Chemopreventive Activity of Plant Flavonoid Isorhamnetin in Colorectal Cancer Is Mediated by Oncogenic Src and β-Catenin

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Abstract
Analysis of the Polyp Prevention Trial showed an association between an isorhamnetin-rich diet and a reduced risk of advanced adenoma recurrence; however, the mechanism behind the chemoprotective effects of isorhamnetin remains unclear. Here, we show that isorhamnetin prevents colorectal tumorigenesis of FVB/N mice treated with the chemical carcinogen azoxymethane and subsequently exposed to colonic irritant dextran sulfate (DSS). Dietary isorhamnetin decreased mortality, tumor number, and tumor burden by 62%, 35%, and 59%, respectively. MRI, histopathology, and immunohistochemical analysis revealed that dietary isorhamnetin resolved the DSS-induced inflammatory response faster than the control diet. Isorhamnetin inhibited AOM/DSS–induced oncogenic c-Src activation and β-catenin nuclear translocation, while promoting the expression of C-terminal Src kinase (CSK), a negative regulator of Src family of tyrosine kinases. Similarly, in HT-29 colon cancer cells, isorhamnetin inhibited oncogenic Src activity and β-catenin nuclear translocation by inducing expression of csK, as verified by RNA interference knockdown of csK. Our observations suggest the chemoprotective effects of isorhamnetin in colon cancer are linked to its anti-inflammatory activities and its inhibition of oncogenic Src activity and consequential loss of nuclear β-catenin, activities that are dependent on CSK expression. Cancer Res; 73(17); 5473–84. ©2013 AACR.

Introduction
Colorectal cancer is the fourth most commonly diagnosed type of cancer in the United States, and it is the second leading cause of cancer-related deaths (1). Chronic inflammation, such as ulcerative colitis and Crohn disease, are associated with increased risk of colorectal cancer (2-5).

The Src family of tyrosine kinases (SFK) are nonreceptor protein tyrosine kinases that are activated in multiple cancers, including colorectal cancer (6). Increased Src activity in primary colorectal cancer is an indicator of poor prognosis (7). C-terminal Src kinase, also known as c-Src kinase (CSK), negatively regulates SFK by phosphorylation of a C-terminal tyrosine (Y530 in c-Src; ref. 8). C-terminal phosphorylation of SFK stabilizes the protein in an inhibitory conformation that prevents autophosphorylation of the activation loop tyrosine (Y419 in c-Src). The receptor-like tyrosine phosphatase CD45 prevents autophosphorylation of Src by removing the C-terminal tyrosine phosphate (9). Activated Src can regulate many downstream pathways including phosphoinositide 3-kinase (PI3K), Ras–Mek–extracellular signal–regulated kinase (ERK), STAT3, and p130 to increase survival, proliferation, angiogenesis, motility, and invasion (6). Activated Src can also phosphorylate β-catenin, causing its release from E-cadherin sequestration at the plasma membrane and enhancing its nuclear localization (10). Colorectal cancer seems to be sensitive to loss of Src regulation. Both the loss of expression of the negative regulator CSK and the overexpression of Src have been implicated in colorectal carcinogenesis (8).

The cost of treating colorectal cancer is estimated to be $6.5 billion per year (11). Dietary change and use of dietary supplements, both feasible and safe, represent a viable and important strategy for preventing colorectal cancer (12). Recent analysis of the Polyp Prevention Trial (PPT), a clinical trial that investigated the role of diet modulation in the prevention of colorectal adenoma recurrence, suggested that consuming an isorhamnetin-rich diet was associated with a decreased risk of advanced adenoma recurrence (13).
Recently, it was shown that isorhamnetin can suppress skin cancer by binding to and inhibiting MAP (mitogen-activated protein)/ERK kinase (MEK) I and PI3K (14). To further investigate the potential role of flavonoids in colorectal cancer prevention, we assessed the effects of four flavonoids commonly consumed by humans, isorhamnetin, quercetin, rutin, and myricetin, in a mouse model for colorectal cancer. We found that supplementing the diet with isorhamnetin significantly reduced colorectal tumorigenesis in these mice. The isorhamnetin diet seemed to resolve the dextran sodium sulfate (DSS)–induced inflammation as measured by MRI, histopathology, and immunohistochemical analysis (IHC). Isorhamnetin also inhibited Src activity and nuclear localization of β-catenin and increased expression of CSK both in vivo and in colorectal carcinoma cells. Finally, we showed that inhibition of Src and nuclear β-catenin was dependent on the upregulation of CSK in colon cancer cells and associated with CSK expression in isorhamnetin-fed mice.

Materials and Methods

Animal studies

All mouse experiments were agreed to and regulated by the Animal Care and Use Committee of the National Cancer Institute (Frederick, MD). Pathogen-free male C57BL/6J mice were purchased from the NCI-Frederick Animal Production Area at 5 weeks of age and were exposed to a 12:12 light/dark cycle. Mice were given ad libitum heat-purified, diet from Harlan Teklad and drinking water 0.1 mL of saline (Fig. 1). Seven days later, mice were treated with azoxymethane (Sigma) at a dose of 10 mg/kg body weight in 0.1 mL of saline (Fig. 1). Seven days after DSS treatment ended, the mice were evenly sorted by change in body weight into five diet groups to ensure that effects of the DSS were evenly represented in each of the different diets. Mice were single caged and started on the indicated diet (day 17; Supplementary Table S1). Isorhamnetin and myricetin were obtained from Jiaan Haohua Industry Co., Ltd. and quercetin and rutin were obtained from Sigma-Aldrich Co., LLC. Purified diets were prepared by Harlan Laboratories. Mice were allowed to eat ad libitum. One day before euthanasia (days 20, 25, and 33), 12 mice per group and time point were imaged by MRI as described previously (15). Mice were euthanized at indicated times and colon tissue was collected.

Histopathology

Colonic tissue was harvested from mice at various times after azoxymethane or AOM/DSS treatment, fixed in 4% formaldehyde overnight, and stored in 70% ethanol. Fixed sections of colon tissues were embedded in paraffin, cut into 5-μm sections, and put onto microscopic slides by Histoserv Inc. Slides were either stained with hematoxylin and eosin (H&E) for histologic analysis or light microscopy or deparaffinized in xylene and rehydrated in graded ethanol for IHC. For histopathology analysis, a section of approximately 5 mm in length of the distal colon was evaluated for each animal. The tissues were blinded and scored by the pathologist semiquantitatively from 0 to 15 (Supplementary Table S2). Colon sections were evaluated beginning at the colorectal junction and proceeding adorally 5 mm to the middle colon. Briefly, the severity of the leukocytic infiltrate in the mucosa was subjectively assessed as mild, moderate, or severe (1, 2, and 3, respectively); the distribution was evaluated and denoted as focal/locality extensive, multifocal, or diffuse (1, 2, and 3, respectively); and the depth was evaluated and denoted as mucosa only, extends to submucosa, or extends to muscularis mucosa or serosa (1, 2, and 3, respectively). The distribution of atypical glandular hyperplasia (AGH)/squamous (sq) metaplasia was assessed as focal, multifocal, or diffuse (1, 2, and 3, respectively). The distribution

Figure 1. Isorhamnetin (IsR) decreased AOM/DSS–induced colorectal tumorigenesis. A, time line showing that mice were treated with azoxymethane on day 0 and with 2% DSS from day 7 to 14. On day 17, mice were blocked by weight loss and randomly started within weight blocks on experimental diets. On days 20, 25, and 33, mice were imaged using MRI and 1 day later they were euthanized and tissue was collected. At later times (days 59 and 102), mice were euthanized and tissue was collected without MRI. B–D, analysis of colorectum collected at day 102. B, number of tumors per mouse (geometric mean ± SE). C, tumor burden in mm3/mouse (geometric mean ± SE). D, tumor size in mm3/tumor (geometric mean ± SE). Ct, control diet, N = 13; isorhamnetin, N = 12; IQR, interquartile range.
of ulceration was assessed as focal, multifocal, or diffuse (1, 2, and 3, respectively). If necrosis was present it was subjectively assessed as mild, moderate, or severe and scored accordingly (1, 2, and 3, respectively). A score of 0 was assigned for each criterion not represented in the section, which was the case for the majority of mice for ulceration and necrosis. Total disease score per mouse was calculated by summation of the six parameters for each mouse.

Immunohistochemistry

For IHC, the sections were placed in low-pH–modified citrate buffer (Dako) and subjected to antigen retrieval via pressure chamber. The sections were preincubated in 3% H2O2 for 20 minutes and blocked in normal goat serum for 1 hour. Sections were incubated overnight in primary antibody solution diluted as follows: Ki-67, phospho-β-catenin854 (Abcam Laboratories) 1:50, CD45 (BD Biosciences) 1:50, pSrcY417 and β-catenin (Cell Signaling Technology) 1:100 and 1:1,000, respectively. Slides were incubated for 1 hour in a biotin-conjugated secondary antibody diluted 1:200. The sections were treated with a Vectorstain ABC kit (Vector Laboratories); the resulting activity was detected with diaminobenzidine (Sigma) and counterstained in hematoxylin. For CSK (Abcam Laboratories), tissues were incubated 1:50 for 1 hour and detected with a Ventana iView DAB detection kit (Ventana Medical Systems) on a Ventana BenchMark.

Cell culture

Human colon cancer HT29 cell line was obtained from American Type Culture Collection and cultured as described previously (16).

Western blot analysis

HT29 cells were seeded on 100-mm plates and treated with either dimethyl sulfoxide (DMSO) vehicle (<1%) or isorhamnetin (10, 20, and 40 µmol/L) for 24 hours. The media was removed and cells were washed with cold PBS. Cells were lysed with complete radioimmunoprecipitation assay (RIPA) lysis buffer (Santa Cruz Biotechnology). Protein concentration was calculated using standard bovine serum albumin (BSA) curve. Approximately, 30 to 80 µg of protein was loaded and separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the following antibodies at indicated dilutions: CSK (1:1,000), pSRC417 (1:1,000), pSRC529 (1:1,000), β-catenin (1:2,000), pAKT (1:1,000), pERK (1:2,000), E-cadherin (1:1,000), pGSK3β (1:1,000; Cell Signaling Technology), and pβ-catenin854 (Abcam Laboratories) overnight at 4°C, followed by incubation in horseradish peroxidase–labeled secondary antibodies for 1 hour. Cells were developed with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Each membrane was probed with β-actin (1:5,000; Sigma) to ensure consistent protein loading.

Real-time PCR

Total RNA from HT29 cultured cells was isolated using TRIzol RNA extraction method. Briefly, chloroform was added to the sample, and after shaking and centrifugation the aqueous layer was isolated. Isopropanol was next added to the sample to precipitate the RNA. Following centrifugation, the RNA pellet was washed with 70% ethanol. RNA was further purified using an RNaseasy kit (Qiagen). The cDNA was synthesized using Bio-Rad iScript reverse transcriptase, and the PCR reactions were carried out using Bio-Rad SYBR Green Master Mix carried out in Bio-Rad iCycler (Bio-Rad Laboratories). The human csk splicing variant 1 and 2 were purchased from IDT and sequence is as follows: sense, 5’–TCGGCCCGCTCTCTCTTTG and antisense, 5’–ACCTCACGGGAGGAGCAG. The PCR included nontemplate control and glyceraldehyde-3-phosphate dehydrogenase (gapdh) as control primer set. The csk transcript activity level was calculated using 2ΔΔCT, where ΔCT = C T csk − C T gapdh and Δ (ΔCT stimulated − ΔCT control).

CSK siRNA construct

HT29 cells were stably transfected with three unique 27mer siRNA duplexes—2 nmol each against csk siRNA (Trilencer-27; Origene Technologies) or a scrambled control siRNA using the manufacturer’s transfection reagent and recommendations. After transfection, cells were incubated for 36 hours. The total protein was isolated and subjected to Western blot analysis.

Soft agar assay

HT29 cells were seeded onto 100-mm plates and pretreated with either DMSO vehicle (<1%) or isorhamnetin (10, 20, and 40 µmol/L) for 24 hours. Cells were trypsinized and seeded at 30,000 cells in 2 × Dulbecco’s Modified Eagle Medium (DMEM). Cell suspension was added 1:1 with 0.5% agarose (2 ml/well in a 6-well plate) and constitutes the top layer. The bottom layer consisted of 2 ml of 1.2% agarose. The cells were maintained in an incubator for 14 days and the colonies were scanned and counted with GelCount (Oxford Optronix Ltd.).

Statistical analyses

Statistical analyses were done using SAS, version 9.2 (SAS, Inc.) software. A Student unpaired t test was used to compare treatment group averages for histopathology and cell culture data. Generalized linear models were used to compare treatment group averages for mouse morbidity (binomial; PROC GLIMMIX) and tumor data (PROC GLM). In addition to diet, their SEs; tumor data are shown as least-squares mean and log transformed, after adding a 1 to prevent values below zero. Histopathology and cell culture data are shown as raw mean and their SEs; tumor data are shown as least-squares mean and their SEs, which were back transformed to their original scale. All statistical tests were two-sided. The P values were not adjusted for multiple comparisons. Significance was declared at P ≤ 0.05 and a tendency at 0.05 to 0.10.

Results

Isorhamnetin prevents colon tumorigenesis in AOM/DSS–treated mice

We previously showed that a high intake of flavonol-enriched foods and beverages, specifically isorhamnetin, kaempferol, and quercetin, was associated with decreased risk...
of advanced adenoma recurrence (13, 17). To verify the chemoprotective effects of individual flavonoids on colorectal tumorigenesis, we fed FVB/N male mice previously treated with azoxymethane and DSS, diets enriched with four primary flavonoids consumed by humans (Table 1 and Supplementary Table S1). FVB/N mice are sensitive to the AOM/DSS and only require one cycle of DSS to promote tumorigenesis. Three days after the DSS treatment, mice were started on an AIN93-G control diet (18) or one supplemented with the indicated flavonol. Sensitive strains of mice treated with azoxymethane and 1 week of DSS will develop colon dysplastic lesions, adenomas, and adenocarcinomas; unfortunately some of these mice will have to be euthanized prematurely due to the large tumor burden and colorectal prolapse (15, 19). In our study, feeding mice isorhamnetin after the DSS exposure produced a significantly higher survival rate (80%) compared with the mice fed the control diet (48%, P = 0.02; Table 1). The isorhamnetin-fed mice also developed 35% (P = 0.03) fewer tumors and a 59% (P = 0.04) smaller tumor burden than mice fed the control diet (Table 1). Because the decreased mortality and tumor number in the quercetin intervention was not significant and we saw no beneficial effects with rutin or myricetin diets, we conducted an in-depth study of the isorhamnetin intervention. A repeat of the isorhamnetin intervention showed 63% decrease in tumor number (P = 0.002) and an 83% decrease in tumor burden (P = 0.02; Fig. 1). In this study, tumors did not progress to adenomas, and adenocarcinomas; unfortunately some of these mice will have to be euthanized prematurely due to the large tumor burden and colorectal prolapse (15, 19). In our study, feeding mice isorhamnetin after the DSS exposure produced a significantly higher survival rate (80%) compared with the mice fed the control diet (48%, P = 0.02; Table 1). The isorhamnetin-fed mice also developed 35% (P = 0.03) fewer tumors and a 59% (P = 0.04) smaller tumor burden than mice fed the control diet (Table 1). Because the decreased mortality and tumor number in the quercetin intervention was not significant and we saw no beneficial effects with rutin or myricetin diets, we conducted an in-depth study of the isorhamnetin intervention. A repeat of the isorhamnetin intervention showed 63% decrease in tumor number (P = 0.002) and an 83% decrease in tumor burden (P = 0.02; Fig. 1). In this study, tumors did not progress to adenocarcinoma on either of the diet interventions. These data indicate that a diet supplemented with isorhamnetin prevents colorectal tumorigenesis in a mouse model of colitis-associated colorectal cancer.

**Isorhamnetin resolves colitis faster in AOM/DSS–treated mice**

*FVB/N* mice exposed to one cycle of DSS will develop colitis that can be detected by MRI (15). Mice treated with AOM/DSS were imaged on day 20, 25, and 33 (Fig. 1) using MRI before and after i.v. administration of 0.2 mmol/kg gadolinium diethyletrenitriamine-pentaacid (Gd-DTPA) contrast agent (Fig. 2). In normal colons, little to no contrast was detected in the colonic epithelium (15), whereas in DSS-treated mice, the contrast agent clearly reached the epithelial lining of the colon, indicating colitis at day 20 in both diet groups (Fig. 2A, left). By day 25, colitis increased in mice fed both diets; however, colitis was less severe in mice fed isorhamnetin (Fig. 2A, middle). By day 33, isorhamnetin-fed mice had less uptake of contrast agent in the colonic epithelium as compared with mice fed the control diet, indicating colitis had begun to resolve in these mice (Fig. 2A, right).

Histopathology analysis of the colonic tissue collected 1 day after MRI (days 21, 26, and 34) confirmed the results of the imaging (Fig. 2B). Regardless of day of sacrifice (no time × diet interaction at P < 0.20), dietary isorhamnetin decreased leukocyte distribution (P = 0.003), depth (P = 0.07), and severity (P = 0.11) of inflammation, and the distribution of AGH or squamous metaplasia (P = 0.02), resulting in an overall lower histopathology score for isorhamnetin-fed mice (P = 0.02). The severity and distribution of inflammation was similar in the mice on days 21 and 26, irrespective of the diet. By day 34, however, the mice fed the isorhamnetin diet showed only localized or multifocal distribution of leukocytes with mild to moderate severity, whereas the mice fed the control diet still exhibited moderate to severe inflammation, which was distributed diffusely within the section of colon evaluated (Fig. 2B). Colons collected at days 21 and 26 from mice fed both the control and isorhamnetin diet showed AGH and squamous metaplasia confined to the colorectal area in most sections. By day 34, the colons of the mice that were fed the control diet had a higher rate of microadenoma formation and a greater degree of hyperplasia and squamous metaplasia, which are considered premalignant lesions, as compared with the mice fed the isorhamnetin diet. Unlike in our previous study (15), no adenocarcinoma was detected in this study in either group. These results suggest that the mice on the isorhamnetin diet recovered from colitis faster than the mice on the control diet and further suggest a chemopreventive effect of isorhamnetin on colorectal tumorigenesis.

**Table 1.** Effect of feeding flavonols at equimolar concentrations (ppm) for 85 days to AOM/DSS–induced male FVB mice on morbidity, tumor number, and tumor burden (N = 32/group)

<table>
<thead>
<tr>
<th>Dietary flavonol</th>
<th>Morbidity (%)a</th>
<th>Tumor number (n/mouse)</th>
<th>Tumor burden (mm3/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg ± SEb P vs. C</td>
<td>Avg ± SE P vs. C</td>
<td>Avg ± SE P vs. C</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>19.6 ± 1.2</td>
<td>172.7 ± 2.9</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>552</td>
<td>12.7 ± 1.1</td>
<td>74.5 ± 2.9</td>
</tr>
<tr>
<td>Myricetin</td>
<td>556</td>
<td>17.3 ± 1.1</td>
<td>178.2 ± 2.9</td>
</tr>
<tr>
<td>Quercetin</td>
<td>591</td>
<td>14.8 ± 1.1</td>
<td>132.5 ± 2.9</td>
</tr>
<tr>
<td>Rutin</td>
<td>1,099</td>
<td>17.6 ± 1.2</td>
<td>122.3 ± 2.9</td>
</tr>
</tbody>
</table>

*Morbid mice had to be sacrificed because they either lost more than 10% of their maximum body weight or their intestines had prolapsed.

Values are shown as geometric mean and their SEs. To achieve normality, tumor number had to be once natural log-transformed and tumor burden had to be twice natural log-transformed.

P vs. C indicates P values of natural log-transformed data when diet groups are compared with control diet.
Isorhamnetin decreases CD45-positive leukocytes infiltration in AOM/DSS--treated mice

The DSS-induced inflammation resulted in infiltration of the colon by leukocytes, which was quantified by expression of the cell surface marker of leukocytes, CD45. The leukocyte distribution in the colonic mucosa and submucosal tissue was similar at days 21 and 26, irrespective of diet (data not shown). By day 34, however, dietary isorhamnetin decreased infiltration of...
CD45-positive cells compared with mice on the control diet (Fig. 2C). The expression of CD45 was predominantly localized to the gut-associated-lymphoid tissue (GALT) in mice fed isorhamnetin, similar to what is seen in mice not exposed to DSS. The GALT provides immune protection to the gut (20). These results suggest that isorhamnetin prevents the release of CD45-positive leukocytes. Consistent with the histopathology and MRI data (Fig. 2A and B), the decrease in CD45-positive leukocyte infiltration indicates that mice on the isorhamnetin diet recover more quickly from the colitis than do mice fed the control diet.

**Isorhamnetin reduces cell proliferation in AOM/DSS–treated mice**

To assess the effects of the isorhamnetin diet on cell proliferation, Ki-67 expression was evaluated by IHC on day 34 in the colon tissue of AOM/DSS–treated mice. Mice fed the isorhamnetin diet had a 16% crypt proliferation fraction compared with mice on the control diet with a crypt proliferation fraction of 63% ($P < 0.001$; Fig. 3). Furthermore, Ki-67 expression in the isorhamnetin-treated mice was predominantly in the basal portion of the colon crypt that contains multipotent stem cells (21), whereas in mice fed the control diet, Ki-67 expression showed aberrant cell proliferation throughout the crypts. Similar results were seen at day 59 (Fig. 3), indicating that the mice on the control diet never completely recovered from the DSS-induced proliferation. The decrease in proliferation suggests that the mice on the isorhamnetin diet recover more quickly from the inflammation-induced cell proliferation response in the colon.

**Isorhamnetin inhibits anchorage-independent growth of human colorectal carcinoma HT29 cells**

To assess the effects of isorhamnetin in a human relevant model, human colorectal carcinoma cell lines were treated with varying concentrations of isorhamnetin. Treatment of HT29 cells showed no effect on cell viability with up to 40 μmol/L isorhamnetin and very little effect with up to 100 μmol/L. HCT116 colorectal cell viability was more sensitive to...
isorhamnetin than viability of HT29 cells (Supplementary Fig. S2). At 40 μmol/L, isorhamnetin significantly inhibited anchor- age-independent growth of HT29 cells by 62% ($P = 0.001$) compared with solvent control (Supplementary Fig. S2). These results suggest that isorhamnetin at 40 μmol/L can inhibit tumorigenesis without significantly affecting cell viability.

Isorhamnetin inhibits ERK, Akt, and Src activity

Previous studies have shown that isorhamnetin inhibits MEK and PI3K in squamous cell carcinoma skin cancer (14). Here, we show a similar dose-dependent inhibition of phosphorylation of Akt, a PI3K target, and of ERK1/2, a MEK target in HT29 colorectal cancer cells (Fig. 4A). Interestingly, we did not see inhibition of S6kinase phosphorylation, a target of Akt. The tyrosine phosphatase CD45 dephosphorylates Src tyrosine 529, leading to autophosphorylation at tyrosine 417 and activation of Src. Here, we show a dose-dependent inhibition of Src Y417 phosphorylation by isorhamnetin (Fig. 4A). Conversely, a dose-dependent increase was shown in Src Y529 phosphorylation. Src activation was inhibited by isorhamnetin in a dose-dependent manner with a 34% inhibition (increase in Y529 phosphorylation) at 40 μmol/L.

Figure 4. Isorhamnetin (IsR) inhibition of ERK, Akt, and Src activity is CSK dependent. A, dose-dependent inhibition of ERK, AKT, and Src activity. Western blot analysis of whole-cell extracts from HT29 cells treated with indicated dose of isorhamnetin for 24 hours. p, phospho-specific antibody targeting Akt phosphorylated at S473 (pAkt), p70S6K (pp70S6K), ERK1/2 (pERK1/2), activated Src phosphorylated at Y417 (pSrc417), inactivated Src phosphorylated at Y529 (pSrc529). B, dose-dependent activation of CSK mRNA and protein expression. Top, real-time PCR analysis of cDNA from csk mRNA transcript in response to isorhamnetin pretreatment for 24 hours. The data are expressed as fold changes relative to gapdh control. The values are the mean ± SEM of three independent experiments. Isorhamnetin treatment at 20 and 40 μmol/L resulted in significantly higher csk mRNA transcript levels (*, $P < 0.05$; **, $P < 0.01$). Bottom, Western blot analysis against CSK with a 2.6-fold increase at 40 μmol/L as determined by densitometry. C, isorhamnetin inhibition reversed by siRNA knockdown of csk. Western blot analysis of HT29 cells transfected with siCtl or siCSK and 24 hours later treated with the indicated concentration of isorhamnetin. α indicates antibody.
Isorhamnetin induces csk expression
Because tyrosine 529 on Src is phosphorylated by CSK, we examined the effect of isorhamnetin on CSK. Isorhamnetin induced a dose-dependent increase in CSK mRNA (2.2-fold change at 40 μmol/L) and protein (2.6-fold change at 40 μmol/L; Fig. 4B). Isorhamnetin at 40 μmol/L also induced CSK expression in the breast cancer cell line MCF7 (data not shown). These results indicate that isorhamnetin activates gene expression of csk, leading to inhibition of Src activity.

Isorhamnetin inhibition of ERK, Akt, and Src activity is CSK dependent
To determine if the isorhamnetin inhibition of Src is dependent on CSK expression, we knocked down csk expression with siRNA and assessed Src activity. Transfection with the control siRNA (siCt) did not affect the dose-dependent increase of CSK protein in the isorhamnetin-treated cells, whereas knockdown of the csk gene blocked the isorhamnetin-induced increase in CSK protein (Fig. 4C). Knockdown of csk expression reversed the inhibition of Src activation by isorhamnetin. Transfection with control siRNA had no effect on the isorhamnetin-dependent decrease in Src Y417 phosphorylation. Conversely, transfection of HT29 cells with siRNA against csk reversed the isorhamnetin-induced decrease in Y417 phosphorylation on Src. In fact, siRNA knockdown of csk resulted in a slight increase in Y417 phosphorylation (Fig. 4C), showing that inhibitory effects of isorhamnetin on Src is dependent on CSK in HT29 cells.

Transfection of HT29 cells with siCSK also reversed the isorhamnetin inhibition of Akt and ERK activation seen in cells transfected with the control siRNA (Fig. 4C), indicating that activation of the MAPK and PI3K pathways is in part regulated by Src or an Src family kinase in HT29 cells. Src can activate both the ERK and the PI3K pathways (22–24). These observations show that in HT29 colorectal cancer cells isorhamnetin induces CSK expression, leading to inactivation of Src by phosphorylation at Y527 and decrease in ERK1/2 and AKT activity.

Isorhamnetin inhibits Src-dependent β-catenin nuclear translocation
The Wnt/β-catenin pathway is activated in most colorectal cancer. β-Catenin can complex with APC and GSK3 in the cytoplasm. Perturbation of this complex releases a stabilized β-catenin. β-Catenin can also be tethered to the plasma membrane by binding with α-catenin to E-cadherin (10). We
found a dose-dependent reduction in nuclear β-catenin with a corresponding accumulation of cytoplasmic β-catenin in HT29 cells treated with isorhamnetin (Fig. 5A). Interestingly, isorhamnetin did not change GSK3 activity or E-cadherin levels (Fig. 5C). Alternatively, isorhamnetin inhibited tyrosine phosphorylation at Y654 on β-catenin (Fig. 5A, nuclear). Tyrosine 654 is phosphorylated by Src, which results in release of β-catenin from its complex with E-cadherin allowing nuclear localization (10). The corresponding increase in cytoplasmic β-catenin did not show an increase in phosphorylation at Y654, consistent with a decrease in Src-regulated nuclear translocation of β-catenin. To assess whether nuclear translocation of β-catenin was Src-dependent, we transfected HT29 cells with csk siRNA. Knockdown of csk expression reversed the inhibitory effects of isorhamnetin treatment on Y654 phosphorylation and nuclear translocation of β-catenin (Fig. 5B). These results indicate that nuclear localization of β-catenin in HT29 colorectal cancer cells is a consequence of Src phosphorylation of Y654 on β-catenin and that isorhamnetin induces expression of csk leads to inhibition of Src phosphorylation of Y654 on β-catenin, blocking its release from E-cadherin and its nuclear localization.

**Isorhamnetin induces CSK expression and inhibits Src activation in vivo**

To determine if the effects of isorhamnetin on Src activation and β-catenin nuclear localization seen in HT29 cells can be translated to the mouse colon, we measured CSK levels, Src activity, and β-catenin levels by IHC in AOM/DSS–treated mice. Similar to the HT29 cells, mice consuming isorhamnetin showed higher levels of CSK in both the DSS-treated and untreated groups of mice compared with the mice fed the control diet (Fig. 6A). CSK expression was higher in the lamina propria surrounding the crypts compared with the epithelial tissue. CSK was also detected, although at lower levels, in normal human colon tissue. Furthermore, activated Src (phosphorylated at Y417) was higher in the crypts of AOM/DSS–treated mice fed the control diet at both day 34 and 59 (Fig. 6B). Conversely, inactivated Src (phosphorylated at Y529) was higher in crypts of the isorhamnetin-fed mice (Fig. 6B). These results indicate that isorhamnetin induces expression of CSK and inhibits Src activation in vivo in the colons of AOM/DSS–treated mice.

**Isorhamnetin inhibits β-catenin nuclear localization in vivo**

Nuclear β-catenin levels were increased in AOM/DSS–treated mice on the control diet, consistent with activation of Src (Fig. 6C, middle). By day 34, AOM/DSS–treated mice on the control diet showed a diffuse staining for β-catenin throughout the cells, indicating both cytoplasmic and nuclear accumulation of β-catenin. Furthermore, IHC staining with the Y654 localization of β-catenin in AOM/DSS–treated animals (middle). In isorhamnetin-fed mice, β-catenin staining is predominantly cytoplasmic and with limited p-β-catenin (bottom). Representative serial sections of colonic tissue are taken from mice treated with azoxymethane only (day 31) or AOM/DSS (day 34).

**Figure 6.** Isorhamnetin (IsR) induces CSK expression and inhibits Src phosphorylation at Y417, Src phosphorylation of β-catenin at Y654, and β-catenin nuclear localization in vivo. A, IHC analysis of CSK levels showed expression was elevated in lamina propria surrounding the crypts and epithelial tissue of mice fed isorhamnetin diet after azoxymethane (day 31; top) and after AOM/DSS (days 34 and 59; middle). Images are representative photomicrographs of colonic sections. Normal colon (no azoxymethane, DSS, or isorhamnetin treatment) from mouse (top left) and human colon tissue (bottom right). B, DSS-induced phosphorylation of Src Y417 was reduced in isorhamnetin-fed mice. Representative photomicrographs depicting activated Src in crypts of AOM/DSS–treated mice as measured using IHC analysis of pSrc Y417 (brown staining). Blue counterstaining of nuclei indicates activated Src is not nuclear. Isorhamnetin supplementation reduced the amount of Src activity as measured at days 34 and 59 (middle left). C-terminal phosphorylated Src (pSrcY529) was elevated in isorhamnetin-fed mice at day 34 (bottom). C, nuclear localization and phosphorylation of β-catenin was reduced in isorhamnetin-fed mice. IHC analysis of β-catenin and Y654 phosphorylated β-catenin. β-Catenin and pβ-catenin colocalized with nuclear counterstaining, indicate phosphorylation and nuclear localization.
phospho-specific β-catenin antibody indicated a high level of Src-induced phosphorylation. In contrast, in the isorhamnetin-fed mice, β-catenin remained predominantly in the cytoplasm (Fig. 6C, bottom), with lower amounts of Y654 phosphorylated β-catenin and decreased nuclear localization. These results suggest that Src regulated phosphorylation of β-catenin and that its nuclear translocation can be inhibited with an isorhamnetin-supplemented diet.

Discussion

In a mouse model of colorectal cancer, we evaluated the effects of dietary isorhamnetin for colorectal cancer prevention. In this model, FVB mice treated with azoxymethane and DSS develop tumors that will progress to adenoma and adenocarcinomas (Table 1; refs. 15, 19). Dietary isorhamnetin reduced inflammation, neutrophil infiltration, cell proliferation, tumor burden, and mortality associated with the AOM/DSS treatment. Src activation and β-catenin nuclear localization induced by AOM/DSS were also reduced in the isorhamnetin-fed mice and in HT29 colon cancer cells treated with isorhamnetin. Isorhamnetin induced the expression of CSK, a negative regulator of Src. In HT29 cells isorhamnetin-induced inhibition of Src activity, and nuclear localization of β-catenin was dependent on CSK expression (Fig. 4). Isorhamnetin did not affect the expression of E-cadherin, activation of GSK3, or activation of S6K kinase. These results suggest that the anti-inflammatory and anti-cancer activities of isorhamnetin are linked to inhibition of oncogenic Src activity, which can phosphorylate β-catenin at Y654, leading to its dissociation from the membrane and its nuclear localization.

β-Catenin signaling in colorectal cancer

The Wnt/β-catenin pathway is activated in most colorectal cancer (25, 26). This pathway is also activated in the AOM/DSS mouse model (19, 27). β-Catenin can complex with α-catenin and E-cadherin at the cytoplasmic membrane, providing adherin junction communication (10). Activated Src can phosphorylate β-catenin at Y654, releasing it and α-catenin from the E-cadherin complex. Dissociation of β-catenin from the cadherin complex will cause dysregulation of tyrosine kinase signaling, will affect cell-cell communication, will affect β-catenin-regulated gene expression, and can lead to transformation and survival of β-catenin-driven cancer (10).

Activated c-Src in colorectal cancer is an indicator of poor prognosis

Members of the SFK family are nonreceptor tyrosine kinases that are recruited to the membrane by integrin or receptor tyrosine kinase–induced phosphorylation of focal adhesion kinase (FAK). Recruitment of SFK to the membrane provides a molecular switch that is important for regulating proliferation, differentiation, cell adhesion, and cell mobility (28). Activated Src in primary colorectal cancer is an indicator of poor prognosis (7), and elevated Src activity can be detected in the majority of human colon cancer (29). Inhibition of Src or SFK can enhance cell-cell adhesion and can suppress migration and invasion in vitro and metastasis in vivo, suggesting an anti-invasive role for Src inhibitors (10). Inhibitors of Src are currently being tested in clinical trials (10).

CSK negatively regulates Src

The CSK is a nonreceptor tyrosine kinase that serves as a negative regulator of Src and SFK. CSK phosphorylates the C-terminal regulatory site of SFK, resulting in a conformation change and inactivation of the kinase activity (30–33). A reduction in CSK mRNA, protein, and kinase activity in colorectal carcinoma has been shown to be correlated with an increase in Src activity, suggesting that a loss in CSK may influence transformation of colorectal carcinoma (8). Rengifo-Cam and colleagues have shown that CSK regulates signaling from integrin–SFK–mediated cell adhesion, which can influence the metastasis of cancer cells (34). Recruitment to the membrane by scaffolding proteins such as CSK-binding protein (Cbp) is required for inactivation of Src by CSK and is crucial for preventing tumorigenesis (31, 35). The fact that none of the other flavonoids tested, including mycetin, quercetin, and rutin, induced expression of csk in HT29 cells (results not shown) and were not effective for inhibiting carcinogenesis in vivo is consistent with our conclusion that the chemoprotective effects of isorhamnetin in colon cancer are linked to its anti-inflammatory activities and its inhibition of oncogenic Src activity and consequential loss of nuclear β-catenin activities that are dependent on csk expression. Although csk expression can be regulated translationally (36), very little is known about how csk is regulated transcriptionally. Further investigation is needed to learn how csk expression is lost in colorectal cancer and how isorhamnetin restores csk expression.

Dietary isorhamnetin has chemoprotective properties

Isorhamnetin, quercetin, kaempferol, and myricetin are flavonoids that are present in a wide variety of fruits and vegetables and have anticancer activity (37). Computational and binding assays have shown that isorhamnetin can bind directly to MEK1 and to PI3K and quercetin can bind to BSK2 (14, 38). Lee and colleagues (39) reported that kaempferol can bind to Src, and Jung and colleagues (40) showed that myricetin can bind to the SFK Fyn. Our findings show that isorhamnetin can inhibit Src activity, but that this inhibition is dependent on CSK expression, suggesting that unlike the structurally similar flavonoids, kaempferol, and myricetin, isorhamnetin does not bind directly to Src. The mechanism of how isorhamnetin is upregulating CSK expression is currently under investigation. Our results and those of others (8, 34, 35) show the importance of CSK as a negative regulator of SFK and as a tumor suppressor, suggesting that preventing the loss of or restoring the expression of CSK would be beneficial for preventing tumorigenesis, tumor progression, and tumor metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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