Enhanced expression of proapoptotic and autophagic proteins involved in the cell death of glioblastoma induced by synthetic glycans

Laboratory investigation

AHMAD FARIED, M.D., PH.D.,1 MUHAMMAD ZAFRULLAH ARIFIN, M.D., PH.D.,1 SHOGO ISHIUCHI, M.D., PH.D.,2 HIROYUKI KUWANO, M.D., PH.D.,3 AND SHIN YAZAWA, PH.D.3,4

1Department of Neurosurgery, Faculty of Medicine, Universitas Padjadjaran–Dr. Hasan Sadikin Hospital, Bandung, Indonesia; 2Department of Neurosurgery, Faculty of Clinical Medicine, the University of Ryukyus, Nakagami-gun, Okinawa; 3Department of General Surgical Science, Faculty of Medicine, Gunma University, Maebashi; and 4Tokushima Research Institute, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan

Object. Glioblastoma is the most aggressive malignant brain tumor, and overall patient survival has not been prolonged even by conventional therapies. Previously, the authors found that chemically synthesized glycans could be anticancer agents against growth of a series of cancer cells. In this study, the authors examined the effects of glycans on the growth of glioblastoma cells both in vitro and in vivo.

Methods. The authors investigated not only the occurrence of changes in the cell signaling molecules and expression levels of various proteins related to cell death, but also a mouse model involving the injection of glioblastoma cells following the administration of synthetic glycans.

Results. Synthetic glycans inhibited the growth of glioblastoma cells, induced the apoptosis of the cells with cleaved poly (adenosine diphosphate-ribose) polymerase (PARP) expression and DNA fragmentation, and also caused autophagy, as shown by the detection of autophagosome proteins and monodansylcadaverine staining. Furthermore, tumor growth in the in vivo mouse model was significantly inhibited. A dramatic induction of programmed cell death was found in glioblastoma cells after treatment with synthetic glycans.

Conclusions. These results suggest that synthetic glycans could be a promising novel anticancer agent for performing chemotherapy against glioblastoma.

(key word) • synthetic glycan • glioblastoma • apoptosis • autophagy • oncology

This article contains some figures that are displayed in color online but in black-and-white in the print edition.

Glioblastoma is the most aggressive and lethal malignancy of the CNS, and patients with glioblastoma have an average life expectancy of 1 year after the standard treatment of surgery followed by radiation therapy.26,45 Recently, clinical studies have shown that chemotherapy in addition to radiation therapy could increase patient survival up to 2 years.45 The continuing problems caused by glioblastoma and the failure of conventional therapy for this advanced invasive brain tumor indicate that novel strategies and anticancer drugs are critically needed to improve the prognosis.

Glioblastoma cells are naturally resistant to cell death,16,26 which has been considered to be attributable to the activation of phosphatidylinositol 3-kinase (PI3K) by growth factors and the subsequent hyperactivation of its downstream targets, the serine/threonine kinases protein kinase B (Akt) and mammalian target of rapamycin (mTOR). These targets are known to release a variety of...
Cell death of glioblastoma induced by glycans

antiapoptotic signals, thereby promoting the proliferation of the tumor cells. Growing evidence is accumulating that glioblastoma cells exploit glutamate for their proliferation and migration ability. The released glutamate may stimulate glioblastoma cell growth and migration through the autocrine and/or paracrine activation of glutamate receptors. In addition, the expression of Rho GTPase family members has been demonstrated in a wide variety of malignancies and in high-grade glioma as a hallmark of cell migration and as a predictor of the clinical prognosis.

Programmed cell death plays an important role during tissue development and homeostasis. Aberrations in this process result in the pathology of numerous disorders, such as malignancy. Apoptosis is the most common form of programmed cell death, but recently, alternative cell death programs have received increased attention, with autophagy proposed as an important nonapoptotic cell death mechanism.

In our previous studies, using chemically synthesized glycans consisting of sugar cholestanols with mono-, di-, and trisaccharides attached to cholestanols, we showed both strong inhibitory activity against the proliferation of a series of mouse and human cancer cells from the digestive system and antitumor effects in a mouse model of peritoneal dissemination. The sugar cholestanols added to the culture were rapidly taken up via the lipid rafts/microdomains on the cell surface. The uptake of sugar cholestanols in mitochondria increased gradually and was followed by the activation of apoptotic signals via the caspase cascade, leading to apoptotic cell death. Furthermore, the examination of sugar cholestanols in a mouse model of peritoneal dissemination showed a dramatic reduction of tumor growth and a prolonged survival time of the mice. The sugar cholestanols described in our previous studies, therefore, appeared to have clinical potential as novel anticancer agents. However, the cell death pathways in malignant glioma cells induced by the same compounds remain an open question. In this study, we investigated the programmed cell death induced by the sugar cholestanols in glioblastoma cells and its anticancer effect on growth in nude mice.

Methods

Cell Lines and Culture Condition

Human glioblastoma cell lines, CGNH-89 and CGNH-NM, were established as described previously. The morphology of CGNH cells is epithelial and adherent type, and their doubling time is 24 hours. CGNH cells were established through resection from the tumor at the right frontal lobe of female patients according to the explant method by Nichols et al. It has been demonstrated that the CGNH cells have glioblastoma morphological characteristics, and they grow very fast (highly cellular) and are relatively monotonous, while some are multinucleated giant cells with slight nuclear pleomorphism, marked atypical nucleus, and brisk mitotic activity. The cells were maintained at 37°C in DMEM (Nissui) supplemented with 10% fetal bovine serum (Invitrogen) and 3% l-glutamine in a humidified atmosphere of 5% CO₂ in air. When they were confluent, the cells were exposed in 0.05% trypsin and subcultured in the same growth medium.

Compounds

N-acetyl-d-glucosamine (GlcNAc) β1,3 d-galactose (Gal) β cholesterol, or GGChol, and GlcNAc β cholestanol, or GChol, were synthesized and prepared as an inclusion complexation with 20% of hydroxypropyl-β-cyclodextrin (HP-β-CD; BICO) and used for the experiment as previously described.

Antibodies and Chemical Reagents

Anti-GluR1 (glutamate receptor 1) and GluR4 (glutamate receptor 4) were obtained from Chemicon. Anti-RhoA, RhoC, Beclin-1, and LC3 were obtained from Santa Cruz Biotech, Inc. Anti-pAkt at ser473, pmTOR at ser2448, p53 at ser46, Bcl-2 family, caspase family, and poly (adenosine diphosphate-ribose) polymerase (PARP) were obtained from Cell Signaling. 3-Methyladenine (3-MA; Sigma), was used as an inhibitor of autophagy. 3-MA (30 mg) was dissolved in 1 ml dH₂O to make a 200 mM stock solution and kept at room temperature. Benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-FMK, or just Z-VAD; BD Biosciences), a general caspase inhibitor, was used to inhibit apoptosis. Z-VAD was dissolved in dimethylsulfoxide for a stock solution. And 1 mM of 3-MA and 10 μM of Z-VAD were diluted separately in DMEM to obtain the desired concentration. The autofluorescent agent monodansylcadaverine (MDC; Sigma) was introduced as a specific autophagolysosome marker to analyze the autophagic process. Fluorescence of MDC has been reported to be a specific marker for autophagic vacuoles. Monodansylcadaverine was dissolved in methanol (10 mg/ml) and used to observe autophagy.

Cell Proliferation Inhibition and Nuclear Fragmentation Assays

Cell proliferation inhibition with each compound was conducted in the presence of serially diluted compounds as described previously. DNA binding dyes, Hoechst 33342 (HO342), in addition to propidium iodide (PI)–fluoromethylketoine (Z-VAD-FMK, or just Z-VAD; BD Biosciences), a general caspase inhibitor, was used to inhibit apoptosis. Z-VAD was dissolved in dimethylsulfoxide for a stock solution. And 1 mM of 3-MA and 10 μM of Z-VAD were diluted separately in DMEM to obtain the desired concentration. The autofluorescent agent monodansylcadaverine (MDC; Sigma) was introduced as a specific autophagolysosome marker to analyze the autophagic process. Fluorescence of MDC has been reported to be a specific marker for autophagic vacuoles. Monodansylcadaverine was dissolved in methanol (10 mg/ml) and used to observe autophagy.

Protein Extraction and Western Blot Analysis

All cells were harvested at approximately 80% confluent growth. Protein concentrations of the cell lysate were determined with a bicinchoninic acid protein assay kit (Pierce) using bovine serum albumin as a standard. Each sample (50 μg protein/lane) was run on a 5%–20% ReadyGel (Bio-Rad) and the gel was then electrotransferred to a hybond-enhanced chemiluminescence nitrocellulose
membrane (Amersham Pharmacia Biotech). Changes in expression levels of corresponding (apoptosis and autophagy) proteins after treatment with sugar cholestanol were analyzed by Western blotting; β-Chol was used as a loading control. Bands on the membrane were detected using an enhanced chemiluminescence detection system, and horizontal scanning densitometry was performed using Photoshop software (version 3.0, Adobe), and analyzed by Quantity One software (BioRad).

Analysis of Autophagy

The analysis of autophagy was performed with the aid of MDC and counted as previously described. Autophagic vacuoles were labeled with MDC, and the fluorescent images were obtained with an epifluorescence microscope (BX-50, Olympus). The quantification of intracellular MDC accumulation was measured by fluorescence. Cells (2 × 10^5) were incubated with 0.05 mM MDC in phosphate-buffered saline at 37°C for 10 minutes and collected in 10 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100. Fluorescence was measured at a 380-nm excitation wavelength with a 530-nm emission filter, using an MTP-600 microplate reader (Corona Electric). Monodansylcadaverine expression was measured using a relative unit to show the ratio of the amount on intensity from fluorescence imaging.

Antitumor Effect of Sugar Cholestanols on Nude Mice Injected With CGNH-89 Cells

The effect of sugar cholestanols on CGNH-89 cell growth was evaluated quantitatively in a subcutaneous tumor. Cell suspensions (2 × 10^7 cells/200 μl) were injected subcutaneously in the flanks of 5- to 6-week-old nude mice (Clea Laboratories). One hundred microliters of 2 μmol of GChol dissolved in HP-β-CD was administered intratumorally 3 times (at 14, 15, and 16 days) after tumor inoculation with a 27-gauge needle. The same treatment of HP-β-CD without GChol was conducted as control. Tumor volume was calculated as follows: (length × width^2)/2.

At the end of each experiment, tumor tissues were subjected to histological analysis. Five mice were used for each group, and the experiment was approved by the Animal Care and Experimentation Committee of Gunma University. Experiments using patient tissues from glioblastoma cells were approved by the Ethical Committee of Gunma University.

Statistical Analysis

Statistical analysis was performed using StatView software (version 5.0, SAS Institute). Differences were considered significant when p was < 0.05.

Results

Cell Proliferation Inhibition of Glioblastoma Cells by Sugar Cholestanols

The effects of sugar cholestanols on the viability of glioblastoma cells were evaluated at various concentrations. Sugar cholestanols such as GGChol and GChol showed considerable inhibiting activities against the proliferation of glioblastoma cells in a dose-dependent manner (Fig. 1). However, β-Chol itself, without the sugar moiety, showed very low activity only at a high concentration in CGNH cells (data not shown). The minimum concentrations of sugar cholestanols producing 50% cell proliferation inhibition (CPI50) were determined in the glioblastoma cells, and no clear differences were observed (Table 1). The sugar cholestanols clearly induced cell death in glioblastoma cells.

Nuclear Fragmentation

Nuclear fragmentation was clearly observed in CGNH cells treated with GGChol but not in the control cells (Fig. 2 left). Staining of the glioblastoma cells (CGNH-89 and CGNH-NM) with Hoechst 3342 and propidium iodide indicated that GGChol induced nuclear fragmentation (a hallmark of apoptosis) in approximately 17% and 23% of the total cells, respectively, and were counted as apoptotic (Fig. 2 right).

Western Blot Analysis of Caspase Cascade and PARP Activation

Caspase signaling pathways consisting of a death receptor–dependent extrinsic pathway and death receptor–independent intrinsic pathway were examined in the glioblastoma cells treated with GGChol. The expression levels of active caspase-8 for the extrinsic pathway, caspase-9 for the intrinsic pathway, and caspase-3 were found to increase in the CGNH-89 and CGNH-NM cells in a time-dependent manner (Fig. 3). The expression levels of PARP, one of the best biomarkers of apoptosis, were analyzed in CGNH cells during the 24 hours after the treatment with GGChol. The N-terminal fragment of PARP, possessing an 89-kDa peptide cleaved from the full-sized PARP (116 kDa), was detected as early as 2 hours in the CGNH cells after the treatment with sugar cholestanols (Fig. 3). These results suggested that GGChol induced apoptotic cell death through both extrinsic and intrinsic pathways.

Analysis of Autophagy, Apoptosis, and the Inhibition of Both

We examined the changes in autophagy activity in both CGNH-89 and CGNH-NM cells treated with GGChol. The treatment of both cell types with GGChol induced not only apoptosis but also an autophagic response (Fig. 4). In both cell types, the number of distinct dot-like structures distributed within the cytoplasm or localized in the perinuclear regions was higher than in the control (Fig. 4A and B, left). The level of MDC incorporated into the CGNH-89 and CGNH-NM cells was increased 1.4- and 1.5-fold, respectively, after being treated with GGChol compared with that in the untreated cells (Fig. 4A and B, right). The cell viability of glioblastoma cells was reduced in the presence of GGChol up to 60% but was restored after the addition of 3-MA and Z-VAD to the culture medium (Fig. 4C). Our results showed that 3-MA and Z-VAD can block autophagy and apoptosis from 17%–20% and 38%–41%, respectively. The combination of inhibitors against both autophagy and apoptosis can fully block the cell death induced by GGChol (45%–
Cell death of glioblastoma induced by glycan

48% increase. When 3-MA and Z-VAD were added at the same time to the cell culture, the cell viability in the GGChol-treated cells was as high as that of the untreated control cells. However, no effect was observed when either agent was added individually to the cell culture (Fig. 4C).

Western Blot Analysis of the Bcl-2 Family

The expression levels of Bcl-2 family members, consisting of both proapoptosis and antiapoptosis factors, were then analyzed in the CGNH cells treated with GGChols. A slightly increased expression of Bax (proapoptosis) was detected in the CGNH-89 and CGNH-NM cells in a time-dependent manner, and a slightly decreased expression of Bcl-xL (antiapoptosis) was detected in the same cells (Fig. 5). We also evaluated the expression level of p53 (ser46), one of the initiators that activates Bax and/or downregulates Bcl-xL. Our results showed that glioblastoma cells treated with GGChol increased the expression of p53 (ser46) in a time-dependent manner (Fig. 5).

Western Blot Analysis of Autophagy

Using Western blot analysis and MDC staining, we found that GGChol increased the expression of apoptosis-related proteins and slightly increased the expression of LC3-II and Beclin-1 (Fig. 5). All these results suggest that sugar cholestanols induced both apoptosis and autophagic cell death in glioblastoma cells.

Western Blot Analysis of Survival Pathways

The expression of survival signaling proteins was evaluated in glioblastoma cells in response to sugar cholestanols. The treatment of both CGNH cell types with GGChol indicated inhibition of Akt activation and expression of both phosphorylated Akt (ser473) and phosphorylated mTOR (ser2448), the downstream targets of Akt in glioblastoma cells (Fig. 6A). The expression levels of the upstream molecules related to Akt/mTOR were also analyzed in CGNH cells treated with GGChol, and the decreased expression of both GluR1 and GluR4 was detected in CGNH cells treated with GGChol in a time-dependent manner (Fig. 6A). However, the expression levels of RhoA and RhoC in CGNH cells treated with GGChol were revealed to be suppressed in a time-dependent manner (Fig. 6B).

Antitumor Effect of Sugar Cholestanols in a Mouse Model

Nude mice were subcutaneously inoculated with CGNH-89 cells and tumors formed within 2 weeks in all

**TABLE 1: Minimum amounts of each compound producing 50% cell proliferation inhibition of various cells**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CGNH-89</th>
<th>CGNH-NM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGChol</td>
<td>14.8</td>
<td>15.6</td>
</tr>
<tr>
<td>GChol</td>
<td>15.6</td>
<td>17.2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

* The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted after 24 hours of incubation under the presence of each compound diluted from 500 μM to 0.98 μM (in a gradual manner).
mice. Tumor formation was significantly suppressed (p < 0.05) in the mice treated with GChol in HP-β-CD intra-tumorally 3 times at 14, 15, and 16 days after inoculation of tumor cells. However, no significant suppression was observed in the mice treated only with HP-β-CD (Fig. 7). The histological analysis of GChol-treated mice revealed the presence of high degrees of tumor anaplasia including nuclear and cytoplasmic pleomorphism, tumor necrosis, and vascular proliferation. However, in the control mice, large numbers of mitotic cells were observed (data not shown), as hallmarks of the glioblastoma cells.

Discussion

Temozolomide is commonly used in the treatment of primary or recurrent high-grade gliomas, including anaplastic astrocytoma and glioblastoma. To date, the prognosis of patients with malignant gliomas has been poor. It is clear that tumor cells with drug-resistant ability will not respond to chemotherapy treatment. The mechanism by which temozolomide mediates cell death in malignant tumor cells has been characterized, and it was shown to induce autophagy, not apoptosis, in glioblastoma. In the cancer field, autophagy is a new concept for the defense mechanisms of malignant cells, and they are eliminated, in some cases, due to the induction of a nonapoptotic mechanism, also known as autophagic cell death. However, the triggers for the induction of autophagy and apoptosis and their roles remain unclear.

In our previous studies, novel glycans consisting of a series of sugar cholestanols were chemically synthesized and evaluated as anticancer drugs in both in vitro and in vivo experiments. In this study, the expression levels of a series of molecules related to programmed cell death (apoptosis and autophagy) were investigated in glioblastoma cells treated with the same sugar cholestanols. We used CGNH-type glioblastoma cells, cell lines showing epithelial morphology and adhesive capacity. These cell lines possess glial fibrillary acidic protein, vimentin, A2B5, O4, and myelin basic protein. The mRNAs for the glutamate-AMPA receptors (GluR1 and GluR4) were analyzed in CGNH cells using reverse transcriptase–polymerase chain reaction; the cells expressed GluR1 and GluR4. As previously described, these cell lines have the same profile as that of the primary glioblastoma cells de novo.
In glioblastoma cells treated with sugar cholestanols, the activation of the initiator caspases (extrinsic caspase-8 and intrinsic caspase-9) followed by the activation of the executor caspase (caspase-3) occurred in the glioblastoma cells after treatment with sugar cholestanols. Accordingly, the activation of the cascade involving such caspases induced PARP cleavage, resulting in nuclear fragmentation. Furthermore, the induction of the apoptosis signaling pathway in glioblastoma cells treated with sugar cholestanols appeared to suppress the expression of Bcl-xL and to enhance the expression of Bax in antiapoptotic and proapoptotic manners, respectively. Therefore, the induction of apoptosis appeared to be caused by the disruption of a balance between these anti- and proapoptotic molecules, as described previously.8,14,15

One of the most important survival-signaling pathways is mediated by PI3K and its downstream targets, such as Akt and mTOR.29 Recently, Akt was reported to play an important role in determining the chemosensitivity of many types of cells.7,10,35 The induction of autophagy requires the activation of Beclin-1 and its interacting partner, Class III PI3K, resulting in the generation of phosphatidylinositol-3′phosphates. This induction is negatively regulated by Class I PI3K via the Akt/mTOR pathway.41,44,46 In contrast, Beclin-1, a mammalian homolog of the yeast autophagy-related gene Atg6, was observed to be

**Fig. 4.** Fluorescence microscope images showing induction of autophagic cell death in CGNH-89 (A) and CGNH-NM (B) cells. Original magnification ×200. Bar graph (C) demonstrates cell viability in the glioblastoma cells treated with GGChol measured in the presence of antiapoptosis and antiautophagy reagents. Monodansylcadaverine incorporation was quantified and presented as the fold increase ± SEM compared with the control (bar graph, upper right). The figures and values are from 3 independent experiments.
deleted in breast and prostate cancer cells, and its expression was shown to induce autophagy and inhibit tumorigenicity in MCF-7 breast cancer cells.\(^{27}\) Furthermore, the microtubule associated protein 1 light chain 3, designated as LC3, exists in 2 forms, which are LC3-I and LC3-II, located in the cytosol and autophagosomal membranes, respectively. LC3 is the first protein that was reported to specifically localize to autophagosome membranes and was later designated as LC3-II (16 kDa), the inner limiting membrane of the autophagosome. During the process of autophagy, cleaved LC3-I conjugates with phosphatidylethanolamine to form LC3-II, which is an important step for autophagosome formation.\(^{25}\)

Immunofluorescence staining of endogenous LC3 can detect autophagy (Fig. 4). The expression of Beclin-1 in glioblastoma cells was slightly increased after treatment with sugar cholestanols along with the decreased expression of the members of the Akt/mTOR pathway. In addition, LC3-II expression was increased, and this hallmark could be used to estimate the abundance of autophagosomes before they are destroyed via fusion with lysosomes.

Recently, p53 has also been revealed to activate autophagy.\(^{25}\) Several groups have reported the localization of p53 to the outer layer of the mitochondrial membrane and the activation of apoptosis through direct binding to the Bcl-2 family members Bax, Bak, or Bcl-xL.\(^{5,30}\) The overexpression of p53 was also reported to increase Bax expression in several cell types following the induction of apoptosis.\(^{31,43}\) The binding of p53 to p53AIP1, which appears to be important for the apoptotic response, is selectively enhanced by the phosphorylation of ser46.\(^{37}\) We also observed that, in fact, p53 at ser46 was increased in glioblastoma cells after treatment with sugar cholestanols. In addition, the stimulation of cell death controlled by apoptosis and/or at least partially by autophagy was observed in glioblastoma cells treated with sugar cholestanols and cotreated with inhibitors of caspases and autophagy. Therefore, we provided evidence that sugar cholestanols induced apoptosis and autophagic cell death in the same glioblastoma cells. The occurrence of cell death induced by apoptosis was also observed in colorectal cancer cells treated with the same sugar cholestanols (S. Yazawa et al., unpublished observation, 2008).

The mechanism of drug-induced cell death has been accepted to be governed not only by the upregulation of proapoptotic, proautophagic factors or tumor suppressors, but also by the modulation of the survival-signaling pathways.\(^{11}\) As we previously showed, CGNH cells express Ca\(^{2+}\)-permeable AMPA receptors assembled mainly from the GluR1 and/or GluR4 subunits, which contribute to the

![Western blot analysis in the glioblastoma cells treated with GGChol. Changes in the expression of the autophagy activation, Bcl-2 family members, p53 (ser46) in the CGNH-89 and CGNH-NM cells are shown. The cells were treated with 30 \(\mu\)M of GGChol for 24 hours, and values given below each figure indicate the calculation of each band, and the LC3 active form band (16 kDa), after normalization of their expression to that of \(\beta\)-actin, shown as a percentage compared with the control. There was a significant increase in the active form of LC3 (16 kDa) measured using densitometric analysis.](image-url)
invasive and aggressive behavior of glioblastoma. Cell growth appeared to be suppressed in cancer cells treated with the sugar cholestanols, particularly through the activation of the Akt/mTOR pathway (A. Faried et al., unpublished observation, 2009). As reported previously, there is an important survival-signaling pathway that is mediated by the Akt/mTOR pathway and its upstream target, the AMPA receptors.

Our results demonstrated that the sugar cholestanols inhibit the activation of the Akt/mTOR pathway, as shown by the downregulation of phosphorylated Akt at ser473 and phosphorylated mTOR at ser2448. Therefore, we analyzed the expression of the glutamate-AMPA receptors as an upstream target of Akt/mTOR in glioblastoma cells. As expected, we found that the sugar cholestanols inhibited the activation of the glutamate-AMPA receptors, GluR1 and GluR4, in both glioblastoma cell types tested. Taken together, our results suggest that the activation of the glutamate-AMPA receptors–Akt/mTOR pathway was downregulated after treatment with sugar cholestanols.

Ca²⁺-permeable AMPA receptors and Rho GTPase family members facilitate the migration ability of human glioblastomas. In addition, we also evaluated the expression of Rho GTPases (RhoA and RhoC) because they were reported to be related to the degree of malignancy in glioblastoma. Furthermore, the inhibition of Rho GTPase signaling has been reported to decrease glioblastoma cell migration. In this study, we showed that the expression of both RhoA and RhoC was decreased after treatment with the sugar cholestanols in a time-dependent manner. Overall, our results showed that different processes of cell death were induced by the sugar cholestanols and that the survival, proliferation, or metastatic properties of glioblastoma cells were affected by some other oncogenic factors (Fig. 8).

Our in vivo experiment using nude mice showed that the sugar cholestanols suppressed tumor growth of CGNH-89 cells that were injected into subcutaneous tissue, possessing the features of human glioblastomas in terms of histological tissue organization. This experiment may provide a reliable in vivo model for studying the response of human glioblastomas to our potential synthetic...
glycans (sugar cholestanols). The sugar cholestanol injections reduced the incidence of intratumoral bleeding in the treated mice compared with the untreated mice, accompanied by the suppression of tumor growth and induction of apoptosis. These results indicate that programmed cell death controlled by apoptosis and/or at least partially by autophagy in CGNH cells was stimulated by treatment with our novel synthetic glycans (sugar cholestanols). It remains to be seen whether the sugar cholestanols could be applicable to an in vivo experiment using an intracranial glioma model to investigate their usefulness in chemotherapy against the expected blood-brain barrier.

**Conclusions**

The activation of programmed cell death in human malignant brain tumor cells induced by treatment with the sugar cholestanols may be involved in not only apoptosis, as we previously demonstrated in several tumor cell lines, but also autophagy, which was demonstrated here for the first time. The sugar cholestanols represent potential pharmaceutical agents against glioblastoma cells.

**Disclosure**

This work was supported partly by the 21st Century COE
Cell death of glioblastoma induced by glycans

Program, Japan; the Japan Society for the Promotion of Science; and a research grant for collaboration research to Dr. Faried from the Faculty of Medicine at Universitas Padjadjaran.

Author contributions to the study and manuscript preparation include the following. Conception and design: Faried, Arfin, Yazawa. Acquisition of data: Faried, Yazawa. Analysis and interpretation of data: Faried, Yazawa. Drafting the article: Faried, Yazawa. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Faried.

References


12. Fritz G, Just I, Kaina B: Rho GTPases are over-expressed in a multiforme invasive phenotype points to new therapeutic targets. Neoplasia 7:7–16, 2005


