

Anthocyanin-Rich Extract From *Aronia meloncarpa* E. Induces a Cell Cycle Block in Colon Cancer but Not Normal Colonic Cells

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Abstract: Anthocyanin-rich extracts, potent antioxidants and commercially available food coloring agents, have been reported to inhibit growth of various cancer cell lines. We investigated the effect of semipurified anthocyanin-rich extract from fruits of *Aronia meloncarpa*, on normal colon and colon cancer cell lines. A 24-h exposure to 50 µg monomeric anthocyanin/ml of *Aronia* extract resulted in 60% growth inhibition of human HT-29 colon cancer cells. The treated cells showed a blockage at G1/G0 and G2/M phases of the cell cycle. The cell cycle arrest coincided with an increased expression of the p21^{WAF1} and p27^{KIP1} genes and decreased expression of cyclin A and B genes. Prolonged exposure to the extract resulted in no further change in the cell number, indicating a cytostatic inhibition of cell growth. NCM460 normal colon cells demonstrated <10% growth inhibition at the highest concentration of 50 µg/ml extract. A 35% decrease in the cyclooxygenase-2 gene expression was observed within 24 h of exposure of HT-29 cells but did not translate into decreased protein levels or protein activity. These results support the need for further research to identify the specific component(s) in this extract that suppress cancer cell growth and the genes affected by these natural compounds.

Introduction

Diets rich in fruits and vegetables have been associated with decreased colon cancer incidence (1). Identification of the components of these foods that can be evaluated as anticancer compounds has been the focus of considerable research. Fruits and vegetables are rich in polyphenols, with certain fruits containing as high as 1–2 g total phenolics/100 g fresh weight (1). The average intake of phenolics is estimated at 1 g/day (2). Anthocyanins belong to the flavonoid group of phenolics, and the red to blue coloring of flowers and fruits is mostly because of these natural compounds. They also are often used as colorants in processed foods (3). The intake of anthocyanins in the United States is estimated to be between 180 and 215 mg/day (4). Anthocyanins exhibit antioxidant activity (5) and antiinflammatory properties (6). Crude methanolic extracts containing anthocyanins were reported to reduce the incidence of esophageal tumors (7), reduce pancreatic swelling (8), and inhibit cell transformation (9), indicating

potential cancer chemopreventive activity. Inhibition of growth of tumor cells derived from intestinal carcinoma and malignant lymphoma by anthocyanin fractions (10) is affected by the sources and glycosylation pattern of anthocyanins and other flavonoids (11–13). Antitumor activity of anthocyanin-containing sources was demonstrated in vivo (11,14). Hagiwara et al. (14) demonstrated a decrease in incidence of early colon cancer lesions, aberrant crypt foci, in rats fed anthocyanin-rich PCC (purple corn color), indicating a protective effect against 2-amino-1-methyl-6-phenylimidazol[4,5-*b*]pyridine (PhIP) induced colon carcinogenesis.

We have demonstrated in our laboratory (Zhao et al., unpublished data) that the growth of human HT-29 colon cancer cells in vitro was inhibited by food grade anthocyanin-rich extracts (AREs) from *Vitis vinifera* (grape), *Aronia meloncarpa* (chokeberry), and *Vaccinium myrtillus* (bilberry). Of these three AREs, chokeberry ARE was the most potent inhibitor of HT-29 cell growth. The current study further examined the inhibitory effects of chokeberry ARE on HT-29 colon cancer cells and NCM460 normal colon epithelial cells.

Materials and Methods

Cell Culture

Human HT-29 colon cancer cells (ATCC, HTB 38) were grown in McCoy's 5A medium (BioWhittaker Inc., Walkersville, MD), and normal colon epithelial cells, NCM460 (INCELL Corp. LLC, Austin, TX), were grown in 25% conditioned M3:10 medium (INCELL Corp. LLC). Media were supplemented with 10% fetal bovine serum (FBS) and 1 × antibiotic-antimycotic (Invitrogen Corporation, Carlsbad, CA), and cells were maintained in a humidified atmosphere of 95% air/5% CO₂ at 37°C.

Anthocyanin-Rich Extract

The ARE from *Aronia meloncarpa* E. (chokeberry; Artemis International, Inc., Madera, CA) was semipurified by solid-phase extraction using a C-18 cartridge (Waters Corp., Milford, MA). In brief, 10 g of powdered extract was dissolved in 100 ml 0.01% HCl. The extract was loaded onto a C-18 cartridge that had been pretreated with ethanol (100%), followed by acidified water (0.01% HCl).

Anthocyanins and other phenolics that bind to the C-18 cartridge were recovered with ethanol-containing 0.01% HCl. The alcohol was evaporated at 40°C, and the solutes were redissolved in 10 ml 0.01% HCl-deionized water (vehicle). Monomeric anthocyanin content was determined by the pH-differential method (15).

Cell Proliferation

Freshly trypsinized cells were seeded in 25-cm² (1 × 10⁶ cells) or 75-cm² (2 × 10⁶ cells) flasks in duplicate. The cells were grown for 24 h under normal growth conditions before treatment with chokeberry ARE. Freshly prepared ARE was added in concentrations ranging from 10 to 200 µg monomeric anthocyanin/ml medium. A vehicle control contained acidified water and was added at the same volume as ARE added to treated cells. The addition of the small amount of acidified extract (pH 4.0) did not affect the pH of the media. Cells were also grown in media alone (no-vehicle control) to ensure there was no effect of the vehicle on the growth of the cells.

Before exposure to ARE for 24-, 48-, or 72-h treatments, the medium was replaced with fresh medium (10% FBS) containing either vehicle control (acidified water) or ARE. For each time point, the cells were trypsinized and centrifuged at 400 g for 10 min at room temperature. Aliquots of cells were counted using a hemacytometer and viability measured using Trypan blue dye exclusion.

Cell Cycle Analysis

Cell cycle analysis was performed using flow cytometry. HT-29 cells were seeded (2 × 10⁶ cells/75-cm² flask) in duplicate and incubated for 24 h before exposure to 50 µg monomeric anthocyanin/ml for various time periods of 24, 48, and 72 h. All experimental conditions were as described above for the cell proliferation study. To further analyze if the cells recovered from growth inhibition after initial 24-h exposure to ARE, we grew cells for an additional 24 h in fresh media that did not contain either ARE or acidified water. Approximately 1 × 10⁶ cells were fixed in cold 70% ethanol and analyzed for cell cycle distribution as described by Park and Schoene (16). In brief, the fixed cells were centrifuged, washed with 1 × phosphate buffered saline (PBS), and then stained for DNA content by resuspending in 1 ml staining solution (PBS containing 20 µg propidium iodide and 25 mg ribonuclease A) (16). Cells were stained for 30 min at room temperature and then immediately analyzed by flow cytometry. Data were collected for 10,000 cellular events per sample using a FACSCalibur cytometer and CELLQuest software (Becton Dickinson, San Jose, CA). Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software (Verity Software House, Inc., Topsham, ME). The DNA QC Particle Kit, for verification of instrument performance, was purchased from Becton Dickinson.

Gene Expression Analysis

HT-29 and NCM460 cells (2 × 10⁶ cells/75-cm² flasks) were incubated for 24 h in the presence of 10–50 µg/ml media.

After exposure to ARE for 24, 48, and 72 h, the cells were either directly lysed in the flasks or trypsinized and counted before total cellular RNA was extracted using TRIzol Reagent (Invitrogen, CA) according to the manufacturer's protocol. RNA samples were treated with DNase I enzyme using the DNA-free kit (Ambion Inc., Austin, TX). RNA Nano LabChip and the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) were used to check the quality and the quantity of the isolated total RNA. First-strand synthesis was done using Retroscript kit (Ambion Inc.) and cDNA was measured spectrophotometrically.

Changes in gene expression were determined using multiplex RT-PCR. Table 1 lists the genes of interest and primer sequences. Ribosomal 18S (Ambion Inc.) was used as the internal control. The 18S primer:competimer ratio was optimized according to the manufacturer's instructions before changes in expression of these genes was studied. After optimization of PCR conditions for a multiplex reaction (cell cycle, primer conditions, and annealing temperature), the following parameters were used for the assay: 1 × complete reaction buffer; dNTPs mixture (200 µM each); 18S primers:competimers (1:9) mixture (0.4 µM), p21^{WAF1} primer (0.4 µM) or p27^{KIP1} primer (0.4 µM) or cyclin A (0.2 µM) or cyclin B1 (0.2 µM) and 0.4 U/10 µl *Taq* DNA polymerase. The amplification conditions used were as follows: one 2-min cycle at 92°C followed by 27 cycles of denaturation for 30 s at 92°C, annealing for 40 s at 60°C and extension for 1 min at 72°C. A final extension was given for 5 min at 72°C before analysis of the PCR products.

Changes in expression of cyclooxygenase genes were studied using human COX-1 and COX-2 gene-specific Relative RT-PCR Kits (Ambion Inc.). Ribosomal gene 18S (498 bp) was used as an internal control. PCR assay conditions were optimized before changes in expression of COX-1 gene (401 bp) and COX-2 gene (297 bp) was analyzed. The multiplex reaction contained 1 × complete reaction buffer; dNTPs mixture (200 µM each); 18S primers:competimers (1:9) mixture (0.4 µM), 0.4 µM COX-1 primer or 0.4 µM COX-2 primer and 0.4 U/10 µl *Taq* DNA polymerase. The following thermocycling conditions were used for PCR assays: one 2-min cycle at 92°C followed by 26 cycles of denaturation for 30 s at 92°C, annealing for 30 s at 59°C (COX-1) or 30 s at 60°C (COX-2), and extension for 1 min at 72°C. The final extension was given for 5 min at 72°C. The PCR products from

Table 1. Primer Sequences (5'-3') and Expected Product Size

Gene Name	Sequence of Primers	Product Size (bp)
p21 ^{WAF1}	Forward: 5'-GGAACCTTCGACTTTGTACCCGAG-3' Reverse: 5'-GAACCTCTCATTCAACCCGCTAG-3'	561
p27 ^{KIP1}	Forward: 5'-TGCCTCTAAAAGCGTTGGAT-3' Reverse: 5'-TTTTTGCCCCAAACTACCTG-3'	542
Cyclin B1	Forward: 5'-GGCCAAAATGCCTATGAAGA-3' Reverse: 5'-AAACATGGCAGTGACACCAA-3'	589
Cyclin A	Forward: 5'-CAGCCATTAGTTTACCTGGACC-3' Reverse: 5'-TGTTGGAGCAGCTAAGTCAAAA-3'	542

multiplex reactions were analyzed using DNA 500 or DNA 7500 LabChip and Agilent 2100 bioanalyzer according to the manufacturer's protocol. The changes in the gene expression were represented by the changing ratio between the area of bands representing gene of interest and the band representing 18S gene. A ratio difference in the control versus the treated cells is the measure of change in gene expression.

COX-2 Protein Assay

HT-29 and NCM460 cells seeded in 25-cm² flasks (1 × 10⁶ cells) and exposed to 50 µg/ml ARE for 24, 48, and 72 h were trypsinized and counted. COX-2 protein was measured using TiterZyme EIA (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's instructions.

Prostaglandin-E2 Assay

The amounts of prostaglandin-E2 (PGE₂) in the culture medium were determined using EIA kit (Cayman Chemical, Ann Arbor, MI) following manufacturer's protocol. In short, the culture medium from each time frame was centrifuged at 800 g for 5 min and the supernatant stored at -80°C. The supernatant was added to 96-well plates, coated with an antimouse antibody, mixed with a PG/acetylcholinesterase tracer and a monoclonal antibody against prostaglandin and incubated at 4°C overnight. Unbound PG/acetylcholinesterase was removed and washed extensively and bound acetylcholinesterase was detected by Ellman's reagent and measured at 405 nm.

Results

Chokeberry ARE Inhibits Growth of Human HT-29 Colon Cancer Cells

Growth of HT-29 cells exposed to 50 µg monomeric anthocyanin/ml was inhibited 60–70% within the first 24 h (Fig. 1). Although continuous exposure of HT-29 cells to ARE for up to 72 h resulted in approximately 90% inhibition of growth relative to the control cells (Fig. 1), the number of cells did not change with time, and >95% of the cells were viable, indicating a cytostatic inhibition.

Inhibition of HT-29 cell growth by chokeberry ARE was concentration dependent up to 50 µg monomeric anthocyanin/ml of medium (data not shown). At higher concentrations of ARE, inhibition of growth reached a plateau and the cells were no longer viable, as analyzed by Trypan blue dye exclusion (data not shown). HT-29 cells incubated in the presence of acidified water (vehicle control) grew at the same rate as cells incubated in media alone (no-vehicle control), indicating that acidified water used in preparation of ARE did not affect the growth of cells (data not shown).

Chokeberry ARE Does Not Inhibit Growth of Human NCM460 Normal Colon Cells

To assess whether ARE inhibits normal cell growth, we exposed NCM460 derived from normal colon epithelial cells

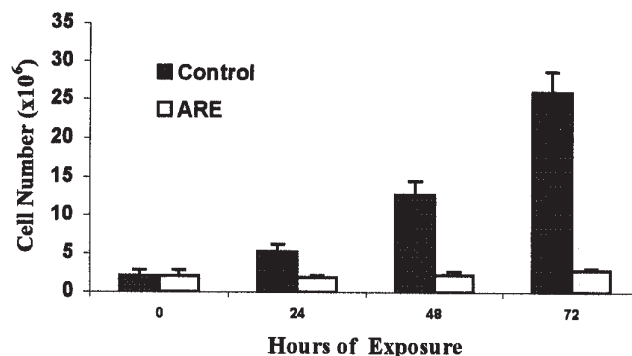


Figure 1. Effect of chokeberry ARE on growth of HT-29 cells. Growth inhibition of HT-29 cells exposed to 50 µg monomeric anthocyanin/ml (ARE, white bars) was observed after 24 h, with no increase in cell number at 48 or 72 h. HT-29 cells grown in control media containing acidified water (vehicle control, black bars) showed normal growth rate. Cells (2 × 10⁶) were plated in 75-cm² flasks in duplicate and allowed to grow for 24 h before being exposed to 50 µg/ml of chokeberry ARE. Every 24 h, fresh media containing acidified water (vehicle) or ARE replaced the used media and thus cells were continually exposed to fresh ARE.

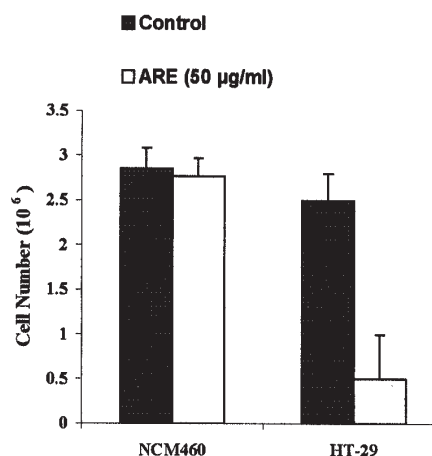


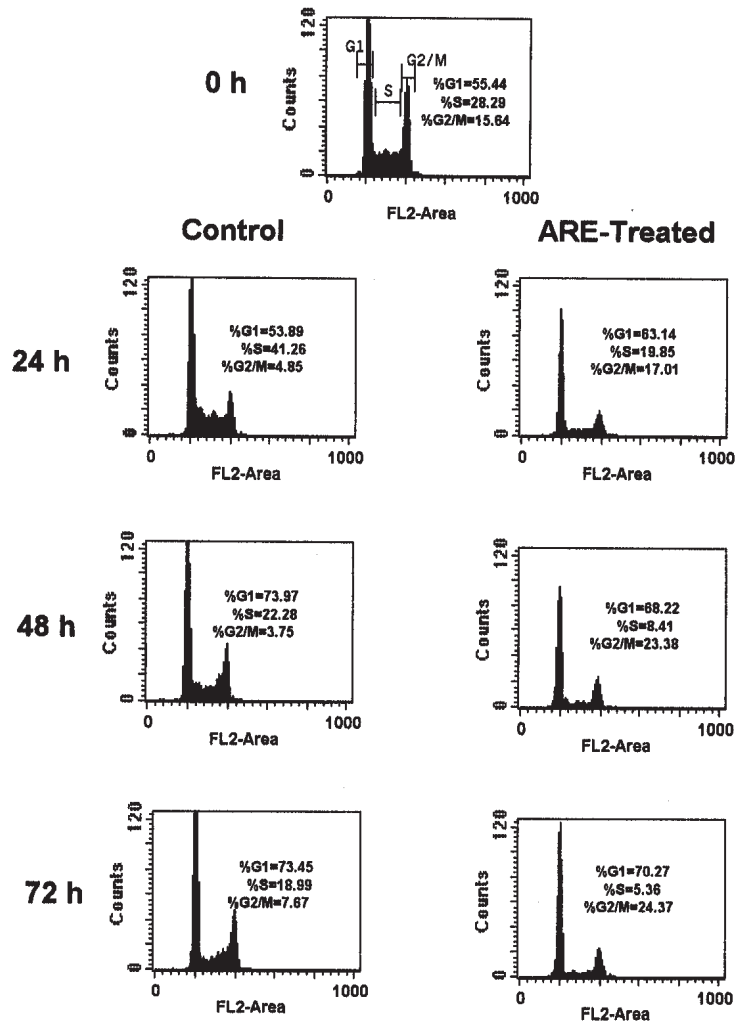
Figure 2. Effect of chokeberry ARE on growth of normal colon cells. Compared with vehicle controls (black bars), NCM460 cells did not show any growth inhibition when exposed to 50 µg monomeric anthocyanin/ml for 24 h, whereas HT-29 cells show almost 65% inhibition (ARE, white bars). The cell viability was more than 95% in each flask.

(17), to the same concentrations of chokeberry ARE. This study shows for the first time that growth of normal human NCM460 colon cells was inhibited less than 10% by exposure to 50 µg/ml of media for 24 h in contrast with HT-29 colon cancer cells where growth inhibition of 65% was observed (Fig. 2).

Chokeberry ARE Induces a Dual Block in Cell Cycle of HT-29 Cells

We investigated whether ARE-induced inhibition of cell growth is through a blockage in progression of cells through the cell cycle. In the current study, chokeberry ARE arrested the HT-29 cells at G1/G0 phase of the cell cycle with an accompanied concomitant decrease of percentage of cells in S phase and an increase in the G2/M phase (Fig. 3A). As shown in Fig. 3B, within 24 h of exposure the percentage of cells in

A



B

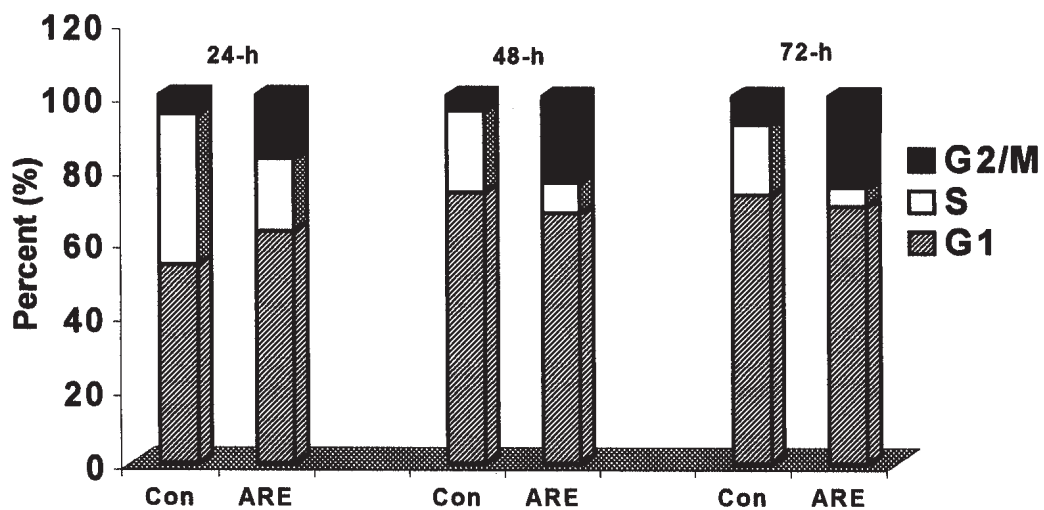


Figure 3. Effect of ARE on cell cycle progression of HT-29 cells. A: Histograms of cell cycle distribution of control (vehicle) and ARE-treated (50 $\mu\text{g/ml}$) HT-29 cells after 24, 48, and 72 h of exposure. HT-29 cells seeded at 2×10^6 cells/flask in duplicate were incubated for 24 h before being exposed to 50 $\mu\text{g/ml}$ ARE. A significant decrease in percentage cells in S phase is observed after 24, 48, and 72 h of exposure to ARE. B: Percentage of cells in each cell cycle phase after 24, 48, and 72 h of exposure to vehicle (con) or chokeberry ARE (ARE) media. A continual decreasing S phase (white box) is indicative of no cell DNA synthesis. An increase in percentage of cells in G2/M phase (black box) is also evident with time.

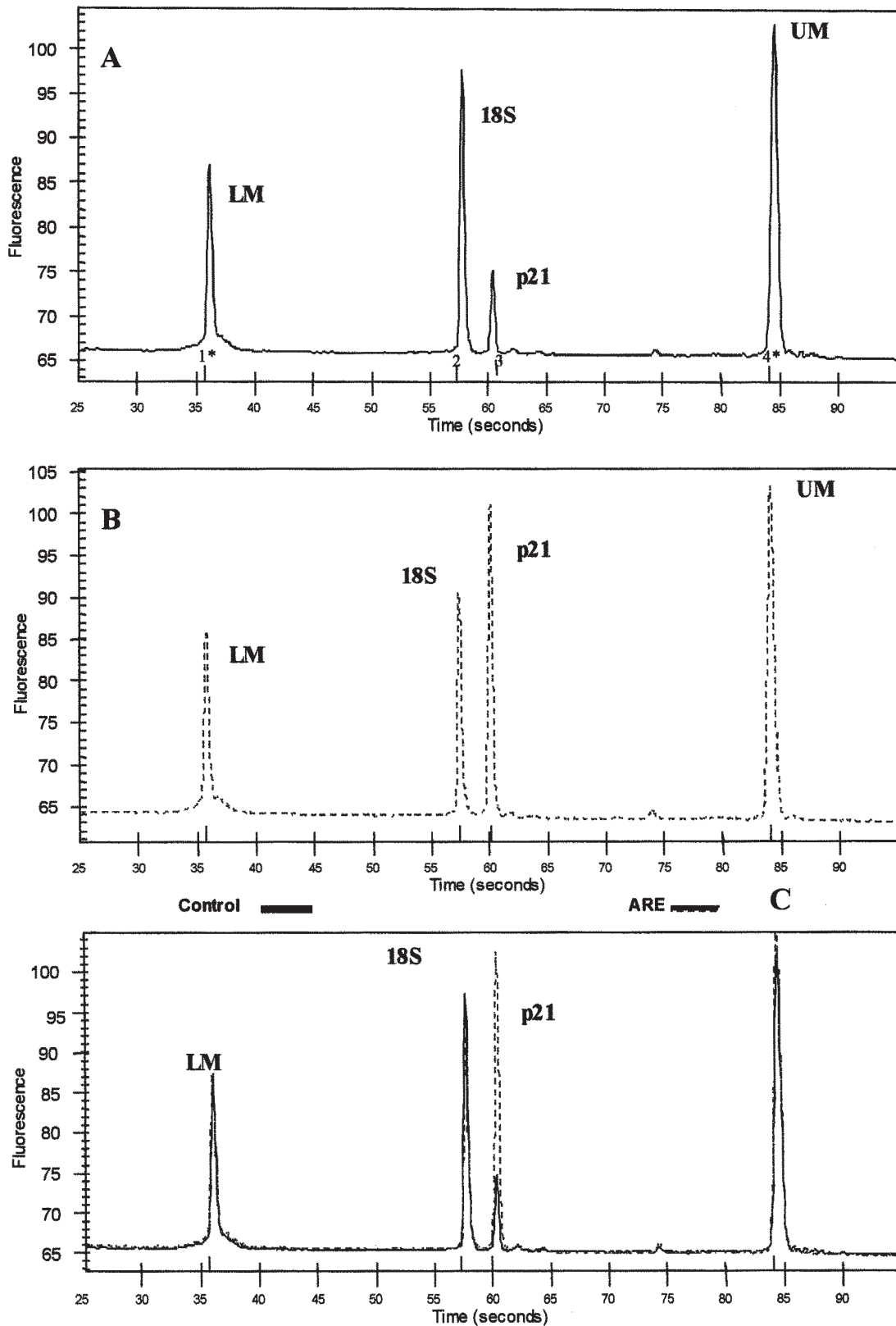


Figure 4. Changes in expression of p21^{WAF1} gene relative to 18S gene analyzed by multiplex RT-PCR. A: Electropherogram (solid line) of control experiment. B: Electropherogram (dashed line) of ARE-treated experiment. The cells in treated experiment demonstrated an increase in the expression of the p21^{WAF1} gene (peak 3), which is represented by the increase in the peak height resulting in an increased peak area. The peak representing 18S (peak 2) show very little change in the peak heights in control versus ARE-treated/exposed experiment. LM (peak 1*) and UM (peak 4*) represent the lower marker and the upper marker that is provided by the manufacturer with the DNA 7500 LabChip. C: Superimposition of electropherograms of treated experiment over control experiment. The change in expression of p21^{WAF1} gene in treated cells can be observed by the increased height of peak 3 represented by dashed line superimposed over the solid peak representing the gene expression in control experiment.

G1/G0 phase increased from 54% to 63%, the percentage of cells in S phase decreased from 41% to 20%, and in G2/M phase the cell percentage increased from 5% to 17%. The increase in percentage of cells in the G2/M phase was more apparent with continued exposure to ARE for up to 48 and 72 h (Fig. 3B). Compared with the controls at 48 and 72 h, there was an almost fivefold increase in accumulation of cells in the G2/M phase with a concomitant decrease of cells in S phase. No sub-G0 peaks indicative of apoptosis were observed in any experiments.

To determine whether the block in the cell cycle was reversible, we removed the ARE after 24 h of exposure and cells were grown for another 24 h in ARE-free medium. These cells recovered from both the G1/G0 and G2/M blocks. The percentage of cells in the S phase increased approximately to the percentage in the control, indicating the occurrence of DNA synthesis, and a corresponding decrease in G1/G0 and G2/M phases was observed (data not shown). There was no change in the cell cycle of NCM460 cells exposed to 50 $\mu\text{g/ml}$ for 24 h (data not shown).

Increased Expression of p21^{WAF1} and p27^{KIP1} Genes in ARE-Treated HT-29 Cells

To evaluate the potential molecular mechanism by which the cell cycle is arrested at G1/G0 phase, we assessed the expression of cyclin kinase inhibitors, p21^{WAF1} and p27^{KIP1} genes. Figure 4A–C illustrates the changes in expression of p21^{WAF1} gene in HT-29 cells exposed to ARE. Figure 4A shows the electropherogram of RT-PCR products from HT-29 cells exposed to vehicle only for 24 h, where the internal control 18S and the p21^{WAF1} genes are represented by peak 2 and peak 3, respectively. Figure 4B (*dashed line*) represents the electropherogram of HT-29 cells treated with 50 $\mu\text{g/ml}$ of chokeberry ARE for 24 h. The Agilent 2100 bioanalyzer allows superimposition of the two electropherograms (Fig. 4C) and the increased expression of p21^{WAF1} gene (increased fluorescence of peak 3, *dashed line*) can be observed. The 18S peak (peak 2), representing the internal control, from exposed as well as the vehicle only control cells show very little change in its expression. The lower marker (LM) and upper marker (UM) provided by the manufacturers are perfectly aligned. The increase in expression of p21^{WAF1} gene in ARE treated HT-29 cells was observed in time-dependent manner, with a 1.5-fold increase seen within 24 h of exposure. At the end of 72 h, the expression of p21^{WAF1} increased by threefold as compared with control cells (Fig. 5). No significant change in p21^{WAF1} expression was observed in NCM460 cells exposed to 50 $\mu\text{g/ml}$ ARE (data not shown).

The p27^{KIP1} gene expression demonstrated a similar pattern of regulation in the HT-29 cells exposed to ARE. As observed in Fig. 5 the expression of p27^{KIP1} gene increased by twofold in HT-29 cells exposed to 50 $\mu\text{g/ml}$ ARE for 48 h, and there was no further increase observed by 72 h of exposure, as compared with cells exposed to vehicle alone. NCM460 cells showed negligible difference in expression of p27^{KIP1} gene in exposed cells compared with control cells (data not shown).

Chokeberry ARE-Treated HT-29 Cells Show a Decreased Expression of Cyclin Genes

Inhibition of expression of cyclin A and cyclin B genes was consistent with the blockage of HT-29 cells at the G2/M phase of the cell cycle (Fig. 6). The inhibition of both genes was observed in a time-dependent manner. As seen in Fig. 6, at the end of 72 h of exposure to ARE HT-29 cells demonstrated three- to fourfold inhibition in the mRNA levels of cyclin B1 and cyclin A genes, respectively.

NCM460 Cells Express Higher Levels of COX-1 and Lower COX-2 as Compared With HT-29 Cells

In the current study, both COX-1 and COX-2 genes were expressed in HT-29 and NCM460 cells as measured by RT-PCR (Fig. 7A). As seen in gel image from Agilent 2100

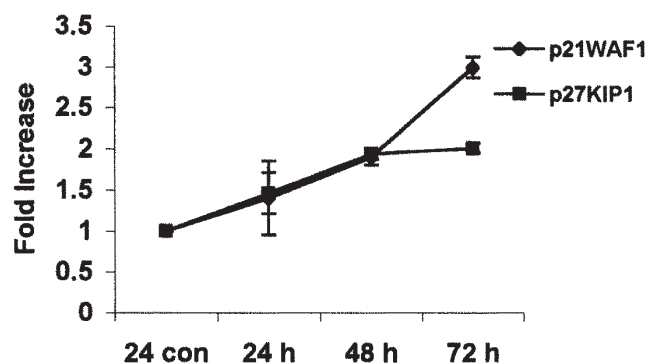


Figure 5. Fold increase in expression of p21^{WAF1} and p27^{KIP1} genes in HT-29 cells exposed to ARE. When compared with expression in cells exposed to vehicle only for 24 h (24 con), both p21^{WAF1} and p27^{KIP1} genes demonstrated increased expression in a time dependent manner for up to 48 h of exposure to 50 $\mu\text{g/ml}$ of ARE. No further increase was observed in expression of p27^{KIP1} gene, whereas p21^{WAF1} gene expression increased fourfold over the control by the end of 72 h of exposure to ARE. Each time point is an average of three replicates and is relative to untreated control.

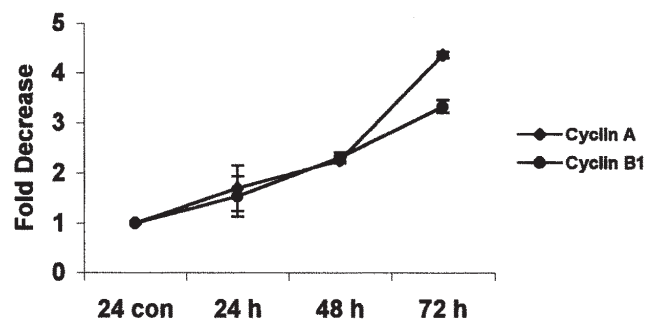


Figure 6. Fold decrease in expression of cyclin A and cyclin B1 genes in HT-29 cells exposed to 50 $\mu\text{g/ml}$ of ARE for 24, 48, and 72 h. The decrease in the expression was compared with cells exposed to vehicle alone for 24 h (24 con). Both the cyclins demonstrated a time-dependent decreased expression for up to 72 h of exposure. Each time point is an average of three replicates.

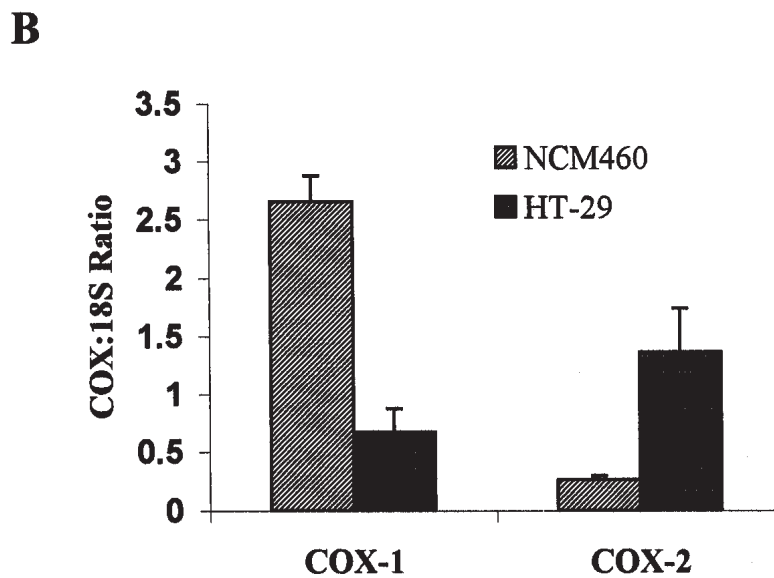
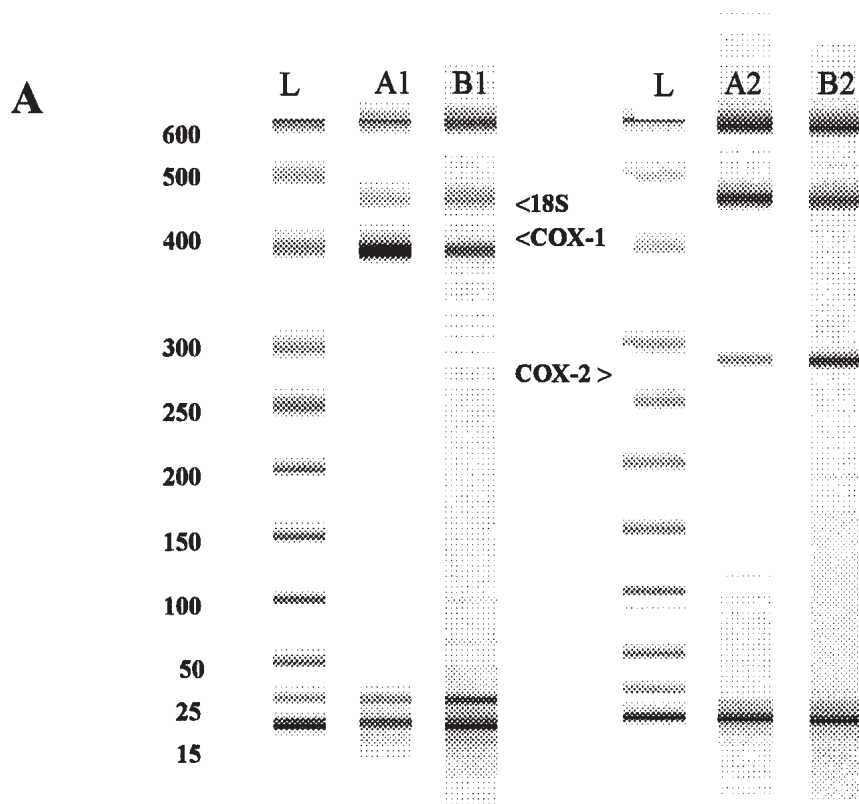


Figure 7. Basal level expression of COX-1 and COX-2 genes in colon cell lines NCM460 and HT-29. A: Gel-like image from Agilent bioanalyzer 2100 of semiquantitative RT-PCR amplification of cyclooxygenase genes in NCM460 (A) and HT-29 (B) demonstrates a higher expression of COX-1 gene (401 bp) in NCM460 normal colon epithelial cells (A1) as compared with the expression in HT-29 cells (B1). COX-2 gene (297 bp) expression in NCM460 (A2) was lower compared with COX-2 expression in HT-29 colon cancer cells (B2). The 498-bp fragment represents the 18S housekeeping gene. Lanes L represents the DNA ladder, supplied by the manufacturer. B: Bar chart depicts the differences in COX-1 and COX-2 gene expression in normal and cancer colon cell lines. The data represented here are from two separate sets of experiments.

bioanalyzer, expression of the COX-1 gene (401 bp, lane A1) in normal NCM460 cells was higher than expression of the COX-2 gene (297 bp, lane A2). In contrast, HT-29 cells derived from colon adenocarcinoma had highly expressed COX-2 gene (lane B2) as compared with the COX-1 gene (lane B1). Figure 7B represents data from two separate experiments. NCM460 cells demonstrate almost fourfold higher expression of COX-1 gene as compared with the expression in HT-29 cells. The normal cells also demonstrate a low level of COX-2 gene expression.

Effect of Chokeberry ARE on COX-2 Gene Expression and Activity

Chokeberry ARE inhibited COX-2 gene expression in HT-29 cells within 24 h in a concentration-dependent manner, with exposures to as low as 10 µg/ml chokeberry ARE (data not shown). At 50 µg/ml chokeberry ARE exposure for 24 h, the inhibition of COX-2 gene expression in HT-29 cells averaged a 32% decrease (Fig. 8A). However, this was a transient effect. With increased time of exposure up to 48 h and 72 h, the COX-2 gene in HT-29 cells showed an increased expression to almost 40%, when compared with cells grown in vehicle control media (Fig. 8A). No significant change in COX-1 gene expression was observed in HT-29 cells (data not shown). Normal colon cells, NCM460, did not show a significant change in expression of either COX-1 or COX-2 genes (data not shown). We further determined whether COX-2 protein expression and PGE₂ production in HT-29 cells exposed to ARE followed a similar pattern as COX-2 gene expression. COX-2 protein (Fig. 8B) and activity as measured by PGE₂ production (Fig. 8C) was elevated in cells treated with chokeberry ARE at all time points.

Discussion

We report that commercially available extract from chokeberry fruits, rich in anthocyanins, can inhibit the growth of colon cancer cells without affecting the growth of normal colon cells *in vitro*. We recently have determined that compared with extracts from grape and bilberry, chokeberry ARE was a potent inhibitor of HT-29 cell growth. HPLC analysis of the commercially available chokeberry ARE illustrated that it is a mixture of phenolic compounds with cyanidin-3-galactoside as the predominant anthocyanin. Almost 70% of total anthocyanins in chokeberry ARE is cyanidin-3-galactoside. The colon cancer cells, HT-29, exposed to chokeberry ARE demonstrated more than 65% growth inhibition within 24 h of exposure and high cell viability, suggesting a cytostatic inhibition. Similar cytostatic inhibition of cell proliferation and differentiation by anthocyanins was reported by Kamei et al. (10), who demonstrated that anthocyanins, such as delphinoidine, cyanidin, and pelargonidin, significantly inhibited HCT-15 intestinal cancer cell growth as compared with other flavonoids. There are few literature reports of the effects of anthocyanins or other flavonoids on growth of cells derived from normal tissue, although growth of WI-38

fibroblasts from normal lung was suppressed by anthocyanin and other flavonoids derived from petals of *Rosa* and *Chrysanthemum* (10). We demonstrated for the first time that NCM460 cells, which are epithelial cells derived from the normal colon (17), grow normally in presence of the chokeberry extract. Less than 10% growth inhibition was observed when the normal cells were exposed for as long as 48 h. This inhibition was not significant when compared with the 90% growth inhibition observed in HT-29 colon cancer cells after 48 h of exposure. Therefore, chokeberry ARE was able to specifically inhibit growth of colonic cancer cells but not normal colonic cells.

One of the mechanisms of cell growth inhibition is the alteration of movement of cells through the cell cycle. Different anthocyanins have been reported to affect the cell cycle at different phases (11,18,19). Exposed to anthocyanidins from grape rind and red rice, HCT-15 cells were arrested at the S phase (11). Anthocyanin fractions extracted from rose petals, and red and white wines, and synthetic cytostatic agents arrested the cells at various stages, including G1, S, and G2/M phases of cell cycle (18,19).

Cell cycle progression is regulated by interaction between cyclin-dependent kinases (CDKs) and cyclins, members of cell cycle regulators, that bind to and activate CDKs. Cyclin-dependent kinase inhibitors (CDKIs) also play a critical role in controlling cell cycle progression by negatively regulating the CDK activities (20–22). Two CDKIs, p21^{WAF1} and p27^{KIP1}, are related proteins, which bind to cyclin-CDK complexes and causes arrest in the G1 phase of the cell cycle (23). We evaluated the effect of chokeberry ARE on cell cycle of HT-29 and NCM460 cells using flow cytometry. Within 24 h of exposure to ARE, an increased percentage of HT-29 cells were observed in G1/G0 phase indicating a block at that cell cycle checkpoint. In our study, ARE-induced G1/G0 cell cycle arrest coincided with a marked increase in expression of p21^{WAF1} and p27^{KIP1} genes and was strongly dependent on time of exposure to ARE. The movement of the cells from the S phase into the G2/M phase appears to continue as observed by the decreasing percentage of cells in S phase. Accumulation of the ARE-exposed cells in the G2/M phase suggests that the cells are not reentering the G1/G0 phase of the cell cycle. The G2/M block can be attributed to increased inhibition of cyclin A and cyclin B1 genes, as observed in the HT-29 cells that were exposed to ARE as compared with the control cells.

Similar changes in cyclins and CDKIs have been reported in colon cancer cells in response to various phytochemicals. Downregulation of cyclin A expression and upregulation of p21^{WAF1} and p27^{KIP1} resulting in cell cycle arrest of various colon cancer cells on exposure to β-carotene, a carotenoid (24), and tangeretin, a flavonoid (25), are some examples. The increased expression of p21^{WAF1} gene due to ARE exposure can also be responsible for block at both the G1/G0 and G2/M phases of the cell cycle. The p21^{WAF1} CDKI has broad specificity and is able to inhibit G1 as well as G2-phase cyclin-CDK complexes, resulting in a block at both phases of the cell cycle

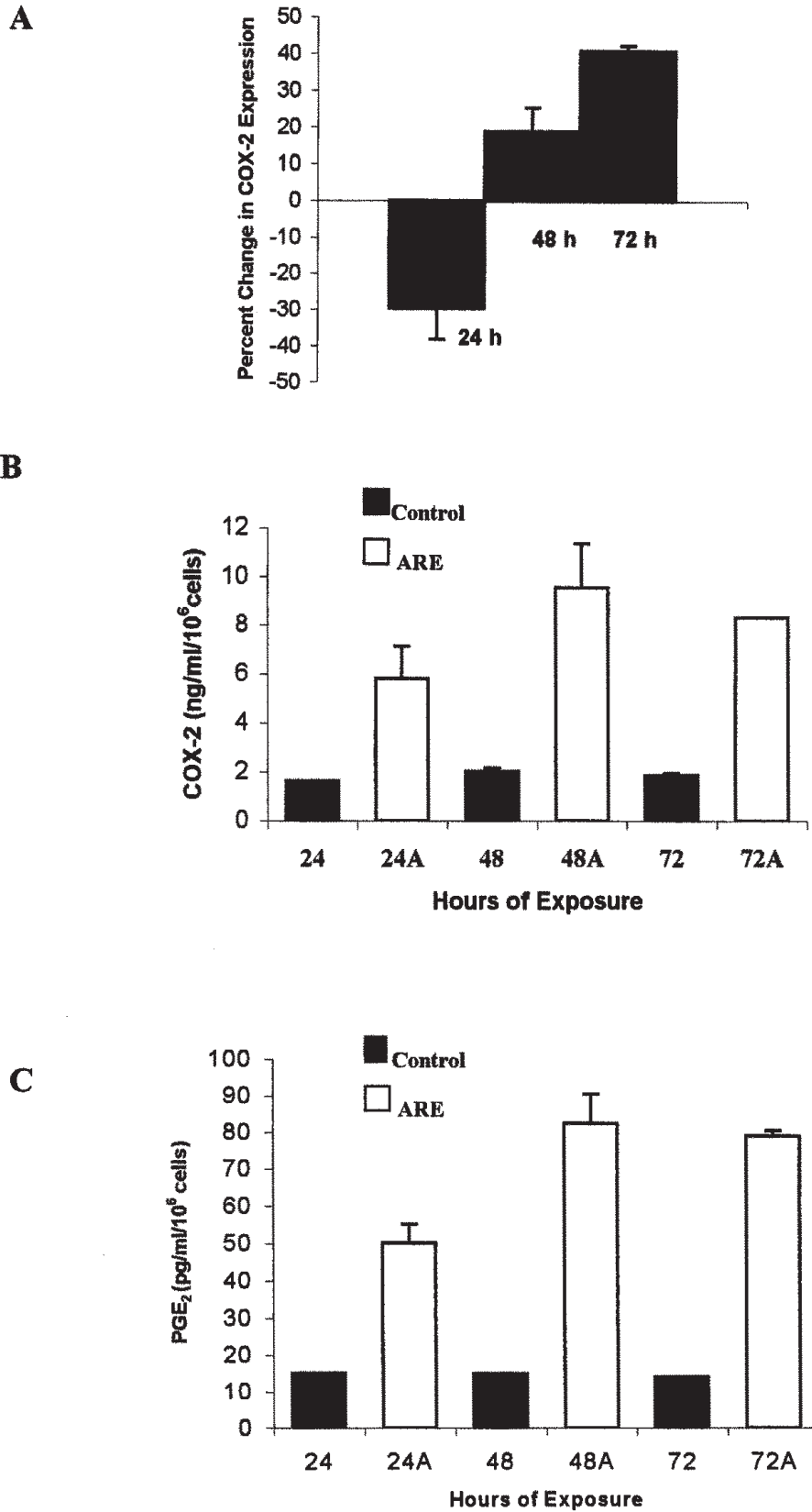


Figure 8. Effect of ARE exposure on COX-2 in HT-29 cells. A: Percentage of change in expression of COX-2 gene induced by ARE exposure in HT-29 cells. The expression of COX-2 gene was inhibited by 32% in HT-29 cells exposed to ARE (50 $\mu\text{g}/\text{ml}$) within the first 24 h. Subsequent increase in the length of exposure resulted in an increase in expression of COX-2 gene. B: Quantitative EIA determination of COX-2 protein in HT-29 cell lysates. The cells were exposed to ARE at the indicated time points, trypsinized, and counted before being lysed. C: The media from COX-2 protein experiment was analyzed for PGE₂ production. The data are presented as mean and SD of two flasks per treatment.

(26). This has been demonstrated in human prostate and mammary cancer cell lines in response to genistein (27,28). The p21^{WAF1} can be upregulated in both p53-dependent and independent manner (29), and because HT-29 cell line is known to have a mutated p53 the induction of p21^{WAF1} gene expression in these cells can be presumed to be independent of p53 status.

Dual blockage at G1/G0 and G2/M phases by chokeberry ARE-exposed HT-29 cells appears to be unique, although further identification of the active compounds in this semipurified extract is required. Interestingly, chokeberry ARE does not appear to induce apoptosis at concentrations of up to 50 µg/ml as was observed by the lack of the apoptotic peak (sub-G0) in the cell cycle analysis. Chokeberry ARE did not affect the cell cycle or expression of any of the cell cycle genes in normal colon cells which supports the hypothesis that cancer cell growth inhibition by ARE is caused by inhibition of cell cycle events.

An upregulation in the expression of COX-2 has been observed in colorectal adenomas and carcinomas with an increase of COX-2 mRNA in 86% of carcinomas compared with normal mucosa (30). Cyclooxygenase enzymes catalyze the oxygenation of arachidonic acid, leading to formation of prostaglandins. There are two isoforms of cyclooxygenase; COX-1 is constitutively expressed in all body tissues, also termed as having a "housekeeping" role. The other isoform is COX-2, which is induced, or upregulated in response to inflammation (31). There is controversy over whether a basal concentration of COX-2 gene is expressed in all tissue (32). We were able to show that cell line NCM460 derived from normal colon mucosa demonstrates a very low level of COX-2 gene expression as compared with the COX-1 gene that is constitutively expressed. On the other hand, high expression of COX-2 gene in colon cancer cell line HT-29 has been shown previously (33,34).

Numerous epidemiological studies have shown that inhibition of COX genes is linked to colon cancer prevention and drugs, especially nonsteroidal antiinflammatory drugs (NSAIDs), which inhibit the COX-2 enzyme, can delay or prevent colon cancer (35,36). Inhibition of the activity of purified cyclooxygenase enzyme, in vitro, by anthocyanin fractions isolated from different berries was reported recently (37,38). Seeram et al. (38) demonstrated that the anthocyanins cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside from raspberries and sweet cherries inactivated COX-1 and COX-2 enzymes. Therefore, we investigated whether the effect of the ARE on HT-29 cell proliferation may be caused by inhibition of cyclooxygenase expression or activity. NCM460 derived from normal epithelial cells of colon showed a basal level of COX-2 RNA that was not altered by exposure to chokeberry ARE. Comparatively, basal levels of COX-2 RNA are higher in HT-29 cells derived from colon adenocarcinoma. The difference in basal levels of cyclooxygenase expression of the two cell lines supported the hypothesis that the different growth response of the two cell lines to chokeberry ARE may be through a COX-dependent mechanism. However, further experimentation illustrated that although 24 h exposure to

ARE decreased the level of COX-2 mRNA in HT-29 cells, this decrease appeared to be a transient effect. An increase in the level of COX-2 gene expression was observed with increased time of exposure of the HT-29 cells to chokeberry ARE. Interestingly, the levels of COX-2 protein and PGE₂ were significantly increased in cells treated with ARE, indicating that the mechanism of growth inhibition was not caused by COX-2 inhibition. The significance of the increased COX-2 activity in the HT-29 cells is not clear at this time. Although anthocyanins may inhibit the activity of purified COX enzymes in vitro (38), the growth inhibition of HT-29 cells by the chokeberry anthocyanin-rich extract does not involve suppression of COX-2 activity and may act in a COX-independent manner. It has been observed that NSAIDs like celecoxib also affect growth of colorectal cancer cells in a COX-independent manner (39), and it is suggested that the mechanism may involve the peroxisome proliferator-activated receptor (PPAR) family of nuclear hormone receptors, inhibition of the NF-κB pathway, or expression of the cell cycle genes (40,41).

Conclusion

In conclusion, anthocyanin-rich extract from chokeberry specifically inhibited the growth and cell cycle progression in colon carcinoma cells mainly through upregulation of CDKIs, p21^{WAF1} and p27^{KIP1}, and downregulation of cyclin A and cyclin B1. Little to no effect of the ARE was observed on the growth of normal colon cells. Additional studies investigating ARE-mediated changes in expression and activity of genes involved in cell cycle are under way. We are also investigating the effect of cyanidin-3-galactoside, the major anthocyanin found in the extract of chokeberry ARE, on growth and cellular mechanisms of colon cancer cell lines.

A survey by the National Academy of Sciences (42) estimated the consumption of the anthocyanin-containing natural colors to be ~70 mg/kg/day. In addition, synthetic FD&C Red No. 40 color consumption was ~127 mg/kg/day. It is possible that if anthocyanins were to completely replace the use of synthetic red dyes, the total daily consumption may be in the range of 14 g/day for a 700-kg person. Therefore, our findings that these compounds have chemopreventive activity are significant and warrant further investigation.

Acknowledgments and Notes

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