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Up-regulation of tumor suppressor carcinoembryonic antigen-related cell adhesion molecule 1 in human colon cancer Caco-2 cells following repetitive exposure to dietary levels of a polyphenol-rich chokeberry juice

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Abstract

Consumption of berries and red fruits rich in polyphenols may contribute to the reduction of colon cancer through mechanisms not yet understood. In this study, we investigated the response of subconfluent Caco-2 cells (a human colon carcinoma model) to repetitive exposure (2 h a day for a 4-day period) of a subtoxic dose of a chokeberry (*Aronia melanocarpa*) juice containing mixed polyphenols. To mimic physiological conditions, we subjected the chokeberry juice to in vitro gastric and pancreatic digestion. The effects on viability, proliferation and cell cycle were determined, and changes in the expression of genes in response to the chokeberry treatment were screened using Affymetrix oligonucleotide microarrays. Exposure to the chokeberry juice inhibited Caco-2 cell proliferation by causing G_2/M cell cycle arrest. We detected changes in the expression of a group of genes involved in cell growth and proliferation and cell cycle regulation, as well as those associated to colorectal cancer. A selection of these genes was further confirmed by quantitative RT-PCR. Among these, the tumor suppressor carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1*), whose expression is known to be reduced in the majority of early adenomas and carcinomas, was up-regulated by the treatment both at the mRNA and protein levels (as shown by flow cytometry analysis). CEACAM1, with a significant regulatory role on cell proliferation of particular interest at early stages of cancer development, may be a potential target for chemoprevention by food components such as those present in polyphenol-rich fruits.

Keywords: Caco-2; Colon cancer; Polyphenols; Chokeberry; Gene expression; CEACAM1

1. Introduction

Colorectal cancer is one of the leading causes of cancer death in developed countries [1]. The complex sequence of events occurring during initiation, development and propagation of tumors is likely the result of lifelong accumulation of a series of mutations [2]. In addition, the modern human diet, which regularly delivers a complex mixture of cancer-promoting agents and inhibitors to the gastrointestinal tract, may exert an important modulating role in the development and progression of this disease [3]. Epidemiological and animal studies have shown a strong association between the consumption of fruits and vegetables and a reduction of the incidence of cancer of the alimentary tract [4], but the mechanisms are not yet fully understood. Various agents in plant foods, such as fiber, vitamins, minerals, folates and

Abbreviations: BMP2, bone morphogenetic protein 2; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; EEF1D, eukaryotic translation elongation factor 1 delta; FGFR2, fibroblast growth factor receptor 2; FITC, fluorescein isothiocyanate; FOX, $FeSO_4$/xylenol orange; GNA13, guanine nucleotide binding protein (G protein) alpha 13; H2AFO, H2A histone family, member O; HNRPA1, heterogeneous nuclear ribonucleoprotein A1; ID1, inhibitor of DNA binding 1, dominant negative helix–loop–helix protein; IFNAR1, interferon (alpha, beta and omega) receptor 1; IL6ST, interleukin 6 signal transducer (oncostatin M receptor); ITGA2, integrin alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor); PBS, phosphate-buffered saline; PLK2/SNK, polo-like kinase 2; POLR2L, polymerase (RNA) II (DNA-directed) polypeptide L, 7.6 kDa; S100A4, S100 calcium binding protein A4 (metastasin); SLC7A8/LAT2, solute carrier family 7 (cationic amino acid transporter, y+ system), member 8.

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polyphenols or most likely a combination of all of them, may play a role in the anticancer properties of these foods [5]. Considerable attention has focused on the anticancer properties of dietary polyphenols, and their beneficial effects have been reported in a plethora of in vitro and animal studies [6]. Also, bioavailability studies in animals and humans have shown that only a small proportion of dietary polyphenols are absorbed but that substantial levels of these compounds remain in the lumen, and thus, cancers of the alimentary tract are potentially the most susceptible to modulation by these dietary components [7].

Among other fruits, berries and red fruits are important dietary sources of polyphenols such as anthocyanins and procyanidins [8]. A number of studies have reported the antiproliferative effects of berry extracts, berry fractions and anthocyanins or procyanidins on human colon and oral cancer cells [9–14]. A chemopreventive role for berries or derived polyphenols extracts has also been shown in animal models [15–17]. However, the specific components in the fruit and the molecular mechanisms by which this may occur remain poorly understood. Many reported chemopreventive effects of polyphenols are based on in vitro cell assays that have studied single compounds at relatively high doses and for extended periods of up to 72 h [9–12]. Although these studies are important to unravel the potential mechanisms involved, the experimental conditions are unlikely to happen in vivo. Polyphenols are normally ingested as mixtures of different compounds immersed in a complex food matrix that undergoes a digestion process as it progresses along the gastrointestinal tract. Epithelial cells lining the gut are therefore more likely to be exposed frequently to these digested polyphenol-rich foods for a reduced number of hours than to single compounds for long periods.

It has been hypothesized that dietary polyphenols or their derived digestion products may exert some of their chemopreventive effects on the gut epithelium by direct or indirect interaction with cellular components such as membrane receptors or transcription factors, altering the expression of groups of genes that may be related to key cellular processes such as cell growth and proliferation or tumor formation [18]. Transcriptional profiling using DNA microarrays becomes a very useful tool to conduct a broad survey of the changes in gene expression that accompany specific cellular responses such as those triggered by food or food components on cells of the gastrointestinal tract and to identify gene products of potential significance that can be further examined in relation to chemoprevention. In this study, we used subconfluent Caco-2 cells (as a model of human colon cancer) to investigate the transcriptional changes that followed exposure of the cells to a subtoxic repetitive dose (2 h a day for a 4-day period) of chokeberry (*Aronia melanocarpa*) juice with high content of polyphenols. Chokeberry originates from North America and has been extensively cultivated in Denmark, Eastern Europe and Russia. It is widely used in the food industry either on its own or blended with other fruits (e.g., juice and soft drink making, wine production, food coloring, natural health products). Chokeberry was selected for this study because of its high content of antioxidants and polyphenols (mostly anthocyanins and procyanidins [8]) and because of its antiproliferative and anticarcinogenic effects on human colon cancer cells [10,12]. To mimic more physiological conditions, we subjected the chokeberry juice to successive in vitro gastric and pancreatic digestion. The effects of the treatment on viability, proliferation and cell cycle were determined, and gene expression changes were studied using oligonucleotide microarrays (Affymetrix) and quantitative RT-PCR. Changes in gene expression are reported, and findings are discussed in relation to previous published data on the gene expression profile of human colon cancer cells and on its modulation by dietary components. Our aim was to gain additional insight into the possible mechanisms by which polyphenol-rich fruits may reduce the risk for colorectal cancer and to identify possible related targets.

2. Materials and methods

2.1. Chokeberry juice

Frozen chokeberry (*A. melanocarpa*) commercial concentrate (1.3 g ml^{-1}) was kindly provided by the juice manufacturing company Juver Alimentación S.A. (Murcia, Spain). The chokeberry concentrate was diluted (1:5) with ultrapure water (Millipore) to obtain a juice with a soluble solids content of ~15°Brix (20°C) and a pH of 3.4.

2.2. In vitro digestion

The chokeberry juice was subjected to successive in vitro gastric and pancreatic digestion as described previously [19]. Briefly, the chokeberry juice was digested with a mixture of pepsin–HCl (pH 2.0) for 2 h (to simulate gastric digestion) followed by a 2.5-h digestion with pancreatin and bile salts (pH 7.5). The incubation was slightly modified as follows: the digestion mixtures were incubated in a flask without the use of a dialysis membrane. After addition of the salts and enzymes, the flasks were flushed with N_2 and were kept closed in the absence of light during incubation at 37°C with shaking. Control samples were run in parallel and consisted of an equivalent volume of ultrapure water subjected to the same in vitro digestion (contains only the mix enzymes+ salts). Immediately after digestion, aliquots of the digested chokeberry were stabilized by acidification (pH 2.0), filtered (0.45 μm) and analyzed via HPLC-diode array–MS–MS [8] to determine the quantity of polyphenols that was added to the culture medium. The stability of the chokeberry polyphenols in the culture media during several hours of incubation was also monitored using the HPLC-diode array.

2.3. Cell line and culture conditions

Human colon cancer Caco-2 cells were grown in EMEM medium supplemented with 10% v/v fetal bovine serum, 1% v/v nonessential amino acids, 1% v/v L-glutamine and

1% v/v antibiotic solution (penicillin, streptomycin; Gibco) at a final pH of 7.2–7.4. Cells were cultured in 10-cm dishes or 6-well plates and maintained at 37°C in an incubator under a 5% CO_2/95% air atmosphere at constant humidity. Cells were routinely tested and found to be negative for mycoplasm contamination. The osmolality of the culture medium was measured using a vapor pressure osmometer 5520 (VAPRO Wescor). The pH of the culture medium was determined using pH indicator paper (Neutralit, pH 5.5–9.0, Merck) inside the incubator.

For the experiments, Caco-2 cells (Passage 25 after recultivation from −80°C stocks) were seeded at 10^4 cells cm^{-2} density, allowed to adhere for 48 h and used on Day 3 after seeding (subconfluent homogeneously undifferentiated cells as a model of colon cancer cells). Digested chokeberry and control samples were freshly prepared, analyzed and filter sterilized (0.2 μm) prior to addition to the culture media. Medium was replaced by fresh medium containing the digested chokeberry juice (at selected concentrations) or the equivalent control sample. In order to simulate repetitive doses, more in consonance with a regular consumption of this fruit, we applied this treatment to cells 2 h daily for 4 consecutive days. After the 2-h incubation period, the medium was replaced again by a fresh medium. Untreated cells were also run in parallel and subjected to the same changes of medium.

2.4. Cell proliferation and viability tests

At the end of 4 days of treatment, trypsinized cells (2.5 g L^{-1} trypsin, 0.2 g L^{-1} EDTA) were suspended in culture medium, counted using a hemacytometer and viability measured using Trypan blue dye exclusion. Results of proliferation and viability in control and chokeberry-treated cells are expressed as percentage of those values obtained for untreated cells. Data are presented as mean values±S.D. from four independent experiments ($n=3$ plates per experiment).

2.5. Measurement of H_2O_2

The concentration of H_2O_2 in the culture medium was determined using ferrous iron oxidation in xylenol orange (the FOX assay) according to the procedure described by Nourooz-Zadeh [20]. The H_2O_2 concentration was calculated from a standard curve with H_2O_2 in the concentration range 0–200 μM. Data are presented as mean values±S.D. from three independent experiments ($n=3$ plates per experiment). Catalase (Sigma, EC 1.11.1.6. bovine liver, 728 U μl^{-1}) was used to revert and confirm the formation of H_2O_2.

2.6. Cell cycle analysis using flow cytometry

Cells (2×10^5) were collected after the 2-h daily treatment during the 4-day experimental period, fixed in ice-cold ethanol/phosphate-buffered saline (PBS) (70:30) for 30 min at 4°C, further resuspended in PBS containing 100 μg ml^{-1} RNAse and 40 μg ml^{-1} propidium iodide and incubated at 37°C for 30 min. DNA content (20,000 events) was analyzed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson) for instrument control and data acquisition. The coefficient of variation according to the FACStation Cell Quest software was always less than 10%. Data are presented as mean values±S.D. of at least three independent experiments for untreated, control and chokeberry-treated cells. Where indicated, comparisons were carried out using the Student's t test.

2.7. RNA isolation

After exposure to the digested chokeberry juice (2% or 5%) or to the control enzyme mix, Caco-2 cells were directly lysed in the plates, and total RNA was isolated using an RNeasy mini kit (Qiagen). RNA was eluted in RNAse free water, aliquoted and stored at −70°C. RNA concentration was calculated from Abs at 260 nm. The integrity of the ribosomal RNA was checked using agarose gel electrophoresis (1%). Protein contamination was further assessed by spectrophotometric determination of the $Abs_{260/280}$ ratio, and only samples with a ratio between 1.8 and 2.1 were used in further experiments.

2.8. Screening of mRNA by oligonucleotide microarrays from Affymetrix

A preliminary search for potential candidate genes for which transcription levels may be altered following exposure to the digested chokeberry was performed using microarray analysis. We created a control and a treated (2% chokeberry) 'pooled' group by mixing the RNA from four separate RNA preparations, each from an independent dish of cells. The quality of the resulting pooled RNA was further tested using the Agilent Bioanalyzer 2100 (Agilent Technologies). Five micrograms of total RNA was used for double-stranded cDNA synthesis and generation of biotin-labeled cRNA, following the manufacturer's one-cycle protocol (Affymetrix). The size distribution of biotin-labeled cRNAs was assessed using the Agilent Bioanalyzer 2100 prior to fragmentation and hybridization onto human Affymetrix HG-U133A 2.0 GeneChip arrays. Fluorescence intensity for each chip was captured with a laser GeneChip Scanner 3000. Affymetrix Gene Chip Operative software with the Expression Default Settings was used for data acquisition and quantification. Data were evaluated as follows: the signal intensities from control and treated arrays were global scaled to an average signal value of 200, and comparison analysis was performed using signals from control samples as baseline. For each probe set, a signal ratio quantifying the change and a change call (increase, marginal increase, not change, marginal decrease or decrease) reflecting the statistical probability and orientation of each change were generated. Only probe sets with changes classified as 'increase' or 'decrease' were used in future analysis. Those probe sets detected as 'absent' or 'marginal' in both control and treated cells were eliminated.

Signal ratios were $\log_2$ transformed and used to calculate fold change (treated vs. control). Genes showing changes in expression ≥ 1.5-fold or ≤ 1.5-fold were selected for further analysis (because modest changes in gene expression may have biological significance depending on the basal expression of the gene). Selected genes were categorized into different functional groups based on gene ontology search using Fatigo, GEPAS 1.1 (http://www.fatigo.org). These categories were further refined based upon the bioprocess and molecular functions described in the annotation tables available for each target at SOURCE (http://source.stanford.edu/cgi-bin/source/sourceSearch) and from existing literature. Genes that were not annotated or not easily classified were excluded.

2.9. Validation of gene expression by quantitative RT-PCR

Changes in the expression of 15 selected genes were assessed by one-step quantitative RT-PCR (Taqman system, Applied Biosystems, ABI) in the same RNA extracted for microarrays and in further independent experiments both at 2% and 5% chokeberry concentrations using Caco-2 cells from Passage 25 and Passage 22 (to determine possible variations at a different passage number). Amplification was performed using a total reaction volume of 25 μl in a MicroAmp Optical 96-well reaction plate covered by optical adhesive covers. Real-time reactions were carried out using TaqMan Universal Master Mix. Primers and probes for each gene were selected from those assays-on-demand available from ABI [carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1*), Hs00236077_m1; fibroblast growth factor receptor 2 (*FGFR2*), Hs00240796_m1; polymerase (RNA) II (DNA-directed) polypeptide L, 7.6 kDa (*POLR2L*), Hs00360764_m1; S100 calcium binding protein A4 (metastasin) (*S100A4*), Hs00243201_m1; integrin alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor) (*ITGA2*), Hs00158127_m1; heterogeneous nuclear ribonucleoprotein A1 (*HNRPA1*), Hs01656228_s1; interferon (alpha, beta and omega) receptor 1 (*IFNAR1*), Hs00265057_m1; interleukin 6 signal transducer (oncostatin M receptor) (*IL6ST*), Hs00174360_m1; solute carrier family 7 (cationic amino acid transporter, y+ system), member 8 (*SLC7A8*), Hs00202051_m1; inhibitor of DNA binding 1, dominant negative helix–loop–helix protein (*ID1*), Hs00357821_g1; bone morphogenetic protein 2 (*BMP2*), Hs00154192_m1; polo-like kinase 2 (*PLK2*), Hs00198320_m1; eukaryotic translation elongation factor 1 delta (*EEF1D*), Hs00260723_m1; guanine nucleotide binding protein (G protein) alpha 13 (*GNA13*), Hs00183573_m1; H2A histone family, member O (*H2AFO*), Hs00358508_s1]. The one-step real-time RT-PCRs were run on the ABI 7500 Real Time RT-PCR with the following thermal conditions: 48°C for 30 min for reverse transcriptase reaction, 95°C for 10 min followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. All RT-PCR assays for a particular gene were undertaken at the same time under identical conditions and carried out in triplicate. The expression levels of target genes were normalized to the levels of the glyseraldehyde-3-phosphate dehydrogenase (Hs99999905_m1), utilizing a standard curve method for quantitation.

2.10. Indirect staining of cell surface CEACAM1 and immunofluorescence analysis by flow cytometry

Untreated, control and chokeberry-treated Caco-2 cells were analyzed for CEACAM1 cell surface expression by flow cytometry using monoclonal antihuman CEACAM-1/CD66a antibody (R & D Systems) as the primary antibody. At the end of the 4-day treatment, cells were trypsinized, washed twice with PBS and suspended in cold flow cytometry buffer (FACS; PBS, 2% fetal bovine serum, 0.05% sodium azide). Cells (2×10^5) and the appropriate diluted primary antibody were gently mixed and incubated on ice. After 2 h, cells were washed with cold FACS buffer followed by incubation for 1 h on ice in the dark with fluorescein isothiocyanate (FITC)-conjugated antimouse (Sigma). Cells were washed again and resuspended in PBS, and the extent of binding of the antibody to its antigen on the surface of the live cells was analyzed (12,000 events were acquired) by flow cytometry using a FACScan flow cytometer (Becton Dickinson). Scan parameters were set up while running unstained cells to discard any fluorescence emitted by the cells. The background fluorescence was determined using only secondary FITC-labeled antibody as a negative control. Data are presented as mean values ± S.D. for five independent experiments. Where indicated, comparisons were carried out using the Student's *t* test.

3. Results

3.1. Establishment of the experimental conditions and stability of chokeberry polyphenols in the culture medium

To discard cytotoxicity caused by a pronounced shift of the osmolality and/or the pH of the culture medium when

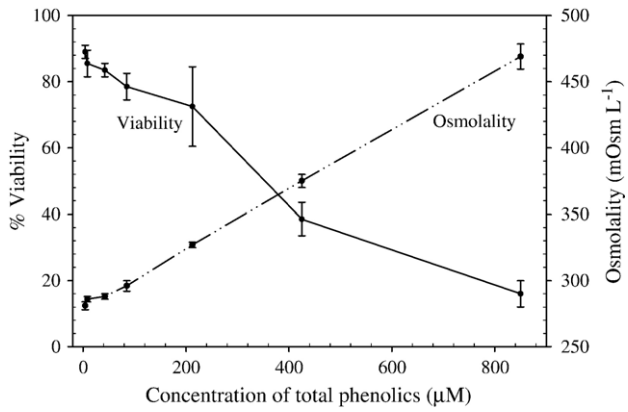

Fig. 1. Osmolality values (in milliosmolars per liter) in EMEM culture medium and viability of Caco-2 cells (after a 2-h incubation period) versus quantity of digested chokeberry juice added to the medium (expressed as initial micromolar concentration of total phenolics).

Table 1
Summary of the experimental conditions applied to human colon cancer Caco-2 cells: phenolic concentration (in micromolars) in the culture medium at the start of the incubation period, pH and osmolality values in the culture medium with added chokeberry juice

	2% (v/v)	Control 2%	5% (v/v)	Control 5%
Anthocyanins (μM)	13.5±1.3[a]	–	36.1±0.9	–
Flavonols (μM)	4.3±0.3	–	11.1±0.6	–
Caffeic acid derivatives (μM)	32.4±0.4	–	79.6±0.3	–
Flavan-3-ols (μM)	33.5±7.5	–	91.2±11.5	–
Total phenolics (μM)	83.8±8.8	–	218.0±13.3	–
pH[b]	7.0	7.0	7.5	7.5
Osmolality (mosM L^{-1})[c]	310±4	299±1	325±5	309±5

[a] Data are presented as mean values±S.D. from six independent experiments.
[b] pH in EMEM culture medium+10% serum from untreated cells was 7.0.
[c] Osmolality in EMEM culture medium+10% serum from untreated cells was 291±2 mosM L^{-1}. Data are presented as mean values±S.D. from five independent experiments.

incubated with excessive quantities of digested chokeberry juice, we measured the osmolality in the medium and the cells' viability after 2 h of exposure to increasing amounts of digested chokeberry juice (Fig. 1). Cell viability decreased dramatically at concentrations of digested chokeberry juice in the medium above 5% (v/v), corresponding to ~220 μM total phenolics. At this concentration, osmolality in the medium (327±2 mosM L^{-1}) was on the upper limit, tolerated by human cells (260–320 mosM L^{-1}). The addition of digested chokeberry juice at concentrations equal or below 5% (v/v) or the addition of the equivalent control digestion mix did not alter appreciably the pH values (7.0–7.5) of the culture medium during the experimental period. Consequently, and for subsequent studies, we selected the two highest concentrations of digested chokeberry juice that did not alter critically the pH and osmolality of the medium or cell viability: that is, 2% and 5% (v/v, 0.2 or 0.5 ml of digested chokeberry juice/10 ml culture medium). Table 1 summarizes the final experimental conditions selected for this study. We estimated that the consumption of a standard 250-ml portion of chokeberry juice (~8°Brix) prepared from the commercial concentrate used in this study corresponds to a polyphenol intake of ~640 mg [8]. After the digestion process and dilution with the digestive secretions (about 3 L per meal) [21], ingested polyphenols may reach intestinal cells at concentrations around 160 mg L^{-1}. Therefore, the concentrations selected for this study, corresponding to ~30 and 80 mg L^{-1} total phenolics in the culture medium at the start of the incubation period, represent possible in vivo luminal concentrations where epithelial cells may be exposed to.

In addition, we monitored the stability of the chokeberry polyphenols added to the culture media. The concentration of anthocyanins, flavonols, caffeic acid derivatives (chlorogenic and neochlorogenic acid) and flavan-3-ols decreased within the first few hours of incubation of the chokeberry juice in the culture medium with Caco-2 cells. The most affected compounds were the anthocyanins, which were only 7.5% of the initial quantity added to the medium after 2 h of incubation and which were no longer detected after 4 h of incubation. Flavonols and caffeic acid derivatives decreased by ~50% after 2 h of incubation. Flavan-3-ols were the most stable compounds, and after 2 h of incubation, ~63% of these compounds were still detected in the culture medium. In the absence of serum or cells, we quantified a similar reduction for all these compounds, confirming that the observed decrease of phenolics and, in particular, of anthocyanins, was mostly due to their instability at the pH of the medium [22]. These results indicate that under the selected culture conditions, a good proportion of the polyphenols was still present in the culture media after 2 h of incubation. The changes observed in the concentrations of these compounds due to their chemical instability may reflect changes produced in the intestinal lumen where the pH conditions are also slightly alkaline.

3.2. Chokeberry juice inhibits Caco-2 cell proliferation

Results of cell viability and cell proliferation are presented in Table 2. No changes in the viability of control cells were detected, although a small decrease (~10%) in the proliferation was observed. Exposure of Caco-2 cells to 2% or 5% of digested chokeberry juice resulted in inhibition of cell growth (~40% and ~70% of inhibition compared with untreated cells). At both concentrations, cell confluence was never reached. There was no difference in the percentage of viable cells between the two treatments (about 80% of viable cells compared with untreated cells).

3.3. H_2O_2 is formed in the culture medium at subtoxic levels

We did not detect H_2O_2 in the culture medium of untreated or control cells. The increase in absorbance

Table 2
Results of proliferation and viability for control and chokeberry-treated Caco-2 cells (at the end of the 4-day experimental period) expressed as percentage of those values obtained for untreated cells

	2% (v/v)	Control 2%	5% (v/v)	Control 5%
% Inhibition proliferation	37±3[a]	11±8	70±1	12±4
% Viability	82±6	101±7	76±1	98±2

[a] Data are presented as mean values±S.D. from four independent experiments (n=3 plates per experiment).

measured in the culture medium from cells treated with 5% chokeberry juice after incubation with the FOX reagent was completely reverted by addition of catalase (~350 units; 1 unit decomposes 1.0 μmol of H_2O_2 min^{-1}, pH 7.0, 25°C), confirming the formation of H_2O_2, which increased from ~5 to 20 μM during the 2-h incubation period (Fig. 2).

3.4. Chokeberry juice induces cell cycle arrest at G_2/M

The effect of the chokeberry treatment on cell proliferation was further evaluated by measuring cell cycle distribution. On Day 1 of the experimental period, untreated cells were primarily in the G_0/G_1 phase (40.5±2.7%) and in the S phase (39.1±1.1%), with half the percentage of cells in the G_2/M phase (20.4±3.4%; Fig. 3A). Within 4 days of the experiment, the proportion of cells in the G_0/G_1 phase increased slowly from 40.5±2.7% to 52.3±6.9% ($P<.05$), and cells in the S phase decreased from 39.1±1.1% to 26.3±5.6% ($P<.01$). There were no apparent changes in the percentage of cells in the G_2/M phase. A similar behavior was observed for the control cells (Fig. 3B). When cells were treated with 2% of digested chokeberry juice, cell cycle distribution profiles were similar to those observed for untreated and control cells (data not shown). However, following treatment with 5% of digested chokeberry juice, there was a twofold increase of cells in the G_2/M phase (from 18.4±3.2% to 40.2±9.1% after the second day of treatment; $P<.01$), which was maintained during the 4 days of treatment (Fig. 3C). The G_2/M phase accumulation was accompanied by a corresponding twofold decrease in the G_0/G_1 phase of the cell cycle (from 41.5±4.1% to 19.5±3.1%; $P<.01$), which slightly recovered throughout the experimental period and reached 27.2±3.8% after the last day of treatment. Cells on the S phase were constant as the treatment progressed. All groups of cells (untreated, control and treated) were allowed to recover in fresh culture media for 1 day after the last exposure, and cell cycle distribution was analyzed once more. In the untreated, control and 2% chokeberry-treated cells, the cell cycle profiles reverted to those of the first day; however, the G_2/M arrest was still observed in cells that had been treated with 5% chokeberry juice.

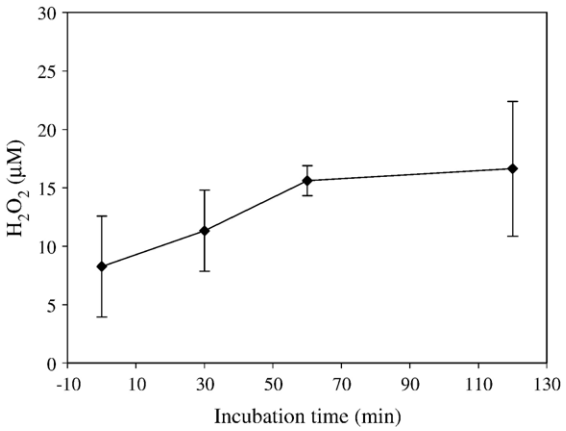

Fig. 2. Generation of H_2O_2 by the addition of digested chokeberry juice (5%) in EMEM during a 2-h incubation period. H_2O_2 was measured by the FOX assay.

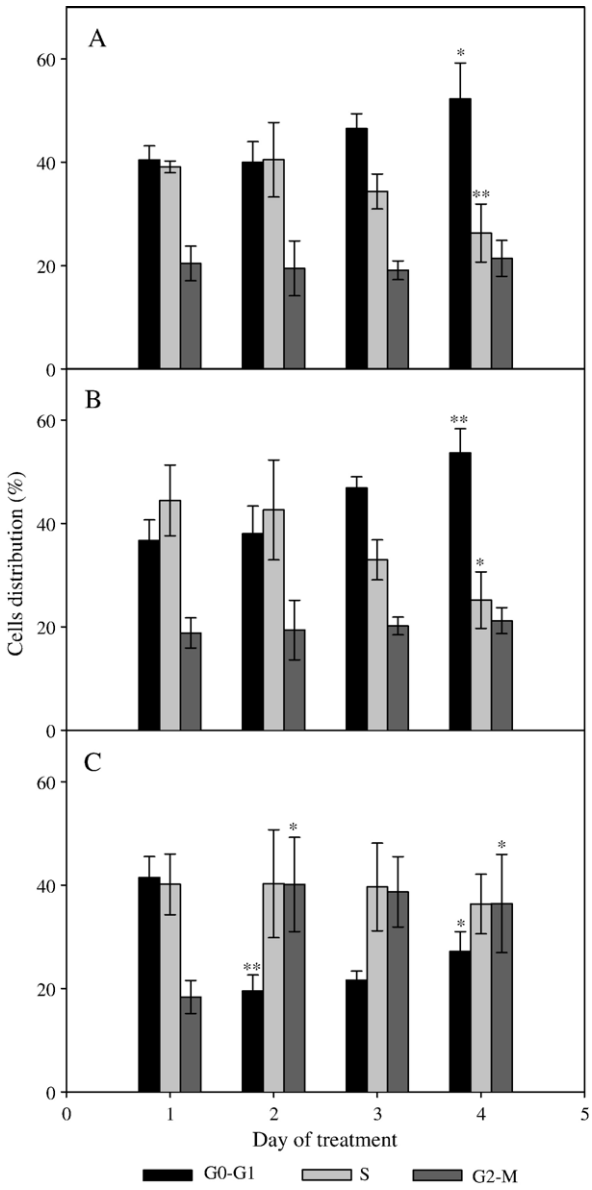

Fig. 3. Effect of digested chokeberry juice on Caco-2 cell cycle. Distribution of cells in the G_0/G_1, S and G_2/M phases at each day of treatment during the 4-day experimental period: (A) untreated cells (only subjected to medium changes), (B) control cells (treated with the enzyme + salt mix only) and (C) cells treated with digested chokeberry juice (5%). The analyses of cell cycle distribution were performed at least in triplicate for each group of cells. Data are presented as mean values±S.D. Where indicated, comparisons were carried out using the Student's t test. Statistical significance is as follows: $*P<.05$, $**P<.01$, which indicates significant differences between a cell cycle phase at Day 2 or Day 4 after treatment and the same phase at Day 1.

3.5. Analysis of gene expression after exposure to chokeberry juice

The observed inhibition of Caco-2 cell proliferation and the cell cycle arrest caused by the treatment with chokeberry may be associated to changes in the expression of genes related to cell growth and proliferation and cell cycle

regulation and/or genes that are connected to colon cancer. Transcriptional differences between control and 2% chokeberry-treated Caco-2 cells were screened using Affymetrix microarrays. Data analysis showed that 58% of the total transcripts represented in the chip were detected as 'present' in both control and treated cells and that the expression of the majority of these genes did not change as a result of the treatment with chokeberry juice. Only about 1% of the total transcripts represented in the chip displayed altered levels of expression in response to the chokeberry treatment, mostly in the range between 1.5- and 2.0-fold. Genes with altered mRNA levels were found to be involved in a wide range of

Table 3
Comparison of changes in gene expression (fold change, treated/control) detected by Affymetrix arrays and real time RT-PCR in Caco-2 cells after exposure to chokeberry juice

Accession number	Gene name	Gene symbol	Affymetrix	RT-PCR[a]		Biological process involved
			2%	2%	5%	
NM_006572	Guanine nucleotide binding protein (G protein) alpha 13	*GNA13*	3.0	1.1±0.1	1.4±0.1	Regulation of cell proliferation
NM_006622	Polo-like kinase 2	*PLK2/SNK*	1.6	2.3±1.0	3.3±0.4	Cell cycle regulation
NM_002184	Interleukin 6 signal transducer (oncostatin M receptor)	*IL6ST*	3.0	−1.2±0.1	−1.2±0.1	Cell growth and proliferation Expressed in colorectal cancer
NM_000629	Interferon (alpha, beta and omega) receptor 1	*IFNAR1*	−2.3	−1.7±0.1	1.1±0.0	Cell growth, immune response and apoptosis
NM_003516	H2A histone family, member O	*H2AFO*	−1.9	1.0±0.0	1.0±0.0	DNA package Role in cell cycle progression
AI144007	Heterogeneous nuclear ribonucleoprotein A1	*HNRPA1*	−3.0	−1.1±0.1	1.5±0.5	Processing and transport of RNA to the cytosol
BC005903	Polymerase (RNA) II (DNA-directed) polypeptide L, 7.6 kDa	*POLR2L*	−1.7	−2.0±0.5	−1.7±0.1	General gene transcription
BF690020	Eukaryotic translation elongation factor 1 delta	*EEF1D*	2.3	1.2±0.1	1.3±0.1	Mediates GDP/GTP exchange on EEF1alpha Associated to gastrointestinal cancer
NM_001712	Carcinoembryonic antigen-related cell adhesion molecule 1	*CEACAM1*	2.1	2.6±1.0	12.2±4.5	Reduced expression in colon cancer Tumor suppressor involved in cell–cell adhesion that regulates apoptosis in colon epithelium
NM_001200	Bone morphogenetic protein 2	*BMP2*	1.6	2.4±1.0	2.0±0.5	Tumor suppressor promoting apoptosis in mature colonic epithelial cells and inhibiting proliferation
D13889	Inhibitor of DNA binding 1, dominant negative helix–loop–helix protein.	*ID1*	−1.6	−1.1±0.1	−1.2±0.2	Cell growth regulation and tumorigenesis Up-regulated in colon cancer Associated to metastasis
NM_022975	Fibroblast growth factor receptor 2	*FGFR2*	−4.6	−1.8±0.2	−2.0±0.2	Implicated in tumor growth and invasion (colon carcinoma)
NM_002203	Integrin alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	*ITGA2*	3.0	2.6±1.0	3.6±0.8	Participates in cell adhesion (hemidesmosomes) Role in cell proliferation and migration Involved in colorectal cancer
NM_002961	S100 calcium binding protein A4 (metastasin)	*S100A4*	−2.5	−1.9±0.1	−1.8±0.5	May function in motility, invasion and tubulin polymerization Significant prognostic marker of colorectal carcinoma Associated with metastasis
AL365343	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	*SLC7A8/LAT2*	8.0	1.7±0.3	3.2±0.3	Transport of cationic amino acids

[a] Results shown as means±S.D. from at least three independent experiments (at two different passage numbers).

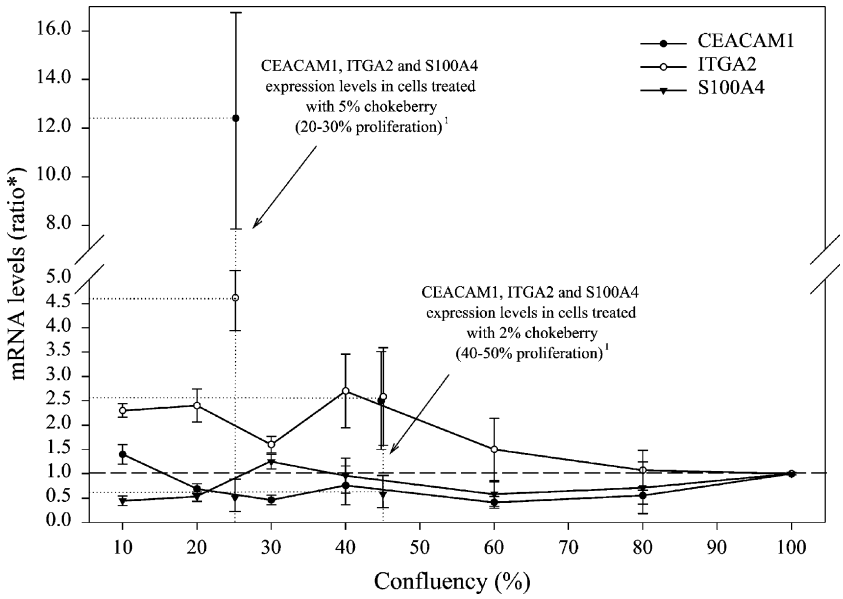

Fig. 4. mRNA levels of *CEACAM1*, *ITGA2* and *S100A4* in Caco-2 cells at different proliferative stages between seeding and confluence (*values expressed as ratio of proliferative subconfluent cells to confluent cells). Changes in the expression levels of these three genes in Caco-2 cells after exposure to 2% or 5% of chokeberry are also indicated ([1]values expressed as ratio of treated cells to control cells). Points represent the mean±S.D. of at least three independent experiments.

physiological functions: (a) cell signaling and signal transduction; (b) DNA processing and transcription; (c) RNA processing; (d) translation, protein processing and degradation; (e) cell growth, proliferation and apoptosis; (f) cell cytoskeleton and extracellular matrix associated; (g) transporters and metabolism; (h) response to various stimuli and other markers of cancer.

Changes in the expression of 15 of these genes were further analyzed by RT-PCR in at least three independent experiments at the two chokeberry concentrations (Table 3). Selected genes had been reported to be involved in cell growth and proliferation and cell cycle regulation and/or had been clearly associated to colorectal cancer. RT-PCR confirmed the up-regulation of five of the genes selected: *PLK2*, a serine threonine kinase with a regulatory role on cell cycle [23]; *CEACAM1*, a tumor suppressor involved in cell–cell adhesion [24]; *BMP2*, a tumor suppressor member of the transforming growth factor β family that inhibits proliferation of mature colonic epithelial cells [25]; *ITGA2*, a cell surface integrin expressed in colon cancer cell lines with a role in cell proliferation and migration [26]; and *SLC7A8*, a membrane transporter for amino acids [27]. RT-PCR also confirmed the down-regulation of the following: *POLR2L*, a subunit of the polymerase (RNA) II responsible for the synthesis of mRNA in eukaryotes [28]; *FGFR2*, a high-affinity receptor for fibroblast growth factor implicated in tumor growth and invasion in colon carcinoma [29]; and the *S100A4*, a significant prognostic marker of colorectal carcinoma associated with the metastasis capacity of cancer cells [30]. The down-regulation of the interferon receptor *IFNAR1* involved in cell growth [31] was only verified by RT-PCR when cells were treated with 2% of chokeberry, but no changes were detected after treatment with 5% chokeberry.

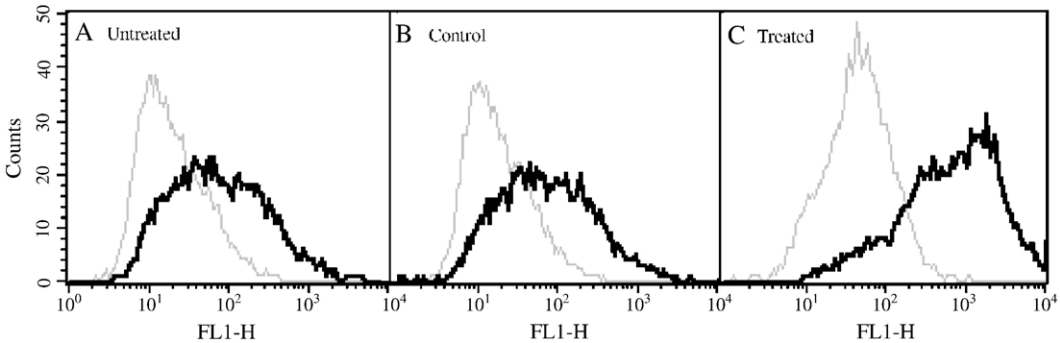

Fig. 5. Flow cytometry profiles indicating levels of cell surface expression of CEACAM1 in (A) untreated cells (only subjected to medium changes), (B) control cells (treated with the enzyme + salt mix only) and (C) cells treated with digested chokeberry juice (5%). Cells were stained with monoclonal antihuman CEACAM-1/CD66a antibodies and analyzed by flow cytometry (dark curves). The background fluorescence (light gray curve) was determined in cells incubated only with secondary FITC-labeled antibody.

Table 4
Flow cytometry analysis of CEACAM1 monoclonal antibody binding on Caco-2 cells by indirect immunofluorescence assay

Day of treatment	Caco-2 cell surface expression (%)		
	Untreated cells	Control cells	Treated (5%) cells
Day 4[a]	37.1±6.3[b]	38.1±6.4	48.9±11.0
Day 5[c]	31.4±6.0	35.1±7.3	57.8±11.9*

[a] Day 4 of treatment with 5% chokeberry juice.
[b] Data are the average of five independent experiments.
[c] Cells were allowed to recover in fresh medium for 1 day after the treatment.
* Statistical comparison between cells treated with chokeberry (5%) and control cells: highly significant difference ($P<.01$).

3.6. Up-regulation of CEACAM1 mRNA in Caco-2 cells is associated to the exposure to chokeberry juice

Of particular interest was the induction of *CEACAM1*, a tumor marker whose reduced expression is an important event in colorectal carcinogenesis and which regulates cell proliferation [24]. The levels of mRNA of this gene were up-regulated in Caco-2 cells up to ~12-fold after exposure to 5% of chokeberry juice. Under normal culture conditions, Caco-2 cells proliferate and reach confluence (~7 days after seeding) followed by spontaneous differentiation [32]. This process is itself accompanied by changes in the expression of multiple genes, including a large number of genes related to proliferation, cell adhesion and signaling [33–35]. In our study, gene expression changes are presented as the ratio between treated and control cells at the end of the 4-day experimental period. At this time point, control cells were ~100% confluent, whereas treated cells did not reach confluence (proliferation was inhibited by the treatment). To ensure that the observed up-regulation of *CEACAM1* was associated to the treatment with chokeberry and not to the different proliferative stages of the two compared groups of cells, we analyzed the expression of *CEACAM1* in proliferative untreated Caco-2 cells from seeding to confluence (Fig. 4). For comparison purposes, we also included mRNA levels for two other selected genes with altered expression: *ITGA2* and *S100A4*. Values are expressed as the ratio of proliferative subconfluent cells to confluent cells. These results show that subconfluent Caco-2 cells exhibited similar expression levels of *CEACAM1* regardless of the percentage of proliferation and that *CEACAM1* expression was predominantly lower than in 100% confluent cells. On sparse cell cultures ~20–30% confluent and ~40–50% confluent (approximately Days 3 and 4 after seeding, respectively), the expression levels of *CEACAM1* were approximately half of those in 100% confluent cells (Day 7 after seeding). However, the expression of *CEACAM1* in cells treated with 5% or 2% chokeberry juice (~20–30% or ~40–50% confluent, respectively) was 12.2- and 2.6-fold higher than in control confluent cells, indicating that up-regulation of *CEACAM1* was associated to the treatment and not to the proliferative stage of the cells. Unlike *CEACAM1*, changes in the expression levels of *S100A4* cannot be attributed to the treatment and may be associated to the different proliferative stages of the compared groups of cells. In the case of *ITGA2*, the expression of this gene may have been affected only by the treatment with 5% chokeberry juice.

It has been reported that at low concentrations, H_2O_2 can act as a signaling molecule, regulating the expression of genes and modulating diverse cellular functions [36]. To ensure that the observed up-regulation of *CEACAM1* in chokeberry-treated cells was not due to the small amounts of H_2O_2 generated under our experimental conditions, we determined the expression levels of *CEACAM1* in Caco-2 cells after repeated exposure (2 h for a 4-day period) to 15 μM H_2O_2. There were no changes in the expression of this gene as compared with untreated cells (1.4±0.2-fold change).

3.7. Up-regulation of cell surface expression of CEACAM1

Using flow cytometry, we analyzed the expression of CEACAM1 on the surface of Caco-2 cells to determine whether the observed changes at the mRNA level were translated into changes at the protein level. The fluorescence profile for CEACAM1 in untreated, control and chokeberry (5%)-treated cells is shown on the overlaid single-parameter histograms of background and test antibody (Fig. 5). The results of the flow cytometry analysis of CEACAM1/CD66a antibody binding on each group of cells, expressed as percentage of cell surface expression, are shown in Table 4. Data are the average of five independent experiments. Untreated Caco-2 cells showed a positive reaction with monoclonal antihuman CEACAM-1/CD66a antibody (37.1±6.3% and 31.4±6.0% for ~80% and ~100% confluent cells, Days 4 and 5 of treatment, respectively). Control cells expressed similar amounts of CEACAM1. Exposure of Caco-2 cells to 5% digested chokeberry juice induced slightly, although not significantly, the surface expression of CEACAM1 by Day 4 of treatment. Because changes at the protein levels may occur at a later stage than changes at the mRNA levels, all groups of cells (untreated, control and treated) were allowed to recover in fresh culture media for 1 day after the last exposure to chokeberry juice, and CEACAM1 surface expression was analyzed once more. A persistent and significant ($P<.01$) up-regulation of CEACAM1 surface expression was observed in the treated cells. We also compared CEACAM1 surface expression levels between untreated ~30% subconfluent cells, ~80% subconfluent cells and ~100% confluent cells and found no significant differences between these groups of cells, corroborating that proliferative subconfluent cells exhibited similar cell surface expression levels of CEACAM1 and that the changes in expression after repetitive exposure to chokeberry juice were associated to the treatment and not to the proliferative stage of the compared groups of cells.

4. Discussion

Much of the research carried out regarding the antiproliferative properties of polyphenols has used experimental

models in which cancerous cultured cells were exposed to individual polyphenols or extracts rich in these compounds for very long periods [9–14]. In vivo, however, gut epithelial cells are more likely to be exposed to complex food containing mixtures of these compounds and for shorter periods. In addition, these fruits undergo a digestion process that may affect the structure and properties of the fruit components. Therefore, the in vitro protective effects of polyphenols do not necessarily equate to in vivo chemoprotection, which is more likely due to the combined actions of all the components in the fruit. In this study, we examined the cellular response of proliferative colon cancer Caco-2 cells after repetitive exposure to a berry juice rich in polyphenols. To mimic more physiological conditions, we subjected the berry juice to in vitro gastric and pancreatic digestion. Following reported requirements for in vitro experiments looking at the anticarcinogenic potential of test substances [37], we selected appropriate subtoxic doses of the chokeberry juice based on (a) the composition in polyphenols after the simulated gastric and pancreatic digestion, (b) the stability of these polyphenols in the culture medium, (c) the pH and osmolality conditions in the culture medium and (d) in vivo realistic luminal exposure levels. Under our selected experimental conditions, Caco-2 cell proliferation was markedly inhibited. It has been established that polyphenols may suffer oxidation in the culture media and generate H_2O_2, which could account (at least partially) for the reported antiproliferative effects ascribed to these compounds [38,39]. The extent of H_2O_2 formation depends on the type of culture medium or phenolic compounds, and therefore, the formation of reactive H_2O_2 needs to be carefully examined for each experimental condition [40]. In our study, the levels of H_2O_2 produced in EMEM culture medium with 5% chokeberry juice were below 25 μM during 2 h of exposure. It has been reported that the proliferation of Caco-2 cells in DMEM is affected at H_2O_2 levels ~100 μM or above [41–43]. In one particular study, it was shown that incubation for 20 h of Caco-2 in EMEM with up to 10 mM of H_2O_2 resulted in less than ~25% loss of cell viability [44]. Under our experimental culture conditions, the levels of H_2O_2 generated are thus below those that may affect proliferation. The toxicity of this molecule depends not only on the concentration but also on the length of exposure and the type of cells [40]. We treated Caco-2 cells with 15 μM H_2O_2 in EMEM culture medium for 2 h a day for 4 days and found no significant effect on cell proliferation as compared with untreated cells (data not shown). These results indicate that the inhibition of proliferation in Caco-2 cells after exposure to chokeberry juice was not due to reactive, derived H_2O_2.

Inhibition of Caco-2 cell proliferation by the chokeberry treatment was linked to G_2/M cell cycle arrest. Regulation of cell cycle progression through cell cycle block at the G_0/G_1 or G_2/M checkpoints is one of the mechanisms of cell growth inhibition and allows normal cells to ensure for correct DNA synthesis or chromosomal segregation [45]. In cancer cells, particularly in colon cancer cells, the mechanisms that regulate cell cycle are defective [45,46]. Given their critical role in cell growth and division and their possible contribution to the transformation of a normal cell into a cancer cell, cell cycle checkpoints and their regulation are important targets for selective anticancer agents [46]. For example, flavopiridol, a synthetic flavone with antitumor properties, is a potent inducer of cell cycle arrest in colorectal cancer, but this compound shows important dose-limiting toxicities [47]. Dietary flavonoids such as genistein or apigenin (with structural similarities to flavopiridol) have also been shown to inhibit human colon cancer cell growth by inducing cell cycle arrest [48,49]. These results suggest that plant food polyphenols at dietary nontoxic levels can contribute to the blocking of the initiation or progression of gastrointestinal cancers by modulating cell cycle checkpoints. The chokeberry juice used in this study contains a mixture of flavonoids, mostly anthocyanins [8]. It has been recently reported that an anthocyanin-rich extract prepared from chokeberry can induce G_2/M cell cycle arrest in colon cancer cells HT29 [50], which suggests that some of the polyphenols present in chokeberry juice may be involved in the observed Caco-2 cell cycle arrest. The specific components in the juice that may be responsible for the observed effects were not evaluated in the present study and deserve further investigations.

To gain additional insight into the possible mechanisms by which exposure of Caco-2 cells to the chokeberry juice may have affected cell proliferation and cell cycle, we used Affymetrix microarrays and RT-PCR to identify possible related targets whose expression may have been altered. We detected a small number of genes related to several major cellular functions with altered expression levels, mostly between 1.5- and 2.0-fold changes. Microarrays have been increasingly used to study gene expression changes in colon cancer cells after exposure to food components such as individual polyphenols [51–53] or food extracts rich in polyphenols [54,55]. In general, these studies show moderate expression changes in a small proportion of genes. These studies also reveal the modulation of genes involved in a wide range of physiological functions such as metabolism, signal transduction, cell proliferation, transcription, apoptosis, cell signaling or cell adhesion. All these results support the general idea that nutrition is not associated with major changes in gene expression but rather induces multiple minor changes. A number of genes differentially expressed in Caco-2 cells after exposure to the chokeberry juice were found to be involved in cell proliferation and cell cycle regulation and associated with colon cancer. Altered expression of genes involved in proliferation may predispose colon cells for colon tumor development, and regulation of their expression may be a potential mechanism of chemoprevention [46,56].

Among those altered genes, we detected a significant induction of *CEACAM1*. This tumor suppressor has an important regulatory role on cell proliferation [24], and its expression is down-regulated in more than 85% of early

colorectal adenomas and carcinomas [57]. Failure to express CEACAM1 is likely to contribute to the early development of hyperplastic lesions prior to neoplastic lesions that may eventually lead to colon cancer development [57,58]. This transmembrane glycoprotein has been identified as a valuable target for cancer therapy as shown, for example, by a significant reduction of cell growth in human metastatic prostate cells after transfection of CEACAM1 [59]. From this perspective, it is plausible that dietary factors may contribute to the reduction of early proliferative lesions on colon cells by up-regulating the expression of CEACAM1 and maintaining a low rate of proliferation. Our results show that regular exposure of proliferative colon cancer cells to dietary levels of polyphenol-rich chokeberry fruit caused inhibition of cell proliferation and induced up-regulation of CEACAM1 both at the mRNA and protein levels. However, the levels of expression and the regulatory effects of CEACAM1 are cell density dependent and cell state specific [60], and thus, it is very important to pay attention to the functional states of the cells that are being investigated. For example, whereas subconfluent prostate epithelial normal cells or bladder carcinoma cells express similar amounts of CEACAM1 at different percentage of confluence, remarkable differences were found in the expression of CEACAM1 between proliferating and nonproliferating quiescent cells [24]. Also, in subconfluent Caco-2 BBe cells, CEACAM1 expression level was lower than in postconfluent cells [33]. We showed that the levels of expression of CEACAM1 in untreated subconfluent Caco-2 cells did not change very much during proliferation and were predominantly lower in subconfluent 10–80% cells than in confluent cells, indicating that the up-regulation of CEACAM1 observed after the treatment with chokeberry juice in subconfluent Caco-2 cells was associated to the treatment and not to the proliferative stage of the compared groups of cells.

The mechanisms by which CEACAM1 regulates cell proliferation are not yet fully understood, but CEACAM1 may participate in controlling the cell cycle [24,60]. The differences in CEACAM1 expression between proliferating and quiescent cells suggest that CEACAM1 may have a role in contact inhibition [60]. On contact inhibition, Caco-2 cells spontaneously undergo cell cycle arrest, with cells accumulating in the G_0/G_1 phase as a function of time in culture [34]. It has been suggested that the switch in CEACAM1 expression may take place when cells enter/exit the cell cycle from/to the quiescent G_0/G_1 phase [24]. Up-regulation of CEACAM1 in the chokeberry-treated subconfluent Caco-2 cells is concomitant with cells being arrested into the G_2 phase, indicating that inhibition of Caco-2 cell proliferation by exposure to chokeberry may occur through different mechanisms than those taking place in normal differentiating cells. It has also been reported that CEACAM1 can influence the expression levels of proteins involved in the regulation of the G_1/S and G_2/M transitions, through tyrosine-dependent signaling pathways [61]. The cell-proliferation-related signaling function of CEACAM1 is known to be influenced by factors like the serine/threonine phosphorylation of CEACAM1 or the binding to calmodulin [60]. Our results of the Affymetrix screening of treated Caco-2 cells also detected up-regulation of Ser/Thre kinases such as the polo-like kinases, PLK2 and PLK4 (twofold induction) or down-regulation of the phosphorylase kinase calmodulin 1 (1.8-fold). It has also been suggested that in cells with high abundance of cell–cell contacts, CEACAM1 may preferentially activate tyrosine phosphatases and inhibit cell proliferation [60]. Interestingly, the protein tyrosine phosphatase nonreceptor type 12, known to interact with cell adhesion molecules and involved in the regulation of cell growth, was also up-regulated in the chokeberry-treated Caco-2 cells (twofold induction as estimated by Affymetrix microarrays). The mechanisms by which cell signaling and cell cycle may be modulated in Caco-2 cells by exposure to the chokeberry juice and the involvement of proteins such as kinases and phosphatases related to CEACAM1 are currently being investigated.

In conclusion, these results demonstrate that repeated exposure to dietary levels of a polyphenol-rich chokeberry has a potent in vitro antiproliferative effect toward the human colorectal cancer cell line Caco-2 and that this antiproliferative effect may be mediated through arrest of the cells at the G_2/M checkpoint. Through analysis of gene expression, we have detected changes in the mRNA levels of several tumor markers typical for colon cancer and of proteins involved in proliferation and cell cycle that may be associated to the treatment. Among those, CEACAM1, with a significant regulatory role on cell proliferation of particular interest at early stages of cancer development, may be a potential target for chemoprevention by food components such as those present in polyphenol-rich fruits.

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References

[1] Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. CA Cancer J Clin 2005;55:10–30.
[2] Umar A, Viner JL, Hawk ET. The future of colon cancer prevention. Ann NY Acad Sci 2001;952:88–108.
[3] Reddy BS. Novel approaches to the prevention of colon cancer by nutritional manipulation and chemoprevention. Cancer Epidemiol Biomarkers Prev 2000;9:239–47.
[4] Riboli E, Norat T. Epidemiologic evidence of the protective effect of fruit and vegetable on cancer risk. Am J Clin Nutr 2003;78(Suppl): 559S–69S.
[5] Rui HL. Health benefits of fruit and vegetable are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr 2003; 78(Suppl):517S–20S.
[6] Singh B, Bhat TK, Singh B. Potential therapeutic applications of some antinutritional plant secondary metabolites. J Agric Food Chem 2003; 51:5579–97.

[7] Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans: I. Review of 97 bioavailability studies. Am J Clin Nutr 2005; 81(Suppl):230S–42S.
[8] Bérmudez-Soto MJ, Tomás-Barberán FA. Evaluation of commercial red fruit juice concentrates as ingredients for antioxidant functional juices. Eur Food Res Tech 2004;219:133–41.
[9] Han C, Ding H, Casto B, Stoner GD, D'Ambrosio S. Inhibition of the growth of premalignant and malignant human oral cell lines by extracts and components of black raspberries. Nutr Cancer 2005; 51:207–17.
[10] Olsson ME, Gustavsson KE, Andersson S, Nilson A, Duan RD. Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. J Agric Food Chem 2004;52:7264–71.
[11] Seeram NP, Adams LS, Hardy L, Heber D. Total cranberry extract versus its phytochemical constituents: antiproliferative and synergistic effects against human tumor cell lines. J Agric Food Chem 2004; 52:2512–7.
[12] Zhao C, Giusti MM, Malik M, Moyer MP, Magnuson BA. Effects of commercial anthocyanin-rich extracts on colonic cancer and non-tumorigenic colonic cell growth. J Agric Food Chem 2004;52:6122–8.
[13] Glei M, Matuscheck M, Steiner C, Böhm V, Persin C, Pool-Zobel BL. Initial in vitro toxicity testing of functional foods rich in catechins and anthocyanins in human cells. Toxicol In Vitro 2003;17:723–9.
[14] Gossé F, Guyot S, Roussi S, Lobstein A, Fischer B, Seiler N, et al. Chemoprotective properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. Carcinogenesis 2005;26:12291–5.
[15] Kang SY, Seeram NP, Nair MG, Bourquin LD. Tart cherry anthocyanins inhibit tumor development in Apc^{Min} mice and reduce proliferation of human colon cancer cells. Cancer Lett 2003; 194:13–9.
[16] Harris GK, Gupta A, Nines RG, Kresty LA, Habib SG, Frankel WL, et al. Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydroxy-2′ -deoxyguanosine levels in the Fischer 344 rat. Nutr Cancer 2001;40(2):125–33.
[17] Kresty LA, Morse MA, Morgan C, Carlot PS, Lu J, Gupta A, et al. Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries. Cancer Res 2001;61:6112–9.
[18] Bode AM, Dong Z. Targeting signal transduction pathways by chemopreventive agents. Mutat Res 2004;555:33–51.
[19] Gil-Izquierdo A, Gil MI, Ferreres F, Tomás-Barberán F. In vitro availability of flavonoids and other phenolics in orange juice. J Agric Food Chem 2001;49:1035–41.
[20] Nourooz-Zadeh J. Ferrous ion oxidation in presence of xylenol orange for detection of lipid hydroperoxides in plasma. Methods Enzymol 1999;300:58–62.
[21] Laurent C, Besançon P, Auger C, Rouanet JM, Caporiccio B. Grape seed extract affects proliferation and differentiation of human intestinal Caco-2 cells. J Agric Food Chem 2004;52:3301–8.
[22] Markakis P. Anthocyanins and their stability in food. CRC Crit Rev Food Technol 1974;4:437–56.
[23] Eckerdt F, Yuan J, Strebhardt K. Polo-like kinases and oncogenesis. Oncogene 2005;24:267–76.
[24] Singer BB, Scheffrahn I, Öbrink B. The tumor growth-inhibition cell adhesion molecule CEACAM1 (C-CAM) is differently expressed in proliferating and quiescent epithelial cells and regulates cell proliferation. Cancer Res 2000;60:1236–44.
[25] Hardwick JCH, van der Brink GR, Bleuming SA, Ballester I, van der Brande JMH, Keller JJ, et al. Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon. Gastroenterology 2004;126:111–21.
[26] Beaulieu JF. Integrins and human intestinal cell functions. Front Biosci 1999;4:310–21.
[27] Yoon JH, Kim YB, Kim MS, Park JC, Kook JK, Jung HM, et al. Expression and functional characterization of the system L amino acid transporter in KB human oral epidermoid carcinoma cells. Cancer Lett 2004;205:215–26.
[28] Kadonaga JT. Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. Cell 2004;116:247–57.
[29] Thiery JP. Role of growth factor signaling in epithelial cell plasticity during development and in carcinogenesis. Bull Acad Natl Med 2001;185:1279–94.
[30] Gongoll S, Peters G, Mengel M, Piso P, Klempnauer J, Kreipe H, et al. Prognostic significance of calcium-binding protein S100A4 in colorectal cancer. Gastroenterology 2002;123:1478–84.
[31] Piehler J, Roisman LC, Schreiber G. New structural and functional aspects of the type I interferon–receptor interaction revealed by comprehensive mutational analysis of the binding interface. J Biol Chem 2000;275:40425–33.
[32] Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol 2005;21:1–26.
[33] Fleet JC, Wang L, Vitek O, Craig BA, Howard JE. Gene expression profiling of Caco-2 BBe cells suggests a role for specific signaling pathways during intestinal differentiation. Physiol Genomics 2003; 13:57–68.
[34] Mariadason JM, Arango D, Corner GA, Arañes MJ, Hotchkiss KA, Yang W, et al. A gene expression profile that defines colon cell maturation in vitro. Cancer Res 2002;62:4791–804.
[35] Hauck W, Stanners CP. Control of carcinoembryonic antigen gene family expression in a differentiating colon carcinoma cell line, Caco-2. Cancer Res 1991;51:3526–33.
[36] Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. FEBS Lett 2000;486:10–3.
[37] Venhagen H, Aruoma OI, van Delft JHM, Dragsted L, Ferguson LR, Knasmüller S, et al. The 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in in vitro experiments and animal studies in vivo. Food Chem Toxicol 2003;41:603–10.
[38] Long LH, Clement MV, Halliwell B. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. Biochem Biophys Res Commun 2000; 273:50–3.
[39] Chai PC, Long LH, Halliwell B. Contribution of hydrogen peroxide to the cytotoxicity of green tea and red wines. Biochem Biophys Res Commun 2003;304:650–4.
[40] Wee LM, Long LH, Whiteman M, Halliwell B. Factors affecting the ascorbate- and phenolic-dependent generation of hydrogen peroxide in Dulbecco's modified Eagles medium. Free Radic Res 2003; 37:1123–30.
[41] Wijeratne SSK, Cuppet SL, Schlegel V. Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells. J Agric Food Chem 2005;53:8768–74.
[42] Lee KW, Hur HJ, Lee HJ, Lee CY. Antiproliferative effects of dietary phenolic substances and hydrogen peroxide. J Agric Food Chem 2005;53:1990–5.
[43] Liu RH, Sun J. Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation. J Agric Food Chem 2003;51:1718–23.
[44] Manna C, Galleti P, Cucciolla V, Moltedo O, Leone A, Zappia V. The protective effect of the olive oil polyphenol (3,4-dihydroxyphenyl)-ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. J Nutr 1997;127:286–92.
[45] Damia G, Broggini M. Cell cycle checkpoint proteins and cellular response to treatment by anticancer agents. Cell Cycle 2004;3:46–50.
[46] McWilliams RR, Erlichman C. Novel therapeutics in colorectal cancer. Dis Colon Rectum 2005;48:1632–50.
[47] Murthi KK, Dubay M, McClure C, Brizuela L, Boisclair MD, Worland PJ, et al. Structure–activity relationship studies of flavopiridol analogues. Bioorg Med Chem Lett 2000;10:1037–41.

[48] Chen AC, Donovan SM. Genistein at a concentration present in soy infant formula inhibits Caco-2BBe cell proliferation by causing G2/M cell cycle arrest. J Nutr 2004;134:1303–8.
[49] Wang W, Heideman L, Chung CS, Pelling JC, Koehler KJ, Birt DF. Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. Mol Carcinog 2000;28:102–10.
[50] Malik M, Zhao C, Schoene N, Guisti MM, Moyer MP, Magnuson BA. Anthocyanin-rich extract from *Aronia melanocarpa* E. induces a cell cycle block in colon cancer but not normal colonic cells. Nutr Cancer 2003;46:186–96.
[51] van Erk MJ, Roepman P, van der Lende TR, Stierum RH, Aarts JMMJG, van Bladeren PJ, et al. Integrated assessment by multiple gene expression analysis of quercetin bioactivity on anticancer-related mechanisms in colon cancer cells in vitro. Eur J Nutr 2005; 44:143–56.
[52] van Erk MJ, Teuling E, Staal YCM, Huybers S, van Bladeren PJ, Aarts JMMJG, et al. Time- and dose-dependent effects of curcumin in gene expression in human colon cancer cells. J Carcinog 2004;3:8.
[53] McLoughlin P, Roengvoraphoj M, Gissel C, Hescheler J, Certa U, Sachinidis A. Transcriptional responses to epigallocatechin-3 gallate in HT 29 colon carcinoma spheroids. Genes Cells 2004;9:661–9.
[54] Noé V, Peñuelas S, Lamuela-Raventós RM, Permanyer J, Ciudad CJ, Izquierdo-Pulido M. Epicatechin and cocoa polyphenolic extract modulate expression in human Caco-2 cells. J Nutr 2004;134: 2509–16.
[55] Dolara P, Luceri C, De Filippo C, Femia AP, Giovannelli L, Cardeni G, et al. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. Mutat Res 2005;591:237–46.
[56] Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nat Med 2004;10:789–99.
[57] Nittka S, Günther J, Ebisch C, Erbersdobler A, Neumaier M. The human tumor suppressor CEACAM1 modulates apoptosis and is implicated in early colorectal tumorigenesis. Oncogene 2004;23: 9306–13.
[58] Shively JE. CEACAM1 and hyperplasic polyps: new links in the chain of events leading to colon cancer. Oncogene 2004;23: 9303–5.
[59] Okegawa T, Pong RC, Li Y, Hsieh JT. The role of cell adhesion molecule in cancer progression and its application in cancer therapy. Acta Biochim Pol 2004;51(2):445–57.
[60] Scheffrahn I, Singer BB, Sigmundsson K, Lucka L, Öbrink B. Control of density-dependent, cell state-specific signal transduction by the cell adhesion molecule CEACAM1, and its influence on cell cycle regulation. Exp Cell Res 2005;307:427–35.
[61] Pagano M. Control of DNA synthesis and mitosis by the Skp2–p27–Cdk1/2 axis. Mol Cell 2004;14:414–6.