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Ginger ingredients reduce viability of gastric cancer cells via distinct mechanisms

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Abstract

Ginger has been used throughout the world as spice, food and traditional herb. We found that 6-gingerol, a phenolic alkanone isolated from ginger, enhanced the TRAIL-induced viability reduction of gastric cancer cells while 6-gingerol alone affected viability only slightly. 6-Gingerol facilitated TRAIL-induced apoptosis by increasing TRAIL-induced caspase-3/7 activation. 6-Gingerol was shown to down-regulate the expression of cIAP1, which suppresses caspase-3/7 activity, by inhibiting TRAIL-induced NF-κB activation. As 6-shogaol has a chemical structure similar to 6-gingerol, we also assessed the effect of 6-shogaol on the viability of gastric cancer cells. Unlike 6-gingerol, 6-shogaol alone reduced the viability of gastric cancer cells. 6-Shogaol was shown to damage microtubules and induce mitotic arrest. These findings indicate for the first time that in gastric cancer cells, 6-gingerol enhances TRAIL-induced viability reduction by inhibiting TRAIL-induced NF-κB activation while 6-shogaol alone reduces viability by damaging microtubules.

Keywords: Herbal medicine; Stomach neoplasms; TRAIL; NF-κB; Microtubules

Development of gastric cancer depends on complex interactions between several bacterial, host genetic and environmental factors such as *Helicobacter pylori*, polymorphisms of cytokine genes and nitrosamines [1–3]. Gastric cancer still remains a major health problem despite recent advances of techniques and technologies of endoscopy [4]. Although chemotherapy significantly improves survival in patients with established gastric cancer [5], additional agents effective against gastric cancer are still needed to increase available regimens and advance the efficacy of chemotherapy.

Herbs are generally defined as any forms of a plant or plant product including leaves, stems, flowers, roots and seeds [6]. Herbal medication is now being used for treatment of various diseases in many countries. However, further research is required to identify bioactivities of herbal ingredients and clarify molecular mechanisms of their actions for rational applications of herbs and herbal ingredients. In this context, we expect that herbal ingredients not only exert some biological activities by themselves but also reinforce host defenses such as cancer surveillance. In the cancer surveillance, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) plays a critical role by inducing apoptosis in a wider range of cancer cells than tumor necrosis factor α or Fas ligand [7–9]. Furthermore, TRAIL has a huge potential for cancer therapy [8]. Therefore, it is important to identify herbal ingredients enhancing the TRAIL-induced viability reduction of cancer cells for preventing cancer development.

In the present study, we screened six ingredients of herbs using gastric cancer cells and identified 6-gingerol,

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a phenolic alkanone isolated from ginger. Although 6-gingerol enhanced TRAIL-induced viability reduction dramatically, 6-gingerol alone affected viability only slightly. We also assessed the effect of 6-shogaol, another phenolic alkanone isolated from ginger, on the viability of gastric cancer cells and found that 6-shogaol reduced the viability by itself unlike 6-gingerol. Finally, we elucidated the distinct actions of 6-gingerol and 6-shogaol in gastric cancer cells.

Materials and methods

Reagents and cells. We obtained all herbal ingredients (purity of each $\geqslant$ 98%) from Wako Pure Chemical (Osaka, Japan), TRAIL from PeproTech (London, UK), Z-DEVD-FMK from BioVision (Mountain View, CA), anti-caspase-3 and anti-caspase-7 antibodies from Lab Vision (Fremont, CA), anti-cIAP1 and anti-IκBα antibodies from Imgenex (San Diego, CA), anti-actin antibody from Sigma–Aldrich (St. Louis, MI), anti-p65 antibody from Cell Signaling (Beverly, MA), anti-retinoblastoma antibody from NeoMarkers (Fremont, CA), human gastric cancer-derived HGC cells from RIKEN BRC cell bank (Ibaragi, Japan), AGS cells and KATO III cells from ATCC (Rockville, Maryland).

Cell viability assay. HGC, AGS or KATO III cells (1×10^4) were seeded in 96-well plates. One day later, the cells were incubated with TRAIL, each of herbal ingredients or both for additional 24 h. Viability was determined by CellTiter96 cell proliferation assay (Promega, Madison, WI), a colorimetric method, and expressed as the ratio of optical density in the presence versus absence of the agents.

Tumor growth assay. HGC cells (2.5×10^6) were subcutaneously inoculated into the flank of 7-week-old athymic nude mice obtained from SLC (Hamamatsu, Japan). After 3 weeks when the tumors had reached an average volume of approximately 40 mm^3, the tumor-bearing mice were administered 200 μl of physiological saline alone or containing 6-gingerol (1 mg/ml), TRAIL (10 μg/ml) or both subcutaneously near the tumor every day. Alternatively, the tumor-bearing mice were administered 400 μl of physiological saline containing 6-shogaol (0, 100, or 200 μg/ml) intraperitoneally every day. The tumor volume in mm^3 was calculated as $(\text{width})^2 \times \text{length}/2$ [10], and tumor growth was calculated as the volume 1 day after the 8th administration/the volume before the first administration. Animal experiments were performed according to the guidelines of the Institute for Laboratory Animal Research in Nagoya University School of Medicine and the approval of the ethics committee in Nagoya University School of Medicine.

Apoptosis assay. HGC cells (1.5×10^5) were seeded in 24-well plates. One day later, the cells were incubated with 6-gingerol or 6-shogaol in the absence or presence of TRAIL for additional 18 h, and then stained with annexin V-FITC (BioVision) and 7-amino-actinomycin D (7-AAD) (BD Pharmingen, San Diego, CA). A total of 1×10^4 cells were counted with a flow cytometer (Beckman, Fullerton, CA), and the annexin V-FITC-positive/7-amino-actinomycin D negative cells were considered undergoing apoptosis [11]. Apoptosis induction was expressed as the relative number of the cells undergoing apoptosis after each treatment to no treatment.

Caspase activity assay. HGC cells (5×10^3) were seeded in 96-well plates. One day later, the cells were incubated with 6-gingerol or 6-shogaol in the absence or presence of TRAIL for additional 1–6 h. Caspase-8, -9, or -3/7 activity in the cell lysates was determined with Caspase-Glo 8, 9, or 3/7 assay kit (Promega) according to the manufacturer's instructions. Caspase-induced cleavage of the proluminogenic substrate containing the optimal peptide sequence for each caspase generates luminescent signal, which is detected with a luminometer (ATTO, Tokyo, Japan).

RT-PCR. Total RNA was isolated with RNeasy Mini kit (Qiagen, Valencia, CA). cDNA synthesis was performed from 3 μg of the total RNA with SuperScriptIII First-Strand Synthesis System (Invitrogen, Carlsbad, CA). PCR was conducted for 22 cycles with 30 s at 95 °C, 1 min at 54 °C and 30 s at 72 °C to detect cIAP1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, or for 23 cycles with 30 s at 95 °C, 1 min at 52 °C and 30 s at 72 °C to detect XIAP cDNA. The primers were 5′-ggttaaaggaaatgctgcgg-3′ and 5′-acccttgattatacccctgc-3′ for cIAP1 cDNA, 5′-aatggggttcagtttcaagg-3′ and 5′-aatgactgtgtagcacatgg-3′ for XIAP, and 5′-tcccatcaccatcttccag-3′ and 5′-atgagtccttccacgatacc-3′ for GAPDH.

Luciferase reporter assay. HGC cells (1.5×10^5) were seeded in 24-well plates. One day later, the cells were transfected with 500 ng of NF-κB-dependent firefly luciferase reporter [12] and 5 ng of phRL-TK construct expressing *Renilla* luciferase (Promega) using 0.5 μl of TransFectin (Bio-Rad, Hercules, CA). Twenty hours later, the cells were stimulated with TRAIL (40 ng/ml) in the absence or presence of 6-gingerol (50 μg/ml) for 4 h, and then lysed to determine luciferase activities using Dual Luciferase Reporter Assay System (Promega). Reporter activity was assessed by normalization of firefly luciferase activity to *Renilla* luciferase activity.

Immunocytochemical staining. HGC cells were incubated with 6-shogaol (0 or 2 μg/ml) for 6 h, and then fixed with 4% paraformaldehyde and 0.1% Tween 20 in phosphate-buffered saline. The cells were incubated with Alexa Fluor 568 phalloidin (Molecular Probes, Eugene, OR) or with rabbit anti-tubulin β antibody (Lab Vision) followed by incubation with anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes). The cells were viewed under a fluorescence microscope (Keyence, Tokyo, Japan).

Cell cycle analysis. HGC cells (2×10^5) were seeded in 12-well plates. One day later, HGC cells were incubated with 6-shogaol (0 or 2 μg/ml) for 16 h, and then with bromodeoxyuridine (BrdU) (10 μM) for additional 2 h. The cells were stained to measure the incorporated BrdU and total DNA contents with BrdU flow kit (BD Pharmingen) according to the manufacturer's instructions, and then a total of 1×10^4 cells were analyzed with the flow cytometer. The cells containing a large amount of DNA and little amount of BrdU were considered to be in G2 + M phases.

Statistical analysis. Synergy of TRAIL with 6-gingerol or 6-shogaol was assessed with two-way factorial analysis of variance using StatView (SAS Institute, Cary, NC), and $p < 0.05$ were considered statistically significant.

Results

Synergy between 6-gingerol and TRAIL regarding the viability reduction of gastric cancer cells

To determine the effect of herbal ingredients on TRAIL-induced viability reduction, we decided to incubate gastric cancer cells with TRAIL at concentrations inducing approximately 20% reduction of viability compared with the control (incubation without TRAIL). Therefore, HGC cells were incubated with 40 ng/ml TRAIL in the absence or presence of 50 μg/ml aucubin isolated from Plantago Herb (*Aucuba japonica* Thunb.), catalpol from Rehmannia Root (*Rehmannia glutinosa* Libosch var.), gentiopicroside from Gentian (*Gentiana lutea* L.), 6-gingerol from ginger (*Zingiber officinale* Roscoe), quercetin, a common flavonoid in plants, or swertiamarin from Swertia Herb (*Swertia japonica* Makino) (Fig. 1A). 6-Gingerol enhanced TRAIL-induced viability reduction the most dramatically among them (Fig. 1A). Interestingly, 6-gingerol alone affected viability only slightly, and statistically significant synergy was observed between 6-gingerol and TRAIL regarding viability reduction (Fig. 1B). Statistically significant synergy was also observed in two other gastric cancer-derived cells, AGS cells and KATO III cells (data not shown). Furthermore, we observed statistically significant synergy *in vivo* between 6-gingerol and TRAIL regarding the inhibition of tumor growth (Fig. 1C). TRAIL-induced

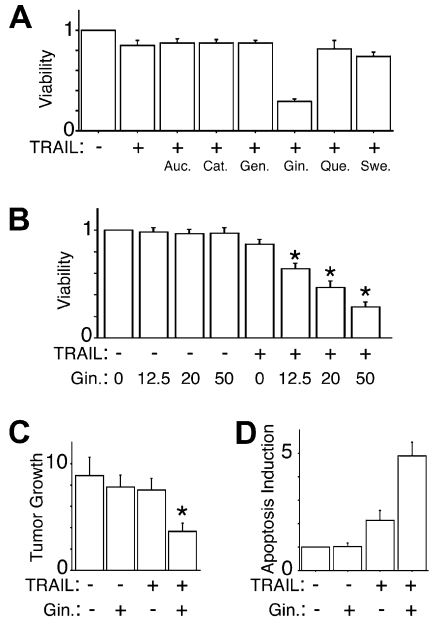

Fig. 1. 6-Gingerol enhanced the TRAIL-induced viability reduction of gastric cancer cells. (A) HGC cells were incubated with each of herbal ingredients (50 μg/ml, 134–170 μM) in addition to TRAIL (40 ng/ml) for 24 h, and viability was determined as described in Materials and methods; $n = 3$. Histograms represent means ± SD. Auc., aucubin; Cat., catalpol; Gen., gentiopicroside; Gin., 6-gingerol; Que., quercetin; Swe., swertiamarin. (B) HGC cells were incubated with 6-gingerol (12.5–50 μg/ml), TRAIL (40 ng/ml) or both for 24 h, and viability was determined; $n = 4$. $^{*}p < 0.01$, two-way factorial analysis of variance at each concentration of 6-gingerol. (C) Mice bearing tumors were given subcutaneously 200 μl of 6-gingerol (1 mg/ml), TRAIL (10 μg/ml) or both every day for 8 days. Tumor growth was determined as described in Materials and methods; $n = 4$. (D) HGC cells were incubated with 6-gingerol (50 μg/ml), TRAIL (40 ng/ml) or both for 18 h, and apoptosis induction was evaluated as described in Materials and methods; $n = 3$.

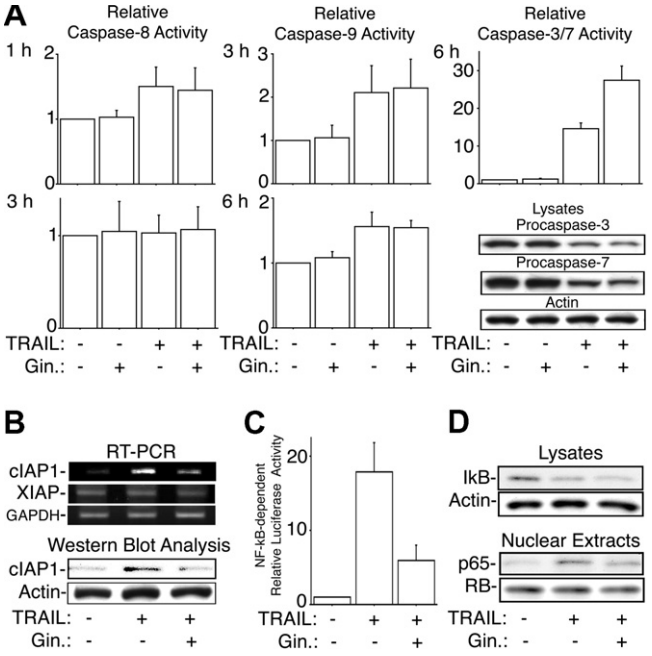

Fig. 2. 6-Gingerol elevated TRAIL-induced caspase-3/7 activation by inhibiting NF-κB activation. (A) HGC cells were incubated with 6-gingerol (50 μg/ml), TRAIL (40 ng/ml) or both for 1–6 h, and caspase activities were determined; $n = 3$. (B) HGC cells were incubated with 6-gingerol (50 μg/ml), TRAIL (40 ng/ml) or both for 6 h. RT-PCR and Western blot analysis were performed to determine cIAP1 and XIAP expression. (C) HGC cells were incubated with TRAIL (40 ng/ml) in the absence or presence of 6-gingerol (50 μg/ml) for 4 h, and NF-κB-dependent luciferase activity was determined; $n = 3$. (D) HGC cells were incubated with TRAIL (40 ng/ml) in the absence or presence of 6-gingerol (50 μg/ml) for 30 min. IκB degradation and p65 nuclear translocation were determined by Western blot analysis. RB, retinoblastoma.

apoptosis and the apoptosis induction was elevated with the co-treatment of TRAIL and 6-gingerol while 6-gingerol alone did not induce apoptosis (Fig. 1D). These findings indicate that 6-gingerol enhances the TRAIL-induced viability reduction of gastric cancer cells by facilitating TRAIL-induced apoptosis.

6-Gingerol elevates TRAIL-induced caspase-3/7 activation by inhibiting NF-κB activation

To evaluate the effect of 6-gingerol on caspase activation, HGC cells were incubated with 6-gingerol, TRAIL or both, and then caspase activities were determined. TRAIL activated caspase-8 slightly and caspase-9 up to approximately 2-fold induction. 6-Gingerol did not affect the TRAIL-induced activation of caspase-8 and -9 (Fig. 2A). We also observed that 6-gingerol did not affect the TRAIL-induced release of cytochrome *c* (Cyt *c*) from mitochondria (data not shown). On the other hand, 6-gingerol elevated the TRAIL-induced activation of caspase-3/7 without dramatic effect on the TRAIL-induced processing of procaspase-3 and -7 (Fig. 2A). In HGC cells, TRAIL-induced cIAP1 expression rather than XIAP expression, and 6-gingerol suppressed the mRNA and protein expression of cIAP1 (Fig. 2B).

TRAIL activated NF-κB (Fig. 2C), which induces cIAP1 expression [13]. Therefore, we assessed the effect of 6-gingerol on NF-κB activation and found that 6-gingerol inhibited the TRAIL-induced NF-κB activation (Fig. 2C). During the TRAIL-induced NF-κB activation, IκB was degraded and p65, a subunit of NF-κB, was translocated into the nucleus (Fig. 2D). Although the TRAIL-induced IκB degradation was not inhibited with 6-gingerol, the nuclear translocation of p65 was impaired (Fig. 2D).

6-Shogaol alone reduces the viability of gastric cancer cells in a caspase-3/7-independent manner

6-Shogaol is another ingredient of ginger, and has a very similar chemical structure to 6-gingerol (Fig. 3A). Therefore, we also assessed the effect of 6-shogaol on the viability of gastric cancer cells. Unlike 6-gingerol, 6-shogaol alone reduced the viability of HGC cells and did not show statistically significant synergy with TRAIL (Fig. 3B) ($p = 0.15$, between 1 μg/ml 6-shogaol and 40 ng/ml TRAIL; $p = 0.22$, between 2 μg/ml 6-shogaol and 40 ng/ml TRAIL). 6-Shogaol also reduced the viability of AGS cells and KATO III cells by itself (Fig. 3C). Moreover, 6-shogaol alone

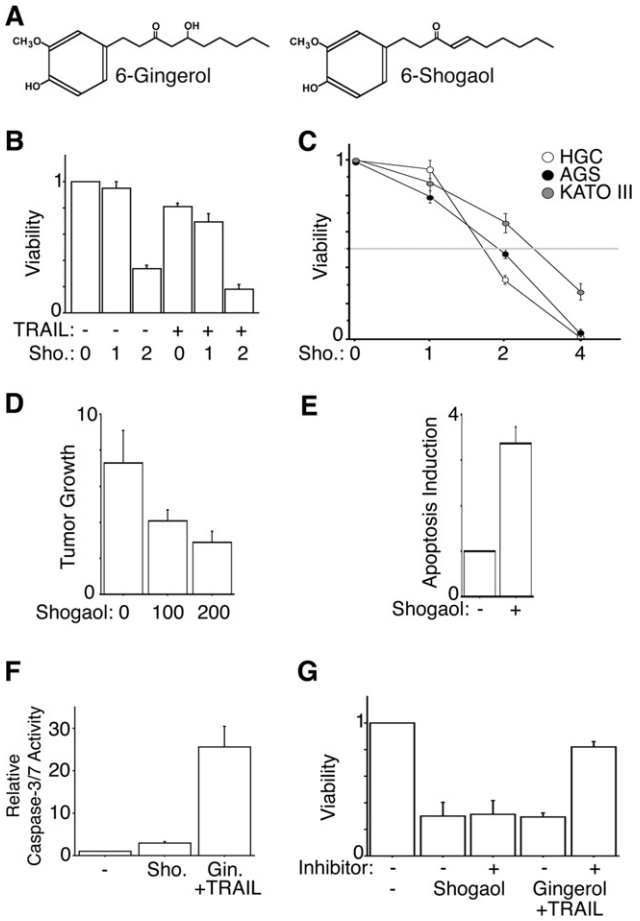

Fig. 3. 6-Shogaol alone reduced the viability of gastric cancer cells. (A) Chemical structures of 6-gingerol and 6-shogaol. (B) HGC cells were incubated with 6-shogaol (1 or 2 μg/ml), TRAIL (40 ng/ml) or both for 24 h, and viability was determined; $n = 4$. Sho., 6-shogaol. (C) HGC, AGS or KATO III cells were incubated with 6-shogaol (1-4 μg/ml) for 24 h, and viability was determined; $n = 4$. (D) Mice bearing tumors were given intraperitoneally 400 μl of 6-shogaol (100 or 200 μg/ml) every day for 8 days, and tumor growth was determined; $n = 4$. (E) HGC cells were incubated with 6-shogaol (2 μg/ml) for 18 h, and apoptosis induction was evaluated; $n = 3$. (F) HGC cells were incubated with 6-shogaol (2 μg/ml) or 6-gingerol (50 μg/ml) + TRAIL (40 ng/ml) for 6 h, and caspase-3/7 activity was determined; $n = 3$. (G) HGC cells were incubated with 6-shogaol (2 μg/ml) or 6-gingerol (50 μg/ml) + TRAIL (40 ng/ml) in the absence or presence of Z-DEVD-FMK (20 μM), a caspase-3/7 inhibitor, for 24 h, and viability was determined; $n = 3$.

suppressed tumor growth *in vivo* (Fig. 3D). Although 6-shogaol-induced apoptosis (Fig. 3E), 6-shogaol activated caspase-3/7 much less than 6-gingerol + TRAIL (Fig. 3F) under the experimental conditions showing similar viability reduction (Fig. 3G). Z-DEVD-FMK, a caspase-3/7 inhibitor, hampered 6-gingerol + TRAIL-induced viability reduction but not 6-shogaol-induced viability reduction (Fig. 3G).

6-Shogaol damages microtubules

We found that 6-shogaol treatment induced cellular shrinkage (Fig. 4A). Therefore, we assessed the effect of

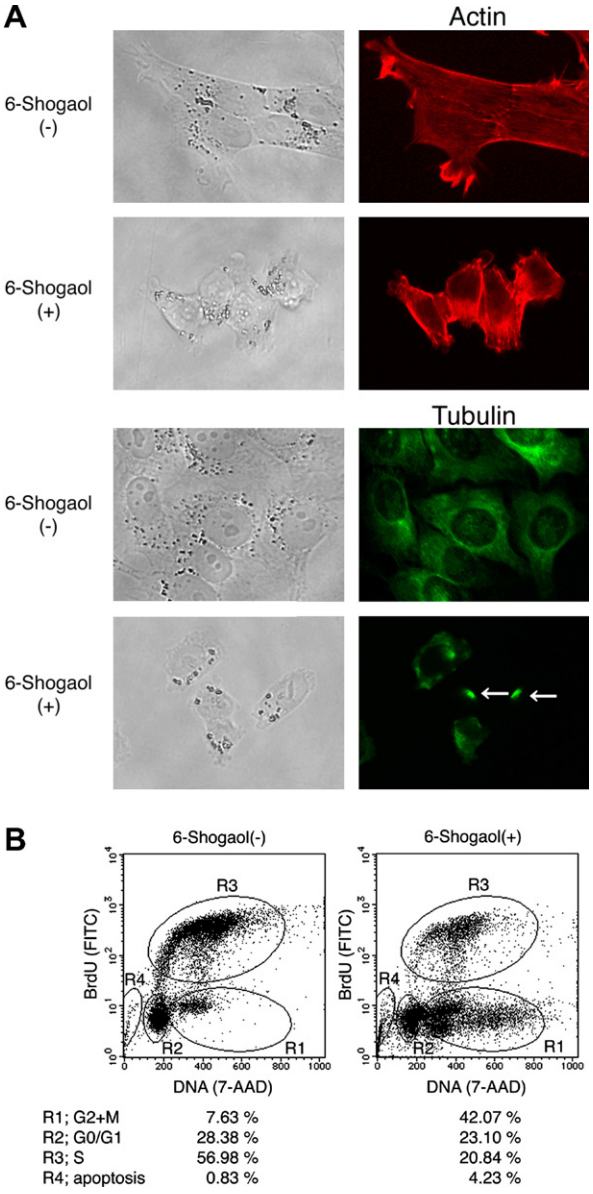

Fig. 4. 6-Shogaol disrupted tubulin distribution and induced mitotic arrest. (A) HGC cells were incubated with 6-shogaol (0 or 2 μg/ml) for 6 h, and actin or tubulin β was stained. Three independent experiments showed similar results. Arrows indicate the aggregation of tubulin β. Original magnification, 1000×. (B) HGC cells were incubated with 6-shogaol (0 or 2 μg/ml) for 16 h, and then with BrdU for additional 2 h. The cells were stained to measure the incorporated BrdU and total DNA contents, and analyzed with the flow cytometer. Three independent experiments showed similar results.

6-shogaol on cytoskeleton, actin and microtubules. Actin was distributed cortically, and the cortical distribution of actin was not affected with 6-shogaol treatment (Fig. 4A). On the other hand, tubulin β, a major constituent of microtubules, was distributed reticulately (Fig. 4A). 6-Shogaol disrupted the reticulate distribution of tubulin β and induced the aggregation of tubulin β (Fig. 4A, arrows), suggesting that 6-shogaol damaged microtubules. We also observed that 6-shogaol treatment dramatically increased a population of the cells in G2 + M phases (Fig. 4B), indicating that 6-shogaol induced mitotic arrest.

Discussion

In this study, we demonstrate for the first time that the similar ingredients isolated from ginger, 6-gingerol and 6-shogaol, affect the viability of gastric cancer cells in quite different manners.

6-Gingerol enhances the TRAIL-induced viability reduction by up-regulating TRAIL-induced apoptosis. TRAIL activates caspase-8 to an extent, which is enough to activate caspase-3/7, in type I cells, or TRAIL-induced caspase-8 activation is limited and a small quantity of active caspase-8 triggers the release of Cyt *c* from mitochondria, which induces caspase-9 activation, in type II cells [14,15]. In gastric cancer cells, TRAIL activates casapase-8 slightly, but induces the release of Cyt *c* and caspase-9 activation. 6-Gingerol affects neither TRAIL-induced caspase-8 activation, the release of Cyt *c* nor caspase-9 activation. However, 6-gingerol increases TRAIL-induced caspase-3/7 activation, suggesting that 6-gingerol down-regulates the expression of molecules inhibiting caspase-3/7 activity such as cIAP1 [14,16]. In fact, 6-gingerol suppresses TRAIL-induced cIAP1 expression. Previous studies have already demonstrated that cIAP1 expression is induced by NF-κB, especially p65 subunit [13,17]. TRAIL activates NF-κB, and 6-gingerol inhibits the TRAIL-induced NF-κB activation by impairing p65 nuclear translocation. These findings suggest that 6-gingerol enhances TRAIL-induced apoptosis, at least partially, by inhibiting TRAIL-induced NF-κB activation. The nuclear transport of proteins is mediated by a superfamily of transport receptors such as importins/karyopherins [18]. Fagerlund et al. have indicated the involvement of importin α3 and α4 in NF-κB nuclear translocation induced by tumor necrosis factor α [19]. Although the molecular mechanism of NF-κB nuclear translocation induced by TRAIL remains unknown, 6-gingerol may interfere with TRAIL-induced association between NF-κB and transport receptors.

Unlike 6-gingerol, 6-shogaol alone reduces the viability of gastric cancer cells. 6-Shogaol activates caspase-3/7 much less than 6-gingerol + TRAIL under the experimental conditions showing similar viability reduction. Furthermore, the caspase-3/7 inhibitor hampers 6-gingerol + TRAIL-induced viability reduction but not 6-shogaol-induced viability reduction, indicating clearly that 6-shogaol and 6-gingerol affect the viability of gastric cancer cells in quite different manners. 6-Shogaol disrupts the intracellular tubulin distribution and induces mitotic arrest. Tubulin is the main constituent of microtubles, and the microtubules are involved in a number of cellular functions including mitosis and cellular scaffolding [20]. Microtubule-damaging agents induce mitotic arrest followed by apoptosis and, hereby, represent an important class of anticancer drugs [20]. These findings suggest that 6-shogaol reduces the viability of gastric cancer cells by damaging microtubules. The processes coupling microtubule damage to apoptosis induction are very complicated. Previous studies have indicated that the microtubule damage affects activities of various protein kinases and the expression of upstream genes of apoptosis, leading to apoptosis via a caspase-3/7-dependent or independent pathway [20,21]. Further studies are needed to clarify how 6-shogaol damages microtubules and induces apoptosis.

Ginger has been used as traditional herb, and several biological activities of its ingredients have been suggested [22–24]. Kim et al. have indicated that 6-gingerol inhibits NF-κB activation induced by phorbol ester through the suppression of IκB degradation in the mouse skin [22]. Although 6-gingerol also inhibits NF-κB activation induced by TRAIL in gastric cancer cells, IκB degradation is not affected and the nuclear translocation of NF-κB is impaired with 6-gingerol. Kyung et al. have reported that 6-shogaol attenuates apoptosis in the experimental spinal cord injury [23] while Chen et al. have indicated that 6-shogaol induces apoptosis by depleting glutathione (GSH) in Mahlavu cells, a human hepatoma subline [24]. In gastric cancer cells, 6-shogaol induces apoptosis by damaging microtubules, but does not deplete GSH (data not shown). These findings suggest that the pharmacological effects of 6-gingerol and 6-shogaol may depend on cell types.

In conclusion, we present a model for the actions of 6-gingerol and 6-shogaol in gastric cancer cells. 6-Gingerol inhibits TRAIL-induced NF-κB activation by impairing the nuclear translocation of NF-κB, suppresses cIAP1 expression, and increases TRAIL-induced caspase-3/7 activation. 6-Shogaol damages microtubules, arrests cell cycle in G2 + M phases, and reduces viability in a casepase-3/7-independent manner.

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