Dietary Consumption of Black Raspberries or Their Anthocyanin Constituents Alters Innate Immune Cell Trafficking in Esophageal Cancer

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Abstract

Freeze-dried black raspberries (BRB), their component anthocyanins (AC), and a metabolite of BRB ACs, protocatechuic acid (PCA), inhibit the development of esophageal cancer in rats induced by the carcinogen, N-nitrosomethylbenzylamine (NMBA). All three components reduce inflammation in the esophagus and in plasma. The present study determined the relation of changes in inflammatory markers to infiltration of innate immune cells in NMBA-treated esophagus. Rats were injected with NMBA (0.35 mg/kg) for 5 weeks while on control diet. Following NMBA treatment, rats were fed diets containing 6.1% BRB powder, an AC-rich fraction of BRBs (3.8 mol/g), or 500 ppm PCA. At weeks 15, 25, and 35, inflammatory biomarker expression in the plasma and esophagus was quantified, and infiltration of immune cells in the esophagus was examined. At all three time points, BRB, AC, and PCA similarly affected cytokine production in the esophagus and plasma of NMBA-treated rats, relative to the NMBA-only control. These included decreased expression of the proinflammatory cytokine IL1β and increased expression of the anti-inflammatory cytokine IL10. Moreover, all three diets also increased the expression of IL12, a cytokine that activates both cytolytic natural killer and CD8+ T cells. In addition, the three diets also decreased infiltration of both macrophages and neutrophils into the esophagus. Overall, our results suggest that another mechanism by which BRBs, ACs, and PCA inhibit NMBA-induced esophageal tumorigenesis is by altering cytokine expression and innate immune cell trafficking into tumor tissues.

Introduction

Esophageal cancer continues to be the third most common gastrointestinal malignancy as well as the sixth most frequent cause of cancer in the world. The two types of esophageal cancer include squamous cell carcinoma (SCC) and adenocarcinoma, with SCC being the predominant form of the disease worldwide (1, 2). Preclinical studies in our laboratory have shown that a chemopreventive approach using whole fruits and vegetables or their natural constituents has potential for prevention of esophageal SCC. Specifically, we have demonstrated the effectiveness of black raspberries (BRB) and their bioactive components in preventing the development of N-nitrosomethylbenzylamine (NMBA)-induced esophageal squamous cell papillomas in rats, some of which progress to carcinomas (3–6).

BRBs have significant concentrations of many polyphenolic compounds that are chemopreventive, including anthocyanins (AC), ellagic acid, quercetin, and ferulic acid (4, 5). The mechanisms by which BRBs and their component ACs impede tumorigenesis in the rat esophagus include reducing inflammation, cell proliferation, and angiogenesis, as well as stimulating apoptosis, cell differentiation, and cell adhesion (6). The inhibitory effects that BRBs and ACs have on inflammatory and angiogenic biomarkers in the esophagus are especially profound, including their ability to modulate the mRNA and protein expression of COX-2, inducible nitric oxide synthase (iNOS), NF-kB, IL1β, soluble epoxide hydrolase (sEH), pentraxin-3 (PTX3), CD34, VEGF, and HIFα (6–9). BRB ACs are more effective at reducing inflammation than 5-aminosalicylic acid, a common anti-inflammatory drug in vitro (10). In addition, protocatechuic acid (PCA), a major microbial metabolite of BRB ACs (11, 12), prevents NMBA-induced esophageal tumorigenesis, at least in part, by reducing biomarkers of inflammation and angiogenesis (13). In all studies to date, the reduction of inflammatory and angiogenic biomarkers by BRBs and their constituents correlate with reduced tumor multiplicity and burden in the NMBA-induced rat-esophageal cancer model (3, 4, 6–9, 13).

Inflammatory cytokine expression changes in human esophageal SCC (14, 15). The cytokine PTX3 is downregulated in human esophageal SCC tissue and cell lines (16). PTX3 decreases neutrophil accumulation into sites of localized inflammation by binding P-selectin on endothelial cells, thus blocking the neutrophil rolling adhesion migration process (17). We reported that dietary intake of BRBs, BRB ACs, or PCA increases PTX3 expression.

in NMBA-treated rat esophagi, suggesting that whole berries and their constituents may reduce neutrophil trafficking within the esophagus. In addition, cytokines II.1β and II.6, associated with the proinflammatory macrophage subtype M1 (18–20), are over-expressed in human esophageal SCC and are associated with a poor prognosis (21, 22). BRBs may be protective here through reduction of inflammation in humans by inhibiting II.1β expression (23). In addition, elevated II.4, a marker for M2 tumor-associated macrophages (24), is observed in the plasma of human esophageal SCC patients (25). This macrophage subtype is associated with a more aggressive form of esophageal SCC in humans (26). Because M2 macrophages are generally anti-inflammatory (20), higher numbers in esophageal tissue may inhibit the activity of other killer immune cells, thus preventing immune cell–mediated tumor cell destruction (27). BRB ACs and PCA can down-regulate the expression of II.4 by rat innate immune cells in vitro (28). Although BRBs and BRB ACs can decrease the markers associated with both macrophage subtypes (23, 28), it is not known whether this is due to inhibition of macrophage accumulation.

Finally, BRBs and their component ACs are capable of reducing angiogenesis in the esophagus of NMBA-treated rats by reducing microvessel density (MVD) and VEGF expression (6, 8). This decrease in blood vessel density may lower entry sites for lymphoid cells, leading to reduced accumulation of inflammatory cells in the esophagus. These associations suggest that BRBs and their constituents may be capable of altering immune cell trafficking within the esophagus.

The Fischer-344 (F-344) rat model has been used extensively in our laboratory to investigate the etiology, biology, and chemoprevention of NMBA-induced esophageal tumorigenesis. Esophageal tumors (predominately papillomas and occasionally carcinomas) are induced within 25 to 35 weeks by multiple s.c. injections of NMBA at a concentration of 0.3 to 0.5 mg/kg/injection. Preneoplastic changes, as well as changes in the expression of inflammatory and angiogenic biomarkers, closely resemble changes observed in human esophageal SCC. Lesions follow the progression sequence from normal>hyperplasia>hyperplasia with dysplasia>anaplasia>to esophageal SCC (4, 29). Dysregulated changes in inflammatory biomarker expression, namely COX-2, iNOS, and NF-κB, have been reported during tumor development in this model (6, 7, 9, 29, 30). The present study evaluates whether BRBs, their component ACs, and PCA alter immune cell trafficking within the esophagi of NMBA-treated F-344 rats, and whether these changes are associated with inhibition of tumorigenesis. Initial studies evaluated the expression of 24 pro- and anti-inflammatory cytokines in rat plasma at early time points during the progression of esophageal tumorigenesis. Changes in the expression of these cytokines in the plasma and esophagus at the end of the 35-week bioassay were then confirmed using a smaller subset of markers. Cytokine expression was then related to the presence of specific innate immune cells within the esophagus, including macrophages, neutrophils, and natural killer (NK) cells, all of which have been shown to be associated with esophageal SCC progression in humans (26, 31–33).

Materials and Methods

BRB powder

Freeze-dried BRBs (Rubus occidentalis) were obtained from BerriProducts, Inc. and Decker Farms, Inc. and stored at 4°C in vacuum-sealed bags at the Medical College of Wisconsin (MCW). One hundred grams of powder from each vendor was shipped to Covance Laboratories (Madison, WI) for quantification of the content of minerals, phenolic acids, phytosterols, vitamins, carotenoids, fungicides, pesticides, and herbicides as described in Kresty and colleagues (4). The content of the three major ACs (cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, and cyanidin-3-O-syringoside) in both lots of powder were determined by high-performance liquid chromatography (HPLC) in the laboratory of Dr. Stephen S. Hecht. After analysis, approximately 200 kilograms of powder was shipped from the MCW to Dr. Hecht’s laboratory for preparation of the AC-enriched fraction, and the remaining 150 kg was used to conduct the carcinogenesis bioassay at the MCW.

Preparation of the AC-enriched fraction

The AC-enriched fraction was prepared according to the protocol described in Peiffer and colleagues (13). After preparation, the AC-enriched fraction was shipped on dry ice to the MCW where it was stored at −80°C until used in the bioassay.

Chemicals

PCA (97% pure) was purchased from Sigma-Aldrich. NMBA was purchased from Ash Stevens and was found to be >98% pure via HPLC.

Diet preparation

Each diet was prepared using a Hobart mixer. BRB powder, the AC-enriched fraction, and PCA were added to the American Institute of Nutrition-76A (AIN-76A) synthetic diet (Dyets, Inc.) at the appropriate concentrations and mixed in the diet for 20 minutes. Diets were evaluated for content of ACs and PCA via HPLC to ensure homogeneity.

Animals

Male F-344 rats, 3 to 5 weeks of age, were obtained from Harlan Sprague-Dawley. Two animals were housed per cage under standard conditions (20 ± 2°C, 50 ± 10% relative humidity, 12-hour light/dark cycles). Twice-weekly cage changes were done to maintain hygienic conditions. Food intake and body weights were taken weekly over the course of the study. Animals were kept according to the recommendations of the American Association of Laboratory Animal Care.

Bioassay

Upon their arrival in our animal facility, rats were randomly assigned to five separate groups and placed on an AIN-76A control diet. Rats then received s.c. injections containing 0.2 mL of either 20% DMSO in water (vehicle control) or 20% DMSO + NMBA (0.35 mg/kg body weight) three times per week for 5 weeks. After the injections, rats were fed the following diets for the duration of the bioassay: AIN-76A (Group 1, vehicle control); AIN-76A (Group 2, NMBA control); AIN-76A supplemented with 6.1% BRB powder (Group 3, BRBs + NMBA); AIN-76A supplemented with 3.8 μmol ACs/g (Group 4, ACs + NMBA); AIN-76A supplemented with 500 ppm PCA (Group 5, PCA + NMBA). Note that 6.1% BRB supplemented in the control diet was chosen to match the AC content of previous studies (6–8, 13), while Group 4 was given a diet containing 3.8 μmol/g of AC to match the AC content of Group 3. In addition, the starch content of the Group 3 diet was reduced by 6.1% to match the caloric content of the Group 1, 2, 4,
and 5 diets. At weeks 15, 25, and 35, rats from each group were euthanized, and their plasma and esophagi were obtained for molecular analysis. The tumor data from this study have been reported (13).

**Cytokine measurement in plasma**

Whole blood was collected in heparin-coated tubes (BD Biosciences) and centrifuged at 3,000 × g for 10 minutes. Plasma was collected and stored at −80°C until analysis. At weeks 15 and 25, concentrations of immune markers in the plasma were determined using a rat 24-plex assay. These markers included 13 cytokines (IL1α, IL1β, IL2, IL4, IL5, IL6, IL10, IL12(p70), IL13, IL17α, IL18, TNFα, and IFNγ), 5 chemokines (MIP-1α, MIP-3α, RANTES, MCP-1, and GRO/KC), and 6 growth factors (IL7, G-CSF, GM-CSF, VEGF, EPO, and M-CSF; Bio-Plex Suspension Array System; Bio-Rad) following the manufacturer’s instructions. Plasma samples were diluted 1:4 in sample diluent and incubated for 30 minutes at room temperature (300 rpm agitation) with capture antibody-coupled magnetic beads in the manufacturer-supplied 96-well plates. Following three washes in the Bio-Plex Pro wash station (Bio-Rad), samples were incubated for 30 minutes in the dark at room temperature (300 rpm agitation) with the supplied biotinylated detection antibody. Each marker was detected by the addition of streptavidin–phycoerythrin and quantified using the BioPlex array reader software (Bio-Rad). Week 35 plasma samples were run on separate single-analyte ELISA for IL1β (Sigma-Aldrich), IL12 (p70; Cusabio), and IL10 (Life Technologies).

**Esophagus tissue samples**

The esophagi were cut longitudinally in half; one half was snap frozen in liquid nitrogen for protein extraction and the other half was fixed for 24 hours in 10% neutral-buffered formalin and stored in PBS for subsequent quantification of preneoplastic lesions as well as for immunohistochemical analysis.

**Immunoblotting**

Esophagi were disrupted via sonication and solubilized in modified RIPA buffer (50 mmol/L Tris-HCl, pH 7.3, 150 mmol/L NaCl, 0.25% (v/v) sodium deoxycholate, 1.0% (v/v) NP-40, 0.1% (v/v) SDS, and 1 mmol/L EDTA) supplemented with Protease Inhibitor Cocktail Set III (EMD Biosciences) and 10 mmol/L orthovanadate, 40 mmol/L glycercophosphate, and 20 mmol/L sodium fluoride as phosphatase inhibitors. Lysates were then centrifuged at 10,000 rpm for 10 minutes at 4°C, and the supernatant was collected. Lysates were standardized using a DC protein assay kit (Bio-Rad) to 2 μg/μL. A total of 50 μg of protein was resolved on precasted SDS-PAGE gels (Bio-Rad). Blots were prepared using polyvinylidene difluoride membranes soaked in methanol and run on a Trans-Blot TurboTM Transfer System (Bio-Rad). Blots were blocked in 5% BSA for 60 minutes and then incubated with primary antibody to IL1β (Abcam), IL10 (Life Technologies), or IL12 (R&D Systems, Inc.). An anti-rabbit secondary antibody labeled with horseradish peroxidase (HRP; Cell Signaling Technology) was used in conjunction with an ECL detection kit (GE Healthcare) to detect the presence of IL1β and IL10, whereas an anti-goat secondary antibody labeled with HRP (Santa Cruz Biotechnology) was used with the same ECL detection Kit to visualize the presence of IL12. For each cytokine marker, all animals whose plasma was run at week 35 via ELISA were analyzed for esophageal cytokine expression using Western blot (n = 5 for DMSO control; n = 8 per diet group for NMBA-treated animals). Densitometric analysis of the relative protein abundance compared with β-actin (Cell Signaling Technology) was determined using the ImageLab 4.0.1 software (Bio-Rad).

**Immunohistochemistry**

Based on the literature, the markers CD68 and CD163 were used to mark macrophages, myeloperoxidase (MPO) for neutrophils, and CD161 as well as CD244 for NK cells (34–37). Slides were deparaffinized, hydrated in water and individual antigens stained using a DAKO Autostainer Plus. Tissue sections to be stained for CD163 were treated with a citrate buffer at pH 6 for 40 minutes for antigen retrieval (DAKO Target Retrieval, S1699), and sections to be stained for CD68, MPO, CD161, and CD244 were digested using Enzyme 1 solution (Leica; AR9551) for 10 minutes at room temperature. A standard labeled streptavidin–biotin immunohistochemical approach was used to stain for CD68 (ABD Serotec; MCA341R), CD163 (ABD Serotec; MCA342GA), MPO (Thermo Scientific; PA5-16672), CD161 (Biorbyt; orb100571), and CD244 (BiossUSA; bs-2470R). Following peroxidase (15 minutes; DAKO; S2003), avidin/biotin (15 minutes each; VECTOR; sp-2001), and protein blocking (30 minutes; DAKO; X0909), the primary antibodies (each diluted 1:100) for these antigens were incubated for 1 hour at room temperature. For CD68 and CD163, biotinylated secondary antibody (Donkey anti-Mouse; Jackson Immunoresearch; 715-066-151) was applied for 30 minutes followed by HRP-labeled streptavidin (DAKO; P039701-2). Visualization was achieved using diaminobenzidine (DAB+: DAKO; K3468). All slides were counterstained with Mayer’s hematoxylin (DAKO; S3309) and coverslipped using synthetic mounting media. MPO, CD161, and CD244 were detected using the Rabbit on Rodent HRP-Polymer (Biocare; RMR622). Peroxidase blocking, protein blocking, and primary antibody incubations were completed as described above, without avidin/biotin blocking. Following incubation for 20 minutes with the Rabbit and Rodent-HRP polymer Kit, visualization was achieved using diaminobenzidine. Slides were counterstained with Mayer’s hematoxylin and coverslipped using a synthetic mounting media. All processing and staining for immune cell trafficking was conducted by the Histology CORE at the MCW.

**Determination of immune cell counts**

Immune cell counting was performed under ×200 and ×400 magnification (×20 objective and ×10 and ×40 ocular, respectively). Slides were viewed and photographed using a Nikon microscope with a high-resolution camera and an image analysis software (Nikon: NIS-Elements). Any brown staining from an individual cell was considered a single positive cell for the appropriate marker (CD68, CD163, MPO, CD161, or CD244). The total length of each esophagus varied from 6.5 to 9.5 cm, with the average length of the esophagi being 7.1 cm by ruler. Immune cell counts were calculated by dividing the total number of positive cells of each esophagus by the length of the esophagus. A total of 37 esophagi were stained for each marker (n = 5 for DMSO, n = 8 per diet group for NMBA-treated animals). This yielded the number of cells/cm of esophagus tissue. Immune cell counting was performed by a single investigator blinded to the treatment group. Cells that stained positive for CD68, but did not stain for CD163, were considered CD68+/CD163− cells. Lesion-specific immune cell counts were quantified by first scoring the esophagus for areas of normal epithelium, hyperplasia, dysplasia, and papilloma tissue as indicated by
Kresty and colleagues in 2001 (4). The number of positive immune cells was then counted and recorded for normal, hyperplastic, dysplastic, and papillomatous tissues.

**Quantification of MVD**

Esophageal MVD was measured using the protocol of Chen and colleagues (8). Briefly, slides were stained with rat CD34-specific antibody expressed on vascular endothelial cells and viewed using a Nikon microscope with a high-resolution spot camera linked to computer-loaded image analysis software (Nikon: NIS-Elements). Vessel counting was performed under ×200 magnification (20× objective and 10× ocular). Any brown-stained endothelial cells or cell clusters in the form of vessels that were separate from adjacent blood vessels, tumor cells, or other connective tissue were considered single countable microvessels. Total length of each esophagus ranged from 6.5 to 9.0 cm, with the average being 7.1 cm in length. The MVD was calculated by dividing the total number of microvessels in each esophagus by the length of the esophagus. MVD in 5 esophagi/group, or a total of 25 esophagi, was counted by an investigator blinded to the treatment group.

**Statistical analysis**

Body weight, food consumption, plasma cytokine levels, immunohistochemical staining, and Western blot density were compared using ANOVA via Prism 5 (GraphPad). A *P* value < 0.05 was considered to be statistically significant. The Tukey test was the post-hoc test to which all experimental groups were compared with the AIN-76A diet + NMBA injection group.

**Results**

**General observations**

No significant differences were seen in animal body weights or food consumption between experimental groups throughout the course of the study (*P* > 0.05; data not shown). Before fixing and freezing each esophagus, the tumors on the surface of the esophagus were counted under a dissecting microscope to quantify the tumor response. Lesions with the histologic features of squamous cell papilloma as reported by Wang and colleagues were counted (*P* > 0.05; Fig. 2A). No significant differences were seen in the stroma or muscle tissue of NMBA-treated rats at any time point (*P* > 0.05; ref. 13).

**Effects on plasma cytokine expression**

Cytokines that were significantly altered in NMBA-treated rats fed experimental diets relative to NMBA controls are summarized in Table 1. At week 15, IL2, RANTES (CCL5), TNFα, IL1β, IL4, and VEGF were all significantly reduced in NMBA-treated rats fed BRB, AC, and PCA diets compared with NMBA controls (*P* < 0.05; Table 1). These markers plus IL6 were also reduced at week 25 in the plasma of NMBA-treated rats given these same diets (*P* < 0.05; Fig. 2A). In contrast, IL5, IL12, IL17A, IL18, GM-CSF, IFNγ, and MIP-1α (CCL3) were significantly increased in the plasma of the BRB, AC, and PCA-fed groups compared with NMBA control animals at both weeks 15 and 25 (*P* < 0.05; Table 1). IL10 was also present at higher concentrations in the plasma of these groups at week 25 (*P* < 0.05; Fig. 2A). No significant differences of IL1α, IL13, MIP-3α, MCP-1, GRO/KC, IL7, G-CSF, and GM-CSF in the plasma were observed at any time points (*P* > 0.05, data not shown).

**Effects on immune cell movement in the esophagus**

The proinflammatory cytokines IL1β, IL10, and IL12 were measured in both plasma and esophageal tissues due to limited availability of esophageal tissues. The proinflammatory cytokine IL1β was measured at 35 weeks because of its association with poor prognosis of esophageal SCC (21). IL12 was measured due to its stimulation of CD8+ T cells and NK cells for tumor eradication (38). Finally, we compared expression of these two cytokines with the anti-inflammatory cytokine IL10 at week 35 (39). Results from all of these measurements are presented below.

**Table 1. Plasma cytokine expression**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Week 15 DMSO</th>
<th>Week 15 BRB + NMBA</th>
<th>Week 15 AC + NMBA</th>
<th>Week 15 PCA + NMBA</th>
<th>Week 25 DMSO</th>
<th>Week 25 BRB + NMBA</th>
<th>Week 25 AC + NMBA</th>
<th>Week 25 PCA + NMBA</th>
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<tbody>
<tr>
<td>IL1β</td>
<td>85.6 (3.5)</td>
<td>19.7 (1.8)</td>
<td>12.8 (0.9)</td>
<td>12.9 (5.3)</td>
<td>90.6 (3.1)</td>
<td>27.6 (1.6)</td>
<td>90.6 (3.1)</td>
<td>72.4 (1.2)</td>
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<tr>
<td>IL2</td>
<td>410.4 (9.2)</td>
<td>779.8 (12.4)</td>
<td>314.2 (4.8)</td>
<td>283.1 (6.2)</td>
<td>239.5 (2.0)</td>
<td>485.9 (27.7)</td>
<td>905.1 (9.2)</td>
<td>389.4 (7.6)</td>
</tr>
<tr>
<td>IL4</td>
<td>375.7 (2.8)</td>
<td>75.1 (0.4)</td>
<td>46.9 (0.8)</td>
<td>50.1 (0.2)</td>
<td>35.2 (1.7)</td>
<td>421.5 (1.5)</td>
<td>912.0 (1.0)</td>
<td>469.1 (1.8)</td>
</tr>
<tr>
<td>IL5</td>
<td>183.1 (2.2)</td>
<td>180.7 (5.0)</td>
<td>682.7 (4.8)</td>
<td>698.2 (3.1)</td>
<td>754.6 (10.5)</td>
<td>1815.2 (7.7)</td>
<td>1715.1 (7.7)</td>
<td>6305.0 (17.1)</td>
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<td>IL6</td>
<td>151.3 (3.8)</td>
<td>198.2 (6.6)</td>
<td>2212.5 (5.2)</td>
<td>1881.8 (5.5)</td>
<td>1909.5 (5.0)</td>
<td>1979.7 (9.4)</td>
<td>3072.7 (4.0)</td>
<td>2546.8 (8.6)</td>
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<tr>
<td>IL10</td>
<td>269.0 (9.9)</td>
<td>1662.7 (8.8)</td>
<td>1825.8 (8.0)</td>
<td>1704.5 (5.2)</td>
<td>1745.3 (3.1)</td>
<td>1475.6 (16.9)</td>
<td>1424.3 (4.8)</td>
<td>2901.0 (9.0)</td>
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<td>IL12(p70)</td>
<td>476.2 (7.7)</td>
<td>497.7 (5.3)</td>
<td>1085.8 (0.7)</td>
<td>993.4 (9.9)</td>
<td>1352.4 (4.2)</td>
<td>496.6 (6.5)</td>
<td>388.8 (4.1)</td>
<td>988.8 (5.2)</td>
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<tr>
<td>IL17A</td>
<td>25.4 (3.0)</td>
<td>19.9 (2.6)</td>
<td>87.5 (1.8)</td>
<td>609.0 (8.0)</td>
<td>184.3 (0.6)</td>
<td>334.1 (6.6)</td>
<td>289.0 (2.2)</td>
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<td>IL18</td>
<td>155.1 (26)</td>
<td>2025.2 (22.5)</td>
<td>358.9 (13.8)</td>
<td>356.0 (1.9)</td>
<td>3978.5 (5.7)</td>
<td>3270.4 (4.7)</td>
<td>3099.9 (10.7)</td>
<td>4213.1 (3.3)</td>
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<td>GM-CSF</td>
<td>451.2 (2.7)</td>
<td>356.5 (5.4)</td>
<td>983.1 (0.7)</td>
<td>968.8 (2.9)</td>
<td>1245.5 (5.1)</td>
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<td>405.0 (9.9)</td>
<td>1357.0 (4.9)</td>
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<td>IFNγ</td>
<td>37.1 (2.8)</td>
<td>39.2 (0.6)</td>
<td>139.1 (3.9)</td>
<td>98.5 (1.0)</td>
<td>147.2 (5.7)</td>
<td>59.7 (4.4)</td>
<td>498.9 (8.7)</td>
<td>96.2 (2.6)</td>
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<td>MIP-1α</td>
<td>174.8 (4.0)</td>
<td>1654.4 (9.7)</td>
<td>2441.2 (26.8)</td>
<td>3991.5 (5.1)</td>
<td>3845.15 (15.7)</td>
<td>2797.7 (17.1)</td>
<td>2655.3 (12.8)</td>
<td>4983.1 (5.5)</td>
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<td>RANTES</td>
<td>2689.6 (2.9)</td>
<td>557.7 (4.5)</td>
<td>2013.2 (1.8)</td>
<td>2514.8 (1.1)</td>
<td>3131.3 (3.6)</td>
<td>3524.6 (4.6)</td>
<td>3565.2 (5.7)</td>
<td>3195.9 (5.0)</td>
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<td>TNFα</td>
<td>255.2 (2.5)</td>
<td>729.0 (0.7)</td>
<td>521.0 (8.0)</td>
<td>440.2 (4.7)</td>
<td>570.0 (7.7)</td>
<td>308.0 (9.9)</td>
<td>1063.0 (6.4)</td>
<td>596.0 (7.0)</td>
</tr>
<tr>
<td>VEGF</td>
<td>59.6 (1.0)</td>
<td>585.3 (2.0)</td>
<td>5430.2 (20.0)</td>
<td>591.2 (2.5)</td>
<td>413.3 (1.0)</td>
<td>245.0 (1.1)</td>
<td>645.0 (1.2)</td>
<td>417.4 (5.0)</td>
</tr>
</tbody>
</table>

Note: All diets mixed with AIN-76A.

*Significantly lower relative to NMBA control (Group 2; *P* < 0.05).

*Significantly higher relative to NMBA control (Group 2; *P* < 0.05).
The plasma samples of animals at week 35 were run on Single-Analyte ELISA kits for IL1β, IL10, and IL12 with the results summarized in Fig. 1A. IL1β was significantly lower in the plasma of rats fed BRBs, ACs, or PCA compared with NMBA control rats ($P < 0.05$; Fig. 1A). In contrast, IL10 (Fig. 1A) and IL12 (Fig. 1A) levels were significantly higher in the plasma of the same groups at week 35 following the trend that was observed at week 25 ($P < 0.05$). Together, these results suggest that BRBs, their constituent ACs, and PCA are capable of altering cytokine expression globally in the plasma of NMBA-treated rats.

Cytokine expression in the esophagus. Cytokine levels in the esophagus were determined by Western blot only at week 35 because esophageal tissues at weeks 15 and 25 were insufficient for analysis. A representative blot depicting these cytokines is shown in Fig. 1B. IL1β expression was significantly reduced in the esophagus by BRB, AC, and PCA ($P < 0.05$; Fig. 1C), which correlated with plasma concentrations at week 35 ($P < 0.05$). Together, these results suggest that BRBs, their constituent ACs, and PCA are capable of altering cytokine expression globally in the plasma of NMBA-treated rats.

Innate immune cell trafficking in the esophagus. Immunohistochemistry for each marker was performed on 8 NMBA-treated esophagi/experimental group ($n = 8$, $n = 5$ for DMSO control) to determine if cytokine levels both in the plasma and in the esophagus correlated with altered innate immune cell trafficking. This was done only at week 35 due to a lack of esophageal tissue at weeks 15 and 25. We focused on innate immune cells, as these have been specifically indicated in esophageal SCC in humans (26, 31–33), and the results are described below.
all significantly reduced the migration of inflammatory macrophages into the esophagus (\(P < 0.05\)). Interestingly, as the severity of the lesion increased, the experimental diets had a stronger inhibitory effect on CD68\(^+\) CD163\(^+\) cell accumulation compared with the NMBA control tissue (Fig. 2C and D). BRB, AC, and PCA diets did not reduce CD68\(^+\) CD163\(^+\) cell accumulation in normal or hyperplastic epithelium in the esophagus of rats treated with NMBA (\(P > 0.05\); Fig. 2C), but they significantly reduced the infiltration of inflammatory macrophages into dysplastic lesions and esophageal papillomas (\(P < 0.05\); Fig. 2D).

We also investigated infiltration of CD163\(^+\) macrophages, as they are tumor-associated macrophages (with M2 phenotype). A visual representation of the staining for CD163\(^+\) is provided in Fig. 3A. At week 35, all three diets significantly reduced total CD163\(^+\) macrophage accumulation in the esophagi of NMBA-treated rats (\(P < 0.05\); Fig. 3B). The BRBs, ACs, and PCA caused a significant reduction in CD163\(^+\) macrophages in all histopathologic categories, including normal epithelium, hyperplasia, dysplasia, and papilloma tissue (\(P < 0.05\); Fig. 3C and D). These results indicate that BRB, AC, and PCA can prevent CD163\(^+\) macrophage trafficking into NMBA-treated esophageal tissues.

**Esophageal neutrophils.** Cells that were positive for MPO were considered neutrophils (35). A representative image of the total neutrophil accumulation across all groups is shown in Fig. 4A. The BRB, AC, and PCA diets all significantly reduced total neutrophil accumulation in NMBA-treated esophageal tissue (\(P < 0.05\); Fig. 4B). This reduction was observed across all histopathologic categories of the esophagus, including normal epithelium, hyperplasia, dysplasia, and papilloma tissue (\(P < 0.05\); Fig. 4C and D).

In addition, PCA was significantly more effective at reducing neutrophil accumulation in papilloma tissue than BRBs or their ACs (\(P < 0.05\); Fig. 4D).

**NK cells in the esophagus.** Two separate markers were used to quantify NK-cell accumulation, CD161 and CD244 (24); however, neither the esophagi nor the positive control tissues (thymus and liver) yielded measurable responses (Stoner et al., unpublished data). CD161 (36) and CD244 were reported to be NK-cell activation markers (37). None of the cells in either the esophagus or the positive control tissue were stained for CD161, although CD244-stained tissues had high amounts of nonspecific or background staining, making individual NK-cell identification unreliable (data not shown).

**Angiogenesis**

**MVD via CD34 staining.** Both BRBs and ACs have antiangiogenic properties (6, 8), but it is not known whether this is associated
with changes in immune cell trafficking within the esophagus. The results of CD34 MVD staining are summarized in Fig. 5A and B. A representative image for CD34 staining is illustrated in Fig. 5A. As expected, BRBs and ACs significantly reduce MVD in the esophagus at week 35 ($P < 0.05$; Fig. 5B). In addition, PCA had a similar effect on inhibiting angiogenesis in the NMBA-treated rat esophagus compared with the NMBA control ($P < 0.05$; Fig. 5B). Overall, MVD was reduced by 43.1%, 38.1%, and 34.0% by BRBs, ACs, and PCA, respectively (Fig. 5B).

**Discussion**

Supplementing a synthetic AIN-76A diet with BRBs, ACs, or PCA is effective at preventing NMBA-induced esophageal tumorigenesis in rats, in part, by reducing inflammatory biomarker expression and angiogenesis (6–8, 13). Specifically, biomarkers typically dysregulated in human esophageal SCC (40), including NF-κB, COX-2, and iNOS (41–43), are reduced in expression by BRBs, ACs, or PCA diets in the rat model of esophageal tumorigenesis (13). As expression of these inflammatory markers has been linked to the activity and trafficking of specific innate immune cells (19, 44), we undertook a study to investigate whether BRBs, ACs, and PCA alter immune cell accumulation in the esophagus. Through quantification of cytokines at weeks 15, 25, and 35 in the plasma, and at week 35 in the esophagus, we identified groups of cytokines that were associated with specific innate immune cells. We then determined whether the plasma and esophageal tissue levels of these cytokines correlated with immune cell trafficking in the rat esophagus through immunohistochemistry.

One trend observed was the ability of BRBs and their constituents to increase expression of cytokines associated with NK cells in the plasma at weeks 15 and 25. Of note, NK cells are an effective treatment in human gastrointestinal cancer (45), and higher NK-cell activity in human esophageal SCC is considered protective (31). IL12 and IL18 are associated with NK-cell activation and differentiation (46, 47), whereas IL17A has been linked to increased NK-cell accumulation (31). All three of these markers were upregulated in the plasma by BRBs, ACs, and PCA at weeks 15 and 25, while the expression of IL12 was also significantly
higher in the both the plasma and esophagus at week 35. Further, MIP-1α and IFNγ, cytokines secreted by NK cells (20, 48), were expressed at significantly more in the plasma of rats fed BRBs, ACs, or PCA at weeks 15 and 25, as well. This suggests that BRBs, ACs, and PCA may increase NK-cell activity in NMBA-induced esophageal tumorigenesis in rats. Unfortunately, we were unable to quantify NK-cell migration in esophageal tissue due to ineffective antibodies; however, further studies are currently under way to determine whether NK-cell accumulation is in fact changed by dietary intake of BRBs, ACs, or PCA.

Diets containing BRBs, ACs, or PCA altered macrophage-associated cytokines both in the plasma and the esophagus of NMBA-treated rats. The M1 macrophage–associated makers IL1β, IL6, and TNFα (20) were all significantly reduced by BRBs, ACs, and PCA in the plasma at weeks 15 and 25. In addition, IL1β expression was reduced by the same treatments in both the plasma and esophagus of NMBA-treated rats at week 35. Downregulation of these cytokines correlated with reduced CD68+CD163− macrophage accumulation in the esophagus. As higher CD68+ macrophage staining is associated with human esophageal SCC (33), these results suggest that BRBs, ACs, and PCA may positively alter the tumor microenvironment in the rat esophagus by reducing M1 macrophage accumulation and subsequent proinflammatory effects in the esophagus. In fact, our results show that BRBs, ACs, and PCA had the greatest effect of reducing CD68+CD163− macrophages in the most severe preneoplastic esophageal lesions. Specifically, the greatest reduction of the proinflammatory cells was illustrated in dysplastic lesions and in papillomas. This suggests that BRBs, ACs, and PCA may prevent progression of preneoplastic lesions into papillomas by reducing the accumulation of these proinflammatory immune cells. All three diets also reduced IL4 expression in the plasma of NMBA-treated rats at weeks 15 and 25. Interestingly, IL4 is a distinct marker for M2 or anti-inflammatory macrophages (26), which are commonly found in tumor tissue (20, 27) and are associated with a more aggressive form of human esophageal SCC (26). Correlating with plasma IL4 expression, BRBs, ACs, and PCA significantly reduced total M2 macrophage migration into the rat esophagus compared...
with the NMBA control. This decrease in CD163+ cells was observed across all preneoplastic lesion categories as well as in papilloma tissue.

Neutrophil accumulation in human esophageal SCC is associated with a more aggressive form of the disease and poor prognosis (32). We observed that BRBs, ACs, and PCA all reduced neutrophil accumulation at week 35 in preneoplastic lesions and in papillomas of NMBA-treated rats. This is another way in which these treatments positively alter immune trafficking in the esophagus. We reported recently that PCA is more effective at increasing the expression of PTX3 in NMBA-treated animals compared with rats fed either BRBs or ACs (13). The present data indicate that PCA is also more effective at increasing PTX3 expression in plasma and esophageal tissue, and PCA may be the most effective agent in achieving this. Because PTX3 expression is downregulated in human esophageal SCC tissue and cell lines (16), further studies should be conducted to evaluate whether there is a relationship between PTX3 and neutrophil levels in the esophagus of human esophageal SCC.

Finally, our results on the reductive effect BRBs and its constituents have on angiogenesis are consistent with findings from previous studies (6, 8). Specifically, BRBs, ACs, and PCA all significantly reduced MVD in the esophagus of NMBA-treated rats at week 35. This decrease in blood vessel formation may limit the ability of immune cells and cytokines to accumulate in the esophagus and therefore reduce inflammation in the tissue. The mechanism for this lowering of MVD in the esophagus can be attributed to at least two factors. First, as shown in the plasma at weeks 15 and 25, BRBs, ACs, and PCA reduced blood vessel growth factor VEGF. In addition, as CD163+ macrophages are associated with secreting cytokines that promote blood vessel formation (34, 49), BRBs and their constituents may indirectly reduce angiogenesis in the esophagus by preventing CD163+ cell accumulation in the tissue.

Overall, results from the present study illustrate that BRBs, ACs, and PCA are capable of altering cytokine expression in the plasma and esophagi of NMBA-treated rats, which correlates
with changes in immune cell trafficking. These changes are also correlated with reduction in tumor burden in the esophagus (13), indicating another protective effect BRBs, Acs, and PCA have on NMBA-induced esophageal SCC. We attribute the changes in cytokine and immune cells to a number of factors, including downregulation of immune cell–associated inflammatory biomarkers, such as COX-2 and NF-κB (19, 41, 43, 44); upregulation of inhibitory cytokines, such as PTX3 (13, 17); and the general anti-inflammatory effects of BRBs and their constituents (6, 23). As BRBs have been effective at reducing inflammation in other rodent cancer models, such as the colon (50), more studies are needed to investigate whether BRBs and their constituents alter immune cell trafficking in other types of cancer. In addition, human trials are needed to determine if similar effects of BRB and their constituents occur in human esophageal SCC and other cancers.

Disclosure of Potential Conflicts of Interest

G.D. Stoner has ownership interest in BerriProducts, LLC. No potential conflicts of interest were disclosed by the other authors.

References


Dietary Consumption of Black Raspberries or Their Anthocyanin Constituents Alters Innate Immune Cell Trafficking in Esophageal Cancer

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