The Anthocyanin Metabolites Gallic Acid, 3-O-Methylgallic Acid, and 2,4,6-Trihydroxybenzaldehyde Decrease Human Colon Cancer Cell Viability by Regulating Pro-Oncogenic Signals

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INTRODUCTION

Diets rich in anthocyanins, found in many plants and foods, can inhibit growth of colorectal cancer (CRC) cells in vitro and tumorigenesis in rodent models [1–5]. The in vivo effects of anthocyanins are most likely due to microflora metabolites produced in the gut [6,7]. Anthocyanins are poorly absorbed in the gastrointestinal tract [8] and are largely metabolized by gut microflora to several metabolites including 3-O-methylgallic acid (Megal), gallic acid (Gal), syringic acid, vanillic acid, protocatechuic acid, and 2,4,6-trihydroxybenzaldehyde (THBA) [6,7,9,10]. Among them, it was previously found that Megal, Gal, and THBA are effective in reducing colon cancer cell (Caco-2) viability [11]. The underlying mechanisms of these effects are not fully understood.

A aberrant activation of select transcription factors (e.g., nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and signal transducer and activator of transcription (STAT-1)) that regulate the expression of proteins that promote cell survival, and regulate the cell cycle are often found in cancer. NF-kB is proposed to play a central role in carcinogenesis, regulating the expression of proteins that modulate cell proliferation, survival, angiogenesis, and metastasis [12–14]. STAT-1 has various physiological roles depending on the cell type; yet its over-activation plays a role in cancer cell survival [15–18]. Activator protein 1 (AP-1), a redox sensitive transcription factor [19], can also be pro-oncogenic by promoting cell proliferation and survival [20,21]. Anthocyanins have been shown to inhibit these transcription factors [22,23], yet it is not known if gut microflora metabolites can also exert these inhibitory actions. Of the three anthocyanin metabolites studied in this work, Gal has been widely...
investigated as a natural product for cancer treatment and has been reported to inhibit cell proliferation and induce apoptosis in CRC cells [24–29]; yet there is little mechanistic information about how Gal affects apoptosis and cell proliferation. Furthermore, although we previously showed that Megal, Gal, and THBA decrease CRC cell viability, the involved mechanisms are unknown.

Using physiologically relevant concentrations [30], this work investigated the anticancer effects of Megal, Gal, and THBA in Caco-2 cells as a model of CRC cells. The effects of the metabolites on cell viability were also investigated in other human CRC cells (HCT-116, SW-480, HT-29, and HCT-15). The mechanisms underlying the reduction of Caco-2 cell viability by Megal, Gal, and THBA were studied, including their capacity to regulate the cell cycle and apoptotic cell death. The capacity of these compounds to modulate cell signals with known pro-oncogenic action was investigated.

MATERIALS AND METHODS

Materials

Caco-2, HCT-116, SW-480, HT-29, and HCT-15 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MA). Cell culture media and reagents were from Invitrogen Life Technologies (Carlsbad, CA), and INCCELL (San Antonio, TX). The CellTiter-Glo Luminiscent Cell Viability assay, the oligonucleotides containing the consensus sequence for NF-κB, AP-1, and octamer 1 (OCT-1), and the reagents for the electrophoretic mobility shift assay (EMSA) were from Promega (Madison, WI). The protease inhibitor cocktail was from Roche Applied Science (Mannheim, Germany). The oligonucleotide containing the consensus sequence for STAT-1, and the antibodies for baculoviral IAP repeat-containing protein 3 (c-IAP2; sc-7944), cyclin D1 (sc-718), and β-tubulin (sc-9104) were from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody for bcl-2 (2876) was obtained from Cell Signaling Technology (Beverly, MA). PVDF membranes were obtained from Bio-rad (Hercules, CA) and Chroma Spin-10 columns were obtained from Clontech (Palo Alto, CA). The ECL Western blotting system was from GE Healthcare (formerly Amersham Pharmacia Biotech, Inc., Piscataway, NJ) (Piscataway, NJ). Megal was purchased from Extrasynthese (Genay, France), malvidin-3-glucoside (M3g) was from ChromaDex (Irvine, CA), and THBA was from Alfa Aesar (Ward Hill, MA). Catalase, Gal, propidium iodide, RNase A, and all other reagents were of the highest quality available and were purchased from Sigma (St. Louis, MO).

Cell Culture

Caco-2 cells were cultured in minimum essential medium (MEM) supplemented with 20% (v/v) fetal bovine serum (FBS). HCT-116 and HT-29 cell growth medium consisted of McCoy's 5A supplemented with 10% (v/v) FBS. SW-480 and HCT-15 cell growth medium consisted of RPMI supplemented with 10% (v/v) FBS. All media were supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin).

Stock solutions (1 mM) of the tested compounds (Megal, Gal, THBA, and M3g) were freshly prepared and sterile filtered (0.22 μm filter) before their addition to the medium at final concentrations of 10, 25, 50, and 100 μM. Control and treatment media were replaced every 24 h for the duration of the experiment. THBA was solubilized in DMSO, but DMSO concentration in the medium never exceeded 0.1% (v/v). Controls treated with the highest concentration of DMSO used were run in parallel. Cell viability was similar for control cells incubated with or without DMSO, and in the absence of metabolites.

Cell Viability Assay

The effect of Megal, Gal, THBA, and M3g on cell viability was determined by CellTiter-Glo Luminiscent Cell Viability Assay which measures cellular ATP levels. Briefly, cells (5,000 cells/well) were plated in 96-well plates. The following day they were incubated in the absence or in the presence of 10–100 μM of metabolites for different periods of time (24–72 h). For Caco-2 cell differentiation, cells were seeded as described, allowed to differentiate for 12 d after confluence, and then treated with the metabolites for 72 h. Caco-2 cells were also incubated with the different compounds (100 μM), with or without catalase (135 U/ml media).

Caspase-3 Assay

Cells were seeded (200,000 cells/well) in 6-well plates. After 24, 48, and 72 h of treatment (metabolite final concentration of 50 μM), cells were harvested and pellets were frozen at –80°C until analysis. Cells were lysed prior to analysis and lysates were analyzed for caspase-3 activity using a fluorescence-based kit (Clontech; CA) following the manufacturer's protocol. All readings were normalized for protein content.

DNA Fragmentation

Cells were seeded (5000 cells per well) in 96-well plates. After 48 h of treatment (metabolite final concentration of 50 μM), cells were lysed in the plate, and oligonucleosomes were quantified by an ELISA based assay (Roche, Switzerland) following the manufacturer's protocol. All readings were normalized for cell viability.

Evaluation of Cell Cycle Progression

To synchronize Caco-2 cells, they were serum-starved for 48 h in MEM medium containing 0.1% (v/v) FBS. Re-entry into the G0/G1 phase of the cell cycle was initiated by replacement of the starvation
medium with complete medium containing 50 μM Megal, Gal, and THBA. After 24, 48, and 72 h, cells were collected, washed with PBS, and fixed with 70% (v/v) methanol. At least 3000 propidium iodide-stained nuclei were analyzed by flow cytometry and the percentages of cells in the G0-G1, S, and G2-M phases of the cell cycle were determined by using the Mod Fit LT cell cycle analysis software (Verity Software, Topsham, ME).

Western Blot Analysis

After 24, 48, and 72 h incubation in the absence or presence of 50 μM metabolites with and without addition of catalase (135 U/ml), nuclear fractions were isolated from cells (1 x 10^6 cells/100 mm dish) as previously described [31]. For the Western blot, the oligonucleotides containing the consensus sequences for NF-κB, STAT1, AP-1, and OCT-1, were end labeled with [γ-32P] ATP using T4 polynucleotide kinase, and purified using Chromaspin-10 columns. Samples were incubated with the labeled oligonucleotide (20 000–30 000 cpm) for 20 min at room temperature in 1× binding buffer (10 mM Tris–HCl buffer, pH 7.5, containing 4% (v/v) glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 0.05 mg/ml poly(dI–dC). In some cases, control samples were also incubated with cold oligonucleotides for competitive binding. The products were separated by electrophoresis in a 6% (w/v) non-denaturing polyacrylamide gel using TBE (45 mM Tris/borate, 1 mM EDTA, pH 8.3) as the running buffer. The gels were dried and the radioactivity was quantified in a Phosphoimager 840 (Amersham Pharmacia Biotech Inc.).

Electrophoretic Mobility Shift Assay (EMSA)

After the corresponding treatments (72 h incubation in the absence or presence of 50 μM metabolites) with and without addition of catalase (135 U/ml), nuclear fractions were isolated from cells (1 x 10^6 cells/100 mm dish) as previously described [31]. As the running buffer, the gels were dried and the percentages of cells in the G0-G1, S, and G2-M phases of the cell cycle were determined by using the Mod Fit LT cell cycle analysis software (Verity Software, Topsham, ME).

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Newman–Keuls posttest using Graph Pad software package version 5 (La Jolla, CA). A P-value < 0.05 indicates treatments are statistically different compared to controls. Data are shown as mean ± SEM.
in Caco-2 cells given that they are highly susceptible to the effects of both Gal and Megal. Furthermore, they have a well characterized capacity to differentiate into a functional intestinal epithelium, which allowed us to assess the effects of the metabolites both in dividing (CRC model), and differentiated (model of normal intestinal epithelium) cells.

**Effects of Megal, Gal, and THBA on Caco-2 Cell Apoptotic Death**

The observed decrease in cell viability could be due to the capacity of Megal, Gal, and THBA to induce apoptotic cell death or decrease cell proliferation. Apoptosis was evaluated measuring caspase-3 activity and DNA fragmentation. Results from the caspase-3 assay are shown in Figure 4A. The time point that showed the greatest induction of caspase-3 activity was 24 h, yet the effect was only significant ($P < 0.05$). Apoptosis was next evaluated by measuring DNA fragmentation (Figure 4B). This is one of the final steps of apoptosis and is carried out by endonucleases [32]. A significant increase in mono and oligonucleosomes was observed after 48 h of incubation with Megal and Gal treatments, but not with THBA.

**Effects of Megal, Gal, and THBA on Cell Cycle Progression**

The capacity of Megal, Gal, and THBA to inhibit cell proliferation by affecting the cell cycle was next investigated. The Caco-2 cell cycle distribution was analyzed by flow cytometry. Incubation with Megal, Gal, and THBA (Figure 5) caused a significant ($P < 0.05$) arrest of the cell cycle at the G0/G1 checkpoint after 72 h of incubation. Additionally, Gal significantly ($P < 0.05$) increased the fraction of cells in S-phase by 16% after 72 h of treatment. The earlier time points did not show major differences among treatments, given that the doubling time for Caco-2 cells is approximately 62 h [33].
Effects of Megal, Gal, and THBA on the Activation of Transcription Factors NF-κB, AP-1, STAT-1, and OCT-1

We next evaluated the effects of Megal, Gal, and THBA on the activation of transcription factors that regulate the expression of genes involved in cell proliferation and survival. NF-κB, AP-1, STAT-1, and OCT-1 activation was evaluated by EMSA. The incubation of cells with Megal, Gal, and THBA for 72 h caused an 88, 71, and 53% decrease in nuclear NF-κB-DNA binding, respectively (Figure 6).

Although to a lesser extent to that observed for NF-κB, the metabolites also inhibited AP-1-DNA binding. After 72 h of incubation, Megal and Gal caused a 27%, 23%, and 38% decrease in AP-1-DNA binding, respectively (Figure 6). After 72 h of incubation, Megal and Gal also caused a 17% and 30% decrease in nuclear STAT-1-DNA binding, respectively, while THBA had no effect (Figure 6). OCT-1 is another transcription factor that is over expressed in intestinal carcinomas [34]. Gal and THBA significantly decreased (P < 0.05) nuclear OCT-1-DNA binding by 34% and 37%, respectively, while the effect of Megal was not significant (Figure 6). The simultaneous incubation with catalase (135 U/ml) did not alter the effects of the metabolites on NF-κB, AP-1, and STAT-1 activation (data not shown).

We next investigated the expression of NF-κB-regulated proteins involved in the promotion of cell survival (B-cell lymphoma 2 (Bcl-2) and c-IAP2) and in the regulation of the cell cycle (cyclin D1). After incubation, Megal and THBA had no effect on expression of these proteins, while Gal caused a significant decrease in cyclin D1 protein levels as measured by Western blot (Figure 7).

**DISCUSSION**

This work demonstrates that select anthocyanin metabolites (Megal and Gal) have the capacity to decrease CRC Caco-2 cell viability. This decrease was mostly due to the capacity of Megal and Gal to inhibit the cell cycle at the G0/G1 phase. The capacity of these compounds to inactivate transcription factors NF-κB, AP-1, STAT-1, and OCT-1 could in part mediate the anti-carcinogenic actions of Megal and Gal. Importantly, all these effects are observed at metabolite concentrations similar to those found in human feces, a direct indicator of concentrations in...
the colon, after consuming blood orange juice containing 71 mg of cyanidin-glucosides [35] and raspberry puree containing 40.3 mg total anthocyanins [36]. Similar levels are also found in subjects without any dietary restrictions or experimental diets [30], indicating these metabolites may be produced after consuming a wide range of dietary polyphenols. Anthocyanins in particular are poorly absorbed into the bloodstream and largely pass through the intestinal tract [8], where they are metabolized by colonic microbiota to the studied metabolites (Megal, Gal, and THBA) [6,7,9]. Some of these metabolites, including gallic acid, have been shown to have anti-cancer effects in CRC cells [11,25,26,29] and reduce the tumor load in animal models of intestinal carcinogenesis [37,38].

While Megal and Gal caused a dose-dependent decrease of Caco-2 cell viability, THBA was only effective at the highest and non-physiological concentration tested. Megal and Gal were not only effective in Caco-2 cells, but also decreased cell viability in four other cell lines derived from human colorectal cancers. Although Caco-2 cells are a suitable model of colonic cancer when they are actively dividing, when reaching confluence Caco-2 cells differentiate into a colonic epithelium. Of high relevance to cancer treatment, Megal and Gal did not affect the viability of differentiated Caco-2 cells. This finding also suggests that the most important mechanism of action of these compounds would be regulating CRC cell proliferation rather than promoting cell death.

We assessed the potential effects of the metabolites on cell proliferation by evaluating the cell cycle. Cell cycle analysis showed that all three anthocyanin metabolites increased the percentage of cells in the G0/G1 phase. Cyclin D1 regulates the G0/G1 checkpoint of the cell cycle. We observed that Gal caused a significant decrease in cyclin D1 protein levels, while a trend for lower values was observed for Megal and THBA. Cyclin D1 was previously found to be inhibited by tea polyphenols in a mouse lung cancer model, which was linked to the observed reduction in lung lesion cell proliferation [39]. Furthermore, hydroxytyrosol, a major phenolic component of olive oil, can inhibit cell proliferation and decrease cyclin D1 in Caco-2 cells causing cell cycle arrest at the G2/M phase [40]. Treatment with Gal also induced a significant increase in the fraction of cells in S-phase, suggesting that the metabolites could affect cell proliferation at other phases of the cell cycle. Interestingly, Bilodeau et al. [41] previously found that oxidative stress can induce arrest in S-phase which may be an important mechanism underlying our findings.

Caspase-3 activation and oligonucleosome formation results indicate that Megal and Gal may also induce apoptosis in Caco-2 cells. As this parallels
the cell viability results, this suggests that the mechanism of reducing Caco-2 cell viability is partially related to the induction of apoptosis. While initially THBA activated caspase-3, the lack of apoptotic evidence from an increase in DNA breakage indicates that cells treated with THBA do not reach the end stages of the apoptotic process. Accordingly, the expression of the anti-apoptotic proteins Bcl-2 and c-IAP2 were not affected by the metabolites. Thus, evidence noted above from the cell cycle analysis suggests that THBA inhibits cell proliferation as a primary mechanism for a decrease in cell viability.

Induction of apoptosis and inhibition of cell proliferation, and the observed alterations in the expression of cyclin D1 could be in part due to a down regulation of the pro-proliferative and anti-apoptotic transcription factors NF-κB, AP-1, STAT-1, and OCT-1. NF-κB is constitutively active in colon cancer cells, and downregulation of this factor has been shown to inhibit cell proliferation and induce apoptosis in cell models [12]. All three metabolites significantly inhibited the nuclear binding of NF-κB to its consensus sequence. With the strong inhibition of proliferation by both Gal and Megal, and weaker inhibition by THBA, and those effects mirrored in the inhibition of NF-κB activation, this mechanism appears to be the best explanation of the effect of the metabolites on Caco-2 cells, and thus perhaps of the reduction in colon cancer by anthocyanin consumption [1,4,5,42]. In humans, the consumption of ethanolic beverages containing polyphenols inhibit NF-κB in circulating mononuclear cells [43]. Epigallocatechin gallate (EGCG), which has similar redox capabilities compared to Megal and Gal by virtue of its catechol groups, can inhibit NF-κB and cause growth arrest in G0/G1 phase in cancer cells, while having no effect in normal cells [44].

To a lesser extent, inhibitory actions of the anthocyanin metabolites were also observed for AP-1, STAT-1, and OCT-1. AP-1 regulates numerous genes involved in inflammation and proliferation [21], including regulation of cell cycle proteins [45]. STAT-1 is a pro-apoptotic and anti-proliferative transcription factor [15–18], and OCT-1 is transcription factor that is over expressed in intestinal carcinomas [34]. Due to the role of these transcription factors in cell proliferation and apoptosis the above evidence indicates that the anthocyanin metabolites could exert anticarcinogenic actions through the modulation of multiple signaling pathways, but in particular of NF-κB.

Reactive oxygen species have been shown to induce apoptosis in colon cancer cells. Isuzuwaga et al. [46] showed that gallic acid generates hydrogen peroxide, when incubated with hepatocytes. They showed that cells with high amounts of catalase were less affected by Gal treatment, than cells with low amounts of catalase. Our results suggest that these anthocyanin metabolites may induce their effects by at least a partial involvement of oxidants. While hydrogen peroxide production seems to be partially responsible for the activity of Megal, it is largely responsible for the observed inhibition by Gal of CRC cell survival, given that a reduced effect was observed upon co-incubation with catalase.

In conclusion, Megal and Gal both induce apoptosis and inhibit cell proliferation, in part through oxidant-mediated mechanisms. These metabolites also regulate signaling pathways (NF-κB, AP-1, STAT-1, and OCT-1) controlling cell fate. The studied metabolites have a particular inhibitory action on NF-κB, which regulates the transcription of genes involved in the regulation of the cell cycle and in apoptosis. The potential involvement of oxidant species requires further investigation.

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REFERENCES


