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ORIGINAL ARTICLE

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Apoptosis induced by glycoprotein (150-kDa) isolated from *Solanum nigrum* L. is not related to intracellular reactive oxygen species (ROS) in HCT-116 cells

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Abstract This study was carried out to investigate the apoptotic effects of glycoprotein [*Solanum nigrum* L. (SNL) glycoprotein, 150-kDa] isolated from *Solanum nigrum* L., which has been used as an antipyretic and anticancer agent in folk medicine. With the purified SNL glycoprotein, we evaluated the cytotoxic and apoptotic effects of SNL glycoprotein on HCT-116 cells, DNA fragmentation and nuclear staining assays, respectively. SNL glycoprotein has an apparent cytotoxic and apoptotic effect at a concentration of 40 μg/ml after 4 h. To further verify the apoptotic effect, we investigated the changes in activity of the apoptotic-related proteins [Bid, cytochrome *c*, caspases and poly(ADP-ribose)polymerase (PARP)] triggered by SNL glycoprotein, using a western blot analysis. The results in this study indicated that SNL glycoprotein has a stimulatory effect on Bid activation, resulting in the release of cytochrome *c*, the stimulation of caspase-8, -9 and -3 activities, and the cleavage of PARP in HCT-116 cells. However, SNL glycoprotein did not significantly stimulate an increase in levels of intracellular reactive oxygen species (ROS). From the results in this experiment, it is suggested that SNL glycoprotein induces apoptosis through the mitochondrial apoptotic signal pathway in HCT-116 cells, rather than through intracellular ROS.

Keywords Glycoprotein *Solanum nigrum* L. · Bid · Cytochrome *c* · Caspases · Reactive oxygen species · Apoptosis

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Introduction

In the last 3 decades, numerous antitumor polysaccharide-protein complexes (glycoprotein) have been discovered from mushrooms, fungi, yeasts, algae, lichens, and plants. The research for a novel glycoprotein with antitumor properties originates as a result of shortcomings of existing cancer chemotherapy and radiotherapy, although the mechanism of antitumor action of the glycoprotein is not fully understood [1]. A large number of chemical compounds, which have been identified as specific agents for killing cancer cells, are also toxic to normal cells. In particular, many cancer cells are either inherently resistant to chemotherapy, or they develop resistance during the course of therapy [2, 3]. Hence, the discovery and identification of new safe drugs, without severe side effects, has become an important goal of research in the biomedical sciences.

Apoptosis is a programmed cell death process, which is highly regulated and organized by a number of intracellular signal mediators controlling the development and homeostasis of multicellular organisms that result in chromatin condensation, DNA fragmentation, cytoplasmic membrane blebbing, and cell shrinkage [4]. Apoptosis is signaled through several death receptors and ligands stimulated by apoptotic mediators, which are placed mainly in the cell surface, the cytosol, the mitochondria, and the nucleus [5]. Bid (full-length Bid, p21) is a pro-apoptotic Bcl-2 family protein that is cleaved and activated by caspase-8 in response to Fas/TNF-R1 death receptor activation. This activated Bid (truncated Bid, p15) is translocated to the mitochondria and then induces cytochrome *c* release from mitochondria. This process, in turn, activates the downstream caspases, which are cysteine-dependent enzymes [6]. Among these caspases, caspase-3 has been implicated in the execution phase of apoptosis and has been shown to cleave over 100 substrates, including poly(-ADP-ribose) polymerase (PARP), that respond to

DNA strand breaks and eventually lead to apoptosis [7]. Moreover, it is also well known that the generation of intracellular ROS plays an important role in apoptosis occurrence through the disruption of redox homeostasis [8]. The generated ROS can directly or indirectly cause the loss of mitochondrial membrane potential by activating mitochondrial permeability transition, and induce apoptosis by releasing apoptogenic protein such as cytochrome *c* to cytosol [9, 10]. For the purpose of cancer therapeutic strategies, the induction of apoptosis by chemotherapeutic agents through the modulation of mitochondrial apoptotic mediators and intracellular ROS production has been proposed as one of the potential clinical methods to eliminate cancer cells [11, 12].

Recently, we found a glycoprotein with an approximate molecular mass of 150-kDa, isolated from *Solanum nigrum* L. (SNL), which is made up of carbohydrate content (69.74%) and protein content (30.26%). This active substance, designated as SNL glycoprotein, has pharmacological activities such as antitumor, antimicrobial, and antioxidative effects, indicating SNL glycoprotein is a functional substance with multiple biological activities [13–15]. A more recent study revealed that SNL glycoprotein regulates the activity of protein kinase C alpha (PKCα), the DNA binding activation of nuclear factor-kappa B (NF-κB) and the production of inducible nitric oxide (iNO) in 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated HCT-116 cells [16]. However, the apoptosis induced by SNL glycoprotein through mitochondrial death signal pathways in HCT-116 cells is still undefined without the tumor promoter (TPA).

Therefore, with respect to its cytotoxicity, we investigated whether the SNL glycoprotein regulates mitochondrial apoptotic signal mediators and intracellular ROS production, and whether it consequently induces apoptosis in HCT-116 cells.

Materials and methods

Chemicals

All the plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA). Bisbenzimide (H33342, B2261), catalase (CAT, C40), glutathione peroxidase (GPx, G1637), streptomycin (H0447), superoxide dismutase (SOD) (S2515), penicillin G (H0474), neutral red (N7005), phenol solution (P4682), ribonuclease A (R4875), silica gel (28–200 mesh, 22 Å, S4883), and trypsin (T4549) were obtained from Sigma (St. Louis, USA) and nitrobluetetrazolium chloride monohydrate (NBT, 74030) were obtained from Biochemica. Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals and reagents were of the highest quality available.

Preparation of SNL glycoprotein

Samples of *S. nigrum* L. (SNL) were obtained in October 2002 from Naju in the Chonnam province of South Korea, and glycoprotein was isolated and purified as described previously [16]. In this study, we used SNL glycoprotein with a molecular weight of 150-kDa.

Cell culture

HCT-116 cells, the human colorectal carcinoma cells, were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). The HCT-116 cells were incubated in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2 atmosphere, respectively. The medium was renewed two times per week. The cells (1×10^6 cells/ml) were divided into 35-mm culture dishes or 96-well flat bottom plates. The final volume was adjusted to 2 ml/dish for culture dishes and to 100 μl/well for 96-well plates.

Cytotoxicity of SNL glycoprotein

The cellular cytotoxicity induced by SNL glycoprotein was measured using the neutral red assay [17]. Cells were treated either with or without SNL glycoprotein (5–80 μg/ml) for 4 h, and then the cells were incubated in 100 μl of new medium containing 10 mg/ml neutral red for 90 min at 37°C. After the complete removal of the medium, each well was washed three times with 100 μl of phosphate buffered saline (PBS). One hundred microliter of 50% ethanol containing 50 mM sodium citrate (pH 4.2) was added into each well on the 96-well multiple plates. After 20 min, the absorbance was measured at 510 nm using a SpectraCount (Packard Instrument Co. Downers, USA) ELISA reader.

Preparation of the whole cell extracts

HCT-116 cells (1×10^6 cells/ml) were plated in 35-mm culture dishes and maintained at 37°C in an incubator for 48 h. Next, the medium was replaced with serum-free DMEM medium for a further 4 h. After this step, the cells were treated either with or without SNL glycoprotein at different concentrations (5–40 μg/ml) for the indicated times. The cells were rinsed twice with PBS after removing the medium and scraped in 300 μl of buffer A [20 mM HEPES (pH 7.9), 0.4 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40, 25% glycerol] containing a protease inhibitor cocktail. For cytolysis, the cells were freeze-thawed, shaken for 30 min at 4°C, and centrifuged at 14,000 *g* for 30 min at 4°C. The supernatant was designated as a whole cell extract. The amount of protein was measured using the Lowry method [18] and the proteins were stored at −70°C for further experiments.

Preparation of the mitochondrial extracts

As described above, HCT-116 cells were treated either with or without SNL glycoprotein (5, 10, 20, and 40 μg/ml), and rinsed twice with PBS after replacement of the medium. Cell pellets were resuspended in 300 μl of buffer B [20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 1.0 mM dithiothreitol, 250 mM sucrose] containing a protease inhibitor cocktail. The cells were homogenized by ten strokes in a Dounce homogenizer (B. Braun, Melsungen, Germany). To collect nuclei and debris, the homogenates were centrifuged twice at 750 *g* for 5 min at 4°C. The supernatants were centrifuged at 10,000 *g* for 15 min at 4°C, to collect mitochondria-enriched heavy membrane pellets. The resulting supernatants were centrifuged at 100,000 *g* for 1 h at 4°C and the final supernatants are referred to as cytosolic fractions.

Western blot analysis

The whole cell and mitochondrial extracts for immunoblotting of Bid, cytochrome *c*, caspase-3, caspase-8, caspase-9, and PARP were isolated from HCT-116 cells after treatment with a defined concentration of SNL glycoprotein and incubation time. These sample proteins were separated in a 12% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a Mini–PROTEIN II electrophoresis cell (Bio–Rad). After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were incubated for 1 h at room temperature in TBS-T solution [10 mM Tris–HCl (pH 7.6), 150 mM NaCl and 0.1% (v/v) Tween-20] containing 5% (w/v) nonfat dry milk (NFDM). The membranes were subsequently incubated for 2 h at room temperature with rabbit polyclonal antibodies [1:3,000; Bid, cytochrome *c*, caspase-3, caspase-8, caspase-9, and PARP, Santa Cruz Biotechnology, CA, USA] in TBS-T solution containing 5% NFDM. After three washes with TBS-T, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat antirabbit IgG (1:10,000; Santa Cruz Biotechnology, CA, USA) in TBS-T containing 5% NFDM. The protein bands were visualized by incubation with nitrobluetetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma Chemical Co.).

Measurement of intracellular reactive oxygen species (ROS)

The intracellular ROS production was measured by using nonfluorescent 2′, 7′-dichlorofluorescein (DCFH-DA). This compound is deacetylated by intracellular esterases to the nonfluorescent DCFH, which is oxidized to the fluorescent compound DCF by ROS [19]. For the measurement of ROS production, HCT-116 cells were pre-incubated with 10 μM DCFH-DA for 30 min at 37°C, and then the cells were washed twice with PBS to remove the excess DCFH-DA. After that, the cells were treated with or without SNL glycoprotein at different concentrations for 4 h. Finally, the fluorescence of DCF was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescent microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation, Sunnyvale, CA, USA). The values were calculated as a relative of the DCF fluorescence intensities, compared to the control.

Measurement of antioxidant enzymes activities

HCT-116 cells (1×10^6 cells/ml) were treated either with or without SNL glycoprotein (5, 10, 20, 40 μg/ml) for 4 h, as described above. The cells were rinsed twice with PBS after removing the medium. Cell pellets were resuspended in 300 μl of buffer C [130 mM sodium chloride, 5 mM glucose, 1 mM EDTA, 10 mM sodium phosphate buffer (pH 7.0)]. Then, they were freeze-thawed in liquid nitrogen, shaken for 30 min at 4°C and centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant was used for antioxidant enzyme assays as described below and the amount of protein was measured using the Lowry method [18]. The proteins were stored at −70°C.

Superoxide dismutase activity

The SOD activity was measured according to the method of Beauchamp and Fridovich [20]. An adequate amount of the cell supernatant was mixed with reaction mixtures, which contained 0.1 mM EDTA, 25 mM NBT, 0.1 mM xanthine, 50 mM sodium carbonate buffer (pH 10.2), and the final volume of the reaction mixture was brought up to 3 ml with distilled water. The reaction was initiated by the addition of 2 mU/ml xanthine oxidase and was maintained under two 40 W lamps at 25°C. After 15 min, the inhibition rate of NBT reduction was spectrophotometrically determined at 560 nm. One unit of SOD is defined as the amount of enzyme required to reduce the NBT by 50%. The specific activity of SOD was expressed as a unit/mg protein in each supernatant and the values were calculated as a percentage of the control value.

Catalase activity

This assay was conducted according to the method of Thomson et al. [21]. The cell supernatants were mixed with 2.8 ml of 50 mM phosphate buffer (pH 7.4). After equilibration at 30°C for 5 min, the reaction was started by the addition of 200 ml of 100 mM sodium perborate (pH 7.4). The CAT activity, which reduces the sodium perborate as the substrate, was assessed spectrophotometrically following the consumption of hydrogen per-

oxide at 220 nm for 2 min. One unit of CAT was defined as the amount of enzyme required to reduce 1 μM of H_2O_2/min. Results were expressed as a unit/mg protein in each supernatant and the values were calculated as a percentage of the control value.

Glutathione peroxidase (GPx) activity

The GPx activity was measured according to the method of Paglia and Valentine [22]. The cell supernatants were added to the reaction mixtures consisting of 1 mM EDTA, 1 U of glutathione reductase, 1 mM glutathione, 0.25 mM H_2O_2 and 1 mM sodium azide in 50 mM phosphate buffer (pH 7.0). The reaction was initiated by the addition of 0.2 mM NADPH and GPx activity was measured as the rate of NADPH oxidation at 340 nm. One unit of GPx was defined as the amount required to oxidize 1 μM of NADPH/min. Results were expressed as a unit/mg protein in each supernatant and the values were calculated as a percentage of the control value.

DNA fragmentation assay

HCT-116 cells (1×10^6 cells/ml) were treated either with or without SNL glycoprotein (5, 10, 20, 40 μg/ml) for 4 h, as described above. For DNA extractions, HCT-116 cells were harvested by centrifugation at 2,000 *g* for 5 min and washed one time with ice cold PBS. After this step, all operations were carried out on ice, if not otherwise stated. Cell pellets were gently resuspended in 500 μl of lysis buffer [50 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1% NP-40, and 20 μl of 10% SDS] and incubated at 65°C for 30 min. After that, 100 μl of 8 M potassium acetate was added to the suspended mixtures and incubated on an ice bath for 1 h. The lysates were centrifuged at 10,000 *g* for 10 min and the supernatants were transferred to a new Eppendorf tube. Finally, DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and stored at −70°C prior to use. To perform the DNA fragmentation assay, samples were dissolved in 20 μl TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) and incubated at 37°C for 30 min with 1 μl of RNase A (10 μg/ml) and 1 μl of proteinase K (100 μg/ml). Electrophoresis was carried out on 2% agarose gel for 100 V/2 h using Mini Sub DNA Cell (Bio-Rad), and the gels were stained with 5 μg/ml ethidium bromide and photographed under UV light.

Nuclear staining assay

Another analysis for apoptosis was conducted using staining with H33342 and ethidium bromide in HCT-116 cells (1×10^6 cells/ml), which were spread in 35 mm culture dishes and maintained at 37°C in an incubator for 48 h. The medium was replaced with serum-free DMEM medium for a further 4 h. After this step, HCT-116 cells were treated with SNL glycoprotein for the indicated times. Next, 25 μl of the cell suspension (5×10^5 cells/ml) in DMEM was collected and mixed with 1 μl of dye mix (100 μg/ml H33342 and 100 μg/ml ethidium bromide in PBS). The morphological changes of the nuclei were observed by fluorescent microscopy (Carl Zeiss, City, Germany). At least 200 cells with condensed or fragmented nuclei were counted as apoptotic cells. The relative percentages of apoptotic cells was calculated using the following formula:

$$\text{Apoptosis (\%)} = \frac{\text{total number of cells with apoptotic nuclei}}{\text{total number of cells}} \times 100$$

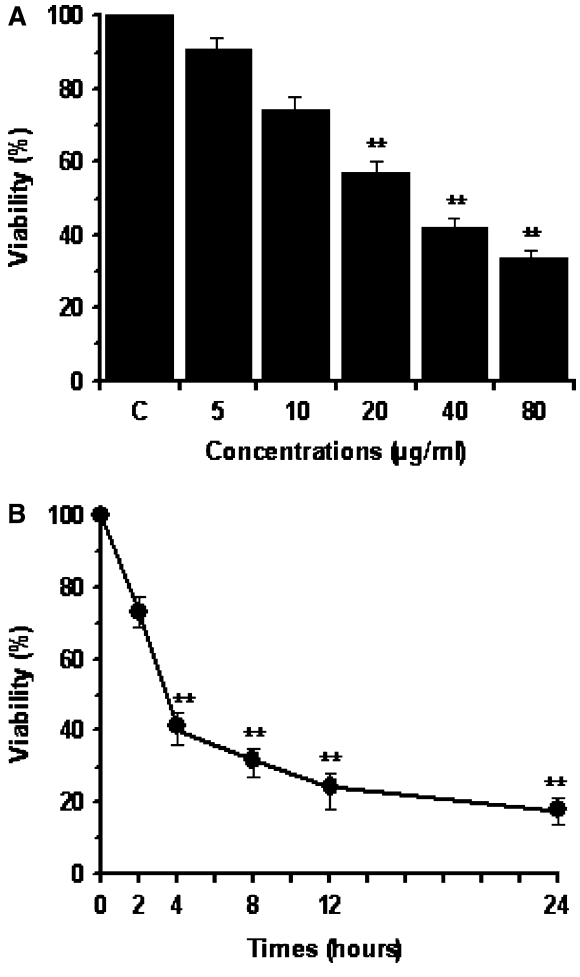

Fig. 1 Cytotoxic effects of SNL glycoprotein. HCT-116 cells were treated with either SNL glycoprotein (5, 10, 20, 40, and 80 μg/ml) alone for 4 h (**a**), or 40 μg/ml of SNL glycoprotein alone for various times (2, 4, 8, 12, and 24 h) (**b**) at 37°C in an atmosphere containing 5% CO_2. Cell viabilities were evaluated by the neutral red assay as described in the section entitled "Materials and methods." The values are relative percentages to the control value, which was treated with culture medium. Each *bar* represents the mean ± SD of triplicate experiments ($n=9$). ** represents a significant difference between the control and concentration of SNL glycoprotein, and between the control and treatment time with SNL glycoprotein (40 μg/ml), $P<0.01$

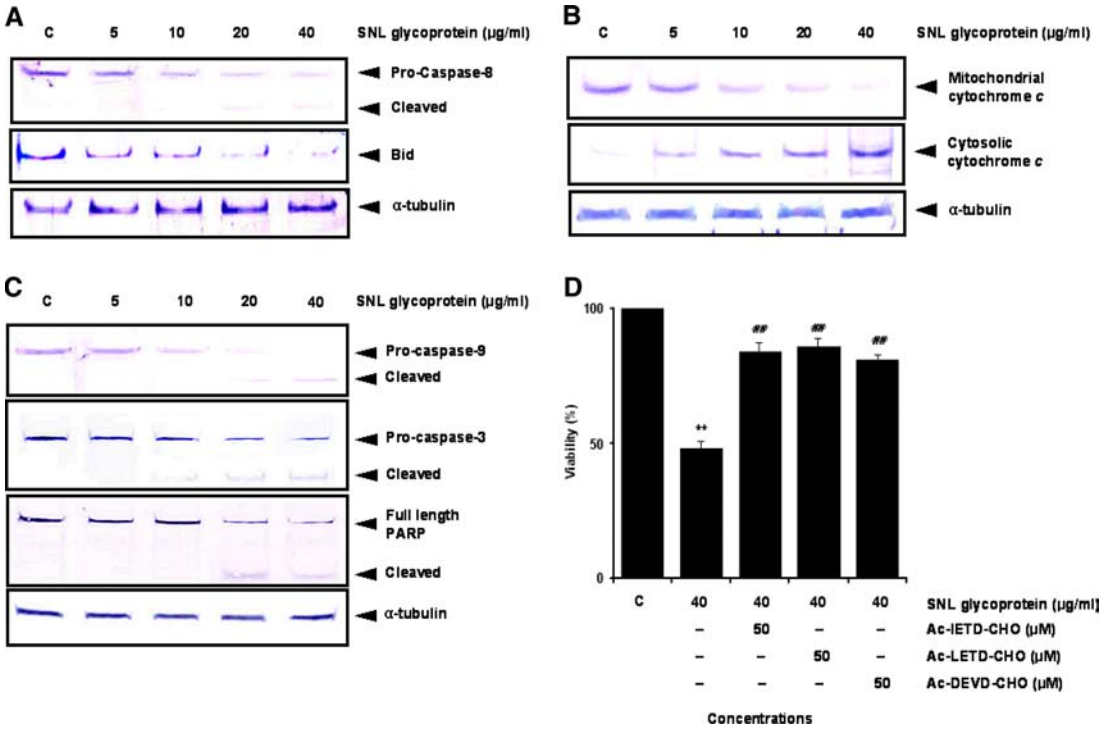

Fig. 2 Effects of SNL glycoprotein on activities of apoptotic related proteins. HCT-116 cells were treated with various concentrations of SNL glycoprotein (5, 10, 20, and 40 μg/ml) alone for 4 h. Whole cell extracts for immunoblotting of caspase-8 and Bid protein (**a**), and caspase-9, caspase-3 and PARP proteins (**c**) were obtained as described in the section entitled "Materials and methods." **b** The cytosolic and mitochondrial extracts for immunoblotting cytochrome *c* protein (**b**) were also isolated from HCT-116 cells. Detection of caspase-8, Bid, cytochrome *c*, caspase-9, caspase-3, and PARP proteins was performed by Western blotting using an anticaspase-8, -Bid, -cytochrome *c*, -caspase-9, -caspase-3, and -PARP polyclonal antibodies. *Lane C* control, *lane 1* 5 μg/ml SNL glycoprotein, *lane 2* 10 μg/ml SNL glycoprotein, *lane 3* 20 μg/ml SNL glycoprotein, *lane 4* 40 μg/ml SNL glycoprotein. α-Tubulin was used as an internal control. **d** Cells were pretreated with either 50 μM inhibitors of caspase-8 (Ac-IETD-CHO), caspase-9 (Ac-LETD-CHO), or caspase-3 (Ac-DEVD-CHO) for 1 h and then treated with 40 μg/ml SNL glycoprotein for another 4 h. Cell viabilities were evaluated by the neutral red assay as described in the section entitled "Materials and methods." The values are relative percentages to the control value, which was treated with only medium. Each *bar* represents the mean ± SD of triplicate experiments ($n = 9$). ** represents a significant difference between SNL glycoprotein (40 μg/ml) and the control, $P < 0.01$. $^{\#\#}$ represents a significant difference between the SNL glycoprotein alone and treatments with caspases inhibitors in the presence of SNL glycoprotein, $P < 0.01$

Statistical analysis

All experiments were done three times in triplicate ($n = 9$), and the results were expressed as means ± SD (standard deviation). A one-way analysis of variance (ANOVA) and the Duncan test were used for multiple comparisons (SPSS program, ver 10.0).

Results

Cytotoxicity of SNL glycoprotein

When the cells were exposed to various concentrations of SNL glycoprotein for suitable incubation times, the cell viabilitiesgradually decreased (Fig. 1a, b). For the SNL glycoprotein-dependent cytotoxicity, the viabilities were 91, 74, 57, 42 and 34% at 5, 10, 20, 40 and 80 μg/ml SNL glycoprotein for 4 h. With respect to the time-dependent cytotoxicity, theviabilities at 40 μg/ml SNL glycoprotein were 74, 41, 32, and 24% for 2, 4, 8, and 12 h at 40 μg/ml SNL glycoprotein, compared to the control. Therefore, this condition (40 μg/ml SNL glycoprotein, 4 h) was chosen in this experiment to study the apoptotic signal pathway, because the cell viability was less than 50% under this experimental condition.

Effects of SNL glycoprotein on apoptotic related proteins (caspases, Bid, cytochrome *c*, and PARP)

The increase in activities of caspases, Bid, cytochrome *c*, and PARP proteins, after the cells were treated with SNL glycoprotein (5–40 μg/ml) for 4 h, caspase-8, Bid, cytochrome *c*, caspase-9, caspase-3, and PARP was apparent (Fig. 2). The cleaved form of caspase-8 and the degradation of Bid protein were observed after the addition of 20 and 40 μg/ml SNL glycoprotein (Fig. 2a). In the cytochrome *c* protein, the intensities of its bands in cytosol markedly increased depending on the concentration of the SNL glycoprotein (5–40 μg/ml), decreasing its intensity in the mitochondria (Fig. 2b). This phenomenon can be explained by the fact that SNL glycoprotein activates to cleave Bid protein by the acti-

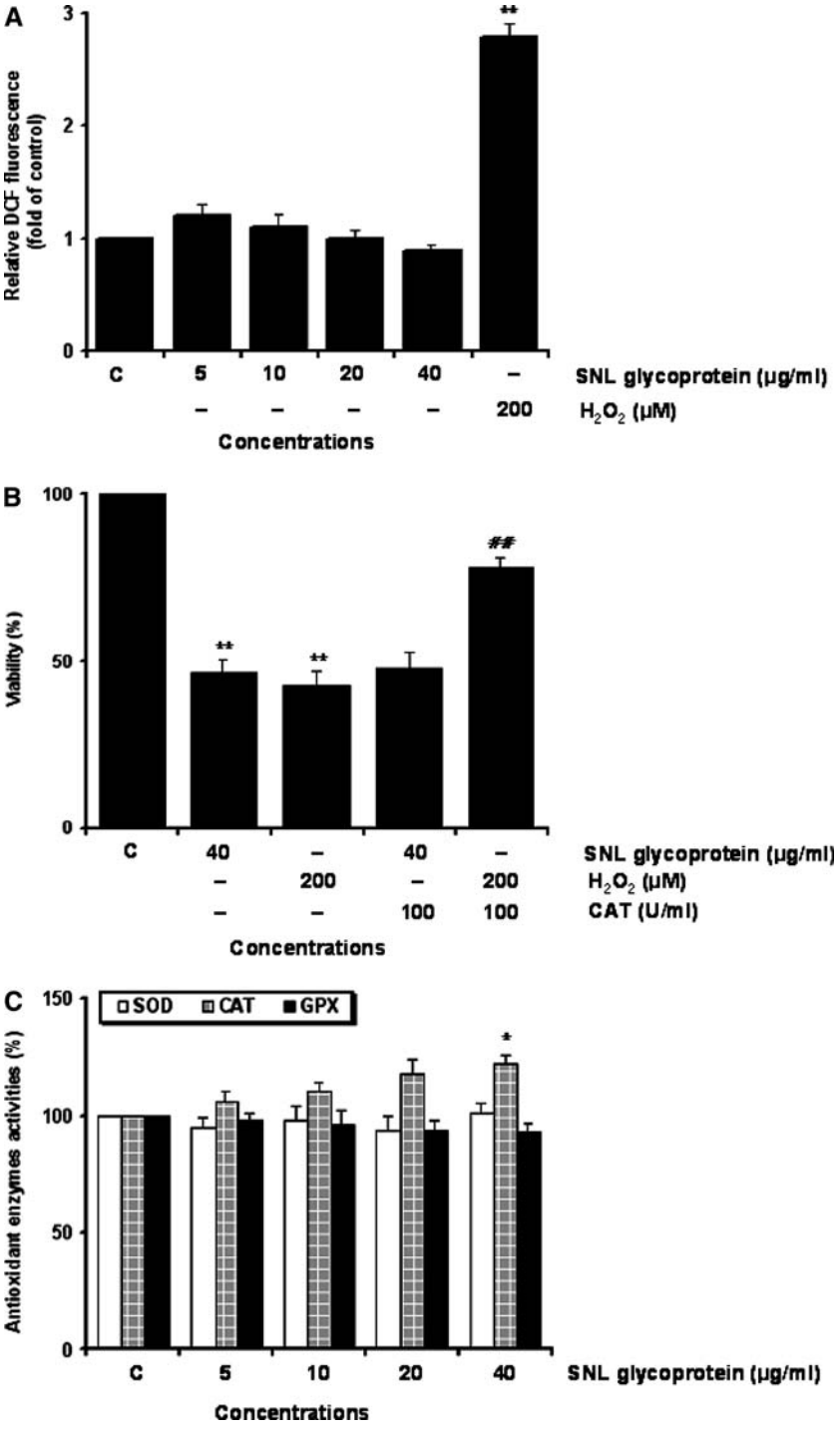

Fig. 3 Intracellular antioxidative character of SNL glycoprotein. **a** HCT-116 cells were treated with either SNL glycoprotein (5, 10, 20, and 40 μg/ml) alone or H_2O_2 (200 μM) alone for 4 h. The intracellular ROS production was measured at an excitation wavelength (485 nm) and an emission wavelength (530 nm) using a fluoresencent microplate reader. The values were calculated as a relative intensities of the DCF fluorescence intensities, compared to the control. H_2O_2 (200 μM) is used as a positive control for ROS generation. ** represents a significant difference between the SNL glycoprotein (40 μg/ml) and H_2O_2, $P < 0.01$. **b** Cells were pretreated with CAT (100 U/ml) for 1 h and then treated with either SNL glycoprotein (40 μg/ml) or H_2O_2 (200 μM) for another 4 h. Cell viabilities were evaluated by the neutral red assay as described in the section entitled "Materials and methods." The values are percentages of the control value, which was treated with only medium. ** represents a significant difference between the control and SNL glycoprotein, and between the control and H_2O_2, $P < 0.01$. ## represents a significant difference between the SNL glycoprotein (40 μg/ml) and H_2O_2, $P < 0.01$. **c** Cells were treated with various concentrations of SNL glycoprotein (5, 10, 20, and 40 μg/ml) alone for 4 h and superoxide dismutase (*SOD*), catalase (*CAT*), and glutathione peroxidase (*GPx*) activities were measured at 560, 220, and 340 nm, respectively. Results were expressed as a unit/mg protein in each supernatant and the values were calculated as a percentage of the control value. Each *bar* represents the mean ± SD of triplicate experiments ($n = 9$). * represents a significant difference between the control and the SNL glycoprotein, $P < 0.05$

vation of caspase-8 to release cytochrome *c* from mitochondria into the cytosol. On the other hand, SNL glycoprotein treatment induced the cleavage of pro-caspase-9, pro-caspase-3, and PARP were detected at 20 μg/ml (Fig. 2c). The cleaved forms of the pro-caspase-9, pro-caspase-3, and PARP were detected at 20 μg/ml SNL glycoprotein. To confirm whether the caspase activations are essential to the SNL glycoprotein-induced cell death pathway, we performed a neutral red assay using caspase-8 (Ac-IETD-CHO), caspase-9 (Ac-LETD-CHO), and caspase-3 (Ac-DEVD-CHO) as inhibitors (Fig. 2d). When the cells were exposed to 40 μg/ml SNL glycoprotein for another 4 h after pretreatment with 50 μM of Ac-IETD-CHO, Ac-LETD-CHO, and Ac-DEVD-CHO for 1 h, cell viabilities significantly increased by 84, 86, and 81%, respectively. These results demonstrated that the caspase-dependent signaling pathway is involved in SNL glycoprotein-induced cytotoxicity in HCT-116 cells.

Effects of SNL glycoprotein on intracellular antioxidative enzymes

In the intracellular ROS production by SNL glycoprotein, SNL glycoprotein did not increase the levels of intracellular ROS atthe various concentrations (5–40 μg/ml) for 4 h in HCT-116 cells (Fig. 3a). In contrast, when the cells were treated with 200 μM of H_2O_2 alone, which was used as a positive control, the levels of intracellular ROS significantly increased by 1.8 after 4 h, compared to the control, indicating that SNL glycoprotein might induce the cytotoxicity via a ROS-independent mechanism in HCT-116 cells. To support the evidence that SNL glycoprotein-induced cytotoxicity is independent of ROS generation, we investigated whether free radical scavengers (catalase, CAT) can protect against SNL glycoprotein-induced cytotoxicity, using a neutral red assay (Fig. 3b). When the cells were treated either with SNL glycoprotein (40 μg/ml) or H_2O_2 (200 μM) for 4 h, the values of cell viability significantly decreased by 53.5 or 57%, compared to the control, whereas, pretreatment with CAT (100 U/ml) for 1 h in these conditions resulted in the recovery of cell viability by 35% in H_2O_2-induced cells, but not in SNL glycoprotein-induced cells. Furthermore, results of the antioxidant enzyme assays in Fig. 3c showed that the CAT activity significantly increased by 24% after the addition of SNL glycoprotein (40 μg/ml) for 4 h, compared to the control, whereas SOD and GPx activities remained unchanged, compared to the control. Based on these results, it seems that SNL glycoprotein might induce cytotoxicity via a ROS-independent mechanism, increasing CAT activity in HCT-116 cells.

Apoptotic effect of SNL glycoprotein

The apoptotic activity of SNL glycoprotein was confirmed by DNA fragmentation (Fig. 4a) and nuclei staining (Fig. 4b) assays. The nucleosomal DNA fragmentations of 180–200 bp were gradually detected after the addition of various concentrations of SNL glycoprotein (10–40 μg/ml) for 4 h (Fig. 4a, lanes 1–3). To confirm whether the caspase activation and ROS independence in the SNL glycoprotein-induced apoptosis pathways, we performed further experiments with CAT and caspase inhibitors. When the cells were pretreated with 50 μM Ac-IETD-CHO, Ac-LETD-CHO, and Ac-DEVD-CHO for 1 h, and then exposed to 40 μg/ml SNL glycoprotein for another 4 h, the DNA fragments (apoptotic band) were not obviously visible (Fig. 4a, lanes 5–7), whereas, the pretreatment with CAT (100 U/ml) could not block the SNL glycoprotein-induced apoptosis (Fig. 4a, lane 4).

The apoptotic activity of SNL glycoprotein was also evaluated using a H33342/ethidium bromide assay for nuclear staining (Fig. 4b). The results showed that the number of apoptotic cells increased by 20,50, and 78% at 10, 20, and 40 μg/ml SNL glycoprotein for 4 h, compared to the control. In addition, when the cells were pretreated with CAT and caspase inhibitors for 1 h, then exposed to 40 μg/ml SNL glycoprotein for another 4 h, the number of SNL glycoprotein-induced apoptotic cells was reduced by 75,77, and 74% at 50 μM Ac-IETD-CHO, Ac-LETD-CHO, and Ac-DEVD-CHO, compared to 40 μg/ml SNL glycoprotein, whereas, pretreatment with CAT (100 U/ml) did not block the SNL glycoprotein-induced apoptosis.

Discussion

We previously reported that SNL glycoprotein (150-kDa) induces apoptosis through serial signal mediators (PKCα, NF-κB and iNO), which are closely related to oncogenesis, resistance and antiapoptosis in HCT-116 cells [16]. From these results it is suggested that SNL glycoprotein can be used as a chemotherapeutic agent for colorectal cancers. To clarify the mechanism of the apoptotic signal pathway, we further investigated whether SNL glycoprotein can stimulate the activities of mitochondrial apoptotic signal mediators in this cell line (HCT-116 cells). With a purified SNL glycoprotein, we first evaluated its cytotoxic effects on HCT-116 cell proliferation using a neutral red assay (Fig. 1). The cell viability of SNL glycoprotein was generally low, indicating a high cytotoxicity. The results indicated that SNL glycoprotein could induce cytotoxicity in HCT-116 cells in a dose- and time-dependent manner. For cytotoxicity with IC_{50} value for 4 h, it was necessary to use 29 μg/ml SNL glycoprotein in these experiments.

To provide evidence to support a possible mechanism of the cytotoxic effect of SNL glycoprotein, we investigated the effects of SNL glycoprotein on the activities of mitochondrial apoptosis mediators in HCT-116 cells (Fig. 2). Our results in this study indicate that SNL glycoprotein has cytotoxic effects which are deduced from stimulating the activities of caspase-8,

Bid, mitochondrial cytochrome *c*, caspase-9/-3, and PARP proteins in HCT-116 cells. Two major apoptosis pathways in the mammaliancells have been defined as the Fas/TNF-R1 death receptor pathway and the mitochondrial death pathway [6, 23–26]. Bid, one of the linkers between Bcl-2 family members and caspases, connects with the Fas/TNF-R1 death receptor and mitochondrial apoptosis pathways [6]. Following the death receptor activation, cytoplasmic full-length Bid (p22) is cleaved and activated by caspase-8. The activated Bid causes mitochondria cytochrome *c* efflux, caspase-9/-3 activations, and PARP cleavage, and finally results in apoptosis [6]. Although we did not determine the interaction between SNL glycoprotein and death ligands/death receptors, our results demonstrated that caspase-8, through death receptor pathways,is first activated by SNL glycoprotein, and then the activated caspase-8 leads to Bid activation. Taken together, SNL glycoprotein induces apoptosis through mitochondrial pathway, which stimulates theactivities of mitochondrial cytochrome *c*, caspase-9/-3, and PARP protein in HCT-116 cells.

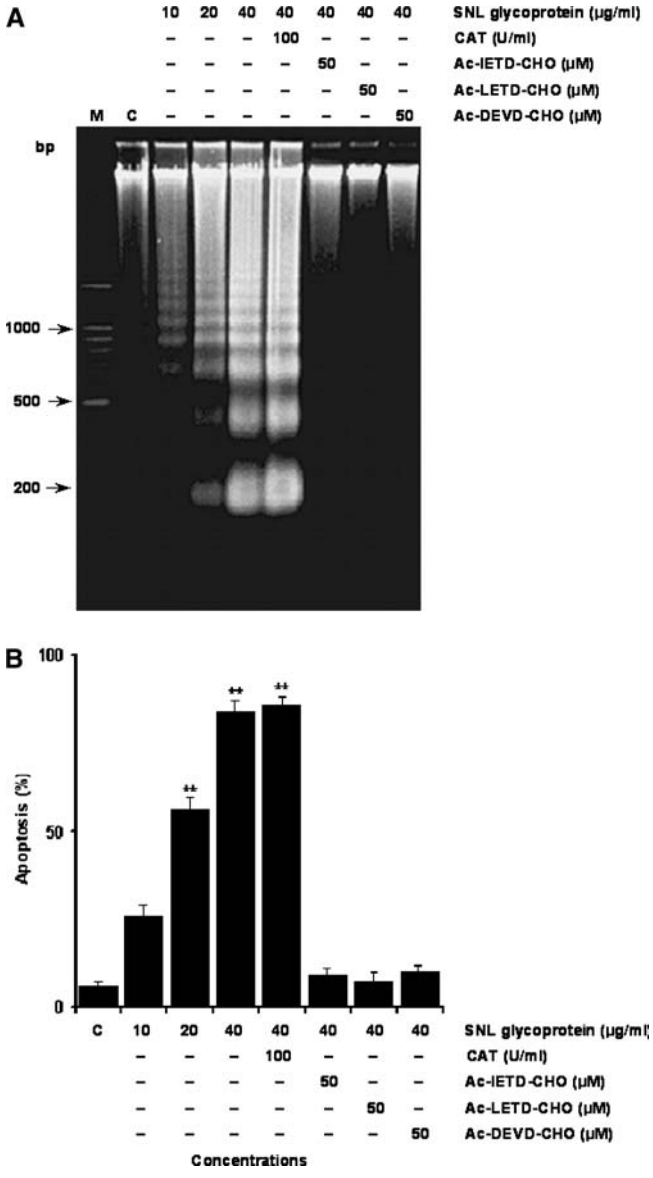

Fig. 4 Apoptotic effects of SNL glycoprotein. **a** HCT-116 cells were treated with various concentrations of SNL glycoprotein (10, 20, and 40 μg/ml) alone for 4 h. Also, cells were pretreated with either 100 U/ml CAT, or 50 μM inhibitors of caspase-8 (Ac-IETD-CHO), caspase-9 (Ac-LETD-CHO), or caspase-3 (Ac-DEVD-CHO) for 1 h and then treated with 40 μg/ml SNL glycoprotein for another 4 h. Electrophoresis was carried out on a 2% agarose gel. *M* molecular weight marker, *C* control, *lane 1* 10 μg/ml SNL glycoprotein, *lane 2* 20 μg/ml SNL glycoprotein, *lane 3* 40 μg/ml SNL glycoprotein, *lane 4* 40 μg/ml SNL glycoprotein + 100 U/ml CAT, *lane 5* 40 μg/ml SNL glycoprotein + 50 μM Ac-IETD-CHO, *lane 6* 40 μg/ml SNL glycoprotein + 50 μM Ac-LETD-CHO, *lane 7* 40 μg/ml SNL glycoprotein + 50 μM Ac-DEVD-CHO. **b** The apoptotic inducing effect of SNL glycoprotein was also evaluated by H33342/ethidium bromide staining. Cells were treated as described above. Results were expressed as a means ± SD of triplicate experiments ($n=9$). ** represents a significant difference between the control and treatments with SNL glycoprotein, $P<0.01$

Reactive oxygen species are constantly generated and eliminated in the biological system, and play important roles in a variety of normal biochemical functions and abnormal pathological processes [27]. Several anticancer agents, including anthracyclines, cisplatin, bleomycin, and irradiation currently used for cancer treatment have been shown to cause increased intracellular ROS generation [28–31]. On the other hand, some studies have reported that anticancer agents such as quercetin and emodin induce apoptosis in HL-60 (promyelocytic leukemia) cells through ROS-independent mechanism [32, 33]. Theresults in the present study indicate that SNL glycoprotein did not increase the levels of intracellular ROS, and antioxidant (exogenic CAT) does not increase SNL glycoprotein-induced cytotoxicity in HCT-116 cells (Fig. 3). Furthermore, theactivity of CAT, among the three intracellular antioxidant enzymes in this study, showed a susceptible dependence on the concentration of SNL glycoprotein in HCT-116 cells. With regards to the relationshipbetween increased intracellular CAT activity and apoptosis, we believe that there are two presumableexplanations. The first is that despite the fact that SNL glycoprotein first stimulates the increase of CATactivity, the amount of induced CAT by SNL glycoprotein is not enough to bring about apoptosis. Thus, itdoes not directly contribute to apoptosis in HCT-116 cells, even in conjunction with SNL glycoprotein. Inthe alternative, the second explanation is that the amount of induced CAT scavenges the intracellular ROS,which might be generated by SNL glycoprotein, and possibly even already existing intracellular ROS. From these results, we speculate that SNL glycoprotein induces cytotoxic activity via a ROS-independent cell death pathway in HCT-116 cells. It should also be noted that H_2O_2, one of the antitumor agents has been shown to inhibit or kill giant cell tumors, breast cancer cells and osteoblasts in vitro, whereas, it has severe side effects on normal cells such as the alteration of cellular functions, genotoxic damage, and tumor initiation through the generation of excessive ROS in vivo [34]. In addition, the

increase of ROS generation in cancer cells can further stimulate cell proliferation, cause DNA mutations, and promote genetic instability and the emergence of drug-resistant cells [35]. Based on these theories, our results demonstrated that SNL glycoprotein does not only stimulate the production of intracellular ROS.Consequently, intracellular ROS is not involved in the cytotoxicity (ROS-independent cytotoxicity) ofHCT-116 cells.

Apoptotic cell death differs from necrosis by distinct morphological and biochemical features, such as chromatin condensation, membrane blebbing, oligo-nucleosomal DNA fragmentation and finally, the breakdown of the cell into a series of smaller units [4, 36]. Although apoptosis induction by SNL glycoprotein has been shown in previous studies, the mitochondiral apoptotic mechanism of SNLglycoprotein has not been confirmed [16]. In the present study, apoptotic DNA fragmentation in size multiples of 180–200 bp was induced by treatment with SNL glycoprotein in HCT-116 cells (Fig. 4a). These results for apoptosis also showed a similarity in the nuclei staining with H33342/ethidium bromide (Fig. 4b). The number of apoptotic cells significantly increased after the addition of SNL glycoprotein (40 μg/ml), compared to the control. Furthermore, our results also showed that the SNL glycoprotein-induced apoptosis was abolished by pretreatments with 50 μM Ac-IETD-CHO, Ac-LETD-CHO, and Ac-DEVD-CHO, whereas pretreatment with CAT (100 U/ml) did not block the SNL glycoprotein-induced apoptosis in HCT-116 cells, demonstrating that the caspase-dependent signaling pathway is involved in SNL glycoprotein-induced apoptosis. Consequently, our findings in this study suggest that SNL glycoprotein stimulates the activation of caspase-8, Bid, cytochrome *c*, caspase-9/-3, and PARP proteins without intracellular ROS production in apoptotic pathways.

In conclusion, the results in this study indicated that apoptosis induced by SNL glycoprotein is attributed to the result of mitochondrial death protein-mediated signals, without intracellular ROS induction in HCT-116 cells. Consequently, we speculate that SNL glycoprotein can be used as a chemotherapeutic agent for colorectal cancers. However, the remaining problem is that further research must be carried out to elucidate the mechanisms of apoptotic signals involving mitogen-activated protein (MAP) kinase, and the expression of apoptotic mRNA at the molecular biological level, such as Bax, p53 and Bcl-2 expressions.

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