Research Article

Trolox induces inhibition of cell pro-
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Trolox induces inhibition of cell proliferation and apoptosis in human colon cancer cells

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Abstract

In the present study, the effect of trolox on human colon cancer cell lines was investigated. The results revealed that trolox treatment caused inhibition of cell growth in T84 and HCT-15 colon cancer cell lines in a dose-dependent manner. The inhibition was significant at 50 µM of trolox after 48 hours in both cell lines. Trolox treatment promoted expression of p38 and inhibited expression of survivin and Akt. It also induced cleavage of PARP and caspase-3 and ultimately induced apoptosis in T84 and HCT-15 cells. The tumor growth was inhibited significantly in the xenotransplanted mice on treatment with trolox compared to the control group. Since trolox treatment exhibits inhibitory effect on the proliferation of colon cancer cells and inhibits tumor growth *in vivo* therefore, can be of therapeutic importance for the treatment of colon cancer.

Introduction

Colon cancer constitutes one among the four commonly observed malignant digestive tract tumors and is a challenge to the clinicians globally. Malignant tumor cells possess the ability of invasion into the neighboring normal tissues including blood and lymph circulatory system from the original site and develop into secondary tumors (Mareel and Leroy, 2003). Invasion of cancer cells leads to tissue remodeling like proteolytic degradation of the extracellular matrix (ECM) along with the basement membrane of normal adjacent tissues (Johnsen et al., 1998). Although various efforts have been made for the development of treatment strategies for colon cancer but the results obtained are inefficient. Therefore, the discovery of new molecules with roles in the treatment of colon cancer is highly desired for the colon cancer treatment.

The plant derived natural products including, taxol, oncovin, navelbine and vumon have played an important role in the treatment of various types of carcinomas (Pezzuto, 1997; Kinghorn et al., 1999; Lee, 1999). Most of the strategies used for cancer treatment induce disruption of cell cycle (Moalic et al., 2001) and activation of factors involved in inducing cell apoptosis (Fan et al., 1998). Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), an analog of vitamin E has been widely investigated for its role in the inhibition of oxidative stress or damage in cancer cells (Lúcio et al., 2009; Barclay et al., 1995; Forrest et al., 1994). Further, its effect has also been investigated in induction of antioxidative effect through expression of MMP in inflammatory diseases and cancers (Haug et al., 2004; Nelson et al., 2006). It has been reported that MMPs play a vital role in the process of human lung and cervical cancer metastasis (Nelson et al., 2000; Sheu et al., 2003). Not only trolox but the role of several other antioxidants which influence MMP expression in these cancers has also been investigated (Woo et al., 2004; Chu et al., 2004).

In the present study, the effect of trolox on antitumor activity in T84 and HCT-15 human colon cancer cell lines was investigated. The results demonstrated that trolox treatment induced cell apoptosis in human colon cancer cell lines and inhibited tumor growth in xenograft model.

Materials and Methods

Reagents

Trolox was obtained from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide to prepare the stock solution which was stored at -20ºC.

Cell lines and culture

T84 and HCT-15, human colon cancer cell lines were obtained from American type of culture collection (Manassas, VA, USA). The cells were maintained in DMEM supplemented with 10% FBS and antibiotics and were grown at 37ºC in humidified 5% CO_2-incubator.

Viability assay

For the measurement of trolox induced cytotoxicity in T84 and HCT-15 colon cancer cell lines, the cells were distributed onto 96-well plates at a density of 2×10^6 cells per well. After culturing the cells overnight at room temperature, the indicated doses of trolox and oxaliplatin were added to each of the well. The cells were then incubated for 24 hours followed by the measurement of cell viability using a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan). For the purpose of cell incubation the procedure was performed according to manual protocol. The spectrometer was employed to read the plates at A_{450}.

Proliferation assay

T84 and HCT-15 cancer cells were seeded at a density of 2×10^6 cells on a 24-well cell culture plate. The cells were cultured with trolox for 24 hours and deprived of serum for 6 hours followed by replacement of medium with DMEM supplemented with 10% FCS. The cell proliferation was determined after 24 hours by using BrdU assay kit (Roche Diagnostics, Penzberg, Germany) following the manufacturer's instructions. The experiments were performed in triplicates. The plate reading was performed at A_{450} using a spectrometer.

Determination of lactate dehydrogenase (LDH) activity

The activity of lactate dehydrogenase (LDH) expressed in the cytosol was also determined. Briefly, the cells were distributed at a density of 2×10^6 cells per mL on 96-well plates. The commercially available kit (Takara Bio, Tokyo, Japan) was used for the determination of absorbance. The values of absorbance were then correlated with the number of viable cells to predict the cytotoxic activity. Triton X-100 (1%) (Sigma) was used as a positive control.

Apoptosis assay

For the analysis of trolox-induced cell apoptosis annexinV-FITC/propidium iodide double staining kit (Genmed Bioscience, China) was used according to the manufacturer's instructions. Briefly, the cells were dis-tributed at a density of 2×10^6 cells per well in six wells plates and cultured for 24 hours. The cells were then harvested, washed with PBS and resuspended in 1 × binding buffer and exposed to 5 µL of annexin V-FITC (20 µg/mL) and 10 µL of propidium iodide (PI; 50 µg/mL). The cells after incubation of 45 min under dark were subjected to a FACScan flow cytometer [equipped with CellQuest and ModFITLT for Mac V1.01 software (Becton Dickinson, USA)]. All the experiments were performed at least in triplicates independently.

Western blot analysis

The trolox treated or untreated cells after washing with ice-cold phosphate-buffered saline (PBS), were lysed in TNN buffer (Sigma-Aldrich). The cell lysates were centrifuged for 30 min to remove the cell debris. Equal amounts of protein were separated using SDS-PAGE and then transferred onto PVDF (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies including, cleaved caspase-3, cleaved poly (ADP-ribose) polymerase (PARP), survivin, phosphoserine 473 Akt, phospho p38 and GAPDH (Cell Signaling Technology, Beverly, MA, USA) overnight for immunostaining of the blots. The membranes were washed with PBS followed by incubation with secondary antibodies conjugated to horseradish peroxidase, Amersham Biosciences, Inc., (Piscataway, USA). The blots were detected by using enhanced chemiluminescence reagent (ELPS, Korea) and visualized using the ECL Plus system (Amersham Pharmacia Biotech, Inc., Piscataway, USA).

Tumor xenograft study

Male nude mice (6 weeks; weight, 20-22 g) were purchased from the Central Animal Laboratory, Inc. (Seoul, Korea). All the animal care and experimental procedures were performed in accordance with the approval and guidelines of the Inha Institutional Animal Care and Use Committee (INHA IACUC) of the Medical School of Inha University (Incheon, Korea). The animals were kept under 12 hours dark and light cycle at 25ºC and had free access to standard rat chow and tap water *ad libitum.* The mice were randomly assigned to three groups; control, treatment trolox 125 mg/kg and trolox 250 mg/kg groups. HCT-15 cells were harvested, mixed with PBS and then inoculated into one flank of each nude mouse (2×10^6 cells). The mice received daily intraperitoneal injection of trolox (125 and 250 mg/kg) or vehicle (200 µL PBS, control group) for 28 days after the tumors attained palpable stage. Digital caliper was used for the measurement of tumor volume on alternate days. The mice were sacrificed after the completion of treatment to extract the tumors.

Statistical analysis

All the quantitative data presented are recorded as the

means ± SD. Student's *t*-test was used to perform comparison among the two groups and the differences between multiple groups were analyzed using one-way ANOVA. For the relevance analysis of ordinal data was cross χ2 test performed. A statistically significant difference was considered as p<0.05.

Results

Trolox inhibits proliferation of colon cancer cell lines

Treatment of T84 and HCT-15 colon cancer cell lines with trolox induced a concentration dependent inhibition of cell proliferation. The cells were exposed to a range of trolox concentrations from 10 to 50 µM and the WST-8 and BrdU assays were used to determine inhibition of cell proliferation after 24 hours. The results revealed that trolox treatment induced a significant decrease in the proliferation of both the tested cell lines compared to untreated cells at all the concentrations. However, the inhibition of cell proliferation was maximum at 40 µM concentration of trolox after 24 hours (Figure 1). After 24 hours, the rate of cell proliferation was 28 and 98%, respectively in the trolox treated and control T84 cells. Similarly, the proliferation

rate in trolox treated and control HCT-15 cells was 32 and 99%, respectively after 24 hours (Figure 1).

Trolox does not induce necrosis

It is reported that in necrotic cells degradation of the cell membrane induces secretion of LDH along with various other substances from cytoplasm into the culture medium (Korzeniewski and Callewaert, 1983). Comparison of the secreted LDH in the control and trolox-treated T84 and HCT-15 cells revealed no significant difference following 24 hours of the treatment (Figure 2). These findings suggest that the decrease in cell proliferation in T84 and HCT-15 cells on treatment with trolox is not associated with the process of cell necrosis.

Trolox induces apoptosis in colon cancer cells

Exposure of HCT-15 cells to trolox led to a significant decrease in the expression of survivin following 24 hours of the treatment at 40 µM concentrations. However, the expression of Akt and p38 was increased markedly compared to the untreated cells. Furthermore, the trolox treatment for 24 hours also induced expression of cleaved PARP and caspase-3 (Figure 3A). The results from TUNEL staining analysis showed a significant increase in the proportion of apoptotic cells
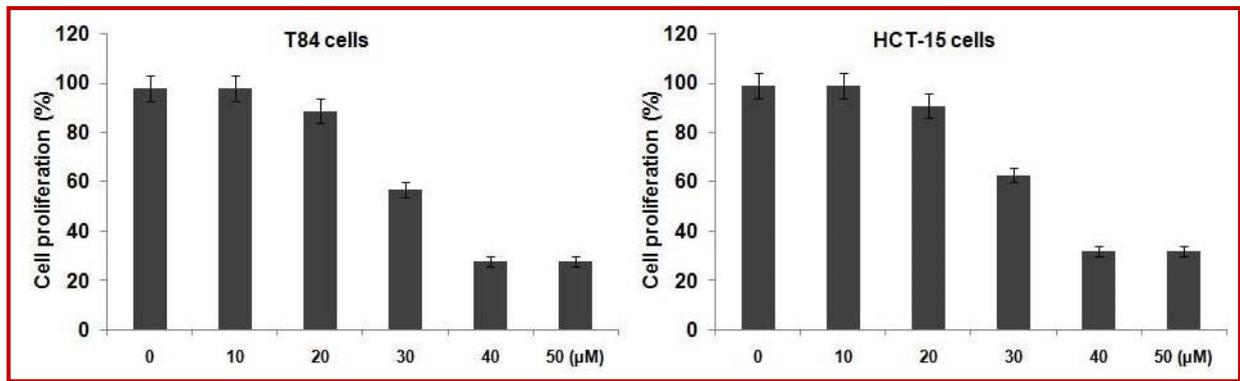

Figure 1: Trolox treatment inhibits proliferation of T84 and HCT-15 human colon cancer lines after 24 hours. The cell lines were exposed to a range of trolox concentrations from 10-50 µM for 24 hours
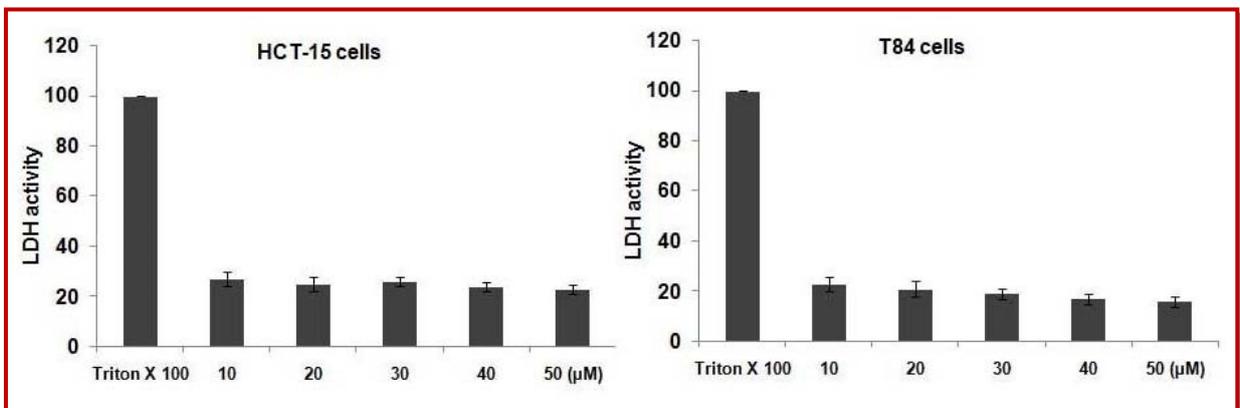

Figure 2: Trolox treatment exhibits no significant effect on the secretion of LDH into the medium in colon cancer cell lines. The values were compared to triton X 100
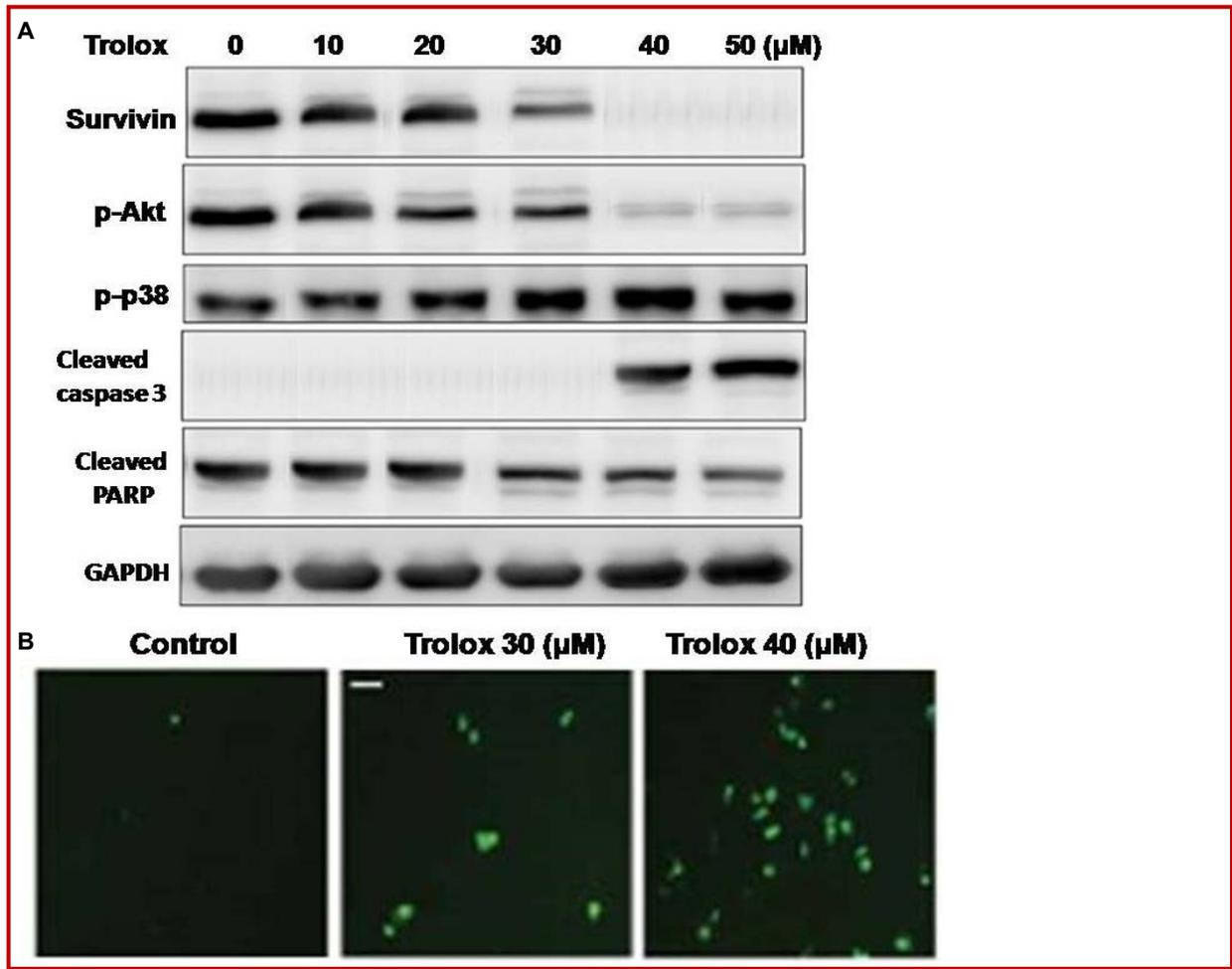
Figure 3: HCT-15 cells were treated with 10-50 µM of trolox for 24 hours followed by western blot analysis using GAPDH as the internal control (A) . HCT-15 cells were subjected to TUNEL staining following treatment with 0, 30 and 40 µM doses of trolox (B)

in HCT-15 cells on treatment with 30 and 40 µM doses of trolox compared to untreated cells (Figure 3B).

Inhibition of tumor growth of HCT-15 xenografts by trolox

Examination of the effect of trolox on tumor xenograft model of HCT-15 cells revealed a marked decrease in the volume of tumor in trolox treated mice compared to the untreated group (Figure 4).

Discussion

The present study demonstrates the effect of trolox, an analog of vitamin E on the growth and proliferation of human colon cancer cell lines *in vivo* and tumor volume *in vitro*. Treatment of human colon cancer cell lines, T84 and HCT-15 with trolox induced a concentration dependent inhibition of cell proliferation.

Cell apoptosis is inhibited by various factors like survivin protein which is one type of inhibitor of apoptosis (IAP) and its function is to suppress apoptosis. It is reported that expression of IAPs is enhanced during malignancies which inhibit apoptosis to avoid the death of neoplastic cells (Altieri, 2003). In case of tumor cells decrease in the apoptosis index, enhanced resistance to drugs and the low rate of prognosis have been found to be associated with the increase in expression of survivin (Carrasco et al., 2011). It has been demonstrated in various types of cell lines that knockdown of the expression of survivin induces apoptosis and arrests cell cycle in the G_2/M phase (Carrasco et al., 2011). The results from the present study demonstrated that treatment of colon cancer cell lines with trolox inhibited the expression of survivin.

When cells are under different types of stresses MAP kinases including p38 MAP are activated (Rouse et al., 1994; Han et al., 1994; Lee et al., 1994; Freshney et al., 1994; Raingeaud et al., 1995). After activation, p38 MAP kinase plays an important role by targeting various factors including reduction in the expression of survivin and resulting in apoptosis (Hsiao et al., 2007; Hsu et al., 2012). Our results demonstrated that trolox treatment induced activation of p38 in the colon cancer cells. In
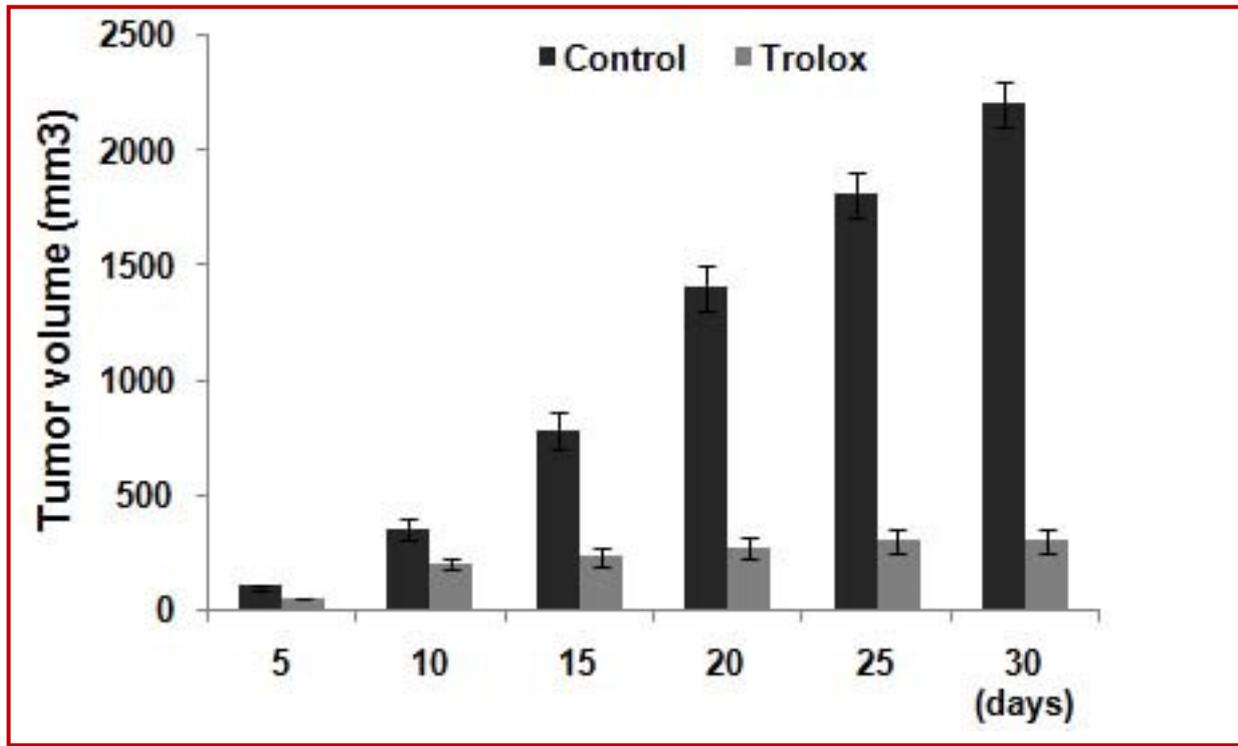
Figure 4: Effect of trolox treatment on the tumor volume of HCT-15 tumor xenograft in mice following 30 days of the treatment

cancer cells the phosphatidylinositol-3-kinase (PI3K)/Akt pathway which proliferation and decreases rate of cell apoptosis (Toker and Yoeli-Lerner, 2006) is constitutively active. The results from our study showed that treatment of the colon cancer with trolox induced dephosphorylation of Akt. Trolox induced increase in the expression of activated p38 and Akt dephosphorylation resulted in cleavage of caspase-3 and PARP. These alterations led to the induction of colon cancer cell apoptosis.

Treatment of the mice with xenotransplant with trolox for 30 days led to a significant decrease in the tumor volume in the treatment group compared to untreated group of the mice. Trolox treatment also did not induce any disorder in the skin of the treatment group of mice.

Conclusion

Trolox treatment inhibits proliferation of the colon cancer cells *in vivo* by inducing apoptosis and suppesses tumor volume *in vitro*. Therefore, trolox can be a potential agent for the treatment of colon cancer.

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