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Inhibition of liver fibrosis by sensitization of human hepatic stellate cells by combined treatment with galtanin and TARIL

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Abstract Liver fibrosis is caused by metabolic problems such as cholestasis, genetic problems, or viral infections. Inhibiting hepatic stellate cell (HSC) activation or inducing selective apoptosis of activated HSCs is used as a treatment strategy for liver fibrosis. It has been reported that when HSCs are activated, their apoptosis sensitivity to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is enhanced because the expression of death receptor 5 is elevated. Finding a natural compound that can enhance the apoptotic effect of TRAIL on HSCs is a necessary strategy for liver fibrosis treatment. It was confirmed here that mangosteen-derived gartanin increased the effect of TRAIL-induced apoptosis by increasing the expression of DR5 in a p38-dependent manner in the hepatic stellate cell line LX-2. Combined treatment with gartanin and TRAIL accelerated DNA cleavage through caspase-3 activation and enhanced antifibrotic effects in LX-2 cells.

Keywords DR5 · Gartanin · Liver fibrosis · TRAIL

Introduction

Liver fibrosis occurs when liver cell damage persists over a long period, as is the case in chronic hepatitis [1]. Hepatic stellate cells (HSCs) are the main cells involved in hepatic fibrosis, producing extracellular matrix (ECM) [2,3]. Activated HSCs increase the expression of α-smooth muscle actin (α-SMA), a cytoskeletal protein, and adopt a myofibroblast-like phenotype with increased proliferation and production of various ECM components, including collagen I and II [4,5]. Several receptors and intracellular signal transduction systems activate and sustain HSCs, providing potential therapeutic targets for antifibrotic treatment [6]. Inducing apoptosis of activated HSCs is a therapeutic target in hepatic fibrosis treatment, as it prevents the progression of fibrosis and restores normal liver function [7,8].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in tumor cells and has been shown to induce apoptosis in activated HSCs [9-14]. TRAIL-mediated apoptosis in HSC is mainly induced by caspase activation via DR5, also known as TRAIL-R2, and also occurs by down-regulation of XIAP (X-linked inhibitor of apoptosis protein) via the Smad2 pathway [13,15]. However, TRAIL's low stability and short half-life in vivo limit its therapeutic use. Research is ongoing to find a combination agent that can enhance the effect of TRAIL-induced HSC apoptosis.

Gartanin, a xanthone compound found in mangosteen, has been shown to arrest the cell cycle and induce apoptosis in various cancer cells [16,17]. Studies have reported that gartanin can regulate the AKT, MAPK, and NF-κB pathways, induce autophagy by inhibiting the mTOR pathway, and increase the sensitivity of TRAIL by inducing an increase in DR5 through Chop activation [18-20]. However, it is not clear whether gartanin plays a role in TRAIL-mediated apoptosis of activated HSCs. The hypothesis of this experiment is that gartanin induces the overexpression of DR5 in HSCs and enhances the apoptotic effect of TRAIL to reverse liver fibrosis. To test this hypothesis, the effect of gartanin was verified in activated HSCs.

Materials and Methods

Cell culture

The Hepatic stellate cell line LX-2 was cultured in DMEM

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supplemented with 10% fetal bovine serum, 100 U/mL streptomycin, and 100 U/mL penicillin, and treated with 10 ng/mL of Transforming Growth Factor-β (TGF-β) in serum-free DMEM for activation.

Reagents

Gartanin, extracted from mangosteen, was obtained from Prof. Young-Won Chin at Dongguk University, and primary antibodies including anti-caspase-3 antibody (cat no. AB1899) purchased from Calbiochem, anti-GAPDH (cat no. #5174), anti-p38 MAPK (cat. no. #9212), and anti-phosphorylated-p38 MAPK (Thr180/Tyr182) antibodies (cat. no. #9211) from Cell Signaling Technology, Inc., and anti-α-SMA from Abcam were used in this study. Primary antibodies were diluted at a ratio of 1:500 to 1:2000 before use.

Cell viability (MTT)

For cell viability (MTT) assay, LX-2 cells were seeded at a density of 5×10^4 cells/mL in a 96-well culture plate and treated with gartanin and TRAIL, followed by incubation with MTT solution (Sigma-Aldrich Corp., St. Louis, MO, USA), and measurement of absorbance at 570 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA) after 24 h.

DAPI and α-SMA staining

For DAPI and α-SMA staining, LX-2 cells were seeded and supplemented with TGF-β (10 ng/mL), stained with 300 nM DAPI solution and FITC-conjugated α-SMA mAb after 24 h, and observed and analyzed for expression fluorescence intensity of α-SMA using a fluorescence microscope (Carl Zeiss Meditec AG, Jena, Germany) and Image J program (National Institutes of Health, Bethesda, MD, USA).

Western blot

Total protein extracts were obtained using PRO-PREP protein extraction solution (iNtRON Biotechnology, Sungnam, Republic of Korea), and used for Western blot analysis after quantitation of protein and separation by SDS-PAGE.

Flow cytometric analysis

Flow cytometric analysis was performed by collecting and fixing cells with 70% ethanol, treating with RNase and PI, and staining with FITC conjugated annexin-V (R&D Systems, Minneapolis, MN, USA) for 1 h.

Picro-Sirius red staining

Picro-Sirius red staining was carried out using Picro Sirius Red Stain Kit (Connective Tissue Stain) (ab150681) purchased from Abcam, and collagen type I production induced by TGF-β treatment was measured by picro-Sirius red staining.

Statistical analysis

Data analysis was conducted using SPSS version 14.0 (SPSS, Inc., Chicago, IL, USA), and statistical comparisons were performed using a two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test, with p values less than 0.05 and 0.01 considered significant.

Results

TGF-β activates LX-2 cell

α-SMA is a significant marker protein that exhibits increased expression in activated HSCs. To confirm the effect of TGF-β on LX-2 cell activation, cells were treated with 10 ng/mL of TGF-β, and proteins were extracted at different time points and analyzed by Western blotting. As demonstrated in Fig. 1A, α-SMA expression was elevated in a time-dependent manner by TGF-β. Subsequently, immunostaining was conducted to observe changes in α-SMA expression in LX-2 cells. In agreement with the Western blotting results, α-SMA expression (green color) was seen to increase in LX-2 cells 12 hours after TGF-β treatment (Fig. 1B).

Gartanin sensitizes TRAIL-induced apoptosis in activated HSC

Next, we verified the combined treatment effect of TRAIL and

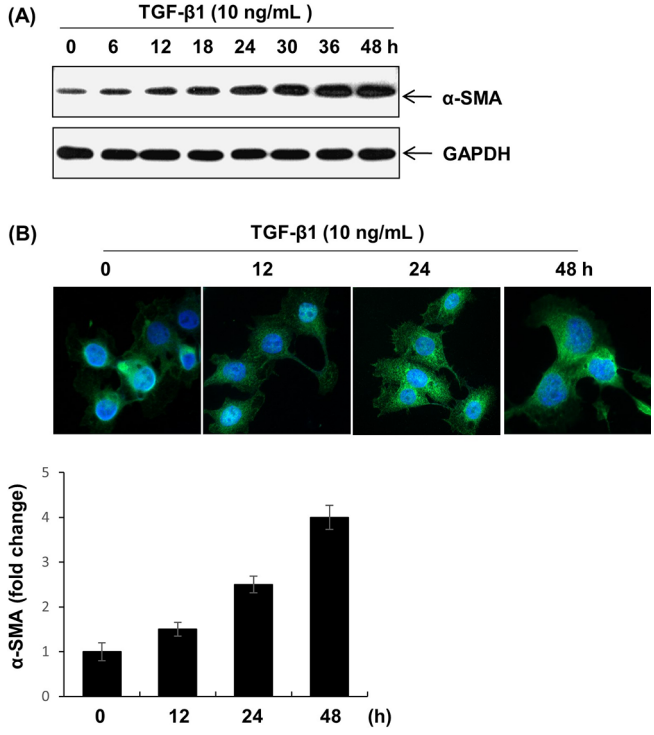

Fig. 1 TGF-β upregulates α-SMA expression in activated LX-2 cells. To analyze changes in the expression of α-SMA protein, activated LX-2 cells were exposed to 10 ng/mL of TGF-β for the indicated time. Total protein was extracted, and western blotting was performed, using GAPDH as a loading control. α-SMA expression level was also analyzed in HX-2 cells at each time point through the immunofluorescence staining method. A graph was created by normalizing α-SMA intensity to the number of nuclei in fluorescence staining

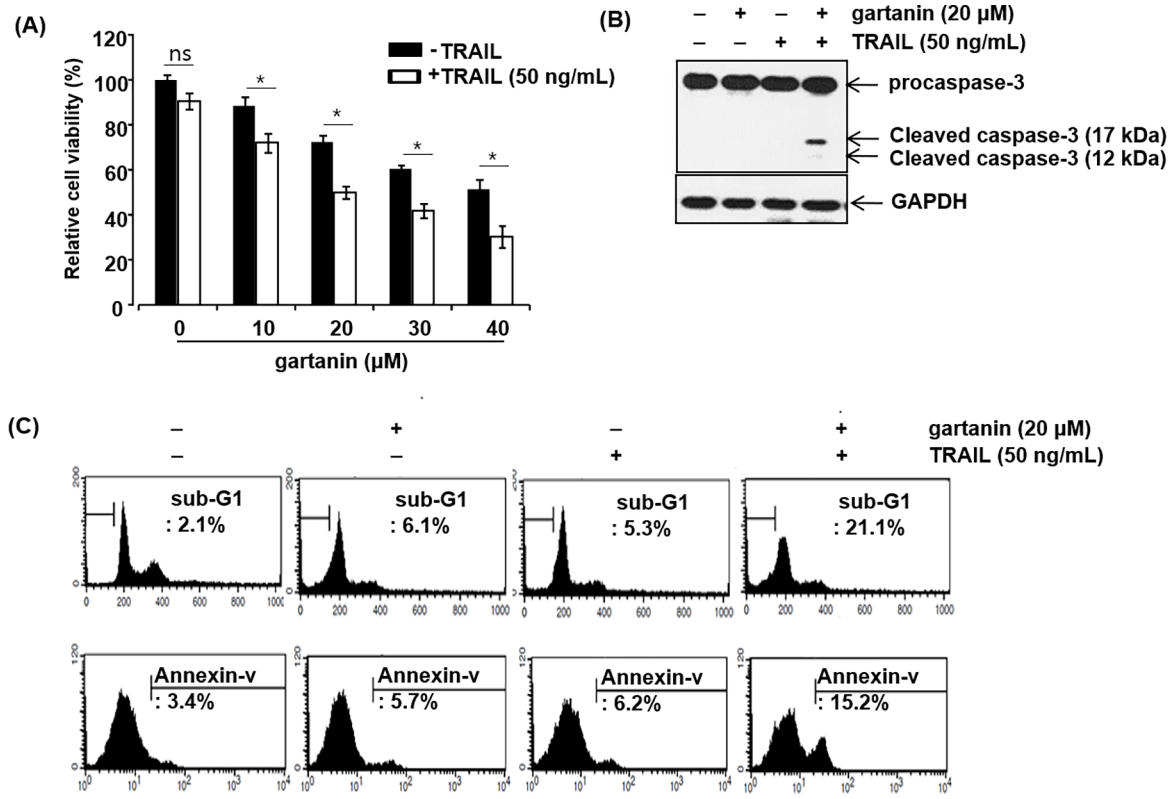

Fig. 2 Gartanin sensitizes TRAIL-induced apoptosis in HSC. Activated LX-2 cells were treated with 10 ng/mL TGF-β and cultured for 24 h. The activated LX-2 cells were then treated with 50 ng/mL TRAIL and different concentrations (10, 20, 30, and 40 μM) of gartanin for 24 h. Cell growth was analyzed by MTT assay, and the data represent the mean (± standard deviation, SD) of three independent experiments. *p <0.05 vs. Control. In addition, caspase-3 was confirmed by Western blotting in activated LX-2 cells treated with 50 ng/mL TRAIL and 20 μM gartanin for 24 h. GAPDH was used as a loading control. Cell cycle analysis and Annexin-V analysis were also performed by flow cytometry

gartanin on LX-2 cells activated by TGF-β treatment for 24 h. As demonstrated in Fig. 2A, gartanin sensitized the cell growth inhibitory effect of TRAIL in a concentration-dependent manner. To confirm whether the cell growth inhibitory effect of TRAIL and gartanin combined treatment was due to apoptosis, we examined changes in caspase-3 by Western blotting. Caspase-3 is a protease that is cleaved and activated during apoptosis, and the active form of caspase-3 cleaves many intracellular proteins. As shown in Fig. 2B, we identified 12KDa and 17KDa fragments of cleaved caspase-3 in the cell group treated with TRAIL and gartanin combined. When apoptosis occurs, caspase activated DNase is activated and induces DNA fragmentation. When the DNA is fragmented, the cell cycle analysis shows an increase in the sub-G1 cell group. As confirmed in Fig. 3C, the sub-G1 cell population of the cells treated with the combination of TRAIL and gartanin increased by about 21%. Another characteristic of apoptosis is the migration of phosphatidylserine from the inner layer of the cell membrane to the outer layer. Phosphatidylserine binds to specific receptors on macrophages or dendritic cells and transmits the “eating me” signal. The amount of phosphatidylserine in the outer layer was analyzed by FITC-conjugated Annexin-V. As demonstrated in Fig. 2C, the combined treatment of TRAIL and gartanin increased the number of phosphatidylserine-positive cells to about 15%.

p38 MAPK upregulates DR5 expression in activated HSC

p38 MAPK is a signaling molecule that regulates the expression of DR5, a TRAIL receptor. We conducted Western blotting to determine whether gartanin regulates the activity of p38 MAPK. As illustrated in Fig. 3A, phosphorylation of p38 MAPK was strongly induced 15 minutes after gartanin treatment. SB203580 is a compound that inhibits p38 MAPK activity by preventing ATP binding to p38 MAPK. We pretreated cells with SB203580 for 1 h before treating them with gartanin to investigate the effect of p38 MAPK activated by gartanin on DR5 expression. After 24 h, we analyzed DR5 expression by flow cytometry. As shown in Fig. 3B, the gartanin-treated group expressed DR5 about 5 times more than the control group. Additionally, we found that pretreating cells with SB203580 partially reduced the expression of DR5 induced by gartanin by about 10%. Thus, we discovered that the p38 MAPK signaling pathway is involved in the expression of DR5 induced by gartanin. To further investigate the functional role of p38 MAPK in sensitizing TRAIL-mediated apoptosis by gartanin, we used flow cytometry to detect sub-G1 and annexin-

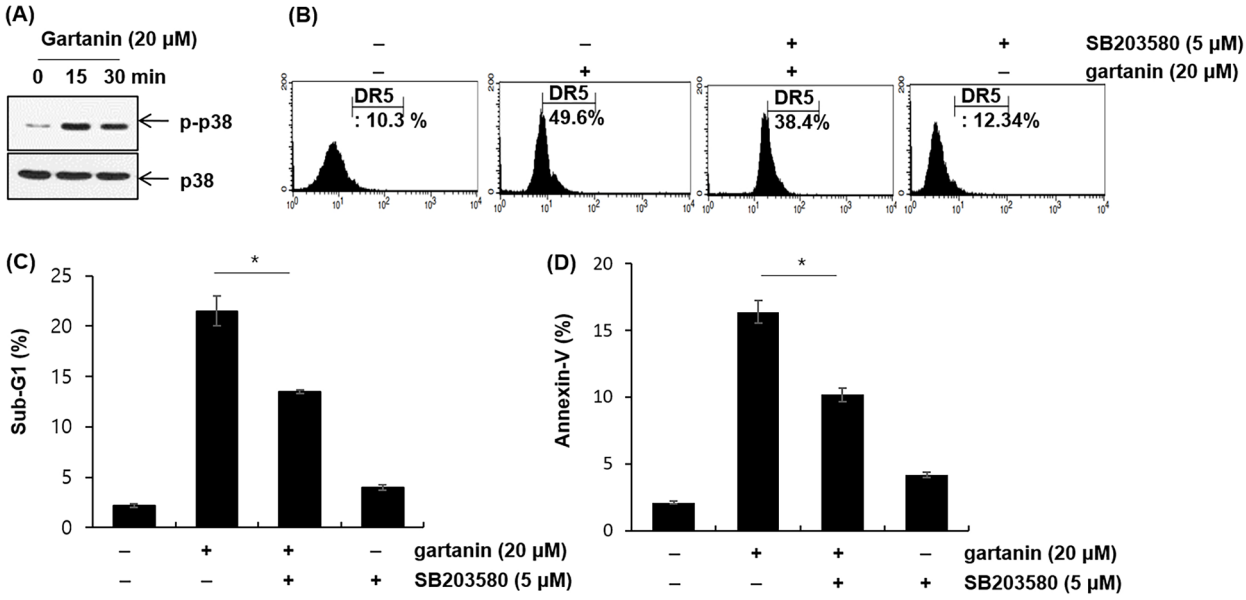

Fig. 3 Gartanin upregulates DR5 expression via p38 MAPK. Activated LX-2 cells were treated with 20 μM gartanin for 30 min and 60 min, and then subjected to cell lysis for Western blotting. In addition, a p38 MAPK inhibitor, SB203580, was pretreated at a concentration of 5 μM for 1 h, followed by treatment with 20 μM gartanin for 24 h. Expression level of DR5 was analyzed by flow cytometry. Cell cycle analysis and Annexin-V analysis were also performed by flow cytometry after pretreating with SB203580 at a concentration of 5 μM for 1 h, and then treating with TRAIL and gartanin at the indicated concentrations for 24 h

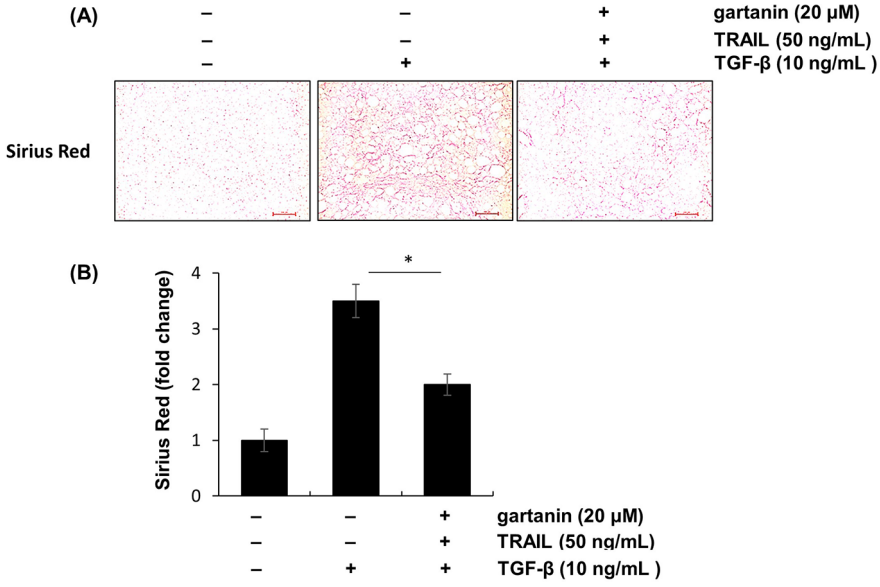

Fig. 4 TRAIL and gartanin inhibit collagen I production. Activated LX-2 cells were treated with 10 ng/mL TGF-β and cultured for 24 h. The activated LX-2 cells were then treated with 50 ng/mL TRAIL and 20 μM gartanin. Collagen type I secretion was measured by picro-Sirius red staining of LX-2 cells, and relative quantification of picro-Sirius red staining was performed

V positive cells. SB203580 treatment inhibited sub-G1 cells and increased the number of annexin-V positive cells induced by combined gartanin and TRAIL treatment in activated LX-2 cells (Fig. 3D).

TRAIL and gartanin reduced collagen type I production

Picro-sirius staining was performed next to investigate the effect of combined treatment with TRAIL and gartanin on the production of collagen I, an indicator of hepatic fibrosis, in

activated LX-2 cells. The red-stained area was measured, and it was found to be significantly increased in the TGF-β treatment group compared to the control group. However, treatment with TRAIL and gartanin was shown to suppress the effect of TGF-β, as shown in Fig. 4A and 4B.

Discussion

Liver fibrosis is caused by the excessive secretion of extracellular matrix due to chronic intrahepatic inflammation [1]. If the excessive deposition of extracellular matrix persists, it can induce structural changes in the liver and reduce the number of hepatocytes, resulting in chronic liver disease [21]. Hepatic stellate cells, Kupffer cells, and endothelial cells are closely related to hepatic fibrosis. Among them, hepatic stellate cells are the main producers of extracellular matrix, and activated hepatic stellate cells secrete various types of extracellular matrix, including collagen. Cytokines that affect hepatic fibrosis include transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and others. TGF-β is the most potent cytokine that promotes fibrosis by contributing to the activation of hepatic stellate cells, and hepatic stellate cells themselves are the primary source of TGF-β production. PDGF is the most potent cytokine that promotes the division and proliferation of hepatic stellate cells [22].

TRAIL is a useful therapeutic agent for inducing apoptosis of activated hepatic stellate cells, which are a major cause of liver fibrosis and liver cirrhosis. Recent studies have shown that when hepatic stellate cells are activated, death receptors DR4 and DR5 are overexpressed. This study confirmed that gartanin, a mangosteen-derived xanthine class compound, enhances the effect of TRAIL-mediated apoptosis by increasing the expression of DR5 in hepatic stellate cells. The synergistic effect of TRAIL by gartanin was confirmed by the activation of caspase-3, the increase of sub-G1 cells, and annexin-V positive cell population. In addition, gartanin induced phosphorylation of p38 MAPK, and pretreatment with SB203580, a p38 MAPK inhibitor, partially suppressed gartanin-induced DR5 expression.

A lot of research has been conducted recently on combined treatment agents to increase the effect of TRAIL. Agents that enhance the activity of Smad or inhibit the activity of NF-κB activated by TRAIL were effective in TRAIL-mediated hepatic stellate cell apoptosis [15,23]. Another recent study showed that PEGylated TRAIL (TRAILPEG), which increases the half-life of TRAIL, is more likely to induce apoptosis in activated hepatic stellate cells [24]. This study verified that gartanin, which can increase the expression of TRAIL receptors, increases the sensitivity of TRAIL-mediated apoptosis and is effective in liver fibrosis. In future studies, it will be necessary to identify more specifically the role of gartanin in TRAIL-mediated signal transduction.

Competing interests The authors declare no conflicts of interest.

Authors' contributions Conceptualization, Funding acquisition, Investigation, Methods development, Supervision, Writing, review, and editing: Dong-Oh Moon

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