Digestive Diseases and Sciences, Vol. 50, No. 4 (April 2005), pp. 696–707 (© 2005)
DOI: 10.1007/s10620-005-2560-6

Systemic Iron Supplementation Replenishes Iron Stores Without Enhancing Colon Carcinogenesis in Murine Models of Ulcerative Colitis: Comparison with Iron-Enriched Diet

DARREN N. SERIL, PhD, JIE LIAO, MD, CHUNG S. YANG, PhD, and GUANG-YU YANG, MD, PhD

Ulcerative colitis (UC) patients frequently require iron supplementation to remedy anemia. The impact of systemic iron supplementation (intraperitoneal injection) on UC-associated carcinogenesis was assessed in mice subjected to cyclic dextran sulfate sodium (DSS) treatment and compared with dietary iron enrichment. Systemic iron supplementation, but not a twofold iron diet, remedied iron deficiency as indicated by the histochemical detection of splenic iron stores. A twofold iron diet, but not systemic iron, increased iron accumulation in colonic luminal contents, at the colonic mucosal surface, and in superficial epithelial cells. Colitis-associated colorectal tumor incidence after 15 DSS cycles was not affected by systemic iron (2/28; 7.1%) compared to nonsupplemented controls (4/28; 14.1%) but was significantly increased by the twofold iron diet (24/33; 72.7%) ($P < 0.001$). Mechanistic study revealed that systemic iron had no effect on DSS-induced inflammation, or colonic iNOS and COX-2 protein levels, compared to controls. Systemic iron supplementation for 16 weeks replenished splenic iron in a spontaneous colitis model (interleukin-2-deficient mice) and significantly reduced colonic inflammation compared to interleukin-2 (−/−) controls without increasing hyperplastic lesions. These results suggest that iron supplemented systemically could be used to remedy anemia in UC patients without exacerbating inflammation or enhancing colon cancer risk. These findings need to be verified in clinical studies.

KEY WORDS: ulcerative colitis; colon carcinogenesis; iron supplementation; oxidative stress.

Ulcerative colitis (UC) is a chronic inflammatory condition of the large intestine that is quite prevalent in the United States and other Western countries (1, 2). One of the most common and troublesome complications of this disease is iron-deficiency anemia, a result of chronic bleeding due to persistent injury to the colonic mucosa (3). UC patients are suggested to maintain a diet rich in protein and iron in order to balance losses resulting from chronic bleeding and diarrhea (4). The Western diet is characteristically rich in iron sources, including red meat and iron-fortified foods. Iron-deficiency anemia in UC patients is remedied by oral or parenteral iron supplementation (5). Body iron levels are tightly regulated such that iron losses, primarily due to epithelial cell sloughing, are matched by iron gains through intestinal absorption (6). Accordingly, the intestinal uptake of iron is limited to approximately 10% of the dietary iron content, which means

Manuscript received August 6, 2004; accepted September 24, 2004.

From the Susan L. Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey, 08854-8020, USA.

This work was supported by Research Project Grant RPG-00-034-01-CNE from the American Cancer Society and NIH Grant RO1CA104741-01A1 (G.-Y.Y.).

Address for reprint requests: Dr. Guang-Yu Yang, Susan L. Cullman Laboratory for Cancer Research, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854-8020, USA; gyyang@rci.rutgers.edu.

that the vast majority of dietary iron goes unabsorbed and is lost in the feces. Iron introduced into the body systemically, such as via intravenous injection, is not subject to the regulations placed on dietary iron and is entirely available for utilization or storage through the actions of the reticulendothelial system (7). However, both routes of iron supplementation can be accompanied by problems. Oral iron is the preferred route of supplementation based on ease of administration and cost but is often poorly tolerated. Intravenous or subcutaneous iron injections can cause injection site inflammation and iron overload (8). In addition, iron supplementation, as well as iron-rich foods, may have the potential to enhance inflammation and mucosal damage during UC disease flare-ups. Such an effect has been suggested but not well-studied (4).

It has been found that conditions of iron overload can exacerbate chronic inflammatory conditions, such as arthritis. Recent studies in animal models suggest that iron may exacerbate UC as well. Iron supplementation by dietary iron enrichment or injection in rodents with chemically induced colitis increases the degree of colorectal inflammation and epithelial damage (9–11). Oxidative stress resulting from the overproduction of reactive oxygen and nitrogen species (RONS) by activated phagocytic leukocytes likely plays an important role in the pathogenesis of UC (12). RONS are normally produced to combat invading microorganisms but can have deleterious effects on surrounding host cells when produced in an uncontrolled manner. Iron catalyzes the production of reactive oxygen species and takes part in the oxidative burst of activated phagocytic leukocytes (13). The enhancement of RONS production and subsequent increased cellular damage may explain the colitis-exacerbating effects of dietary iron supplementation in experimental models. Most ingested iron passes directly through the colon, where it can take part in the generation of potentially cytotoxic and genotoxic reactive oxygen species (14).

UC is complicated by an increased risk of colorectal malignancy. UC patients are 10 times more likely to develop colorectal dysplasia and adenocarcinoma than the general population (15–17). Many diseases of chronic epithelial infection and inflammation are linked with an elevated cancer risk; however, the mechanistic basis for the association between chronic inflammation and cancer remains unclear. Several aspects of the inflammatory and wound healing processes have been suggested to be contributing factors. Inflammatory mediators and growth factors elicited by activated leukocytes or surrounding stromal cells, and the overproduction of RONS, may be involved at various stages of inflammation-driven colorectal carcinogenesis (14, 18, 19). For example, the increases in cyclooxygenase (COX)-2 expression and associated arachidonic acid metabolites characteristic of UC may promote colorectal carcinogenesis by stimulating cell proliferation and new blood vessel formation (20). Nitric oxide produced by inducible nitric oxide synthase (iNOS) is thought to contribute to the pathogenesis of UC, as well as facilitate UC-associated carcinoma development, via direct or indirect mutagenic and mitogenic effects (21).

High body iron levels, such as those associated with iron overload, have long been correlated with increased cancer incidence at different organ sites (22). It is important to assess the effects of iron on inflammatory disease severity and associated carcinogenesis in UC patients given their frequent requirement for iron supplementation and the recommendation that they consume an iron-rich diet. Recent studies in our laboratory using the dextran sulfate sodium (DSS) model of UC in mice have shown that the consumption of iron-enriched diet, in addition to exacerbating active UC, significantly enhances the development of chronic UC-associated colorectal carcinoma with a concomitant increase in oxidative stress (11). Based on these observations, it is important to determine if iron supplementation administered systemically poses the same risk to UC patients. In the present study, the DSS-induced and interleukin-2 (Il-2) gene knockout models of UC in mice were used to compare the effects of iron supplementation administered via intraperitoneal injection or the diet on depleted iron stores, inflammation, and colitis-associated adenocarcinoma development.

MATERIALS AND METHODS

Animals, Chemicals, and Diets

Female C57BL/6 mice (6 weeks of age) and *Il*-2 (+/−) breeder pairs (B6.129P2-*Il2*^{tm1Hor}) (23) were obtained from The Jackson Laboratory (Bar Harbor, ME). After weaning and during the experiments, the mice were housed five per cage and maintained in air-conditioned quarters with a room temperature of $20 \pm 2^{\circ}$C, a relative humidity of $50 \pm 10\%$, and an alternating 12-hr light/dark cycle. Body weights and food consumption were monitored every other week throughout the experiment.

DSS (MW: ≈40,000) was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Iron–dextran, EDTA–iron, hematoxylin, eosin, and 3,3′-diaminobenzidine (DAB) were purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal rabbit anti-COX-2 antibody was from Cayman Chemical Co. (Ann Arbor, MI), and polyclonal rabbit anti-iNOS antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The diets were purchased from Research Diets, Inc. (New Brunswick, NJ). The AIN76A diet contains 45 mg iron/kg diet. AIN76A/2XFe diet contains two times the amount of iron in the control AIN76A diet (90 mg iron/kg diet).

Genotyping of *Il-2*–Deficient Mice

Mice homozygous null for the Il-2 gene were obtained by heterozygote breeding. DNA extracted from mouse-tail clippings

was subjected to PCR analysis using a three primer approach following the conditions available on The Jackson Laboratory Web site (http://jaxmice.jax.org). The primers are as follows: oIMR0041, 5′ TCG AAT TCG CCA ATG ACA AGA CGC T-3′; and oIMR0042, 5′-CTA GGC CAC AGA ATT GAA AGA TCT-3′; and oIMR0043, 5′-GTA GGT GGA AAT TCT AGC ATC ATC C-3′.

Animal Experiments

Long-Term Experiment Using DSS-Treated Mice. A total of 135 female C57BL/6 mice were randomized into five groups. Group 1 (water control group; $n = 10$) was administered water and AIN76A control diet continuously. Group 2 (DSS control group; $n = 30$) was administered DSS and AIN76A diet. Group 3 (iron injection group 1; $n = 30$) was administered DSS and AIN76A diet and given weekly intraperitoneal injections of iron–dextran (6 mg/kg bw) in normal saline. Group 4 (iron injection group 2; $n = 30$) was administered DSS and AIN76A diet and given weekly intraperitoneal injections of iron–dextran (12 mg/kg bw) in normal saline. Group 5 (twofold iron diet group; $n = 35$) was administered DSS and AIN76A/2X-iron diet (twofold iron diet). DSS-treated mice (Groups 2, 3, 4, and 5) were administered 1.0% DSS in distilled water for 15 DSS "cycles." A cycle consisted of 7 days of DSS treatment followed by tap water administration for 10 days (11, 24). Control mice (Group 1) were administered distilled water for 7 days, followed by tap water for 10 days. The animals in Group 5 were given twofold iron diet *ad libitum* starting 1 week before the start of the experiment and continuing until the end of the experiment. All other groups were administered AIN76A control diet starting 1 week before the start of the experiment.

Short-Term Experiment Using DSS-Treated Mice. A total of 220 female C57BL/6 mice was randomized into six treatment groups. Group 1 (negative control; $n = 20$) was administered water and AIN76A diet. Group 2 (iron injection control; $n = 20$) received water, AIN76A diet, and weekly intraperitoneal. injections of iron–dextran (12 mg/kg bw). Group 3 (twofold iron diet control; $n = 20$) was given water and twofold iron diet. Groups 4 to 6 ($n = 30$ per group) were administered 1% DSS. In addition, Group 4 was administered AIN76A diet, Group 5 was administered AIN76A diet and weekly intraperitoneal injections of iron–dextran (12 mg/kg bw), and Group 6 was administered the twofold iron diet. Groups 2 and 5 were administered iron–dextran injections starting on day −2 (2 days before the start of DSS treatment) and subsequently every 7 days (e.g., Day 5, Day 12, Day 19, etc.) for the duration of the experiment. Ten mice from each of the DSS-treated groups were sacrificed after 7 days of DSS treatment and 1 day of recovery (Day 8), after 1 DSS cycle (Day 17), and after 5 DSS cycles (Day 85). Ten mice from the non-DSS treated control groups were sacrificed on Day 17 and Day 85.

Long-Term Experiment Using Il-2 (−/−) Mice. *Il-2* (−/−) mice have been reported to develop colitis between 6 and 15 weeks of age (23). All mice were fed AIN76A diet from weaning until 8 weeks of age. Male and female *Il-2* (−/−) mice were divided into three groups. Group 1 was given AIN76A diet starting at 8 weeks of age and continuing for 16 weeks. The mice in Group 2 were given AIN76A diet continuously and administered intraperitoneal injections of iron-dextran (12 mg/kg bw) once per week starting at 8 weeks of age. Group 3 was administered twofold iron diet starting at 8 weeks of age and continuing for 16 weeks. All mice were sacrificed after 16 weeks of treatment (24 weeks of age). Only those mice that survived for 16 weeks of treatment were included in the analysis. All of the mice that survived for 16 weeks exhibited histological signs of colon inflammation.

Tissue Preparation and Histopathological Evaluation

The mice were sacrificed by CO_2 asphyxiation after the completion of 15 DSS cycles or 16 weeks of treatment (*Il-2* [−/−] mice). Colons were removed, opened longitudinally, and examined for gross abnormalities and macroscopic tumors. Three perpendicular diameter measurements were taken for colorectal tumors using calipers, and tumor volume was calculated by the formula: $4/3\pi r^3$, where r is the tumor radius. The colon was rinsed free of feces with normal saline and fixed in 10% buffered formalin for 24 hr before being transferred to 80% ethanol. The tissues were prepared for routine processing and embedding as a "Swiss roll." Five μm serial tissue sections were made and mounted on glass slides. Serial tissue slides were used for histopathological and histochemical analyses. Four slides from the series from each tissue were stained with hematoxylin and eosin for histopathological examination. Macroscopic colorectal tumors were confirmed and classified microscopically. Tumors were diagnosed as colorectal adenocarcinoma based on the observation of dysplastic cell invasion through the basement membrane.

UC was graded histologically based on inflammation severity, ulceration, hyperplasia, and area of inflammatory involvement, as described previously (11, 25). The score for each criterion was the mean of the scores of 5–12 mice from each treatment group. A total score, or UC index, was generated for each sample by adding the individual criterion scores for that sample. The mean UC index was generated from the mean of the UC indices from 5 to 12 mice per group. The criterion and UC index scores for the total colon were obtained by averaging the scores for the three colon regions.

Tissue Iron Levels

Colonic, hepatic, and splenic iron levels were assessed using Perl's Prussian blue. In brief, paraffin-embedded tissue sections were dewaxed, rehydrated, and incubated in 3% H_2O_2 for 30 min. Then the slides were incubated in a ferrocyanide solution (2% potassium ferrocyanide and 2% aqueous hydrochloric acid) for 1 hr. For colon samples, the slides were immersed in a DAB solution for 30 sec to intensify iron staining before counterstaining lightly with Mayer's hematoxylin.

Western Blot Analysis

Colon samples were minced with scissors and homogenized in ice-cold homogenization buffer using a Polytron homogenizer. Cytosolic proteins were heated at 95°C in sample buffer, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes. The blots were checked for equal protein loading by staining with Ponceau S solution. The membranes were incubated in 10% nonfat milk in TBS/0.1% Triton X-100 for 2 hr at room temperature to minimize nonspecific protein–protein interactions and washed between incubations with TBS/0.1% Triton X-100. Inducible NOS and COX-2 were detected by overnight incubation in

TABLE 1. EFFECTS OF SYSTEMIC VERSUS DIETARY IRON SUPPLEMENTATION ON COLORECTAL CANCER DEVELOPMENT IN DSS-TREATED MICE*

	Treatment group			
	Group 2: DSS only	*Group 3: DSS + 6 mg iron/kg bw, i.p.*	*Group 4: DSS + 12 mg iron/kg bw, i.p.*	*Group 5: DSS + 2-fold iron diet*
Total no. of mice	28	28	28	33
No. tumor-bearing mice	4	0	2	24
Tumor incidence (%)	14.3	0.0	7.1	72.7†
Tumor multiplicity‡	1.5 ± 0.3	NA	1.0 ± 0.0	1.6 ± 0.1
Tumor volume (cm^3)‡	0.007 ± 0.001	NA	0.03 ± 0.03	0.04 ± 0.01§

*All mice were administered 1.0% DSS for 15 cycles. Groups 2 to 4 were administered AIN76A diet continuously. Group 5 was administered AIN76A-based diet supplemented with 45 mg iron/kg diet. Groups 3 and 4 were administered injections of 6 mg iron–dextran/kg bw or 12 mg iron–dextran/kg bw once per week for the duration of the experiment.
†Statistically significant difference from Groups 2, 3, and 4 ($P < 0.001$, Fisher exact test).
‡Data are expressed as mean ± SE. NA, not applicable.
§Statistically significant difference from Group 2 ($P < 0.05$, Mann–Whitney rank sum test).

primary antibody (1:500 dilution for anti-iNOS and anti-COX-2) diluted in TBS/0.5% nonfat mik/0.1% Triton X-100 at 4°C. After washing, the membranes were incubated in horseradish peroxidase-conjugated anti-rabbit IgG (1:2000; Cell Signaling Technology, Beverly, MA). The blots were visualized by the enhanced chemiluminescence method (ECL-plus, Amersham, Piscataway, NJ).

Statistical Analysis

Tumor incidences were analyzed by Fisher exact test using SigmaStat Version 1.01 statistical software (Jandel Technology). Tumor multiplicity and tumor volume were analyzed using the Mann–Whitney rank sum test. Analysis of the UC index was performed using one-way ANOVA test.

RESULTS

General Observations: Long-Term DSS Experiment

During the DSS treatment period, all of the DSS-treated mice exhibited bloody, mucoid diarrhea and slight reductions in body weight. However, the body weights typically returned to water-treated control levels within 10 days after the cessation of DSS treatment. There were no significant difference in average body weight among Group 1, the water control group; Group 2, the DSS control animals; and the iron-supplemented groups. Food consumption was also unchanged among controls and iron-supplemented mice at the end of the experiment (data not shown). Two mice from each of Groups 2, 3, 4, and 5 died prior to the completion of 15 DSS cycles. Necropsy showed that the probable cause of death was colitis-associated toxicity. The mice exhibited significant weight loss, severe rectal bleeding and diarrhea, and extensive ulceration of the colon, but no colorectal tumors. None of these mice were considered in the final results of the carcinogenesis experiment.

Effect of Systemic Iron Supplementation on Colitis-Associated Carcinogenesis

The results for tumor incidence, tumor multiplicity, and tumor volume are presented in Table 1. Four of 28 (14.3%) Group 2 mice administered cyclic DSS and AIN76A diet developed macroscopic colorectal tumors, with a tumor multiplicity of 1.5 ± 0.3 (mean ± SE) tumors per tumor-bearing mouse. Gross colorectal tumors were not observed in Group 3 mice that received AIN76A diet and weekly iron–dextran injections (6 mg/kg bw). Two of 28 (7.1%) Group 4 mice (AIN76A diet plus weekly 12 mg/kg bw iron–dextran injections) exhibited colon tumors. The tumor incidences of Groups 3 and 4, and the tumor multiplicity of Group 4 (1.0 ± 0.0), were not significantly different from that of Group 2. Gross colorectal tumors were observed in 24 of 33 (72.7%) Group 5 mice administered DSS plus iron-enriched AIN76A diet, a significant increase compared to the DSS control and iron injection groups ($P < 0.001$; Fisher exact test). Dietary iron supplementation did not significantly affect tumor multiplicity in this experiment (1.6 ± 0.1 tumors per tumor-bearing mouse). Colorectal lesions were not observed in Group 1 mice administered water throughout the experiment.

Histopathological Analysis of UC-Associated Colorectal Tumors

The macroscopically observed colorectal tumors were confirmed by histopathological analysis and classified as well-differentiated adenocarcinomas. Besides the gross tumors (confirmed histopathologically), one animal in Group 2 also carried a microscopic adenocarcinoma. The overall tumor incidence for Group 2 (after histopathological analysis) was 17.9% (5/28), with a tumor multiplicity of 1.4 tumors per tumor-bearing mouse. Four of the

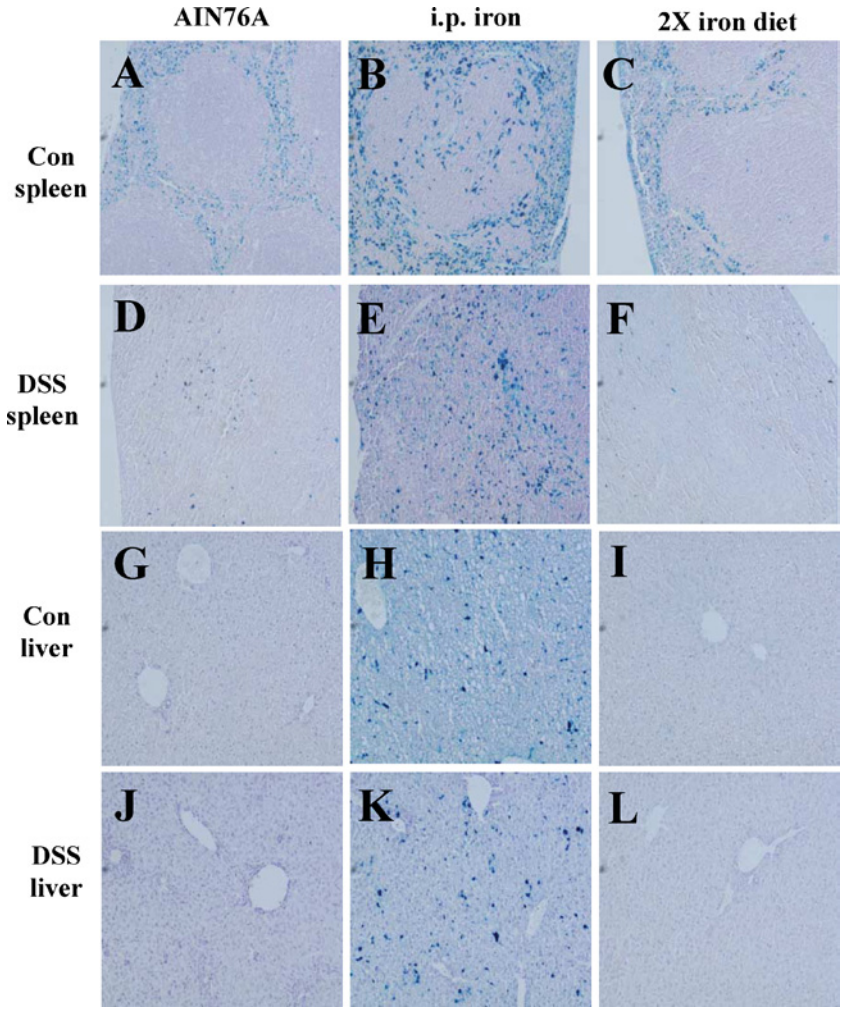

Fig 1. Histochemical staining for splenic and hepatic iron in DSS-treated mice. Ferric iron was detected by Prussian blue staining in tissues from mice subjected to five DSS cycles (day 85). Blue staining for iron was detected in the splenic red pulp of water-treated mice administered AIN76A diet (A), iron–dextran injections (B), and twofold iron-supplemented diet (C). Scattered splenic iron staining was observed in DSS control mice (D) and in DSS-treated mice fed twofold iron diet (F), and numerous iron deposits were detected in the white and red pulp in DSS-treated mice given iron injections (E). Hepatic iron deposits were rare in water-treated and DSS-treated mice fed the control diet (G and J) or the twofold iron diet (I and L). Numerous iron deposits were detected in water-treated and DSS-treated mice given iron injections (H and K). (Original magnifications: 125×.)

seven adenocarcinomas in Group 2 were mucinous, and the remaining three were tubular. The tumor incidence for Group 4 after microscopic examination was 14.3% (4/28), with a tumor multiplicity of 1.0 ± 0.0. Two of the tumors were classified as mucinous adenocarcinomas, and two were tubular adenocarcinomas. In Group 5, 28 of 33 (84.8%) mice exhibited colorectal adenocarcinomas upon microscopic examination, a significant difference from Groups 2, 3, and 4 (Fisher exact test, $P < 0.001$). Forty-two colorectal adenocarcinomas were observed in Group 5, of which 36 were mucinous and 6 were tubular adenocarcinomas.

Tissue Iron Deposition and the Effect of Iron Supplementation

Ferric iron was detected in tissues from mice subjected to five DSS cycles by Prussian blue histochemistry. Typical results for the spleen and liver are shown in Figure 1. In the spleens of control mice administered AIN76A diet

and water, blue staining for iron was visible in the red pulp surrounding lymphoid follicles (Figure 1A). In mice administered weekly iron injections (12 mg/kg bw), more intense blue staining was observed in macrophages in the red pulp, and numerous iron-staining cells were observed within the lymphoid follicles (Figure 1B). Mice fed the twofold iron-enriched diet exhibited a splenic iron staining pattern similar to that of control mice (Figure 1C). Iron staining showed a scattered pattern in the spleens of DSS-treated mice given control diet and was markedly decreased compared to water-treated controls (Figure 1D). In contrast, DSS-treated mice administered weekly iron injections showed intense staining for ferric iron in all regions of the spleen (Figure 1E). Similar to the DSS plus control diet group, the spleens of DSS-treated mice given the twofold iron diet exhibited scattered, weak iron staining (Figure 1F).

Iron staining was occasionally seen in a scattered pattern in the livers of water-treated and DSS-treated mice administered the control diet (Figures 1G and J, respectively). Intense hepatic iron staining was seen in the mice administered weekly iron injections plus water or DSS (Figures 1H and K, respectively). Similar to the control diet groups, the mice administered water or DSS and the twofold iron diet showed small amounts of hepatic iron staining (Figures 1I and L, respectively).

Prussian blue staining with DAB intensification was used to assess iron deposition in the colon. In control mice, iron staining was infrequently observed. Iron staining was increased in lamina propria inflammatory cells, predominantly in the proximal colon, in water control mice administered iron injections. Mice administered DSS and control diet for five cycles showed scattered iron-containing macrophages in the lamina propria of the inflamed mucosa (Figure 2A). Colonic iron staining in the mucosa and submucosa was increased in DSS-treated mice administered iron injections (Figures 2B and C). Iron-containing inflammatory cells were also seen in the mucosa and submucosa throughout the colons of DSS-treated mice administered the twofold iron diet (Figure 2D). Dietary iron supplementation was associated with increased iron accumulation at the surface of the mucosa (Figure 2E), as well as iron staining in the colonic lumen contents and in superficial epithelial cells (Figure 2F).

Effect of Systemic Iron Supplementation on DSS-Induced Colitis

The UC index results for Days 8 and 17 (cycle 1), Day 85 (cycle 5), and Day 255 (cycle 15), and the effects of systemic and dietary iron supplementation, are shown in Figure 3. On Day 8, the UC index was significantly greater in the twofold iron diet group (mean $\pm$ SE, 6.2 ± 0.2)

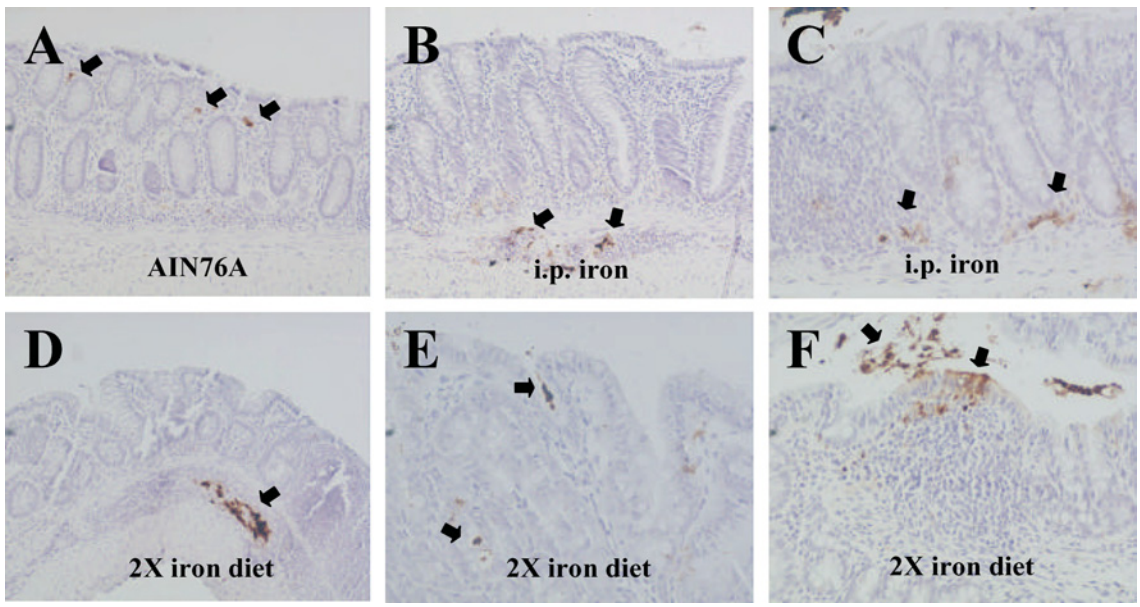

Fig 2. Colonic iron accumulation in DSS-treated mice and the effect of iron supplementation. Ferric iron was detected by Prussian blue staining with DAB intensification (arrows) of colon sections from controls and mice subjected to five DSS cycles. (A) Iron staining in lamina propria inflammatory cells in a mouse administered AIN76A diet. (B, C) Increased iron staining in submucosal and lamina propria inflammatory cells in the colon of a mouse administered weekly iron injections. Iron accumulation in submucosal inflammatory cells (D), at the epithelial surface (arrows) (E), and in luminal contents and superficial epithelial cells (F) in mice administered the twofold iron diet. (Original magnifications: A–D, 125×; E and F, 250×.)

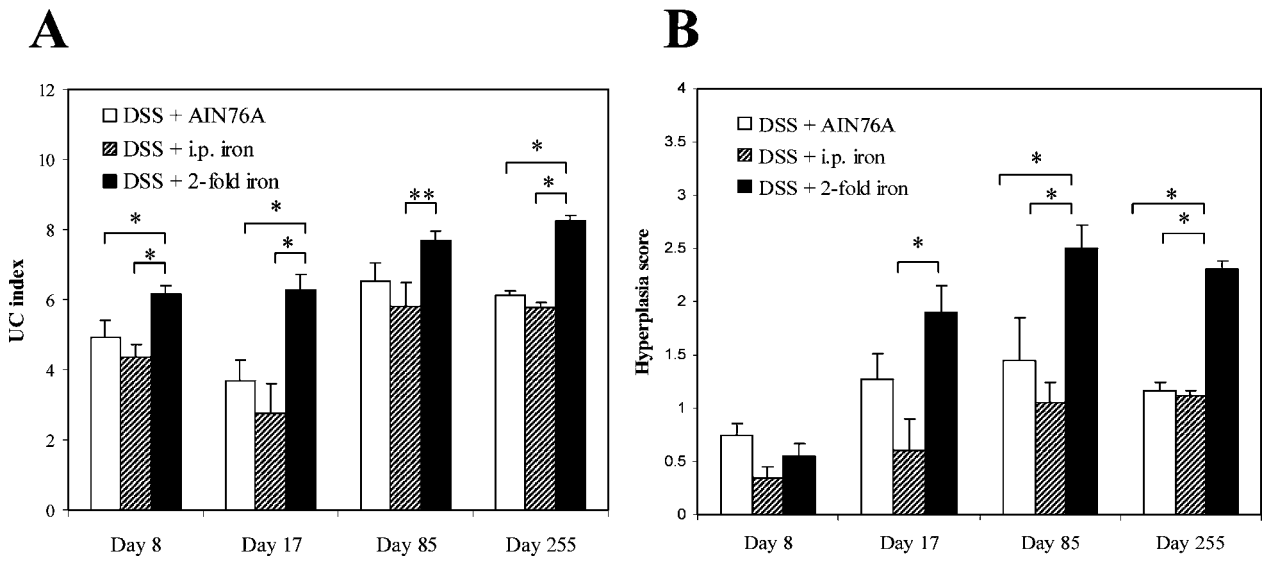

Fig 3. Effect of systemic iron supplementation on DSS-induced colitis. The UC index (A), which includes the hyperplasia score (B), was graded in the colons of mice subjected to 7 days of DSS administration and 1 day of recovery (Day 8), after 1 DSS cycle (Day 17), after 5 cycles (Day 85), and after 15 cycles (Day 255). The mice were administered AIN76A diet, AIN76A plus weekly intraperitoneal injections of 12 mg/kg bw iron–dextran, or twofold iron diet. $*P < 0.05$, one-way ANOVA test. $**P < 0.05$, Student's t test. Data are expressed as mean ± SE ($n = 5$–10 per group and time point).

compared to the diet control group and the iron injection group (4.9 ± 0.5 and 4.4 ± 0.4, respectively; $P < 0.05$). The twofold iron diet group exhibited more severe mucosal injury on Day 8, including more frequent and extensive ulceration. The twofold dietary iron supplementation group also exhibited significantly greater UC indices on Day 17 (10 days after the cessation of DSS treatment) and Day 85 (5 DSS cycles) than the AIN76A diet control group and the iron injection group (Figure 3A). Branched and distorted glands exhibiting pleiomorphic and hyperchromatic cells were occasionally observed in the twofold iron diet group on Day 17, and frequently observed on Day 85, but were rare in the AIN76A and iron injection groups. The two fold iron diet group exhibited significantly greater hyperplasia scores on Days 17, 85, and 255. The hyperplasia scores for the iron injection group were decreased compared to the AIN76A diet control group on Days 17 and 85, but not significantly so (Figure 3B). Similarly, there were no differences in the UC indices of the AIN76A diet group and the iron injection group at any of the time points. The colons of water control animals administered AIN76A diet, AIN76A diet plus weekly iron injections, or twofold iron diet were morphologically normal.

Effects of Iron Supplementation on Colonic iNOS and COX-2 Expression

The expressions of the pro-inflammatory enzymes, iNOS and COX-2, were assessed in the colons of mice treated with DSS for one cycle. Inducible NOS protein expression was detected by Western blot in colon homogenates from DSS-treated mice administered AIN76A control diet (Figure 4A) but was absent in the colons of water-treated mice (not shown). COX-2 protein levels were elevated in the colons of DSS-treated controls versus water-treated mice. The colons of DSS-treated mice administered iron injections exhibited similar iNOS and COX-2 levels compared to the DSS control group (Figures 4A and B). The protein levels of iNOS were increased more than twofold in the colons of DSS plus twofold iron diet-treated mice compared to both the DSS control and the iron injection groups. Colonic COX-2 protein levels were also increased nearly twofold in DSS-treated mice administered twofold iron diet compared to the other groups.

Effect of Iron Supplementation on Iron Stores and Colitis in *Il-2*(−/−) Mice

Prussian blue histochemical staining was performed on spleen and liver sections from 24-week-old *Il-2* (−/−) mice and wild-type littermates (Figure 5). Blue-staining ferric iron was observed in the splenic red pulp of *Il-2* (+/+) littermates fed the AIN76A control diet (Figure 5A). Ferric iron staining was absent in the spleens of 24-week-old *Il-2* (−/−) mice (Figure 5B) but was visible in the red pulp of *Il-2* (−/−) mice administered iron supplementation by weekly iron–dextran injection

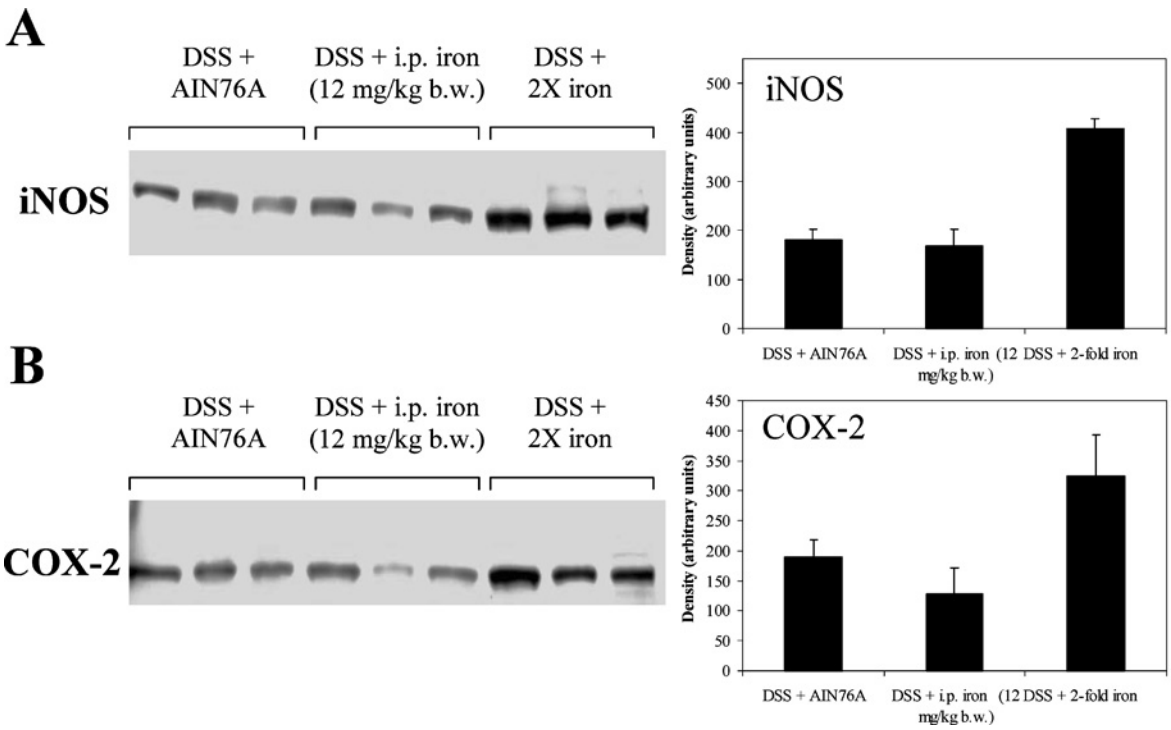

Fig 4. Expression of iNOS and COX-2 proteins in the colons of DSS-treated mice and the effects of iron supplementation. Inducible NOS (A) and COX-2 (B) proteins were detected by Western blot analysis of colon homogenates from control and iron-supplemented mice at the end of one DSS cycle. Band densitometry is expressed as arbitrary units.

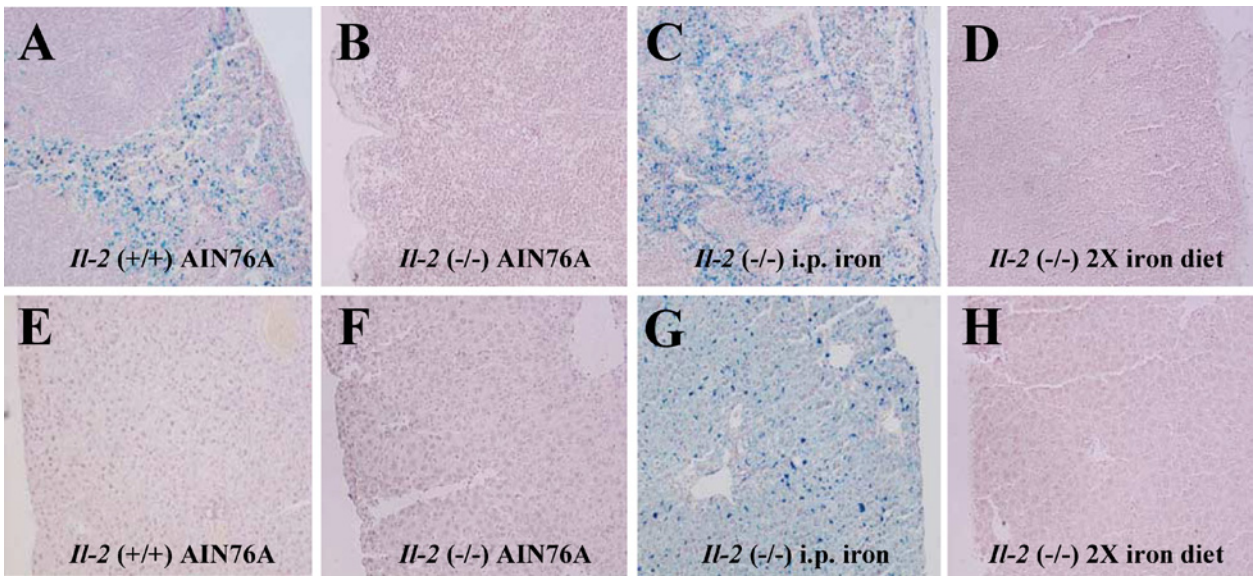

Fig 5. Histochemical staining of splenic and hepatic iron in *Il-2* (−/−) mice. Prussian blue staining was performed on spleen (A to D) and liver (E to H) sections from 24-week-old *Il-2* (−/−) mice and wild-type littermates. (A) Large, blue-staining iron deposits were observed in the splenic red pulp of *Il-2* (+/+) mice administered AIN76A control diet. (B) Iron staining was absent in the spleens of *Il2* (−/−) mice fed the control diet. (C) Iron staining was present in *Il-2* (−/−) mice administered iron injections for 16 weeks. (D) Iron staining was not observed in *Il-2* (−/−) mice given the twofold iron diet. Large hepatic iron deposits were rare in (E) *Il-2* (+/+) and (F) *Il-2* (−/−) mice fed the control diet, as well as *Il-2* (−/−) mice fed the twofold iron diet (H). (G) Large iron deposits were observed in the livers of *Il-2* (−/−) mice given weekly iron injections. (Original magnifications: 125×.)

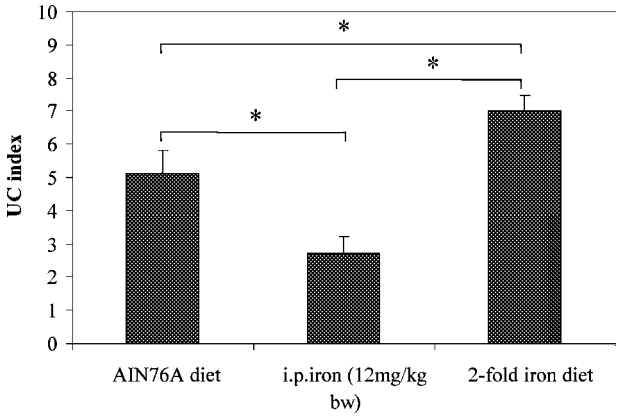

Fig 6. Effects of iron supplementation on colitis in *Il-2* (−/−) mice. *Il-2* (−/−) mice were administered AIN76A diet, AIN76A plus weekly injections of 12 mg/kg bw iron–dextran, or two fold iron diet starting at 8 weeks of age. The UC index was determined in the colons of mice sacrificed at 24 weeks of age. The data are expressed as the mean ± SE (n = 10–12 mice). $^{*}P < 0.05$, One-way ANOVA test.

(Figure 5C). Similar to *Il-2* (−/−) controls, iron staining was absent in the spleens of *Il-2* (−/−) mice fed the twofold iron diet (Figure 5D). Significant iron deposits were rarely observed in the livers of *Il-2* (+/+) mice, *Il-2* (−/−) mice fed the control diet, and *Il-2* (−/−) mice fed the twofold iron diet (Figures 5E, F, and H, respectively). The livers of *Il-2* (−/−) mice supplemented with iron via intraperitoneal injection showed marked ferric iron staining (Figure 5G).

Inflammation was assessed histologically in the colons of *Il-2* (−/−) mice at 24 weeks of age and expressed as a UC index (Figure 6). The mean UC index for *Il-2* (−/−) mice fed the control diet was 5.1 ± 0.9 (mean ± SE). The colons of mice consuming the control diet were characterized by mild to moderately severe inflammation. Inflammatory cell infiltration was mild and restricted to the mucosa. Goblet cell depletion was evident, and crypt abscesses were common. Hyperplastic epithelia was observed and classified as mild (Figure 7A). *Il-2* (−/−) mice administered iron supplementation via intraperitoneal injection exhibited significantly lower UC indices compared to the AIN76A control diet group (2.6 ± 0.6; $P < 0.05$). The inflammation in the iron injection group was mild and characterized by focal regions of epithelial injury, goblet cell depletion, and mild inflammatory cell involvement. Hyperplasia was less common than in the mice fed control diet only and was mild in grade (Figure 7B). The colitis in the mice administered twofold iron diet was moderately severe. The UC index for the dietary iron supplementation group was 7.0 ± 0.52, a significant increase compared to the AIN76A diet and iron injection groups ($P < 0.05$) (Figure 6). Epithelial injury in the twofold iron diet group occasionally involved small ulcers. Mucosal inflammatory cell infiltration was often moderately intense

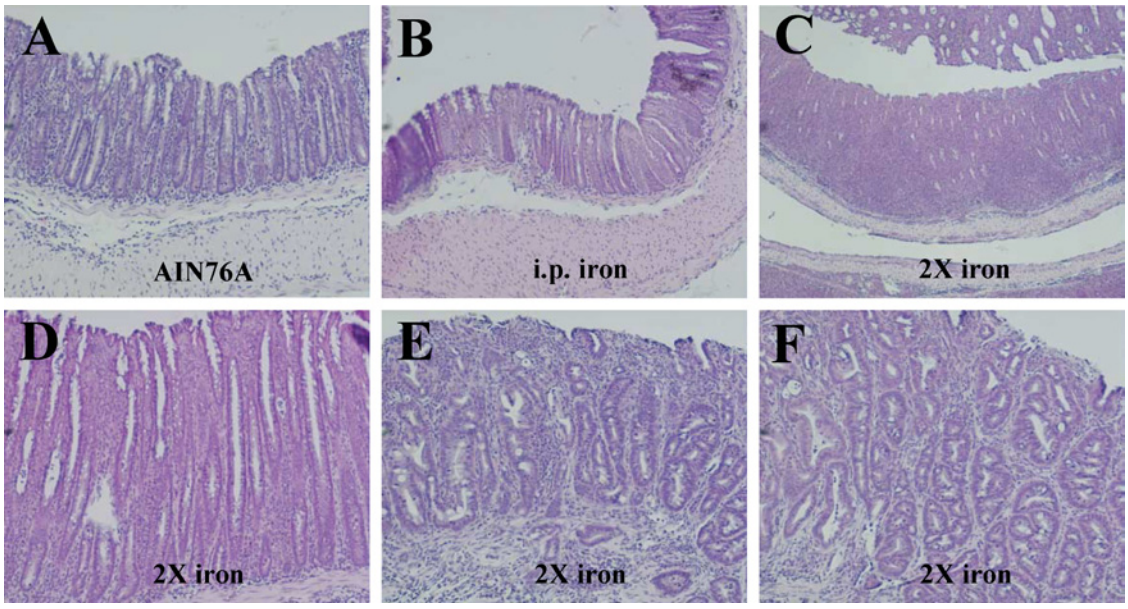

Fig 7. Histopathology of colitis and associated dysplasia in iron-supplemented *Il-2 (−/−)* mice. (A) Mild to moderate chronic colitis was observed in *Il-2* (−/−) mice fed AIN76A control diet. (B) Mild colitis was observed in mice administered weekly iron injections (12 mg/kg bw). *Il-2 (−/−)* mice administered the twofold iron diet exhibited moderate colitis with moderate epithelial hyperplasia (C), with moderate to severe hyperproliferation (D), and dysplasia (E, F). (Original magnifications: A–E, 125×; F, 250×.)

(Figure 7C) and moderate to severe hyperplasia, characterized by branching crypts, hyperchromatic cells, and significantly elongated glands, was observed frequently (Figure 7D).

Effect of Iron Supplementation on Colitis-Associated Carcinogenesis in the *Il-2* (−/−) Mouse

The occurrence of hyperplastic lesions was assessed by histopathological examination of the colons of *Il-2* (−/−) mice at 24 weeks of age. Dysplastic colorectal lesions, signified by loss of epithelial cell polarity, hyperchomatic and pleiomorphic nuclei, and an increased number of mitotic figures, were observed in the colons of 1 of 12 (8.3%) *Il-2* (−/−) mice administered AIN76A diet. Dysplasia was not observed in the colons of the 12 mice that received weekly iron injections. Dysplasia was detected in 4 of 11 (36.4%) mice administered the twofold iron diet (Figures 7E and F). Colorectal adenocarcinoma was not observed in the tissue slides from any of the three treatment groups at 24 weeks of age.

DISCUSSION

UC patients commonly experience iron-deficiency anemia as a result of persistent colonic blood loss, as well as chronic disease (5, 14, 26) It is recommended that these patients fortify their diets with iron-rich foods to balance losses due to bleeding. In addition, iron is supplemented in the form of oral iron supplements or parenteral iron injections (5, 27). Using the DSS model in mice, we have shown that the consumption of iron-enriched diet, in addition to exacerbating UC, significantly enhances the development of UC-associated colorectal carcinoma (11). Thus, iron supplementation and iron-enriched diet consumption may be contributing factors to elevated cancer risk in UC patients. In the present study, chemically induced and genetic models of colitis in mice were used to study the effects of iron supplementation administered by different routes on colitis and associated carcinoma development. Systemic iron supplementation, in the form of intraperitoneal injections of iron–dextran, did not enhance colitis-associated carcinoma development, in contrast to dietary iron supplementation. Similarly, systemic iron administration did not exacerbate inflammation in the DSS model, and decreased inflammation in *Il-2* (−/−) mice, a genetic model with histology closely resembling UC (23). These results may have implications for the management of UC patients, as well as provide mechanistic insights into the previously observed effects of dietary iron supplementation on colitis-associated carcinogenesis.

The effects of iron supplementation on colorectal cancer development are likely associated with the effects on acute inflammation. Unlike the twofold iron diet, intraperitoneal iron injection did not exacerbate UC in the DSS model and significantly decreased inflammation in the *Il-2* (−/−) mouse model. Similarly, iron injection, unlike iron-enriched diet, did not affect the colonic levels of the pro-inflammatory enzymes iNOS and COX-2, both of which are up-regulated during UC. Others have reported that the intraperitoneal administration of iron–dextran (1000 mg/kg bw) increases colitis disease activity and lipid peroxidation in the DSS model in rats (28). However, the much lower doses of iron–dextran used in the present study (6 or 12 mg/kg bw) adequately restored depleted splenic iron levels in DSS-treated mice and *Il-2* (−/−) mice without increasing inflammation. Even at these relatively low doses, iron deposition in the spleen and liver was significant.

It is interesting that systemic iron supplementation did not affect the severity of colitis in either model despite marked increases in iron accumulation in phagocytic leukocytes of the mucosa and submucosa. Iron is known to catalyze the generation of reactive oxygen species and has been shown to increase the activity of phagocytic leukocytes (13, 14, 29). Our findings suggest that the effects of iron on colitis and cancer development *in vivo* are not due to the direct enhancement of leukocyte activities, including cytokine release and the oxidative burst. Rather, the effects of dietary iron may be enacted locally at the surface of the colonic mucosa. Approximately 5 to 10% of dietary iron is absorbed by the small intestine daily. Therefore, 90 to 95% of dietary iron passes through the colon in the feces. Fecal iron may catalyze RONS formation near the surface of the colonic mucosa in conjunction with adherent inflammatory cells, bacteria, or epithelial cells, leading to oxidative injury to the surface mucosa. The local enhancement of RONS production would serve to further increase the inflammatory response by increasing mucosal and vascular permeability and increasing the recruitment and activities of inflammatory cells (14, 30). Such indirect effects would likely contribute to UC-associated colorectal carcinogenesis as well. Interaction with negatively charged colonic mucins could bring dietary iron in contact with the mucosal surface (14). Alternatively, superficial epithelial cells may directly take up iron available in the feces. The iron staining patterns in the colons of DSS-treated mice administered the twofold iron diet are suggestive of both of these possibilities.

In both the DSS model and the *Il-2* knockout mouse, dietary iron supplementation was associated with branched and hyperplastic epithelial lesions that were rarely noted in control animals or those administered iron injections. These lesions may be a result of the more severe and extensive inflammatory injury caused by dietary iron

supplementation. However, we have observed that dietary iron supplementation enhances mucosal thickness and colonic epithelial proliferation in the absence of inflammation, an effect not seen with systemic iron supplementation (unpublished data). The increase in proliferation with the twofold iron diet is not associated with histological signs of epithelial injury, so it does not appear to be a response to cytotoxicity as has been described for dietary heme administration (31). Thus, dietary iron may also contribute to UC-associated carcinogenesis via effects on epithelial proliferation.

The results in the *Il-2* (−/−) mice, as well as in DSS-treated mice after 1 DSS cycle, indicate that systemic iron supplementation can decrease the severity of acute colitis. Systemic iron is taken up by macrophages in the reticuloendothelial system (8). Iron and oxidative stress may act to down-regulate macrophage activity in a negative feedback fashion. Our preliminary observations have indicated that the rate of apoptosis in colonic lymphoid follicles is higher in systemic iron-supplemented mice than in DSS control and dietary iron supplemented animals. However, the mechanistic basis for the possible UC-ameliorating effects of systemic iron suppementation requires closer examination.

It is important to determine what forms of iron exposure present the greatest risk to UC patients. Oral iron supplementation is preferred over the parenteral route because it is easy to administer, relatively inexpensive, and associated with fewer side effects (5, 8). The present study using chemically induced and genetic models of UC in mice suggests that dietary iron and oral iron supplementation are risk factors for carcinoma development in UC patients, while parenteral iron supplementation is not. Iron injections may circumvent the exacerbation of acute inflammation and enhancement of colorectal carcinogenesis in UC patients associated with oral iron consumption. However, care must be taken in the use of intravenous or intramuscular iron injections. Both are associated with hypotension, nausea, headache, and pain at the injection site (8). In addition, long-term systemic supplementation of iron can lead to iron overload, a risk factor for increased cancer development at various organ sites (22, 32). It is also important to consider if it is advisable for UC patients to consume a high-iron diet. In this study, a twofold iron diet was ineffective in replenishing iron stores in mice with colitis, most likely due to the exacerbation of colitis and increased rectal bleeding. It may be less harmful for UC patients to periodically take iron supplements to remedy anemia rather than continuously consuming foods high in iron. This would avoid persistent elevations in oxidative damage and epithelial proliferation that may contribute to colorectal carcinogenesis.

ACKNOWLEDGMENT

The authors are grateful to Ms. Kwok-Lam K. Ho for her technical assistance.

REFERENCES

1. Fiocchi C: Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 115:182–205, 1998
2. Farrell RJ, Peppercorn MA: Ulcerative colitis. Lancet 359:331–340, 2002
3. Wilson A, Reyes E, Ofman J: Prevalence and outcomes of anemia in inflammatory bowel disease: a systematic review of the literature. Am J Med 116(Suppl 7A):44S–49S, 2004
4. Truelove SC: Medical management of ulcerative colitis. Br Med J 2:605–607, 1968
5. Barrison IG, Roberts PD, Kane SP: Oral or parenteral iron treatment in chronic ulcerative colitis? Br Med J (Clin Res Ed) 282: 1514, 1981
6. Pietrangelo A: Physiology of iron transport and the hemochromatosis gene. Am J Physiol Gastrointest Liver Physiol 282:G403–G414, 2002
7. Knutson M, Wessling-Resnick M: Iron metabolism in the reticuloendothelial system. Crit Rev Biochem Mol Biol 38:61–88, 2003
8. Burns DL, Mascioli EA, Bistrian BR: Parenteral iron dextran therapy: a review. Nutrition 11:163–168, 1995
9. Reifen R, Matas Z, Zeidel L, Berkovitch Z, Bujanover Y: Iron supplementation may aggravate inflammatory status of colitis in a rat model. Dig Dis Sci 45:394–397, 2000
10. Carrier J, Aghdassi E, Platt I, Cullen J, Allard JP: Effect of oral iron supplementation on oxidative stress and colonic inflammation in rats with induced colitis. Aliment Pharmacol Ther 15:1989–1999, 2001
11. Seril DN, Liao J, Ho KL, Warsi A, Yang CS, Yang GY: Dietary iron supplementation enhances DSS-induced colitis and associated colorectal carcinoma development in mice. Dig Dis Sci 47:1266–1278, 2002
12. Grisham MB: Oxidants and free radicals in inflammatory bowel disease. Lancet 344:859–861, 1994
13. Morris CJ, Earl JR, Trenam CW, Blake DR: Reactive oxygen species and iron—A dangerous partnership in inflammation. Int J Biochem Cell Biol, 27:109–122, 1995
14. Babbs CF: Oxygen radicals in ulcerative colitis. Free Radical Biol Med 13:169–181, 1992
15. Lashner BA, Silverstein MD, Hanauer SB: Hazard rates for dysplasia and cancer in ulcerative colitis. Results from a surveillance program. Dig Dis Sci 34:1536–1541, 1989
16. Sugita A, Sachar DB, Bodian C, Ribeiro MB, Aufses JAH, Greenstein AJ: Colorectal cancer in ulcerative colitis. Influence of anatomical extent and age at onset on colitis-cancer interval. Gut 32:167–169, 1990
17. Lashner BS, Provencher KS, Bozdech, JM, Brzezinski A: Worsening risk for the development of dysplasia or cancer in patients with chronic ulcerative colitis. American Journal of Gastroenterology 90:377–380, 1995
18. Fitzpatrick FA: Inflammation, carcinogenesis and cancer. International Immunopharmacology 1:1651–1667, 2001
19. Seril DN, Liao J, Yang G-Y, Yang CS: Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. Carcinogenesis 24:353–362, 2003
20. Agoff SN, Brentnall TA, Crispin DA, Taylor SL, Raaka S, Haggitt RC, Reed MW, Afonina IA, Rabinovitch PS, Stevens AC, Feng Z,

Bronner MP: The role of cyclooxygenase 2 in ulcerative colitis-associated neoplasia. Am J Pathol 157:737–745, 2000
21. Jaiswal M. LaRusso NF, Gores GJ: Nitric oxide in gastrointestinal epithelial cell carcinogenesis: linking inflammation to oncogenesis. Am J Physiol Gastrointest Liver Physiol 281:G626–G634, 2001
22. Stevens RG, Graubard BI, Micozzi MS, Neriishi K, Blumberg BS: Moderate elevation of body iron level and increased risk of cancer occurrence and death. Int J Cancer 56:364–369, 1994
23. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I: Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75:253–261, 1993
24. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R: A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 98:694–702, 1990
25. Mahler M, Bristol IJ, Leiter EH, Workman AE, Birkenmeier EH, Elson CO, Sundberg JP: Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. Am J Physiol 274:G544–G451, 1998
26. Weiss G: Iron and anemia of chronic disease. Kidney Int Suppl 69:S12–S17, 1999
27. Burns DL, Pomposelli JJ: Toxicity of parenteral iron dextran therapy. Kidney Int Suppl 69:119–124, 1999
28. Aghdassi E, Carrier J, Cullen J, Tischler M, Allard JP: Effect of iron supplementation on oxidative stress and intestinal inflammation in rats with acute colitis. Dig Dis Sci 46:1088–1094, 2001
29. Herbert V, Shaw S, Jayatilleke E, Stopler-Kasdan T: Most free-radical injury is iron-related: it is promoted by iron, hemin, holoferritin and vitamin C, and inhibited by desferoxamine and apoferritin. Stem Cells 12:289–303, 1994
30. Grisham MB, Granger DN: Neutrophil-mediated mucosal injury: role of reactive oxygen metabolites. Dig Dis Sci 33:6S–15S, 1988
31. Sesink AL, Termont DS, Kleibeuker JH, Van der Meer R: Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. Cancer Res 59:5704–5709, 1999
32. Stevens RG, Jones DY, Micozzi MS, Taylor PR: Body iron stores and the risk of cancer. N Engl J Med 319:1047–1052, 1988