**Note**

Characterization of Apoptosis Induced by Fucoxanthin in Human Promyelocytic Leukemia Cells

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Apoptosis induced by fucoxanthin in HL-60 cells was associated with a loss of mitochondrial membrane potential at an early stage, but not with an increase in reactive oxygen species. Fucoxanthin treatment caused cleavages of procaspase-3 and poly (ADP-ribose) polymerase without any effect on the protein level of Bel-2, Bel-X1, or Bax. Apoptosis induction by fucoxanthin may be mediated via mitochondrial membrane permeabilization and caspase-3 activation.

Key words: apoptosis; caspase; fucoxanthin; HL-60; mitochondria

Epidemiological studies suggest that fruit and vegetable consumption reduces the risk of certain cancers and other degenerative diseases. Phytochemicals such as carotenoids, flavonoids, and isothiocyanates present in such foods have been thought to have beneficial effects on human health. Carotenoids, which are pigments with conjugated polyene structure, are considered to work as antioxidants with radical scavenging and singlet oxygen quenching activities in biological tissues. Moreover, the cancer-preventive potential of carotenoids has been indicated in studies with cultured cells as well as experimental animals. Carotenoids have been shown to suppress the propagation of cancer cells in vitro by inducing differentiation and apoptosis, enhancing gap-junctional communication, and arresting the cell cycle.

Since Muto et al. discovered that β-carotene induces apoptosis in cervical dysplastic cells via down-regulation of an epidermal growth factor receptor, several carotenoids such as lycopene, β-cryptoxanthin, lutein, and canthaxanthin have been reported to induce apoptosis in certain cancer cells. Hosokawa et al. found that HL-60 human promyelocytic leukemia cells underwent apoptosis by fucoxanthin, which has characteristic structures of 5,6-monoepoxide and an allenic bond. In our previous studies, fifteen dietary carotenoids were evaluated for their effects on propagation of PC-3 human prostate cancer cells. In addition to fucoxanthin, neoxanthin, which has a structure similar to fucoxanthin, remarkably reduced cell viability by inducing apoptosis. These two carotenoids have also been shown to suppress chemically induced carcinogenesis in experimental animals. Fucoxanthin and neoxanthin are the major carotenoids present in chloroplasts of brown algae and higher plants respectively. Our recent studies indicate that these carotenoids orally administered to mice were absorbed and underwent metabolic conversion. Thus fucoxanthin and neoxanthin are noteworthy dietary carotenoids with respect to cancer prevention, but the detailed mechanisms underlying apoptosis induction by these carotenoids remain unknown. In the present study, we characterized the apoptosis induced by fucoxanthin in HL-60 cells.

HL-60, HP50-2, and HP100-1 human promyelocytic leukemia cells were obtained from the Riken Gene Bank (Tsukuba, Japan). HP50-2 and HP100-1 cells are hydrogen peroxide-resistant cells derived from HL-60 cells and have 3.9 and 17.6-fold higher catalase activity than HL-60 cells respectively. These cell lines were cultured in an RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine, and antibiotics (40 U/ml penicillin and 40 μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO2 in air. Treatment with fucoxanthin was conducted by incubating the cells at a density of 2 × 105 cells/ml medium containing 10 μM fucoxanthin for the indicated period. Fucoxanthin, prepared as previously reported, was dissolved in distilled tetrahydrofuran (THF) and added to the culture medium. The final concentration of THF was 0.25% (v/v), and the control culture received only THF (vehicle alone). The percentage of apoptotic cells among those treated with 10 μM fucoxanthin for 24 h was estimated by fluorescence-activated cell sorting (FACS) and DNA ladder formation by agarose gel electrophoresis, as described previously.

The intracellular levels of reactive oxygen species (ROS) were evaluated with diacetoxymethyl 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF) as a fluorescence probe. Briefly, HL-60 cells were preincu...
bated with the medium containing 10 μM DCF at 37°C for 30 min. Thereafter, the culture was diluted two-fold with the medium containing 20 μM fucoxanthin, and the cells were further incubated at 37°C for 1 h. The treated cells were washed with phosphate-buffered saline (PBS) and resuspended in 0.25 ml lysis solution (0.5% SDS in PBS). The fluorescence intensity (Ex. 510 nm, Em. 534 nm) of the oxidized DCF in the lysate was then measured.

The effect of fucoxanthin on mitochondrial membrane potential was evaluated with rhodamine 123 as a probe. Cells treated with 10 μM fucoxanthin for 1 h were incubated with 10 μM rhodamine 123 in PBS at 37°C for 15 min. Thereafter, the cells were suspended in 0.25 ml of 0.1% bovine serum albumin solution. They were subjected to FACS. Data were analyzed with CellQuest software (BD Immunocytometry Systems, San Jose, CA, U.S.A.).

The effect of fucoxanthin on caspase activities was evaluated with substrates specific to the individual caspases. HL-60 cells treated with 10 μM fucoxanthin for 24 h were lysed in 50 μl of lysis buffer (50 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 100 mM EDTA, 10% glycerol, 1 mM PMSF, and 10 mg/ml each of antipain, chymostatin, leupeptin, and pepstatin A (Peptide Institute, Osaka, Japan). Aliquots (50 μg protein) of the cell lysates were incubated in the dark at 37°C for 1 h with 10 μM caspase substrates as follows: Ac-DEVD-MCA (MCA: 4-methyl-1-coumaryl-7-amide) for caspase-3, Ac-IETD-MCA for caspase-8, and Ac-LEHD-MCA for caspase-9. Afterwards, fluorescence intensity (Ex. 360 nm, Em. 465 nm) was measured.

The effect of fucoxanthin on the levels of apoptosis-related proteins was evaluated by western blot analysis. The proteins of the cells treated with fucoxanthin for 24 h as described above were extracted with a lysis buffer (0.5 mM Tris–HCl, pH 6.8, 10% SDS, 10% glycerol, and 0.6% mercaptoethanol). Total proteins (50 μg) of the lysate were subjected to western blot analysis as described previously.4 The primary antibodies were as follows: mouse anti-Bcl-2 monoclonal antibody (1:1000; B3170, Sigma Aldrich, St. Louis, MO, U.S.A.), rabbit anti-Bcl-XL polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), rabbit anti-Bax polyclonal antibody (1:200; N-20, Santa Cruz Biotechnology), rabbit anti-caspase-3 polyclonal antibody (1:1000, H-227, Santa Cruz Biotechnology), rabbit anti-cleaved caspase-3 polyclonal antibody (1:1000; #9661, Cell Signaling Technology, Beverly, MA, U.S.A.), rabbit anti-poly (ADP-ribose) polymerase (PARP) polyclonal antibody (1:1000; #9542, Cell Signaling Technology), and mouse anti-β-actin monoclonal antibody (1:50000; AC-15, Sigma Aldrich). The proteins were probed with alkaline phosphatase-conjugated secondary antibodies.

The prooxidant action of β-carotene and enhancement of intracellular ROS associated with an activation of NF-κB has been reported to mediate apoptosis in several cancer cells, including HL-60 cells.8 ROS in the biological tissues play pivotal roles in mediating some cellular signals. In particular, enhancement of ROS has been associated with apoptosis induced by certain stimuli. With regard to HL-60 cells, the food components curcumin and quercetin as well as the cytotoxic drugs duocarmycin A and cisplatin have been reported to induce apoptosis via enhanced generation of ROS at the early stage. In order to elucidate the possible involvement of ROS in apoptosis induction by fucoxanthin, HP50-2 and HP100-1 cells, which are hydrogen peroxide-resistant cell lines derived from HL-60 cells, were exposed to 10 μM fucoxanthin for 24 h. Fucoxanthin treatment induced apoptosis in the resistant cells as well as in HL-60 cells, although the percentage of apoptotic cells evaluated as SubG1 phase was lower in the resistant cell lines than in the parental cells (Fig. 1A). DNA ladder formation was also confirmed in the three cell lines treated with fucoxanthin (Fig. 1B). However, the apoptosis induced by 50 μM hydrogen peroxide was abolished in HP50-2 and HP100-1 cells (Fig. 1A). We examined whether exposure of HL-60 cells to fucoxanthin could enhance the intracellular level of ROS. TheDCF-treated HL-60 cells were exposed to fucoxanthin for 1 h. The relative fluorescence intensity of the oxidized DCF in the cells treated with vehicle alone, 50 μM hydrogen peroxide, and 10 μM fucoxanthin was 100 ± 7.1, 386.4 ± 26.4, and 94.7 ± 5.1% (n = 3), respectively. Thus fucoxanthin treatment did not affect the ROS level at all. These results suggest that ROS is not involved in a major pathway of apoptosis induced by fucoxanthin.

The mitochondria play an important role in regulating the apoptotic process. Mitochondrial membrane permeabilization (MMP) followed by the loss of membrane potential occurs at the early stage of apoptosis via the mitochondria9 and leads to the release of apopptotic proteins such as cytochrome c, AIF, and EndoG to cytosol. Oxidative stress is known to cause MMP-associated apoptosis via an enhancement of ROS. In fact, treatment with 50 μM hydrogen peroxide reduced the mitochondrial membrane potential in HL-60 cells after 1 h incubation, but had no effect in the H2O2-resistant cell lines in the present study (data not shown). On the other hand, treatment with 10 μM fucoxanthin for 2 h reduced the mitochondrial membrane potential in both HL-60 cells and the resistant HP100-1 cells (Fig. 2). Therefore, these results suggest that fucoxanthin causes the loss of mitochondrial membrane potential at an early stage in a manner different from oxidative stress, which thereafter leads to apoptotic pathways via the mitochondria.

Bcl-2 family proteins regulate MMP and the release of cytochrome c from mitochondria to cytosol. The released cytochrome c leads to apoptotic cell death via activation of caspase-9 and -3. In the present study, treatment of HL-60 cells with 10 μM fucoxanthin for 24 h did not affect the levels of the anti-apoptotic

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proteins, Bcl-2 and Bcl-X\textsubscript{L}, or the pro-apoptotic protein Bax (Fig. 3). Thus the Bcl-2 family proteins tested were not involved in MMP and apoptosis induced by fucoxanthin. The fucoxanthin treatment induced cleavages of procaspase-3 and PARP. The activities of caspase-3, -8, and -9 were respectively higher by 8.52 ± 0.36, 2.74 ± 0.26, and 5.11 ± 0.65 times (n = 3) in HL-60 cells treated with 10μm fucoxanthin for 24 h than in the cells treated with the vehicle alone. The remarkable enhancement of caspase-3 and the cleavage of procaspase-3 to an active fragment (Fig. 3) indicate that the apoptosis induced by fucoxanthin was caspase-3 dependent. Furthermore, the marked enhancement of caspase-9 activity suggests the release of cytochrome c from the mitochondria to cytosol and the subsequent activation of the caspase cascade. Taken together, the results in the present study suggest that apoptosis induced by fucoxanthin in HL-60 cells is mediated via the reduction of mitochondrial membrane potential, followed by caspase-9 and -3 activation. The reduction of mitochondrial membrane potential was not associated with the Bcl-2 family protein levels and ROS generation. Fucoxanthin, which was taken up readily by the cells (data not shown), may directly cause dysfunction of mitochondria by highly accumulating in mitochondrial membranes, leading to the reduction of its membrane potential. The action of fucoxanthin on mitochondria and the detailed mechanism of subsequent apoptosis induction deserve future study.
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References


