Red raspberries have antioxidant effects that play a minor role in the killing of stomach and colon cancer cells

Jason Goda, Patricia L. Tate, Lyndon L. Larcoma,b,*

aDepartment of Nursing, Healthcare Genetics Program, and Department of Biological Sciences, Clemson University, Clemson, SC 29634, USA
bDepartment of Physics and Astronomy, Clemson University, Kinard Laboratory, Clemson, SC 29634, USA

Received 10 June 2010; revised 7 October 2010; accepted 8 October 2010

Abstract

Berries and berry extracts possess properties that make them important in the prevention of cancer. The high antioxidant levels of these extracts play a role, but components of the berries can have other effects on cell replication and survival. We chose to test the hypothesis that (i) although the antioxidant capacity of raspberry extracts is important for inhibiting the proliferation of tumor cells, other characteristics of the berry extracts are responsible for a major part of their antiproliferative activity, and that (ii) the relative importance of the antioxidant effect can depend on the cell type being studied. The aim of this study was to assess the relative roles of low pH and high antioxidant levels in the killing of 3 cell types by an aqueous extract from Meeker red raspberries. Stomach, colon, and breast cancer cells were treated with berry extract and with HCl and ascorbic acid solutions of the same pH. A dilution of 7.5% ascorbic acid solution, of the same pH and slightly higher antioxidant concentration than the berry extract, killed less than 10% of the stomach and colon cancer cells. In contrast, the berry extract at this same dilution killed more than 90% of these cells. Antioxidants played a more significant role in the killing of breast cancer cells, however. For these cells, approximately 50% of the killing could be attributed to antioxidant effects. We conclude that the antioxidant effect plays a minor role in the killing of 2 gastrointestinal cell types, but its role in inactivating a breast cancer cell line is much more significant. No evidence of apoptosis was observed, and caspase activation did not contribute to cell killing by the extract.

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Keywords: Cancer prevention; Berry extracts; Antioxidants; Raspberries; Caspases; AGS stomach cancer cells; LoVo colon cancer cells; MCF-7 breast cancer cells

Abbreviations: z-VAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) plus phenazine methosulfate; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); NF-κB, nuclear factor-kappa B.

1. Introduction

The anticarcinogenic and antiinflammatory effects of fruits and berries have been well documented. Several plant extracts inhibit the replication of cancer cells in culture, and many plant compounds are highly effective in pure form—the most thoroughly studied of these being black raspberries [1,2] and resveratrol [3-8]. Although the focus has been mainly on cancer prevention by whole fruit extracts, it is also important to consider these as sources of compounds which could be of therapeutic value. Therefore, it is important to identify those properties of fruits that could maximize their anticancer effects. One such property that has received much attention is the high antioxidant level of most berries and fruits. Because red raspberries are reported to be among the
most effective berries for inhibiting the growth of cancer cells [9], we examined the role of antioxidants in their killing of tumor cells.

In this laboratory, Smith et al [10] found extracts from raspberries, strawberries, and blueberries to inactivate mutagenesis by benzo[a]pyrene. Tate et al [11] reported similar suppression by blackberries for mutagenesis by 2-amino anthracene and found the magnitude of the effect to vary with the variety of berries grown in the same orchard under identical conditions. God et al [12] also found muscadine grapes to be highly effective in suppressing 2-amino anthracene mutagenesis. In this case, all 4 varieties tested caused approximately the same amount of suppression (98%), although they differed significantly in antioxidant activity. The inactivation of metabolically activated carcinogens is caused by components of the extracts that inhibit the cytochrome P-450 system responsible for converting carcinogenic compounds into forms capable of covalent binding with DNA [13]. Less understood is the ability of blackberry extracts to inhibit ultraviolet-induced mutagenesis [14]. In addition to their effects on mutagenesis, raspberries, blackberries, and muscadine grapes almost completely inhibit the activity of the matrix metalloproteinases 2 and 9 involved in the invasion and metastasis of cancer cells [11,15].

Although antioxidants can clearly protect cells from mutagenesis at the initiation stage of carcinogenesis, their role in the destruction of transformed cells is less clear. Boivin et al [9] measured the antioxidant activity and anti-proliferative activities of 13 types of berries and found red raspberries to be among the most effective in blocking proliferation of 5 different types of cancer cells; however, they found no correlation between antioxidant activity and the ability to inhibit cancer cell replication. Likewise, Liu et al [16] found no correlation between antioxidant activity and anti-proliferative activity when comparing 4 different varieties of raspberries. In addition to its antioxidant effect, each extract also contains numerous compounds that can affect cell survival and replication by interacting with different replicative and metabolic pathways. The most clearly demonstrated of these effects is suppression of the Nuclear factor-kappa B (NF-κB) pathway [9]. To help clarify the role of antioxidants, we chose to test the hypothesis that (i) the antioxidant capacity of raspberry extracts is important for inhibiting the proliferation of tumor cells, but other components of the berry extracts can be responsible for a significant part of their antiproliferative activities, and (ii) the relative importance of the antioxidant effect can depend on cell type. To avoid complications involved in comparing different berry extracts, we compared the effects of the water-soluble components of red raspberries on cell survival with those of simple ascorbic acid solutions of known antioxidant capacity. Because the berry extracts and ascorbic acid solutions are acidic, we compared these with HCl solutions of the same pH. Three tumor cell lines were used: AGS stomach and LoVo colon cancer cells (representing tumors of the gastrointestinal tract) and MCF-7 breast cancer cells (representing a hormonally responsive tumor). At the concentrations of berry extract used, any effects of pH were undetectable. At the same pH, the total antioxidant capacity of the ascorbic acid solution was slightly higher than that of the berry extract, but the berry extract was much more effective at inhibiting cell survival of the stomach and colon cancer cells. The MCF-7 cells proved to be much less sensitive than the AGS or LoVo cells to berry extract killing.

2. Methods and materials

2.1. Cell culture

AGS stomach cancer (ATCC CRL-1739), LoVo colon cancer (ATCC CRL-229), and MCF-7 breast cancer (ATCC HTB-22) cell lines were obtained from the American Type Culture Collection (Manassas, Va). AGS and LoVo cells were maintained in an F-12K medium supplemented with 10% fetal bovine serum, and MCF-7 cells were maintained in a DMEM (Dulbecco’s modified Eagle’s medium) medium with 10% fetal bovine serum. An extract of Meeker red raspberries (Van Drunen Farms, Momence, Ill) was prepared by blending 22 g of freeze-dried berries with 200 mL of ddH₂O. The blended product was then centrifuged, and the supernatant was collected and filter sterilized through a 0.2-μm syringe filter.

2.2. Cell proliferation assay

Fresh solutions of berry extract, ascorbic acid, and HCl with the same pH were prepared before each assay and diluted to the test concentrations of 5%, 7.5%, and 10%. Cell survival was measured with the CellTiter 96 Assay from Promega (Madison, Wis), according to instructions provided by the manufacturer. Cells were plated in 50 μL of complete medium at the following densities: AGS, 2 × 10⁴ cells/well; LoVo, 6.25 × 10⁴ cells/well; MCF-7, 2.5 × 10⁴ cells/well. Then, 50 μL of the appropriate test solution at 10%, 15%, or 20% dilution was added to give the test concentrations given above. Because the test solutions were water solutions of extract, ascorbic acid or HCl, 50 μL of water were added to the control cells to maintain the same number of cells and the same growth medium composition as the test solutions. Each sample was plated in quintuplicate for each cell line. The plates were incubated for 48 hours at 37°C in an atmosphere of 5% CO₂.

In the assays for cell survival, the medium was removed and replaced by 50 μL of fresh medium without extract. Twenty microliters of MTS reagent were added to each well, and the plate was incubated for an additional 4 hours. The absorbance was read at 490 nm. The absorbance values for the 5 wells containing a particular sample were averaged. A surviving fraction was calculated for the cells in each test solution by dividing the average absorbance of a particular test culture by the average absorbance of the control culture. To determine whether the effect of the extract on cell survival depended upon the time of addition to the culture,
we did the experiments as described above, but the extract was added 24 hours after the cells had been plated and allowed to adhere. For each condition, 3 independent experiments were performed, and the surviving fractions of the 3 were averaged.

In separate assays, cells from each cell line were plated and treated with the extract as described above and incubated for 48 hours. The culture medium was removed and the cells were treated with trypan blue stain to assess the effects of the extract on cell viability. Microscopic observation indicated that most of the cells had taken up the dye.

2.3. Caspase activity analysis

To determine whether cell killing involved caspase activation, we used the Caspase-Glo Kit from Promega to assay the activities of caspases 3/7, 8, and 9 in the AGS cell line. The assay was performed according to the manufacturer’s instructions. The cells were treated for 1 hour with 10% extract as described above. The medium was removed and 100 μL of Caspase-Glo reagent were added to each well. After a 30-minute incubation at room temperature, the luminescence was measured. Because no caspase activation was detected, the effect of caspase inhibition on cell death was tested. Cells from all 3 cell lines were treated with 10% extract as described above for the MTS viability assays, but with the addition of 50 μL of z-VAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) per well. The plates were incubated for 24 hours, and viability was measured as described for the MTS assay.

2.4. Antioxidant assay

The Antioxidant Status Kit from Calbiochem (San Diego, Calif) was used to measure the total antioxidant capacity of the ascorbic acid and extract solutions. Here, the antioxidant capacity is measured as the capacity to inhibit oxidation of ABTS to ABTS’ by metmyoglobin. Fresh extract and ascorbic acid solutions were prepared and diluted to 10% concentration. Absorbance was measured at 600 nm with a 37°C temperature-controlled spectrophotometer. The assay was performed in triplicate, and in each assay, the blank, standard, extract, and ascorbic acid solutions were measured in triplicate.

2.5. Statistical analyses

In each experiment, there were 5 wells for each condition: untreated cells and cells in 5%, 7.5%, or 10% test solution. All wells were loaded with the same number of cells. Absorbance values for the 5 wells representing each condition were averaged, and surviving fractions were calculated by dividing the mean absorbance of a particular test solution by that of the control that contained untreated cells. The surviving fractions for 3 separate experiments were averaged (Tables 1 and 2). Surviving fractions of the test solutions were compared with those of the control culture with Student’s t test. The same comparison was performed between the cultures treated with the berry extract and those treated with the ascorbic acid solution. A difference was taken to be significant for values of *P* < .05.

### 3. Results

The aim of the experiments reported herein was to compare the effects of an aqueous extract of Meeker raspberries with those of control solutions having the same pH and similar antioxidant capacity on cancer cell lines from different tissues of origin. Stoner’s group has successfully treated precancerous oral lesions in humans [17] with black raspberry extract and found prevention of esophageal cancer by this extract in rats [18-20], but similar results have not been documented for other types of tissues.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Comparison of ascorbic acid and berry extract effects on cells treated from the time of plating</td>
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<tr>
<td><strong>Ascorbic acid treatment of replicating cells</strong></td>
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<td><strong>Cell lines</strong></td>
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<td><strong>AGS</strong></td>
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<td><strong>LoVo</strong></td>
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<td><strong>MCF-7</strong></td>
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<td><strong>Raspberry extract treatment of cells</strong></td>
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<tr>
<td><strong>AGS</strong></td>
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<td><strong>LoVo</strong></td>
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<tr>
<td><strong>MCF-7</strong></td>
</tr>
</tbody>
</table>

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<sup>a</sup> Values are means ± standard error of the mean.
<sup>b</sup> *P* values are given in parentheses for means significantly different from untreated cells and ascorbic acid–treated cells.
<sup>c</sup> Surviving fractions significantly different from untreated cells: *P* < .05.
<sup>d</sup> Surviving fractions of berry extract treated cells significantly different from ascorbic acid–treated cells: *P* < .05.
Table 2
Comparison of ascorbic acid and berry extract effects on adhered cells

Ascorbic acid treatment of adhered cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Acid concentration&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>7.5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS</td>
<td>0.98 ± 0.23</td>
<td>0.91 ± 0.14</td>
<td>0.66 ± 0.10 (0.02)</td>
</tr>
<tr>
<td>LoVo</td>
<td>0.98 ± 0.17</td>
<td>0.98 ± 0.023</td>
<td>0.86 ± 0.066 (0.03)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0.93 ± 0.21</td>
<td>0.66 ± 0.55</td>
<td>0.35 ± 0.39</td>
</tr>
</tbody>
</table>

Raspberry extract treatment of adhered cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Concentration of extract</th>
<th>7.5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS</td>
<td>1.05 ± 0.001</td>
<td>0.25±0.057 (0.02, 0.003&lt;sup&gt;–&lt;/sup&gt;)</td>
<td>0.06±0.011 (0.003&lt;sup&gt;–&lt;/sup&gt;, 0.008&lt;sup&gt;–&lt;/sup&gt;)</td>
</tr>
<tr>
<td>LoVo</td>
<td>0.36&lt;sup&gt;–&lt;/sup&gt; ± 0.009 (0.003&lt;sup&gt;–&lt;/sup&gt;, 0.007&lt;sup&gt;–&lt;/sup&gt;)</td>
<td>0.097±0.016 (5 × 10&lt;sup&gt;–5&lt;/sup&gt;; 3 × 10&lt;sup&gt;–6&lt;/sup&gt;)</td>
<td>0.091±0.016 (5 × 10&lt;sup&gt;–5&lt;/sup&gt;; 2 × 10&lt;sup&gt;–3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>1.09 ± 0.094</td>
<td>0.90 ± 0.17</td>
<td>0.27±0.22 (0.02)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± standard error of the mean.
<sup>b</sup> P values are given in parentheses for means significantly different from untreated cells and ascorbic acid–treated cells.
<sup>c</sup> Surviving fractions significantly different from untreated cells: P < .05.
<sup>d</sup> Surviving fractions of berry extract treated cells significantly different from ascorbic acid–treated cells: P < .05.

One might ask whether gastrointestinal tumor cell types are inherently susceptible to the berry extracts. Alternatively, the response of these tumors might result from the fact that the extracts can come into direct contact with the affected tissue. We compared susceptibilities of cancer cells from the stomach, colon, and breast to different concentrations of red raspberry extract. Because the berry extract is acid (pH 3.3), an HCl solution of the same pH was used to treat the cells in the same way. Ascorbic acid dissolved in water to give a pH of 3.3 was also used at the same concentrations as the berry extract to examine the effects of both low pH and high antioxidant capacity. The antioxidant concentrations of the ascorbic acid and raspberry extract were 1.86 and 1.45 mmol/L, respectively. The results obtained with ascorbic acid are indicative of the role of a simple antioxidant effect in cancer cell killing. In contrast, the berry extract contains many antioxidant species but can also affect the cells by acting directly on pathways regulating cell replication and survival (eg, NF-κB, hormone receptors, and regulators of apoptosis). To determine whether the time of extract addition would affect susceptibility of the cells, we added the extract at 2 different times: at the time of plating (before the cells had a chance to adhere to the plate) and 1 day after plating (when the cells were adhered to the substrate).

Cell survival was measured as the ability to reduce a colorless MTS solution to formazan dye with an absorbance maximum at 490 nm (CellTiter 96 Assay from Promega). Because the absorbance of the untreated control cells could differ slightly from one experiment to another, the absorbance readings observed for treated cells were normalized to the absorbance reading for the untreated control cells of that type. In a particular experiment, a surviving fraction was calculated as the mean absorbance of the treated samples divided by the mean absorbance of the control. At least 3 experiments were performed for each treatment condition, and the surviving fractions for these were averaged. The pH of the extract had essentially no effect on survival of the cells because of the buffering capacity of the growth medium and the low acid concentrations used. A solution of HCl with the same pH as the berry extract was diluted to the same percentage concentrations that were used for the extract. For solutions containing 5%, 7.5%, and 10% of this HCl solution, surviving fractions of 0.92, 0.97, and 1.00 were observed for AGS cells and 0.95, 0.97, and 0.97 for LoVo cells treated at the time of plating. When cells were split and then cultured for 1 day to allow them to adhere before addition of the extract, the surviving fractions were 0.98, 1.00, and 1.03 for AGS and 0.98, 1.07, and 1.07 for LoVo at extract concentrations of 5%, 7.5%, and 10%.

Surviving fractions observed after treatment with ascorbic acid or berry extract are given in Table 1 for the cells treated at the time of plating. The results for the cells treated 24 hours after plating are given in Table 2. For AGS and LoVo cells, the raspberry extract was considerably more effective than the equivalent concentration of ascorbic acid at inhibiting cell growth. MCF-7 breast cancer cells proved to be much more resistant to killing by the berry extract, and for these, the antioxidant effect played a larger role in cell killing.

Cells from all 3 lines were examined by trypan blue staining 48 hours after plating. Untreated cells completely excluded trypan blue, whereas a large fraction of those treated with raspberry extract accumulated the stain, which confirmed that the observed decreases in MTS absorbance were the result of cell death. To determine whether the mechanism of killing was caspase-induced apoptosis, we did a preliminary assay using the AGS stomach cancer cells. Cells treated with 10% raspberry extract were tested for activities of caspases 3/7, 8, and 9. Because the caspase activities for the treated and untreated cells were essentially
the same for these caspases, the 3 cell lines were tested for the effect of a caspase inhibitor on berry extract-induced cell death. For cultures treated with 10% berry extract, the pan-caspase inhibitor z-VAD-fmk was found to have no effect on cell death (Fig. 1).

AGS cells treated with 10% berry extract were stained with 4′-6-diamidino-2-phenylindole and Texas Red-X phalloidin to visualize the chromatin and F-actin, respectively. Examination with confocal microscopy indicated condensation of the chromatin and disorganization of the cytoskeletal structure.

4. Discussion

The data presented clearly indicate that an aqueous extract of Meeker red raspberries is capable of killing cancer cells from different tissues of origin. These data are in agreement with the results of others [9,16,21]. Here, we show that although part of this effect can be attributed to antioxidants present in the berry juice; for colon and stomach cancer cells, antioxidant activity probably plays a minor role in the killing. Ascorbic acid is capable of killing the 3 cell types and may even be the dominant factor in the inactivation of the MCF-7 breast cancer cells. This results from its antioxidant properties because addition of HCl of the same pH had no effect on cell survival. The stomach and colon cancer cells proved to be much more sensitive to the berry extract, even though the antioxidant effect played a much smaller role in cell killing of these cells. Data presented elsewhere (submitted) show that AsPC-1 pancreatic cancer cells were killed by the extract also, with sensitivity similar to that of the MCF-7 cells. Previous studies have reported a lack of correlation between antioxidant capacity and antiproliferative capacity in comparisons with different berry extracts. However, in such studies, it is impossible to evaluate the contribution of the antioxidant effect alone because various components of the extracts can affect other aspects of cell survival and proliferation by binding to cellular receptors and altering regulatory pathways (eg, NF-κB). Ascorbic acid should only affect antioxidant capacity and pH. The buffering capacity of the medium minimizes any effect of the pH of the ascorbic acid or the berry extract on cell survival as indicated by the lack of killing by HCl solutions at the same pH.

Halvorsen et al [22] performed an extensive survey of the antioxidant capacities of various types of dietary plants, among which were 19 different types of berries. Raspberries were among those with the highest levels of antioxidants. Liu et al [16] compared the antioxidant content of 4 different varieties of raspberries and measured the ability of acetone extracts from these to inhibit growth of Hep G2 liver cancer cells. They found that the antioxidant activity was directly correlated with the total amount of phenolics and flavonoids, but there was no relationship between this and antiproliferative activity. McDougall et al [21] examined raspberry extracts enriched for the polyphenol component by column chromatography. They found inhibition of HeLa cervical cancer cell proliferation by these extracts. The ellagitannin-rich fraction was found to be much more effective than the anthocyanin fraction for reducing proliferation. Seeram et al [23] compared the growth inhibition by methanol-HCl extracts of several types of berry on oral, colon, breast, and prostate cells and found the relative IC50 values to vary with cell type for each particular type of berry. Using clarified but unfractionated plant juices, Boivin et al [9] compared the antioxidant activities and antiproliferative activities of 14 different types of berries. Raspberries were among the most effective at blocking growth of several types of tumor cells, but when extracts from the various types of berry were compared, no correlation was found between antioxidant activity and antiproliferative activity.

Although trypan blue staining confirmed that the decrease in MTS absorbance resulted from cell death, the mechanism of killing remains to be determined. Ross et al [24] studied the antiproliferative effects of raspberries on HeLa cells and hypothesized that ellagic acid released by degradation of the ellagitannins could have caused apoptosis in this system. However, in the experiments reported here, the treated cells did not exhibit morphological changes that are characteristic of apoptosis. Also, caspases 3/7, 8, and 9 were not activated, and the pan-caspase inhibitor z-VAD-fmk had no effect on the extent of cell death. Boivin et al [9] also found no evidence for caspase-dependent apoptosis. They did observe suppression of the tumor necrosis factor–induced NF-κB pathway. One possible mechanism for berry-induced cancer cell death could be autophagy, which is a major factor in the killing of MCF-7 cells by resveratrol [25].

In summary, our data support the hypothesis that the antioxidant capacity of a water extract from red raspberries is capable of inhibiting proliferation of tumor cells, but other...
components of the extract are responsible for much of its antiproliferative effect. In addition, the relative contributions of these 2 effects are dependent on cell type. Although in vitro data can clearly show that extracts of this type can kill cancer cells in culture, extrapolation to in vivo systems is cautioned. Many components of the extracts will be metabolically altered after consumption. Also, many effects of the extract are probably indirect. Changes in gene expression through epigenetic effects induced by the extracts or enhancement of immune attack on tumor cells are among the changes in the host that could be induced by these extracts.

Acknowledgment

Funding for this study was provided by the Oregon Raspberry and Blackberry Commission, the Washington Red Raspberry Commission, the Cancer Research Fund donated to Clemson University by James and Carolyn Creel and by the Healthcare Genetics Program, Department of Nursing, Clemson University, Clemson, SC.

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