

Research Article

American Ginseng Suppresses Colitis through p53-Mediated Apoptosis of Inflammatory Cells

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Abstract

Ulcerative colitis is a dynamic, chronic inflammatory condition associated with an increased colon cancer risk. Inflammatory cell apoptosis is a key mechanism regulating ulcerative colitis. American ginseng (AG) is a putative antioxidant that can suppress hyperactive immune cells. We have recently shown that AG can prevent and treat mouse colitis. Because p53 levels are elevated in inflammatory cells in both mouse and human colitis, we tested the hypothesis that AG protects from colitis by driving inflammatory cell apoptosis through a p53 mechanism. We used isogenic p53^{+/+} and p53^{-/-} inflammatory cell lines as well as primary CD4⁺/CD25⁻ effector T cells from p53^{+/+} and p53^{-/-} mice to show that AG drives apoptosis in a p53-dependent manner. Moreover, we used a dextran sulfate sodium (DSS) model of colitis in C57BL/6 p53^{+/+} and p53^{-/-} mice to test whether the protective effect of AG against colitis is p53 dependent. Data indicate that AG induces apoptosis in p53^{+/+} but not in isogenic p53^{-/-} cells *in vitro*. *In vivo*, C57BL/6 p53^{+/+} mice are responsive to the protective effects of AG against DSS-induced colitis, whereas AG fails to protect from colitis in p53^{-/-} mice. Furthermore, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling of inflammatory cells within the colonic mesenteric lymph nodes is elevated in p53^{+/+} mice consuming DSS + AG but not in p53^{-/-} mice consuming DSS + AG. Results are consistent with our *in vitro* data and with the hypothesis that AG drives inflammatory cell apoptosis *in vivo*, providing a mechanism by which AG protects from colitis in this DSS mouse model. *Cancer Prev Res*; 3(3); 339–47. ©2010 AACR.

Introduction

Dysfunction of the intestinal immune system has been implicated as a major mechanism by which chronic inflammation occurs in colitis. Immune responses are initiated when either CTL CD8⁺ cells or CD4⁺ T helper cells in the intestinal lumen recognize a foreign antigen. This initiates the immunologic cascade responsible for eradicating the antigenic material. This includes the production of proinflammatory and anti-inflammatory cytokines and the infiltration of macrophages and neutrophils. Once the antigen has been eradicated, T lymphocytes of the intestinal mucosa require a method to attenuate the local

immune response. Failure to regulate T-cell responses in the intestinal or colonic mucosa leads to an inappropriate and sustained injurious immunologic reaction (1, 2). A key mechanism for immune suppression is apoptosis of overly aggressive effector T cells, and defects in mucosal T-cell apoptosis are likely to be critical in the pathogenesis of colitis.

Apoptosis can proceed through two major pathways. There is an intrinsic (mitochondrial-mediated) pathway and an extrinsic (death receptor-mediated) pathway (3). A key molecule involved in apoptosis (mainly in the intrinsic pathway) is p53, which is activated in epithelial and inflammatory cells during the process of colitis. This is well documented by others and ourselves (4–8). Mechanistically, levels are elevated either due to a stabilization of the wild-type form of p53 by posttranslational modification (often phosphorylation at Ser¹⁵; ref. 6) or through p53 mutation, leading to abnormally elevated levels of an inactivated form (4, 5). Consistent with this hypothesis is that patients with ulcerative colitis have an elevated p53 mutation load in noncancerous colon epithelial cells (9, 10).

The finding that p53 levels are elevated in inflammatory cells during colitis (7, 8) is interesting and significant because it identifies a potential molecular target for apoptosis by drugs and/or complementary and alternative medicines, such as American ginseng (AG). Although the mechanisms for the increased p53 observed in

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inflammatory cells are not fully delineated, many key inflammatory players have been shown to induce p53. A nonexhaustive list includes reactive nitrogen species (6, 11), reactive oxygen species (12), tumor necrosis factor- α (13), and other cytokines (14).

There are several different species of ginseng: two of the most commonly used are *Panax ginseng* (Asian ginseng) and *P. quinquefolius* (AG; ref. 15). Here, we use *P. quinquefolius* (AG; root, harvested at 3-5 years, dried, and ground to a powder). AG, although less studied, has similar ginsenosides as the Asian ginseng. Studies indicate that ginseng can improve end points associated with prevalent diseases in Western society, such as cardiovascular disease and cancer, as well as autoimmune diseases, such as diabetes (reviewed in refs. 16–18). Mechanisms include inhibition of DNA damage, induction of apoptosis, inhibition of cell proliferation, and inhibition of immune cell activity (19–21). Interestingly, ginseng has not been documented as a standard complementary and alternative medicine in patients with inflammatory bowel diseases. In addition to massage, meditation, and Tai chi, probiotics, such as acidophilus, and herbal therapies, such as flax seed, aloe vera, and garlic, are common alternatives used in this population.

There is extensive evidence that (a) Ginseng induces p53 (22–24), (b) p53 is elevated in activated inflammatory cells and plays a role in their apoptosis, (c) p53 is activated and activated in inflammatory cells during colitis (4–8), and (d) p53 protects from colitis-driven colon cancer (25, 26). In addition, AG induces apoptosis in inflammatory cells. For these reasons, here, we tested the hypothesis that AG protects from mouse colitis through a p53 mechanism that is involved in driving apoptosis of inflammatory cells. We describe results consistent with this hypothesis.

Materials and Methods

AG extract

The *P. quinquefolius* (AG) extract has been described previously in detail by our laboratory (7). Briefly, AG extract was purchased from the National Research Council of Canada. This extract was cultivated by Chai-Na-Ta Farms Ltd. and processed by Canadian Phytopharmaceuticals Corp. Following grinding to pass 80 mesh, 35 kg of the root material were extracted with aqueous ethanol (75% ethanol/25% water) in a recirculating filter extraction system for 4 h at a temperature of 60°C under vacuum. The ratio of solvent to root was 8:1 (v/w). After extraction, the filtrate was partially dried *in vacuo* to yield a concentrated extract. Maltodextrin (2.8 kg; 40% of final weight) was then blended as a support, and the resultant slurry spray was dried to yield 7 kg of free-flowing powder. Analysis by Canadian Phytopharmaceuticals by high-performance liquid chromatography–UV against pure standards determined the total ginsenoside content (as the sum of Rg1, Re, Rb1, Rc, Rb2, and Rd) of the finished material to be 10.1% (w/w) and confirmed by high-performance liquid

chromatography–mass spectrometry at the National Research Council of Canada. The final powder form of AG extract also contained 2% additional ginsenosides (made up of F11, Ro, isomers of Rd, and traces of malonyl ginsenosides) and 40% of maltodextrin derived from hydrolyzed cornstarch. The remaining 48% of the powder was made up of ginseng root–derived polysaccharides/ligosaccharides and proteins and up to 5% of moisture. The lot used was screened and found to be free of heavy metals and contaminants. It should be noted here that regular AIN-93M chow fed to mice contains 12.5000% maltodextrin. The addition of 75 ppm AG in the chow equates to 30 mg/kg final concentration of maltodextrin added to 12.5000% already in the chow. Therefore, there is 12.5000% maltodextrin in the AIN-93M chow and 12.5003% of maltodextrin in the AIN-93M chow supplemented with 75 ppm AG extract.

Chemicals and reagents

Dextran sulfate sodium (DSS) was purchased from MP Biomedicals (molecular weight, 36,000–50,000). Concanavalin A (2.5 μ g/mL) was purchased from Sigma-Aldrich. The following antibodies were used in these studies: anti-poly(ADP-ribose) polymerase (polyclonal, 1:500; Cell Signaling), anti-caspase-3 (polyclonal, 1:500; Cell Signaling), anti-caspase-7 (polyclonal, 1:1,000; Calbiochem), anti-p53 (monoclonal, 1:500, clone PAB 421; Calbiochem), anti-p53-Ser¹⁵ phosphorylation (polyclonal, 1:500; Cell Signaling), anti-Bax (polyclonal, 1:500; Cell Signaling), anti-caspase-9 (polyclonal, 1:500; Cell Signaling), and anti-actin (monoclonal, 1:2,000, clone 1A4; Calbiochem).

Cells

TK6 (p53^{+/+}) and NH32 (p53^{-/-}) were a kind gift from Curtis Harris (National Cancer Institute), originally derived from Dr. William Thilly's and Howard Liber's labs. TK6 cells are a lymphoblastoid cell line derived from the spleen more than 30 y ago (27). NH32 cells are an isogenic derivative of TK6 cells in which both alleles of the *p53* gene were knocked out (28). Jurkat cells are an immortalized line of T lymphocyte cells derived in the late 1970s from the peripheral blood of a 14-y-old boy with T-cell leukemia (29). Jurkat cells have a defective p53 pathway due to a mutation in the COOH-terminal domain responsible for transactivation (30, 31). CD4⁺/CD25⁻ cells from C57BL/6 p53^{+/+} or C57BL/6 p53^{-/-} mice were purified from the spleens using nylon wool columns (Polysciences) followed by depletion of B cells and macrophages. The purity of T cells was 90% as determined by flow cytometry (Cytomics FC 500, Beckman Coulter). CD4⁺/CD25⁻ T cells were then isolated using a MACS mini separator and CD4 and CD25 microbeads according to the manufacturer's instructions (Miltenyi Biotec) by depletion of CD4⁻CD25⁺ T cells (negative selection). CD4⁺/CD25⁻ effector T cells (1 \times 10⁶) were cultured in six-well plates overnight followed by experimentation as indicated. All cells were maintained in exponentially

growing suspension culture at 37°C in a humidified 5% CO₂ atmosphere in RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine.

Flow cytometry analysis for Annexin V

Cells were seeded at 1×10^6 per well into six-well dishes for 24 h. Following this, fresh medium or medium containing concanavalin A (2.5 µg/mL) was added to cells and cultured for 12 h. Concanavalin A was then washed off, and fresh medium or fresh medium containing freshly dissolved indicated concentrations of AG (0-1,000 µg/mL) was added for 24 to 72 h, as indicated. Cells were then harvested for Annexin V according to instructions provided by the kit manufacturer (BD Biosciences). Annexin V/propidium iodide (PI) staining was examined using a Beckman Coulter Cytomics FC500 flow cytometer.

DSS mouse model of colitis

We have described the DSS mouse model of colitis previously (7). Both p53^{-/-} and p53^{+/+} were of C57BL/6 background. All males were used in the present study because we have found that there are rarely females in p53^{-/-} litters. Briefly, 8-wk-old C57BL/6 mice received either water *ad libitum* or 1% DSS. All mice were on an AIN76A high-iron diet. AG extract was mixed into the chow of indicated groups at 75 ppm (Research Diets, Inc.), which is the human equivalent dose of ~58 mg daily. Our calculation of the human equivalent amount of AG consumed by mice uses the body surface area normalization method (32), with the following assumptions: a typical mouse eats 3.5 g chow daily and weighs 22 g; the average adult human weighs 60 kg. More specifically, here, chow contains 75 ppm AG extract. This equates to 75 mg/kg of chow. A mouse consumes 3.5 g chow daily. Therefore, 75 mg/1,000 g chow \times 3.5 g chow/day = 0.2625 mg AG extract daily. If a mouse weighs on average 22 g, then 0.2625 mg/22 g \times 1,000 g/1 kg = 11.93 mg/kg daily. As discussed by Reagan-Shaw (32), the human equivalent dose (mg/kg) = animal dose (mg/kg) \times [animal Km/human Km]. As such, human equivalent dose (mg/kg) for mouse = 11.93 mg/kg/[3/37] = 0.967 mg/kg. If an average human adult weighs 60 kg, this equates to 0.967 mg/kg \times 60 kg = 58 mg daily for humans. Mice consumed the same amount of chow daily (on average, 3.5 g) regardless of it containing AG extract (data not shown).

To determine whether AG extract prevents colitis onset, it was given to indicated groups of mice 1 wk before and during DSS or water treatment. Organs were harvested from the treated mice after either 2.5 cycles, where each cycle in the DSS groups consisted of 1% DSS in drinking water for 7 d followed by a 7-d interval with normal drinking water. Mice were then euthanized at one-cycle intervals (another 7 d water then 7 d DSS). For pathology and immunohistochemistry, colon tissue samples were washed with PBS (Mediatech, Inc.), cut longitudinally, Swiss rolled, then formalin fixed, and paraffin embedded.

Disease activity index

The disease activity index (DAI), which monitors weight loss, stool consistency, and blood in the stool as a measure of disease severity, was scored for each animal every other day throughout the experiment. The DAI was calculated for each animal as described by Maines et al. (33) according to the system described in Table 1. With this scoring system, the DAI is calculated by scoring each animal for weight loss, stool consistency, and blood in the stool and then dividing the total score by 3. For example, an animal that lost 12% of its body weight (score of 3) with evidence of loose stool (score of 2) plus gross rectal bleeding (score of 4) would have a calculated DAI of 3.

Quantification of inflammation

The colon was transected, pinned open, and rinsed with PBS, and the colon length was recorded. The tissue was then Swiss rolled and fixed in formalin overnight followed by embedding in paraffin, sectioning, and staining with H&E. The sections were microscopically examined for histopathologic changes using the following scoring system. Histology score was determined by multiplying the percent involvement for each of the three following histologic features by the percent area of involvement (33, 34): inflammation severity (0, none; 1, minimal; 2, moderate; 3, severe), inflammation extent (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), crypt damage (0, none; 1, one third of crypt damaged; 2, two thirds of crypt damaged; 3, crypts lost, surface epithelium intact; 4, crypts lost, surface epithelium lost), percent area involvement (0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%). Therefore, the minimal score is 0 and the maximal score is 40.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

A terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was carried out to assess apoptosis *in vivo*, according to the manufacturer's directions (DeadEnd Colorimetric TUNEL System, Promega). Briefly, this is a modified TUNEL assay designed to provide simple, accurate, and rapid detection of apoptotic cells *in situ* at the single-cell level. The system measures nuclear DNA fragmentation, an important biochemical indicator of apoptosis. It end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assay.

Table 1. Scoring system for DAI

Score	Weight loss	Stool consistency	Blood in stool
0	None	Normal	Negative
1	1-5%		
2	6-10%	Loose stool	Positive
3	11-15%		
4	>15%	Diarrhea	Gross rectal bleeding

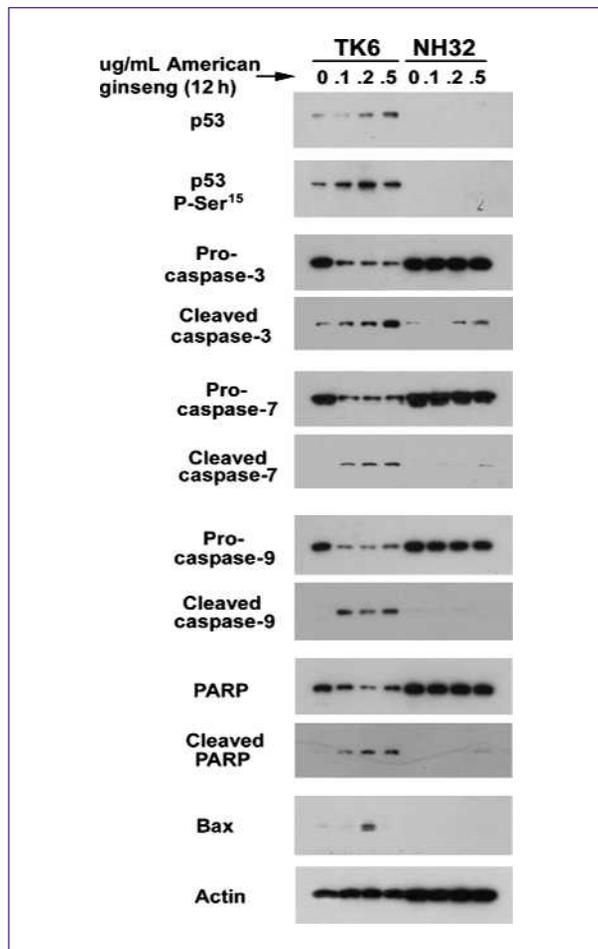


Fig. 1. AG drives apoptosis of lymphoblasts through the p53 pathway. The TK6 human lymphoblast line and NH32 isogenic p53 knockout cell line were cultured in RPMI 1640 + 10% FCS. Cells were exposed to indicated doses of AG for 12 h and then harvested for protein. Following separation by 10% SDS-PAGE electrophoresis, gels were blotted onto nitrocellulose and then probed with the indicated antibodies. Results indicate that caspase-3, caspase-7, caspase-9, and poly(ADP-ribose) polymerase (PARP) cleavage and induction of Bax by AG are p53 dependent. p53 and phosphorylated p53 are also shown, indicating that p53 is activated by AG.

Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the enzyme TdT. Horseradish peroxidase-labeled streptavidin is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen 3,3'-diaminobenzidine. Using this procedure, apoptotic nuclei are stained dark brown. The counterstain was CAT Hematoxylin (Biocare Medical). Labeling was carried out on serial sections to that we used to score inflammatory index (Fig. 3). TUNEL labeling in 10 separate sections from 10 individual mice was quantified in both the epithelial areas and the mesenteric lymph nodes (MLN). For the epithelial areas, 10 random fields were evaluated per slide. Because there are relatively fewer MLNs, we eval-

uated each MLN for TUNEL labeling. Labeled tissues were examined for intensity of staining using a method similar to that previously described (35). Briefly, intensity of staining was evaluated independently by blinded investigators. For each tissue section, the percentage of positive cells in either the epithelium or the MLNs was scored on a scale of 0 to 4 for the percentage of tissue stained: 0 (0% positive cells), 1 (<10%), 2 (11-50%), 3 (51-80%), or 4 (>80%). Staining intensity was scored on a scale of 0 to 3: 0, negative staining; 1, weak staining; 2, moderate staining; or 3, strong staining. The two scores were multiplied, resulting in an immunoreactivity score (IRS) value ranging from 0 to 12.

Statistical analysis

Statistical analysis was done using one-way ANOVA with Scheffe's post hoc test for TUNEL scores or the Kruskal-Wallis test when comparing histology 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. The *P* value chosen for significance in this study was 0.05.

Results

AG causes apoptosis in inflammatory cells in a p53-dependent manner

We had recently shown that AG prevents and treats mouse colitis (7). As well, we and other have shown that p53 levels are elevated in inflammatory cells in mouse and human colitis (6-8). Still, others have shown that AG can drive apoptosis and induce p53 in leukemia cells (36, 37). We therefore decided to test the hypothesis that AG drives apoptosis of inflammatory cells in a p53-dependent manner. We initially used the isogenic lymphoblastoid cell lines TK6 and NH32. TK6 cells are a lymphoblastoid cell line and NH32 cells are an isogenic derivative of TK6 cells in which both alleles of the *p53* gene were knocked out (28). Figure 1 shows that caspase 3, 7, 9, and PARP cleavage and induction of Bax by AG are p53 dependent. Complimentary to this finding, Table 2 and Supplementary Fig. S1 show that AG induces apoptosis (identified as Annexin V-positive/PI-negative cells) in TK6 cells but not in NH32 cells. As well, we were unable to induce significant apoptosis in Jurkat T cells, which have a mutant and dysfunctional p53 pathway (Table 3). A minimum of 10,000 cells was counted from three separate plates for each time and dose indicated. Representative experiments are shown.

A key mechanism for immune suppression is apoptosis of overly aggressive effector T cells, and defects in mucosal T-cell apoptosis are likely to be critical in the pathogenesis of colitis (1, 2). Therefore, as a penultimate measure that p53 is key to the AG-driven apoptosis of inflammatory cells that drive colitis, we isolated CD4⁺/CD25⁻ effector T cells from p53^{+/+} and p53^{-/-} mice. Four mice from each genotype were used. Table 4 and Supplementary Fig. S2 show that following exposure to AG for 24 hours, CD4⁺/CD25⁻ effector T cells from p53^{+/+} undergo apoptosis.

Table 2. Percentage of cells from a representative experiment staining positive for Annexin V and negative for PI (early apoptotic cells) in TK6 (p53^{+/+}) and NH32 (p53^{-/-}) isogenic lymphoblastoid cells

Dose AG (μg/mL)	TK6 (p53 ^{+/+})			NH32 (p53 ^{-/-})		
	24 h	48 h	72 h	24 h	48 h	72 h
0	3.20%	3.20%	2.40%	1.30%	1.50%	1.00%
50	ND	3.50%	2.80%	ND	1.30%	1.30%
100	3.70%	3.60%	3.20%	1.50%	1.40%	1.40%
200	ND	4.20%	4.40%	ND	1.20%	1.30%
300	4.20%	4.70%	5.50%	1.60%	1.00%	1.50%
500	4.70%	5.30%	8.20%	1.70%	1.20%	2.20%
800	5.10%	ND	ND	1.50%	ND	ND
1,000	6.60%	ND	ND	1.60%	ND	ND

Abbreviation: ND, not done.

However, CD4⁺/CD25⁻ effector T cells from p53^{-/-} are resistant to apoptosis.

AG prevents mouse colitis in p53^{+/+} mice but not in p53^{-/-} mice

We have previously shown that AG can be used to prevent and treat mouse colitis (7). Here, we repeated such experiments but concomitantly tested the ability of AG (again 75 ppm in chow) to prevent colitis in p53^{-/-} mice. The DAI, which monitors weight loss, stool consistency, and blood in the stool as a measure of disease severity, was scored for each animal every other day. DAI increased in both p53^{+/+} and p53^{-/-} mice consuming DSS and chow without AG. However, only p53^{+/+} mice consuming AG had a significantly lower DAI. Significance ($P < 0.05$) was reached at day 21 and continued until the end of the experiment (Fig. 2).

On day 35, the animals were sacrificed by cervical dislocation, and the entire colon was Swiss rolled, formalin

fixed, sectioned, and examined histologically on a blinded basis. Histologic examination of colon sections from the various treatment groups was consistent with the DAI end point, revealing marked damage in the DSS-alone groups (both p53^{+/+} and p53^{-/-} mice). Figure 3 shows representative sections from each group. Importantly, it shows that AG protected from DSS-induced colitis in the p53^{+/+} mice but not in the p53^{-/-} mice. The panel shows a severely inflamed and damaged colon from DSS-treated animals (p53^{+/+} and p53^{-/-} as indicated). Numerous neutrophils were present throughout the section, along with severely damaged crypts, and moderate to severe inflammatory infiltration with submucosal edema. A section from p53^{+/+} mice treated with AG + DSS shows a healthier colon, with minimal infiltrating inflammatory cells and largely intact crypts. However, AG was not protective in this way in p53^{-/-} mice. Figure 3 also shows quantitative data reflecting these observations. The colons were graded for their histology score, which is based on inflammation severity, inflammation extent, crypt damage, and percentage of surface area showing the characteristic. These morphologies were scored on a blinded basis by two separate investigators.

Table 3. Percentage of cells from a representative experiment staining positive for Annexin V and negative for PI (early apoptotic cells) in Jurkat T cells, which have a dysfunctional p53 pathway

Dose AG (μg/mL), 72 h	Jurkat T cells	
	No Con A	Con A
0	1.40%	1.80%
50	2.30%	2.40%
100	2.50%	3.00%
200	2.60%	3.00%
300	3.00%	3.20%
500	3.30%	3.50%

Abbreviation: Con A, concanavalin A.

Table 4. Percentage of cells from a representative experiment staining positive for Annexin V cells from p53^{+/+} and p53^{-/-} C57BL/6 mice and negative for PI (early apoptotic cells) in CD4⁺/CD25⁻ effector T cells

AG (μg/mL), 24 h	p53 ^{+/+}		p53 ^{-/-}	
	No Con A	Con A	No Con A	Con A
0	1.50%	0.90%	2.60%	3.70%
50	4.60%	4.30%	2.30%	2.30%
100	3.90%	6.50%	2.70%	2.40%
200	9.00%	29.20%	3.40%	3.40%
300	12.70%	52.20%	3.90%	4.60%

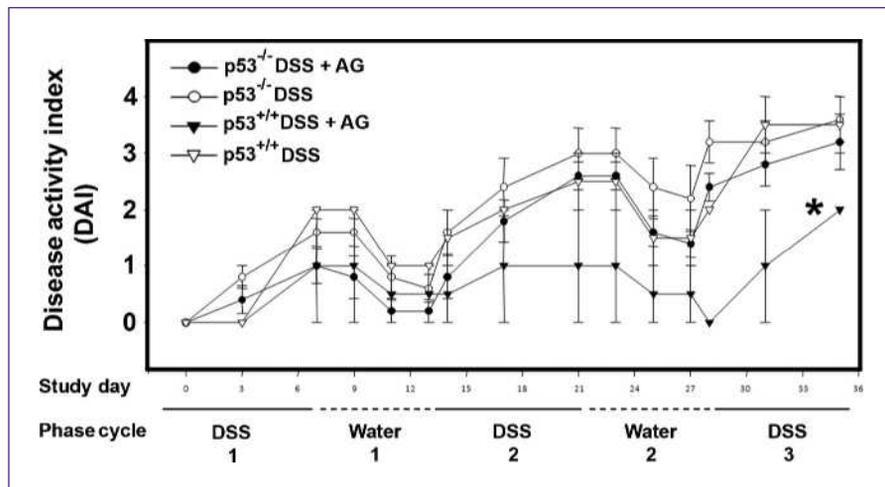


Fig. 2. Effects of AG on the DAI in p53^{+/+} and p53^{-/-} mice in the chronic DSS colitis model. Mice received 2.5 cycles (7 d DSS followed by 7 d water per cycle) of DSS with and without AG in chow, as indicated. ○, p53^{-/-} mice on DSS and on chow without AG; ●, p53^{-/-} mice on DSS and on chow with 75 ppm AG; ▽, p53^{+/+} mice on DSS and on chow without AG; ▼, p53^{+/+} mice on DSS and on chow with 75 ppm AG. *, $P < 0.005$, significant difference from other groups from day 21 to the end of the experiment.

As indicated in Fig. 3, p53^{+/+} and p53^{-/-} animals receiving DSS in their drinking water had substantially higher histology scores (representing moderate-to-severe inflammatory bowel disease) than animals receiving normal drinking water. Protection by AG from damage was significant only in the p53^{+/+} genotype but no in the p53^{-/-} genotype.

AG stimulates apoptosis of lymphocytes *in vivo*

To examine the effects of AG on apoptosis *in vivo*, we carried out a TUNEL assay on serial sections used for quantifying inflammation (Fig. 3). As shown in Fig. 4, for the epithelial cells, there is a significantly higher IRS (i.e., TUNEL label) in both the p53^{+/+} and p53^{-/-} mice treated with DSS than in mice treated with water. However, the IRS in the epithelial cells only decreased in the p53^{+/+} but not the p53^{-/-} mice when they were treated with both DSS + AG. This observation is consistent with the data from the inflammatory index (Fig. 3), indicating that AG protects epithelial cells from DNA damage *in vivo* only when p53 is present. However, this observation was the opposite

in the lymphocytes in the MLNs. Although there was an increase in IRS in the MLNs in both the p53^{+/+} and the p53^{-/-} mice when treated with DSS alone, AG exacerbated the apoptotic response only in the p53^{+/+} mice. Such results are consistent with our *in vitro* data and with the hypothesis that AG drives apoptosis in inflammatory cells *in vivo*, providing a mechanism by which AG protects from colitis in this DSS mouse model.

Discussion

In this current study, we have identified the p53 pathway as key to the ability of AG to suppress colitis. Previous studies have shown that p53 is mutated in noncancerous and precancerous epithelial lesions of patients with chronic ulcerative colitis (9, 10, 38, 39). Studies done on p53^{+/+} versus p53^{-/-} mice have shown that although the intermediate inflammatory index (i.e., colitis) is not significantly different between these two genotypes (consistent with our findings; Fig. 3; ref. 26), colitis-associated neoplasias are

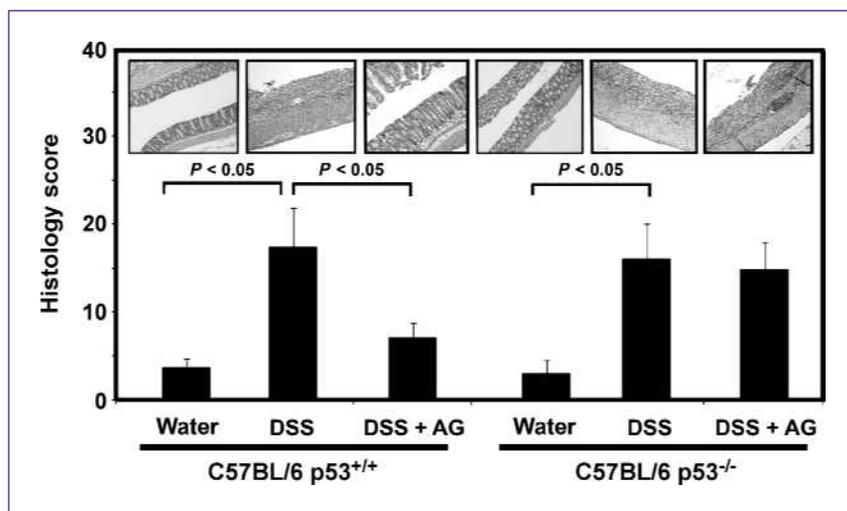


Fig. 3. Effects of AG on the colon histology score in the acute DSS colitis model. Ten mice from each group described in Fig. 2 were sacrificed on day 35, and the colon was harvested from each animal and the histology score was determined. Values represent the mean \pm SE. Representative H&E-stained colons are shown for each group. Significant differences are indicated ($P < 0.05$).

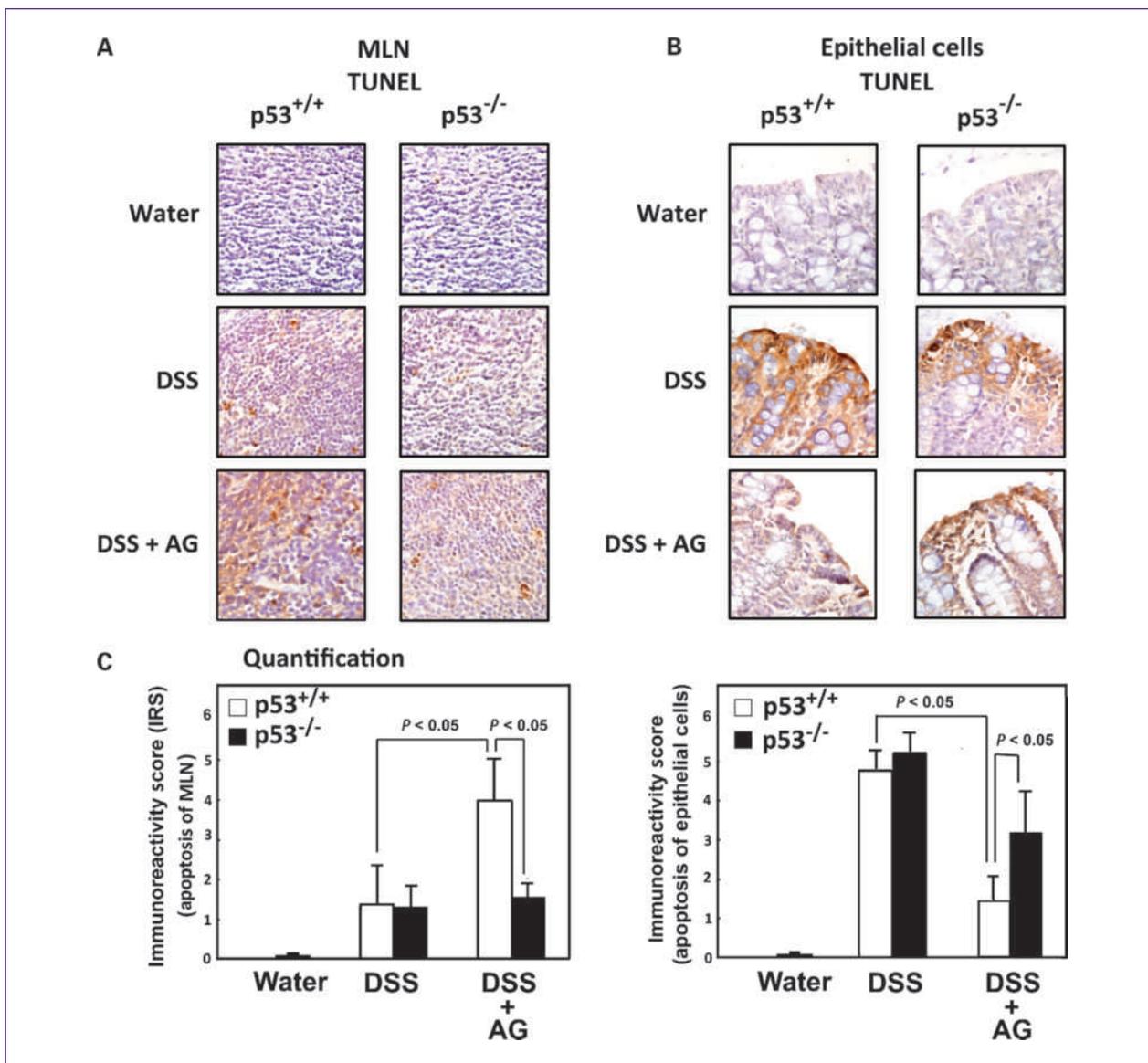


Fig. 4. Effects of AG on apoptosis in cells of the epithelium and the MLNs. A, IRS (TUNEL staining) in MLN cells of indicated groups. B, IRS (TUNEL staining) in epithelial cells of indicated groups. C, quantification of staining in the MLNs (left) and epithelium (right) of indicated groups.

significantly elevated in p53^{-/-} mice versus p53^{+/+} mice (25, 26). Although this indicates that the level of colon inflammation may not play a key role in driving colon carcinogenesis in such models, we are currently examining whether AG has different capabilities to prevent colon cancer associated with colitis in p53^{+/+} versus p53^{-/-} mice.

Because of the complicated nature of AG, it is difficult to pin down the specific ingredients within the extract capable of interacting with p53. We feel it is important to present our present results on the entire AG extract. However, although beyond the scope of this current study, we are currently trying to identify possible mechanisms of p53 activation by AG, including the possibility of minor, targeted DNA damage, the induction of hypoxia, altered glu-

cose metabolism, thymidylate synthase inhibition, interference with ribosome biogenesis, and cytokine stimulation (40, 41). In addition, because the activation of p53 is associated with an increase in reactive oxygen species, facilitating the ability to selectively kill off p53-overexpressing cells (42, 43), it is possible that AG not only increases p53 in itself but selectively kills off CD4⁺/CD25⁻ effector T cells by facilitating the release of reactive oxygen species from p53-overexpressing activated CD4⁺/CD25⁻ effector T cells. Other future studies we are carrying out are bioassay-guided fractionation of AG to delineate ingredients active against colitis and capable of interacting with p53. In human arterial smooth muscle cells, it has been shown that Rg1 induces p53 transcription and increases p53 protein levels,

thought to play a role in the inhibition of proliferation of such cells (44). Similarly, mRg2, a mixture of ginsenosides containing 60% Rg2, was capable of inducing p53 and p21 in response to UVB exposure in NIH 3T3 cells, thereby protecting such cells from genotoxicity (45). Ming et al. (24) showed that 20-O-(β -D-glucopyranosyl)-20(S)-protopanaxadiol (IH-901), a novel intestinal bacterial metabolite of ginseng protopanaxadiol saponins, upregulates p53 and drives apoptosis through a mitochondrial pathway by increasing the levels of the proapoptotic molecule Bax in hepatocellular carcinoma cells. Similarly, Huang et al. (23) showed that a new triterpenoid isolated from *P. ginseng* was potent in stimulating p53-mediated cell cycle arrest, leading to apoptosis via activation of the caspase signaling pathway in HepG2 cells. As well, ginsenoside-Rs4 and ginsenoside-Rs3, isolated from *P. ginseng*, can selectively elevate protein levels of p53 and p21^{WAF1} and induce apoptosis in SK-HEP-1 human hepatoma cells (46, 47). More recently, Wang et al. (22) showed that steaming AG increased Rg3 and Rh2 content, and AG was capable of inducing apoptosis of colon cancer cells (SW-480) through the mitochondrial pathway, involving the activation of p53. The finding that Rh2 isolated from AG was potent in the induction of apoptosis of colon cancer cells was confirmatory to a previous study showing similar findings (48).

Although others have reported an apoptotic effect of ginseng on blood cells (36), ours is the first study reporting a p53-dependent induction of apoptosis in isogenic lymphoblastoid and primary CD4⁺/CD25⁻ primary T cells. Indeed, although there is relatively little induction of apoptosis in the lymphoblastoid cells (Table 2), these data provided useful initial data indicating a p53-dependent mechanism to apoptosis by AG. We recognized that the more relevant experiment is that which is presented in Table 4. Importantly, AG not only induces apoptosis in CD4⁺/CD25⁻ effector T cells from p53^{+/+} mice but preferentially induces apoptosis in stimulated CD4⁺/CD25⁻ effector T cells from p53^{+/+} mice. It is recognized that CD4⁺ cells play a critical role in driving colitis in a DSS model (49) and that the lack of a capability to control apoptosis of CD4⁺/CD25⁻ T cells is thought to play an intricate role in colitis (2). Because p53 plays a key role in apoptosis, it is reasonable that the ability of AG to drive apoptosis in activated p53^{+/+} CD4⁺/CD25⁻ effector T cells *in vitro* is a key mechanism toward its ability to protect from colitis in this genotype. Results shown here are consistent with this hypothesis. Importantly, we also show that AG drives apoptosis of inflammatory cells of the MLNs *in vivo* (Fig. 4). The finding that DSS induces apoptosis of wild-type mice in the epithelium is

consistent with other studies (50–52) and the understanding that DSS causes DNA damage to epithelial cells. The finding that apoptosis is reduced in the epithelium by AG only in the wild-type mice is confirmatory that AG protects the epithelial tissues in a p53-dependent manner. Based on our *in vitro* data (Tables 2–4; Supplementary Figs. S1 and S2), and our *in vivo* data (Fig. 4), we suggest that this protection is at least in part due to the ability of AG to target the apoptosis of inflammatory cells.

Overall, we have presented data underpinning a key role for the p53 pathway in the protection from colitis by AG. Although p53 is mutated and the pathway is dysfunctional in patients with long-standing colitis, it is not completely clear whether this mutation is in epithelial cells and/or in inflammatory cells. A functional p53 pathway in inflammatory cells seems to be a necessary prerequisite to the ability of AG to protect from colitis. In this regard, p53 levels are induced under inflammatory stress (7), as well as by exposure to AG under nonstressed conditions (Fig. 1). In inflammatory conditions, we suggest that AG has the capability to facilitate p53-driven apoptosis in inflammatory cells, thereby killing them off. Although we have no evidence to date, it would also be interesting to test the ability of AG to protect from p53 mutation in epithelial cells, thereby reducing the risk of colon cancer associated with colitis.

Disclosure of Potential Conflicts of Interest

L.J. Hofseth has a patent pending for an antibody amplifier used in immunohistochemistry. The other authors disclosed no potential conflicts of interest.

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