Diosgenin, a Steroid Saponin of *Trigonella foenum graecum* (Fenugreek), Inhibits Azoxymethane-Induced Aberrant Crypt Foci Formation in F344 Rats and Induces Apoptosis in HT-29 Human Colon Cancer Cells

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Abstract

*Trigonella foenum graecum* (fenugreek) is traditionally used to treat disorders such as diabetes, high cholesterol, wounds, inflammation, and gastrointestinal ailments. Recent studies suggest that fenugreek and its active constituents may possess anticarcinogenic potential. We evaluated the preventive efficacy of dietary fenugreek seed and its major steroidal saponin constituent, diosgenin, on azoxymethane-induced rat colon carcinogenesis during initiation and promotion stages. Preneoplastic colonic lesions or aberrant crypt foci (ACF) were chosen as end points. In addition, we assessed the mechanism of tumor growth inhibition of diosgenin in HT-29 human colon cancer cells. To evaluate the effect of the test agent during the initiation and postinitiation stages, 7-week-old male F344 rats were fed experimental diets containing 0% or 1% fenugreek seed powder (FSP) or 0.05% or 0.1% diosgenin for 1 week and were injected with azoxymethane (15 mg/kg body weight). Effects during the promotional stage were studied by feeding 1% FSP or 0.1% diosgenin 4 weeks after the azoxymethane injections. Rats were sacrificed 8 weeks after azoxymethane injection, and their colons were evaluated for ACF. We found that, by comparison with control, continuous feeding of 1% FSP and 0.05% and 0.1% diosgenin suppressed total colonic ACF up to 32%, 24%, and 42%, respectively (\(P \leq 0.001\) to 0.0001). Dietary FSP at 1% and diosgenin at 0.1% fed only during the promotional stage also inhibited total ACF up to 33% (\(P \leq 0.001\)) and 39% (\(P \leq 0.0001\)), respectively. Importantly, continuous feeding of 1% FSP or 0.05% or 0.1% diosgenin reduced the number of multicrypt foci by 38%, 20%, and 36% by comparison with the control assay (\(P \leq 0.001\)). In addition, 1% FSP or 0.1% diosgenin fed during the promotional stage caused a significant reduction (\(P \leq 0.001\)) of multicrypt foci compared with control. Dietary diosgenin at 0.1% and 0.05% inhibited total colonic ACF and multicrypt foci formation in a dose-dependent manner. Results from the *in vitro* experiments indicated that diosgenin inhibits cell growth and induces apoptosis in the HT-29 human colon cancer cell line in a dose-dependent manner. Furthermore, diosgenin induced apoptosis in HT-29 cells at least in part by inhibition of bcl-2 and by induction of caspase-3 protein expression. On the basis of these findings, the fenugreek constituent diosgenin seems to have potential as a novel colon cancer preventive agent. (Cancer Epidemiol Biomarkers Prev 2004;13(8):1392–8)

Introduction

Colon cancer is considered a preventable disease (1). However, there seems to be no decline in the incidence of colon cancer, and many of the risk factors associated with colon cancer prevail (2). Diet-based strategies hold promise for both prevention and treatment of colon cancer (1, 3). In this regard, plant-derived diets containing phytochemicals could be used in preventive strategies to reduce the risk and inhibit or retard the development of colon cancer. *Trigonella foenum graecum*, commonly called fenugreek, is a leguminous plant native to many Asian, Middle Eastern, and European countries (4). The seeds and leaves of fenugreek are edible and are used as condiments and as Ayurvedic medicine in the Indian subcontinent to treat diabetes, high cholesterol, wounds, inflammation, and gastrointestinal ailments (4). Fenugreek seeds have been successfully tested in laboratory animals and in humans with type 1 and type 2 diabetes as a hypoglycemic agent (5-7). The potential of fenugreek seeds to modulate several enzymes, including those associated with glucose and lipid metabolism, has been described earlier (8). Among bioactive compounds isolated from fenugreek seeds are protodioscin, trigonoside, diosgenin, yamogenin, and others (9, 10).

Extracts of fenugreek seeds and some of their saponin constituents have been found to have anticarcinogenic potency in different settings (11, 12). Fenugreek seed extract has been evaluated in the Ehrlich ascites carcinoma model in BALB/c mice, where it effected 70% inhibition of tumor cell growth compared with controls (11). The findings of Hibasami et al. (12) suggest that growth inhibition of human leukemia HL-60 cells by...
protodioscin, isolated from fenugreek seeds, results from the induction of apoptosis. Diosgenin [(25R)-5-spirosten-3β-ol], a steroid sapogenin constituent of fenugreek seeds, is a precursor of steroid hormones, such as progesterone, and anti-inflammatory steroids, such as cortisone (13). Figure 1 illustrates the chemical structure of diosgenin. Moalic et al. (14) reported that diosgenin inhibits cell proliferation in the human osteosarcoma 1547 cell line by induction of apoptosis and G1 phase cell cycle arrest. Furthermore, in the osteosarcoma 1547 cell line, it was showed that diosgenin caused cell cycle arrest and apoptosis principally by increasing the expression of the tumor suppressor oncoprotein p53 (15). On the basis of the information described above, diosgenin and other fenugreek seed constituents possess anticarcinogenic properties, suggesting their potential role as suitable phytochemicals for colon cancer prevention. However, to our knowledge, the colon cancer inhibitory properties of diosgenin and other fenugreek seed constituents have not been studied in detail, and there is no single study to ascertain that the anticancer capabilities of diosgenin showed earlier in vivo will prevail in an in vitro setting.

Colon carcinogenesis is a multistep process involving sequential change of normal colonic epithelial cells into preneoplastic, neoplastic, and metastatic states (16). Aberrant crypt foci (ACF) are preneoplastic lesions of the colon in mice, rats, and humans that are regarded as valuable biomarkers in screening potential chemopreventive agents (reviewed in refs. 17, 18). The molecular basis for inhibition of preneoplastic and neoplastic lesions by potential chemopreventive agents is currently being explored. Changes pertaining to aberrant cell growth and proliferation that lead to tumorigenicity have been identified as early as in the formation of ACF per se (19-22). Mechanisms involved in the inhibition of cell proliferation and induction of apoptosis are recognized as being pivotal. Bcl-2 and caspase-3 among others have been implicated as molecular mediators of apoptosis (reviewed in refs. 23, 24). Colon tumors are characterized by an overexpression of bcl-2, whereby apoptosis is down-regulated (25), and chemopreventive agents decrease the expression of bcl-2 in human colon cancer cells, thus augmenting apoptosis (26, 27). By contrast, the proapoptotic caspase-3 is down-regulated in colon tumors, and its expression is increased by cancer preventive agents (reviewed in ref. 24). Several studies have used the measurement of apoptosis in colon cancer cells induced by chemopreventive agents to assess the efficacy of such agents (26-29). Whether diosgenin inhibits colon tumor cell growth by the induction of apoptosis is not known. The present study was therefore designed to evaluate the efficacy of dietary fenugreek seed and its major steroid saponin constituent diosgenin in inhibiting or retarding ACF formation during initiation/postinitiation and promotional stages of azoxymethane-induced rat colon carcinogenesis. In addition, we determined the effect of diosgenin on inhibiting cell growth and modulating the expression of bcl-2 and caspase-3 in HT-29 human colon cancer cells.

**Materials and Methods**

**Animals, Care, and Diets.** Seven-week-old male F344 rats were procured from Charles River Laboratories (Kingston, NY) and housed in suspended cages ~10 cm above bedding trays with a 12-hour light/dark cycle in the animal housing facility of the Institute for Cancer Prevention (Valhalla, NY). Temperature and relative humidity were controlled at 21°C and 55%, respectively. All animals were acclimatized to the above conditions for 1 week with free access to standard laboratory rodent chow and drinking water until initiation of the experiment. Animals were cared for according to the guidelines of the American Council on Animal Care. Diets were based on modified AIN-76A containing 5% corn oil by weight (30). Fenugreek seeds were a gift from Dr. Peter R. Chang (Agriculture and Agro-Food Canada Research Center, Saskatoon, SK), and diosgenin was purchased from Sigma Chemical Co. (St. Louis, MO). The control diet contained no fenugreek seed powder (FSP) or diosgenin. The experimental diets contained 1% FSP or 0.05% or 0.1% diosgenin (w/w). Diets were prepared twice each week and were stored at 4°C until used. Rats were allowed ad libitum access to the respective diets and tap water, and food cups were replenished with fresh diets thrice weekly. The stability of diosgenin in the diet was established by testing the experimental diet kept at room temperature for a period of 1 week. Each day, diet samples were collected, extracted, and analyzed by high-performance liquid chromatography according to the method of Artuno et al. (31). Based on the results, even after 7 days at room temperature, >95% diosgenin were recoverable from the feed, suggesting the reasonable stability of diosgenin at room temperature.

**Experimental Design.** The experimental protocol is shown in Fig. 2. Rats were randomized into groups receiving either the control diet (n = 30) or diets containing 1% FSP or 0.05% or 0.1% diosgenin (n = 10 per group). Beginning 1 week later, all rats were s.c. injected with azoxymethane once a week for 2 weeks at a dose of 15 mg/kg body weight. Four weeks after the second injection, rats intended for promotion stage testing were switched from control diet to experimental diets, in this case, either 1% FSP or 0.1% diosgenin (n = 10 per group). All animals were sacrificed by CO2 asphyxiation 8 weeks after azoxymethane injection. The colons were removed, flushed with ice cold PBS, and slit open along the length from the anus to the cecum on an ice-cold glass plate. The colons were examined for any macroscopic changes and were fixed flat between filter papers in 70% ethanol and coded for blind scoring.

**Quantification of ACF.** Topographical analysis of the colon mucosa according to Bird (32) was done after a minimum of 24 hours in 70% ethanol. Colons were...
Bromide Staining. HT-29 cells treated with 0, 20, 40, or 100 \( \mu \)g/mL diosgenin for 24 hours were washed with PBS and trypsinized. Twenty-five microliters of the cell suspension (\( \sim 0.5 \times 10^6 \) per mL) were incubated with 1 \( \mu \)L of acridine orange/ethidium bromide (one part each of 100 \( \mu \)g/mL acridine orange and 100 \( \mu \)g/mL ethidium bromide in PBS) just prior to microscopy. A 10 \( \mu \)L aliquot of the gently mixed suspension was placed on microscope slides, covered with glass slips, and examined under an Olympus AX70 microscope (Tokyo, Japan) connected to a digital imaging system with SPOT RT Software version 3.0. Acridine orange is a vital dye that will stain both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity. Live cells stain uniformly green and can be distinguished from apoptotic cells as they exhibit yellow to orange coloration depending on the degree of loss of membrane integrity due to containing with ethidium bromide.

Western Blot Analyses. Whole cell lysates of treated and untreated HT-29 cells were prepared with lysis buffer containing protease inhibitors. Total proteins were quantified using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA). Volumes of whole cell lysates containing 100 \( \mu \)g protein were heated for 4 minutes at 80°C with 2 \( \times \) Laemmli sample buffer (Sigma Chemical) and separated by 10% SDS-PAGE using the Mini-Protean Bio-Rad II System. The separated proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham Life Sciences, Arlington Heights, IL). These membranes were blocked for 1 hour at room temperature with 5% skim milk powder and probed with primary antibodies of interest, incubated with secondary anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:2,500 dilution for 1 hour at room temperature. After washing, the blots were incubated with secondary anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at 1:2,500 dilution for 1 hour at room temperature. After washing, the blots were incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL) for 5 minutes and exposed to photographic film to detect protein bands.

Results

General Observations. To ascertain that dietary fenugreek seed or diosgenin had no negative effect on body weight gain or eating habit, all rats were monitored on a routine basis. The initial body weight (mean ± SE) before dietary interventions with fenugreek seed or diosgenin and azoxymethane injection was 117.20 \( \pm \) 2.18. At the time of termination, there was no significant difference in body weights of control and treated rats (Table 1). The food intake of animals in the experimental groups did not vary. Fenugreek seed at 1% and diosgenin at 0.05% or 0.1% were well tolerated and caused no adverse effects in F344 rats.

Effect of FSP and Diosgenin on Colonic ACF during Initiation/Postinitiation Stages. We used the well-established, short-term protocol of the azoxymethane-induced rat colon carcinogenesis model to determine...
the efficacy of fenugreek seed and diosgenin to inhibit the formation or retard the development of ACF. Dietary 1% FSP and 0.05% and 0.1% diosgenin given continuously for 8 weeks suppressed total colonic ACF to 32%, 24%, and 42% (*P < 0.001 to 0.0001), respectively, compared with control group (Fig. 3). Importantly, the continuous feeding of 1% FSP or 0.05% or 0.1% diosgenin significantly lowered the number of multicrypt foci or large ACF (with crypt multiplicity ≥4) by 38%, 20%, and 36% compared with control (P ≤ 0.001; Fig. 4). Dietary diosgenin inhibited total colonic ACF and multicrypt ACF formation in a dose-dependent manner (Figs. 3 and 4).

Effect of FSP and Diosgenin on Colonic ACF during Promotion Stages. To determine the effects of fenugreek seed and diosgenin at the promotional stages, groups of rats were given dietary 1% FSP and 0.1% diosgenin 4 weeks after azoxymethane injections for 4 weeks. In this protocol, 1% FSP and 0.1% diosgenin inhibited total ACF up to 33% (*P ≤ 0.001) and 39% (P ≤ 0.0001), respectively (Fig. 3). Similar to the effects observed in the initiation/postinitiation stages, 1% FSP and 0.1% diosgenin resulted in 25% and 32% lower (P ≤ 0.001) incidence of multicrypt ACF than were seen in positive controls (Fig. 4).

Effect of Diosgenin on HT-29 Human Colon Cancer Cell Proliferation and Apoptosis. To explore the anticancer potential of diosgenin in human colon cancer cells, we conducted several in vitro experiments. We examined the cytotoxic effects of 0 to 100 μmol/L diosgenin (24-hour treatment) on HT-29 cells using the MTT cytotoxicity assay. A dose-dependent MTT reduction (or color change from yellow to purple) was observed in diosgenin-treated cells (Fig. 5). On 24-hour exposure to diosgenin, MTT activity reduced by ≥50% was achieved at the higher concentrations (i.e., ≥80 μmol/L). However, compared with the control, 20 to 60 μmol/L diosgenin reduced the MTT activity only by ~5% to 30% (Fig. 5). Next, we examined dose-dependent and time-dependent effects of diosgenin on the proliferation of HT-29 cells using the trypan blue dye exclusion method. Diosgenin caused a significant time-dependent and dose-dependent decrease in the proliferation of HT-29 cells (Fig. 6). Twenty-four-hour exposure to diosgenin (20 to 100 μmol/L) inhibited cell proliferation compared with untreated cell growth (taken as 0%; Fig. 6). This inhibition was 21%, 68%, 82%, and 100% for 20, 40, 60, and 80 μmol/L diosgenin, respectively (Fig. 6).

To determine whether the inhibition of cell proliferation by diosgenin was due to the induction of apoptosis, we assessed the latter with the acridine orange/ethidium bromide method. Figure 7 summarizes the apoptotic effects of diosgenin in HT-29 cells. A dose-dependent increase in induction of apoptosis was observed when HT-29 cells were treated with diosgenin.
at 0, 20, 40, and 60 μmol/L for 24 hours. Compared with the control, 42% and 62% of the cell population in 40 and 60 μmol/L diosgenin-treated cells displayed apoptosis, respectively.

**Diosgenin Modulates the Protein Expression of bcl-2 and Caspase-3 in HT-29 Cells.** Western blot analyses of the apoptosis regulatory proteins bcl-2 and caspase-3 were conducted using HT-29 cells treated with or without 24-hour diosgenin. We found that bcl-2 protein was significantly decreased in a dose-dependent manner by 0, 20, 40, and 60 μmol/L diosgenin (Fig. 8); by contrast, these doses of diosgenin significantly increased caspase-3 expression in HT-29 cells again in a dose-dependent manner (Fig. 8).

**Discussion**

The main objective of this study was to evaluate the potential efficacy of fenugreek seed, a commonly used herb, and its steroid saponin constituent, diosgenin, in preventing colon carcinogenesis in vivo and to understand the anticancer mechanisms of diosgenin in vitro. To our knowledge, this is the first study demonstrating that fenugreek seed and diosgenin have the potential to prevent colon cancer. Using the azoxymethane-induced rat colon carcinogenesis model, we show that dietary fenugreek seed and diosgenin reduce or retard the appearance of colonic ACF when given during the initiation/postinitiation stages and even when given only during the promotional stage. In addition, diosgenin inhibits cell proliferation and induces apoptosis in HT-29 human colon cancer cell lines. The induction of apoptosis by diosgenin is in part affected by its ability to suppress the expression of the antiapoptotic bcl-2 while increasing the expression of the proapoptotic caspase-3.

Fenugreek seeds and their active constituents have been reported to be excellent antidiabetic agents based on several in vivo studies, including human intervention studies (reviewed in ref. 5), and their possible mechanisms of action as antidiabetics have been described (8, 33). In a 90-day subchronic study, rats fed fenugreek seeds, at doses between 1% and 10% in pure diet, had no toxic effects (34). In the present study, 1% fenugreek seed was used in the bioassay with rats. The level of diosgenin in fenugreek seeds ranges from ~0.42% to 0.75% depending on the cultivars and seed quality (35). Because the entire seed was used, active seed contents other than diosgenin might influence ACF modulation; therefore, we selected doses at higher levels of diosgenin (0.1% and 0.05%) than are actually found in 1% fenugreek diet (~0.004% to 0.007%). Moreover, in a previous study, no acute toxic effects were reported when rats were given dietary diosgenin at 1%, 0.2%, or 0.05% doses (36). As our results indicate, no acute or chronic distress was observed in diosgenin-treated or fenugreek seed–treated animals.

Colon carcinogenesis is a multistep event; it involves the transformation of normal colonic epithelial cells into a preneoplastic state and progresses toward advanced neoplasia (16). We observed a significant inhibition of the initiation and development of total and large colonic ACF when dietary fenugreek seed and diosgenin were given (Fig. 4). We also observed that colonic ACF in the azoxymethane-induced rat colon carcinogenesis model were inhibited by dietary fenugreek seed and diosgenin when given either during the initiation/postinitiation stages or even when given only during the promotional stage (Fig. 5).
ACF when 1% fenugreek or 0.1% or 0.05% diosgenin were given during either initiation/postinitiation or promotion stage. The ability to cause regression or retard the appearance of ACF in either stage marks fenugreek seed and diosgenin as likely colon cancer preventive agents. The latter is being confirmed through tumorigenesis studies in our laboratory using the azoxymethane-induced colon cancer model in rats with intervention strategies directed toward initiation/postinitiation and promotion/promotion stages. In the present study, ACF were classified according to their size; ACF with one to three crypts and those with four or more crypts were designated as either "small" or "large" (multi-crypt), respectively. Fenugreek seed and diosgenin significantly reduced the number of large ACF in both intervention strategies used. This suggests that these agents would be effective not only in preventing the appearance of ACF but plausibly also in retarding the growth and progression of large ACF, including those of the intermediate and advanced type. This aspect is very important considering that a large portion of the population at risk for colon cancer is characterized by the presence of polyps and large ACF in their colons (37, 38). It is also important with regard to the dose levels of diosgenin used that there were no differences in ACF numbers between rats treated with 0.1% and those receiving 0.05% of the agent; hence, the lower dose seems sufficient to block ACF formation during the early stages of colon carcinogenesis. However, the dose responses to 0.05% or 0.1% diosgenin in inhibiting tumor parameters require further evaluation.

Our in vitro data indicate dose-dependent inhibition of HT-29 human colon cancer cell proliferation by diosgenin. Diosgenin exhibited cytotoxicity only at the highest concentrations (80 to 100 μmol/L); however, concentrations as low as 40 μmol/L were capable of inhibiting cell proliferation by ≥50%. Furthermore, diosgenin induced apoptosis in HT-29 cells; this is at least in part mediated by the suppression of bcl-2 and the induction of caspase-3 proteins. As mentioned earlier, colonic tumors potentiate their growth and survival by suppressing apoptosis (23, 24). How diosgenin regulates bcl-2 or caspase-3 expression during colon carcinogenesis in vivo is unclear and warrants further investigation. Other mechanism(s) of diosgenin that could possibly be involved in the inhibition of HT-29 cells could be those relating to modulation of cyclooxygenase-2 and the activation of nuclear factor-κB, p53, or p21 expression as shown earlier in the inhibition of osteosarcoma cells (14).

In summary, our results show for the first time that (a) fenugreek seed and diosgenin inhibit early events of azoxymethane-induced colon cancer when given during either initiation/postinitiation or promotion stage and (b) diosgenin exhibits anticancer effects by blocking the proliferation of HT-29 human colon cancer cells and induces apoptosis in part by modulating bcl-2 and caspase-3 expression in vitro. How much of the dietary diosgenin is absorbed into the system and how it is retained, metabolized, or excreted by rats during azoxymethane-induced colon carcinogenesis are currently under investigation in our laboratory. Phytochemicals or food-based compounds hold promise for novel strategies in cancer chemoprevention and control (reviewed in ref. 39). Whereas the clinical potential of fenugreek seeds and their active constituents in the control of hypercholesterolemia or diabetes has been documented (reviewed in ref. 5), the findings of this study and those of earlier ones demonstrating the anticancer properties of fenugreek constituents and diosgenin (11, 12, 14, 15) have potential clinical relevance for cancer prevention and control. Thus, the role of fenugreek seed and its main active constituent diosgenin as new supplements in diet-based preventive/therapeutic strategies to potentially alleviate human colon cancer remains an important field of study for future investigations.

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References


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