Halocynthiaxanthin and Peridinin Sensitize Colon Cancer Cell Lines to Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand

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Abstract

Carotenoids are compounds contained in foods and possess anticarcinogenic activity. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a promising candidate for cancer therapeutics due to its ability to induce apoptosis selectively in cancer cells. However, some tumors remain tolerant to TRAIL-induced apoptosis. Therefore, it is important to develop agents that overcome this resistance. We show, for the first time, that certain carotenoids sensitize cancer cells to TRAIL-induced apoptosis. Combined treatment with halocynthiaxanthin, a dietary carotenoid contained in oysters and sea squirts, and TRAIL drastically induced apoptosis in colon cancer DLD-1 cells, whereas each agent alone only slightly induced apoptosis. The combination induced nuclear condensation and poly(ADP-ribose) polymerase cleavage, which are major features of apoptosis. Various caspase inhibitors could attenuate the apoptosis induced by this combination. Furthermore, the dominant-negative form of a TRAIL receptor could block the apoptosis, suggesting that halocynthiaxanthin specifically facilitated the TRAIL signaling pathway. To examine the molecular mechanism of the synergistic effect of the combined treatment, we did an RNase protection assay. Halocynthiaxanthin markedly up-regulated a TRAIL receptor, death receptor 5 (DR5), among the death receptor–related genes, suggesting a possible mechanism for the combined effects. Moreover, we examined whether other carotenoids also possess the same effects. Peridinin, but not alloxanthin, diadinoximate, and pyrrhoxanthin, induced DR5 expression and sensitized DLD-1 cells to TRAIL-induced apoptosis. These results indicate that the combination of certain carotenoids and TRAIL is a new strategy to overcome TRAIL resistance in cancer cells.


Introduction

Carotenoids are biologically active compounds contained in plants and microorganisms, but they cannot be synthesized in animals. We obtain carotenoids from our diet, and the carotenoids in our blood and peripheral tissue reflect our dietary intake (1, 2). Various natural carotenoids have been proven to have anticarcinogenic activity by epidemiologic studies and animal studies of colon, liver, skin, and lung cancer models (2, 3). Colon cancer is considered to be related to dietary habits, and numerous studies suggest that a high intake of fruits and vegetables that are rich in carotenoids has been associated with decreased risk of colon cancer (4, 5).

Halocynthiaxanthin is a kind of carotenoid isolated from sea squirt Halocynthia roretzi and inhibits the growth of human malignant tumor cells (6). Moreover, halocynthiaxanthin possesses an inhibitory effect on EBV activation activity of a tumor promoter in Raji cells, which is widely used as a primary screening test for antitumor-promoting activity (7) and shows the highest suppressive effect on the generation of free radicals among 19 natural carotenoids (8).

A death ligand, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), is one of the most promising new candidates for cancer therapeutics, due to its ability to induce apoptosis selectively in cancer cells in vitro and in vivo, with little or no toxicity toward normal cells (9-11). Death receptor 5 (DR5; also called TRAIL-R2) is a receptor for TRAIL (12-15). Interaction between TRAIL and DR5 forms a protein complex with an adapter protein Fas-associated death domain (FADD) and activates initiator caspases containing caspase-8 and caspase-10 (16). Initiator caspases cleave and activate effector caspases, like caspase-3, and the activated effector caspases disrupt a variety of substrates and lead to apoptosis (16). In some cell types, as a secondary effect, initiator caspases cleave BID and the truncated BID stimulates mitochondria followed by caspase-9 activation, effector caspase activation, and apoptosis (17). Soluble recombinant TRAIL is in a phase I clinical trial for...
the treatment of solid tumors (18), but some tumor types exhibit resistance to TRAIL (19). Thus, it is important to overcome this resistance in tumor cells. Conventional antitumor agents, such as doxorubicin, cisplatin, and 5-fluorouracil, sensitize malignant tumor cells to TRAIL-induced apoptosis (20-22), although these agents lead to DNA damage in cells. Therefore, to develop more safe TRAIL sensitizers, we have been searching for candidates among food factors.

**FIGURE 1.** Halocynthiaxanthin (Halocyn) sensitizes DLD-1 cells to TRAIL-induced apoptosis. A. DLD-1 cells were treated with 40 μmol/L halocynthiaxanthin for 24 h and subsequently incubated with 10 ng/mL TRAIL for 12 h. The DNA contents of cells were analyzed by flow cytometry. M1 shows the populations of apoptotic cells with sub-G₁ DNA contents. Percentages of sub-G₁. Columns, mean (n = 3); bars, SD. B. Analysis of the synergistic effect of the combination with halocynthiaxanthin and TRAIL.
In this report, we show for the first time that dietary carotenoids act as sensitizers for TRAIL-induced apoptosis.

Results

Dietary Carotenoid Halocynthiaxanthin Sensitizes Colorectal Cancer DLD-1 Cells to TRAIL-Induced Apoptosis

We investigated the effect of combined treatment with halocynthiaxanthin and TRAIL on apoptosis by measuring the sub-G1 population (Fig. 1A). Halocynthiaxanthin or TRAIL as single agents slightly induced apoptosis in colorectal cancer DLD-1 cells. However, interestingly, the combination of halocynthiaxanthin and TRAIL drastically induced apoptosis. These results suggest that halocynthiaxanthin functions as a sensitizer for TRAIL-induced apoptosis. In this study, we used recombinant human TRAIL without any tag protein because histidine-tagged TRAIL is toxic to normal hepatocytes (11). To examine whether the apoptosis induced by the combined treatment of halocynthiaxanthin and TRAIL is synergistic or additive effect, combination index (CI) values were analyzed by the method of Chou and Talalay (Fig. 1B; Tables 1 and 2; ref. 23). CI value was 0.45 or 0.102, when fraction affected by the dose (Fa) was 0.2 or 0.8, respectively. An additive effect is reflected by CI = 1, a synergistic effect is reflected by CI < 1, and an antagonistic effect is reflected by CI > 1 (24). Thus, the CI values suggest that the combination of halocynthiaxanthin and TRAIL possesses synergistic effect.

Combination of Halocynthiaxanthin and TRAIL Induces Cleaved Caspases

Next, we examined the statuses of various caspases by Western blotting. The cleaved forms of caspase-8, caspase-10, caspase-9, and caspase-3 were strikingly detected following combined treatment with halocynthiaxanthin and TRAIL (Fig. 3A). Moreover, all procaspases were decreased by the combined treatment. Bid is cleaved by caspase-8, and subsequently, the cleaved Bid mediates the apoptotic signal from the TRAIL receptor to mitochondria (17). Indeed, combined treatment with halocynthiaxanthin and TRAIL cleaved Bid (Fig. 3B). A dominant-negative form of the TRAIL receptor, DR5/Fc chimeric protein, attenuated the cleaved caspases and Bid (Fig. 3A and B).

Caspase Inhibitors and DR5/Fc Chimeric Protein Block Apoptosis Induced by the Combination of Halocynthiaxanthin and TRAIL

We investigated whether caspase inhibitors could block the apoptosis induced by the combined treatment of halocynthiaxanthin and TRAIL. Inhibitors of caspase-3, caspase-10,
caspase-8, and pan-caspase blocked the apoptosis (Fig. 4). These results indicate that the apoptosis depends on caspases. In addition, DR5/Fc chimeric protein markedly blocked the apoptosis, indicating that the apoptosis is induced through a specific interaction of TRAIL and its receptor.

Halocynthiaxanthin Specifically Induces DR5 Expression among Death Receptor–Related Genes

Next, we examined the mechanism for the sensitization of TRAIL by halocynthiaxanthin. Previous reports have shown that the exogenous expression of TRAIL receptor using expression vector can overcome TRAIL resistance (26, 27). Therefore, we hypothesized that halocynthiaxanthin may modulate the expression of TRAIL receptor. We carried out an RNase protection assay to examine the expressions of death receptor–related genes. Consequently, halocynthiaxanthin specifically up-regulated DR5, a receptor of TRAIL, among death receptor–related genes (Fig. 5A). We confirmed the increase of DR5 mRNA by halocynthiaxanthin using Northern blotting. Halocynthiaxanthin up-regulated DR5 mRNA in a dose- and time-dependent manner (Fig. 5B). Furthermore, halocynthiaxanthin increased DR5 protein in a dose- and time-dependent manner (Fig. 5C and D). We prepared subcellular fractions and carried out Western blotting of DR5. Halocynthiaxanthin markedly induced DR5 protein in membrane fraction (Fig. 5E). Interaction between TRAIL and DR5 forms a protein complex with FADD (16). We did a pull-down assay in DLD-1 cells treated with halocynthiaxanthin and recombinant TRAIL and examined TRAIL-induced complex. Halocynthiaxanthin treatment enhanced TRAIL-induced formation of the complex containing DR5 and FADD (Fig. 5F).

DR5 Small Interfering RNA Blocks the Sensitizing Effect of Halocynthiaxanthin to TRAIL-Induced Apoptosis

To examine the relation between DR5 up-regulation and the sensitization to TRAIL by halocynthiaxanthin, we used DR5 small interfering RNA (siRNA). DR5 siRNA blocked the up-regulation of DR5 by halocynthiaxanthin at a total protein level (Fig. 6A). In addition, DR5 siRNA decreased cell surface DR5 protein (Fig. 6B). DR5 siRNA effectively prevented the sensitizing effect to TRAIL (Fig. 6C), suggesting that the sensitizing effect of halocynthiaxanthin on TRAIL-induced apoptosis at least partly depends on the
up-regulation of DR5 by halocynthiaxanthin. Next, we investigated the effect of halocynthiaxanthin in other colon cancer HT29 cells. Halocynthiaxanthin induced DR5 expression and enhanced TRAIL-induced apoptosis in HT29 cells as well as DLD-1 cells (Fig. 7).

**Carotenoids Induce DR5 Expression and Sensitize DLD-1 Cells to TRAIL-Induced Apoptosis with Specificity**

We next investigated whether carotenoids universally induce DR5 expression. We used four additional carotenoids contained in corbicula clams (Fig. 8A; ref. 28). Interestingly, peridinin also up-regulated DR5 expression, but alloxanthin, diadinochrome, and pyrrhoxanthin did not induce DR5 expression (Fig. 8B). It has been reported that peridinin also exhibits antitumor and anticarcinogenic activity (29, 30). These results indicate that carotenoids do not universally, but rather, specifically, up-regulate DR5 expression. Furthermore, we examined the sensitizing effect of carotenoids for TRAIL-induced apoptosis. Halocynthiaxanthin and peridinin enhanced TRAIL-induced apoptosis (Fig. 8C). However, alloxanthin, diadinochrome, and pyrrhoxanthin only showed an additive effect or less than that when together with TRAIL. Of interest, these carotenoids, which sensitized DLD-1 cells to TRAIL-induced apoptosis, accorded with those that up-regulated DR5 expression (Fig. 8B and C).
Discussion

In this study, we showed for the first time that dietary carotenoids sensitize cancer cells to TRAIL-induced apoptosis. Moreover, we first showed that dietary carotenoids up-regulate the expression of DR5, a TRAIL receptor. Of interest, only some carotenoids possess these activities. The carotenoids that induced DR5 expression are the same as those that sensitized DLD-1 cells to TRAIL, suggesting that the DR5 up-regulation is a possible mechanism for the sensitization to TRAIL by carotenoids. Moreover, DR5 siRNA partly prevented the enhancement of TRAIL-induced apoptosis by halocynthiaxanthin accompanied with the inhibition of DR5 up-regulation by halocynthiaxanthin. Indeed, DR5 overexpression enhances the sensitivity of cancer cells to TRAIL (26, 27). In addition, our results suggest the possibility that more effective carotenoids than halocynthiaxanthin or peridinin could be detected among several carotenoids and that more effective agents may be developed through insight into the common chemical structure.

To date, it has been reported that combined treatment with TRAIL and conventional antitumor agents, such as doxorubicin, cisplatin, and 5-fluorouracil, is a promising strategy against malignant tumors (20-22). However, the action of these conventional antitumor agents is accompanied by DNA damage. In this regard, carotenoids are considered to be safer than these conventional antitumor agents. Thus, carotenoids may substitute for conventional antitumor agents in combined treatment with TRAIL against malignant tumors.

We show here that DR5 expression is specifically induced by carotenoids among death receptor–related genes. To our knowledge, reports of carotenoid-regulated genes in human cells are rare. Especially, this is the first report about genes regulated by halocynthiaxanthin and peridinin. Therefore, our
results may be useful for the elucidation of the molecular mechanisms for the function of carotenoids in humans. Some carotenoids, such as β-carotene, possess provitamin A activity (2, 3). Previous reports showed that retinoic acids also up-regulate DR5 expression (22). Because the carotenoids we used in this study do not possess provitamin A activity, the mechanism of DR5 induction by carotenoids may be different from that by retinoic acids. Moreover, as an important point, a previous report showed that DR5 down-regulation by siRNA promotes tumor growth in an in vivo xenograft model and confers resistance to the antitumor agent 5-fluorouracil (31). From this point of view, it may be important for cancer prevention that carotenoids up-regulate DR5 expression, as shown in our present study.

DR5 is a downstream gene of the tumor suppressor p53 gene (32-34). However, halocynthiaxanthin up-regulates DR5

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**FIGURE 6.** Down-regulation of DR5 by siRNA partially prevents the sensitizing effect of halocynthiaxanthin on TRAIL-induced apoptosis. **A.** Western blotting. DR5 siRNA or control LacZ siRNA were transfected into DLD-1 cells. Twenty-four hours after the transfection, 40 μmol/L halocynthiaxanthin or DMSO was treated for 24 h. OF, treated with Oligofectamine without any siRNA. β-Actin is a loading control. Arrowheads, DR5 protein bands. *, a nonspecific signal because the band was not affected by DR5 siRNA. **B.** Cell surface expression. Forty-eight hours after transfection of DR5 siRNA or control LacZ siRNA with FITC-oligonucleotides, cell surface levels of DR5 and DR4 were detected with phycoerythrin-conjugated anti-DR5 (DR5 Ab), anti-DR4 (DR4 Ab), or anti-mouse IgG1 isotype control (CT Ab) antibody by flow cytometry. Cell surface level was analyzed in FITC-positive cells. Clear histograms, control LacZ siRNA–transfected cells; gray histograms, DR5 siRNA–transfected cells. **C.** DLD-1 cells were treated with 40 μmol/L halocynthiaxanthin and/or 10 ng/mL TRAIL after transfection of siRNA and FITC-oligonucleotides. The DNA contents of cells were analyzed by flow cytometry. M1 shows the populations of apoptotic cells with sub-G1 DNA contents in FITC-positive cells. Percentages of sub-G1. Columns, mean (n = 3); bars, SD. *, P < 0.05.
independent of p53 because we used DLD-1 cells, which contain a mutation of the p53 gene. More than half of malignant tumors possess an inactivating mutation in the p53 gene (35, 36). Halocynthiaxanthin and peridinin slightly induced apoptosis in DLD-1 cells, indicating that the apoptosis by these carotenoids is independent of p53. Furthermore, these carotenoids can sensitize cancer cells to TRAIL-induced apoptosis in a p53-independent manner. Thus, our results suggest that the combination of carotenoids and TRAIL is useful for cancer treatment, regardless of the p53 status.

Recently, we reported that flavonoid luteolin sensitizes cancer cells to TRAIL-induced apoptosis (37). Flavonoids and carotenoids are abundantly contained in foods and have been shown to have anticarcinogenic effects (3, 38). It is remarkable that both of these famous food factors are capable of enhancing TRAIL activity. TRAIL is a cytokine existing at concentrations in the order of nanograms per milliliter in the blood or urine of the human body due to the stimuli of IFN or Bacillus Calmette-Guerin (39, 40). These and our data together raise the possibility that TRAIL-DR5 signaling may be responsible for the anticarcinogenic effects of carotenoids and flavonoids.

**Materials and Methods**

**Reagents**

Halocynthiaxanthin was derived from fucoxanthin according to the method described previously (41), and peridinin, alloxanthin, diadinochrome, and pyrrhoxanthin were prepared as described previously (28). These carotenoids were dissolved...
in DMSO. Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech. Human recombinant DR5 (TRAIL-R2)/Fc chimera protein and caspase inhibitors, zVAD-fmk, zDEVD-fmk, zIETD-fmk, and zAEVD-fmk, were purchased from R&D Systems.

**Cell Culture**

Human colorectal cancer DLD-1 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Human colon cancer HT29 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 4 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

**Detection of Apoptosis**

Cells stained with 4',6-diamidino-2-phenylindole were analyzed using a fluorescent microscope as described previously (42). For the detection of sub-G₁, cells were harvested from culture dishes and washed with PBS. The cells were suspended with PBS containing 0.1% Triton X-100 and RNase A (Sigma Chemical). The nuclei were stained with propidium iodide before the DNA content was measured using FACSCalibur.

**FIGURE 8 Continued. C.**

DLD-1 cells were treated with 40 µmol/L of the indicated carotenoids for 24 h, then treated with 10 ng/mL TRAIL, and incubated for the following 12 h. The DNA contents of cells were analyzed by flow cytometry. M₁ shows populations of apoptotic cells with sub-G₁ DNA contents. Percentages of sub-G₁. Columns, mean (n = 3); bars, SD.
(Becton Dickinson). For each experiment, 10,000 events were collected. The CellQuest software (Becton Dickinson) was used to analyze the data.

**Northern Blot Analysis and RNase Protection Assay**

Total RNA from the cells was extracted using the Sepasol-RNA I (Nacalai Tesque). Total RNA (10 μg) was separated with electrophoresis on a 1% agarose gel and transferred to a nylon membrane (Biodyne B, Pall). A full-length DR5 cDNA was used as a probe for the Northern blot analysis. Hybridization was carried out with a 32P-labeled probe in PerfectHyb PLUS Hybridization buffer (Toyobo) at 68°C for 16 h and the membrane was washed at 68°C in 2x SSC containing 0.1% SDS. The blot was exposed to X-ray Films (Kodak). The RNase protection assay was done with an RPA III kit (Ambion). hAPO3d (death receptor and death ligand) template sets (BD Biosciences PharMingen) were labeled with [α-32P]UTP and MAXI script (Ambion). The labeled RNA probes were hybridized with 10 μg total RNA from DLD-1 and digested with RNase. The remaining probes were analyzed on 5% urea-polyacrylamide gel.

**Western Blot Analysis**

The cell lysate was prepared as described previously (43). Cell lysate containing 50 μg protein was separated on 10% or 12.5% SDS-polyacrylamide gel for electrophoresis and blotted onto polyvinylidene difluoride membranes (Millipore). Rabbit polyclonal anti-DR5 antibody (Cayman Chemical); mouse monoclonal anti-Bid, anti-caspase-8, anti-caspase-9, and anti-caspase-10 antibodies (MBL); mouse monoclonal anti-pro-caspase-3 antibody (Immunotech); rabbit monoclonal cleaved caspase-3 antibody (Cell Signaling); mouse monoclonal anti-PARP antibody (Santa Cruz Biotechnology); and mouse monoclonal anti-β-actin antibody (Sigma Chemical) were used as the primary antibodies. The signal was detected with an enhanced chemiluminescence Western blot analysis system (Amersham Biosciences). For subcellular fractionation, subcellular proteome extraction kit (Calbiochem) was used.

**Analysis of TRAIL-Induced Complex Formation**

DLD-1 cells were treated with 40 μmol/L halocynthiaxanthan or DMSO for 24 h. Cells were harvested and 7 × 106 cells were treated with 1 μg/mL recombinant human histidin-tagged TRAIL (R&D Systems) at 37°C for 1 h with gentle shake. Cells were washed with PBS and lysed for 15 min on ice with lysis buffer containing 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.2% NP40, and 10% glycerol. The lysate was cleared by centrifugation at 13,000 rpm for 10 min at 4°C. The soluble fraction was incubated with Ni-NTA agarose (Qiagen) and 10 mmol/L imidazol (Qiagen) at 4°C for 30 min. After three washes with lysis buffer containing 10 mmol/L imidazole, the bound proteins were eluted by boiling for 3 min in loading buffer and resolved by SDS-PAGE. DR5 and FADD proteins were detected by Western blotting.

**siRNA**

The DR5 and control LacZ siRNA (100 nmol/L) were transfected into DLD-1 cells with Oligofectamine (Invitrogen) as described previously (42). The volume of Oligofectamine was two thirds the recommended volume in the manufacturer’s protocol. For flow cytometry analysis, 100 nmol/L FITC-oligonucleotides (FITC-5′-gacgccgcegagttgaaggt-3′) was cotransfected with siRNA. FITC-positive cells were selected and analyzed.

**Detection of DR5 and DR4 Expressions on Cell Surface**

DLD-1 cells were transfected with 100 nmol/L FITC-oligonucleotides and 100 nmol/L DR5 siRNA or control LacZ siRNA. Forty-eight hours after transfection, cells were harvested, washed with PBS, and suspended in PBS containing 1% bovine serum albumin. Then, 3 μL of phycoerythrin-conjugated anti-DR5, anti-DR4, or mouse IgG1: isotype control antibody (eBiosciences) were added to cells and incubated on ice for 30 min. After wash with PBS, DR5 and DR4 expressions on cell surface were analyzed in FITC-positive cell population by flow cytometry.

**Statistical Analysis**

Data represent means ± SD of triplicate data. Data were analyzed using a Student’s t test and differences were considered significant from controls when P < 0.05. The CI was calculated by the method of Chou and Talalay (23) using CalcuSyn software (Biosoft). Synergism is defined as more than the expected additive effect with CI < 1 (24). An additive effect is reflected by CI = 1 and an antagonistic effect is reflected by CI > 1.

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**References**


