells fixed with 1% glutalaldehyde were treated with Hoechest 33258 and photographed using a fluorescence microscope (×600)

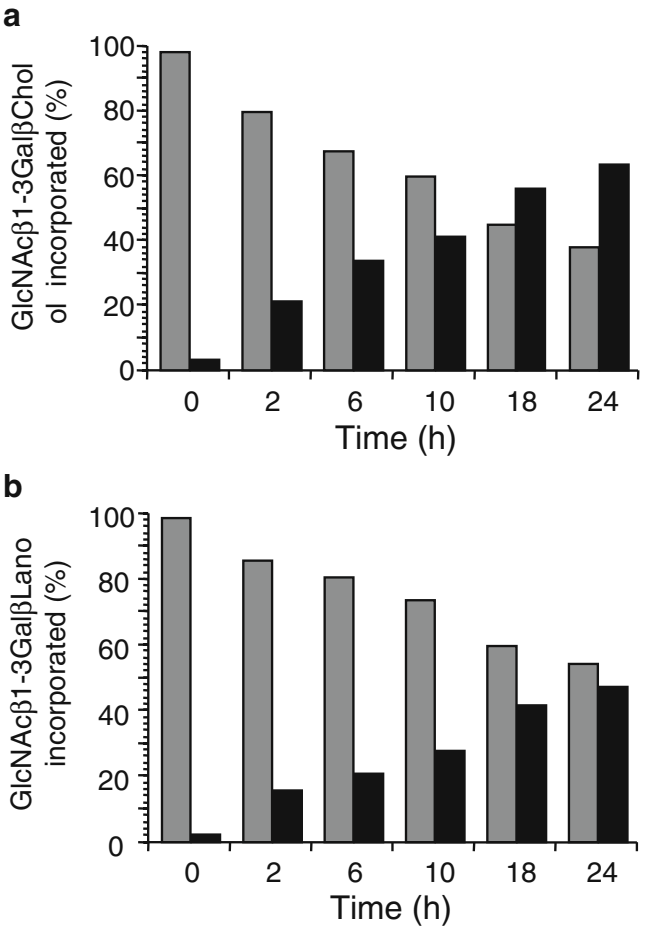

Fig. 5 Levels of GlcNAcβ1-3Galβ attached to cholestanol and lanosterol in culture medium and in colon26 cells. GlcNAcβ1-3GalβChol (**a**, 15 μM) and GlcNAcβ1-3Galβlanosterol (**b**, 20 μM) were added to 10 mL of each culture medium of colon26 cells. The levels of sugar-cholestanol and lanosterol in a culture medium (*gray bars*) and cells (*black bars*) were determined by LC-MS analysis as described in "Materials and methods"

determined in sub-cellular fractions as well after treatment with sugar-cholestanols. The incorporation of GlcNAcβ1-3GalβChol in mitochondria was found to start soon after the addition of the sugar-cholestanol and continued to 20 h (Fig. 8). Interestingly, treatment of the cells with sugar-cholestanol followed the release of cytochrome *c* from mitochondria into the cytoplasm and, at the same time, the levels in the cytoplasm increased up to 24 h (Fig. 9). However, when colon26 cells were treated with cholestanol without sugar moieties, no morphological changes or apoptosis occurred, and no release of mitochondrial cytochrome *c* was observed during the same period.

Expression of Bcl-2 family, caspase-related molecules, and PARP in colon26 cells

Since apoptotic cell death seemed to be induced in cancer cells that had been treated with sugar-cholestanols, in particular, via the activation of mitochondria-dependent pathways, Western blot analyses of colon26 cells that had been treated with sugar-cholestanols were conducted to investigate changes in the expression levels in molecules relating mainly to the death receptor-independent and caspase-dependent pathways (Fig. 10). When the expression levels of molecules in the Bcl-2 family, which are related to the pro- and anti-apoptosis, were analyzed in the colon26 cells that had been treated with GlcNAcβ1-3GalβChol, GlcNAcβChol, and βChol, the expressions of both tBid (15 kDa) and Bax (20.5 kDa) increased in a time-dependent manner under treatment with sugar-cholestanols. Conversely, the expression of anti-apoptotic Bcl-xL (30 kDa) was found to be suppressed, but the levels of Apaf-1 (140 kDa) increased in the same cells under the same condition. Furthermore, a procaspase-9/Apaf-1 complex (52 kDa) seemed to be formed simultaneously, and then the complex processed procapase-9 into a large active fragment (38 kDa) and a small fragment. Most of these changes had been observed only in the cells that had been treated with sugar-cholestanols in a time-dependent manner before 10 h but not during 24 h when the cells had been treated with cholestanol. Analyses of the molecules relating to the caspase cascade indicated that activation of the initiator caspase (caspase-9) leading to activation of the effector caspase (caspase-3) had occurred in the cells in a time-dependent manner when the cells were exposed to sugar-cholestanols. These two caspases could be detected as fragments with 38 and 20 kDa, respectively. Furthermore, the activation of PARP was suggested by an increased level of a large fragment (96 kDa) in the cells that had been treated with sugar-cholestanols. Changes in the expressions of these molecules in the caspase cascade also occurred within 10 h, but not when the same cells were treated with cholestanol.

The anticancer effect of sugar-cholestanol in the peritoneal dissemination model

Balb/c mice were intraperitoneally inoculated with colon26 cells and then administered sugar-cholestanol intraperitoneally three times at 0, 24, and 48 h after inoculation. The weights of the mesenterium and omentum in mice treated with sugar-cholestanols were measured (Fig. 11). Many tumors were formed within 18 days in the omentum and mesentery of untreated mice; however, tumor formation was significantly suppressed, and tumor-free mice were observed in the presence of GlcNAcβ1-3GalβChol ($P=0.0075$) and GlcNAcβChol ($P=0.003$). In addition, significantly increased survival times were found in mice that had been treated with sugar-cholestanol in comparison with those that had not been treated ($P<0.0001$; Fig. 12). The results indicated that sugar-cholestanols had a strong antitumor effect, as seen in the peritoneal dissemination of colon26 cells.

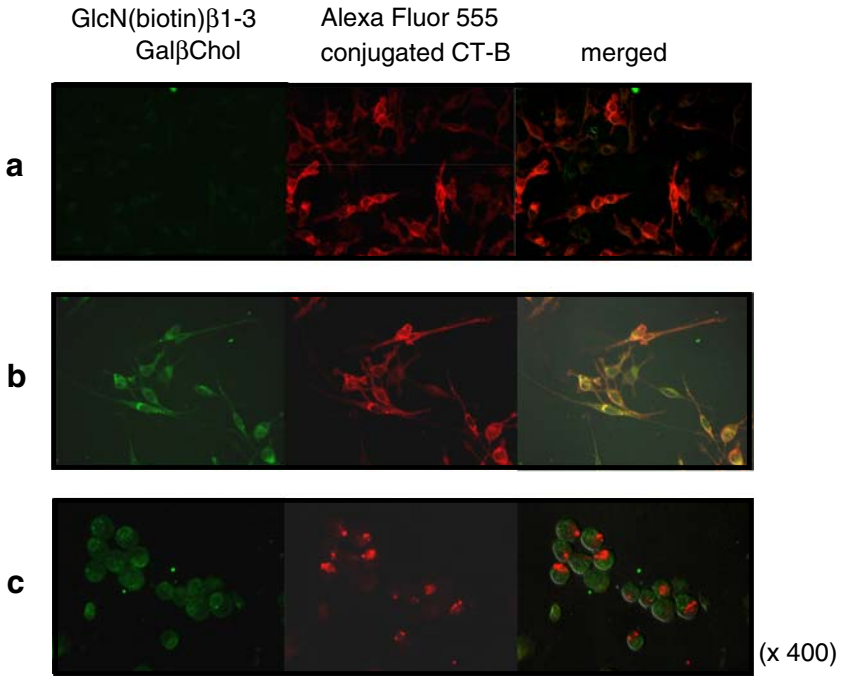

Fig. 6 Colocalization of sugar-cholestanols and G_{M1} on the lipid rafts/microdomains on the surface of colon26 cells treated with GlcN(biotin)β1-3Galβ-Chol. The colon26 cells (1×10^6) were treated with GlcN(biotin)β1-3Galβcholestanol (15 μM) for 1 (**a**), 3 (**b**) and 17 h (**c**). Double immunofluorescence stainings of biotin-labeled GlcN-(biotin)β1-3Galβcholestanol and G_{M1} with Alexa Fluor 488-conjugated streptavidin and Alexa Fluor 555-conjugated cholera toxin subunit B, respectively were conducted as described in "Materials and methods" and photographed using a fluorescence microscope (×400)

Discussion

Sphingolipid metabolites and certain gangliosides are known to have roles in controlling apoptotic cell death in various cancer cell types [26–34]. Similarly, disialogangliosides such as GD3 (NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer) and GD1b (Galβ1-3GalNAcβ1-4[NeuAcα2-8NeuAcα2-3]-Galβ1-4Glcβ1-1Cer) have been shown to induce apoptosis in cancer cells [35, 36]. These findings indicate that certain glycolipids could be novel anticancer agents. In contrast, studies on glycosylation inhibitors, in particular, substrates for intercellular glycosyltransferase-based inhibitors [37–39], have enabled us to develop new approaches for blocking the syntheses of specific glycoconjugates in cancer cells, including tumor-associated glycoconjugates and SLX and sialyl Le^a (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAc), which endow cells with the ability to adhere to selectins present on endothelia, platelets, and leukocytes [15, 40, 41]. It was then demonstrated that the peracetylated disaccharide, GlcNAcβ1-3Gal, attached to naphthalenemethanol was taken up by colorectal cancer cells, deacetylated, and primed by the cells as a substrate for their glycosyltransferases, which mediated the assembly of oligosaccharides, such as the SLX active structure (NeuAcα2-3Galβ1-4[Fucα1-3]

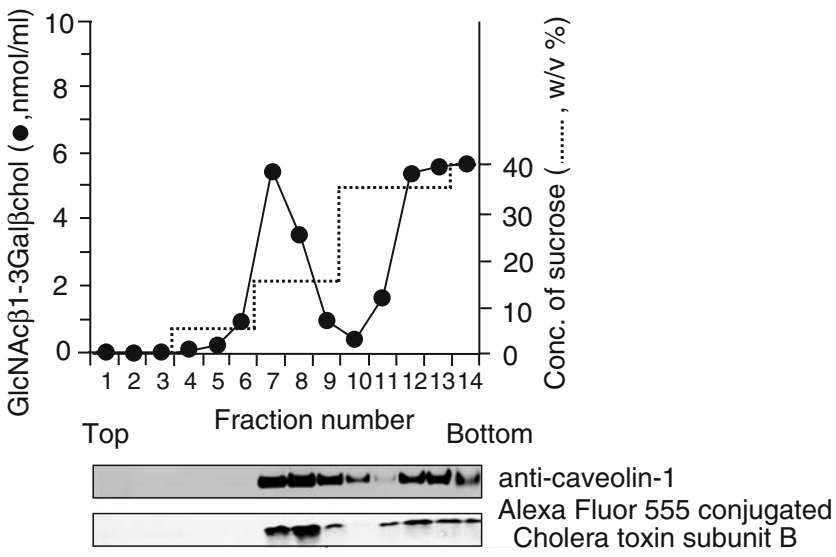

Fig. 7 Sub-cellular fractionation of colon26 cells after treatment with sugar-cholestanol by the ultra-centrifugal method using a sucrose gradient solution and Western blot analysis of caveoline-1 and G_{M1} in each fraction. The colon26 cells (1×10^6) were treated with GlcNAcβ1-3Galβcholestanol (30 μM) overnight and harvested, and sucrose gradient ultracentrifugation was conducted as described in "Materials and methods." The levels of sugar-cholestanol in each fraction were determined by LC-MS analysis (*upper*) and immunodetection of caveoline-1 and G_{M1} in each fraction

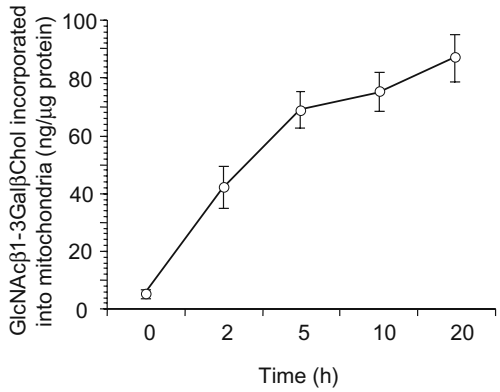

Fig. 8 Levels of GlcNAcβ1-3Galβcholestanol in the mitochondria of colon26 cells after treatment with GlcNAcβ1-3Galβcholestanol. The colon26 cells (1×10^6) were treated with GlcNAcβ1-3Galβcholestanol (15 μM), and the levels of sugar-cholestanol in mitochondria prepared as descried in "Materials and methods" were determined by LC-MS analysis. Values are mean±SD from three experiments at each time

GlcNAcβ1-3Gal), on the disaccharide and then secreted them [15, 41]. This cell-mediated priming of the disaccharide led to a decoy or divert glycosylation away from endogenous glycoconjugates in cancer cells, concomitant reduction of SLX antigen expression on the cell surface, a reduced interaction with selectins and an increased susceptibility to leukocyte-mediated lysis followed by a reduction of the metastatic potential of cancer cells [16].

Recently, we [17, 18] showed that GlcNAcβ1-3GalβChol had the same priming effect on various cancer cells and that sugar-cholestanol could be actively taken up by cancer cells without any change in the sugar structure. Furthermore, the amounts of tumor-associated antigens including α1-2fucosylated antigens and the SLX antigen expressed on the cancer cell surface, could be selectively suppressed, and this enhanced the susceptibility of cancer cells to anticancer treatments [5]. More recently, the same primers were shown to have strong inhibiting actions on the proliferation of cancer cells when the concentrations of the primers were increased [17, 18]. Based on these findings, in the present study, a series of chemically synthesized sugar-sterols was investigated for its cyototoxic activity against various cancer cells. The CPI assay clearly indicated that sugar-cholestanols and sugar-cholesterol have strong cytotoxic effects against several cancer cell lines, including colorectal and gastric cancer cells, in a time- and dose-dependent manner. Among these, sugar-cholestanols with GlcNAc derivatives seemed to be more potent than those with other sugar moieties irrespective of the linkages attached to the sub-terminal residue. Since the sugar moieties without sterols were completely ineffective against the proliferation of cancer cells, the hydrophobic aglycones seemed to be essential for the activity, while the attached sugar moieties appeared to important as well. Although the mechanism of the cytotoxicity of sugar-cholestanols has not been fully understood yet, it is of particular interest that they show stronger activity against various cancer cells than other compounds having the same sugar structure but different aglycones. Furthermore, GlcNAcβ1-3Gal disaccharides with different aglycones, such as cholestanol and lanosterol, were incorporated almost equally into colon26 cells in spite of their CPI_{50} values being different by more than ten times. Presumably, these sugar-sterols in the colon26 cells are fed into different metabolic pathways with the cells. Work on understanding the possible pathways is currently ongoing by using sugar-sterols and colon26 cells.

Measurement of GlcNAcβ1-3GalβChol and GlcNAcβ-Chol with the aid of LC/MS analyses and immunostaining of biotinylated GlcNAcβ1-3GalβChol in cancer cells suggested that the sugar-cholestanols were rapidly taken up by cancer cells. Colocalization of caveoline-1 and G_{M1}, which are the most common markers of cholesterol- and glycolipid-enriched rafts/microdomains on the cell membrane, and biotinylated sugar-cholestanol at an early period of incubation indicated that the sugar-cholestanols were taken

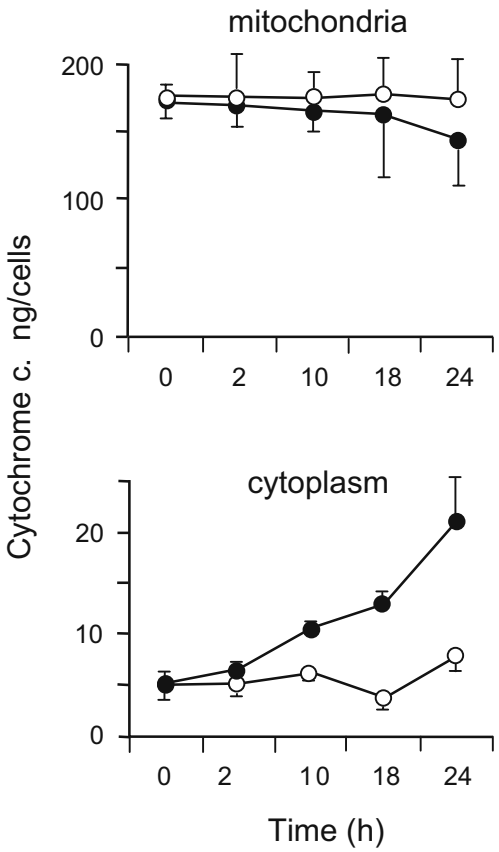

Fig. 9 Levels of cytochrome *c* in the mitochondria and cytoplasm of colon26 cells after treatment with GlcNAcβ1-3Galβcholestanol and βcholestanol. The colon26 cells (1×10^6) were treated with GlcNAcβ1-3GalβChol (*filled circles*) and βChol (*empty circles*; 15 μM each) and levels of cytochrome *c* in the mitochondria and cytoplasm fractionated by the method described in "Materials and methods" were measured by a Cytochrome *c* ELISA kit. Values are mean±SD from three experiments at each time

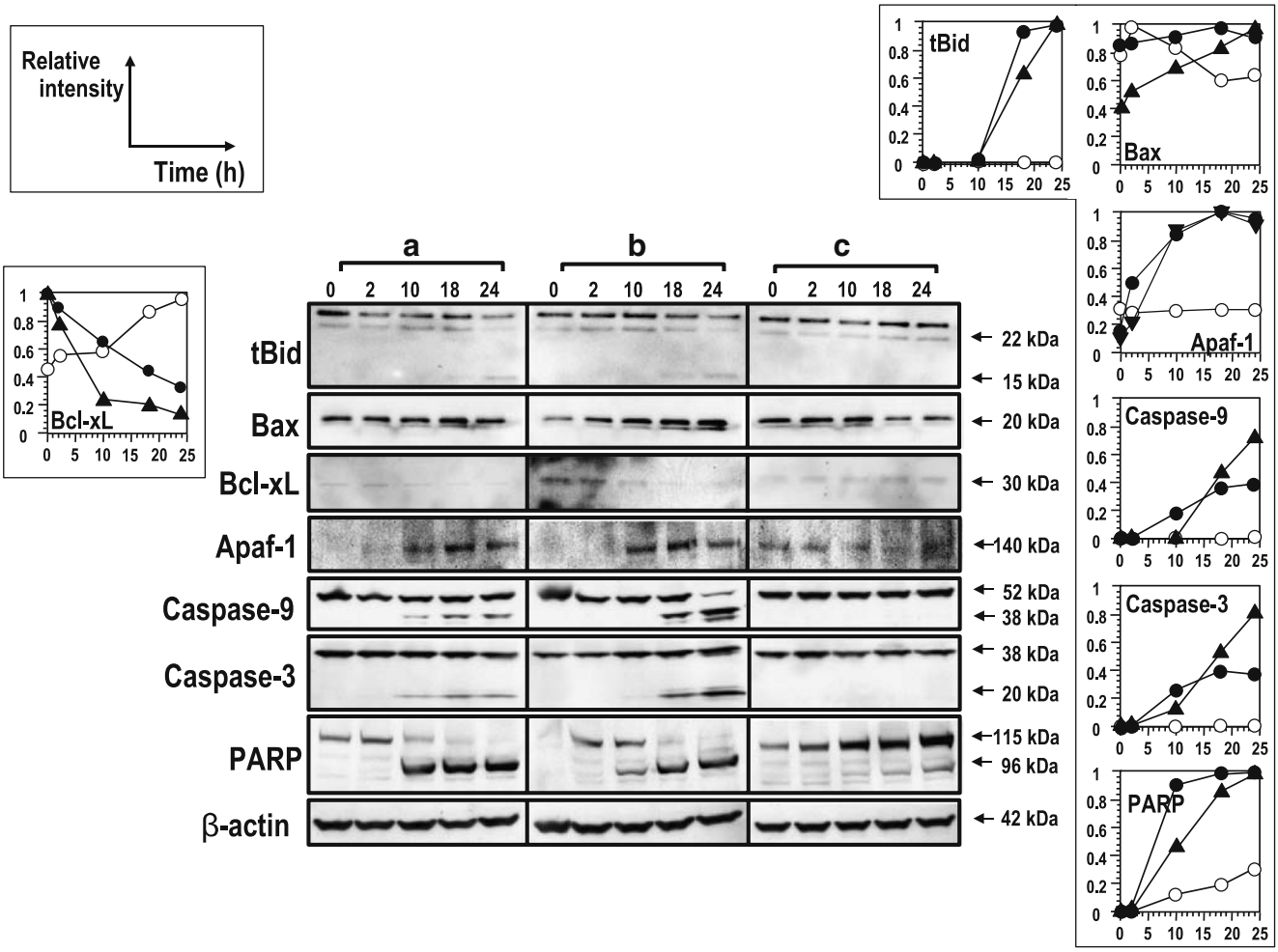

Fig. 10 Western blot analyses of the Bcl-2 family and caspase cascade activation in colon26 cells after treatment with sugar-cholestanols and cholestanol. The colon26 cells (1×10^6) were treated with GlcNAcβ1-3Galβcholestanol (**a**, *filled circles*), GlcNAcβcholestanol (**b**, *filled triangles*) and βcholestanol (**c**, *empty circles*; 15 μM each) for 24 h. Changes in the expression levels of apoptosis-related molecules were analyzed by Western blotting of treated cells as a function of time. The arbitrary densitometric unit of bands measured in each molecule is plotted in the *left* (down-regulated with sugar-cholestanols) and *right* (up-regulated with sugar-cholestanols) *figures* with time (*transverse*) and relative intensity (*vertical*)

up by the cells primarily via the lipid rafts/microdomains. Since cholesterol is abundant in the microdomains [42], it is very likely that sugar-cholestanols and/or sugar-cholesterol readily passes through the lipid rafts/microdomains.

The sugar-sterols used in this study are hardly soluble in water and are available as cyclodextrin clathrate- and liposome-encapsulated [17] forms to investigate their inhibiting activities on the proliferation of various cancer cells. Cyclodextrins have been used as vehicles to deliver hydrophobic drugs because chemical modification of hydrophobic groups on cyclodextrins enhanced their solubility in water and the ability to dissolve hydrophobic compounds [43, 44]. It has been demonstrated that cholesterol is readily depleted from lipid rafts/microdomains on the cell membranes with the aid of cyclodextrins including methyl-β-cylodextrin, followed by induction of apoptosis [43]. HDβCD has also been used to promote chemical efflux from cells, although such an effect was found to be weak compared with methyl-β-cyclodextrin [45]. However, in this study, HPβCD clathrate cholestanol did not show any cytotoxic activity against cancer cells. Since HPβCD without any clathrate sugar-cholestanols did not have any inhibiting activity against the proliferation of cancer cells, it is valid to assume that the apoptotic cell death observed in this study was not induced by HPβCD.

Recently, we found that sugar-cholestanols containing GlcNAcβ1-3GalβChol and GlcNAcβChol had strong cytotoxicity against esophageal cancer cells and that, surprisingly, non-cancerous esophageal cells responded poorly to the same treatment [18]. In contrast, in this study, sugar-cholestanols composed of GlcNAc derivatives including the same compounds showed strong inhibiting activities against the proliferation of various gastric and colorectal cancer cells in a dose- and time-dependent manner irrespective of their sugar structures. No significant difference in their inhibiting activities was seen even

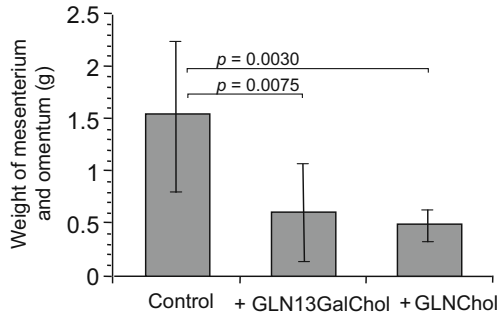

Fig. 11 Anticancer effect of sugar-cholestanols on the peritoneal dissemination of cancer cells. Balb/c mice were intraperitoneally inoculated with 5×10^4 of colon26 cells, and 100 μL of 2 μmol of GlcNAcβ1-3Galβcholestanol (+*GLN13GalChol*), GlcNAcβcholestanol (+*GLNChol*) or PBS only (*Control*) was administered intraperitoneally three times (at 0, 24 and 48 h). The weights of the mesenterium and greater omentum were measured on day 18. Values represent the mean of ten mice±SD

though there seemed to be some orders among mono-, di-, and tri-saccharides containing GlcNAc residues.

Based on DNA ladder formation and nuclear fragmentation, sugar-cholestanols rapidly induced apoptotic cell death in gastric and colorectal cancer cells. Two main pathways that are involved in mediating apoptosis in cancer cells by anticancer drugs have been reported [46, 47]: mitochondria-dependent (intrinsic) and death receptor-dependent (extrinsic) pathways. The present results from Western blot analyses of molecules related to apoptosis showed that treatment of cancer cells with various sugar-cholestanols induced the cleavage of the cytoplasmic BiD protein to a truncated BiD (tBiD); this then resulted in an increased expression of tBiD, which translocates into the mitochondrial membrane. As yet, however, the involvement of such a treatment in the death receptor-dependent extrinsic pathway has not been fully elucidated. In the aforementioned two pathways, mitochondria play an important role in the response to DNA damage. In fact, along with changes in pro-apoptotic events, a significant release of cytochrome *c* from mitochondria [48, 49] was observed in colon26 cells together with the incorporation of sugar-cholestanols into mitochondria. Furthermore, the expression levels in a series of molecules pertaining to apoptotic cell death, in particular, those in the mitochondria-dependent intrinsic pathway, were investigated. It has been demonstrated that mitochondrial damage resulted in cytochrome *c* release and the formation of the apoptosome, consisting of a multimeric protein with the apoptotic protease activating factor-1 (Apaf-1), cytochrome *c*, and procaspase-9 [50–52]. Treatment of colon26 cells with sugar-cholestanols in this study resulted in increased Apaf-1 levels shortly after the treatment. The formation of the apoptosome has also been reported to trigger a cascade of effector caspase activation and proteolysis, leading to apoptotic cell death. In fact, activation of the initiator caspase (caspase-9), which leads to the activation of the effector caspase (caspase-3), occurred in the same cancer cells that had been treated with sugar-cholestanols simultaneously when Apaf-1 levels started to rise. In addition, activation of the caspases successively led to PARP cleavage accompanied by DNA ladder formation and nuclear fragmentation. The expression of the anti-apoptotic molecule, Bcl-xL, was suppressed only in the cells that had been treated with sugar-cholestanols. It is therefore evident that this molecule-based induction of apoptotic signaling mainly involving the mitochondria-dependent intrinsic pathway occurred rapidly in cancer cells in the presence of sugar-cholestanols in line with the down-regulation of anti-apoptotic molecules and up-regulation of apoptotic molecules in the caspase cascade. It is likely that the induction of apoptotic cell death somehow contributes to the disruption of a balance between pro-apoptotic and anti-apoptotic molecules, as mentioned previously [18]. It remains to be examined whether certain signal transduction pathways other than caspase-dependent pathways, such as death receptor-dependent and death receptor-independent pathways, could proceed under identical conditions. In our preliminary results, caspase-independent apoptosis as well as autophagic cell death was also observed in colorectal cancer cells that had been treated with sugar-cholestanols (author's unpublished observations).

The present *in vivo* experiments using a mouse model of the peritoneal dissemination demonstrated that sugar-cholestanols suppressed tumor growth at the omentum and the mesenterium in the peritoneum, where most peritoneal dissemination has been demonstrated to occur [53, 54], and, furthermore, that treatment of mice with sugar-cholestanols was associated with a significantly increased survival time. In earlier studies, we developed new *ex vivo* methods for

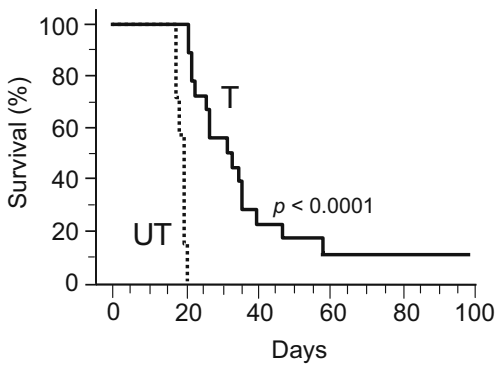

Fig. 12 Probability of survival in mice injected i.p. with colon26 cells and GlcNAcβ1-3GalβChol. Balb/c mice were intraperitoneally inoculated with 5×10^4 of colon26 cells, and 100 μl of 2 μmol of GlcNAcβ1-3Galβcholestanol (*T*) or PBS (*UT*) was administered intraperitoneally three times (at 0, 24, and 48 h). Values represent the mean of 20 mice±SD

assaying the adhesion of gastric and colorectal cancer cells to the mouse mesothelium [12, 14] and found that the presence of anti-adhesion glycoconjugates inhibited cancer cell adhesion [13, 14]. In contrast, cell-mediated priming by certain glycoconjugates in colorectal cancer cells showed that novel antitumor agents might exist among such glycoconjugates [5]. However, it is plausible that treatment of cancer cells with the present sugar-cholestanols achieves more potent advantages as an anticancer agent.

In conclusion, treatment of cancer cells with sugar-cholestanols in this study was shown to have an anti-proliferation action in *in vitro* and *in vivo* experiments. In addition, for the first time, we report a new strategy for evaluating anticancer agents and identify more potent anti-peritoneal dissemination agents in the mouse than those discovered hitherto. Sugar-cholestanols, in particular, GlcNAcRβcholestanols possess strong apoptotic cell death activity on cancer cells. Accordingly, novel therapeutic agents against peritoneal dissemination are quite likely to be found among sugar-cholestanols.

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