Pre-treatment with α-tocopherol (α-Toc) potentiated cytotoxicity induction by benzyl isothiocyanate (BITC). Biochemical events related to apoptosis, such as DNA ladder formation and caspase-3 activation, were also enhanced by α-Toc. These results suggest a significant role of the caspase-3 pathway in apoptosis induction regulated by α-Toc in combination.

Key words: benzyl isothiocyanate; α-tocopherol; apoptosis; caspase-3; HL-60 cells

Isothiocyanates (ITCs), which occur naturally and abundantly in cruciferous vegetables such as broccoli, watercress, cabbage, and Japanese radish, may play a significant role in affording the cancer chemopreventive potential of these vegetables. Among them, our group has recently focused on benzyl ITC (BITC), isolated from an extract of papaya (Carica papaya) fruits. We have found that BITC potently induces phase 2 enzyme and apoptosis. More recently, BITC, as well as phenethyl ITC and sulforaphane, were identified as metabolites in serum from a human subject eating broccoli, garden cress, and watercress, suggesting that BITC can be consumed from a diet containing cruciferous vegetables.

The ITC concentrations required to elicit anticancer activity have been found to be much higher than the peak plasma concentrations of ITCs. One study indicated that the ITC concentration required for inhibition of the growth of human cancer xenografts on mice was 4.4 mg/kg per day, which corresponds to 308 mg ITCs daily upscaled to a 70 kg person. This amount of ITCs is hard to reach by intake of cruciferous vegetables, since, for example, more than 2.5 kg standard broccoli would have to be eaten daily. Moreover, adverse effects of high concentrations of ITCs have been reported. Hence, it is important to enhance the pharmacologic effects of ITCs to obtain health benefit at reasonable concentrations in daily life.

This study was initially designed to identify a component that can be added to foodstuffs and that can be used effectively in combination with ITCs, and to determine the molecular mechanism underlying this effect. We have reported that α-tocopherol (α-Toc) promotes the expression but not the activation of caspase-3 in various human cell lines. Using an in vitro system, we examined to determine whether α-Toc enhances the apoptosis-inducing activity of BITC. We identified in this study apoptosis sensitization by α-Toc with an antioxidative effect.

Acute promyelotic leukemia HL-60 cells (Health Science Research Resources Bank, Osaka) were maintained in RPMI 1640 media from Gibco-Invitrogen (Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Trace Scientific, Melbourne, Australia). 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) was obtained from Sigma (St. Louis, MO). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

To evaluate the cell viability, we performed a trypan blue dye (Gibco-Invitrogen) exclusion assay. Briefly, HL-60 cells (5 × 10$^5$) were pre-incubated with and without α-Toc (50 or 100 μM) for 12 h, followed by treatment with BITC for 24 h. Each cell suspension was mixed with 0.4% trypan blue stain. Viable cells (those that excluded the blue dye) were counted with a hemocytometer under a light microscope (TMS-1, Nikon, Tokyo).

DNA fragmentation and cleavage of caspase-3 and poly-(ADP-ribose) polymerase (PARP) were detected as previously reported. Caspase-3 and PARP antibodies were from Cell Signaling Technology (Beverly, MA). An antibody to actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as loading control.

The intracellular peroxide level was determined by H$_2$DCF-DA as intracellular fluorescence probe, as previously reported. HL-60 cells (4 × 10$^5$ cells/mL) were pre-treated with α-Toc (100 μM) for 12 h at 37°C, and then incubated with BITC (50 μM) for 1 h. After additional incubation with H$_2$DCF-DA for 1 h, the cells were harvested and washed twice with PBS (—). A flow cytometer (Coulter Epics XL, Beckman Coulter, Brea, CA) was used to measure dichlorofluorescein (DCF). Data were collected and analyzed with the Win MDI 2.9 Software Program.

Statistical significance was assessed by Student’s paired two-tailed t-test and by analysis of variance on untransformed data, followed by a comparison of group.
Apoptosis, HL-60 cells were treated with 50 or 100 µM BITC, even at 1 µM, tended to reduce the pro-caspase-3 level. Caspase-3 activation by BITC treatment due to proteolytic processing (cleaved caspase-3 upregulation) was also enhanced by α-Toc pre-treatment (Fig. 2B), which is consistent with previous findings using other apoptosis-inducing stimuli such as etoposide and Fas ligand. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. Although PARP is not essential for cell death, cleavage of PARP is one of the representative hallmarks of apoptosis. The cleaved PARP induced by α-Toc alone was as much as that of the control (Fig. 2A), but was significantly enhanced by α-Toc + BITC-treatment (Fig. 2B). These results clearly indicate that enhanced expression of caspase-3 correlates positively with enzyme activity and thus with apoptosis-inducing pathway activation by BITC.

BITC has been reported to induce intracellular reactive oxygen species (ROS) production in several cell lines. Hence, to determine whether α-Toc modulates the intracellular ROS level enhanced by BITC, we did flow cytometric analysis using H2DCF-DA, a general fluorescent probe for detection of intracellular ROS. As shown in Fig. 3, when cells were treated with 50 µM BITC for 1 h, the intracellular ROS level increased averages by contrast analysis, using the Super ANOVA statistical program (Abacus Concepts, Berkeley, CA). A p value of 0.05 was considered to be statistically significant.

To determine the effect of α-Toc on BITC-induced apoptosis, HL-60 cells were treated with 50 or 100 µM α-Toc for 12 h, washed 3 times to remove α-Toc from the culture medium, and then exposed to BITC. As shown in Fig. 1A, pre-treatment with α-Toc at each concentration did not show significant effect on cell viability. Pre-treatment with α-Toc dose-dependently increased the cytotoxic effect of BITC on HL-60 cells. Furthermore, α-Toc significantly enhanced the DNA ladder formation induced by 1 µM BITC (Fig. 1B), suggesting that the potentiation of the apoptosis pathway is involved in this cytotoxic effect.

Since α-Toc has been reported to enhance caspase-3 expression without activation, we examined the effect of α-Toc on caspase-3 by immunoblot analysis. When α-Toc was added to the culture medium, a significant increase in the pro-caspase-3 protein level was observed, as previously reported (Fig. 2A). BITC, even at 1 µM, tended to reduce the pro-caspase-3 level. Caspase-3 activation by BITC treatment due to proteolytic processing (cleaved caspase-3 upregulation) was also enhanced by α-Toc pre-treatment (Fig. 2B), which is consistent with previous findings using other apoptosis-inducing stimuli such as etoposide and Fas ligand. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. Although PARP is not essential for cell death, cleavage of PARP is one of the representative hallmarks of apoptosis. The cleaved PARP induced by α-Toc alone was as much as that of the control (Fig. 2A), but was significantly enhanced by α-Toc + BITC treatment (Fig. 2B). These results clearly indicate that enhanced expression of caspase-3 correlates positively with enzyme activity and thus with apoptosis-inducing pathway activation by BITC.

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2.5-fold as compared to control, but we observed no significant alteration in ROS at 1 μM BITC (data not shown). This result is relevant to apoptosis induction. α-Toc pre-treatment almost completely inhibited the ROS production induced by 50 μM BITC, whereas it did not inhibit BITC-induced cytotoxicity (data not shown). Although previous studies have suggested that intracellular redox alteration plays a role in ITC-induced apoptosis, an unfavorable oxidative stress-related effect of higher concentrations of ITCs has also been demonstrated. Thus, the present data imply that α-Toc in combination eliminates at least excessive ROS, which might be dispensable for apoptosis induction by BITC but responsible for undesirable oxidative stress on the surrounding cells.

We found that α-Toc potentiates apoptosis induction by BITC, possibly through caspase-3 expression enhancement. α-Toc concentrations in human plasma vary from 20 to 60 μM, depending on the amount of dietary intake. This α-Toc order is comparable to effective concentration for caspase-3 upregulation (Fig. 2A), and thus appears to be physiologically relevant. As for the concentration for caspase-3 upregulation (Fig. 2A), and enhancement.

Furthermore, a considerable number of papers have indicated that α-Toc shows inhibitory effects on various forms of oxidative stress-induced apoptosis. The timing of treatment and the residual intracellular concentration cannot be ruled out as determinants of apoptosis potentiation.

We have found that the potentiation of caspase-3 expression by α-Toc is mediated by an Sp1-dependent pathway and occurs in various human cell lines, suggesting that these phenomena might not be cell-specific. Some studies have found that caspase-3 is a biochemical and prognostic key molecule in controlling cancer cell proliferation in humans. For example, patients with caspase-3 positive tumors (strong expression) exhibited a significantly better overall survival rate than patients with caspase-3 negative tumors (weak expression). In this respect, non-cytotoxic α-Toc should be explored as a potentiator of anti-cancer agents as well as of BITC-induced apoptosis.

In summary, the results found here provide biological evidence for an enhancing effect of α-Toc on BITC-induced activation of apoptotic biochemical events. Further relevant information on the anti-cancer activity of combined α-Toc and BITC treatment will emerge from in vivo study.

References