Gomisin A Enhances Tumor Necrosis Factor-α-Induced G1 Cell Cycle Arrest via Signal Transducer and Activator of Transcription 1-Mediated Phosphorylation of Retinoblastoma Protein

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Gomisin A, a dibenzocyclooctadiene lignan isolated from the fruit of Schisandra chinensis, has been reported as an anti-cancer substance. In this study, we investigated the effects of gomisin A on cancer cell proliferation and cell cycle arrest in HeLa cells. Gomisin A significantly inhibited cell proliferation in a dose-dependent manner after 72 h treatment, especially in the presence of tumor necrosis factor-α (TNF-α), due to cell cycle arrest in the G1 phase with the downregulation of cyclin D1 expression and Retinoblastoma (RB) phosphorylation. In addition, gomisin A in combination with TNF-α strongly suppressed the expression of signal transducer and activator of transcription 1 (STAT1). Inhibition of STAT1 pathways by a small-interfering RNA against STAT1 and AG490 Janus kinase (JAK) kinase inhibitor AG490 reduced the cyclin D1 expression and RB phosphorylation, indicating that JAK-mediated STAT1 activation is involved in gomisin A-induced G1 cell cycle arrest.

Key words gomisin A; tumor necrosis factor-α; cell cycle; cyclin D1; Retinoblastoma

Cancer cells often have a selective growth advantage due to deregulation of cell cycle proteins, causing aberrant growth signaling that drives tumor development.1–4) The eukaryotic cell cycle is regulated through the sequential activation and inactivation of cyclin-dependent kinases (CDKs). Deregulation of G1 to S-phase transition is implicated in the pathogenesis of most human cancers.5) Retinoblastoma protein (RB) is a critical target protein in G1-S checkpoint control. Phosphorylation of RB by CDK/cyclin complex results in the release of active E2F transcription factor to stimulate the transcription of genes involved in DNA synthesis and S-phase progression.6,7) Cyclin D1 can accelerate the progress of cells through the G1 phase by binding to CDKs and subsequently phosphorylating RB. Overexpression of cyclin D1 has been shown to shorten the G1 phase in cancer cells and is most frequently associated with human cancer.8,9) Cyclin D1 is rarely mutated, but its overexpression confers a selective growth advantage and hence acts as a driver of neoplastic growth in various cancers.10,11) The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays a significant role in various physiological processes, including immune function, cell growth, differentiation, and hematopoiesis.12) Constitutive activation of STAT3 correlates with cell proliferation in breast carcinoma13) and non-small-cell lung cancer.14) It has been revealed that inhibition of JAK-STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells.15) Activated STAT3 correlates with elevated cyclin D1 protein in primary breast tumors and breast cancer-derived cell lines.16) Our recent study demonstrated that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis was enhanced by STAT1 knockdown.17) Consequently, regulation of STATs can be considered as a key major target in controlling the growth and differentiation of cancer cells. Several phytochemicals, including dietary plant products, have been shown to have anti-tumor properties.18,19) The fruits of Schisandra chinensis have been traditionally used in Japan, Korea and China to treat coughs etc., and have also been employed in the treatment of some chronic diseases such as inflammation, hepatitis and cancer.20,21) Pharmacological studies revealed that lignans isolated from Schisandra chinensis show anti-cancer, anti-hepatotoxic, anti-oxidative, and anti-inflammatory activities.22–24) Gomisin A, for example, has several pharmacological activities, including anticancer and cancer chemopreventive potential in animal models.25–27)

Tumor necrosis factor-α (TNF-α) causes a hemorrhagic necrotic effect against implanted solid tumors.28–31) Nuclear factor (NF)-κB has been well understood to prevent the pro-apoptotic effect of TNF-α.32,33) We previously demonstrated that gomisin N, but not gomisin A, inhibited the TNF-α-induced NF-κB and epidermal growth factor receptor (EGFR) signaling pathways in HeLa cells, which resulted in enhanced pro-apoptotic events.34) However gomisin A also inhibited cell proliferation; therefore, in this study, we investigated the molecular mechanisms of the antiproliferative effect of gomisin A in TNF-α-treated HeLa cells.

MATERIALS AND METHODS

Antibodies and Reagents Phospho-specific antibodies against p65 (Ser-536), STAT1 (Tyr-701), STAT3 (Tyr-705) and RB (Ser-807/811), and anti-poly(A)ADP-ribose polymerase (PARP)-1, anti-cyclin D1 and anti-STAT3 antibodies were purchased from Cell Signaling Technology. Anti-phospho-TAK1 antibody was generated as described previously.35) Antibodies against p65 (C-20-G), TAK1, STAT1 and Actin (C-11) were obtained from Santa Cruz Biotechnology. Recombinant human TNF-α was obtained from R&D systems. Gomisin A, N and cycloheximide (CHX) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell Culture and Proliferation Assay HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO2 atmosphere. Cell proliferation was determined by the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) as per the manufacturer’s instructions. For the assay, cells were seeded at 1 × 104 cells per well in a 96-well plate and treated with various compounds for 72 h. Cell proliferation was expressed as a percentage of growth compared with untreated control. For cell cycle analysis, cells were fixed with ethanol and stained with 4′,6-diamidino-2-phenylindole (DAPI) plus propidium iodide (PI) (Sigma-Aldrich, St Louis, MO).

The authors declare no conflict of interest.

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maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal calf serum, 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO₂. The number of cells was directly counted under microscope (Fig. 1). Cell viability was quantified using the cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-tetrazolio]-1,3-benzene disulfonate) (Dojindo, Kumamoto, Japan). HeLa cells were plated in 96-well microplates at 6×10³ cells/wells, and then incubated for 24 h. Gomisin-containing medium was added to the wells, and cells were incubated for 30 min and then stimulated with TNF-α. After 24 h-incubation, 10 µL WST-1 solution was added, and the absorbance at 450 nm was measured.

Cell Cycle Analysis Cell cycle was analyzed using the Nuclear-ID Red cell cycle kit (Enzo, Life Science). Cells were harvested, cell count adjusted to 10⁶ cells/mL, washed with phosphate buffered saline (PBS), and fixed with 70% ethanol on ice for 1 h. Subsequently, cells were centrifuged and resuspended in DNA staining solution for 30 min at 37°C. The DNA content and percentage of cells in each phase of the cell cycle were analyzed by flow cytometry.

Small Interfering RNAs (siRNAs) and Transfection The siRNA for human STAT1 and non-targeting siRNA were purchased from Invitrogen Life Technologies. HeLa cells were transfected with siRNAs at a final concentration of 20 nM using LipofectAMINE reagents (Invitrogen Life Technologies). At 72 h after transfection, cells were stimulated.

Preparation of Cell Extracts Cells were treated with gomisin A and TNF-α, and whole cell lysates were prepared with lysis buffer (25 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) (pH 7.7), 0.3 mM MgCl₂, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 10% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 µg/mL aprotinin, and 10 µg/mL leupeptin). Cell lysate was collected from the supernatant after centrifugation at 14000 rpm for 10 min.

Immunoblotting Cell lysate was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon-P-nylon membrane.
Effect of Gomisin A on TNF-α-Mediated HeLa Cell Proliferation

To understand the mechanism by which gomisin A exerts its anti-proliferative activities, we first tested the effect of gomisin A on cell proliferation by treating cells with gomisin A in the presence or absence of TNF-α. Figure 1 shows that gomisin A alone slightly inhibited cell growth, whereas, in combination with TNF-α, it induced a significant inhibition. These results indicated that gomisin A inhibits cell growth in the presence of TNF-α in a concentration-dependent manner.

Effect of Gomisin A on TNF-α-Induced Apoptotic Response

The decrease in cell number could result from reduced cell proliferation and/or apoptosis. To distinguish these effects, we next investigated the effect of gomisin A on TNF-α-induced cleavage of caspase-3 and PARP, cellular pro-apoptotic responses. Phase-contrast microscopy demonstrated that gomisin A induced cell growth inhibition without significant cell death, while the cell morphology was slightly changed (Fig. 2A). Although gomisin A has a similar structure to gomisin N, it induced marginal apoptotic responses (cleavage of caspase-3 and PARP-1) in HeLa cells (Fig. 2B). In addition, population in the Sub-G1 phase was slightly increased by gomisin A (data not shown). In consideration of our previous results that gomisin A in combination with TNF-α induced moderate cleavage of caspase-3 and PARP-1 (34), we concluded that gomisin A has a marginal potential to induce apoptotic responses; however, the decreased cell number after 72 h incubation with gomisin A and TNF-α is mainly due to...
Fig. 4. STAT1-Mediated RB Phosphorylation in HeLa Cells

(A) HeLa cells were pretreated with gomisin A at 100 µM in the presence or absence of TNF-α for 24 h. (B) HeLa cells were transfected with siRNAs against firefly luciferase (Luc) and STAT1. Whole cell extract was prepared and analyzed by Western blotting using anti-STAT1, cyclin D1, Actin and anti-phospho-RB antibodies. (C) Cells were transfected with Luc and STAT1 siRNA. Cell viability was determined using a WST-1 reagent. *p<0.05. (D) Cells were pretreated with AG490 (100 µM) in the presence or absence of TNF-α for 24 h. Whole cell extract was prepared and analyzed by Western blotting using anti-phospho-RB, JAK2 and cyclin D1, STAT1, and actin antibodies.

Fig. 5. Effect of Sodium Butyrate on Apoptotic Response in Cells Treated with Gomisin A and TNF-α

HeLa cells were pretreated with gomisin A (100 µM) in the presence or absence of TNF-α for 6 h and then treated with 5 mM sodium butyrate for 24 h. Cell viability (WST-1 assay) (A) and cell morphology (B) were examined. *p<0.005. C, Cells pretreated with gomisin A and TNF-α for 6 h were treated with 5 mM sodium butyrate (NaBu) for 4 h. Whole cell extract was prepared and analyzed by Western blotting using anti-PARP and actin antibodies. Arrow indicates cleaved forms of PARP.
cell growth inhibition.

**Gomisin A Enhances G1/S Cell Cycle Arrest** Since gomisin A enhanced TNF-α-mediated cell growth inhibition without an obvious apoptotic effect, this raised the possibility that it might be related to the regulation of the cell cycle; therefore, we analyzed cell cycle distribution by flow cytometry following gomisin A treatment with or without TNF-α. Figure 3A demonstrates that treatment with TNF-α or gomisin A alone resulted in enrichment of cancer cells in the G1 phase at 12 and 24 h. Interestingly, gomisin A in the presence of TNF-α significantly increased the ratio in the G1 phase, accompanied by a concomitant decrease in the number of cells in the G2 phase. Altogether, these findings demonstrated that gomisin A had cytostatic properties against cancer cells.

To investigate the mechanistic basis of growth inhibitory effects of gomisin A, we next examined the effect on the expression of key proteins involved in G1 cell cycle regulation. Cells were untreated or pretreated with gomisin A in the presence or absence of TNF-α for 24 h, and then RB phosphorylation and cyclin D1 expression were examined by Western blotting. Compared with the control, co-treatment with gomisin A and TNF-α could inhibit RB phosphorylation and cyclin D1 expression in a dose-dependent manner (Fig. 3B). These results indicated that gomisin A collaborated with TNF-α to inhibit RB phophorylation and cyclin D1 expression, which led to G1 cell cycle arrest.

**Role of STAT1 in G1 Cell Arrest by Gomisin A and TNF-α** In order to elucidate the molecular mechanism of the anti-proliferative effect of gomisin A, we assessed several intracellular signaling pathways. Interestingly, we found that gomisin A or TNF-α, especially in combination with these agents, could inhibit the expression of STAT1 as well as cyclin D1 expression and RB phosphorylation (Fig. 4A), suggesting that G1 cell cycle arrest by gomisin A and TNF-α is mediated through inhibition of the STAT1 pathway. To investigate the functional significance of STAT1, we next tried to suppress STAT1 expression by siRNA. As expected, RNAi-based downregulation of STAT1 resulted in reduced cyclin D1 expression and RB phosphorylation (Fig. 4B). In addition, this correlated with significant cell growth inhibition (Fig. 4C). Similarly, AG490, a JAK/STAT inhibitor, could block the phosphorylation of RB and expression of cyclin D1 (Fig. 4D). Altogether, these results indicate that STAT1 is involved in the anti-proliferative activity of gomisin A.

**Apoptotic Cell Death by Sodium Butyrate in Combination with Gomisin A and TNF-α** Sodium butyrate (NaBu), a histone deacetylase inhibitor, induces apoptosis in a variety of tumor cells, especially non-proliferating cells including G1 phase-arresting cells. The results in Fig. 3 showing G1/S cell cycle arrest encouraged us to examine the effect of NaBu on cell viability and apoptosis. In the absence of NaBu, both gomisin A and TNF-α and their combination showed no cytotoxicity. Interestingly, in the presence of NaBu, gomisin A and TNF-α were able to strongly induce cell death (Figs. 5A, B). As shown in Fig. 5C, NaBu enhanced TNF-α-induced cleavage of PARP-1 in cells pretreated with gomisin A. In contrast, although NaBu in combination with TNF-α or gomisin A also inhibited cell viability, PARP-1 cleavage was not detected. These results indicate that NaBu induced apoptotic death of G1/S-arrested cells.

**DISCUSSION**

Natural products are well recognized as sources of drugs in several human ailments including cancers.46,47 Our previous study investigated the anticancer activity of gomisin A and N, which are diabenzo[a,c]cyclooctadiene lignans isolated from *Schisandra chinensis*. We found that gomisin N inhibited the TNF-α-induced NF-κB and EGFR signaling pathways, which resulted in enhanced pro-apoptotic events. Unlike gomisin N, no gomisin A-induced cytotoxicity was observed. Although gomisin A did not induce apoptotic cell death, it was able to suppress cell proliferation. It is well known that one of the main mechanisms of action of chemotherapeutic drugs is interruption of the cell cycle.38–41 In this study, we investigated the role of gomisin A and TNF-α in cell proliferation and growth arrest and found that gomisin A or TNF-α alone moderately inhibited cell growth and G1 cell cycle arrest. When combined, these anti-cancer effects were markedly increased.

The cell cycle is regulated by the concerted actions of cyclins, CDKs and CDK inhibitors.42,43 Cyclin D1 and its catalytic partner CDK4 dominate in the G1 phase. Previous studies have shown that cyclin D1 is the dominant regulatory protein of cell cycle progression and a potential chemopreventive and chemotherapeutic target of cancer. Cyclin D1 is a sufficient target for inducing G1 arrest of cancer cells in chemoprevention and therapeutic strategies for cancer.44 RB is a critical target protein that is phosphorylated via these CDK-cyclin D1 complexes; therefore, the RB pathway is consistently altered in cancer cells to promote deregulated cell proliferation.45–47 In our investigation of the underlying mechanisms of the effect of gomisin A on TNF-α-mediated cyclin D1 expression and RB phosphorylation, we found that gomisin A enhanced TNF-α-induced downregulation of cyclin D1 expression and RB phosphorylation.

It has been demonstrated that cyclin D1 overexpression, at least in part, involved in the activation of STAT3 in mouse hepatic carcinogenesis.48 STAT proteins transduce signals through the cytoplasm and function as transcription factors in the nucleus. STAT3 binds to a conserved element in the enhancers of genes, mostly involved in cell proliferation or survival. STAT1 binds to the same DNA sequence as STAT3; however, in contrast to STAT3, it activates a different set of genes and usually promotes cell cycle arrest and apoptosis. There are some reports defining STAT1 as a tumor promoter in cancer cell that promotes cell proliferation.49–52 However, to date, the roles of STAT1 in the cell cycle remain to be investigated. The present study demonstrated that gomisin A enhanced TNF-α-mediated STAT1 expression. In addition, this study investigated the functional interaction of STAT1 on RB phosphorylation and found that downregulation of STAT1 decreased both cyclin D1 expression and RB phosphorylation. Our findings indicate that gomisin A can potentiate the anti-tumor activity of TNF-α and inhibit cell growth by inducing G1 cell cycle arrest via suppression of STAT1-dependent RB phosphorylation. Future characterization of STAT1 function in cell cycle arrest will provide information for new molecular targets of anti-cancer therapy.

Histone deacetylase inhibitors (HDIs) are a new class of drugs with anticancer potential. It has previously been demonstrated that HDIs have tumor-selective cytotoxicity. HDIs are able to kill cells by two pathways, a rapid pathway that
involves transit through the defective G2 checkpoint and undergoing an aberrant mitosis, and a slow pathway that is activated in G1 phase arrest cells. Sodium butyrate (NaBu) is a histone deacetylase (HDAC) inhibitor that can lead to hyper-acetylation of chromatin components and alterations in gene expression. It has been shown that NaBu induced cell cycle arrest and apoptosis. Our data suggest that the combination of NaBu with gomisin A and TNF-α could induce apoptosis in cancer cells, strongly supporting our finding that gomisin A induces G1 cell cycle arrest; therefore, gomisin A in collaboration with TNF-α or HDAC inhibitor is a new promising agent in the treatment of cancers.

Acknowledgements This work was supported in part by Grants-in-Aid for Scientific Research on Innovative Areas (No. 23117516) and Scientific Research (C) (No. 23590071) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and a Grant from the Research Foundation for Pharmaceutical Sciences.

REFERENCES


