Inhibition of the Growth of Premalignant and Malignant Human Oral Cell Lines by Extracts and Components of Black Raspberries

ChunHua Han, Haiming Ding, Bruce Casto, Gary D. Stoner, and Steven M. D’Ambrosio

Abstract: Black raspberries are a rich natural source of chemopreventive phytochemicals. Recent studies have shown that freeze-dried black raspberries inhibit the development of oral, esophageal, and colon cancer in rodents, and extracts of black raspberries inhibit benzo(a)pyrene-induced cell transformation of hamster embryo fibroblasts. However, the molecular mechanisms and the active components responsible for black raspberry chemoprevention are unclear. In this study, we found that 2 major chemopreventive components of black raspberries, ferulic acid and β-sitosterol, and a fraction eluted with ethanol (RO-ET) during silica column chromatography of the organic extract of freeze-dried black raspberries inhibit the growth of premalignant and malignant but not normal human oral epithelial cell lines. Another fraction eluted with CH₂Cl₂/ethanol (DM:ET) and ellagic acid inhibited the growth of normal as well as premalignant and malignant human oral cell lines. We investigated the molecular mechanisms by which ferulic acid and β-sitosterol and the RO-ET fraction selectively inhibited the growth of premalignant and malignant oral cells using flow cytometry and Western blotting of cell cycle regulatory proteins. There was no discernable change in the cell cycle distribution following treatment of cells with the RO-ET fraction. Premalignant and malignant cells redistributed to the G2/M phase of the cell cycle following incubation with ferulic acid. β-sitosterol treated premalignant and malignant cells accumulated in the G0/G1 and G2/M phases, respectively. The RO-ET fraction reduced the levels of cyclin A and cell division cycle gene 2 (cdc2) in premalignant cells and cyclin B1, cyclin D1, and cdc2 in the malignant cell lines. This fraction also elevated the levels of p21WAF1/CIP1 in the malignant cell line. Ferulic acid treatment led to increased levels of cyclin B1 and cdc2 in both cell lines, and p21WAF1/CIP1 was induced in the malignant cell line. β-sitosterol reduced the levels of cyclin B1 and cdc2 while increasing p21WAF1/CIP1 in both the premalignant and malignant cell lines. These results show for the first time that the growth inhibitory effects of black raspberries on premalignant and malignant human oral cells may reside in specific components that target aberrant signaling pathways regulating cell cycle progression.

Introduction

The consumption of fruits and vegetables has been associated with a reduced risk for many different types of human cancer (1–3). A high consumption of fruits is associated with a large reduction in the risk for cancers of the oral cavity and pharynx (1). The protective effect of fruits and vegetables is related to multiple anticancer components including dietary fiber, carotenoids, vitamins C and E, selenium, glucosinolates, indoles, isothiocyanates, flavonoids, polyphenols, protease inhibitors, and plant sterols. Among fruits, black raspberries have a relatively large content of these chemopreventive agents (4–12). Freeze-dried black raspberries inhibit N-nitrosomethylbenzylamine (NMBA) induced tumorigenesis in the rat esophagus (13,14), reducing by 40–60% esophageal tumor multiplicity, when administered at dietary concentrations of 5% and 10% before, during, and after treatment of the rats with NMBA (15). Another study showed that black raspberries given postinitiation inhibited azoxymethane-induced tumors in the rat colon, indicating that berries are effective in inhibiting the progression stages of cancer (16). Recently, the feeding of lyophilized black raspberries to hamsters significantly reduced the number of 7,12-dimethylbenz(a)anthracene-induced tumors in the cheek pouch (5). These animal studies have indicated that the phytochemicals in berries can inhibit multiple types of cancer during both the initiation and promotion stages. In vitro studies using a Syrian hamster embryo transformation assay showed that a methanol extract of black raspberries inhibited benzo(a)pyrene diol-epoxide-induced cell transformation (17). The same organic fraction of black raspberries inhibited benzo(a)pyrene diol-epoxide-induced transactivation of AP-1 and NFκB in mouse epidermal JB6 CI 41 cells (18). These studies have suggested that selective...
phytochemicals may represent the active chemopreventive components of the berries. They also provide a rationale for combining identified phytochemicals to mimic active berry extracts. Although it is clear that whole berries and berry extracts elicit significant chemopreventive effects, the components and mechanisms by which the berries and their components inhibit multiple stages of cancer are not well understood.

In the United States, cancer of the oral cavity is one of the 10 most common cancers representing 2–3% of all cancers in men and women (19,20). Of these cancers, 90% are squamous cell carcinomas, and the prognosis is poor, with an overall mortality rate of approximately 50%. Because current therapeutic protocols are relatively ineffective for oral cancer, it is important to identify and develop chemopreventive strategies to prevent this disease. Although most chemopreventive studies in the oral cavity have focused on demonstrating the inhibition of tumor formation, few studies have determined the effects in normal cell phenotypes and the mechanisms of chemopreventive activity in premalignant and malignant oral cell types. It is important to include normal human cells in these studies to better identify and understand molecular mechanisms leading to agents that selectively target premalignant and malignant cells.

To determine whether black raspberries and their components are effective chemopreventive agents in human oral cancer, we used a previously described human oral cancer cell model consisting of primary normal cells and premalignant and malignant human oral cell lines. Our results show that ferulic acid and β-sitosterol and a fraction of freeze-dried berries (eluted from a silica gel column with ethanol) inhibit the growth of both premalignant and malignant but not normal human oral cell lines. Growth inhibition was due to inhibition of cell cycle regulatory proteins leading to the blocking of cells in the G0/G1 (β-sitosterol in premalignant cell line) and G2/M (ferulic acid in premalignant and malignant and β-sitosterol in malignant cell lines) phases of the cell cycle.

**Materials and Methods**

**Cell Lines and Culture**

The previously described (21) in vitro model consisting of the normal (TE1177), premalignant (SCC-83-01-82), and malignant (83-01-82CA) human oral epithelial cell lines was used in this study. The characteristics of the premalignant and malignant oral cell lines (both kindly provided by Dr G. Milo of The Ohio State University) have been previously described (22). The cell lines were maintained in modified minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 5 U/ml penicillin, and 5 μg/ml streptomycin (23). TE1177 cells were grown in a modified alpha MEM medium supplemented with epithelial growth factor, insulin, transferrin, hydrocortisone, and 10% fetal bovine serum.

**Black Raspberry Extract, Fractions, and Components**

Black raspberries [Rubus occidentalis (RO), Jewel variety] were supplied by the Stokes Raspberry Farm (Wilmington, OH) and shipped frozen to Van Drunen Farms (Momence, IL) for freeze-drying. The composition of the berry powder was determined by Covance Laboratories (Madison, WI) and has been previously described (4,5). Black raspberry extracts were prepared and fractionated into RO-F001, RO-F003, RO-F004, RO-DM, and RO-ET fractions as previously described (17,18) except that ethanol was substituted for methanol in the extraction protocol. Ellagic acid, ferulic acid, β-sitosterol, and 2-hydroxypropyl-β-cyclodextrin (HBC) were purchased from Sigma (St. Louis, MO). The β-sitosterol-cyclodextrin complex was prepared according to the method of Greenberg et al. (24). The molar ratio of β-sitosterol to cyclodextrin was maintained at 1:300.

**Growth Inhibition Assay**

Normal human oral cells were seeded at 300 cells, and both premalignant and malignant cells were seeded at 200 cells per well in 96-well plates 24 h prior to treatment. Black raspberry fractions, ellagic acid, ferulic acid, and β-sitosterol and their respective solvents were added to the culture at the indicated concentrations. The concentrations of water, dimethyl sulfoxide (DMSO; <0.2%), ethanol (<0.4%), and HBC (<4.5 mM) used as solvents did not inhibit the growth of the cell lines. On Day 5, the medium was replenished with fresh medium-containing agent. On Day 7, the medium was removed and the cells washed twice with phosphate-buffered saline (PBS), air dried, and fixed with 4% paraformaldehyde. A 100 μl solution containing 1% methylene blue was added for 30 min to stain the cells. The dye was extracted with 100 μl of methanol/glacial acetic acid/water (100:1:99) solution, and the optical density was measured at 660 nm using a BioTex microplate reader (Houston, TX). The number of cells following treatment was compared to the solvent and control groups and used to determine growth inhibition as follows: GI = (Abtreated/Abcontrol) × 100, where Ab represents the mean absorbance. Mean values ± standard error of measurement (SEM) were calculated from 4–6 experiments. Student’s 2-tailed t-test was used to determine significance. Cytotoxicity was assayed following 24 h of treatment with agent using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In this assay, normal human oral cells were seeded at 7,000, and both premalignant and malignant cells were seeded at 4,000 cells per well in 96-well plates. MTT was added at a final concentration of 0.5 mg/ml and incubated during the 20th to 24th h of treatment. The supernatant was removed, and the cells were washed with PBS twice, dried in air, and 100 μl DMSO was added. The optical density was measured at 570 nm. A decrease in MTT activity compared to solvent and untreated controls was used as an indicator and measure of cytotoxicity.
Cell Cycle Analyses Using Flow Cytometry

Cells (5–10 × 10^4 per 75 cm^2 flask) were treated on the 2nd and 5th days with 200 µg/ml of either the berry RO-ET fraction or with DMSO (solvent control). Cells were treated with 200 µg/ml ferulic acid, ethanol (solvent control), 15 µM β-sitosterol, and HBC (solvent control) for 3 days. At the end of treatment, cells were harvested with a 0.01% trypsin/0.1 mM ethyleneglycetraetraetic acid (EGTA) solution, fixed in 70% ethanol and stored at −20°C as previously described (25). Prior to fluorescence-activated cell sorter analyses, the fixed cells were washed with PBS and incubated with a solution containing 20 µg/ml propidium iodide, 200 µg/ml ribonuclease A, 0.1% Triton-x100 for 30 min in the dark. Flow cytometry analysis was performed by the Analytical Cytometry Laboratory of The Ohio State University Comprehensive Cancer Center using a Beckman-Coulter EPICS Elite flow Cytometer (BD FACS Calibur, Bedford, MA) equipped with a 488 nm 15 mW Argon laser collecting at least 30,000 events. Multicycle DNA modeling software and Modfit software (version 3.0, Verity Software House, Topsham, ME) were used to estimate cell cycle distribution. The DNA profile indicated the relative abundance of the cell population in subG0, G0/G1, S, and G2/M phases. The coefficient of variance and χ² value were used to determine significance of the distribution of cells in each phase of the cell cycle.

Western Blot Analyses

Cells were treated with RO-ET, ferulic acid, β-sitosterol, and their corresponding solvent controls in the same concentrations and times described previously for flow cytometry. Immediately following treatment, cells were washed twice with PBS and removed from the surface of the flask using a 0.01% Trypsin/0.1 mM EGTA solution. Cells were collected by centrifugation, washed twice with PBS, and lysed by boiling for 10 min in a buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 10 mM dithio-DL-threitol, 62 mM Tris-HCl pH 6.8, 10 µg/ml peptatin, and 1 mg/ml leupeptin. Cell debris was removed by centrifugation and protein concentration determined using the Bio-Rad assay (Hercules, CA). Equal amounts (30–70 µg) of protein were subjected to gel electrophoresis in 12% polyacrylamide slab gels (InVitrogen, Carlsbad, CA) in the presence of SDS. The protein was electrophoretically transferred to a nitrocellulose membrane and dried. The membrane was preincubated with blocking buffer (5% nonfat milk in TBST buffer; 150 mM NaCl, 10 mM Tris, and 0.1% Tween-20) for 2 h and then incubated with primary antibody at 4°C overnight. The primary antibodies used were rabbit polyclonal anti-cyclin D1 (Cell Signaling Technology, Beverly, MA; 1:500), mouse monoclonal anti-tubulin (Oncogene Research Products, San Diego, CA; 1:1000), rabbit polyclonal anti-cyclin A and anti-p21waf1/cip1, and mouse monoclonal anti-cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000). After washing the membrane three times with TBST for 10 min, the membrane was incubated with mouse or rabbit secondary antibody labeled with horseradish peroxidase for 1 h at 37°C. The Western blots were developed using a chemiluminescence kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was then exposed to X-ray film and the image documented and the relative density measured using AlphaEase software from Alpha Innotech Corporation (San Leandro, CA). α-Tubulin or actin (used as loading controls) was detected on the same membrane following incubation with a stripping buffer (20 mM Tris-HCl at pH 7.4, 2% SDS, 500 mM NaCl, 100 mM 2-mercaptoethanol) for 1 h at 37°C.

Statistical Analyses

Each experiment was performed three to four times as indicated in the legends. Where appropriate, the data are presented as the mean ± SEM. Student’s t-test was used to assess the statistical significance of the differences between the control and treatment groups. A statistically significant difference was considered to be P < 0.05.

Results

Black Raspberry Fractions and Components Selectively Inhibit the Growth of Premalignant and Malignant Oral Cell Lines

To identify the active chemopreventive components in black raspberries, previously described fractions of raspberry extracts and identified components of lyophilized berries were tested for growth inhibition of normal, premalignant, and malignant oral cell lines (4, 17, 18). The RO-F001 fraction, isolated following overnight extractions of the freeze-dried berries with ethanol three times, was not growth inhibitory to the oral cell lines at its highest soluble concentration for 6 days. The water soluble RO-F003 fraction and the CH2Cl2 soluble RO-F004 fraction isolated following partitioning of the RO-F001 fraction with CH2Cl2 and water were also inactive. Following chromatography of the RO-F001 fraction on a silica gel column, the RO-DM (CH2Cl2:ethanol, 1:1 eluate) and RO-ET (ethanol eluate) fractions were obtained. At the 200 µg/ml concentration, the RO-DM fraction inhibited 80% of normal, premalignant, and malignant cell growth (Table 1). In contrast to the unselective growth inhibition by the RO-DM fraction, the RO-ET fraction selectively inhibited the growth of the premalignant and malignant cell lines 45% (P < 0.001) and 27% (P < 0.005), respectively, at 200 µg/ml and 35% (P < 0.05) and 25% (P < 0.05), respectively, at 100 µg/ml and the premalignant cell line by 23% (P < 0.05) at 50 µg/ml (Table 1). There was no discernable growth inhibition of the normal cell line. The RO-DM and RO-ET fractions were not cytotoxic to the cell lines as determined by MTT and trypan blue dye exclusion assays (data not shown).
The chemical compositions of cultivars of black raspberries used in previously published animal chemoprevention studies indicate that varying levels of sterols, polyphenols, and carotenoids are present that have demonstrated chemopreventive activity (5,15). Among these, ellagic acid (166–200 mg/100g), ferulic acid (18–21 mg/100g), and β-sitosterol (72–89 mg/100g) were found in high quantities.

To determine the contribution of these components to the growth-inhibitory activity of the berries, the oral cell lines were treated with various concentrations of these three components over a 6-day period for growth inhibition. Ellagic acid (as ellagitannins in the insoluble residue after the initial ethanol extraction) inhibited the growth of all three cell lines, producing 50% growth inhibition at the 25 µg/ml concentration (Fig. 1A). Ellagic acid was also observed to be cytotoxic to the cell lines, which presumably accounts for its growth inhibition. Ferulic acid was a selective growth inhibitor of both the premalignant and malignant oral cell lines (Fig. 1B). At the highest concentration (200 µg/ml) tested, ferulic acid significantly (P < 0.05) reduced the growth of all three cell lines, producing 50% growth inhibition at 200 µg/ml ferulic acid (Fig. 1B). Ferulic acid was not cytotoxic to the cell lines.

Table 1. Growth Inhibitory Effects of the RO-DM (DM) and RO-ET (ET) Berry Fractions on Normal, Premalignant, and Malignant Oral Cell Lines

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Normal</th>
<th>Premalignant</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>17.0 ± 0.6</td>
<td>4.7 ± 0.2</td>
<td>−0.3 ± 0.0</td>
</tr>
<tr>
<td>100</td>
<td>57.2 ± 8.3</td>
<td>14.4 ± 0.1**</td>
<td>21.4 ± 1.1**</td>
</tr>
<tr>
<td>200</td>
<td>78.2 ± 1.1</td>
<td>79.4 ± 4.2</td>
<td>79.2 ± 3.8</td>
</tr>
<tr>
<td>ETb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>3.1 ± 0.1</td>
<td>23.3 ± 0.9#</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>100</td>
<td>−4.5 ± 0.2</td>
<td>35.2 ± 0.8#</td>
<td>24.7 ± 1.2#</td>
</tr>
<tr>
<td>200</td>
<td>−7.3 ± 0.3</td>
<td>45.1 ± 2.2*</td>
<td>27.4 ± 0.9**</td>
</tr>
</tbody>
</table>

a: Data are expressed as the percent mean ± standard error of measurement of solvent control from four to six replicates.
b: Statistical significance is as follows: *, P < 0.001; **, P < 0.005; and #, P < 0.05; indicates significant differences from the normal cell line.
c: µg/ml, treated twice over 6 days as described in Materials and Methods.

The chemical compositions of cultivars of black raspberries used in previously published animal chemoprevention studies indicate that varying levels of sterols, polyphenols, and carotenoids are present that have demonstrated chemopreventive activity (5,15). Among these, ellagic acid (166–200 mg/100g), ferulic acid (18–21 mg/100g), and β-sitosterol (72–89 mg/100g) were found in high quantities. To determine the contribution of these components to the growth-inhibitory activity of the berries, the oral cell lines were treated with various concentrations of these three components over a 6-day period for growth inhibition. Ellagic acid (as ellagitannins in the insoluble residue after the initial ethanol extraction) inhibited the growth of all three cell lines, producing 50% growth inhibition at the 25 µg/ml concentration (Fig. 1A). Ellagic acid was also observed to be cytotoxic to the cell lines, which presumably accounts for its growth inhibition. Ferulic acid was a selective growth inhibitor of both the premalignant and malignant oral cell lines (Fig. 1B). At the highest concentration (200 µg/ml) tested, ferulic acid significantly (P < 0.05) reduced the growth of all three cell lines, producing 50% growth inhibition at 200 µg/ml ferulic acid (Fig. 1B). Ferulic acid was not cytotoxic to the cell lines.

Ferulic Acid and β-Sitosterol Induce Cell Cycle Arrest in Premalignant and Malignant Cells

Flow cytometry was used to gain further insight into the mechanisms of black raspberry chemoprevention and the selective growth inhibiting effects of the RO-ET fraction, ferulic acid, and β-sitosterol. Figure 2 compares the cell cycle distribution patterns in the premalignant and malignant oral cell lines following incubation with the RO-ET fraction for 6 days and either ferulic acid or β-sitosterol for 3 days. Although the RO-ET fraction induced significant growth inhibition of both the premalignant and malignant cell lines, there was no significant difference in the cell cycle distribution between the DMSO control and RO-ET fraction (Fig. 2A). Although there was no difference in the cell cycle distribution following incubation with DMSO and HBC solvent, ethanol, used as the solvent for ferulic acid, increased the number of cells in the G2/M phase. Compared to the ethanol solvent control, treatment of the cell lines with 200 µg/ml ferulic acid led to a further accumulation of
Figure 2. Cell cycle distribution of premalignant and malignant oral cell lines following incubation with the RO-ET berry fraction, ferulic acid (FA), and β-sitosterol (SIT). Flow cytometry analyses and cell cycle distribution of cells treated with A: 0.2% dimethyl sulfoxide (DMSO) and 200 µg/ml RO-ET (ET) for 6 days, B: 0.4% ethanol and 200 µg/ml FA for 72 h, and C: 4.5 mM 2-hydroxypropyl-β-cyclodextrin (HBC) and 15 µM SIT) for 72 h. Data are representative of three independent experiments. Dotted shading indicates cells in G1, hatched shading denotes cells in S, and solid shading are cells in the G2/M (G2) phase of the cell cycle. #P < 0.05 indicates significant increase from solvent control.
both the premalignant (58.6% vs 24.7%) and malignant (75.0% vs 30.2%) cell lines in the G2/M phase of the cell cycle (Fig. 2B). There was a corresponding decrease in the number of cells in the G0/G1 phase (16.8% vs 28.6% in premalignant cells and 7.6% vs 29.9% in malignant cells). These results suggest that ferulic acid arrests both the premalignant and malignant oral cell lines in the G2/M phase of the cell cycle. Compared to the HBC-treated control, 15 μM β-sitosterol treatment led to a moderate accumulation of cells in the G0/G1 phase of the cell cycle in the premalignant cell line (56.3% vs 47.7%; Fig. 2C). However, treatment of the malignant cell lines with β-sitosterol led to a marked depletion of cells in the G0/G1 (43.7% vs 26.2%) and corresponding increases in the G2/M (18.2% vs 34.3%) phases of the cell cycle.

**Effect of the RO-ET Berry Fraction, Ferulic Acid, and β-Sitosterol on Cell Cycle Regulatory Proteins in the Human Oral Cell Lines**

Because ferulic acid and β-sitosterol redistributed the premalignant and malignant cells in either the G0/G1 or G2/M phases of the cell cycle, we evaluated their effects and the effects of the RO-ET fraction on proteins that regulate progression through the cell cycle. Although there was no discernible change in the cell cycle distribution of premalignant and malignant cells following treatment for 6 days with 200 μg/ml of the berry RO-ET fraction, a number of changes were noted in the levels of cyclins, cell division cycle gene 2 (cdc2), and p21waf1/cip1 (Fig. 3A). In the premalignant cell line, cdc2 protein levels were reduced 30% by the RO-ET fraction, and cyclin A was slightly (17%) reduced. There were increases in the levels of cyclin B1 (11%) and cyclin D1 (48%), whereas p21waf1/cip1 remained unchanged. When the malignant cell line was treated with the RO-ET fraction, the levels of cyclin A appeared unchanged, whereas there were increases in the levels of cyclin B1 (33%), cyclin D1 (28%), and cdc2 (30%) and increased (24%) levels of p21waf1/cip1. The changes in the levels of these cell cycle regulatory proteins were much larger in the cell lines treated with ferulic acid (Fig. 3B) and β-sitosterol (Fig. 3C) than with the RO-ET fraction. This may be expected due to the higher concentrations of these two chemicals used in these experiments compared to that found in the black raspberry extracts. As described previously, treatment of both the premalignant and malignant cell lines with ferulic acid led to an accumulation of cells in the G2/M phase of the cell cycle. In both the premalignant and malignant cell lines, ferulic acid increased the levels of the G2/M proteins, cyclin B1 (45%, 19%), and cdc2 (16%, 37%; Fig. 3B). The levels of cyclin A, induced during the S phase, and cyclin D1, required for the transition of cells into the G1 phase, were not affected by ferulic acid. p21waf1/cip1, an inhibitor of the cdk/cyclin complex, was reduced 28% in the premalignant and increased twofold in the malignant cell line. β-Sitosterol, which increased the number of premalignant and malignant cells in the G0/G1 and G2/M phase of the cell cycle, respectively, inhibited the expression of cyclin B1 (38%, 73%) and cdc2 (39%, 40%) and increased p21waf1/cip1 (39%, 287%) levels in both the premalignant and malignant cell lines (Fig. 3C). Cyclin D1 was 36% lower in the premalignant and 45% higher in the malignant cell lines following treatment. Cyclin A was not affected by β-sitosterol.

**Discussion**

The consumption of fruits and vegetables has been strongly associated with the reduced risk of many different types of human cancers including the oral cavity and pharynx (1–3,26). The identification of fruits and vegetables and their components with chemopreventive activity provides a “food-based” approach toward chemoprevention. Among the fruits, black raspberries have demonstrated chemopreventive effects in animal models, and clinical trials are currently being initiated for prevention of oral, esophageal, and colon cancer (4,5,14–16,18). Black raspberries contain large amounts of compounds with demonstrated chemopreventive activity including vitamins, selenium, anthocyanins, β-sitosterol, ellagic acid, and ferulic acid (5,15). However, there is little information as to the active components and mechanisms of chemoprevention. In this study, we observe that among the six fractions isolated from freeze-dried berries, only the fraction eluted with ethanol (RO-ET) from the silica gel chromatography of the F001 fraction exhibited selective growth inhibition of premalignant and malignant human oral cell lines. Of the many components identified in lyophilized black raspberries, ferulic acid and β-sitosterol were selective inhibitors of premalignant and malignant oral cell line growth. Although these are found in high quantities in the freeze dried berries (5), only small amounts (<5 μg/g) are observed in the RO-ET fraction. Growth inhibition by ferulic acid and β-sitosterol was due to their modulation of key signaling proteins regulating the progression of cells through the cell cycle. Ferulic acid treatment led to increased levels of cyclin B1 and cdc2 in both cell lines and higher levels of p21waf1/cip1 in the malignant cell line. β-Sitosterol reduced the levels of cyclin B1 and cdc2 while increasing p21waf1/cip1 in both the premalignant and malignant cell lines. These results indicate that the chemopreventive effect of black raspberries may reside in specific components targeting cell cycle regulatory signaling pathways in human premalignant and malignant oral cells.

The lack of observable cell cycle delay by the RO-ET extract may account for its modest effect on growth inhibition of the premalignant and malignant cell lines. However, we were able to observe RO-ET induced reductions in cdc2 protein in both the premalignant and malignant cell lines, cyclin B1 and cyclin D1 proteins in the malignant cell line, and elevation of p21waf1/cip1 protein in the malignant cell line. These effects are independent of ferulic acid and β-sitosterol, as these two compounds are found in very low quantities in the RO-ET extract. Studies are ongoing to identify the active com-
ponents of the RO-ET extract. The lack of growth inhibition by the first ethanol extraction, RO-F001, and the other organic and aqueous fractions suggests that these may contain suboptimal levels of growth-inhibiting chemicals or the presence of growth-stimulatory compounds. This may be revealed with further chemical, biological, and molecular analyses of the fractions.

The delays in the progression of the premalignant and malignant cells through the cell cycle by ferulic acid and β-sitosterol were correlated with changes in the levels of proteins regulating cell cycle progression. Our previous study and this study have indicated that cyclin A, cyclin B1, and cdc2 are expressed at high and p21waf1/cip1 at low levels in both the premalignant and malignant oral cell lines compared to the normal oral epithelial cell line (25). These are consistent with other studies that have shown elevated levels of cyclins and cyclin kinases and low levels of cyclin kinase inhibitors in most head and neck tumor cells (27–32). The treatment of premalignant and malignant oral cell lines with ferulic acid increased the levels of cdc2 and cyclin B1 proteins, which may facilitate the movement of cells from S into the G2 phase of the cell cycle. However, they were not able to go through mitosis. The accumulation of cells in either the G0/G1 or G2/M phases following β-sitosterol treatment may be due to increases in the level of p21waf1/cip1 inhibiting the cdk2/cyclin E complex for exiting G1 and cdc2/cyclin B complex for progression through G2 into mitosis (33–37). Further studies are needed to determine possible inhibitory interactions of these compounds directly or indirectly via upstream signals regulating cyclins and cyclin-associated kinases.

Figure 3. Expression of cell cycle regulatory proteins in premalignant and malignant human oral cell lines treated with the RO-ET berry fraction, ferulic acid, and β-sitosterol. Cells were treated with A: 0.2% dimethyl sulfoxide (DMSO) and 200 µg/ml RO-ET (ET) for 6 days; B: medium (med), 0.4% ethanol (et), and 150 and 200 µg/ml ferulic acid for 72 h; and C: med, 4.5 mM 2-hydroxypropyl-β-cyclodextrin (HBC), and 15 µM β-sitosterol (sit) for 72 h. Cell lysates were evaluated for the levels of cyclin A, cyclin B1, cyclin D1, cell division cycle gene 2 (cdc2), and p21waf1/cip1 by Western blotting as described in Materials and Methods. The blots are representative of three independent experiments. The blots were scanned using Scan Maker 5900 (Alpha Innotech, San Leandro, CA), and the density was quantified using AlphaEase software (ver. 3.0). The values for the medium control were set to 100.
Ferulic acid and β-sitosterol are found in a wide variety of fruits. Ferulic acid was effective in inhibiting the growth of cultured human breast and colon cancer cell lines, although the mechanisms were not determined (38). The selectivity of ferulic acid to the premalignant and malignant oral cell lines suggests that this compound may target aberrantly expressed cyclins and kinases regulating the cell cycle. Ferulic acid is also readily absorbed in the human intestinal tract and persists in human blood (39,40), suggesting that the high concentrations of ferulic acid found in black raspberries could account for some of the berry chemopreventive activity.

β-sitosterol, a plant sterol, has been identified as a growth inhibitor and inducer of apoptosis in a number of cellular systems (41–48). In the MDA-MB-231 cell line, the down regulation of cholesterol synthesis by β-sitosterol leads to the induction of the MAP kinase pathway, inhibition of cyclin B/cdc2, and G2/M arrest (43,49). Other studies have indicated that the growth inhibition of HT-29 cells by β-sitosterol may be due to decreased levels of sphingomyelin and increased levels of phosphatidylcholine altering ceramide second messaging signaling (50). These data suggest multiple targets for growth inhibition by β-sitosterol depending on cell type. Like ferulic acid, β-sitosterol is well absorbed from the intestinal tract, reaching physiological ranges between 4–70 µmol/L in the blood (50,51). These levels are similar to experimental concentrations in cell culture.

In summary, we have identified some of the active components of black raspberries. Ferulic acid and β-sitosterol, both found in large amounts in freeze-dried black raspberries, and
the RO-ET fraction selectively inhibit the growth of premalignant and malignant oral cells by targeting cell cycle regulatory proteins. The further identification and characterization of these and other active components of black raspberries should help to better establish the nutritional components responsible for food-based chemopreventive activity.

Acknowledgments and Notes

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