Biochemical and Biophysical Research Communications 375 (2008) 129–133

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Kaempferol sensitizes colon cancer cells to TRAIL-induced apoptosis

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ARTICLE INFO

Article history:
Received 25 July 2008
Available online 3 August 2008

Keywords:
Kaempferol
Flavonoid
DR5
TRAIL
Apoptosis
Caspase
Cancer
Combination

ABSTRACT

Kaempferol is a natural compound contained in edible plants, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anti-cancer agent. Here, we show for the first time that the combined treatment with kaempferol and TRAIL drastically induced apoptosis in human colon cancer SW480 cells, compared to single treatments. Kaempferol markedly up-regulated TRAIL receptors, DR5 and DR4. DR5 but not DR4 siRNA efficiently blocked apoptosis induced by the co-treatment with kaempferol and TRAIL, indicating that DR5 up-regulation by kaempferol helps to enhance TRAIL actions. Moreover, we examined the combined effect on normal human cells. The co-treatment induced no apoptosis in normal human peripheral blood mononuclear cells and little apoptosis in normal human hepatocytes. These results suggest that kaempferol is useful for TRAIL-based treatments for cancer.

Introduction

Flavonoids are contained in many fruits, vegetables, and beverages. Kaempferol is a natural flavonoid which has been isolated from tea, broccoli, Witch-hazel, propolis, grapefruit, and other plant sources [1–3]. Kaempferol is considered to have anti-cancer potential and exerts cytotoxic effects in many types of cancer cells [4–6]. The cytotoxic effects are due to induction of apoptosis. Moreover, kaempferol strengthens the toxic effects of chemotherapeutic agents such as doxorubicin and cytarabine [6,7], making it a useful agent against cancer.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is considered a promising anti-cancer agent. TRAIL induces apoptosis in various types of cancer cells *in vitro* and *in vivo*, but has little or no toxicity against normal cells [8,9]. TRAIL interacts with specific pro-apoptotic receptors including death receptor 5 (DR5, also called TRAIL-R2) and DR4 (also called TRAIL-R1) [10–12]. TRAIL and DR5 form a protein complex and activate caspase-8 and caspase-10 [12]. The active caspase-8 and caspase-10 cleave and activate caspase-3, which is followed by the disruption of a variety of substrates resulting in apoptosis. A soluble recombinant TRAIL is undergoing a phase I clinical trial for the treatment of solid tumors [13]; however, some tumor types remain resistant to TRAIL [14]. Thus, it is important to develop strategies to overcome this resistance in tumor cells.

In this report, we show for the first time that kaempferol sensitizes cancer cells to TRAIL-induced apoptosis and overcomes TRAIL-resistance in colon cancer cells.

Materials and methods

Reagents. Kaempferol was purchased from Extrasynthese (Genay, France) and dissolved in DMSO. Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech (London, UK). Human recombinant DR5 (TRAIL-R2)/Fc chimera protein and caspase inhibitors, zVAD-fmk, zDEVD-fmk, zIETD-fmk, zLEHD-fmk, and zAEVD-fmk, were purchased from R&D Systems (Minneapolis, MN).

Cell culture. Human colon cancer SW480 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented

Abbreviations: DR5, death receptor 5; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PARP, poly(ADP-ribose)polymerase.

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doi:10.1016/j.bbrc.2008.07.131

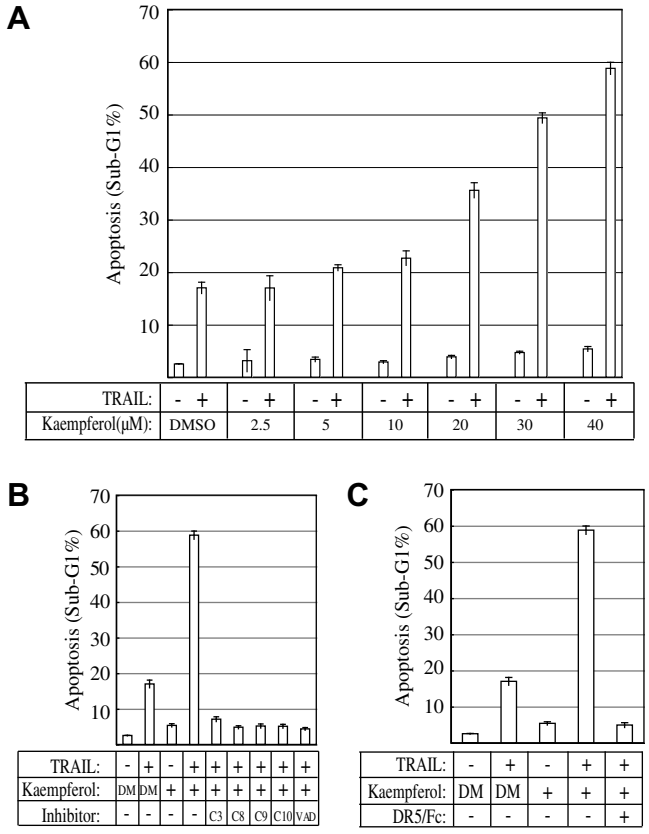

Fig. 1. Kaempferol sensitizes SW480 cells to TRAIL-induced apoptosis. (A) Colon cancer SW480 cells were treated with 20 ng/ml TRAIL and the indicated concentrations of kaempferol for 24 h. The sub-G1 population was analyzed by flow cytometry. (B) SW480 cells were cultured in the presence or absence of 20 ng/ml TRAIL and/or 40 μM kaempferol with or without 20 μM of the caspase inhibitors. The sub-G1 population was analyzed by flow cytometry. C3, caspase-3 inhibitor; C8: caspase-8 inhibitor; C9: caspase-9 inhibitor; C10, caspase-10 inhibitor; VAD, pan-caspase inhibitor, zVAD. (C) Cells were treated as shown in (B) with or without 1 μg/ml of DR5/Fc protein. The sub-G1 population was analyzed by flow cytometry. The values shown are means ($n = 3$); bars, ±SD.

with 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human colon cancer DLD-1 and prostate cancer PC3 cells were maintained in RPMI-1640 Medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Normal human peripheral blood mononuclear cells were prepared as described previously [15]. Normal human hepatocytes were obtained from Cell Systems (Kirkland, WA). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Detection of apoptosis. For the detection of the sub-G1 population, cells were harvested from culture dishes, washed with PBS, and suspended with PBS containing 0.1% Triton X-100 and RNase A (Sigma, St. Louis, MO). The nuclei were stained with propidium iodide (PI). The DNA content was measured using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ). For each experiment, 10,000 events were collected. The data was analyzed by the Cell Quest software (Becton Dickinson).

Western blot analysis. Western blotting was performed as described previously [15]. Rabbit polyclonal anti-DR5 and DR4 (Prosci, Poway, CA), Bcl-XL (Santa Cruz, Santa Cruz, CA) and survivin (R&D systems) antibodies and mouse monoclonal anti-caspase-8, -9 and -10 (MBL, Nagoya, Japan), pro-caspase-3 (Immunotech, Marseile, France), PARP (Santa Cruz), X-linked inhibitor of apoptosis protein (XIAP) (R&D systems) and β-actin (Sigma) antibodies

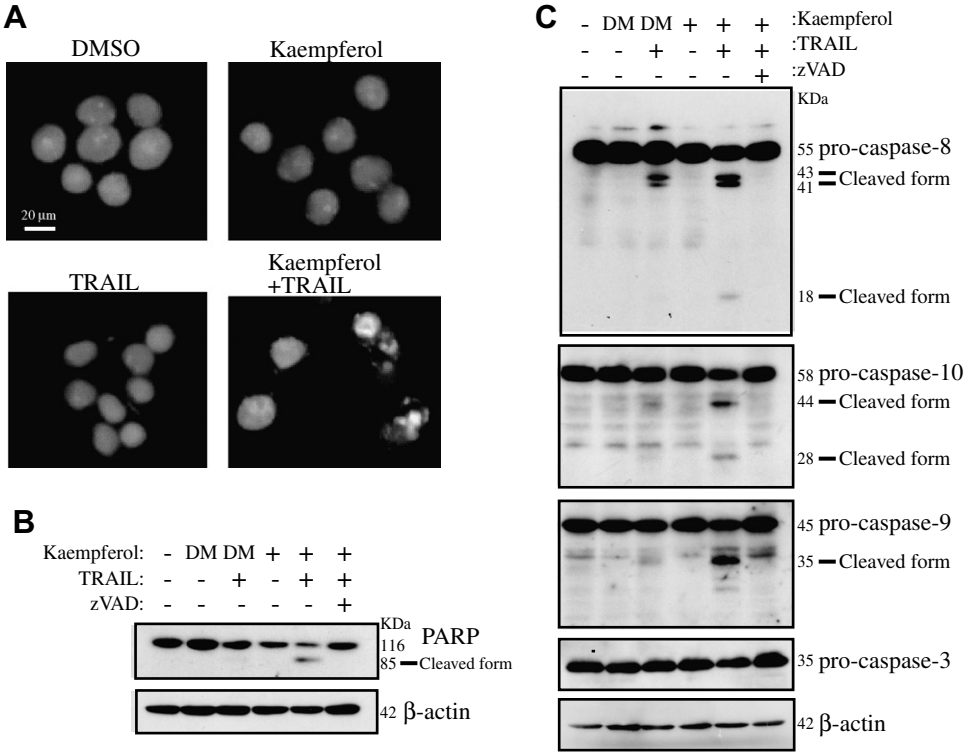

Fig. 2. The combination of kaempferol and TRAIL induces nuclear condensation and fragmentation and cleaves caspases and a substrate of caspases PARP. (A) Nuclei in cells stained with DAPI. SW480 cells were treated with 20 ng/ml of TRAIL and/or 40 μM kaempferol. (B) Western blotting of PARP. (C) Western blotting of caspase-3, -8, -9 and -10. β-Actin is a loading control. –, no treatment; DM, solvent DMSO treatment.

were used as the primary antibodies. The signal was detected with an ECL Western blot analysis system (GEhealthcare, Piscataway, NJ).

siRNA. The DR5, DR4, and control LacZ siRNA (40 nM) were transfected into SW480 cells with oligofectamine (Invitrogen, Carlsbad, CA) as described previously [16].

Statistical analysis. Data represent means ± SD for triplicate experiments and were analyzed using Student's *t*-test. Differences were considered significant from controls when $P < 0.05$.

Results and discussion

Kaempferol sensitizes colon cancer SW480 cells to TRAIL-induced apoptosis

We investigated the effect of kaempferol on apoptosis in colon cancer SW480 cells (Fig. 1A). Treatment with kaempferol did not induce apoptosis in SW480 cells. In addition, TRAIL only weakly induced apoptosis in SW480 cells as a single agent. Next, we exam-

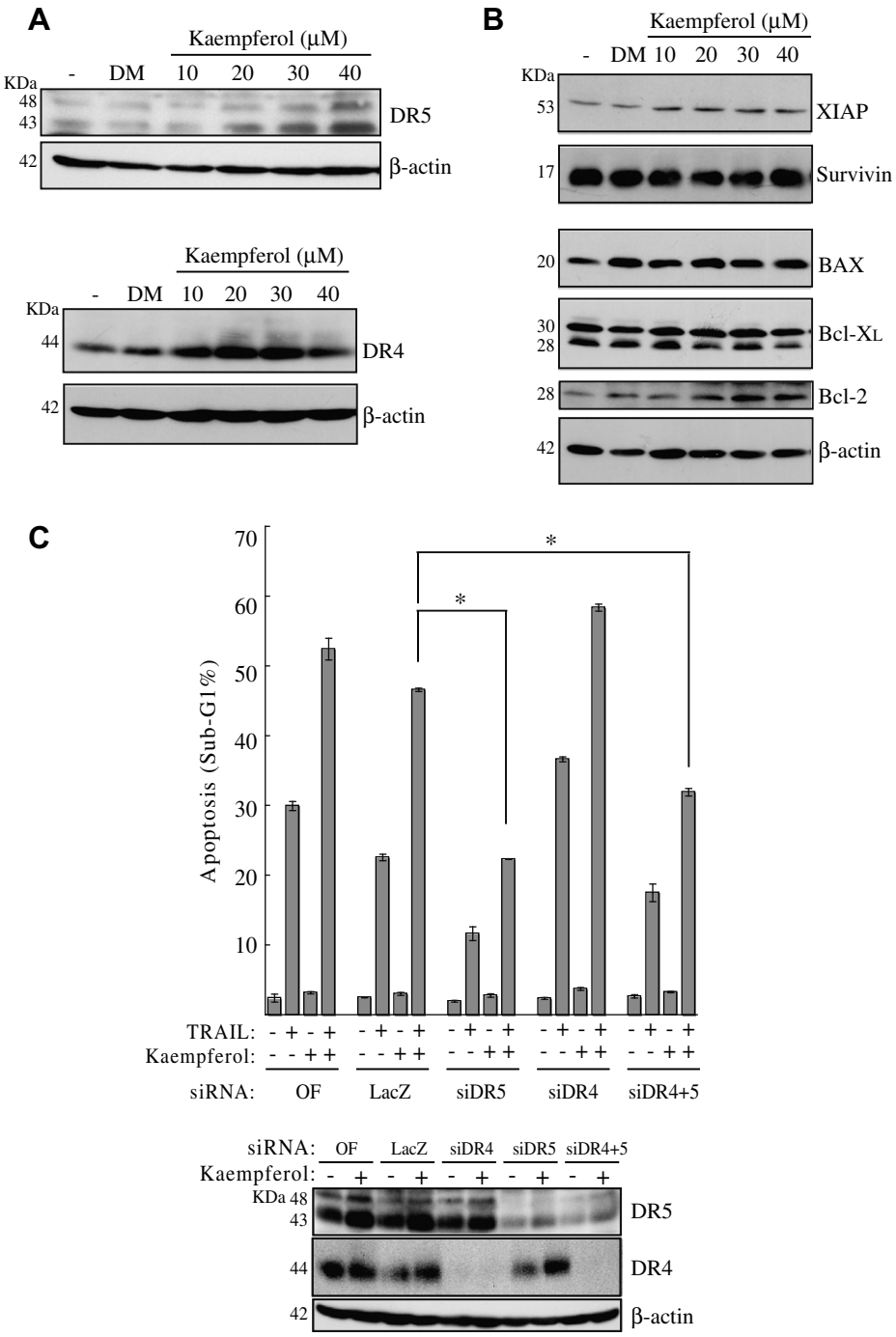

Fig. 3. Kaempferol up-regulates the expression of the TRAIL receptors DR5 and DR4, and DR5 siRNA blocks the sensitizing effect of kaempferol on TRAIL-induced apoptosis. (A) Western blotting of DR5 and DR4. SW480 cells were treated with the indicated concentrations of kaempferol for 24 h. β-Actin is a loading control. (B) Western blotting of XIAP, survivin and bcl-2 family proteins. –, no treatment; DM, solvent DMSO treatment. (C) SW480 cells were transfected with DR5, DR4 or control LacZ siRNA (40 nM). Twenty-four hours after transfection, cells were treated with 20 ng/ml of TRAIL and/or 40 μM kaempferol for 24 h. Sub-G1 populations were analyzed by flow cytometry. The values shown are means ($n = 3$); bars, ±SD. $^{*}P < 0.05$. To verify the knockdown efficiency, Western blotting was carried out after the siRNA and kaempferol treatments.

ined the combined effect of kaempferol and TRAIL on apoptosis. The combined treatment drastically induced apoptosis in a kaempferol dose-dependent manner. To confirm that the sub-G1 population represents apoptosis triggered through caspases, we employed caspase inhibitors (Fig. 1B). A pan-caspase inhibitor and specific caspase-3, -8, -9 and -10 inhibitors effectively blocked the apoptosis induced by the combined treatment, indicating that kaempferol sensitizes SW480 cells to TRAIL-induced apoptosis in a caspase-dependent manner. Furthermore, a dominant negative form of the TRAIL receptor, DR5/Fc chimeric protein, also blocked apoptosis by the combined treatment (Fig. 1C). The results indicate that kaempferol enhances TRAIL actions through the interaction of TRAIL and TRAIL receptors.

The combination of kaempferol and TRAIL induces nuclear condensation and fragmentation and cleaves caspases and a substrate of caspases, PARP

We examined nuclei after staining with DAPI (Fig. 2A). The nuclei in cells treated with kaempferol or TRAIL were similar to those in cells treated with the solvent DMSO. The combination of kaempferol and TRAIL caused nuclear fragmentation and condensation, which are characteristics of apoptotic cells. Next, the status of caspases was investigated. Poly(ADP-ribose)polymerase (PARP) is a substrate of caspases and is cleaved by caspases in apoptotic cells [17]. The cleaved PARP is used as a marker of apoptosis. We performed Western blotting of PARP in SW480 cells treated with kaempferol and/or TRAIL (Fig. 2B). The cleaved form of PARP was detected on combined treatment, but not on single treatment with kaempferol or TRAIL. These results indicate that drastic apoptosis is caused only by the combined treatment with kaempferol and TRAIL which correlated with the results in Figs. 1 and 2A. Moreover, we examined the cleavage of caspases, since caspases are activated by cleavage resulting in apoptosis. Caspase-3, -8, -9 and -10 were clearly cleaved on the treatment with kaempferol and TRAIL, in comparison with single treatments (Fig. 2C). The pan-caspase inhibitor zVAD blocked the cleavage of PARP and caspases by the combined treatment (Fig. 2B and C).

Kaempferol up-regulates expression of the TRAIL receptors DR5 and DR4

To elucidate the molecular mechanism underlying the enhancement of TRAIL-induced apoptosis by kaempferol, we examined the expression of TRAIL receptors. As shown in Fig. 3A, kaempferol increased the DR5 and DR4 protein level in a dose-dependent manner. Previous reports showed that XIAP and survivin modulate sensitivity to TRAIL in cancer cells [14]. Thus, we examined the expression of XIAP and survivin in kaempferol-treated cells, but levels were unchanged (Fig. 3B). In addition, bcl-2 family proteins,

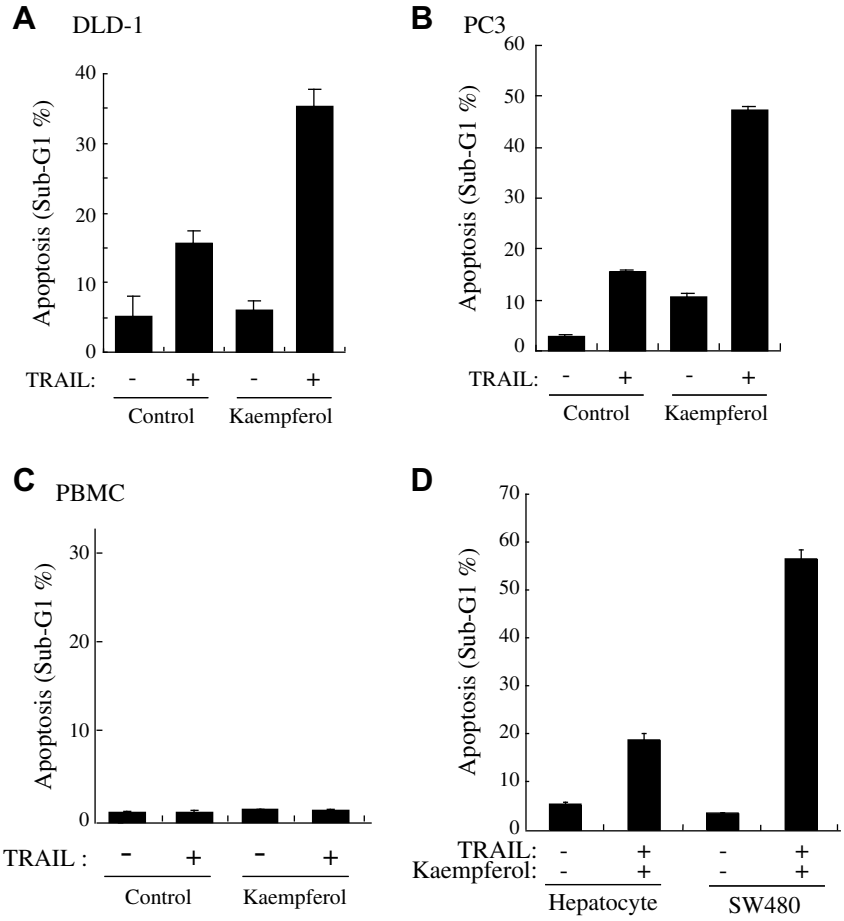

Fig. 4. The combination of kaempferol and TRAIL exerts low toxicity in normal cells. Human colon cancer DLD-1 cells (A) and prostate cancer PC3 cells (B) were treated with 40 μM kaempferol and/or 5 ng/ml of TRAIL for 24 h. (C) Normal human peripheral blood mononuclear cells (PBMC) were treated with 40 μM kaempferol and/or 20 ng/ml of TRAIL for 24 h. Sub-G1 populations were analyzed by flow cytometry. (D) Normal human hepatocytes and colon cancer SW480 cells were treated as shown in (C). Sub-G1 populations were analyzed by flow cytometry and compared with data for SW480. The values shown are means ($n = 3$); bars, ±SD.

Bcl-2, Bcl-XL, and Bax, are also reported to regulate TRAIL-induced apoptosis [14,18]. As shown in Fig. 3B, kaempferol did not significantly change Bcl-XL and Bax protein levels. Conversely, Bcl-2 protein was increased by kaempferol treatment, which is consistent with a previous report [19]. These results indicate that the up-regulation of DR5 and DR4 proteins may be a key mechanism by which kaempferol sensitizes SW480 cells to TRAIL-induced apoptosis.

DR5 siRNA blocks the sensitizing effect of kaempferol on TRAIL-induced apoptosis

To elucidate whether DR5 and DR4 up-regulation by kaempferol contributes to the dramatic apoptosis induced by the combination of kaempferol and TRAIL, we used siRNA to prevent the up-regulation of DR5 and DR4 expression. We performed Western blotting and confirmed that DR5 and DR4 siRNAs could actually provoke the knockdown of target genes (Fig. 3C). The DR5 but not DR4 siRNA effectively abrogated the apoptosis induced by the combined treatment. Furthermore, the DR4 siRNA and DR5 siRNA together had a similar effect to DR5 siRNA alone. These results indicate that DR5 but not DR4 up-regulation by kaempferol plays an important role in the apoptosis induced by kaempferol and TRAIL.

The combination of kaempferol and TRAIL exerts low toxicity in normal cells

To eliminate a possibility that the combined effect with kaempferol and TRAIL is in a SW480 cells specific manner, we examined the combined effect in other cancer cells. Kaempferol enhanced TRAIL-induced apoptosis in human colon cancer DLD-1 and prostate cancer PC3 cells as well as in SW480 cells (Fig. 4A and B). Inducing apoptosis is an effective way to eliminate cancer; however it also causes cell death in normal tissues. Discriminating between cancer and normal cells is important in the development of anti-cancer agents. In this regard, TRAIL is a promising candidate because it induces apoptosis in cancer cells but not normal cells [8,9]. Therefore, enhancing TRAIL efficacy with kaempferol is also expected to have little toxic effect in normal human cells. Thus, peripheral blood mononuclear cells (PBMC) were treated with kaempferol and/or TRAIL (Fig. 4C). The combined treatment did not induce apoptosis in PBMC. A histidine-tagged version of recombinant TRAIL but not non-tagged TRAIL had cytotoxic effects on normal human hepatocytes [20]. Thus, we used non-tagged TRAIL in this study. To eliminate the possibility that kaempferol enhances TRAIL-induced apoptosis in normal human hepatocytes as well as colon cancer SW480 cells, we examined the combined effect on human hepatocytes. As shown in Fig. 4D, the combination caused very little apoptosis in normal human hepatocytes compared with colon cancer SW480 cells.

We showed here that a flavonoid, kaempferol, sensitizes colon cancer cells to TRAIL-induced apoptosis due to the induction of DR5. Moreover, we showed that the combination of kaempferol and TRAIL had little cytotoxic effects in normal cells in comparison with colon cancer SW480 cells. These results suggest that the combination of TRAIL with kaempferol is a useful strategy of cancer therapeutics.

Acknowledgments

This work was supported in part by The Japanese Ministry of Education, Culture, Sports, Science and Technology. We thank Miss Ayumi Nakata and Mr. Yusuke Higuchi for their experimental help.

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