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Increased Susceptibility of Nrf2 Knockout Mice to Colitis-Associated Colorectal Cancer

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Abstract

The nuclear factor-erythroid 2-related factor 2 (Nrf2) plays a critical role in protecting various tissues against inflammation, which is a potential risk factor for colorectal and other cancers. Our previously published mouse model work showed that Nrf2 helps protect against dextran sulfate sodium (DSS)-induced colitis/inflammation, and others have shown that Nrf2 helps protect against inflammation-associated colorectal carcinogenesis (aberrant crypt foci). The present study extended these important earlier findings by exploring the role of Nrf2 in colitis-associated colorectal cancer in a mouse model involving azoxymethane/DSS-induced colorectal carcinogenesis in Nrf2 knockout mice. Azoxymethane/DSS-treated Nrf2 knockout mice had increased incidence, multiplicity, and size of all colorectal tumors, including adenomas, versus treated wild-type (WT) mice, and the proportion of tumors that were adenocarcinoma was much higher in knockout (80%) versus WT (29%) mice. Compared with WT mice, knockout mice also had increased markers of inflammation in tumor tissue (cyclooxygenase-2 and 5-lipoxygenase expressions and prostaglandin E₂ and leukotriene B₄ levels) and in inflamed colonic mucosa (nitrotyrosine expression), supporting the association of knockout mouse tumor formation with inflammation. The phase II detoxifying/antioxidant enzymes NAD(P)H-quinone reductase 1 and UDP-glucosyltransferase 1A1 were elevated in the normal mucosa of WT, but not Nrf2 knockout, mice treated with azoxymethane/DSS. Our findings show that Nrf2 plays a critical role in protecting against inflammation-associated colorectal cancer.

Chronic inflammation has been identified as a potential risk factor for colorectal and other cancers. Inflammatory bowel diseases are forms of chronic, recurrent colitis, most commonly Crohn's disease and ulcerative colitis, and many epidemiologic and clinical

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

studies have shown that inflammatory bowel disease increases the risk of colorectal cancer (1-5). Inflammation, or colitis, can be triggered by environmental insults such as a gastrointestinal microbial infection or toxic chemical irritation. Driven by a cascade of cytokine and chemokine interactions, inflammatory cells such as neutrophils, macrophages, and lymphocytes infiltrate the wounded colonic mucosa and create oxidative stress, which in turn generates reactive oxygen and nitrogen species. Reactive oxygen and nitrogen species are thought to be a major factor underlying the contribution of chronic inflammation to neoplastic transformation (5).

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a basic leucine zipper redox-sensitive transcriptional factor that plays a central role in transcriptional regulation of antioxidant and/or detoxifying genes. We recently reported that Nrf2-deficient mice are more susceptible to dextran sulfate sodium (DSS)-induced inflammation/colitis compared with wild-type (WT) mice (6). This increased susceptibility was associated with decreased expression of antioxidant/phase II detoxifying enzymes and concomitant increases in proinflammatory cytokines and other biomarkers (6). These findings illustrated the importance of Nrf2 in protecting or restoring intestinal integrity after a wound to the colonic mucosa. Others have shown that Nrf2-deficient mice are also more susceptible to inflammation-associated preinvasive colorectal carcinogenesis (aberrant crypt foci) versus WT mice (7). We conducted the present study to extend these previous findings by addressing the question of whether colitis-associated colorectal cancer risk is increased in Nrf2 knockout mice.

Materials and Methods

Reagents

DSS was purchased from MP Biomedicals. Azoxymethane was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute). Rabbit polyclonal antibodies against NAD(P)H-quinone reductase 1 and UDP-glucosyltransferase 1A1 and goat polyclonal antibodies against actin and heme oxygenase 1 were purchased from Santa Cruz Biotechnology. Anti-cyclooxygenase-2 (COX-2) and anti-5-lipoxygenase (5-LOX) antibodies were purchased from Cayman Chemical.

Animal care and treatment

The male Nrf2(-/-) knockout mice used in this study were essentially of the C57BL/6 strain (through backcrossing) and have been described in detail elsewhere (6). Control mice were age-matched male C57BL/6 WT mice purchased from Hilltop Lab Animals, Inc. Five- to six-week-old mice were used and housed at Rutgers Animal Facility and maintained under 12-h light/dark cycles. All animals received water and food *ad libitum*. After 1 wk of acclimatization, mice were divided into four groups: (a) control WT ($n = 6$); (b) control Nrf2(-/-) ($n = 6$); (c) WT + azoxymethane/DSS ($n = 15$); and (d) Nrf2(-/-) + azoxymethane/DSS ($n = 15$). Mice were injected s.c. with a single dose of azoxymethane (10 mg/kg body weight) in 100 μ L of normal saline or vehicle alone (100 μ L normal saline, control group 1) and water *ad libitum*. Two weeks after the azoxymethane injection, the mice were given 1% DSS (w/v) in water as the sole source of drinking fluid for 1 wk, after which the DSS was stopped and drinking fluid was shifted back to drinking water, which continued until the end of experiment (20 wk). All animal use procedures were in accordance with the National Academy of Sciences *Guide for the Care and Use of Laboratory Animals* and were approved by the Rutgers Institutional Animal Care and Use Committee, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Immunohistochemistry

Immunohistochemical staining of nitrotyrosine has previously been described (6). The expression of nitrotyrosine was scored as the number of positive cells per field at $\times 400$ magnification. Four fields were randomly selected for the scoring.

Western blotting

Large bowel tissues and tumors dissected from mice of each treatment group were weighed and pooled. The protein extraction and Western blotting methods were done as previously described (6).

Measurement of prostaglandin E₂ and leukotriene B₄ levels in the small intestine

Frozen intestinal polyps and normal mucosa were quickly excised from the small intestine, stored at -80°C , cut in the microtome at -20°C , and homogenized using a Polytron homogenizer in an ice-cold homogenizing buffer containing protease inhibitors (0.2 mmol/L phenyl methyl sulfonyl fluoride and 2.5 $\mu\text{g}/\text{mL}$ of pepstatin, aprotinin, and leupeptin), 10 $\mu\text{mol}/\text{L}$ nordihydroguaiaretic acid (a LOX inhibitor), and 10 $\mu\text{mol}/\text{L}$ indomethacin (a COX inhibitor; ref. 8). The LOX and COX inhibitors were added to stabilize the levels of prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) during sample processing. The homogenates were then transferred to a clean tube and centrifuged at $14,000 \times g$ for 10 min at 4°C . The supernatant was aliquoted and stored at -80°C until further analysis. To analyze the PGE₂ and LTB₄ levels, a 100- μL homogenate supernatant was acidified with 15 μL of 0.1 N HCl, vortexed for 1 min after adding 1 mL of ethyl acetate, and then centrifuged at 10,000 rpm for 3 min at room temperature. The organic layer was collected and evaporated under nitrogen gas. The dried sample was reconstituted in a 1-mL enzyme immunoassay buffer. The reconstituted samples were diluted appropriately and the concentrations of PGE₂ and LTB₄ were measured using an enzyme immunoassay kit (Cayman Chemical) according to the manufacturer's protocol.

Statistical considerations

Student's *t* test was used for simple group means analysis (nitrotyrosine score), and Fisher's exact test was used to analyze the incidence of tumors and anal bleeding. For between-group comparisons, one-way ANOVA and post-hoc Tukey's test were used. $P < 0.05$ was considered to be statistically significant.

Results

Susceptibility to azoxymethane/DSS-induced colon carcinogenesis and cancer increase in Nrf2 knockout mice

Nrf2 knockout mice were more susceptible to azoxymethane/DSS-induced colorectal carcinogenesis and cancer compared with WT mice. The incidence of colonic tumors increased by $\sim 40\%$ in Nrf2 knockout mice in association with increased rectal bleeding and prolapsed rectums ($\sim 60\%$ increased) compared with WT counterparts (Fig. 1). The number of tumors per mouse also increased significantly in Nrf2 knockout mice (Table 1). Subsequent histologic evaluations of tumors ($>4 \text{ mm}^2$) revealed that 80% of tumors in Nrf2 knockout mice were adenocarcinomas, versus 29% in the control mice (Table 2). The number of nitrotyrosine-positive cells (primarily stroma cells) was significantly higher in the colonic mucosa of Nrf2 knockout mice versus WT mice after azoxymethane/DSS exposure (Fig. 2A and B). These collective results strongly indicate that Nrf2 plays an indispensable role in protecting colonic mucosa against inflammation and carcinogenesis.

Association of increased susceptibility of Nrf2 knockout mice to azoxymethane/DSS–induced colon carcinogenesis with increased expressions of COX-2, 5-LOX, and proinflammatory metabolites of arachidonic acids

Proinflammatory biomarkers such as COX-2 and 5-LOX were overexpressed in tumors compared with their expression in adjacent apparently normal mucosa. Furthermore, COX-2 and 5-LOX expressions were higher in tumors of Nrf2 knockout mice than in tumors of WT mice (Fig. 3A). The expressions of PGE₂ and LTB₄, two downstream products of COX-2 and 5-LOX metabolism of arachidonic acid, were also higher in tumors of Nrf2 knockout mice than in tumors of WT mice (Fig. 3B and C).

Expressions of antioxidant/phase II detoxifying enzymes in normal mucosa versus tumors of azoxymethane/DSS–treated mice

As in our previous studies (6, 9), the expressions of the antioxidant/phase II detoxifying enzymes NAD(P)H-quinone reductase 1, UDP-glucosyltransferase 1A1, and heme oxygenase 1 were elevated in the normal mucosa of azoxymethane/DSS–treated WT mice, but only heme oxygenase 1 was elevated in the normal mucosa of azoxymethane/DSS–treated Nrf2 knockout mice (Fig. 3D). The expressions of these markers were either totally diminished [NAD(P)H-quinone reductase 1] or reduced (UDP-glucosyltransferase 1A1) in the tumors of both WT and Nrf2 knockout mice (Fig. 3D).

Discussion

Our present data clearly show that Nrf2 played a critical role in protecting against colitis/inflammation-associated neoplastic transformation. Nrf2 knockout mice had a significantly greater incidence, multiplicity, and size of colorectal cancers than did control WT mice. Prolapsed rectums and rectal bleeding also were significantly more frequent in Nrf2 knockout mice. The striking significantly higher proportion of adenocarcinomas among Nrf2 knockout tumors (80%) than among WT tumors (29%) further confirms the greater severity of colorectal carcinogenesis in Nrf2 knockout mice. Our findings show that Nrf2 plays a critical role in protecting against inflammation-associated colorectal cancer and thus support and extend a recently reported study showing that Nrf2 knockout increases susceptibility to inflammation-associated aberrant crypt foci (7).

Although strong data support the association between chronic inflammation and cancer, the biological basis of this association is not fully understood; our present data throw some light on this association. Positive associations have been found between the inflammatory diseases hepatitis and hepatocellular carcinoma (10), pancreatitis and pancreatic cancer (11), *Helicobacter pylori* infection and gastric cancer (12), schistosomiasis and bladder cancer (13), primary sclerosing cholangitis and cholangiocarcinoma (14), and between inflammatory bowel diseases and colorectal cancer (4). Oxidative stress has been identified as a possible culprit in the influence of inflammation on cancer development. Our present study joins other recent studies (6, 15, 16), including our study showing that Nrf2-deficient mice were more susceptible to DSS-induced colitis (6), in suggesting that Nrf2 may play a central role in regulating inflammation because Nrf2 is an integral regulator of detoxification and antioxidation mechanisms.

Our findings on the inflammation markers COX-2, 5-LOX, PGE₂, and LBT₄ (19, 20) support the association of knockout mouse tumor formation with inflammation. COX-2 and 5-LOX expressions and PGE₂ and LBT₄ levels were increased in Nrf2 knockout versus WT tumor tissue. These markers also were higher in tumor versus normal tissue either in knockout or WT mice. Furthermore, expression of the inflammation marker nitrotyrosine, which indicates cell damage, inflammation, and nitrite oxide production (data not shown),

was significantly increased in the colonic mucosa of mice treated with azoxymethane/DSS (versus not treated) overall and, more importantly, of Nrf2 knockout (versus WT) mice. Increased COX-2 expression has been observed in colitis-associated cancers of ulcerative colitis patients (20).

A growing body of evidence indicates that the loss of antioxidant/phase II detoxifying enzymes, coupled with increases in reactive oxygen and nitrogen species, is detrimental to normal colonic homeostasis. We previously showed that decreased expression of antioxidant/phase II detoxifying enzymes could help explain why Nrf2 knockout mice are more susceptible to DSS-induced colitis (6). The present study also showed that the expressions of some antioxidant/phase II detoxifying enzymes, including NAD(P)H-quinone reductase 1 and UDP-glucosyltransferase 1A1, were elevated in the normal mucosa of WT, but not Nrf2 knockout, mice treated with azoxymethane/DSS. The expression of heme oxygenase 1 was increased in the normal mucosa of treated WT and Nrf2 knockout mice. It is not surprising that heme oxygenase 1 was induced in Nrf2 knockout mice because other transcription factors (besides Nrf2) that regulate heme oxygenase 1, such as hypoxia-inducible factor 1 (17) and activator protein 1 (18), were presumably still present in Nrf2 knockout mice. In summary, these data suggest that decreased expression of the detoxifying enzymes NAD(P)H-quinone reductase 1 and UDP-glucosyltransferase 1A1, coupled with enhanced inflammation (described above), could be important events that accelerate neoplastic transformation in the colonic tissues of Nrf2 knockout mice, further suggesting that these defensive enzymes are important for protecting colonic mucosa against colitis-associated colorectal carcinogenesis.

The present study indicates that Nrf2 knockout mice were more susceptible to colitis/inflammation-associated colorectal cancer in our model of colitis and cancer induction. Primary contributors to this increased susceptibility were impaired antioxidant/detoxifying mechanisms coupled with enhanced proinflammatory arachidonic acid metabolism. This work adds an important link to the chain of related recent findings on the effects of Nrf2 on the development of colitis/inflammation (6), colitis-associated aberrant crypt foci (16), and colitis-associated colorectal cancer.

Acknowledgments

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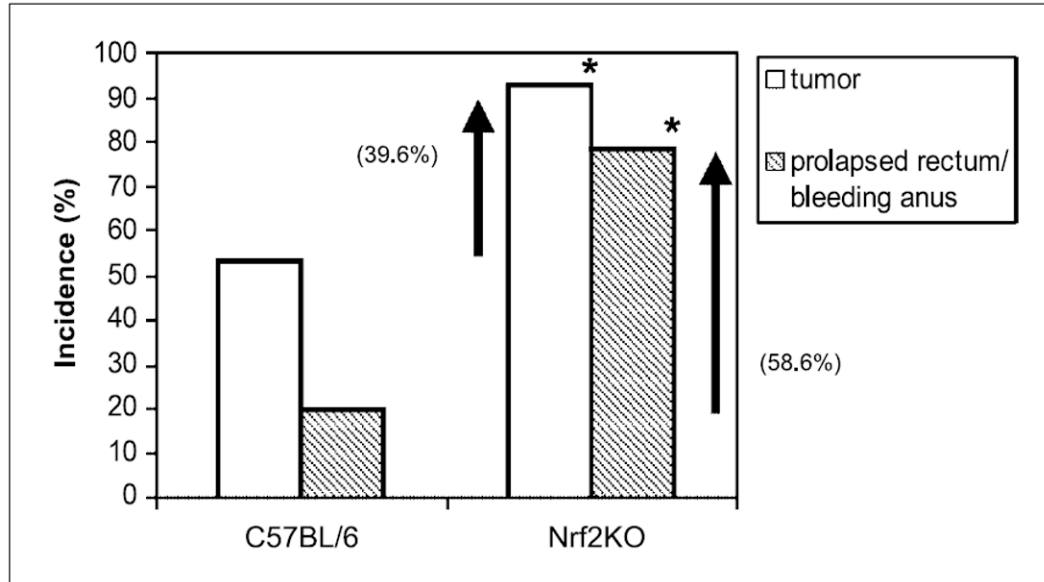


Fig. 1. Incidence of colonic tumors and prolapsed rectum/bleeding anus in C57BL/6 versus Nrf2 knockout (*KO*) mice after azoxymethane (*AOM*)/DSS treatment (20 wks). The incidence of colonic tumors per mouse and prolapsed rectum/bleeding anus was significantly higher in Nrf2 knockout mice compared with WT C57BL/6 mice. Fisher's exact test was used to compare the difference between groups.
*, $P < 0.01$.

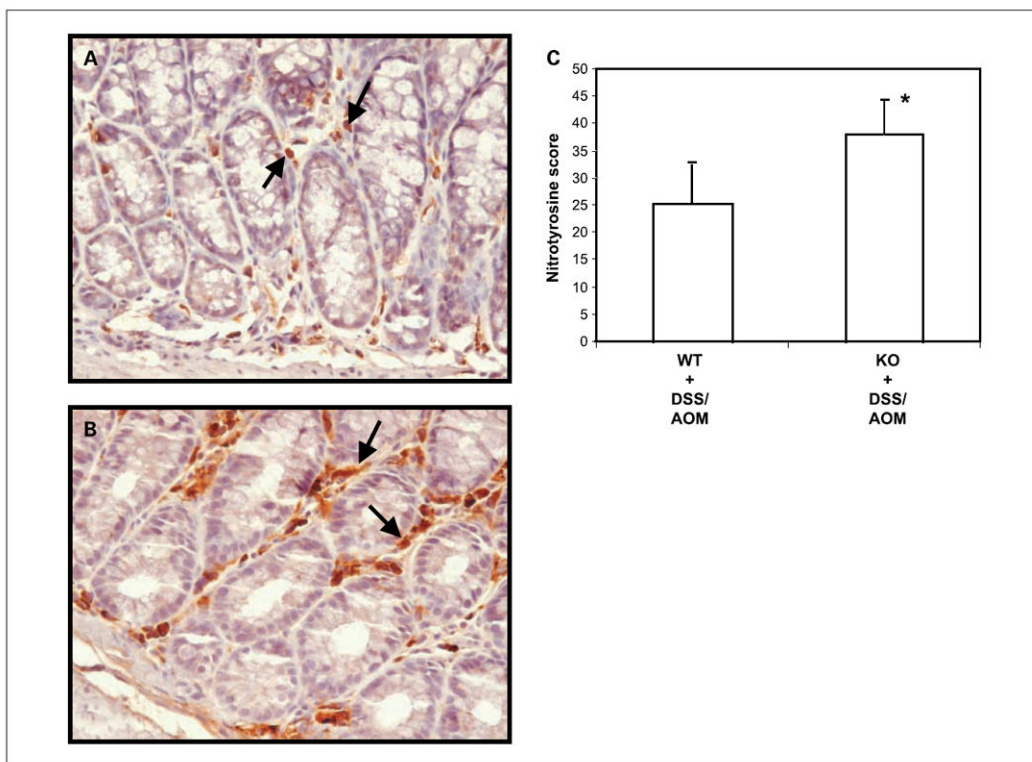
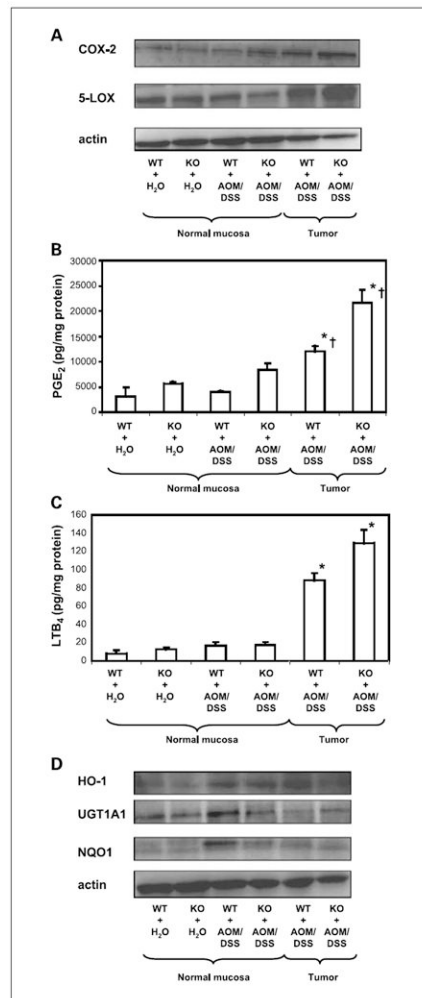


Fig. 2. Nitrotyrosine staining of colonic mucosa. Representative slide showing the expression of nitrotyrosine in colonic tissues of C57BL/6 (A) and Nrf2 knockout (B) mice after azoxymethane/DSS treatment (20 wk). Positive immunoreactivity of nitrotyrosine was observed predominantly in the stroma cells (arrow). Increased nitrotyrosine expression was observed in Nrf2 knockout mice as compared with WT C57BL/6 mice. Columns, mean of number of positive nitrotyrosine cells per field (40 \times); bars, SD. *, $P < 0.05$ (Student's t test).

**Fig. 3.**

The expression of proinflammatory and phase II detoxifying/antioxidant enzymes in the normal mucosa and tumors of Nrf2 knockout mice compared with WT C57BL/6J mice. *A*, expressions of COX-2 and 5-LOX were significantly elevated in the tumors of Nrf2 knockout mice when compared with normal tissues of knockout mice and tumors of WT mice. The levels of PGE₂ (*B*) and LTB₄ (*C*) were also higher in the tumors of Nrf2 knockout mice. *Columns*, mean; *bars*, SD.

*, significantly different from normal mucosa of mice with same phenotype.

†, significantly different from tumors of mice with different phenotypes.

D, expression of phase II detoxifying/antioxidant enzymes in the normal mucosa and tumors of Nrf2 knockout mice compared with WT C57BL/6J mice.

Table 1

Incidence, tumor multiplicity, tumor size, and percentage of prolapsed rectum/anus bleeding after AOM/DSS treatment (20 wk)

Group (no. mice)	Tumor incidence (%)	Tumor/mouse (mean ± SE)	Tumor size (mm ²)			Prolapsed rectum/bleeding of anus (%)
			<4	4-16	>16	
WT + H ₂ O (6)	0	0.00 ± 0.00	0	0	0	0
Nrf2 KO + H ₂ O (6)	0	0.00 ± 0.00	0	0	0	0
WT + AOM/DSS (15)	53.3	1.00 ± 0.40	7	4	4	20.0
Nrf2 KO + AOM/DSS (15)	92.9*	1.86 ± 0.33	9	11	6	78.6*

* $P < 0.05$ compared with WT + AOM/DSS

Abbreviations: KO, knockout; AOM, azoxymethane.

Table 2

Pathologic classification of tumors

Group	Adenomas (%)	Adenocarcinomas (%)
WT + AOM/DSS	71	29
Nrf2 KO + AOM/DSS	20	80