Vitamin E-like molecules potentiate the curcumin-induced suppression of Caco-2 cell proliferation

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Abstract
We previously reported that curcumin inhibited the proliferation of a human colorectal cancer cell line Caco-2 by both apoptosis and G2/M cell cycle arrest. A variety of biological functions of curcumin have been suggested to be involved in its modulatory capacity to oxidative stress. The aim of this study was to investigate the modulation of antioxidants, N-acetyl-L-cysteine (NAC), α-tocopherol (TOC) and trolox, on the suppression of Caco-2 cell proliferation via G2/M cell cycle arrest induced by curcumin. NAC (2 mM) reduced the curcumin-induced suppression of Caco-2 proliferation. Inversely, both TOC (0.2 mM) and trolox (0.2 mM) potentiated the curcumin effect. Trolox at the same concentration amplified both G2/M cell cycle arrest and p21 elevation induced by curcumin independent of oxidative stress status. These results suggest that Vitamin E-like molecules have the potential to amplify the inhibitory effect of curcumin on Caco-2 proliferation through reactive oxygen species-independent cell cycle modulation, and could offer new insights for preventive measures against colorectal cancer incidence.

Keywords: curcumin; trolox; α-tocopherol; colon cancer; cell cycle arrest.

1. Introduction
In spite of significant advances over the last half century in our understanding of the genesis of colorectal cancer and the application of targeted drug therapy, cancer of the colon and rectum remains a leading cause of cancer deaths [1]. New chemopreventative and chemotherapeutic approaches to the prevention and treatment of colon cancer are needed to reduce mortality, since many patients with advanced colon cancer fail to respond to current treatment regimens [2]. In this regard, possible clinical application of dietary polyphenol phytochemicals that prevent carcinogenesis and inhibit the growth of colon carcinoma cells has generated intense interest among both clinicians and the cancer research community [3].

Curcumin (diferuloylmethane) is an active ingredient of turmeric, a well known Indian spice that is derived from the dried roots of the plant Curcuma Longa. Curcumin is considered to be responsible for a low incidence of colorectal cancer [4]. In rats, absorption of curcumin from the intestine was reported to be about 60% [5]. Curcumin and its metabolites formed in intestine and liver are mostly excreted in the feces [6,7], so the colon is a likely target for the anticarcinogenic activity of curcumin. Moreover, the fact that humans are able to consume up to 8 grams of curcumin per day without toxic effects [8] makes curcumin a very interesting chemopreventive agent for colon cancer. However, many aspects of the curcumin effect need to be further investigated.

We previously reported that curcumin decreased the cell viability of a colorectal cancer cell line, Caco-2, through both activation of the apoptotic pathway (an increase in the Bax/Bcl-2 ratio and activation of caspase-3/7) and inhibition of the cell cycle (an arrest of the G2/M phase)[9]. It is generally accepted that curcumin has a potent protective activity against oxidative stress [10-12]. However, it is noteworthy that studies have also shown the capacity of curcumin to impair the cellular redox balance, by up-regulating reactive oxygen species (ROS) production, principally in tumor cells[13-16]. Therefore, the aim of this study was to investigate the modulation of antioxidants, N-acetyl-L-cysteine (NAC), α-tocopherol (TOC) and trolox, on the suppression of Caco-2 cell proliferation via G2/M cell cycle arrest induced by curcumin.
2. Materials and Methods

2.1 Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ribonuclease A (RNase A), propidium iodide (PI), TOC and tert-butyl hydroperoxide (TBHP) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Curcumin and NAC were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Merck Millipore Corporation (Billerica, MA, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Life Technologies Corporation (Carlsbad, CA, USA). Primary antibodies for p21 and β-actin protein and secondary antibody (HRP-linked antibody) were purchased from Cell Signaling Technology Japan, K.K. (Tokyo, Japan). DCFH-DA, curcumin, trolox and TOC were dissolved in dimethyl sulfoxide (DMSO) and added to medium; the DMSO concentration (0.25%) used in the present study had no significant effect on Caco-2 proliferation.

2.2 Cell culture

Caco-2 cells were purchased from the European Collection of Cell Cultures (Salisbury, Wilts, UK) and cultured in Minimum Essential Medium (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) and 1% non-essential amino acids (Life Technologies Corporation, Carlsbad, CA, USA). The cells were maintained in a humidified atmosphere of 5% carbon dioxide at 37°C.

2.3 Measurement of Cell viability

Cell viability was measured by MTT assay, as we described previously [17,18]. Briefly, the cells were incubated with the test reagents at a density of 5.0×10⁶ cells/9.5 cm² well for 48 h. After incubation, the medium was removed and the cells were incubated with 1.1 mL of MTT solution (0.1 mL of 5 mg/mL MTT in 1 mL of medium) for 4 h. The formazan produced was dissolved by the addition of 20% sodium dodecyl sulfate (SDS)/0.01 M hydrogen chloride, and the absorbance at 595 nm was determined using an SH-1200Lab microplate reader (Corona Electric Co. Ltd., Ibaraki, Japan).

Cell viability was calculated according to the following equation:

cell viability (%) = (absorbance of experimenst group/absorbance of control group) × 100.

2.4 Cell cycle analysis

Cell cycle analysis was performed by flow cytometry as we reported previously [9]. Briefly, the cells were incubated with the test reagents at a density of 1.0×10⁶ cells/55 cm² dish for 24 h, and then collected by centrifugation. The pellet was fixed with 70% ethanol cooled at -20°C on ice for 30 min. Following fixation, the cells were incubated with RNase A (100 μg/mL) at 37°C for 30 min. The cells were treated with PI (50 μg/mL) in a dark place on ice for 30 min. The samples were filtrated through a nylon mesh (37 μm), and subjected to flow cytometry.

2.5 Measurement of ROS

The measurement of ROS was performed by flow cytometry with the oxidation-sensitive fluorescent dye DCFH-DA. The cells were incubated with the test reagents at a density of 1.0×10⁶ cells/55 cm² dish for 6 h, and then DCFH-DA was added at a final concentration of 10 μM. After a 30 min-incubation, the cells were collected by centrifugation and washed twice. The samples were filtrated through a nylon mesh (37 μm), and subjected to flow cytometry.

2.6 Measurement of p21 protein expression

After treatment for 12 h, the cells were collected by centrifugation, and then lysed. Cell lysates were subjected to SDS-PAGE using a 12.5% polyacrylamide gel. Proteins were transferred to PVDF membranes by electroblotting and the membranes were incubated overnight in Tris buffered saline-Tween® 20 containing the respective primary antibody to p21 or β-actin, and 3% skim milk. After incubation, the membranes were incubated with the secondary antibody for 1 h, followed by chemiluminescent detection by ECL Prime Western Blotting Detection Reagent (GE Healthcare Japan Corporation, Tokyo, Japan).

2.7 Statistics: Results are the means ± SEM. The significance of differences between two groups was assessed using the Student’s t-test. P-values less than 0.05 were considered significant.

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<th>Table 1: Effect of curcumin on the proliferation of Caco-2 Cells</th>
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<td><strong>Treatment</strong></td>
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Caco-2 cells were treated with various concentrations of curcumin (10-100 μM) for 48 h. Data are expressed as the mean ± SEM (n = 9).

³p < 0.01; significantly different from control.
Caco-2 cells were treated with curcumin (10 and 20 µM) in the presence or absence of N-acetyl-L-cysteine (NAC; 2 mM), α-tocopherol (TOC; 0.2 mM) or trolox (0.2 mM) for 48 h. Data are expressed as the mean ± SEM (n= 3-6). *p<0.01; significantly different from control. †p<0.05, ‡p<0.01; significantly different from the respective curcumin alone.

3. Results

3.1 NAC inhibited, but both TOC and trolox potentiated, the curcumin-induced suppression of Caco-2 cell proliferation

Table 1 shows the effect of curcumin on the cell viability of Caco-2. When Caco-2 cells were incubated with curcumin at concentrations ranging from 10 to 100 µM, a dose-dependent suppression was seen, and the inhibition reached 50% of the control value at a concentration between 10 and 20 µM. This observation is in accordance with our previous report [9]. Therefore, curcumin at 10 and 20 µM was used in subsequent experiments. Fig. 1 shows the effects of NAC, TOC or trolox on the 10 and 20 µM curcumin-induced suppression of Caco-2 cell proliferation. 2 mM NAC, 0.2 mM TOC or 0.2 mM trolox alone exhibited no significant effects on the Caco-2 cell growth. Growth inhibitions induced by 10 and 20 µM curcumin were significantly inhibited by the addition of 2 mM NAC (Fig. 1A; 32 and 33 % inhibition). In contrast, the addition of either 0.2 mM TOC (Fig. 1B) or 0.2 mM trolox (Fig. 1C) synergistically increased the 10 and 20 µM curcumin-induced cell growth inhibitions (0.2 mM TOC, 1.1 and 1.3 fold; 0.2 mM trolox, 1.3 and 1.7-fold), and tentatively trolox being more potent than TOC. Fig. 2 shows the effects of TBHP or curcumin with or without NAC, TOC or trolox on the intracellular ROS generation of Caco-2 cells. 20 µM curcumin increased the ROS generation measured by DCF fluorescence intensity, with almost the same potency as 30 µM TBHP. The up-regulation of ROS levels by curcumin was dose-dependent at concentrations ranging from 10 to 50 µM (data not shown). The TBHP-induced ROS generation was reduced by the addition of all three antioxidants, NAC, TOC and trolox. In contrast, only NAC potently reduced the ROS generation by curcumin, whereas neither TOC nor trolox had any effect on the ROS generation. These results indicate that TOC and trolox, but not NAC, in combination with curcumin synergistically suppress Caco-2 cell proliferation, and that this synergistic effect is independent of oxidative stress.
Figure 2: Effects of TBHP or curcumin with NAC, TOC or trolox on the intracellular reactive oxygen species generation of Caco-2 cells.

Caco-2 cells were treated with tert-butyl hydroperoxide (TBHP; 30 µM) or curcumin (20 µM) in the presence or absence of N-acetyl-L-cysteine (NAC; 2 mM), α-tocopherol (TOC; 0.2 mM) or trolox (0.2 mM) for 6 h. Data are representative of two or three independent results.

Figure 3: Effects of curcumin with trolox on the cell cycle of Caco-2 cells

Caco-2 cells were treated with curcumin (10 µM) in the presence or absence of trolox (0.2 mM) for 24 h. (A) Representative histograms of cell cycle distribution. (B) Percentage of cells in each cell cycle phase. Data are expressed as the mean ± SEM (n= 6). *p<0.01; significantly different from control. †p<0.05; significantly different from the curcumin alone.
Figure 4: Changes in p21 protein expression levels in Caco-2 cells treated with curcumin and trolox.

Caco-2 cells were treated with curcumin (10 µM) in the presence or absence of trolox (0.2 mM) for 12h. (A) Representative detection bands of p21 and b-actin proteins using western blotting. (B) Calculated data of the density (p21/b-actin). Data are expressed as the mean ± SEM (n= 3). *p<0.01; significantly different from control. †p<0.05; significantly different from the curcumin alone.

3.2 Trolox increased both G2/M cell cycle arrest and p21 protein upregulation induced by curcumin

P21 is a cyclin-dependent kinase inhibitor that mediates cell cycle arrest [19]. Therefore, we observed changes in the population of G2/M in the cell cycle and p21 protein expression levels by the addition of 0.2 mM trolox in the presence of 10 µM curcumin (Fig. 3 and 4). 10 µM curcumin alone significantly increased the population of G2/M and p21 protein expression levels. Co-addition of trolox further enhanced both of the curcumin effects. These findings show that trolox enhances the curcumin-induced inhibition of Caco-2 cell proliferation by a synergistic inhibition of cell division at the G2/M cell cycle stage.

4. Discussion

The present study found that NAC blocked the curcumin-induced inhibition of Caco-2 cell proliferation, while TOC and trolox enhanced the curcumin effect. DCF fluorescence studies revealed that, at concentrations used in the proliferation experiment, only NAC quenched the ROS generation caused by curcumin. This study also showed that trolox enhanced the G2/M arrest and p21 protein expression levels induced by curcumin.

It has been reported that curcumin suppresses retardation of tumor cell growth via up-regulation of ROS levels [13,14,16]. Up-regulation of ROS above basal levels has been reported to activate apoptotic processes, including a decrease in Bcl levels in mitochondria [13,14]. On the other hand, Kelkel et al.[20] and Zang et al.[21] have reported that curcumin leads to G2/M cell cycle arrest independent of ROS generation. Therefore, it is possible that NAC blocks the mitochondrial apoptotic pathways and then growth inhibition via reduction of ROS generation induced by curcumin, whereas trolox and TOC enhanced the G2/M arrest triggered by curcumin independent of ROS levels.

Further studies are needed to clarify the mechanism by which trolox and TOC, but not NAC, potentiate the curcumin-induced inhibition of Caco-2 proliferation. However, the present study indicates that combined exposure to curcumin and vitamin E-like molecules causes more extensive cell growth inhibition by G2/M cell cycle arrest as a result of p21 overproduction that likely contributes to ROS-independent cytotoxicity. The therapeutic application of a vitamin E-like molecule with curcumin may be effective against colon cancers.

Conflict of interest

The authors have declared that there is no conflict of interest.

References

Phytochemicals.


