A Hexane Fraction of American Ginseng Suppresses Mouse Colitis and Associated Colon Cancer: Anti-inflammatory and Proapoptotic Mechanisms

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Abstract
Ulcerative colitis is a chronic inflammatory condition associated with a high colon cancer risk. We have previously reported that American ginseng extract significantly reduced the inflammatory parameters of chemically induced colitis. The aim of this study was to further delineate the components of American ginseng that suppress colitis and prevent colon cancer. Among five different fractions of American ginseng (butanol, hexane, ethylacetate, dichloromethane, and water), a hexane fraction has particularly potent antioxidant and proapoptotic properties. The effects of this fraction were shown in a mouse macrophage cell line (ANA-1 cells), in a human lymphoblastoid cell line (TK6), and in an ex vivo model (CD4⁺/CD25⁻ primary effector T cells). A key in vivo finding was that compared with the whole American ginseng extract, the hexane fraction of American ginseng was more potent in treating colitis in a dextran sodium sulfate (DSS) mouse model, as well as suppressing azoxymethane/DSS-induced colon cancer. Furthermore, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) labeling of inflammatory cells within the colonic mesenteric lymph nodes was elevated in mice consuming DSS + the hexane fraction of American ginseng. Results are consistent with our in vitro data and with the hypothesis that the hexane fraction of American ginseng has anti-inflammatory properties and drives inflammatory cell apoptosis in vivo, providing a mechanism by which this fraction protects from colitis in this DSS mouse model. This study moves us closer to understanding the molecular components of American ginseng that suppress colitis and prevent colon associated with colitis. Cancer Prev Res; 5(4); 685–96. ©2012 AACR.

Introduction
Inflammatory bowel disease (IBD; ulcerative colitis and Crohn’s disease) is a group of chronic disorders of unknown etiology characterized by inflammation in the gastrointestinal tract (1) and associated with an increased risk of colon cancer (2). The histopathogenesis of ulcerative colitis–associated colorectal cancer involves a stepwise progression from inflamed, hyperplastic epithelia to flat dysplasia to adenocarcinoma (3). Colitis-associated colorectal cancer accounts for up to 5% of all colorectal cancers (4), and the incidence of colitis-associated colorectal cancer in patients with ulcerative colitis increases with age. The azoxymethane (AOM) tumor model has been used extensively to identify molecular mechanisms involved in the multistage progression of sporadic colorectal cancers (5). The addition of dextran sodium sulfate (DSS) to AOM has been used frequently because of its reproducibility, and the cyclical, dynamic nature of colitis replicates the flare-ups characteristic of human ulcerative colitis (6–8).

American ginseng (Panax quinquefolius) is a perennial native of North America, and ginseng is one of the most popular medicinal herbs used in the world (9). American ginseng has antioxidant properties and targets many key players involved in inflammation, including inducible nitric oxide synthase (iNOS), COX-2, and NF-κB (10). In a series of studies, we have recently reported that American ginseng suppresses the expression of inflammatory markers of colitis and prevents colon cancer associated with colitis (10–12). In general, active or inactive chemical entities obtained from ginseng species can be classified into 5 categories: saponins, polysaccharides, polyynes, flavonoids,...
and volatile oils (13). Ginseng’s saponins (generally called ginsenosides) and acidic polysaccharides of American ginseng have been the main focus of its pharmacologic activities (14–19). Water-soluble polysaccharides also have medicinal properties, including immunomodulating and antiinflammatory effects (13). Other putative active components of American ginseng, include polycyclics such as panaxynol and panaxol, which are nonpolar compounds (20). In contrast to ginsenosides and polysaccharides; polyynes, flavonoids, and volatile oils have been less studied and therefore less is known about their medicinal properties. To further delineate the putative active components of American ginseng against colitis, we have used bioassay-guided fractionation. In doing so, we show here that a hexane fraction of American ginseng is a potent antioxidant, can drive inflammatory cell apoptosis, and is more effective in its ability to ameliorate colitis and prevent colon cancer in mice than the whole American ginseng extract.

Materials and Methods

Bioassay-guided fractionation of American ginseng extract

The *P. quinquefolius* extract has been described previously in detail by our laboratory (10). For bioassay-guided fractionation, 10 g of American ginseng extract was dissolved in 150 mL of water and sequentially partitioned against 3 aliquots of hexane, dichloromethane, ethyl acetate, water, and butanol. The fractions were reduced to near dryness on a vacuum centrifuge, freeze dried, and their respective dry weights determined: water fraction, 7.320 g (i.e., 73% of the original material); butanol fraction, 0.064 g; dichloromethane fraction, 0.062 g; and hexane fraction, 0.044 g. Each fraction was then redissolved in a small volume of solvent to facilitate blending with the appropriate amount of maltodextrin to give a final weight of 10 g after a second round of evaporation by vacuum centrifuge and freeze drying. Thus, the original extract was subdivided on the basis of polarity and reconstituted with maltodextrin to give an equivalent weight as the starting material for bioassay. All fractions were thoroughly vortexed to give a free flowing powder and split into two: one set was retained at National Research Council (Ottawa, ON, Canada) as a reference and the other used for bioassay. Neat maltodextrin was used as a negative control.

Analysis of the hexane fraction of American ginseng

Details are provided in the Supplementary Text.

Fatty acid analysis by gas chromatography–mass spectrometry and flame ionization detector

Details are provided in the Supplementary Text.

Liquid chromatography-UV analysis

Details are provided in the Supplementary Text.

Cell culture and treatment

ANA-1 murine macrophage cells (a kind give from Dr. Michael Espey, National Cancer Institute, Bethesda, MD), TK6 lymphoblastoid cells (a kind give from Dr. Curtis Harris, National Cancer Institute, Bethesda, MD), and mouse primary CD4⁺/CD25⁻ effector T cells were cultured and treated as described in detail in the Supplementary Text. Although no authentication of the ANA-1 or TK6 cell lines was done by the authors, cells looked and behaved as we have observed for more than a decade.

DSS mouse model of colitis

We followed our previous protocol for our DSS (MP Biomedicals: 36,000–50,000 MW) mouse model of colitis (10). Whole American ginseng extract (11.9 mg/kg) or the hexane fraction of American ginseng were dissolved in 100 μL 1× PBS per mouse and administered daily by oral gavage (per os); 11.9 mg/kg daily, which is the human equivalent dose of 58 mg daily (21). Of note, currently, the use of ginseng in human clinical trials can range anywhere from 200 mg to 9 g daily (22, 23). The control group of mice was given 100 μL of maltodextrin dissolved in 1× PBS by oral gavage. All procedures conducted were in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, Washington, DC) and approved by the Animal Resource Facility, University of South Carolina (Columbia, SC). Institutional Animal Care and Use Committee. Additional details are provided in the Supplementary Text. Supplementary Figure S1 outlines the time line of the protocol.

Disease activity index

The disease activity index (DAI) was calculated for each animal as done previously (12). Additional details are provided in the Supplementary Text.

Quantification of inflammation to examine effects on colitis

Paraffin-embedded tissues were serially sectioned, and one section from each mouse was stained with hematoxylin and eosin (H&E). Sections were microscopically examined for histopathologic changes using the system described in Supplementary Text and as we described previously (12). Sections were evaluated independently by 2 blinded investigators (D. Poudyal and A. Chumanевич).

AOM/DSS-induced colon cancer model

We carried out experiments with the AOM/DSS model of colitis-driven colon cancer as we have described previously (24). A total of 11.9 mg/kg of the hexane fraction of American ginseng, whole American ginseng extract, and vehicle groups (1× PBS) were given to the mice at day 14 (after AOM and first week of DSS) by oral gavage and continued daily throughout the course of the experiment. The mice were euthanized at day 35 (1.5 cycles) and day 50 (2 cycles). Additional details are provided in the Supplementary Text. Supplementary Figure S2 outlines the time line of the protocol.
Definition of terms to quantify the effects of treatment on precancerous and cancerous lesions in the AOM/DSS mouse model
All lesions were examined blindly by a trained pathologist, specializing in mouse tissues. Details are provided in the Supplementary Text.

Immunohistochemical staining, Western blot analysis and antibodies, real-time PCR, Annexin V and TUNEL assays
We followed the same protocol as outlined by our group previously (24, 25). Additional details are provided in the Supplementary Text.

Statistical analysis
Statistical analysis was done using one-way ANOVA with Schef’s post hoc test for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) scores or the Kruskal–Wallis test when comparing histology inflammatory scores. A two-way ANOVA for repeated measures was used to test for group and time effects on clinical data (e.g., DAI) over successive days of observation. For flow cytometric data, differences between groups were compared using a two-tailed paired Student t test or an unpaired Mann–Whitney U test. Results were analyzed using the StatView II statistical program (Abacus Concepts, Inc.) and Microsoft Excel (Microsoft) for Macintosh computers. Single-factor variance ANOVA analyses were used to evaluate groups. A Fisher’s exact test was used to test the significance of association between treatments and classifications (inflammation, ulceration, polyps, low-grade dysplasia, high-grade dysplasia, and adenocarcinomas). The P value chosen for significance in this study was 0.05.

Results
The hexane fraction of American ginseng suppresses iNOS and COX-2 expression
We have previously shown that whole American ginseng extract suppresses the expression of inflammatory markers in ANA-1 mouse macrophages (10). To better delineate the active ingredients in American ginseng, we first screened ANA-1 mouse macrophage cells for suppression of IFN-γ–induced iNOS expression by various American ginseng fractions obtained through bioassay-guided fractionation. Interestingly, only the hexane fraction of American ginseng (260 µg/mL) was able to suppress the induction of iNOS protein to an extent similar to that of the whole American ginseng extract (Fig. 1A and C; Supplementary Fig. S3). To confirm these anti-inflammatory properties, we also examined lipopolysaccharide-induced expression of COX-2 protein in the ANA-1 cells. Figure 1B and C indicate that COX-2 protein expression was also suppressed by the hexane fraction of American ginseng. To determine whether the hexane fraction of American ginseng regulates iNOS and COX-2 expression at the transcriptional level, we conducted real-time PCR analysis of these 2 genes. Figure 1D and E indicate that the hexane fraction of American ginseng (260 µg/mL) suppresses the induced transcription of both iNOS and COX-2, respectively.

The hexane fraction of American ginseng induces apoptosis in inflammatory cells
We have previously shown that American ginseng drives apoptosis of inflammatory cells (12), providing a mechanism by which American ginseng suppresses inflammation associated with colitis. To further delineate the active ingredient responsible for apoptosis and complement our screen of American ginseng fractions (Fig. 1; Supplementary Fig. S3), we treated TK6 cells with the increasing concentrations (0–1,000 µg/mL) of whole American ginseng extract and the hexane fraction of American ginseng for 24 hours. Results are shown in Supplementary Table SI and Fig. S4A. Interestingly, although the whole American ginseng fraction had a modest effect on apoptosis of these cells (3.4-fold increase in number of cells undergoing early apoptosis when exposed to 1,000 µg/mL; consistent with our previous findings; ref. 12), there was extensive apoptosis (10.4-fold increase in number of cells undergoing early apoptosis when exposed to 1,000 µg/mL) induced by the hexane fraction of American ginseng. Notably, although there was also a modest induction of apoptosis (6.1-fold increase in number of cells undergoing early apoptosis when exposed to 1,000 µg/mL) by the butanol fraction of American ginseng, there was little to no apoptosis caused by all other American ginseng fractions (Supplementary Table SII).

To complement results with Annexin V, we carried out another dose–response experiment with the hexane fraction of American ginseng and processed cells for Western blot analysis. Results suggest that apoptotic markers, including p53, phospho-serine-15, PUMA, and cleaved PARP, are induced by the hexane fraction of American ginseng in TK6 lymphoblastoid cells (Supplementary Figs. S5 and S6). Interestingly, the oncogenic phosphatase, wild-type p53-induced phosphatase (Wip1), is decreased by the hexane fraction of American ginseng, correlating with induction of p53 phosphorylation (Supplementary Fig. S6), consistent with its (Wip1) capacity to dephosphorylate and deactivate p53 (26).

The hexane fraction of American ginseng induces apoptosis in CD4+/CD25− effector T cells
Overly aggressive CD4+/CD25− T cells are thought to contribute to colitis, and defects in mucosal T-cell apoptosis are likely to be critical in the pathogenesis of colitis (12, 27, 28). We therefore isolated CD4+/CD25− effector T cells from spleens of C57BL/6 mice, then exposed unactivated or activated (preincubated for 12 hours with 2.5 µg/mL concanavalin A) cells to either whole American ginseng extract or the hexane fraction of American ginseng (0–300 µg/mL). Supplementary Table SII and Fig. S3B show that the hexane fraction of American ginseng induces apoptosis of CD4+/CD25− effector T cells to a similar extent to that of the whole American ginseng extract (6.2-fold by American ginseng and 6.3-fold by the hexane fraction of American ginseng in unactivated cells). Apoptosis was induced to a greater extent in the activated effector T cells (10.2-fold by...
American ginseng and 13.6-fold by the hexane fraction of American ginseng).

The hexane fraction of American ginseng suppresses inflammation in the DSS model of colitis

We have previously shown that the whole American ginseng extract can be used to prevent and treat mouse colitis (10–12). The mechanism is, at least in part, due to the induction of inflammatory cell apoptosis (12). Given our in vitro results indicating substantial anti-inflammatory and proapoptotic properties of the hexane fraction of American ginseng (Fig. 1; Supplementary Figs. S4–S6 and Tables S1 and SII), we hypothesized that this fraction can be used to treat DSS-induced mouse colitis. Here, mice were given 1% DSS for 1.5 cycles (7 days DSS, 7 days water, and 7 days DSS), then fed vehicle control (1× PBS by oral gavage), the whole American ginseng extract (11.9 mg/kg/d by oral gavage), or the hexane fraction of American ginseng (11.9 mg/kg/d by oral gavage) for the duration of the experiment (outlined in Supplementary Fig. S1). Figure 2 shows results. The colons were graded for histology scores as described in Materials and Methods and Supplementary Text. A total of 1% DSS stimulates colitis. When mice were fed the hexane fraction of American ginseng, there was a significant reduction in colon inflammation (“histology score”) at 3.5 cycles. Although there was also significant suppression of colitis at 5.5 cycles, the reduction was less than that at 3.5 cycles, but more than that of the whole American ginseng extract at 5.5 cycles. Representative hematoxylin and eosin sections are shown.
Mouse colon length shrinks with stress, inflammation, and ulceration (10). Therefore, as an additional indicator of inflammation and inflammatory stress, mouse colon lengths were measured. The control group had an average colon length of 8.5 ± 0.5 cm. There was a significant decrease in the length of the colon from 1.5-cycle DSS group (7.3 ± 0.2 cm) and 3.5-cycle DSS group (7.3 ± 0.3 cm). In contrast, there was no significant decrease in colon length in the 3.5-cycle DSS + the hexane fraction of American ginseng group (8.1 ± 0.2 cm). Similarly, in the 5.5-cycle groups, there was a significant decrease in colon length in the DSS-only group (7.3 ± 0.4 cm) compared with the DSS + whole American ginseng extract group (8.4 ± 0.3 cm) and the DSS + the hexane extract of American ginseng group (8.6 ± 0.2 cm). This is consistent with the hypothesis that the hexane fraction of American ginseng is a potent anti-inflammatory agent in the DSS mouse model of colitis.

The DAI, which monitors weight loss, stool consistency, and blood in the stool as a measure of disease severity, was also scored for each animal at 0, 1.5, 3.5, and 5.5 cycles. As shown in Supplementary Fig. S7, the DAI increased with 1% DSS exposure, but this was suppressed by both the whole American ginseng extract and the hexane fraction of American ginseng. Significance ($P < 0.05$) was reached at day 21 and continued until the end of the experiment.

**Markers of inflammation and inflammatory stress are reduced in DSS + hexane fraction of American ginseng–treated mice**

To further assess the impact of the hexane fraction of American ginseng on inflammatory markers in vivo, we examined iNOS, COX-2, and p53 expression. Immunohistochemical staining was accomplished by rocking slides using the Antibody Amplifier (ProHisto, LLC) to ensure even, consistent, sensitive, and reproducible staining. Figure 3A shows representative sections of each endpoint as indicated. Figure 3B shows quantification of each endpoint. Overall, iNOS, COX-2, and p53 levels were elevated in DSS-treated mice, with most of the staining appearing in the inflammatory cells. iNOS and COX-2 staining were statistically significantly reduced in the DSS + hexane fraction of American ginseng–treated mice; there was also a trend to decreasing p53 levels. Such results reflect a reduction in the number of inflammatory cells (that otherwise are expressing these inflammatory markers) and complement our H&E pathology results.

**The hexane fraction of American ginseng stimulates apoptosis of lymphocytes in vivo**

To examine the effects of the hexane fraction of American ginseng on apoptosis in vivo, we conducted a TUNEL assay on serial sections used for quantifying inflammation (Fig. 2, 3.5 cycles). As shown in Fig. 4, there was significantly higher immunoreactivity score (IRS; i.e., TUNEL label) in both epithelium (Fig. 4A and C) and the mesenteric lymph nodes (MLN; Fig. 4B and D) of mice treated with DSS, compared with water-treated mice. The IRS in the epithelial cells decreased when they were treated with both DSS and hexane fraction of American ginseng. This observation is consistent with data from the inflammatory index (Fig. 2), indicating that the hexane fraction of American ginseng protects epithelial cells from DNA damage in vivo. Alternatively, in the MLNs, there was an increase in IRS in the MLNs in mice when treated with DSS + hexane fraction of American ginseng. Such results are consistent with our in vivo data, and with the hypothesis that the hexane fraction of American ginseng drives apoptosis in inflammatory cells in vivo, providing a mechanism by which the hexane fraction of American ginseng protects from colitis in this DSS mouse model.
The hexane fraction of American ginseng suppresses colon cancer associated with colitis

We have shown that the hexane fraction of American ginseng suppresses DSS-induced colitis (Fig. 2). Mechanistically, this appears to be mediated, at least in part, by the ability of this fraction to induce apoptosis of lymphocytes (Fig. 4; Supplementary Tables SI and SIII). Because both mice and humans with chronic colitis are at a high risk for colon cancer, we next tested the hypothesis that the hexane fraction of American ginseng prevents the onset of colon cancer in a mouse model of colitis-driven colon cancer. Tables 1 and 2 show results that are consistent with this hypothesis. We first examined the levels of inflammation, ulceration, precancerous, and cancerous lesions at an intermediate point during the experiment (day 35). As shown in Table 1, both the hexane fraction of American ginseng and the whole American ginseng extract reduced the severity of microscopic lesions. There was a significant reduction in total number of inflammatory/ulcerative lesions from 44 in the control group (AOM + DSS + 1× PBS, per os) to 24 and 28 in the American ginseng (AOM + DSS + American ginseng, per os) and the hexane fraction of American ginseng (AOM + DSS + American ginseng, per os), respectively. There was also a shift in the severity of lesions, with more lesions being classified as mild inflammatory lesions in the American ginseng and hexane fraction of American ginseng groups. There was also a drop in the number of ulcerative lesions compared with the control group; with a greater drop in the hexane fraction of American ginseng (36.4% vs. 7.2%) than the American ginseng (36.4% vs. 16.7%) group (Table 1, Day 35: analysis of inflammatory and ulcerative lesions). Similarly, there was a significant reduction in the total number of precancerous/cancerous lesions from 11 in the control group (AOM + DSS + 1× PBS, per os) to 1 and 0 in the American ginseng (AOM + DSS + American ginseng, per os) and the hexane fraction of American ginseng (AOM + DSS + Hex-American ginseng, per os), respectively. Interestingly, most lesions (91%) in the AOM + DSS + PBS (control) group were of high-grade dysplasia or invasive adenocarcinoma (Table 1, Day 35: analysis of precancerous and cancerous lesions).

We next examined levels of inflammation, ulceration, precancerous, and cancerous lesions at a later time point during the experiment (day 50). As shown in Table 2, both the hexane fraction of American ginseng and the whole American ginseng extract reduced the severity of microscopic lesions. There was a significant reduction in the total number of inflammatory/ulcerative lesions from 22 in the control group (AOM + DSS + 1× PBS, per os) to 14 and 17 in the American ginseng (AOM + DSS + American ginseng, per os) and the hexane fraction of American ginseng (AOM + DSS + Hex-American ginseng, per os), respectively. There was again a shift in the severity of lesions, with 2.7- and 4.2-fold lesions being classified as mild inflammatory lesions in the American ginseng group versus the control (PBS) group and the hexane fraction of American ginseng group versus the control (PBS) group, respectively. There was also a dramatic drop in the number of ulcerative lesions compared with the control group compared with the hexane fraction of American ginseng (27.3% vs. 5.8%) than the American ginseng (27.3% vs. 0%) group (Table 2, Day 50: analysis of inflammatory and ulcerative lesions).

Figure 3. iNOS, COX-2, and p53, markers of inflammation and inflammatory stress, are reduced in DSS + hexane fraction of American ginseng (AG)-treated mice. Tissues from experiments carried out (3.5 cycles) were examined for iNOS, COX-2, and p53 by immunohistochemistry, using the Antibody Amplifier (ProHisto, LLC) rocked on a laboratory rocker to ensure even staining and reproducible results. A, representative staining of indicated end points in serial sections from water (n = 11), DSS (n = 15), and DSS + hexane fraction of American ginseng (n = 11) groups. Positive staining is brown colored, ×100 magnification. B, quantification of indicated endpoints. All 3 markers were elevated in the DSS-treated group and suppressed when the DSS-treated group was fed the hexane fraction of American ginseng. P values are indicated.
there was not as dramatic of a drop in the total number of precancerous or cancerous lesions. However, similar to the 35-day time point, the severity of lesions was reduced at 50 days. More lesions (33%) in the AOM + DSS + PBS (control) group were of high-grade dysplasia or invasive adenocarcinoma. Almost half (17%) of the American ginseng fed group, and only 4% of the hexane fraction of American ginseng group were in this classification (Table 2, Day 50: analysis of precancerous and cancerous lesions).

Preliminary chemical analysis of the hexane fraction of American ginseng

Given the potency of the hexane fraction of American ginseng, we initiated experiments to better understand the components of this fraction. The amounts of fatty acids determined from the analysis of the hexane fraction of American ginseng are given in Supplementary Table SIV and account for greater than 40% w/w of the total extract. Strikingly, linoleic acid (18:2n6) was the major fatty acid, accounting for approximately 50% of the total fatty acids detected, followed by palmitic (16:0) and palmitoleic (16:1) acids. Liquid chromatography/mass spectrometric (LC/MS) analysis did not detect either protopanaxadiol or protopanaxatriol; however, low levels of ginsenosides Re, F11, Rb1, and Rd were found but amounted to less than 0.1% w/w of the hexane fraction of American ginseng.

Descriptive LC-UV Diode Array Detector analysis of the hexane fraction of American ginseng gave 3 major UV active peaks, one eluting at 22.7 minutes with UV maxima at 220, 230, 243, and 257 nm and another eluting at 26.7 minutes with UV maxima at 230, 243, and 257 nm which match the UV maxima reported for the polycetylenes, panaxadiol, and falcarinol, respectively (29). The third peak eluting at 20.7 minutes also exhibited that multiple UV maxima at 215, 242, 255, 269, and 284 nm may be a related compound. Confirmation of identity and precise quantification of these compounds awaits individual isolation and structural elucidation, which is currently underway and will be reported in detail separately.

Discussion

We have previously shown that whole American ginseng root extract suppresses colitis and prevents colon cancer associated with colitis in mice (10–12). To better delineate the ingredients responsible for these findings, we carried out bioassay-guided fractionation, using multiple solvents.
Interestingly, it appears that one fraction (the hexane fraction of American ginseng) is particularly potent in its anti-inflammatory and proapoptotic properties. As well, this hexane fraction of American ginseng appears to be more effective than the whole American ginseng extract in treating DSS-induced mouse colitis and modestly more effective at reducing the number and severity of precancerous and cancerous lesions of the colon in the AOM/DSS mouse model.

Specifically, from our in vitro results, the hexane fraction of American ginseng was most effective in suppressing IFN-γ-induced expression of iNOS in ANA-1 mouse macrophages (Fig. 1A, C, and D; Supplementary Fig. S3). iNOS, which is responsible for the high-output production of NO, is upregulated within the inflammatory infiltrate of the lamina propria and in the cytoplasm of the epithelial cells in patients with IBD (30). Large amounts of COX-2 have also been found in inflamed areas, producing most of the prostaglandins (31) and it has been reported that the increased prostaglandin production during acute colitis is dependent upon the activity of COX-2 (32–34). Therefore, suppression of the inflammatory response may be reached through the inhibition of prostaglandin E₂ (PGE₂) production and COX-2 activation (31). Comparing untreated cells, COX-2 expression was also suppressed by the hexane fraction of American ginseng in ANA-1 cells (Fig. 1B, C, and E). However COX-2 protein expression was affected minimally by the whole American ginseng extract (Fig. 1B and C). Interestingly, Ichikawa and colleagues have reported that American ginseng extract has minimal effects on COX-2 protein expression in Raw 246.7 murine macrophages (35). Jeong and colleagues have also reported that ginsenoside Rd induces COX-2 expression in Raw 264.7 cells and other ginsenosides (Rg1, Rg3, Rb1, and Re) did not induce COX-2 (36). Our original American ginseng extract supplied to us by the Canadian Phytopharmaceutical Corporation contains 23.5 mg/g of Rd. This is consistent with the hypothesis that Rd may be one of the ingredients that prevents the whole American ginseng extract from suppressing COX-2 expression, as the hexane fraction of American ginseng has a minimal ginsenoside content, including very little Rd (Supplementary Table SIV). Also, at this time, we can only speculate on the specific molecules targeted by the hexane fraction of American ginseng. Because COX-2 and iNOS transcription is regulated by STAT-1, hypoxia-inducible factor-1α (HIF-1α), NF-κB, and IFN regulatory factor-1 (IRF-1; refs. 37–42), such molecules remain candidates.

There also may be indirect mechanisms, such as targeting growth factors, including TNFα and IFN-γ, both of which regulate iNOS and COX-2 levels (43). Another hypothesis takes into account the fatty acid content of the hexane fraction of American ginseng (Supplementary Table SIV). Fatty acids are known to readily react with nitric oxide species to form nitro-fatty acid derivatives (NO₂-FA; ref. 44). NO₂-FA signals through anti-inflammatory mechanisms that inhibit neutrophil activation, platelet aggregation, and macrophage activation (45). It is therefore possible that the formation of NO₂-FA plays a key role in the anti-

<table>
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<th>Group</th>
<th>No. of animals</th>
<th>Total no. of precancerous/cancerous lesions</th>
<th>Polyps Low-grade dysplasia</th>
<th>High-grade dysplasia</th>
<th>Invasive adenocarcinoma</th>
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<td>1⁴</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Hex-AG</td>
<td>4</td>
<td>0⁴</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Abbreviation: AG, American ginseng.
⁴Significant decrease compared with the control (PBS) treated group (P < 0.05).

Table 1. Percentage of inflammatory and ulcerative lesions and of precancerous and cancerous lesions in mice treated with AOM/DSS ± AG ± hexane fraction of AG (Hex-AG) at days 35

Day 35: analysis of inflammatory and ulcerative lesions

<table>
<thead>
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<th>Group</th>
<th>No. of animals</th>
<th>Total no. of inflammatory/ ulcerative lesions</th>
<th>Inflammatory lesions Mild</th>
<th>Severe</th>
<th>Ulcerative mucosa</th>
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<td>AGa</td>
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<td>24²</td>
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<td>16.7%</td>
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<td>Hex-AGb</td>
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<td>28²</td>
<td>71.4%</td>
<td>21.4%</td>
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</table>

Abbreviation: AG, American ginseng.
²Whole American ginseng extract.
²Hexane fraction of American ginseng.
²Significant decrease compared with the control (PBS) treated group (P < 0.05).
²Significant decrease compared with the control (PBS) treated group (P < 0.05).
inflammatory properties of the hexane fraction of American ginseng. We are currently trying to better understand these mechanisms and will report results in future studies. Nevertheless, the finding that the hexane fraction of American ginseng is effective in suppressing both iNOS and COX-2 expression in vitro led us to hypothesize that this fraction could be potent in suppressing mouse colitis. Results are consistent with this hypothesis (Fig. 2).

In IBD, lymphocytes (both B and T cells) infiltrate the mucosa to eradicate the foreign antigen (46, 47). Once the antigen has been eliminated, T lymphocytes of intestinal mucosa require a mechanism to attenuate the local immune response (12), and the failure to do so results in chronic immunogenic reactions. A key mechanism of immunosuppression is the apoptosis of overly aggressive effector T cells and we have shown that the whole American ginseng extract induces apoptosis of such cells (12). In this study, we show that the hexane fraction of American ginseng and, to a lesser extent, the butanol fraction of American ginseng also have proapoptotic properties. The whole American ginseng extract has only a modest impact on TK6 apoptosis (Supplementary Table SI) but is as potent as the hexane fraction of American ginseng in CD4^+^/CD25^+^ effector T cells (Supplementary Table SIII). Because of the potential impact of differential cellular sensitivity to apoptosis, one of several hypotheses' being explored is that this may be a result of the heterogeneity of the potency of American ginseng based on the cell type. This is consistent with other studies that have reported proapoptotic properties of some of the ingredients we have determined to be in the hexane fraction of American ginseng (48–54).

Another finding that deserves further attention is that American ginseng (whole American ginseng extract and the hexane fraction of American ginseng) both suppresses iNOS and induces apoptosis. This is especially apparent in ANA-1 mouse macrophages, where we measured both iNOS expression (Fig. 1) and apoptosis by TUNEL labeling (Supplementary Fig. S8 and Table SV). Although this is consistent with finding of studies that suppression of iNOS induces apoptosis (55), other groups have found an induction of apoptosis by nitric oxide from iNOS in T cells (56). These findings may again be explained by cell type selectivity, as well as many other factors such as NO output by iNOS. Although our findings here indicate both an anti-inflammatory and proapoptotic effect of American ginseng, it is likely that there are mechanisms of apoptosis by American ginseng other than through iNOS in T cells, which we are exploring.

It is currently unclear which component(s) in the hexane fraction of American ginseng suppresses colitis and drives apoptosis of inflammatory cells. While we are subfractionating the hexane fraction of American ginseng to address this question, at this time, we can only speculate. Full details of the chemical analysis and spectroscopic identification of the major components of the hexane fraction will be reported in a separate manuscript. However, from our initial analysis (Supplementary Table SIV), it is unlikely that the ginsenosides are responsible, as these comprise a

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<th>Table 2. Percentage of inflammatory and ulcerative lesions and of precancerous and cancerous lesions in mice treated with AOM/DSS ± AG ± hexane fraction of AG (Hex-AG) at day 50</th>
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<td><strong>Day 50: analysis of inflammatory and ulcerative lesions</strong></td>
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Abbreviation: AG, American ginseng.
^cSignificant decrease compared with the control (PBS) treated group (P < 0.05).
^dSignificant decrease compared with the control (PBS) treated group (P < 0.05).
very minor portion of this fraction. More likely are either the fatty acids or the polyacetylenes which, combined, comprise approximately 70% of the total hexane fraction by weight (Supplementary Table SIV). Conjugated linoleic acid, for example, has been shown by others to attenuate colitis in animals (57–59). However, in humans, linoleic acid (18:2n6), the fatty acid found in greatest abundance in the hexane fraction of American ginseng, may exacerbate colitis (60). Others have shown that fatty acids, such as oleic acid, have no effect in suppressing colitis (61). The effects of the other fatty acids that we detected in the hexane fraction of American ginseng on colitis are, to our knowledge, unknown but worth exploring. Interestingly, trilinolein, a triglyceride isolated from *P. notoginseng* where glycerol is esterified at all 3 positions with linoleic acid (18:2n6), has been shown to have antioxidative and cardioprotective effects in animal models (62). The high relative levels of 18:2n6 found in the hexane fraction of American ginseng after transesterification suggest that trilinolein may be present in American ginseng also. Eight polyacetylenes have now been reported from American ginseng (63), a class of compound with potent anti-inflammatory activities (64). On the basis of LC-UV, 1H-NMR, and high-resolution mass spectra, 3 C17 polyacetylenes: panaxadiol, panaxydol, and panaxynol were identified and comprised more than 25% of the hexane fraction of American ginseng (Supplementary Table SIV). Therefore, this class of compound is another candidate responsible for the observed activity against colitis.

In the DSS model of colitis, the hexane fraction of American ginseng was found to be very effective in suppressing colon inflammation (Fig. 2). At 3.5 cycles, the DSS + hexane fraction of American ginseng group was able to reverse the inflammation to almost basal levels. At 5.5 cycles, the hexane fraction of American ginseng was less potent against colitis than it was at 3.5 cycles. One reason for this observation is that the increased cycles of DSS (2 more cycles) were able to cause much more damage to the colon to the point where complete recovery was unattainable. Regardless, at 5.5 cycles, the hexane fraction–treated mice had significantly less colon inflammation than the DSS-only–treated groups of mice. It therefore appears that the hexane fraction of American ginseng is more effective during the short-term inflammation (acute colitis) than the long-term inflammation (chronic colitis). It also appears to be more potent than the whole American ginseng extract (Fig. 2). Interestingly, others have found that an n-hexane extract of red ginseng is particularly potent in inhibiting the growth of human lung tumor xenografts in nude mice (65).

Many studies have shown anticancer effects of American ginseng *in vitro* and in vivo, which we have described in detail previously (11). As an extension of that study, it appears that although the hexane fraction of American ginseng has a similar potent effect of suppressing colon cancer associated with colitis in the AOM/DSS model, the severity of precancerous and cancerous lesions is modestly reduced with the hexane fraction of American ginseng compared with the whole American ginseng extract (Tables 1 and 2). Similar to the colitis data (Fig. 2), the reduction in severity of such lesions is greater at the earlier time period (35 days) than at 50 days. Again, one reason for this observation is that the increased time was able to cause much more damage to the colon to the point where complete recovery was unattainable.

In summary, we have identified through various endpoints that the hexane fraction of American ginseng is at least one component of American ginseng extract responsible for the suppression of DSS-induced colitis, and apoptosis of inflammatory cells is a mechanism by which it acts. This hexane fraction of American ginseng is also modestly more potent than the whole American ginseng extract in suppressing the severity of AOM/DSS-induced colon cancer. This finding represents a significant advancement in the field, as it has previously been thought that ginsenosides, extremely minor elements of this fraction, are key anti-inflammatory and anticancer agents in American ginseng (66). To this end, it is currently unclear what component within the hexane fraction of American ginseng suppresses colitis and colon cancer associated with colitis. However, many of the fatty acids detected in our hexane fraction of American ginseng can induce apoptosis in various cell types (48–54), and conjugated linoleic acid and oleic acid have been shown to suppress colitis in other studies (57–59, 61). This is consistent with the hypothesis that at least one of these ingredients may be responsible for the activity of American ginseng root extract against colitis and associated colon cancer. Further bioassay-guided fractionation of the hexane extract of American ginseng is ongoing to extend these current results to further pinpoint this active ingredient(s).

**Disclosure of Potential Conflicts of Interest**

L.J. Hofseth has patent pending for antibody amplifier used in IHC here. No potential conflicts of interests were disclosed by other authors.

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**Writing, review, and/or revision of the manuscript:** D. Poudyal, M.J. Wargovich, P.S. Nagarkatti, A. Windust, L.J. Hofseth

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**Study supervision:** D. Poudyal and L.J. Hofseth

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Hexane Fraction of American Ginseng Suppresses Colitis and Colon Cancer

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A Hexane Fraction of American Ginseng Suppresses Mouse Colitis and Associated Colon Cancer: Anti-inflammatory and Proapoptotic Mechanisms

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