Novel anti-angiogenic effects of formononetin in human colon cancer cells and tumor xenograft

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Abstract. Formononetin is a novel herbal isoflavonoid isolated from *Astragalus membranaceus*, a medicinal plant that possesses antitumorigenic properties. Our previous findings demonstrated that formononetin initiates growth-inhibitory and pro-apoptotic activities in human colon cancer cells. In the present study, we aimed to further examine the potential of formononetin in controlling angiogenesis and tumor cell invasiveness in human colon cancer cells and tumor xenografts. The results showed that formononetin downregulated the expression of the key pro-angiogenic factors, including vascular endothelial growth factor (VEGF) and matrix metalloproteinases. We also discovered that the invasiveness of metastatic colon cancer cells was alleviated following drug treatment. The potential anti-angiogenic effect of formononetin was examined in nude mouse xenografts. The tumor size and the number of proliferating cells were reduced in the tumor tissues obtained from the formononetin-treated group. The serum VEGF level was also reduced in the drug-treated animals when compared to the controls. These findings suggest that formononetin inhibits angiogenesis and tumor cell invasion, and thus support its use in the treatment of advanced and metastatic colon cancers.

Introduction

Angiogenesis is essential in the process of carcinogenesis to facilitate tumor progression and metastasis. The vascular endothelial growth factor (VEGF) is a well-characterized angiogenic factor known to stimulate angiogenesis within a tumor (1). Additionally, the expression and activity of matrix metalloproteinases (MMPs) are increased in almost every type of human cancer, which may be associated with cell invasion, metastasis and angiogenesis. Among the MMPs, MMP-2 and MMP-9 directly regulate angiogenesis (2). Another important mechanism for the progression of colorectal cancer is the ability of the transformed cells to invade and traverse the basement membrane, which in turn facilitates the conversion of adenomas into carcinomas and then metastases (1). MMPs have been implicated in these processes and a previous study has suggested that the plasma level of MMP-2 and MMP-9 may be viewed as a non-invasive indicator of invasiveness and metastasis in colon cancer (3).

The dried root of *Astragalus membranaceus* (*Radix Astragali*) has a long history of medicinal use in traditional Chinese medicine as an immunomodulating agent in mixed herbal decoctions to treat the common cold, diarrhea, fatigue and anorexia (4). In contemporary pharmacotherapy, *Radix Astragali* has been used to ameliorate the side-effects of cytotoxic antineoplastic drugs (5). Among its different constituents, *Astragalus* polysaccharides have been most widely studied particularly for their immunopotentiating properties, such as stimulation of murine B cell proliferation and cytokine production (6). Apart from these, the results from clinical studies have shown that *Astragalus* polysaccharides counteract against the side-effects of chemotherapeutic drugs, including a significant attenuation of myelosuppression in cancer patients (7). Nevertheless, our laboratory findings did not support any direct anticancer activity by *Astragalus* polysaccharides. In recent years, we focused on investigating the anti-carcinogenic properties of total *Astragalus* saponins (AST) in human colon cancer cells and tumor xenografts. Our results indicated that AST inhibits cell proliferation through phase-specific cell cycle arrest and caspase-dependent apoptosis. The reduction in tumor volume as well as pro-apoptotic and anti-proliferative effects in HT-29 nude mice xenograft are comparable to those produced by 5-FU. In addition, the side-effects associated with the drug combo 5-FU and oxaliplatin may be alleviated by AST (8). These data indicate that AST may be an effective chemotherapeutic agent in colon cancer treatment, and may also be used as an adjuvant in combination with other orthodox chemotherapeutic drugs to reduce the side-effects of the latter compounds. Subsequent study using multiple human cancer cell lines, including those of the colon, stomach and liver, further indicated the universal growth-inhibitory and pro-apoptotic effects of AST. We have also confirmed that non-steroidal anti-inflammatory drug activated gene (NAG-1) is a major molecular target of AST during its antitumorigenic action, which has a

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strong correlation with the PI3K-Akt signaling pathway during its action (9). Among various active constituents, information regarding the bioactivity and anticancerogenic potential of *Astragalus* flavonoids remains inadequate.

Formononetin is one of the major isoflavonoid constituents isolated from *Astragalus membranaceus* in sufficient quantity with >95% purity (10). Apart from *Astragalus*, formononetin can also be found in other commonly used herbs in China, Japan and the Western world, including the root of *Glycyrrhiza glabra* and *Glycyrrhiza uralensis* (licorice) (11). Formononetin has demonstrated diverse pharmacological benefits. As a phytoestrogen, it has demonstrated a metabolic effect by upregulating interleukin-4 production in activated T cells via increased AP-1 DNA binding activity (12). It also possesses anti-inflammatory activity by significantly suppressing arachidonic acid release in HT-29 human colon cancer cells (13). Formononetin also reduces blood pressure and central arterial stiffness, leading to reduced cardiovascular risk (14). Furthermore, its value as a potential antidiabetic agent in regulating lipid metabolism by the activation of PPAR-α and PPAR-γ receptors has been indicated (10). Formononetin has demonstrated analgesic activity in an acetic acid-induced abdominal constriction mouse model (15). Nevertheless, this novel compound has also been shown to induce the proliferation of osteoblasts (16). We recently reported that formononetin, similar to AST, can induce growth inhibition and promote caspase-dependent apoptosis in human colon cancer cells. This involves the inhibition of anti-apoptotic proteins, such as Bcl-2 and Bcl-x<sub>L</sub>, and the activation of the novel pro-apoptotic protein, NAG-1 (17). However, data on the influence of formononetin on tumor angiogenesis are insufficient. In the present study, we extended our ongoing study by investigating the effects of formononetin on angiogenesis and the invasiveness of human colon cancer cells and nude mouse xenografts. The results obtained provide evidence for the broader use of formononetin as a chemotherapeutic agent against advanced and metastatic human colorectal cancers, apart from its use as a pro-apoptotic agent.

**Materials and methods**

**Drugs and chemicals.** The structure of formononetin is illustrated in Fig. 1A. The drug chemical used in this study had the purity of >99% and was purchased from Fluka (Buchs, Switzerland). The types and sources of antibodies used were as follows: anti-proliferating cell nuclear antigen (PCNA), anti-VEGF, anti-MMP-2 and anti-MMP-9 (Santa Cruz, Switzerland); and anti-VEGF, anti-MMP-2 and anti-MMP-9 (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-β-actin (Sigma-Aldrich, St. Louis, MO). The BioCoat Matrigel Cell Invasion Chamber was purchased from BD Biosciences (San Jose, CA). All other chemicals were obtained from Sigma-Aldrich unless specified.

**Cell culture.** HCT-116 human colorectal carcinoma cells (CCL-247) and LoVo human colorectal adenocarcinoma cells from metastatic sites (CCL-229) were purchased from ATCC (Manassas, VA). HCT-116 and LoVo cells were grown in 75-cm<sup>2</sup> flasks and incubated at 37°C under a 5% CO<sub>2</sub> atmosphere in DMEM-1640 medium (Gibco, Carlsbad, CA) and F-12K Medium (ATCC), respectively supplemented with 10% FBS (Gibco) plus 1% penicillin and streptomycin (Gibco). LoVo cells were chosen for the invasion test due to their metastatic properties.

**Tumor xenografts in nude mice and drug treatment.** The experimental procedures were approved by our institutional animal research ethics committee with reference to the European Community guidelines for the use of experimental animals. Five-week-old female Balb/c-nu/nu mice were obtained from the Laboratory Animal Service Unit of the Chinese University of Hong Kong. The animals were kept under sterile conditions and in isolated pathogen-free ventilation chambers under an ambient temperature of 20-22°C and 40-50% relative humidity. The mice were anesthetized and injected subcutaneously near the flank with HCT-116 cells as previously described (8). Ten days after tumor induction, the mice began to receive either DMSO (control) or formononetin (20 mg/kg) treatment by daily intraperitoneal injection for 14 days. Tumor volume (in mm<sup>3</sup>) was measured every other day (8), while tumor mass (in grams) was calculated on the final day of the experiment. The animals were sacrificed on day 24, with their serum collected and tumor tissues excised. Apart from the mainstream experiments, we also obtained whole blood from normal Balb/c mice to measure the total white blood cell (WBC) count. This could allow us to assess the influence of formononetin on the immune system of experimental animals. Blood was collected from the tail vein of mice at the end of the experimental period. The sampled blood (20 µl) was mixed with 176 µl of 3% acetic acid and 4 µl of 1% crystal violet. Stained WBCs were counted in a hemocytometer under a light microscope (magnification, x200). The body weight of all the animals was recorded throughout the entire experimental period as an assessment of drug toxicity. Any mortality during the course of the study was recorded.

**Immunohistochemical analysis of cell proliferation and VEGF in tumor tissues.** The excised tumor samples were fixed for paraffin embedment and tumor sections (5 µm) were prepared on slides. Immunohistochemical analysis for cell proliferation and VEGF expression was performed. The sections were blocked with 2% BSA to minimize non-specific binding, and incubated overnight with anti-PCNA or anti-VEGF monoclonal antibody at 4°C. The positively stained cells were visualized by incubating the sections with 3,3′-diaminobenzidine using Mayer's hematoxylin as the counterstain. The negative control was prepared with similar procedures but without the addition of the primary antibody.

**Western immunoblotting.** The cells (treated for 0-72 h with DMSO or 200 µM formononetin) were lysed in RIPA buffer containing 50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 0.1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aproatin and 5 µg/ml pepstatin A. After the insoluble materials were removed by centrifugation at 14,000 x g for 10 min at 4°C, the proteins were quantified using the Coomassie Plus Protein Assay Reagent kit (Pierce, Rockford, IL). Total cellular proteins (20-40 µg) in the cell lysates were electrophoresed and transferred onto nitrocellulose membranes for western immunoblotting as previously described (9). The results were analyzed using Quantity One version 4.4.1 Basic software (Bio-Rad Laboratories, Hercules, CA).
Real-time reverse transcriptase-PCR (RT-PCR). The cells were lysed in TRIzol® reagent to extract total cellular RNA. Single-stranded cDNA was synthesized from 5 µg of total RNA using M-MLV reverse transcriptase. The sequences of VEGF (NM_001025370, 226 bp) used for PCR were: 5'-AAGGAGGAGGCAGAATCAT-3' (forward) and 5'-ATCTGTTGGTGTTGGA-3' (reverse). Those for GAPDH (595 bp) were used as the internal control: 5'-CCACCCATGGCAAATGTTCCACC-3' (forward) and 5'-TCTAGGGAGGCAGGTCCACC-3' (reverse). The conditions for PCR amplification (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA) were established as follows: 30 cycles of 95˚C for 2 min, 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, followed by an additional 10-min extension at 72˚C. The RNA products were resolved by electrophoresis on a 1.8% TAE agarose gel stained with ethidium bromide. All the reagents used in real-time RT-PCR were purchased from Invitrogen (Carlsbad, CA).

Cell invasion assay. The invasive ability of LoVo cells was assessed using a BioCoat Matrigel Invasion Chamber (BD Falcon) as previously described (18). In brief, cells were trypsinized and suspended in serum-free culture medium at the concentration of 1x10^6/ml in the upper chamber. The lower chamber was filled with culture medium supplemented with 10% FBS as the chemoattractant. After 24 h of drug treatment (200 µM formononetin, or DMSO as the control), the invaded cells that had passed through Matrigel and had adhered to the lower surface of the membrane were fixed with methanol and stained with 0.1% crystal violet. The stained cells were viewed and counted under a microscope (x200 magnification).

Serum VEGF level. The serum VEGF level in tumor xeno-grafted nude mice was determined using a VEGF immunoassay kit (Invitrogen). The procedures for the measurement of the serum VEGF level was carried out according to the protocols listed in the manufacturer’s manual.

Statistical analysis. Numerical data are expressed as the means ± standard error of the mean (SEM). Statistical significance of at least P<0.05 was determined by one-way analysis of variance (ANOVA) followed by a post-hoc least significant difference (LSD) test using SPSS version 10.0 software (SPSS, Chicago, IL).

Results

Effects of formononetin on VEGF and MMP expression in human colon cancer cells. In order to determine whether formononetin exerts anti-angiogenic effects in human colon cancer cells, the protein expression of bFGF, VEGF and MMPs was assessed. Both the gene and protein expression of VEGF were downregulated in the formononetin-treated HCT-116 cells, indicating the suppression of angiogenesis (Fig. 1B). MMP-2 and MMP-9 expression was also investigated as their activation contributes to tumor invasion and metastasis. Our results demonstrated that formononetin inhibited the protein expression of MMP-2 and MMP-9 (Fig. 1C). The formononetin-induced downregulation of pro-angiogenic factors occurred in a time-dependent manner, being more pronounced after 24 h of drug treatment. We then examined the effect of formononetin on tumor cell invasiveness using the
metastatic colon cancer cell line, LoVo. The number of cells that had passed through the Matrigel membrane was counted in both control and formononetin-treated groups. The data are summarized in Fig. 1D. The average number of invaded cells in the formononetin-treated group was significantly decreased when compared with the DMSO control group. Based on these findings, we suggest that the anticarcinogenic effects of formononetin involve the inhibition of angiogenesis and tumor cell invasiveness.

**Formononetin inhibits tumor growth in nude mouse xenografts.** In order to explore the antitumorigenic effect of formononetin against colon cancer development in vivo, HCT-116 nude mouse xenografts were used. No mortality or significant change in body weight was observed throughout the experimental period in both the control- and drug-treated animals. Fig. 2A shows the appearance and relative size of the tumors in the control and formononetin-treated animals. On the final day of the experiment, tumor tissue samples were obtained and the tumor volume in the formononetin-treated group was found to be reduced by 44% (1280±222 mm$^3$ in the DMSO-treated control vs. 718.6±52.6 mm$^3$ in the formononetin-treated group) (Fig. 2B). Tumor mass was also decreased by approximately 37% (Fig. 2C). Our results also showed that the WBC counts were comparable between the DMSO- and formononetin-treated groups (Fig. 2D), indicating that formononetin exerted no neutropenic effect on the experimental animals, as in the case of some conventional chemotherapeutic drugs.

**Formononetin reduces VEGF serum levels and tumor tissue expression and inhibits tumor cell proliferation.** In order to further confirm the effects of formononetin on angiogenesis, serum VEGF levels in the nude mice were measured on the final day of the experiment after the animals were sacrificed. The results showed that formononetin markedly reduced the serum VEGF levels in the nude mice by almost 50% when compared to the control group (Fig. 3A). The excised tumor tissues were then stained for VEGF expression, which showed a reduction in the tissues from the formononetin-treated group when compared with those from the control group (Fig. 3B). In order to determine whether the inhibition of tumor growth was associated with the anti-proliferative effects, an immunohistochemical assessment of PCNA in the tumor samples was also conducted. The results showed that formononetin treat-
inhibiting membrane lipid peroxidation. Formononetin, being a major isoflavone constituent of Radix Astragali, possesses anti-inflammatory properties (20), and has also been proposed to be potentially anticarcinogenic (13). We recently showed that formononetin is a strong pro-apoptotic agent in colon cancer cells (17). Nevertheless, formononetin does not appear to exert any direct effect on the cell cycle. This suggests that the pro-apoptotic effect exhibited by formononetin may not directly correlate with the modulation of mitotic inhibition. In addition to formononetin, we also investigated the potential apoptotic effect exhibited by the total isoflavonoid extract (AFT) (data not shown). We first examined the inhibitory effect exhibited by AFT on colon cancer cell growth and observed that there was a significant decrease in the percentage of viable cells with increasing doses and treatment time. By studying the protein expression of PARP, a key marker of apoptosis, it was determined that AFT exerts a pro-apoptotic effect on colon cancer cells. Through qualitative HPLC analysis, we confirmed the presence of formononetin in AFT as one of the major peaks on the chromatogram. This suggests that the anticarcinogenic activity of AFT may be largely due to formononetin. However, further study is required to determine the exact contribution of formononetin in the anticancer action of AFT.

It is generally known that VEGF facilitates angiogenic activities, while the increased expression of these growth factors has been shown to have a strong correlation with tumor development (21,22). Additionally, cancer cells and tumor stromal cells are involved in the secretion of VEGF in the tumor microenvironment (23). Previous studies have demonstrated that isoflavones help prevent osteoporosis and related menopausal disorders, and reduce cardiovascular risk. Their possible effects on chemoprevention and anti-oxidation have also been demonstrated (24,25). The plant-derived isoflavones, biochanin A and formononetin, are the methylated precursors of genistein and daidzein, respectively. Genistein inhibits endothelial cell proliferation and angiogenesis in vitro (26). It has also found that biochanin A and genistein inhibit the proliferation of gastric, esophageal and colon cancer cells (27). Daidzein has been shown to prevent osteoporosis and has exhibited anticancer activities on myeloid leukemia, breast cancer and pediatric tumors in vitro (28). Nevertheless, little is known about the anti-angiogenic effect of formononetin on colon cancer. Our current findings indicate that formononetin downregulates both the gene and protein expression of VEGF, implicating its involvement in the process of modulating angiogenesis. Tumor angiogenesis is an essential step in cancer growth and metastasis, and thus it is an important therapeutic target being extensively studied over the years (29). In tumors, neovascularization is necessary for both primary and metastatic tumor growth due to the increase in nutrient consumption. The process of angiogenesis involves the separation of endothelial cells from pericytes and the basement membrane upon the action of proteases, such as MMPs, urokinase plasminogen activators (uPAs) and cathepsins. Invasion and migration across basement membranes and subsequent extension into the tumor mass are triggered by the angiogenic growth factors, such as VEGF, basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) (30-32). The transition from the avascular state into a highly angiogenic state (angiogenic switch) is due to the

Discussion

Our ongoing studies have indicated that total AST exerts powerful anticarcinogenic activities against both colon and liver cancer development through target-specific mechanisms (8,9,19). In spite of this, only a few reports (in Chinese) have demonstrated the anti-oxidative properties of Astragalus flavonoids by

Figure 3. Anti-angiogenic and anti-proliferative effects of formononetin in the tumor xenografts. Determination of serum VEGF levels and immunohistochemical assessment of VEGF expression and cell proliferation (PCNA) in tumor tissue sections were performed. Total serum was collected from the nude mice on the day of sacrifice. (A) Tumor tissue VEGF level was measured using an ELISA kit as described in Materials and methods. Values represent the means ± SEM of 3 independent experiments. *P<0.05, statistical significance compared to the DMSO control. (B) VEGF- and (C) PCNA-positive cells were visualized in tumor sections excised from the control- (DMSO-treated) or formononetin-treated nude mice. Representative images from 3-5 sections of the control- or formononetin-treated animals are shown.
imbalance between pro-angiogenic signals and anti-angiogenic signals released by tumor cells and host cells. When pro-angiogenic signals dominate, tumors become vascularized (29). Neovascularization in tumors plays a critical role in colorectal cancer progression, and previous studies have shown that the increase in angiogenesis in the primary tumor correlates with poor prognosis and relapse of the disease (33,34).

In colon cancer, tumor cells must invade through the muscularis mucosa and migrate into the submucosa before they can reach the lymphatic channels or blood vessels. This is an essential process for metastasis to occur (35). An increase in invasion may be related to an increase in cell motility (36) and the secretion of basement membrane-degrading enzymes (e.g., MMPs), which together degrade the extracellular matrix and the basement membrane (36,37). The mechanical pressure generated by the rapidly growing neoplasm eventually pushes tumor cells along tissue planes with minimal resistance (38).

In this study, we demonstrate that formononetin reduces the protein expression of MMP-2 and MMP-9 and suppresses tumor cell invasiveness. These MMPs have been proposed to serve as a marker of colorectal cancer development (3). In fact, a novel antisense-based inhibitor of MMP-9 has been found to be capable of attenuating angiogenesis as well as human prostate cancer cell invasion, leading to the suppression of tumorigenicity (39). MMP-2 (also known as gelatinase A) is localized at specific surface protrusions on the cell membrane and promotes invasion (40), whereas MMP-9 (also known as gelatinase B) causes key processes in tumor cell migration, invasion, angiogenesis and tumor growth (41,42). The expression of MMP-9 in tumor epithelial cells positively correlates with colorectal cancer progression due to its control over essential functions mediating metastasis (43). Other studies have indicated that MMP-9 secreted by colon cancer cells promotes extracellular matrix degradation, and assists tumor cell seeding (44). The facilitation of tumor invasion and neovascularization by MMP-9 is mainly caused by the cleavage of specific factors, such as collagen IV in the extracellular matrix (45). Hence, targeting MMP-9 in tumor epithelial cells is regarded as a key therapeutic strategy for controlling the progression of colorectal cancer (46). MMP-9 is not usually expressed in normal human tissues but is mainly found in invasive tumors. It has been reported that the inhibition of MMP-9 expression leads to ERK-mediated p66 expression, resulting in cell cycle arrest (47) while at the same time inducing apoptosis (48). This suggests that the impact of MMP-9 inhibition is not restricted to the suppression of tumor angiogenesis and invasiveness, but also contributes to the promotion of apoptosis. This proposition coincides with our findings on the pro-apoptotic properties of formononetin, although cell cycle arrest was not directly facilitated.

Our present study provides evidence that formononetin controls colon tumor progression without causing significant toxicity in drug-treated animals, with no mortality or drop in body weight. This observation was confirmed by the fact that formononetin did not exert any neutropenic effect on the drug-treated animals. Therefore, we can conclude that formononetin is capable of inducing tumor growth inhibition as well as anti-angiogenic and anti-invasive effects with minimal adverse drug reactions. These important findings suggest that formononetin could be further developed as a potential chemotherapeutic/adjuvant agent in the treatment of colon cancers, including the advanced and metastatic types.

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References


