Tangeretin, a citrus polymethoxyflavonoid, induces apoptosis of human gastric cancer AGS cells through extrinsic and intrinsic signaling pathways

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Abstract. Tangeretin, a natural polymethoxyflavone present in citrus peel oil, is known to have anticancer activities in breast cancer, colorectal carcinoma and lung carcinoma, yet, the underlying mechanisms of tangeretin in human gastric cancer AGS cells have not been investigated to date. In the present study, the apoptotic mechanisms of tangeretin in AGS cells were explored. It was observed that tangeretin increased the apoptotic rates of AGS cells following treatment with tangeretin for 48 h in a dose-dependent manner by Annexin V-FITC and PI double staining. In addition, characteristic apoptotic morphology such as nuclear shrinkage and apoptotic bodies was observed after Hoechst 33258 staining. Flow cytometric assay showed that treatment of AGS cells with tangeretin decreased the mitochondrial membrane potential (MMP) in a dose-dependent manner, which indicated that mitochondrial dysfunction was involved in the tangeretin-induced apoptosis. Caspase-3, -8 and -9 activities were increased by tangeretin in a dose-dependent manner. Western blotting showed that the protein levels of pro-apoptotic proteins including cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, Bax, Bid, tBid, p53, p21/cip1, Fas and FasL were significantly upregulated by tangeretin. In addition, PFT-α (a p53 inhibitor) reduced the apoptotic rates and the expression of p53, p21, caspase-3 and caspase-9 induced by tangeretin, indicating that tangeretin-induced apoptosis was p53-dependent. In conclusion, these results suggest that tangeretin induces the apoptosis of AGS cells mainly through p53-dependent mitochondrial dysfunction and the Fas/FasL-mediated extrinsic pathway.

Introduction

Tangeretin is a natural polymethoxyflavonoid extracted from citrus fruits (1), which exhibits antiproliferative, anti-invasive, antimetastatic and antioxidant activities (2-4). Recently, many studies have shown that polymethoxylated flavonoids such as tangeretin and nobiletin are more potent inhibitors of tumor cell growth than free hydroxylated flavonoids (3,5,6). Tangeretin inhibits the growth of many cell lines such as breast cancer cell lines MDA-MB-435 and MCF-7, human colon cancer cell line HT-29 and leukaemic HL-60 cells through cell cycle arrest or apoptosis (2,7,8). Tangeretin activates the unfolded protein response and synergizes with imatinib in the erythroleukemia cell line K562 and suppresses IL-1β-induced cyclooxygenase (COX)-2 expression through inhibition of p38 MAPK, JNK and AKT activation in human lung carcinoma cells (9,10). In addition, tangeretin was reported to sensitize cisplatin-resistant human ovarian cancer cells through downregulation of the phosphoinositide 3-kinase/Akt signaling pathway (11). Thus, tangeretin may be a potential plant-derived agent for use in the treatment of gastric cancer.

Gastric cancer is the second leading cause of cancer-related death in the world (12). AGS is a type of human gastric adeno-carcinoma cell line with wild-type p53, which has been used in numerous studies on antineoplastic drugs (13). Wild-type p53 is a tumor-suppressor gene, which plays an important role in cancer cell growth inhibition and mediates either cell cycle arrest or apoptosis (14,15). In response to various forms of stress, p53 is activated and accumulates in the nucleus, where it regulates the transcription of numerous target genes using specific DNA response elements. Beyond its nuclear role, p53 regulates a number of mitochondrial functions. However, the mutant p53 existing in some tumor cells loses its activity and has no antitumor function (16). Thus, as a gastric cancer cell line with wild-type p53, AGS is suitable for investigation of the role of p53 in the anticancer action of tangeretin.

Our previous study showed that tangeretin inhibits the proliferation of human AGS gastric cancer cells and induces

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cell cycle arrest in the S and G2/M phases through inhibition of ERK phosphorylation and upregulation of cyclin B1 protein (17). In the present study, we further examined the effects of tangeretin on the apoptosis of AGS cells and explored the related mechanisms.

Materials and methods

Reagents and antibodies. Tangeretin was obtained from Shanghai Tongtian Biotechnology Co., Ltd. (Shanghai, China). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was obtained from Shanghai Richu Biotechnology Ltd. Propidium iodide (PI) was from Sigma-Aldrich, Inc. (St. Louis, MO, USA). ECL Plus Western Blotting detection reagents were from GE Healthcare (Wauwatosa, WI, USA). Annexin V-FITC apoptosis kit and caspase-3, -8, -9 colorimetric assay kits were obtained from BioVision (Mountain View, CA, USA). All antibodies and secondary antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture. Human gastric cancer cell line AGS from the American Type Culture Collection (ATCC, Rockville, MD, USA) was cultured in HAM's/F12 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) at 37˚C in a 5% CO_2 air atmosphere.

MTT assay for cell viability. Cells were seeded in 96-well plates and treated with different concentrations of tangeretin for 24, 48 and 72 h, respectively. Then, MTT solution was added to each well at a final concentration of 1 mg/ml, and the plates were incubated at 37˚C for another 4 h. After incubation, 150 µl dimethyl sulfoxide (DMSO) was added to each well and the absorbance was read at 570 nm using an absorbance microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA). The experiments were performed in triplicate and at least repeated three times. Inhibition rates = (1 - sample OD/control OD) x 100%.

Analysis of apoptosis. Apoptosis was evaluated using an Annexin V-FITC and PI double staining kit. After treatment with 10, 30 and 60 µM tangeretin or 0.05% DMSO for 48 h, AGS cells were harvested and incubated with 500 µl Annexin V binding buffer containing 5 µg/ml PI and 1 µl FITC-labeled Annexin V for 30 min on ice. Then cells were analyzed by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and the results were indicated as percentages of apoptotic cells. Images of cells were captured under an inverted fluorescence microscope (IX71, Olympus, Tokyo, Japan).

Hoechst 33258 staining. After treatment with tangeretin for 48 h, cells were fixed in 4% paraformaldehyde for 2 h and stained with 20 µM Hoechst 33258 for 20 min after washing with PBS. Cells were then observed and images were captured under an inverted fluorescence microscope.

Caspase-3, -8 and -9 activity assay. Caspase activity was examined by a caspase-3, -8, -9 colorimetric assay kit respectively according to the manufacturer's protocol. Sample readings at 405 nm were conducted using a SpectraMax 190
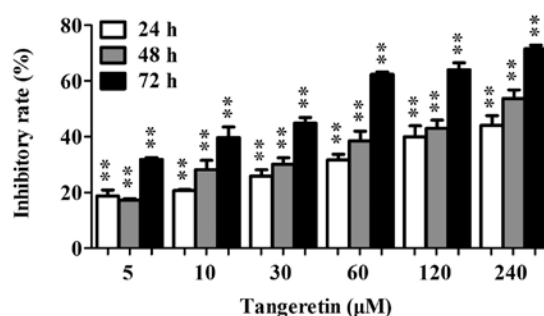

Figure 1. Effect of tangeretin on AGS cell viability. MTT assays showed that tangeretin inhibited the growth of AGS cells in a dose- and time-dependent manner. Data are presented as mean ± SEM, n=6; **P<0.01 vs. control.

microplate reader. Fold-increase in activity was determined by comparing the results with the level of the control.

Mitochondrial transmembrane potential assay. The cells treated with tangeretin were collected by centrifugation at 3,000 rpm for 10 min and suspended in 500 µl PBS. The cells were incubated with Rhodamine 123 (1 µg/ml) for 30 min and examined by a FACSCalibur flow cytometer.

Western blot analysis. After a 48-h exposure to tangeretin at different doses, cells were washed twice with ice-cold PBS and lysed in protein lysis buffer. Protein concentrations were tested by the Bradford method. Proteins were subjected to electrophoresis on 12% polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes by electrotransfer (Bio-Rad Laboratories, CA, USA). After blocking with 5% non-fat milk in PBS buffer containing 0.5% Tween-20 and washed three times, the membranes were incubated with various primary antibodies, respectively. Membranes were washed and incubated with the corresponding secondary antibodies, and then the protein bands were visualized with ECL Plus Western blotting detection reagents.

Statistical analysis. Data are presented as means ± SEM. Statistical analysis of data was carried out with GraphPad Prism 5.0 (Hearne Scientific Software, Chicago, IL, USA). Multiple comparisons were carried out by one-way analysis of variance (ANOVA). A value of P<0.05 was considered to indicate a statistically significant result.

Results

Effects of tangeretin on the viability of AGS cells. The antiproliferative effect of tangeretin on the human gastric cancer AGS cell line was examined by MTT assay. The inhibitory rates of the cells exposed to 5, 10, 30, 60, 120 and 240 µM tangeretin for 24, 48 and 72 h were observed, respectively. The results showed that tangeretin decreased the viability rates of AGS cells in a dose- and time-dependent manner (Fig. 1).

Tangeretin induces AGS cell apoptosis. Flow cytometric assay showed that the early apoptosis rates and late apoptosis (necrosis) rates of AGS cells treated with tangeretin for 48 h increased in a dose-dependent manner (Fig. 2A;
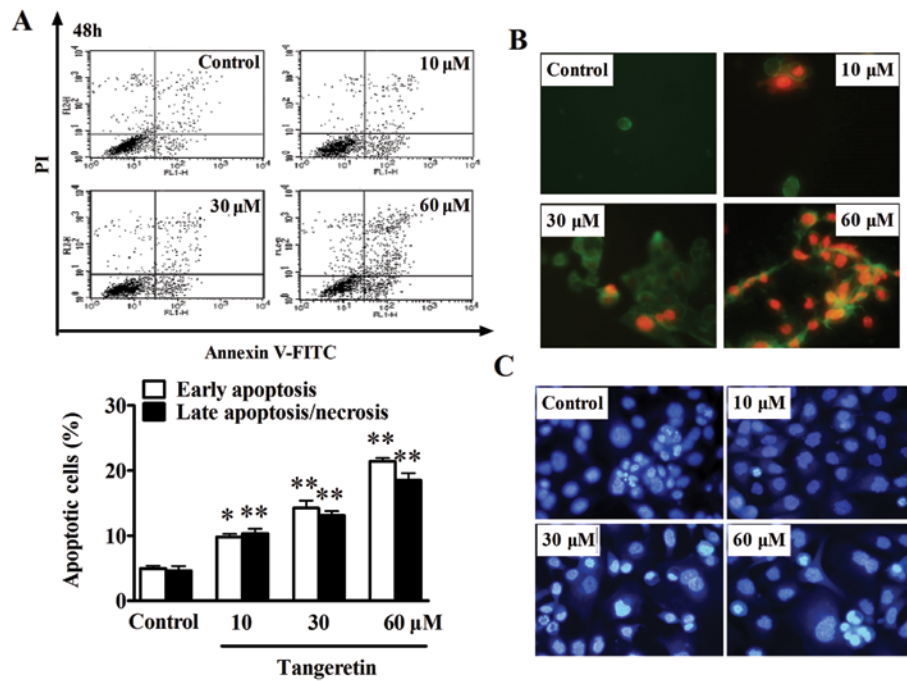
Figure 2. Effect of tangeretin on AGS cell apoptosis. (A) Flow cytometric assay showed that the percentages of early and late apoptotic cells increased in a dose-dependent manner as determined by Annexin V-FITC/PI staining. Data are presented as means ± SEM, n=3; *$P<0.05$, **$P<0.01$ vs. control. (B) The percentages of Annexin V-FITC (green)-labeled early apoptotic cells and PI (red)-labeled late apoptotic or necrotic cells increased with the rise in the concentration of tangeretin. (C) Characteristic apoptotic morphology including cell nuclear shrinkage, chromatin condensation and apoptotic bodies was observed following Hoechst 33258 staining.
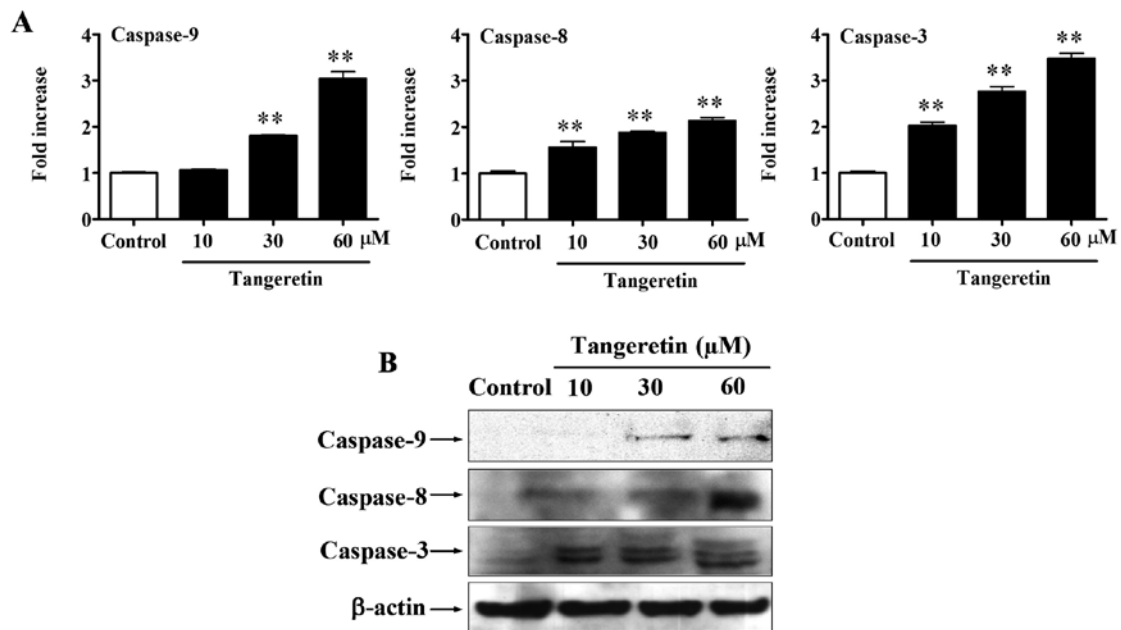

Figure 3. Effect of tangeretin on caspases. (A) Tangeretin increased the activity of caspase-3, -8 and -9 in AGS cells in a dose-dependent manner. Data are presented as means ± SEM, n=3; **$P<0.01$ vs. control. (B) Western blot analysis showed that levels of cleaved caspase-3, -8 and -9 were increased following treatment with tangeretin in a dose-dependent manner.

$P<0.05$, $P<0.01$). Fig. 2B also shows that the percentage of Annexin V-FITC (green)-labeled early apoptotic cells and PI (red)-labeled late apoptotic or necrotic cells increased along with the increase in the concentration of tangeretin. In addition, typical apoptotic morphological changes including cell nuclear shrinkage, chromatin condensation and apoptotic bodies were observed after exposure to different doses of tangeretin by Hoechst 33258 staining (Fig. 2C). These results confirmed that tangeretin induced the apoptosis of AGS cells.

Involvement of caspases in tangeretin-induced apoptosis. As shown in Fig. 3A, the results showed that the activities of
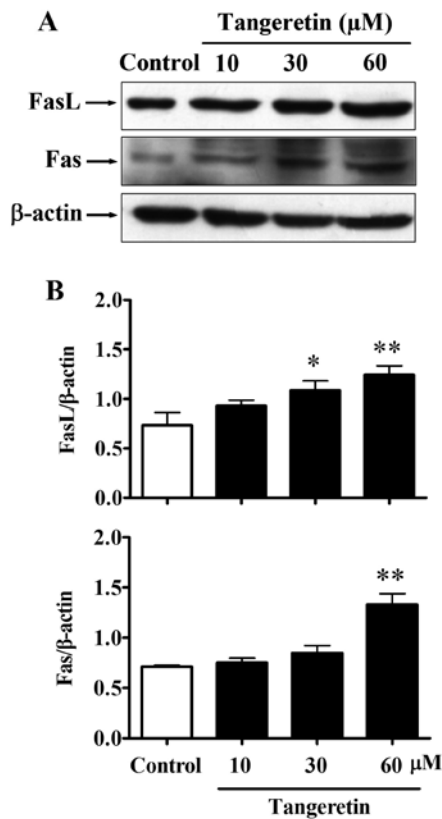
Figure 4. Effect of tangeretin on the levels of Fas and FasL expression. Western blot analysis showed that the expression levels of Fas and FasL increased in AGS cells following treatment with tangeretin for 48 h. Data are presented as means ± SEM, n=4-6; *P<0.05, **P<0.01 vs. control.

Figure 5. Effect of tangeretin on mitochondrial membrane potential (MMP) of AGS cells. (A) Following treatment with 10, 30 and 60 μM tangeretin for 48 h, cells stained with Rhodamine 123 were examined by flow cytometry. (B) Tangeretin decreased the percentages of AGS cells with high MMP. Data are presented as means ± SEM, n=4; **P<0.01 vs. control.

caspase-3, -8 and -9 were increased by tangeretin in a dose-dependent manner after a 48-h exposure (P<0.01). Treatment of cells with 60 μM tangeretin increased the caspse-3 activity ~3.5-fold, caspase-8 ~1.8-fold and caspase-9 ~3.0-fold. Western blot assay showed that the expression levels of cleaved caspase-3, -8 and -9 proteins were upregulated by tangeretin in a dose-dependent manner (Fig. 3B). These results indicate that tangeretin induces both the extrinsic and intrinsic apoptotic pathways with the activation of the caspase cascade.

Fas/FasL is involved in tangeretin-induced apoptosis. To examine the extrinsic signaling pathway through Fas, the expression levels of death receptor Fas and its ligand FasL were assessed. As shown in Fig. 4, the expression of Fas and FasL was increased in a dose-dependent manner (P<0.05, P<0.01). Thus, the binding of FasL to Fas induced Fas trimerization, which recruited and activated initiator caspase-8. Activated caspase-8 stimulates apoptosis via two parallel cascades; it directly cleaves and activates caspase-3, or it cleaves the pro-apoptotic Bcl-2 family protein Bid. Cleaved/truncated Bid (tBid) translocates to the mitochondria, inducing cytochrome *c* release, which sequentially activates caspase-9 and caspase-3.

Mitochondrial signaling pathway in tangeretin-induced apoptosis. Decrease and breakage of mitochondrial membrane potential (MMP) is an important sign of mitochondrial damage. Flow cytometric assay after Rhodamine 123 staining showed that the percentages of cells with high MMP decreased in a
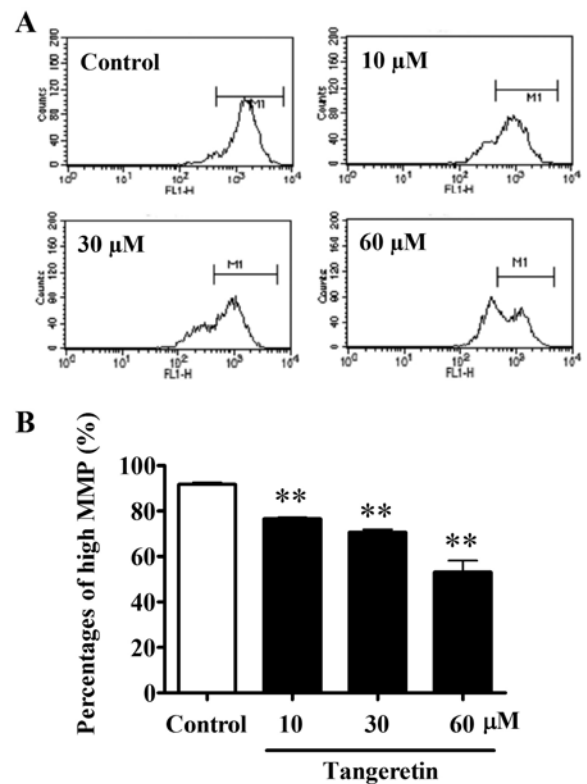
dose-dependent manner (P<0.01), suggesting that tangeretin reduced the levels of MMP and induced mitochondrial damage (Fig. 5). In addition, the protein levels of cytochrome *c*, Bax, Bid and tBid increased as the dose of tangeretin increased (Fig. 6; P<0.05, P<0.01). These results indicate that Bax and tBid activated by upstream proteins mediated mitochondrial dysfunction, induced the release of cytochrome *c* and activation of caspase-9 and caspase-3. In addition, the extrinsic signaling pathway interacted with the mitochondrial signaling pathway through induction of cleavage of Bid by caspase-8.

Tangeretin-induced apoptosis is dependent on p53 activation. As shown in Fig. 7A, significant elevations in the p53 and p21 protein levels were noted in a dose-dependent manner (P<0.01). To determine whether tangeretin-induced apoptosis is p53-dependent, a p53 inhibitor Pifithrin-α (PFT-α) was used. The results showed that 20 μM PFT-α reduced the apoptotic rates induced by tangeretin from 15.8±0.1 to 7.6±1.1% (Fig. 7B; P<0.01, P<0.001). In addition, western blot analysis showed that 20 μM PFT-α reduced the expression of p53, p21, caspase-3 and caspase-9 proteins significantly increased by 60 μM tangeretin (Fig. 7C). These results suggest that tangeretin-induced apoptosis of AGS cells was p53-dependent.

Discussion

The present study showed that tangeretin inhibited the viability and induced the apoptosis of human gastric cancer
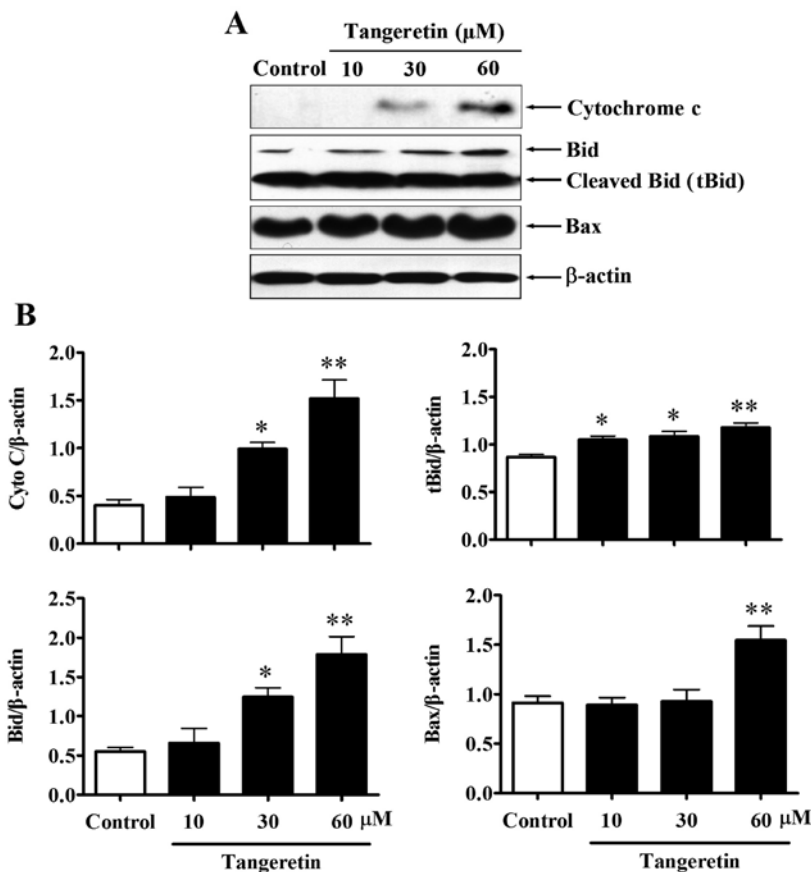
Figure 6. Effect of tangeretin on the expression levels of pro-apoptotic proteins in AGS cells. (A and B) Western blot assay showed that protein expression levels of cytochrome c, Bid, tBid and Bax were increased following tangeretin treatment for 48 h in a dose-dependent manner. Each column in B represents the means ± SEM, n=3; *$P<0.05$, **$P<0.01$ vs. control.
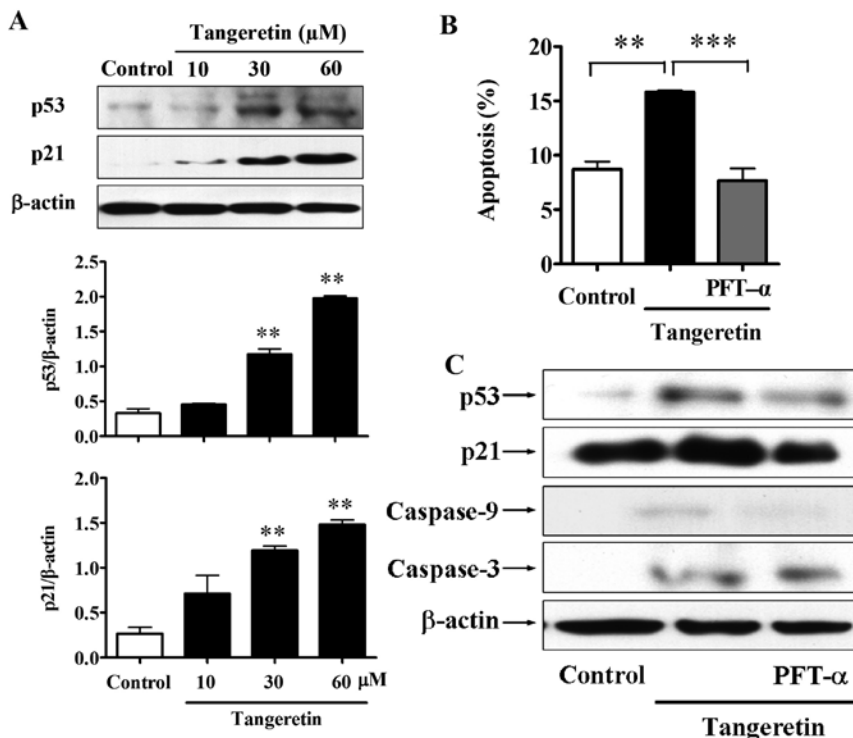

Figure 7. The p53-dependent pathway in tangeretin-induced apoptosis in AGS cells. (A) Western blot analysis showed that p53 and p21 expression in AGS cells was increased in a dose-dependent manner following treatment with tangeretin. (B) PFT-α (a p53 inhibitor) reduced the apoptotic rates of AGS cells induced by 60 μM tangeretin. (C) Western blot analysis showed that PFT-α inhibited tangeretin-induced elevation of p53, p21, caspase-3 and caspase-9 expression levels. Each column represents the means ± SEM, n=3; *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control.
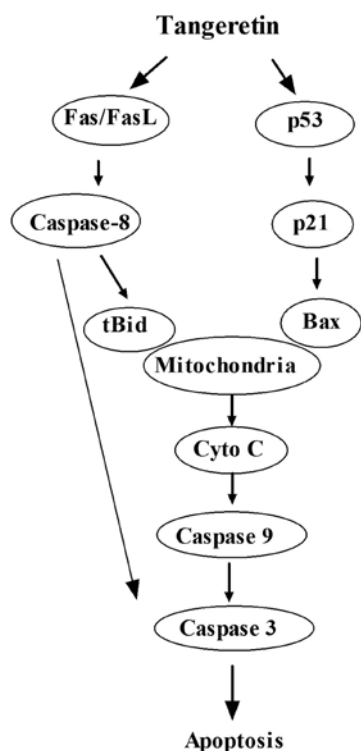
Figure 8. Pathway of tangeretin-induced apoptosis of AGS cells.

AGS cells through both extrinsic and intrinsic signaling pathways through the activation of the caspase cascade. The present study provides important data to support the finding that tangeretin as a natural component widely existing in many fruits or fruit juices exhibits antineoplastic activity in human gastric cancer.

Caspases play a crucial role in apoptosis, and the activation of caspases amplifies apoptotic signaling (18). In the present study, tangeretin increased the activities of caspase-3, -8 and -9 and upregulated the expression of cleaved caspase-3, -8 and -9 proteins. The active forms of caspase-8 and -9 can activate downstream effectors caspase-3, -6 and -7, resulting in the cleavage of several intracellular polypeptides (e.g., PARP-1) as well as activation of DNase, which leads to DNA fragmentation (19).

Apoptosis is mainly mediated by the death receptor-triggered extrinsic pathway, the mitochondrial-initiated intrinsic pathway and the endoplasmic reticulum pathway (20-22). The dissipation of MMP marks the point of no return during the apoptotic program and occurs prior to DNA fragmentation. Thus, the evaluation of MMP depolarization is of critical importance for the assessment of apoptosis (23,24). In the present study, MMP was observed to be reduced by tangeretin in a dose-dependent manner, which suggests that mitochondrial dysfunction contributed to the tangeretin-induced apoptosis. In addition, western blot analysis showed that the expression levels of Fas, FasL, Bid and tBid were increased by tangeretin. It was previously reported that Bid is activated through cleavage by caspase-8 to become cleaved Bid (tBid) in the Fas-induced process of apoptosis, and tBid is necessary for Bax oligomerization (25-27). Therefore, in the present study, tangeretin may have activated caspase-8 through Fas/

FasL, and then the activated caspase-8 cleaved Bid into tBid, which was then transferred to the mitochondria where it probably combined with Bax. Then cytochrome *c* was released to activate caspase-9 and caspase-3 (Fig. 8).

p53 is a transcription factor which plays an important role in the cellular response to DNA damage through either cell apoptosis or cell cycle arrest (13,28,29). In our previous study, tangeretin induced S phase arrest at 24 h and G2/M arrest at 48 h in AGS cells (17,30). In the present study, the results showed that p53 expression was elevated by tangeretin in a dose-dependent manner associated with upregulation of p21$^{waf1/cip1}$ and Bax in AGS cells. p21 plays an essential role in growth arrest after DNA damage, and its overexpression leads to G1 and G2 phase cell cycle arrest (31). Upregulation of p21$^{waf1/cip1}$ is also involved in the modulation of apoptosis in gastric cancer cells (13). Bax expression can be regulated by p53 (32). To investigate the function of p53 in tangeretin-treated AGS cells, we observed the effect of a p53 inhibitor (PFT-α) on the rate of apoptosis of tangeretin-treated AGS cells. The results showed that PFT-α reduced the apoptotic rate of AGS cells following treatment with tangeretin, which confirmed that the apoptosis induced by tangeretin was p53-dependent. Western blot analysis showed that PFT-α inhibited the expression of p21, Bax, caspase-3 and caspase-9, which indicated that p53 was located upstream of these proteins in the apoptotic pathway. Thus, these results suggest that tangeretin induces the apoptosis of AGS cells by p53-activated Bax. Then Bax acts in the mitochondria to cause the release of cytochrome *c*, leading to the activation of caspase-9, and the subsequent activation of caspase-3.

In summary, as shown in Fig. 8, tangeretin induced the apoptosis of AGS cells through extrinsic and intrinsic signaling pathways. Activation of p53 induced mitochondrial-mediated apoptosis through the upregulation of Bax, which contributed to the activation of caspase-9, leading to the activation of downstream caspases in the process. Moreover, the Fas/FasL-mediated death receptor pathway may interact with the mitochondrial signaling pathway through caspase-8-cleaved Bid.

Acknowledgements

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