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ORIGINAL ARTICLE

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A 150-kDa glycoprotein isolated from *Solanum nigrum* L. has cytotoxic and apoptotic effects by inhibiting the effects of protein kinase C alpha, nuclear factor-kappa B and inducible nitric oxide in HCT-116 cells

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Abstract This study was carried out to investigate the anticancer effects of a 150-kDa glycoprotein isolated from *Solanum nigrum* L. (SNL glycoprotein) on spontaneously and experimentally induced tumor promotion in HCT-116 cells. For spontaneously induced tumor promotion, we evaluated the cytotoxic and apoptotic effects in HCT-116 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), DNA fragmentation, and H33342 and ethidium bromide staining assays. SNL glycoprotein had remarkable, dose-dependent cytotoxic and apoptosis-inducing effects at low concentrations. For experimentally induced tumor promotion, we investigated whether the SNL glycoprotein was able to regulate the activity of protein kinase C alpha (PKCα), the DNA binding activation of nuclear factor-kappa B (NF-κB), the activity of NF-κB protein, and the production of nitric oxide (NO) in HCT-116 cells stimulated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) using electrophoretic mobility shift assays (EMSA), Western blot analysis, and NO assays. As expected, SNL glycoprotein dose-dependently inhibited PKCα translocation, NF-κB DNA binding activity, NF-κB protein activity and NO production in HCT-116 cells stimulated with TPA (61.68 ng/ml, 100 n*M*). Collectively, these results suggest that SNL glycoprotein can induce apoptosis through the modulation of signal mediators. Therefore, we speculate that it could be used as a chemotherapy agent even at low concentrations in HCT-116 cells.

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Introduction

One of the major tasks in colorectal cancer prevention and healing is to understand carcinogenic, metabolic enzymes and the transcription factors for biochemical and clinical therapeutic targets. Also, it is important to know about the regulation of the factors responsible for signal pathways and associated with the proliferation and apoptosis of malignant cells. Recently, studies have focused on the regulation of signal mediators—such as reactive oxygen species (ROS), metabolizing enzymes, cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF-κB) and polyamines in cell proliferation and apoptosis—using natural products [1–3]. However, the problem is that the great majority of chemical compounds, which have been identified as specific agents for killing cancer cells, are also toxic to normal cells [4]. In addition, many colorectal cancers are either inherently resistant to chemotherapy, or they develop resistance during the course of therapy [5]. Therefore, the discovery and identification of new, safe drugs that do not have any severe side effects and to which resistance does not develop have become important goals of research in the biomedical sciences [6].

Apoptosis, or programmed cell death, is the result of a highly complex cascade of cellular events that result in chromatin condensation, DNA fragmentation, cytoplasmic membrane blebbing, and cell shrinkage. Apoptosis involves several death receptors and ligands that eventually have an effect on the pleiotropic transcription factor, NF-κB [7]. NF-κB is a ubiquitously expressed transcriptional regulator that controls the expression of genes involved in immune and inflammatory functions [8]. A variety of stimuli can activate the NF-κB pathway, including proinflammatory cytokines, oxidative stress, ionizing radiation, chemotherapeutic agents and phorbol esters [9]. NF-κB has been implicated in the inhibition

of apoptotic cellular pathways. In other words, NF-κB is constitutively expressed in many tumor cell lines, including colorectal cancer cells [5, 10]. Therefore, the inhibition of NF-κB activities by an agent means that the latter induces apoptosis in colorectal cancer cells.

12-*O*-Tetradecanoylphorbol 13-acetate (TPA) is one of the many well-known tumor promoters that induce pleiotropic resistance against anticancer drugs in human colorectal cancers [11]. TPA is not only a potent activator of protein kinase C alpha (PKCα), which plays a role in the malignant transformation process of colon cells [12], but it also stimulates the expression of the iNOS gene, which promotes tumor proliferation and metastasis [13, 14]. For the purpose of cancer therapeutic strategies, we postulate that any kind of agent is able to block TPA-induced cellular responses and will act as a promising antitumor promoter in colorectal cancers.

For the past three decades, many polysaccharide-protein complexes (glycoproteins) have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants. The biological activities of these glycoproteins have been evaluated in terms of their clinical efficacy, such as immunomodulatory, antimetastatic and anticancer effects, although the mechanisms of the biological actions of glycoprotein are still not completely understood [6]. For instance, polysaccharide-Kureha (PSK, glycoprotein) has been used as a supportive cancer therapeutic agent with positive results seen in gastric, esophageal, lung, breast and colorectal cancers. It has been also been demonstrated that certain glycoproteins stimulate natural killer cells and lymphocytes via modulation of cytokine levels. They also possess an antimetastatic property [15]. Recently, we have found a glycoprotein with an approximate molecular mass of 150 kDa [16], isolated from *Solanum nigrum* L. (SNL), which consists of a large carbohydrate (69.74%) and a protein (30.26%) (data not shown). This active substance, designated as SNL glycoprotein, has a strong scavenging effect against hydroxyl and superoxide anion radicals, and growth inhibition effects against JA221 and XL1-Blue [17]. Moreover, it has been reported that SNL glycoprotein has a cytotoxic effect against the MCF-7 breast cancer cell line, even at low concentrations [16].

Therefore, we isolated only the SNL glycoprotein from SNL and studied its apoptotic effects with respect to its cytotoxicity in HCT-116 cells. We investigated whether purified SNL glycoprotein is able to regulate PKCα activation, NF-κB activities and nitric oxide (NO) production by TPA-induced tumor promotion, and whether it has an apoptosis-inducing effect in HCT-116 cells.

Materials and methods

Materials

All chemicals and reagents used were of the highest purity and all the plastics were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, N.J.). Silica gel (28–200 mesh, 22 Å, S4883), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT, M2128), TPA (P8139), penicillin G (H0474), streptomycin (H0447), bisbenzimide (H33342, B2261), sulfuric acid (S1526), phenol solution (P4682), pronase E (P5147), sodium (*meta*) periodate ($NaIO_4$, S1878), Nω-Nitro-L-arginine methylester hydrochloride (L-NAME, N5751) and trypsin (T4549) were from Sigma (St Louis, Mo.), and minimum essential medium (MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, N.Y.). Proteinase K and oligonucleotides containing NF-κB consensus sequences were obtained from Promega (Madison, Wis.). Mouse monoclonal antibodies of β-actin (A5316) were obtained from Sigma, rabbit anti-p50 (H-119) or anti-p65 (Rel A, SC-109) polyclonal antibodies of NF-κB from Santa Cruz Biotechnology (Santa Cruz, Calif.). Other chemicals and reagents were of the highest quality available.

Preparation of SNL glycoprotein

Samples of *S. nigrum* L. were obtained in October 2002 from Naju in Chonnam province, South Korea. They were identified by Dr H.T. Lim (Chonnam National University), and the seeds were chopped into small portions and soaked in 99% ethanol for several months in a dark basement. The ethanol extract was filtered through Whatman filter paper (no. 2) and concentrated with a rotary evaporator (B465; Bunchi, Switzerland). The concentrated solution was dried in a freeze-dryer (SFDS06; Sam Won, Seoul, Korea). Dried-crude ethanol extract, 5 g dissolved in distilled water, was applied to a silica gel column (4×28 cm, 28–200 mesh, 22 Å). An ordered elution was performed using distilled water, 70% ethanol, absolute ethanol, and 5% acetic acid. Only the 70% ethanol eluted solution was collected and lyophilized to 2.0 g (40% of the initial amount), because it had shown a biological function in trials before the experiment. The dried powder was dissolved again with distilled water. The solution was precipitated with 80% ammonium sulfate and then dialyzed with a dialysis membrane (Spectra/por, MWCO 6000-8000) against 20 m*M* Tris-Cl (pH 7.4) at 4°C overnight. After dialysis, the solution was dried in a freeze-dryer and stored at −70°C. After electrophoresis, the SNL glycoprotein was eluted with an electro-eluter (Mini Whole Gel Eluter; Bio-Rad, Hercules, Calif.). The final amount of SNL glycoprotein was 4 mg (0.08%) from the initial SNL sample.

Determination of total carbohydrate and protein content

The carbohydrate content of the SNL glycoprotein was determined using the phenol-sulfuric method [18], with glucose as the standard. An appropriate amount of SNL

glycoprotein was dissolved in 2 ml ddH_2O, mixed well with 1 ml 5% phenol, and then added to 5 ml sulfuric acid. The mixture was incubated at room temperature for 10 min and transferred to a 25–30°C water bath for 10–20 min. The absorbance of the carbohydrate content was measured at 490 nm. To determine the protein content of the SNL glycoprotein, the method of Lowry et al. [19] was used, with bovine serum albumin as the standard. A reading at 280 nm was also used to determine the protein content of SNL glycoprotein.

Treatment with pronase E and $NaIO_4$

The SNL glycoproteins were treated with pronase E [20] and $NaIO_4$ [21] to destroy the protein and the carbohydrate parts of the whole SNL glycoprotein, respectively. Briefly, the SNL glycoproteins were incubated with 0.4 mg pronase E at 30°C in 4 ml 0.1 *M* Tris-HCl buffer (pH 8.0) containing 50 m*M* $CaCl_2$. After 36 h of incubation, an additional 0.2 mg pronase E was added and the incubation was continued for another 36 h. Thereafter, the reaction mixtures were heated at 100°C for 10 min to inactivate the pronase E, and then dialyzed against PBS before being passed through a Sephadex G-150 column. In addition, the SNL glycoproteins were incubated with 100 µl 0.1 *M* $NaIO_4$ at 25°C for 4 h, at which point 250 µl of 20% ethylene glycol was added before the samples were dialyzed and eluted as described above. To compare the biological activities of the protein part with the carbohydrate part of the SNL glycoprotein, the cytotoxic effects of these samples on HCT-116 and NIH/3T3 cells were tested.

Cell culture

HCT-116 cells, human colorectal carcinoma cells, and NIH/3T3 cells, mouse embryonic cells, were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). HCT-116 cells and NIH/3T3 cells were incubated in MEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere containing 5% CO_2. 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 bottomed plates. The final volumes were 2 ml/dish on the 35-mm culture dishes and 100 µl/well on the 96-well flat bottomed plates.

Cytotoxicity of SNL glycoprotein

The cellular cytotoxicity induced by treatment with SNL glycoprotein was measured using the MTT assay [22]. Cells were treated with or without SNL glycoprotein at different concentrations (0–40 µg/ml) for 4 h. Then 2 µl of the MTT solution (5 mg/ml in PBS as stock solution) was added to each well. The cells were then further incubated for 4 h at 37°C. After removing the medium completely, 50 µl acidic isopropanol was added to each well, and the plates were read at 560 nm using a SpectraCount ELISA reader (Packard Instruments, Downers, Ill.).

Preparation of cytosolic and nucleic protein 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 MEM medium for a further 4 h. After this step, HCT-116 cells were treated with 100 n*M* TPA or cotreated with SNL glycoprotein and TPA for the indicated times. The cells were rinsed twice with PBS after removing the medium and scraped in 500 µl of hypotonic buffer A (10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* DTT, 0.5 m*M* PMSF, 1.5 m*M* $MgCl_2$ and 0.5% NP-40), followed by centrifugation at 3000 *g* for 5 min to separate the cytosolic and nucleic protein extracts. The supernatant and pellet were designated as cytosolic and nucleic protein extracts separately. For the immunoblotting of PKCα, the isolated cytosolic protein extracts were then resuspended in 100 µl buffer B (20 m*M* HEPES, pH 7.9, 0.4 *M* NaCl, 0.1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT, 1 m*M* PMSF, 1.5 m*M* $MgCl_2$ and 0.5% NP-40) containing a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) and centrifuged at 12,000 *g* for 15 min at 4°C. For immunoblotting and electrophoretic mobility shift assay (EMSA) of NF-κB, the pelleted nuclear protein extracts were resuspended in 200 µl buffer B, containing a protease inhibitor cocktail, and centrifuged at 12,000 *g* for 1 h at 4°C. The amount of protein was measured by the method of Lowry et al. The cytosolic and nucleic proteins were aliquoted (2 µg/µl) approximately and stored at −70°C prior to use.

Western blot analysis

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, followed by the replacement of the medium with serum-free MEM for another 4 h. The total cytosolic proteins and nuclear proteins were isolated from HCT-116 cells (1×10^6 cells/ml) and treated with TPA alone or with TPA in the presence of various concentrations of SNL glycoprotein for 2 h. For immunoblotting of PKCα and NF-κB, 20 µg/ml of sample protein was separated in a 10% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a Mini-PROTAN II electrophoresis cell (Bio-Rad). After electrophoresis, proteins were electroblotted onto nitrocellulose membranes (Millipore, Bedford, Mass.). The membranes were incubated for 1 h at room temperature in TBS-T solution (10 mM Tris-HCl, pH 7.6, 150 m*M* NaCl and 0.1% v/v Tween-20) containing 5% w/v

non-fat dried milk (NFDM). The membranes were subsequently incubated for 2 h at room temperature with a 1:100 dilution of mouse monoclonal antibodies (PKC, Ab-2; Oncogene Science, Manhasset, N.Y.) and a 1:3000 dilution of rabbit polyclonal antibodies [NF-κBp50, H-119; Santa Cruz Biotechnology, Santa Cruz, Calif.) and a 1:3000 dilution of mouse monoclonal antibodies (β-actin, A5316; St Louis, Mo.) in TBS-T solution containing 5% NFDM. After three washes with TBS-T, the membranes were incubated for 1 h at room temperature in 1:10,000 dilutions of alkaline phosphatase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) in TBS-T containing 5% NFDM. The protein bands were visualized by incubation with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma).

Electrophoretic mobility shift assay

To make the double-stranded oligonucleotide, each strand of NF-κB oligonucleotide was annealed by heating at 37°C for 30 min. It was then labeled with [α-^{32}P]dCTP (0.25 mCi; Amersham Pharmacia Biotech, Little Chalfont, UK) by Klenow polymerase and purified using a QIAquick nucleotide removal kit according to the manufacturer's protocol (LRS Laboratory, QIAGEN Distributor, Seoul, Korea). The following NF-κB oligonucleotide sequences were used for probing: (NF-κB sequence) 5′-AAG GTC CAG GCC AGG GAA AGT CCC GGA GCA CAG G-3′; 3′-A GGT GCC GTC CCT TTC AGG GCC TCG TGT CCG AAG-5′.

The DNA-protein binding reaction was performed by incubation of the NF-κB probes and 10 μg nuclear protein extracts and 0.5 μg/μl poly-dI/dC (Sigma) in a binding buffer comprising 0.2 *M* DTT, 20 mg/ml BSA, buffer D (20 m*M* HEPES, 20% glycerol, 100 m*M* KCl, 0.5 m*M* EDTA, 0.25% NP-40) and buffer F (20% Ficoll 400, 100 m*M* HEPES, 300 m*M* KCl) at room temperature for 1–2 h. To verify the composition of NF-κB, supershift assays were carried out by preincubation with 1 μg rabbit anti-p50 or anti-p65 polyclonal antibodies (NF-κB p50, H-119; NF-κB p65, Rel A, SC-109; Santa Cruz Biotechnology) prior to the addition of NF-κB probe in the DNA-protein binding reaction. The DNA-protein complexes were resolved by applying 4% non-denaturing polyacrylamide gel in 0.5× TBE (45 m*M* Tris-borate, 1 m*M* EDTA). Electrophoresis was carried out at 200 V for 3 h in a cold room. Gels were then dried on 3M blotting papers (Whatman) and exposed to X-ray film at −70°C overnight.

Measurement of nitric oxide production

HCT-116 cells (1×10^6 cells/ml) were treated with either SNL glycoprotein (20 and 40 μg/ml) or TPA (50 and 100 n*M*) alone. To analyze the inhibitory effect of NO, the cells were also treated with either SNL glycoproteins (20 and 40 μg/ml) or L-NAME (1 and 2 μ*M*) in the presence of TPA (100 n*M*) for 24 h in the 96-well multiple plate. After this step, cells were centrifuged at 1000 *g* for 10 min and supernatants were collected. NO production was measured as the concentration of nitrite (NO_2^-) by the method of Green et al. [23]. Supernatants (50 μl) were mixed with 100 μl 1% sulfanilamide and 100 μl 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid at room temperature for 5 min. Absorbance was measured at 540 nm with a MicroReader (Hyperion, USA). The amounts of NO_2^- were quantified using $NaNO_2$ as a standard curve.

DNA fragmentation

HCT-116 cells (1×10^6 cells/ml) were apportioned into 35-mm culture dishes and maintained at 37°C in an incubator for 48 h, followed by replacement of the medium with serum-free MEM for another 4 h. Cells were treated with or without SNL glycoprotein at different concentrations (0–40 μg/ml) for 4 h. For DNA extraction, HCT-116 cells were harvested by centrifugation at 2000 *g* for 5 min and washed once 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 lysis buffer (50 m*M* Tris-HCl, pH 8.0, 20 m*M* EDTA, pH 8.0, 1% NP-40, and 20 μl 10% SDS) and incubated at 65°C for 30 min. After that, 100 μl 8 *M* potassium acetate was added and the suspensions 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 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0) and incubated at 37°C for 30 min with 1 μl RNase A (10 μg/ml) and 1 μl proteinase K (100 μg/ml). Electrophoresis was carried out on a 2% agarose gel at 100 V for 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.

H33342 and ethidium bromide staining

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 MEM 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 MEM was collected and mixed with 1 μl 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,

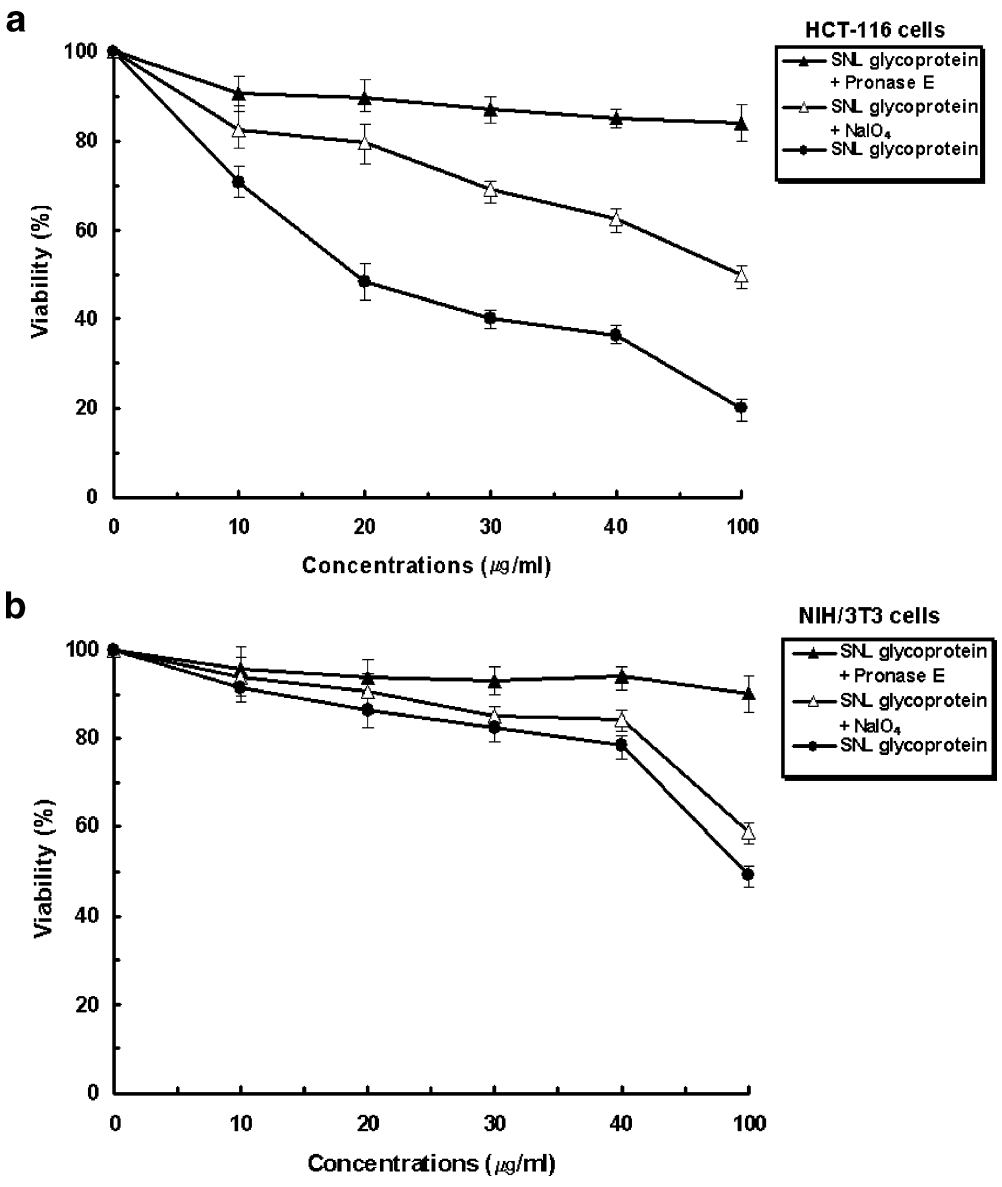

Fig. 1 Cytotoxic effects of SNL glycoprotein. For cytotoxicity, two cell lines (**a** HCT-116, **b** NIH/3T3) were treated with SNL glycoprotein (0–100 μg/ml) alone. The cells were treated with either pronase E or $NaIO_4$ in the presence of SNL glycoprotein at 37°C in an atmosphere containing 5% CO_2 for 4 h. Cell viabilities were evaluated by the MTT assay as described in "Materials and methods." The values are percentages of the control value. Each point represents the mean ± SED of triplicate experiments. At 40 μg/ml SNL glycoprotein, the viabilities are significantly different between treatments in both cell lines ($P < 0.05$)

Germany). At least 200 cells were counted and the number of fragmented nuclei, increased cytoplasm, and condensed chromatin reliably indicated that apoptosis had occurred. The percentage of apoptotic cells was calculated using the following formula:

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

Experiments were performed three times and each value represents the mean ± SED of triplicate experiments.

Statistical analysis

All experiments were done in triplicate, and the results are expressed as means ± SED. A one-way analysis of variance (ANOVA) was used for multiple comparisons (SPSS program, ver 10.0).

Results

Cytotoxicities of SNL glycoprotein

To determine the cytotoxic effects of SNL glycoprotein, HCT-116 cells and NIH/3T3 cells were incubated either in the absence or in the presence of SNL glycoprotein (Fig. 1). When SNL glycoprotein was added to the culture medium of HCT-116 cells for 4 h, the growth of HCT-116 cells was inhibited in a dose-dependent manner. For instance, the viability of HCT-116 cells was 71%, 48%, 40%, 37% and 20% after the addition of 10, 20, 30, 40 and 100 μg/ml SNL glycoprotein, respectively (Fig. 1a). In contrast, the viability of NIH/3T3 cells was more than 79% after the addition of 40 μg/ml SNL glycoprotein. However, after the addition of 100 μg/ml SNL glycoprotein, it was 49% compared to the control (Fig. 1b). Therefore, we chose to maintain the

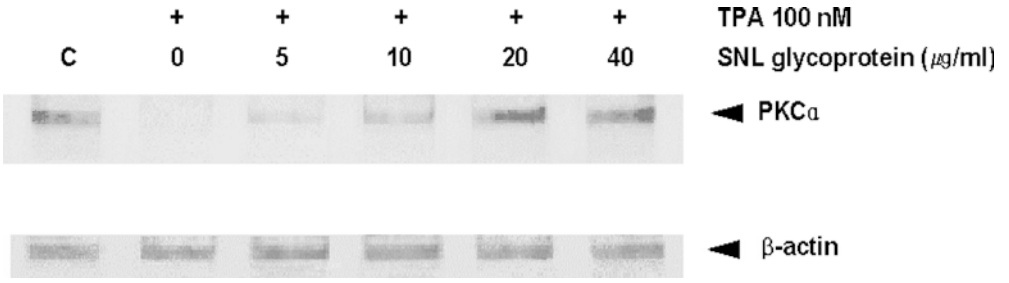

Fig. 2 Inhibitory effect of SNL glycoprotein on PKCα activity. Serum-starved HCT-116 cells were treated with 100 n*M* TPA alone, or with 100 n*M* TPA and various concentrations (5, 10, 20 and 40 μg/ml) of SNL glycoprotein for 2 h, and cytosolic protein extracts were prepared as described in "Materials and methods." Detection of PKCα protein was performed by Western blotting using an anti-PKCα monoclonal antibody. *Lane C* control, *lane 1* 100 n*M* TPA alone, *lane 2* 5 μg/ml SNL glycoprotein + TPA, *lane 3* 10 μg/ml SNL glycoprotein + TPA, *lane 4* 20 μg/ml SNL glycoprotein + TPA, *lane 5* 40 μg/ml SNL glycoprotein + TPA. *β*-Actin was used as an internal control

concentration of SNL glycoprotein between 0 and 40 μg/ml in this experiment.

To compare the activities of the protein and carbohydrate parts of the whole SNL glycoprotein, SNL glycoproteins were treated with pronase E to induce the hydrolysis of the protein or $NaIO_4$ to induce the oxidation of the carbohydrate (Fig. 1). As shown in Fig. 1b, when SNL glycoproteins, cotreated with Pronase E and $NaIO_4$, were added to the culture medium of NIH/3T3 cells, cytotoxic effects were not seen. After the addition of 40 μg/ml SNL glycoprotein, cell survival was more than 80% after cotreatment with Pronase E and $NaIO_4$. As shown in Fig. 1a, however, the cytotoxic effect after treatment with $NaIO_4$ was higher than after treatment with Pronase E in HCT-116 cells in a dose-dependent manner, suggesting that the active site of SNL glycoprotein is the protein part.

Inhibitory effect of SNL glycoprotein on TPA-induced PKCα activity

Since TPA is an activator of PKCα, we studied the inhibitory effects of the SNL glycoprotein on TPA-induced PKCα activity using Western blotting with cytosolic protein extracts from HCT-116 cells. TPA treatment caused the translocation of PKCα to the membrane and the intensities of PKCα were reduced at the cytoplasmic level. This indicates that addition of SNL glycoprotein to the cells pretreated with TPA interfered with the membrane translocation of PKCα. Therefore, its level in the cytoplasmic is only a fraction, as indicated in Fig. 2. Translocation of the PKCα protein from the cytosol to the membrane is a well-known biomarker of TPA-induced PKCα activation. After treatment with TPA at 100 n*M*, PKCα protein was definitely translocated from the cytosolic protein to the membrane protein, while treatment with 20 and 40 μg/ml SNL glycoprotein showed a marked blocking effect of this PKCα translocation. These findings indicate that SNL glycoprotein is a potential inhibitor of TPA-induced PKC activation in HCT-116 cells.

Inhibitory effect of SNL glycoprotein on TPA-induced DNA binding activity of NF-κB (EMSA)

DNA binding activities of NF-κB are located downstream of PKCα activation in the TPA-induced signaling pathway, so we investigated whether SNL glycoprotein could suppress the DNA binding activities of NF-κB by performing an EMSA (Fig. 3). When quiescent HCT-116 cells were treated with various concentrations of TPA (10–200 n*M*) for 2 h, the DNA binding activity of NF-κB was induced at all concentrations and had its maximal activity at 100 n*M* TPA (Fig. 3a). Figure 3b shows the DNA binding activities of NF-κB at 100 n*M* TPA treatment for various times (0.5–4 h). TPA treatment for 2 h resulted in the strongest DNA-binding activity of NF-κB compared to other treatment times. Based on these results, the experimental conditions in this study were fixed at 100 n*M* TPA and 2 h for EMSA. To determine the inhibitory effect of SNL glycoprotein on the DNA-binding activity of NF-κB in HCT-116 cells, cells were exposed to 100 n*M* TPA for 2 h in the absence or presence of SNL glycoprotein. SNL glycoprotein inhibited the DNA-binding activity of NF-κB in a dose-dependent manner (Fig. 3c). In addition, supershift assays were performed with polyclonal antibodies recognizing NF-κB (p50) (Fig. 3d, lane 2) or NF-κB (p65) (Fig. 3d, lane 3) subunits to confirm the specificity of the DNA-binding activity of NF-κB (Fig. 3d). After incubation of TPA-induced nuclear extracts with the antibodies, the NF-κB band was shifted to a higher molecular weight, suggesting that the TPA-induced DNA-binding activity of the NF-κB complex exists as a heterodimer of the above subunits.

Inhibitory effect of SNL glycoprotein on TPA-induced NF-κB activity (Western blot)

Since the transcription factor NF-κB plays a pivotal role in the regulation of apoptosis, we examined the effect of SNL glycoprotein on TPA-induced NF-κB (p50) activity in HCT-116 cells. In this experiment, we used only a nuclear protein to analyze NF-κB activity, because the activated NF-κB translocates from the cytoplasm into

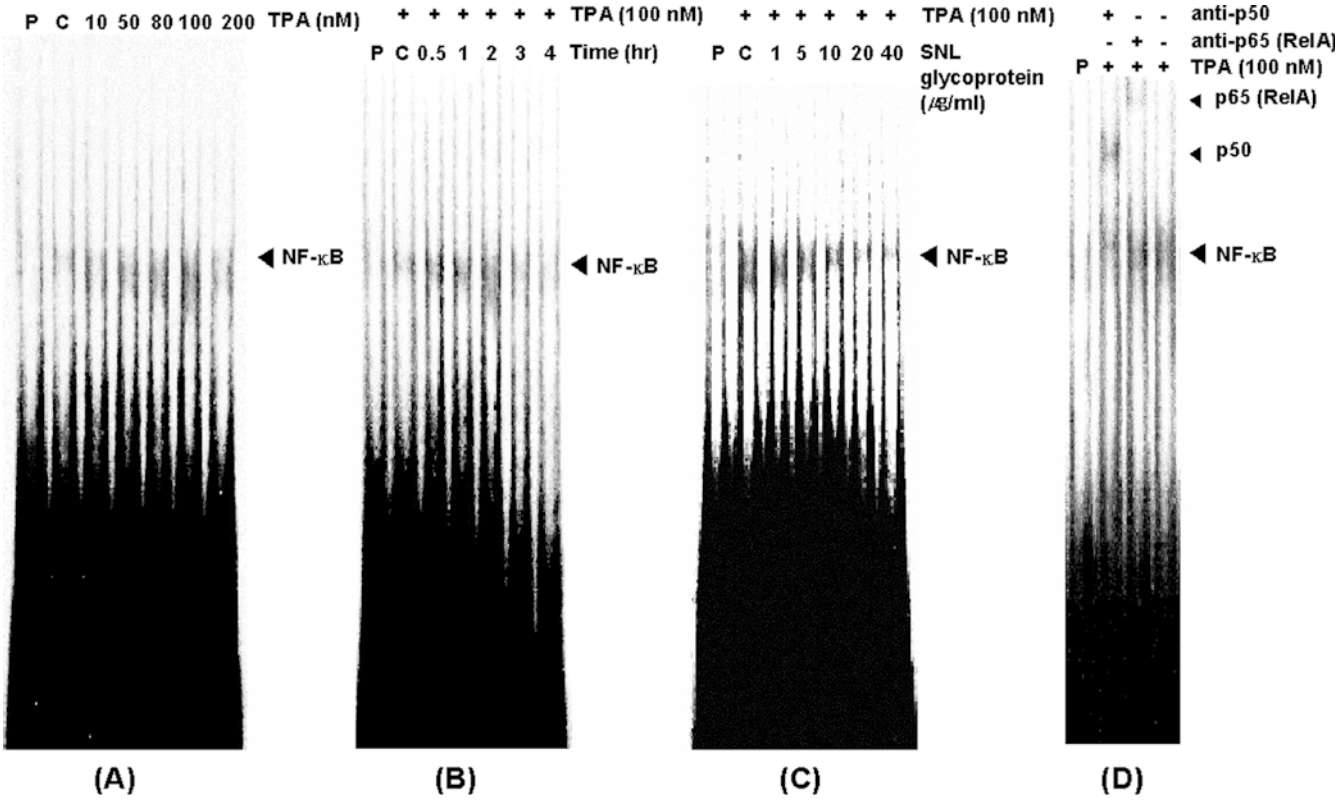

Fig. 3 Inhibitory effect of SNL glycoprotein on TPA-induced DNA-binding activity of NF-κB (EMSA). Serum-starved HCT-116 cells were treated with (**a**) various concentrations of TPA (10, 50, 80, 100 and 200 n*M*) for 2 h, or (**b**) 100 n*M* TPA for various times (0.5, 1, 2, 3 and 4 h). The DNA-binding activity of NF-κB was determined by EMSA. (**c**) Cells were treated with 100 n*M* TPA alone or with various concentrations (1, 5, 10, 20 and 40 μg/ml) of SNL glycoproteins in the presence of 100 n*M* TPA for 2 h. For the supershift assay (**d**), cells were treated with 100 n*M* TPA only or with an antibody against p50 (*lane 2*) or p65 (*lane 3*) in the presence of 100 n*M* TPA for 2 h. *Lane 1* probe alone, *lane C* control

the nuclei of the cells upon activation induced by TPA treatment. As shown in Fig. 4, cells were exposed to 100 n*M* TPA for 2 h in the absence or presence of SNL glycoprotein. In general, when cells were treated without SNL glycoprotein and TPA as a control, NF-κB (p50) was constitutively expressed in HCT-116 (Fig. 4, lane 1). Also, when HCT-116 cells were treated with TPA for 2 h, NF-κB (p50) had its maximal activity at 100 n*M* TPA (Fig. 4, lane 2). However, NF-κB (p50) activities, after treatment with 100 n*M* TPA, were inhibited by SNL glycoprotein in a dose-dependent manner. In addition, activities after treatment with 20 and 40 μg/ml SNL glycoprotein were lower than the activity of the control. This means that the amount of NF-κB (p50) activated by TPA in the cytoplasm was reduced by SNL glycoprotein and was accompanied by its translocation into the nuclei of the cells. The intensity of the NF-κB bands gradually reduced in nuclear extract fractions upon SNL glycoprotein treatment, compared to that following TPA treatment alone (Fig. 4).

Inhibitory effect of SNL glycoprotein on TPA-induced NO production

Next, we examined the effect of SNL glycoprotein on TPA-induced NO production, because increased iNOS expression and/or activity has been reported in several cancers, including colon cancer, and TPA is known to stimulate expression of the iNOS genes [13, 14]. As shown in Fig. 5, when HCT-116 cells were treated with TPA (50 and 100 n*M*), SNL glycoprotein (20 and 40 μg/ml), or TPA (100 n*M*) in the presence of SNL glycoprotein (20 and 40 μg/ml) for 24 h in a 96-well multiple plate, the production of NO was 66 and 82 μ*M* at 50 and 100 n*M* TPA, respectively. On the other hand, NO production was 70 and 49 μ*M* at 20 and 40 μg/ml SNL glycoprotein in the presence of TPA (100 n*M*). In addition, we also compared the levels of inhibition by SNL glycoprotein of TPA-induced NO production with the specific competitive iNOS inhibitor, L-NAME (1 and 2 μ*M*). NO production was 48.8 and 30 μ*M* at 1 and 2 μ*M* L-NAME. The TPA-induced NO production after the addition of 1 and 2 μ*M* L-NAME was clearly inhibited by 33.2 and 52 μ*M*, respectively, compared to treatment with TPA (100 n*M*) alone. The inhibitory effect of 1 μ*M* L-NAME in the presence of TPA (100 n*M*) corresponds to the effect at 40 μg/ml SNL glycoprotein. These findings indicate that TPA-induced NO production is blocked by treatment with SNL glycoprotein in HCT-116 cells. In other words, SNL glycoprotein interfered in the transfer of the TPA signal, necessary for NO production.

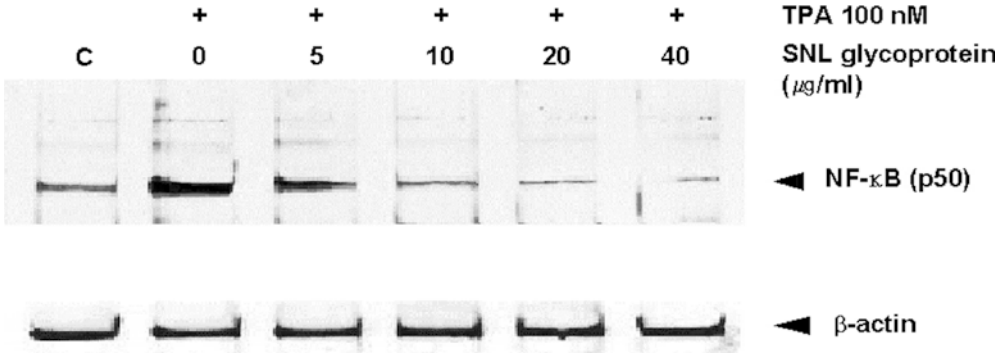

Fig. 4 Inhibitory effect of SNL glycoprotein on TPA-induced NF-κB activity (Western blot). Serum-starved HCT-116 cells were treated with 100 n*M* TPA alone, or with 100 n*M* TPA and various concentrations (5, 10, 20 and 40 μg/ml) of SNL glycoprotein for 2 h, and nucleic protein extracts were prepared as described in "Materials and methods." Detection of NF-κB nuclear protein (p50) was performed by Western blotting using an anti-NF-κB p50 polyclonal antibody. *Lane C* control, *lane 1* 100 n*M* TPA alone, *lane 2* 5 μg/ml SNL glycoprotein + TPA, *lane 3* 10 μg/ml SNL glycoprotein + TPA, *lane 4* 20 μg/ml SNL glycoprotein + TPA, *lane 5* 40 μg/ml SNL glycoprotein + TPA. *β*-Actin was used as an internal control

Apoptosis-inducing effect of SNL glycoprotein

To evaluate the apoptotic effect of SNL glycoprotein in HCT-116 cells, cells were treated with various concentrations of SNL glycoprotein for 4 h (Fig. 6). In this experiment, the total volume of medium in each well was 1 ml and SNL glycoprotein (1, 5, 10, 20 and 40 μg/ml) was added into the culture medium. The SNL glycoprotein induced nucleosomal DNA fragmentation, the cells showing the characteristic features of apoptosis, in a dose-dependent manner. Moreover, as shown in Table 1 and Fig. 6, inducing effects of SNL glycoprotein on apoptosis in nuclear staining with bisbenzimide and ethidium bromide were also observed. For instance, when the HCT-116 cells were treated with 1, 5, 10, 20 and 40 μg/ml SNL glycoprotein, 28%, 45%, 62%, 74% and 88% of cells were apoptotic, respectively, compared to the control cells (Table 1). The intensity of fragmented DNA bands gradually decreased in correspondence with the SNL glycoprotein concentrations, as indicated by the percentages of apoptotic cells which were 30%, 50% and 62% with 10, 20 and 40 μg/ml SNL glycoprotein.

Discussion

The enhancement or potentiation of host defense mechanisms in cancer therapy has been recognized as an optimal means of the inhibition of tumor growth without harming normal cells. From this standpoint, numerous glycoproteins have been screened for their anticancer activity and have been successfully used in

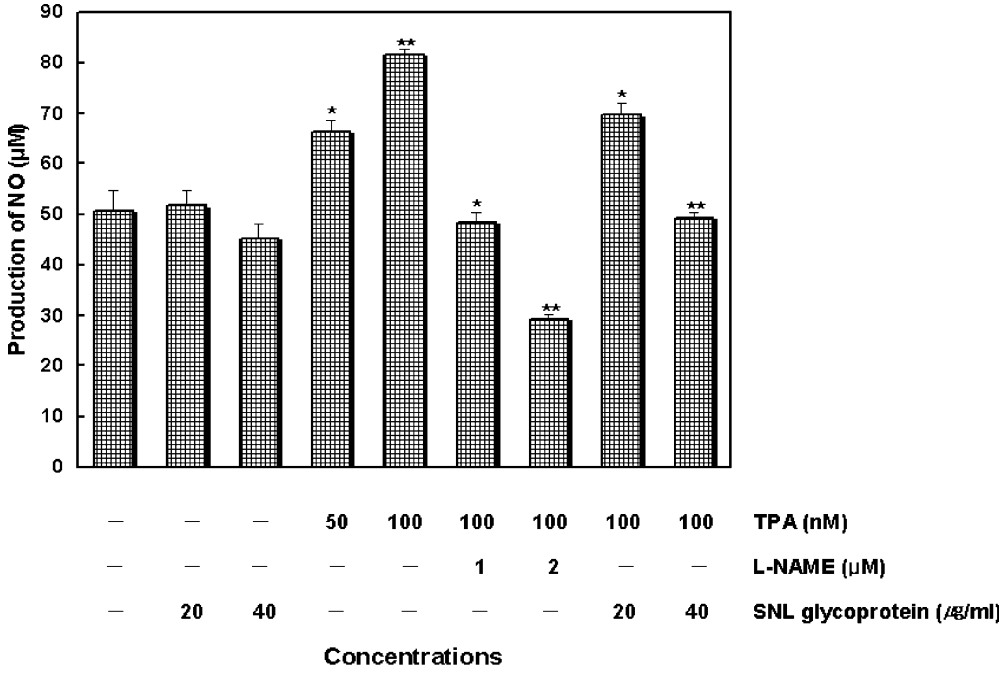

Fig. 5 Inhibitory effect of SNL glycoprotein on TPA-induced NO production. Serum-starved HCT-116 cells were treated with either SNL glycoprotein (20 and 40 μg/ml) or TPA (50 and 100 n*M*) alone, respectively. For the inhibitory effect of NO, the cells were treated with either SNL glycoproteins (20 and 40 μg/ml) or L-NAME (1 and 2 μ*M*) in the presence of TPA (100 n*M*) for 24 h. The supernatants were used for measurements of NO production as described in "Materials and methods." The concentration of NO was assessed using a standard curve of $NaNO_2$. Each bar represents the mean + SED of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, between treatments

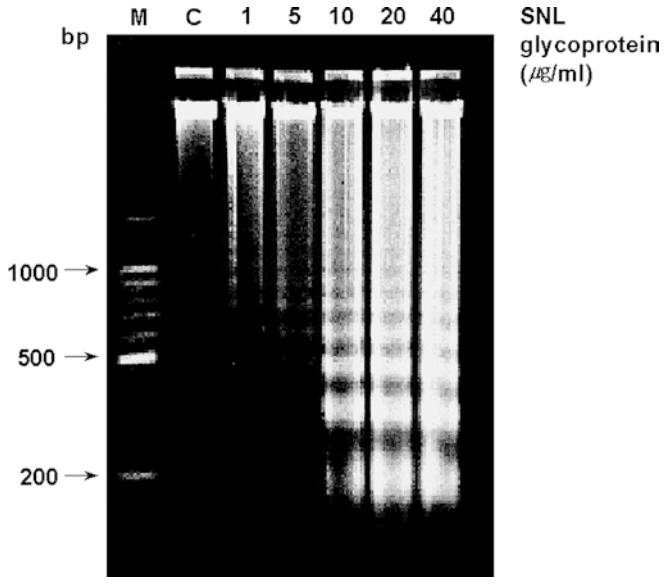

Fig. 6 Apoptosis-inducing effect of SNL glycoprotein on HCT-116 cells. Serum-starved HCT-116 cells were treated with various concentrations (1, 5, 10, 20 and 40 μg/ml) of SNL glycoprotein for 4 h. Electrophoresis was carried out on a 2% agarose gel. *Lane M* molecular weight marker, *lane C* control, *lane 1* 1 μg/ml SNL glycoprotein, *lane 2* 5 μg/ml SNL glycoprotein, *lane 3* 10 μg/ml SNL glycoprotein, *lane 4* 20 μg/ml SNL glycoprotein, *lane 5* 40 μg/ml SNL glycoprotein

clinical treatments. For instance, PSK and PSP (polysaccharide-peptide, glycoprotein) have been reported to inhibit growth and DNA synthesis in vitro in various cell lines such as leukemia P388, sarcoma 180 and human cancer cell lines, including lung, breast and gastric cancers [6]. Moreover, these glycoproteins have antioxidative, antimicrobial, antiviral, immunomodulatory and antimetastatic activities [16, 17, 24–26].

Recently, we have found a glycoprotein isolated from *S. nigrum* L., which has been used as a remedy in various cancers, and as a diuretic and an antipyretic in Korea and China [27], and has been reported to have several biological activities [16, 17, 28]. In this study, we investigated the inhibitory effects of SNL glycoprotein on HCT-116 cell proliferation in order to elucidate its anticancer activity. Cell viability following treatment with SNL glycoprotein was generally low, and ability to lower cell viability is associated with high cytotoxicity. As shown in Fig. 1, SNL glycoprotein not only inhibited the growth of HCT-116 cells, but also had a dose-dependent toxic effect on proliferation of NIH/3T3 cells. The cytotoxicity of SNL glycoprotein showed a similar trend with each different treatment in HCT-116 cells and NIH/3T3 cells. The cytotoxicity of intact SNL glycoprotein was higher than after treatment with either pronase E or $NaIO_4$. Furthermore, because the cytotoxicity after $NaIO_4$ treatment was higher than after pronase E treatment, the specific anticancer active site of SNL glycoprotein is postulated to be located in the protein part (Fig. 1a).

Table 1 Apoptosis-inducing effect of SNL glycoprotein on HCT-116 cells (nuclei staining). Serum-starved HCT-116 cells (5×10^5 cells/ml) were treated with various concentrations (1, 5, 10, 20 and 40 μg/ml) of SNL glycoprotein for 4 h. The apoptosis-inducing effects of SNL glycoprotein were evaluated by H33342/ethidium bromide staining. The values presented are means ± SED ($n=3$)

Treatment	Number of apoptotic cells (%)
Control	0 + 3 (0)
SNL glycoprotein 1 μg/ml	56 ± 7 (28)
SNL glycoprotein 5 μg/ml	90 ± 5 (45)
SNL glycoprotein 10 μg/ml	124 ± 4* (62)
SNL glycoprotein 20 μg/ml	148 ± 5* (74)
SNL glycoprotein 40 μg/ml	172 ± 6* (88)

*$P < 0.05$, difference between SNL glycoprotein treatments

SNL glycoprotein also showed stronger cytotoxicity in HCT-116 cells than in NIH/3T3 cells. Two possible explanations are either that SNL glycoprotein inhibits the activity of the multidrug resistance (MDR) receptor on cancer cell membranes or that the response to exogenic signals from SNL glycoprotein is susceptible to a mutant signal pathway in HCT-116 cells. For instance, the cell viability was 38% in HCT-116 cells, but it was 80% in NIH/3T3 cells after treatment with 40 μg/ml SNL glycoprotein. Although it is difficult to correlate the structure and anticancer activity of glycoprotein, a possible relationship might be justified, because it has been shown that the molecular mass, the degree of branching, conformation, enzymatic digestions and the chemical modification of glycoproteins can significantly affect their anticancer and immunomodulatory activities [29–32]. Also, it has been suggested that glycoproteins with anticancer activity may have some unique structural features generated from the involvement of protein portions, which are associated with the expression of the anticancer activity and/or unique structural configurations, including carbohydrate to carbohydrate linkages, which may be important for the preservation of the structure and activity of the glycoprotein.

We investigated the inhibitory effects of SNL glycoprotein on TPA-induced PKCα activation using cytosolic protein extracts from HCT-116 cells to provide evidence of the mechanism of the cytotoxic effect of SNL glycoprotein (Fig. 2), because TPA is not only a tumor promoter, but also an activator of PKCα [11, 12]. PKCα, which is part of a multigene family of phospholipid-dependent serine-threonine kinases, plays a central role in signal transduction and has been implicated in tumor progression and drug resistance of colorectal cancers [33, 34]. Specifically, PKCα activates the MDR-1 gene product, gp170, by phosphorylation and increases the efflux of drugs from the cell [35]. Therefore, PKCα is a potential target for chemoprevention and therapy of colorectal cancers. In this experiment, TPA-induced PKCα translocation from the cytosol to the membrane was markedly blocked by treatment with SNL glycoprotein, suggesting that SNL glycoprotein is a potent inhibitor of TPA-induced PKCα activation in HCT-116 cells.

To elucidate the molecular mechanisms underlying the suppression of TPA-induced PKCα translocation by

SNL glycoprotein, we examined the effect of this glycoprotein on the activation of NF-κB, which is a ubiquitous eukaryotic transcription factor known to be involved downstream of the TPA-induced PKCα signal transduction pathway [36]. Generally, NF-κB, a nuclear protein of the Rel oncogene family (e.g., Rel A/p65, p50, p52, c-Rel, v-Rel and Rel B), can exist as either a heterodimeric or homodimeric complex, but it is classically composed predominantly of p50 and Rel A/p65 subunits. In a highly simplified scenario of NF-κB activation by an appropriate stimulus, I-κB becomes phosphorylated, and this results in its rapid ubiquitination and subsequent proteolysis by the 26s proteasome. Proteasome-dependent degradation of I-κB causes the translocation of NF-κB to the nucleus, where it initiates gene expression [37]. In particular, since NF-κB has been implicated in cellular proliferation, oncogenesis and malignant transformation, TPA-induced NF-κB activation has been associated with the proliferation and survival of colorectal cancers [38–40] and also has been postulated to cause resistance to apoptosis [41, 42].

Therefore, the inhibition of TPA-induced activation of NF-κB by an agent is closely related to its anticancer, antiresistance and apoptotic activities in colorectal cancer cells. As shown in Fig. 3, TPA-induced activation of NF-κB binding activities in EMSA was generally inhibited by treatment with SNL glycoprotein. Interestingly, the results of this experiment showed that the NF-κB complex exists as a heterodimer with p50 and p65 subunits. Moreover, the inhibitory effects of SNL glycoprotein in Western blot assays showed TPA-induced activation of NF-κB (p50) protein activity, as expected (Fig. 4). Taken together, these findings suggest that suppression of NF-κB DNA binding and NF-κB protein (p50) activities by SNL glycoprotein are likely to contribute to its anticancer, antiresistance and apoptotic activities in HCT-116 cells.

In addition, we evaluated the inhibitory effect of SNL glycoprotein on TPA-induced NO production, because TPA stimulates the expression and/or activity of the iNOS promoter-dependent gene through the activation of NF-κB [13]. Increased iNOS expression and/or activity have been reported in several cancer cells including ovarian, breast and colorectal cancer cells [43–45]. Although the expression of iNOS in colorectal carcinoma tissue is still a controversial issue, numerous studies have shown that the functional roles of iNOS in tumor progression include tumor-cell proliferation, survival, migration and resistance [14]. Therefore, the regulation of NO production by agents such as NOS inhibitors may be useful therapeutically in the treatment of colorectal cancer [46]. In the present study, TPA-induced NO production was significantly reduced in the presence of 40 μg/ml SNL glycoprotein (Fig. 5). Its inhibitory value corresponds to 1 μ*M* L-NAME, suggesting that SNL glycoprotein is a potent inhibitor of iNOS activity as an anticancer agent.

Finally, we investigated the apoptosis-inducing effect of SNL glycoprotein in HCT-116 cells. Unlike necrotic cells, apoptotic cells display chromatin condensation, cell shrinkage, and detachment from neighboring cells and membrane blebbing [47]. In this experiment, apoptotic DNA fragmentation in size multiples of 180–200 bp was clearly induced by treatment with 40 μg/ml SNL glycoprotein in colon cancer cells (Fig. 6). Moreover, such apoptotic activity of SNL glycoprotein was also shown in nuclei stained with H33342/ethidium bromide. Generally, the number of apoptotic cells was significantly increased after the addition of SNL glycoprotein (Table 1).

The results of our recent experiments have shown that one of the most promising activities of glycoproteins are their immunomodulatory and anticancer effects. For instance, *Rhus verniciflua* Stokes (RVS) glycoprotein has the ability to modulate the mitogen-stimulated T/B cell function and the production of cytokines (IL-2 and IL-4), and has also been shown to have marked growth-inhibitory properties in both the HeLa and CT-26 tumor cell lines [24, 48]. Some interesting studies have been carried out to investigate the relationship between the structure and anticancer activity of glycoproteins, and between their immunomodulatory activity and their ability to enhance host defense mechanisms [26, 30]. Based on such characteristics of glycoproteins, this study was carried out to elucidate the apoptotic mechanism of SNL glycoprotein in colorectal cancer, although it does not completely answer all of the questions related to those mechanisms at a biochemical and molecular biological level.

In conclusion, SNL glycoprotein induced apoptosis through the inhibition of the translocation of PKCα, its DNA-binding activity and the protein activities of NF-κB (p50), and the production of iNOS. Such serial signal mediators (PKCα, NF-κB and iNOS) are closely related to oncogenesis, resistance and antiapoptosis in HCT-116 cells. Therefore, we speculate that SNL glycoprotein can be used as a chemotherapeutic agent for colorectal cancers at low concentrations. However, further research must be carried out to elucidate the mechanism of apoptotic signals involving PKCα, NF-κB and iNOS by SNL glycoprotein and to find membrane receptors of SNL glycoprotein at the molecular biological level.

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References

1. Sharma RA, Manson MM, Gescher A, Steward WP (2001) Colorectal cancer chemoprevention: biochemical targets and clinical development of promising agents. Eur J Cancer 37:12–22
2. Greenwald P, Kelloff G, Burch-Whitman C, Kramer BS (1995) Chemoprevention. CA Cancer J Clin 45:31–49
3. Greenwald P, Kelloff GJ, Boone CW, McDonald SS (1995) Genetic and cellular changes in colorectal cancer: proposed targets of chemopreventive agents. Cancer Epidemiol Biomarkers Prev 4:691–702

4. Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME (1999) Mushrooms, tumors, and immunity. Proc Soc Exp Biol Med 221:281–293
5. Lind DS, Hochwald SN, Malaty J, Rekkas S, Hebig P, Mishra G, Moldawer LL, Copeland EM, MacKay S (2001) Nuclear factor-kB is upregulated in colorectal cancer. Surgery 130:363–369
6. Ooi VE, Liu F (2000) Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. Curr Med Chem 7:715–729
7. Negoescu A (2000) Apoptosis in cancer: therapeutic implications. Histol Histopathol 15:281–297
8. May MJ, Ghosh S (1998) Signal transduction through NF-kappa B. Immunol Today 19:80–85
9. Mayo MW, Baldwin AS (2000) The transcription factor NF-kappa B: control of oncogenesis and cancer therapy resistance. Biochim Biophys Acta 1470:M55–M62
10. Foo SY, Nolan GP (1999) NF-kappa B to the rescue: RELs, apoptosis and cellular transformation. Trends Genet 15:229–235
11. O'Brian CA, Ward NE, Gravitt KR, Gupta KP (1995) The tumor promoter receptor protein kinase C: a novel target for chemoprevention and therapy of human colon cancer. Prog Clin Biol Res 391:117–120
12. Davis CD, Johnson WT (2001) Dietary copper and dimethylhydrazine affect protein kinase C isozyme protein and mRNA expression and the formation of aberrant crypts in colon of rats. Biofactors 15:11–26
13. Fukuda K, Akao S, Ohno Y, Yamashita K, Fujiwara H (2001) Inhibition by costunolide of phorbol ester-induced transcriptional activation of inducible nitric oxide synthase gene in a human monocyte cell line THP-1. Cancer Lett 164:7–13
14. Lala PK, Chakraborty C (2001) Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol 2:149–156
15. Fisher M, Yang LX (2002) Anticancer effects and mechanisms of polysaccharide-K (PSK): implications of cancer immunotherapy. Anticancer Res 22:1737–1754
16. Lee SJ, Lim KT (2003) Antioxidative effects of glycoprotein isolated from Solanum nigrum Linne on oxygen radicals and its cytotoxic effects on the MCF-7 cell. J Food Sci 68:466–470
17. Lim KT, Heo KS, Son YO (2002) Antioxidative and antimicrobial effects of glycoprotein isolated from Solanum nigrum Linne. Food Sci Biotechnol 11:484–489
18. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 20:875–882
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin-phenol reagent. J Biol Chem 193:265–275
20. Shan BE, Yoshida Y, Sugiura T, Yamashita U (1999) Stimulating activity of Chinese medicinal herbs on human lymphocytes in vitro. Int J Immunopharmacol 21:149–159
21. Oka S, Shigeta S, Ono K, Jyo T (1987) An epitope residing in carbohydrate chains of a sea squirt antigen termed Gi-rep. J Allergy Clin Immunol 80:57–63
22. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
23. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 126:131–138
24. Lim KT, Lee SJ, Heo KS, Lim K (2003) Effects of glycoprotein isolated from Rhus verniciflua Stokes on TPA-induced apoptosis and production of cytokines in cultured mouse primary splenocytes. Toxicol Lett 145:253–263
25. Tsukagoshi S, Hashimoto Y, Fujii G, Kobayashi H, Nomoto K, Orita K (1984) Krestin (PSK). Cancer Treat Rev 11:131–155
26. Kobayashi H, Matsunaga K, Oguchi Y (1995) Antimetastatic effects of PSK (Krestin), a protein-bound polysaccharide obtained from basidiomycetes: an overview. Cancer Epidemiol Biomarkers Prev 4:275–281
27. Zhongyso D (ed) (1978) Chinese drug dictionary, vol 1. Shanghai Science and Technology, Shanghai, pp 630–631
28. Sultana S, Perwaiz S, Iqbal M, Athar M (1995) Crude extracts of hepatoprotective plants, Solanum nigrum and Cichorium intybus inhibit free radical-mediated DNA damage. J Ethnopharmacol 45:189–192
29. Adachi Y, Ohno N, Ohsawa M, Oikawa S, Yadomae T (1990) Change of biological activities of (1,3)-beta-D-glucan from Grifola frondosa upon molecular weight reduction by heat treatment. Chem Pharm Bull 38:477–481
30. Sakagami H, Aoki T, Simpson A, Tanuma S (1991) Induction of immunopotentiation activity by a protein-bound polysaccharide, PSK (review). Anticancer Res 11:993–999
31. Ohno N, Miura NN, Chiba N, Adachi Y, Yadomae T (1995) Comparison of the immunopharmacological activities of triple and single-helical schizophyllan in mice. Biol Pharm Bull 18:1242–1247
32. Noda K, Ohno N, Tanaka K, Okuda M, Yadomae T, Nomoto K, Shoyama Y (1998) A new type of biological response modifier from Chlorella vulgaris which needs protein moiety to show an antitumour activity. Phytother Res 12:309–319
33. Chakrabarty S, Huang S (1996) Modulation of chemosensitivity in human colon carcinoma cells by downregulating protein kinase C alpha expression. J Exp Ther Oncol 1:218–221
34. Jaken S (1996) Protein kinase C isozymes and substrates. Curr Opin Cell Biol 8:168–173
35. Chakrabarty S, Reynolds S, Wang HM, Rajagopal S (1997) Action of polypeptide growth factors in colon cancer; development of new therapeutic approaches. Front Biosci 2:d460–d470
36. Dekker LV, Parker PJ (1994) Protein kinase C—a question of specificity. Trends Biochem Sci 19:73–77
37. Karin M (1999) How NF-kappa B is activated: the role of the Ikappa B kinase (IKK) complex. Oncogene 18:6867–6874
38. Scaife CL, Kuang J, Wills JC, Trowbridge DB, Gray P, Manning BM, Eichwald EJ, Daynes RA, Kuwada SK (2002) Nuclear factor kappaB inhibitors induce adhesion-dependent colon cancer apoptosis: implications for metastasis. Cancer Res 62:6870–6878
39. Wahl C, Liptay S, Adler G, Schmid RM (1998) Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 101:1163–1174
40. Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, Royer HD, Grinstein E, Greiner A, Scheidereit C, Dorken B (1997) Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J Clin Invest 100:2961–2969
41. Barinaga M (1996) Life-death balance within the cell. Science 274:724
42. Giri DK, Aggarwal BB (1998) Constitutive activation of NF-kappa B causes resistance to apoptosis in human cutaneous T cell lymphoma HuT-78 cells. J Biol Chem 273:14008–14014
43. Thomsen LL, Lawton FG, Knowles RG, Beesley JE, Riveros-Moreno V, Moncada S (1994) Nitric oxide synthase activity in human gynecological cancer. Cancer Res 54:1352–1354
44. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S (1995) Nitric oxide synthase activity in human breast cancer. Br J Cancer 72:41–44
45. Siegert A, Rosenberg C, Schmitt WD, Denkert C, Hauptmann S (2002) Nitric oxide of human colorectal adenocarcinoma cell lines promotes tumor cell invasion. Br J Cancer 86:1310–1315
46. Kojima M, Morisaki T, Tsukahara Y, Uchiyama A, Matsunari Y, Mibu R, Tanaka M (1999) Nitric oxide synthase expression and nitric oxide production in human colon carcinoma tissue. J Surg Oncol 70:222–229
47. Videla LA, Barros SB, Jungueria VB (1990) Lindane-induced liver oxidative stress. Free Radic Biol Med 9:169–179
48. Kitts DD, Lim KT (2001) Antitumorigenic and cytotoxic properties of an ethanol extract derived from Rhus verniciflua Stokes (RVS). J Toxicol Environ Health A 64:357–371