Luteolin Overcomes Resistance to Benzyl Isothiocyanate-Induced Apoptosis in Human Colorectal Cancer HCT-116 Cells

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ABSTRACT

We have previously identified p53, a universal sensor of genotoxic stress, as a negative regulator of the apoptosis induction by benzyl isothiocyanate (BITC) in the normal colon fibroblastoid cells. In the present study, we further confirmed that BITC has a potential to induce cytotoxicity in the p53-mutated colon cancer HT-29 cells in preference to HCT-116 cells with wild-type p53. To obtain effective induction of BITC-stimulated apoptosis in p53-positive cells, we investigated the combination effect of BITC and food ingredient that may overcome resistance to BITC. Pretreatment with luteolin potentiated the cytotoxicity induction by BITC in HCT-116 cells but not in HT-29 cells. The biochemical events related to apoptosis such as DNA ladder formation and caspase-3 activation were also enhanced by luteolin. Luteolin attenuated the expression of $p21^{waf1/cip1}$, a key downstream target of p53. These results suggest the role of $p21^{waf1/cip1}$ pathway in the overcoming BITC resistance by luteolin.

Key words: benzyl isothiocyanate, luteolin, apoptosis, caspase-3, $p21^{waf1/cip1}$, HT-29 cells, HCT116 cells

INTRODUCTION

A number of studies support the idea that certain food phytochemicals protect against cancer. An important group of food compounds that have a cancer preventive property is isothiocyanates (ITCs). ITCs, naturally occurring abundantly in cruciferous vegetables such as broccoli, watercress, cabbage, and Japanese radish, may play a significant role in affording the cancer preventive potential of these vegetables. Among them, our group has recently focused on benzyl ITC (BITC) isolated from the extract of papaya (*Carica papaya*) fruits and demonstrated its potent inducing effects of phase 2 enzymes and apoptosis[(1)]. BITC as well as phenethyl ITC and sulforaphane were found as metabolites in serum from a human subject eating broccoli, garden cress and watercress, suggesting that BITC could be daily consumed from cruciferous vegetables-containing diet[(2)].

The ITC concentrations required to elicit the anticancer activity have been shown to be much higher than the peak plasma concentrations of ITCs[(3)]. Another study indicated that typical ITC concentrations required for growth inhibition of human cancer xenografts on mice are 4.4 mg/kg per day[(4)], which corresponds to 308 mg ITCs daily calculated to a 70 kg person[(3)]. This amount of ITCs is hard to reach by the intake of cruciferous vegetables, since, for example, more than 2.5 kg standard broccoli should be eaten daily. Moreover, adverse effects of high concentrations of ITCs have been reported[(5-7)]. Therefore, it is important to enhance the pharmacologic effect of daily-consumed ITCs to obtain the health benefit in reasonable concentration in daily life.

The p53 tumor-suppressor protein, a universal sensor of genotoxic stress, regulates transcription of several target genes, which facilitates cell-cycle arrest, apoptosis and DNA repair, subsequently to the stabilization and activation of p53 in response to genotoxic stress[(8)]. For example, cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, a key downstream target of p53, mediates both G_1 and G_2/M phase arrest[(9)]. Recent evidence suggests that $p21^{waf1/cip1}$ may also participate in apoptosis in both p53-dependent as well as p53-independent pathways[(10,11)]. We have recently found that BITC induced p53 phosphorylation and accumulation through an ataxia telangiectasia, mutated/ ataxia telangiectasia and Rad3-related (ATM/ATR) kinase signaling pathway[(12)]. Interestingly, down-regulation of

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p53 by a siRNA resulted in the enhancement of susceptibility to undergo apoptosis by BITC, suggesting that the p53 pathway activation maintaining the stay in G_0/G_1 phase might play an important role in the resistance to the cytotoxic effect of BITC. We thus identified p53 as a novel negative regulator of the apoptosis induction by BITC in the quiescent p53 positive cells. The mutation or loss of p53 has been observed in over 50% of all tumors and in almost every tumor type. Although the role of p53 in the ITC-induced apoptosis in cancer cells is controversial[1], these findings implied that BITC has a potential to induce apoptosis in the p53-mutated proliferating pre-cancerous cells in preference to the p53-active normal cells.

In the present study, we further confirmed that BITC has a potential to induce cytotoxicity in the p53-mutated colon cancer HT-29 cells in preference to HCT-116 cells with wild-type p53. To obtain effective induction of BITC-stimulated apoptosis in p53-positive cells, we investigated the combination effect of BITC and food ingredient that may overcome resistance to BITC. We identified luteolin as a potentiator of the cytotoxicity induction by BITC in p53-positive HCT-116 cells. We also implied the role of $p21^{waf1/cip1}$ in the overcoming BITC resistance by luteolin.

MATERIALS AND METHODS

I. *Chemicals*

BITC was purchased from LKT Laboratories, Inc. (MN, USA). Luteolin was purchased from Sigma-Aldrich (MO, USA). McCoy's 5a medium was obtained from Gibco-Invitrogen (CarIsbad, CA, USA). DMEM was from Nacalai tesque (Kyoto, Japan). Fatal bovine serum (FBS) was purchased from Nichirei Corporation (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

II. *Cell Culture*

Human colon tumor cell lines HT-29 (adeno-carcinoma) and HCT-116 (carcinoma) were obtained from American Type Culture Collection (Rockville, MD). HT-29 and HCT-116 cells were grown in DMEM and McCoy's 5a medium, respectively, supplemented with 10% v/v fetal bovine serum (FBS), 50 U/mL penicillin and 50 μg/mL streptomycin (Gibco BRL). Cells were maintained at 37°C in an incubator under 5% CO_2 and 95% air at constant humidity. Cells were plated at 10000 - 20000 cells per well in a 96-well format for 24 h depending on the cell line and then incubated in medium containing 1% FBS for 2 h prior to sample treatment.

III. *Viability and Apoptosis Determination*

The MTT assay was carried out for the quantitative analysis of cell viability. After culturing with BITC at 37°C for 24 h or 48 h, 10 μL of an MTT solution was added to each well, and the fluorescence was measured with excitation at 560 nm and emission at 590 nm according to the manufacturer's instructions after incubation at 37°C. The obtained values were compared with each of the controls incubated with vehicle only.

The DNA fragmentation analysis and determination of caspase-3-like activity were performed as previously reported[13].

IV. *RT-PCR*

Total RNA was isolated with ISOGEN reagent (Nippon Gene, Tokyo, Japan). The RT reaction was performed with 5 μg of total RNA and an oligo (dT) primer using the first strand cDNA synthesis kit. PCR reactions were carried out using 0.5 μL of cDNA 200 μM dNTPs, 1 μM of each forward and reverse primer and 2 units of BioTaq DNA polymerase (Toyobo, Osaka, Japan). The following primers were used: p21, (F) 5′-AAAGGCCCGCTCTACATCTT-3′, (R) 5′-ACAAGTGGGGAGGAGGAAGT-3′; actin, (F) 5′-GTCACCCACACTGTGCCCATCTA-3′, (R) 5′-GCAATGCCAGGGTACATGGTGGT-3′.

V. *Statistical Analysis*

All values were expressed as means ± SD. Statistical significance was assessed by Student's paired two-tailed t-test or analysis of variance on untransformed data, followed by comparison of group 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.

RESULTS AND DISCUSSION

I. *p53-Mutated HT-29 Cells Are More Susceptible to Cytotoxic stimulus by BITC than HCT-116 Cells*

We examined BITC-induced cytotoxicity in the p53-mutated colon tumor HT-29 cells and HCT-116 cells with wild-type p53. Both cells were incubated with different concentrations of BITC for 48 h. The cell viability was measured by a MTT assay. As shown in Figure 1, the cell viability of HT-29 cells with dysfunctional p53 was decreased with the increase in the BITC concentration from 2.5 to 20 μM. As for p53-positive HCT-116 cells, the cell viability decreased with an increase in the BITC concentration from 10 to 40 μM. The value of IC_{50} in

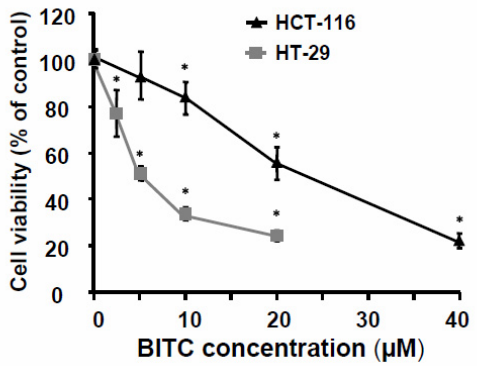

Figure 1. Effect of BITC on cell viability of colon cancer cell lines. The cells were treated with BITC for 48 h. Cell viability was determined by a MTT assay. *, $p < 0.05$ *versus* control.

HCT-116 cells was approximately five times greater than that in HT-29 cells (25 μM versus 5 μM). These results suggested that HT-29 cells were more sensitive than HCT-116 cells to BITC, which is consistent with the previous report using the p53-siRNA-transfected cells[12]. In other words, p53-positive HCT-116 cells exhibit a resistance to the BITC cytotoxic stimulus.

II. *Luteolin Enhances BITC-induced Cytotoxicity in HCT-116 Cells*

It was previously reported that luteolin negatively regulates p53 pathway in a concentration-dependent manner[14]. To determine whether luteolin enhances cytotoxic effect of BITC in p53-positive cells, HCT-116 cells were treated with luteolin for 4 h and then exposed to BITC. Combination treatment with luteolin (10 μM) and BITC (10 μM) significantly suppressed the proliferation of HCT-116 cells to 46% of the control, whereas treatment with BITC or luteolin alone did not significantly inhibit it (Figure 2A). As for p53-mutated HT-29 cells, luteolin did not synergistically enhance BITC-induced cytotoxicity (data not shown). These results suggest that luteolin potentiates BITC-induced cell growth inhibition in a p53 status-dependent manner.

To demonstrate whether apoptosis induction is involved in inhibiting the cell viability in HCT-116 cells, DNA fragmentation and caspase-3 activity were examined. A significant DNA ladder formation (Figure 2B) and increased caspase-3 activity (data not shown) were observed in HCT-116 cells treated with BITC from 2.5 μM to 10 μM. However, no further increase was detected when the cells were treated with more than 15 μM BITC, which might be due to the interference of caspase-3 activity by BITC. The DNA ladder formation was significantly

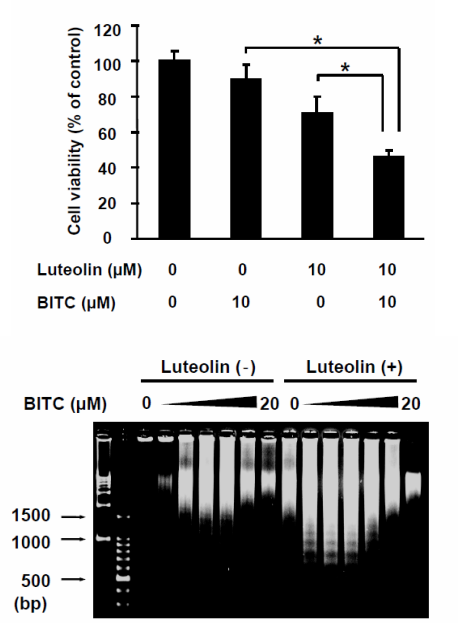

Figure 2. Effect of combination treatment of BITC and luteolin on cell proliferation (A) and DNA ladder formation (B) in HCT-116 cells. (A) HCT-116 cells were pretreated with luteolin (10 μM) for 4 h and then incubated with BITC (10 μM) for 48 h. *, $p < 0.05$. (B) HCT-116 cells were pretreated with 10 μM luteolin for 4 h and then incubated with BITC (0, 2.5, 5, 7.5, 10, 15 and 20 μM) for 24 h. The result is representative of three independent experiments.

enhanced by pretreatment with luteolin. This was coincided with about 4.5-fold increase in caspase-3 activity by luteolin/BITC combination. Although the apoptosis-related biochemical event induction did not completely match the viability data, apoptosis induction might be at least partly involved in the enhancement of cell viability inhibition by luteolin in HCT-116 cells. We initially observed that luteolin did not potentiate higher concentrations of BITC-induced necrosis (data not shown), suggesting that luteolin might overcome p53-dependent resistance to BITC without toxicity.

III. *Luteolin Suppresses $p21^{waf1/cip1}$ Expression*

To investigate the molecular mechanism underlying luteolin-induced potentiation of BITC-induced apoptosis, we examined the effect of luteolin on $p21^{waf1/cip1}$ mRNA expression in HCT-116 cells. As shown in Figure 3, luteolin significantly down-regulated $p21^{waf1/cip1}$ mRNA expression compared to control (vehicle only).

It has been reported that $p21^{waf1/cip1}$ interacts with procaspase 3 by each N-terminal sequence and suppresses its activation by the masking of cytoplasmic serine proteinase-cleaving site[15]. Thus, activation of caspase 3 is

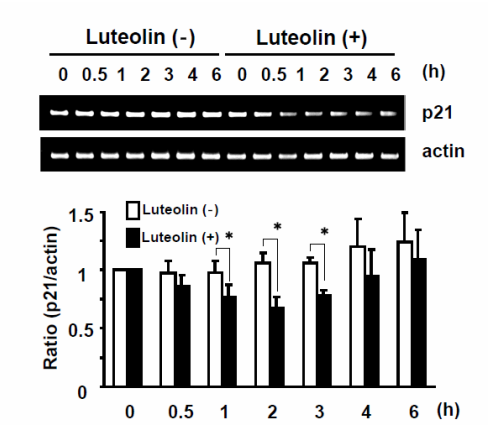

Figure 3. Effect of luteolin on $p21^{waf1/cip1}$ expression in HCT-116 cells. HCT-116 cells were treated with luteolin (10 μM) for the indicated periods. mRNA expression of p21 and actin was determined by RT-PCR. *, $p < 0.05$.

regulated by $p21^{waf1/cip1}$, and procaspase 3/$p21^{waf1/cip1}$ complex formation is an essential system for the cell death such that cell survival is a result of cell death suppression. This information led us to a hypothesis that luteolin might enhance BITC-induced apoptosis through down-regulation of caspase 3 inactivators such as $p21^{waf1/cip1}$. In the meantime, luteolin was reported to induce apoptosis *via* mechanisms involving mitochondria translocation of Bax/Bak by c-Jun-*N*-terminal kinase (JNK) activation[16]. We initially checked the effect of luteolin on BITC-induced JNK activation in HCT-116 cells. However, JNK phosphorylation was significantly enhanced by BITC, whereas luteolin did not affect this phenomenon (data not shown). Thus, JNK-dependent Bcl-2 family protein modification, one of the potential mechanisms underlying BITC-induced apoptosis[17], could be ruled out.

CONCLUSIONS

The present results provide biological evidence for the enhancing effect of luteolin on BITC-induced activation of apoptotic biochemical events. This work also shows that luteolin is a potential regulator of p53 pathway. In this aspect, non-cytotoxic luteolin can be explored as a potentiator for several anti-cancer agents less effective to p53-positive tumors. More relevant information on the anti-cancer activity of combined luteolin and BITC treatment will emerge from *in vivo* study.

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